2. *Transferases*. These catalyze group transfer reactions.
3. *Hydrolases*. Enzymes catalyzing transfer of groups to the HO^- ion of H_2O.
4. *Lyases*. Enzymes promoting addition to double bonds or the reverse.
5. *Isomerases*. Enzymes catalyzing rearrangement reactions.
6. *Ligases* (synthetases). Enzymes that catalyze condensation with simultaneous cleavage of ATP and related reactions.

As an example, chymotrypsin is classified EC 3.4.4.5 according to the IUB system.[210] In this book a more mechanistically based classification is used. Because some official names are quite long, traditional trivial names for enzymes often have been retained. Remember that to be precise it is always necessary to mention the species from which an enzyme was isolated and, if possible, the strain. Also remember that almost every significant genetic difference is reflected in some change in some protein. It is possible that the enzyme you are working with is slightly different from the same enzyme prepared in a different laboratory.

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Study Questions

1. Give two reasons why enzymes are important to living organisms.

2. If an enzyme catalyzes the reaction A→ B + C, will it also catalyze the reaction B + C → A?

3. Can an enzyme use a site to catalyze a reverse reaction different from what it uses to catalyze the reaction in the forward direction? Explain your answer.

4. Define the "steady state" of living cells and contrast with a state of chemical equilibrium.

5. How is the instantaneous initial velocity of an enzymatic reaction measured? What precautions must be taken to ensure that a true instantaneous velocity is being obtained?

6. How is the rate of an enzyme action influenced by changes in:
   a) temperature
   b) enzyme concentration
   c) substrate concentration
   d) pH

7. In what ways are enzymatic reactions typical of ordinary catalytic reactions in organic or inorganic chemistry, and in what ways are they distinct?

8. In general, for a reaction that can take place with or without catalysis by an enzyme, what is the effect, if any, of the enzyme on
   a) standard Gibbs energy change of the reaction
   b) energy of activation of the reaction
   c) initial velocity of the reaction
   d) temperature coefficient of the rate constant

9. What are the dimensions of the rate constant for zero-, first-, and second-order reactions? If a first-order reaction is half completed in 2 min, what is its rate constant?

10. A sample of hemin containing radioactive iron, ^{59}Fe, was assayed with a Geiger-Müller counter at intervals of time with the following results:

Time (days)	Radioactive counts per min
0	3981
2	3864
6	3648
10	3437
14	3238
20	2965

Determine the half-life of ^{59}Fe and the value for the decay constant.

11. The kinetics of the aerobic oxidation of enzymatically reduced nicotinamide adenine dinucleotide (NADH) have been investigated at pH 7.38 at 30°C. The reaction rate was followed spectrophotometrically by measuring the decrease in absorbance at 340 nm over a period of 30 min. The reaction may be represented as

$$NADH + H^+ + \text{riboflavin} \rightarrow NAD^+ + \text{dihydroriboflavin}$$

Time (days)	A (at 340 nm)
1	0.347
2	0.339
5	0.327
9	0.302
16.5	0.275
23	0.254
27	0.239
30	0.229

Determine the rate constant and the order of the reaction.

12. You have isolated and purified a new enzyme (E) which converts a single substrate (S) into a single product (P). You have determined M_r by gel filtration as ~ 46,400. However, in SDS gel electrophoresis, a molecular mass of ~ 23 kDa was indicated for the single protein band observed. A solution of the enzyme was analyzed in the following way. The absorbance at 280 nm was found to be 0.512. A 1.00 ml portion of the same solution was subjected to amino acid analysis and was found to contain 71.3 nmol of tryptophan. N-terminal analysis on the same volume of enzyme revealed 23.8 nmol of N-terminal alanine.

   a) What is the approximate molecular mass of the enzyme? Discuss this answer. Be sure to use an appropriate number of significant figures in this and other calculations.
   b) What is the concentration of your enzyme in moles per liter of active sites?
   c) What is the molar extinction coefficient ε at 280 nm where A = εcl; A = absorbance, c = mol / liter, and l = cell width in cm. Assume that all spectrophotometric measurements are made in 1.00 cm cuvettes.

A second preparation of the enzyme had an absorbance at 280 nm of 0.485. This enzyme was diluted very carefully: 1.00 ml into 250 ml and this diluted enzyme was used for the following experiments (I to III).

Study Questions

Experiment I. A 1.00 ml portion of the diluted enzyme was added to 250 ml of buffered substrate at pH 7.0 and was mixed rapidly. The resulting initial substrate concentration $[S]_o$ was 1.000 mM. This reaction mixture was held at 25.0°C and portions were removed periodically at time t for analysis of the product P formed. The results follow. Plot [P] vs. time.

Time t (s)	[P] (mM)	Time t (s)	Remaining [S] (mM)
200	0.104	2800	0.070
400	0.208	3200	0.040
800	0.392	3600	0.022
1200	0.554	4400	0.0060
1600	0.695	6000	0.00048
2000	0.800		
2400	0.881		

The integrated rate equation corresponding to the Michaelis–Menten equation

$v = V_{max}\,[S] / (K_m + [S])$ is as follows:

$$V_{max} \cdot t = [S]_o - [S] + K_m \ln ([S]_o / [S])$$

d) Plot $([S]_o - [S])/t$ vs. $1/t \times \ln([S]_o/[S])$. From this plot evaluate K_m and V_{max}. Make this and other plots to appropriate scale on good quality graph paper.

e) What is k_{cat}?

Experiment II. In a second experiment, a series of test tubes were set up, each containing a different amount of buffered substrate at pH 7 but each in a volume of exactly 4.00 ml. The same enzyme solution used in part d (absorbance at 280 nm = 0.485) was diluted 2.00 ml in 250 ml as in I, then again 2.00 ml in 200 ml. Portions of 1.00 ml of this twice diluted enzyme were added at $t = 0$ to each of the test tubes of buffered substrate. The reaction was stopped in just 10.0 minutes by adding perchloric acid; a suitable reagent was added to provide for a colorimetric determination of the product. The results were as follows:

[S] (mM)	Amount of product (µmol/tube)	[S] (mM)	Amount of product (µmol/tube)
10.0	2.29	0.600	1.31
5.00	2.18	0.400	1.07
2.50	2.00	0.200	0.686
1.200	1.69	0.100	0.400
0.800	1.48		

f) Plot $1/v$ vs. $1/[S]$ where v is in units of µmol per tube and [S] in millimoles / liter. Evaluate K_m, V_{max}, and k_{cat} from this plot. Fit your data by eye. It is generally agreed that a linear least squares fit is inappropriate unless suitable weighting factors are used.

g) Plot the same data as $v/[S]$ vs. v. Again evaluate K_m and V_{max}.

Experiment III. The preceding experiment was repeated but an inhibitor was present in each tube in a concentration equal to 5.00 mM, 10.0 mM, or 25.0 mM. Two different inhibitors were used, A and B. The following results were obtained.

µmol of product formed in 10 min/tube

[S] (mM)	[I] = 5.00 mM Inhibitor: A	5.00 mM B	25.0 mM Inhibitor: A	10.0 mM B
5.00	2.00	1.09	1.50	0.727
2.50	0.71	1.00	1.09	0.667
1.20	1.31	0.848	0.686	0.565
0.800	1.07	0.739	0.505	0.492
0.600	0.900	0.654	0.400	0.436
0.400	0.686	0.533	0.282	0.356
0.200	0.400	0.343	0.150	0.229
0.100	0.218	0.200	0.077	0.133

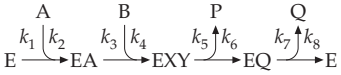
h) Plot $1/v$ vs. $1/[S]$ for each of these sets of data. For each case evaluate V_{max}, apparent K_m, and inhibitor constants $K_I = [I][E]/[EI]$. There may be two K_I values.

i) For uninhibited enzymes, when [S] = 0.4 mM what fraction of the enzyme is ES? Free E?

j) For enzyme in the presence of 10.00 mM inhibitor and 0.8 mM substrate, what fraction of the total enzyme is ES? EI? free E?

k) Recall that $v = [ES]\,k_{cat} = [E][S] \cdot k_{cat}/K_m$ in many cases. What will be the relative rates of product formation from your substrate S and another competing substrate S′ present at the same concentration which also reacts with your enzyme with $K_m = 0.01$ mM and $k_{cat} = 200$ / s if $[S] = [S']$?

13. Using the method of graphs, write the initial rate equation for the following system with A, B, P, and Q present.



Odd-numbered rate constants are for forward reactions; even-numbered constants are for reverse.

a) Satisfy yourself that

$$\frac{-d[A]}{dt} = k_1[A][E] - k_2[EA]$$

$$= \frac{-d[B]}{dt} = k_3[B][EA] - k_4[EXY]$$

$$= \frac{d[P]}{dt} = k_5[EXY] - k_6[P][EQ]$$

$$= \frac{d[Q]}{dt} = k_7[EQ] - k_8[E][Q]$$

The determinants (given by the method of graphs) which provide the steady-state concentrations of [E] and of the various exzyme–substrate and enzyme–product complexes are

$$[E] = k_2k_7[k_4 + k_5] + k_3k_5k_7[B] + k_2k_4k_6[P]$$

$$[EA] = k_1k_7[k_4 + k_5][A] + k_1k_4k_6[A][P] + k_4k_6k_8[P][Q]$$

$$[EXY] = k_1k_3k_7[A][B] + k_2k_6k_8[P][Q] + k_1k_3k_6[A][B][P] + k_3k_6k_8[B][P][Q]$$

$$[EQ] = k_2k_8[k_4 + k_5][Q] + k_1k_3k_5[A][B] + k_3k_5k_8[B][Q]$$

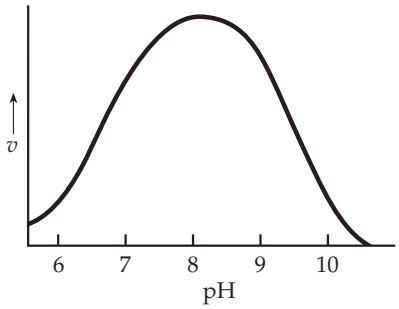
b) To obtain an expression for v_f the expression for $-d[A]/dt$ may be multiplied by $[E]_t$ and divided by $[E] + [EA] + [EXY] + [EQ] = [E]_t$. Then the above determinants may be substituted.

c) What is $V_{max,forward}$ and $V_{max,reverse}$? HINT: $V_{max,forward}$ is obtained when $[P] = [Q] = 0$ and $[A] = [B] = \infty$. What are K_{mA} and K_{mB}? K_{mA} is obtained when $[P] = [Q] = 0$, $[B] = \infty$, and $v = 1/2\ V_{max,forward}$.

d) With a knowledge of the kinetic parameters, indicate how the eight rate constants may be obtained if the total concentration of enzyme, $[E]_t$, is known.

14. Anticooperativity was observed in the plot of velocity vs. substrate concentration for an enzyme. Can this observation be explained by the Monod–Wyman–Changeux model for oligomeric enzymes? By the model of Koshland? Explain.

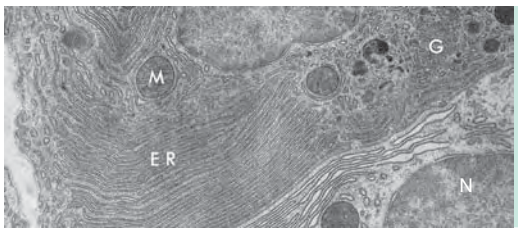
15. Interpret, for each of the following cases, the curve showing measured initial velocity at constant substrate concentration (*not* maximum velocity) against pH for an enzyme-catalyzed reaction.



a) The substrate is neutral and contains no acidic or basic groups. The Michaelis constant is found to be independent of pH over the range studied.

b) The substrate is neutral and contains no acidic or basic groups, and the maximum velocity is found to be independent of pH.

c) The substrate is an α-amino acid, and the maximum velocity is found to be independent of pH.

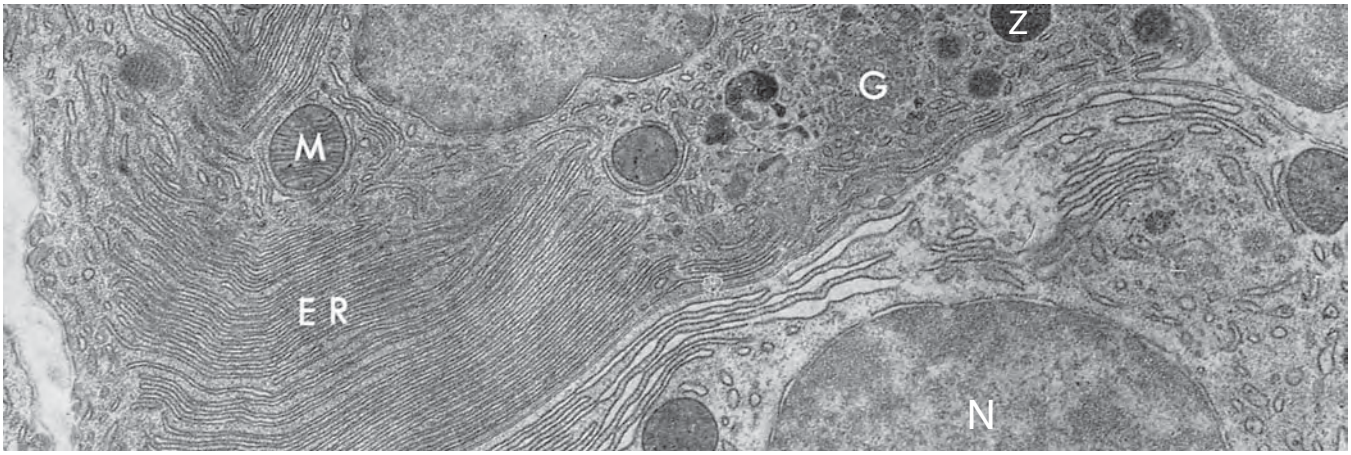


This electron micrograph of a thin section of pancreatic epithelial cells shows parts of two secretory cells that are synthesizing proenzymes (zymogens). Nuclei (N) are seen at the top center and lower right. Numerous ribosomes (barely seen here) line the many membranes of the endoplasmic reticulum (ER) and are most abundant near lateral and basal (left) surfaces of the cells. A few mitochondria (M) are present. The synthesized proteins move toward the apical surfaces (a small piece is seen at upper right), passing through the Golgi region (G) and accumulating in zymogen granules (Z) before secretion. From Porter, K. R. and Bonneville, M. A., *Fine Structure of Cells and Tissues*, Lea and Febiger, Philadelphia 1973. Courtesy of Mary Bonneville.

Contents

An Introduction to Metabolism

10



The first section of this book has dealt with the basic structures and with many of the complexities of the materials formed by living cells. In the next major section we will look at the chemical reactions that build and maintain a cell and that permit it to grow and to be responsive to external stimuli. These reactions are organized into **metabolic sequences** or **pathways** that form a complex, branched, and interconnected network. It would be pointless to try to memorize all of them. However, at this point it will be worthwhile to consider the significance of a few of the major sequences, which describe central pathways of metabolism.

Beginning in the nineteenth century, the investigation of these pathways and of the associated enzymes has provided much of our knowledge about the chemistry of living things. The protein products that are encoded in the structural genes that direct these pathways make up a large fraction of the material in a cell. It will be well worth the reader's effort to examine the chemistry of these metabolic sequences in detail. Regulatory mechanisms that are applicable to them, and to other pathways, are described in Chapter 11 and chemical details of enzyme action are considered in Chapters 12–16. Then, in Chapter 17, the chemical logic of the reaction sequences is considered in more detail.

A. The Starting Materials

All cells of all organisms take in chemical starting materials and give off chemical products. They all have a source of energy and generate heat during their metabolism. They all synthesize complex organic substances and maintain a high degree of organization, that is, a state of relatively low entropy. The materials taken up by cells are often organic compounds which not only supply material for the synthesis of cellular constituents but also may be degraded to provide energy. A characteristic of all cells is the ability simultaneously to synthesize thousands of complex proteins and other materials and, at the same time, to break down (catabolize) the same types of compounds. Since cells both synthesize and catabolize most cellular components there is a continuous turnover of the very structural components of which they are composed. Metabolism encompasses all of these processes.

The most rapid catabolic reactions are usually those that provide the cell's energy. Organisms vary greatly in the materials used for food. Human beings, as well as many other organisms, break down carbohydrates, lipids, and proteins to obtain energy and starting materials for biosynthesis. In contrast, some organisms use only one or a few simple organic compounds while autotrophs satisfy their needs entirely with inorganic materials. Looking at all of the species of living things we find an extraordinary range of specialization in metabolism as well as in structure. Nevertheless, there are many common features of metabolism. For example, most cells utilize glucose or a close relative as a source of biosynthetic intermediates. Pathways for synthesis of nucleic acids and proteins are similar in all species. Even the control of growth and development depends upon proteins whose structures are often conserved throughout the living world.

1. Digestion

We humans must digest most foods before we can utilize them. The same is true for most bacteria, which need amino acids generated by the breakdown of

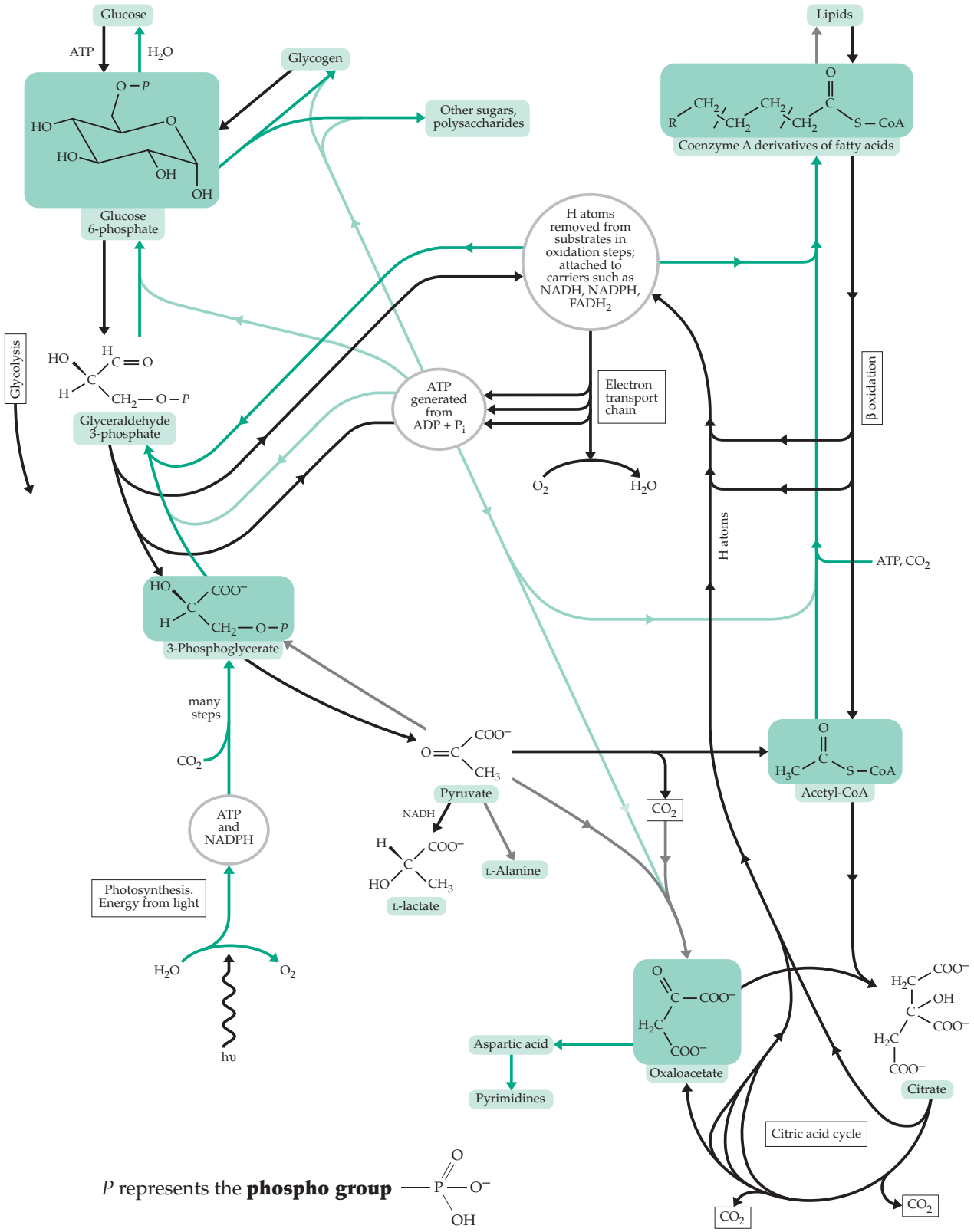
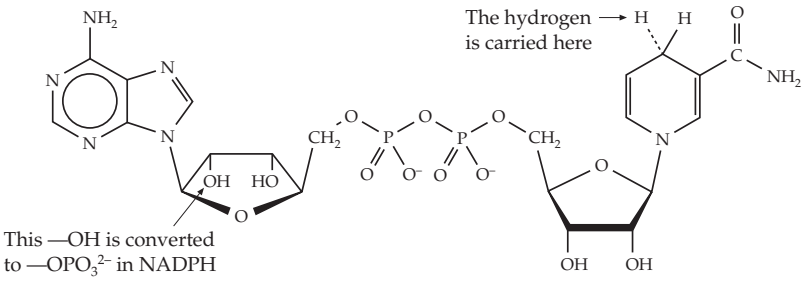


Figure 10-1 An overall view of some metabolic sequences. Several major pathways of catabolism are indicated by heavy lines. The glycolysis pathway, leading to pyruvate and lactate, starts at the top left, while the β oxidation pathway of fatty acids is on the right. Biosynthetic routes are shown in green lines. A few of the points of synthesis and utilization of ATP are indicated by the lighter green lines. Some of the oxidation–reduction reactions that produce or utilize the reduced hydrogen carriers NADH, NADPH, and $FADH_2$ are also indicated. The citric acid cycle, a major supplier of these molecules, is shown at the bottom right, while photosynthesis, the source of reduced hydrogen carriers in green plants, and the source of nearly all energy for life, is shown at the bottom left.

proteins. Our digestive enzymes, which act on carbohydrates, proteins, lipids, and nucleic acids, are synthesized by cells in the salivary glands, stomach, pancreas, and intestinal lining. The properties of these enzymes are described in Chapter 12. Bacteria secrete digestive enzymes into their surroundings. Although most green plants have no need to digest foods, they too have enzymes closely related to our digestive enzymes. These enzymes break bonds in proteins or carbohydrate polymers to allow expansion of leaves and stems, ripening of fruits, and other physical changes. All cells carry out **processing** or **maturation** of their newly synthesized polymers. This often involves the cutting off of one or more pieces of a protein, polysaccharide, or nucleic acid. The processing enzymes are also relatives of digestive enzymes.

2. Sources of Energy

All cells require a source of chemical energy. This is provided to a large extent by ATP (Chapter 6), whose hydrolysis can be coupled to reactions of synthesis, to transport across membranes, and to other endergonic processes. For this reason, all cells have active pathways for the synthesis of ATP. *Biosynthesis often also involves chemical reduction* of intermediates; therefore, cells must have a means of generating suitable reductants. The reagent of choice is often the hydrogen-carrying coenzyme **NADPH**. It is a phosphorylated form of NADH, a derivative of the vitamin **nicotinamide** (Box 15-A), whose structure follows. Reduction of metabolic intermediates by NADPH provides a second mechanism by which chemical energy is harnessed for biosynthesis. A third source of energy, which is considered in Chapter 18, is the gradient of hydrogen ions that is set up across cell membranes by oxidative or photochemical processes.



The reduced coenzyme NADH

B. Catabolism and the Synthesis of ATP

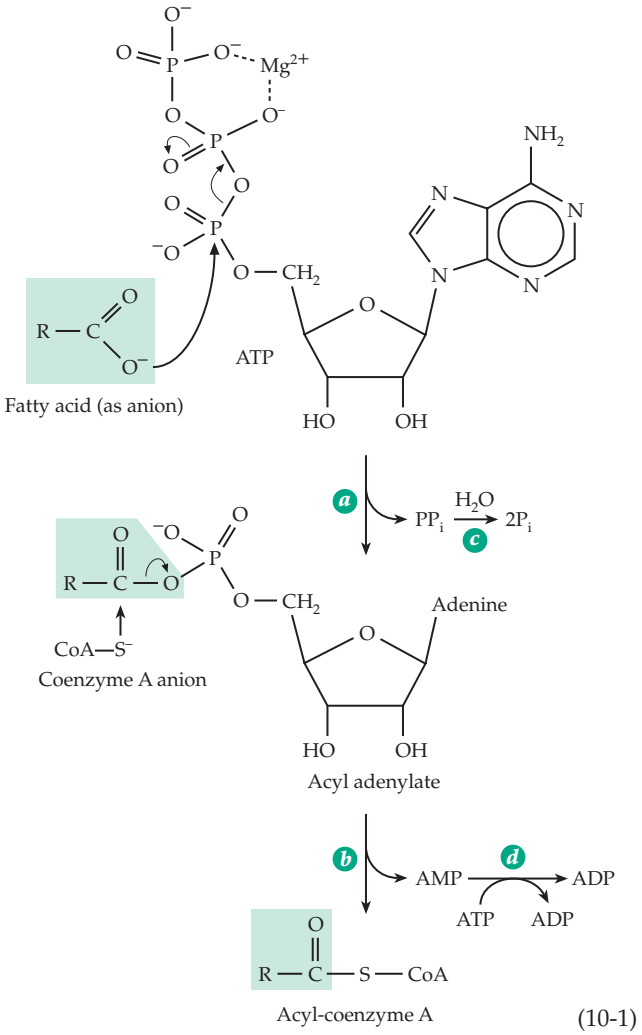
The catabolic sequences by which cells obtain energy often appear dominant. For animals, fungi, and nonphotosynthetic bacteria these pathways are used to metabolize large amounts of food and to produce large amounts of ATP. Because they are also related to biosynthetic pathways they have a central importance in virtually all organisms.

1. Priming or Activation of Metabolites

After a polymeric nutrient is digested and the monomeric products are absorbed into a cell, an energy-requiring **priming reaction** is usually required. For example, the hydrolysis of fats (whether in the gut or within cells) produces free fatty acids. Before undergoing further metabolism, these fatty acids are combined with the thiol compound **coenzyme A** to form **fatty acyl-CoA** derivatives. This requires a thermodynamically unfavorable reaction that necessitates the "expenditure" of ATP, that is, its hydrolysis to AMP and inorganic pyrophosphate (PP_i). Likewise, glucose, when taken into cells, is converted into the phosphate ester glucose 6-phosphate (glucose-6-*P*), again with an associated cleavage of ATP. *The major metabolic pathways often start with one of these two substances: a fatty acyl-CoA derivative or glucose 6-phosphate.* The structures of both are given at the top of Fig. 10-1, which is designed to provide an overall view of metabolism.

The phospho group of glucose-6-*P* is not very reactive but it provides a "handle" which helps enzymes to recognize and to hold onto this glucose derivative. Likewise, the coenzyme A molecule, whose complete structure is given in Chapter 14, provides a large and complex handle for enzymes. However, from a chemical viewpoint, the formation of the thioester linkage of a fatty acid with the –SH group of coenzyme A in the acyl-CoA is more important. This alters the electronic structure of the fatty acid molecule, "activating" it toward the reactions that it must undergo subsequently. Thus, priming reactions often provide chemical changes essential to the mechanisms of subsequent reactions.

Activation of a carboxylic acid by formation of an acyl-CoA derivative (Eq. 10-1) is of special importance because of its widespread use by all organisms. The basic reaction occurs by two steps. The first (Eq. 10-1, step *a*) is a displacement reaction on the phosphorus atom of ATP to form an **acyl adenylate**, a mixed anhydride of the carboxylic acid and a substituted phosphoric acid. Such mixed anhydride intermediates, or **acyl phosphates**, are central to much of cellular energy metabolism because *they preserve the high group-transfer potential of a phospho group of ATP (Chapter 6) while imparting a high group-transfer potential to the acyl group*. This allows the acyl group to be transferred in step *b*



(10-1)

of Eq. 10-1 onto the sulfur atom of coenzyme A to form an acyl-coenzyme A in which the high group transfer potential of the acyl group is conserved (see Table 6-6). Two additional steps are linked to this sequence. In step *c* the inorganic pyrophosphate that is displaced in step *a* is hydrolyzed to two molecules of HPO_4^{2-} (P_i), and in step *d* the AMP formed is phosphorylated by ATP to form ADP. The overall sequence of Eq. 10-1 leads to hydrolysis of two molecules of ATP to ADP + P_i. In other words, two molecules of ATP are spent in activating a carboxylic acid by conversion to an acyl-coenzyme A.

2. Interconversions of Sugar Phosphates

The strategy employed by most cells in the catabolism of several 6-carbon sugars is to convert them to glucose 6-phosphate and, in the several steps outlined in Fig. 10-2, to cleave this hexose phosphate to two equivalent molecules of **glyceraldehyde 3-phosphate**. This triose phosphate can then be metabolized further. Notice the chemical nature of the reactions involved in the formation of glyceraldehyde 3-phosphate. The first step (Fig. 10-2, step *a*) is the transfer of the phospho group from ATP to the 6 position of glucose. Step *b* is the reversible opening of the sugar ring and step *c* the isomerization of an aldose to a ketose. Step *d* is a second transfer of a phospho group from ATP, another priming reaction that provides the second of the phosphate handles for the two molecules of triose phosphate that are formed. Step *e* is an aldol cleavage which breaks the C–C bond in the center of the ketose chain, while step *f* is another sugar isomerization that is chemically similar to the one in step *c*.

We see that in the six steps shown in Fig. 10-2 for conversion of glucose to two molecules of glyceraldehyde 3-phosphate, there are only four kinds of reaction: (1) phospho transfer from ATP; (2) opening of a sugar ring; (3) aldose-ketose isomerization; and (4) aldol cleavage. These types of reactions, which are described in more detail in Chapters 12 and 13, occur in many places in metabolism. As is indicated in Fig. 10-2, glucose 6-phosphate can also be generated from glycogen or starch. The first step (Fig. 10-2, step *g*) is phosphorolysis, cleavage by an inorganic phosphate ion. This leads directly to glucose 1-phosphate. The latter is isomerized by a phospho transfer process (step *h*).

3. Glycolysis and Fermentation

A major route of breakdown of carbohydrates is the **Embden–Meyerhof–Parnas** pathway, often referred to simply as **glycolysis**. It is indicated on the left side of Fig. 10-1 and in more detail in Figs. 10-2 and 10-3. The pathway begins with the reactions of Fig. 10-2, with either free glucose or glycogen as starting materials. Its end products may be reduced materials such as **lactic acid** or **ethanol**, which are formed under *anaerobic conditions* (Fig. 10-3). However, under *aerobic conditions* the product is **acetyl-coenzyme A**, whose acetyl group can then be oxidized to carbon dioxide and water in the **citric acid cycle**.

After the sugar chain is cleaved in the glycolysis sequence, the two resultant molecules of glyceraldehyde 3-phosphate are oxidized to 3-phosphoglycerate (Fig. 10-3, steps *a*,*b*). The oxidant is the hydrogen carrier NAD^+, the oxidized form of NADH. Cells frequently use NAD^+ to dehydrogenate alcohols to aldehydes or ketones, with one atom of hydrogen and an electron from the alcohol becoming attached to the NAD^+ to give NADH and the other hydrogen being released as H^+ (Eq. 10-2).

The oxidation of glyceraldehyde 3-phosphate is considerably more complex. The oxidation of an aldehyde to a carboxylic acid is a strongly exergonic process and the oxidation of glyceraldehyde 3-phosphate by cells is almost always coupled to the synthesis

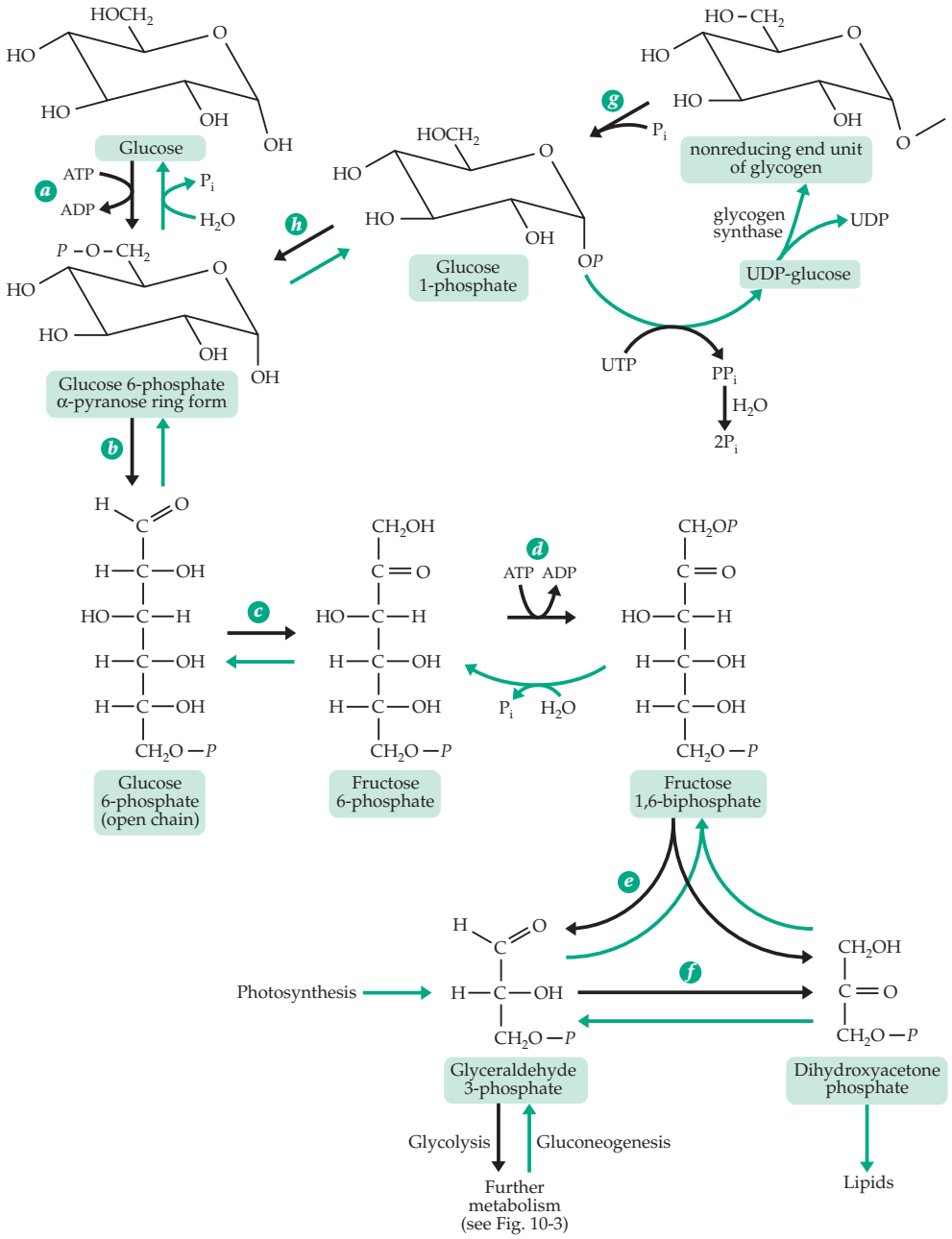
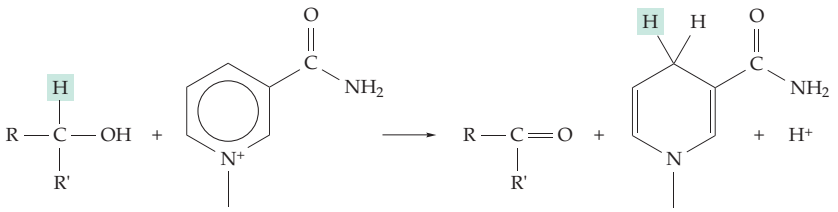


Figure 10-2 The interconversion of glucose, glycogen, and glyceraldehyde-3-phosphate in the pathways of glycolysis, gluconeogenesis, and glycogen synthesis. Pathways of catabolism are indicated with black lines and those of biosynthesis with green lines.

of ATP. The importance of this coupling can be understood by the fact that *for many organisms living by anaerobic fermentation reactions, this one oxidation step provides the sole source of ATP*. It is the mechanism for coupling this oxidation to synthesis of ATP that accounts for the complexity of the glyceraldehyde 3-phosphate dehydrogenase reaction, whose chemistry is presented in detail in Chapter 15. As is indicated in the simplified version of Fig. 10-3 (step *a*) inorganic phosphate (P_i) is a reactant and the product released by the enzyme is 1,3-bisphosphoglycerate. This compound, which has been formed by the oxidative process, is an anhydride of phosphoric acid with 3-phosphoglyceric acid. The phospho group of such **acyl phosphates**, like that of ATP, has a high group transfer potential and can be transferred to ADP to generate ATP (Fig. 10-3, step *b*). Since each glucose molecule is cleaved to two molecules of triose phosphate, two molecules of ATP are

generated in this process by the fermentation of one molecule of glucose. This is enough for a bacterium to live on if it ferments enough sugar.

Further metabolism of 3-phosphoglycerate involves an isomerization by means of a phosphotransferase (mutase reaction, step *c*) to form 2-phosphoglycerate which then loses water in an elimination reaction (step *d*). Elimination of a hydroxyl group in a β position relative to a carboxyl group together with an α-proton is another very frequently used type of metabolic reaction. In this case the product, **phosphoenolpyruvate** (PEP), is a unique and important metabolite. It is a key intermediate in the biosynthesis of aromatic amino acids and in numerous other biosynthetic sequences.



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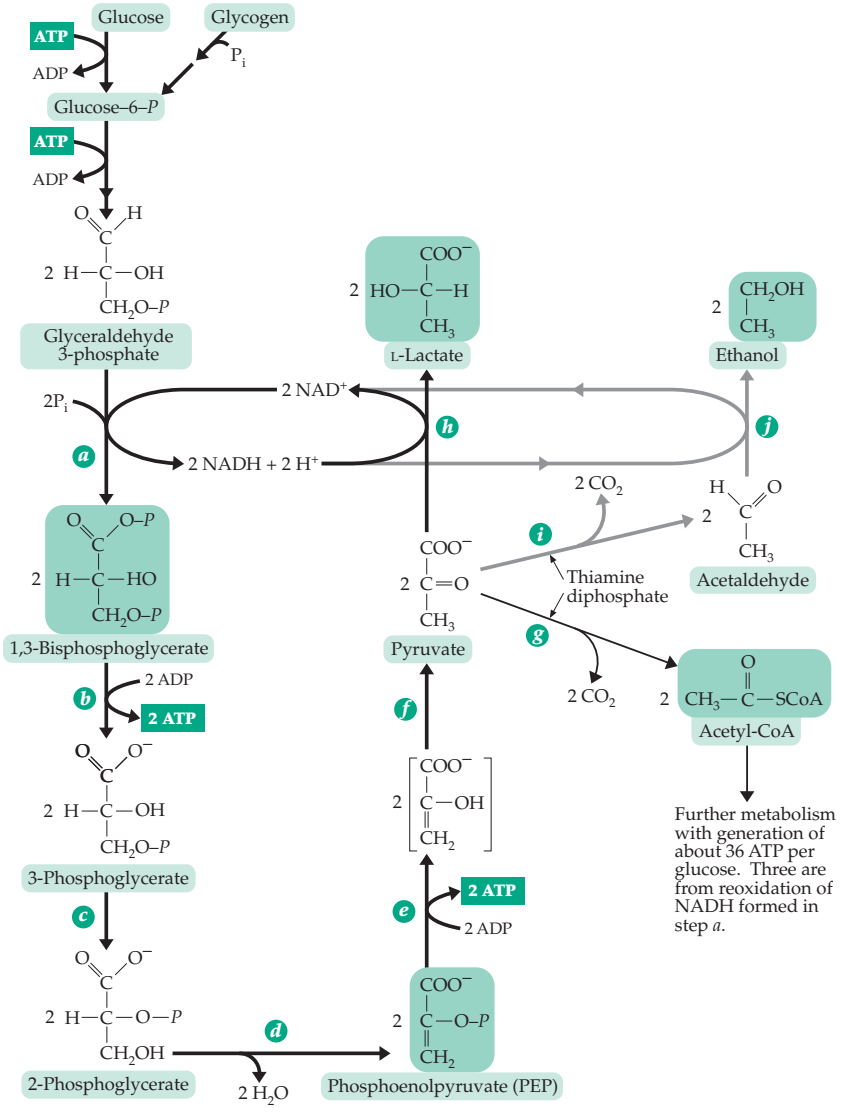


Figure 10-3 Coupling of the reactions of glycolysis with formation of lactic acid and ethanol in fermentations. Steps *a* to *g* describe the Embden–Meyerhof–Parnas pathway. Generation of 2 ATP in step *b* can provide all of the cell's energy.

In the Embden–Meyerhof–Parnas pathway PEP transfers its phospho group to ADP (step *e*) to generate ATP and pyruvate. The latter is shown enclosed in brackets in Fig. 10-3 as the enolic form, which, tautomerizes to the oxo form (step *f*). The fact that the oxo form of pyruvate is much more stable than the enol gives the phospho group of phosphoenolpyruvate its high group transfer potential. The metabolic significance of this step is that the phosphate handles of the glycolytic intermediates, which were initially transferred on from ATP, are now returned to ADP with the regeneration of an equivalent amount of ATP.

The product of this metabolic sequence, **pyruvate**, is a metabolite of central importance. Its fate depends upon the conditions within a cell and upon the type of cell. When oxygen is plentiful pyruvate is usually converted to acetyl-coenzyme A, but under anaerobic conditions it may be reduced by NADH + H^+ to the alcohol **lactic acid** (Fig. 10-3, step *h*). This reduction exactly balances the previous oxidation step, that is, the oxidation of glyceraldehyde 3-phosphate to 3-phosphoglycerate (steps *a* and *b*). With a balanced sequence of an oxidation reaction, followed by a reduction reaction, glucose can be converted to lactate in the absence of oxygen, a **fermentation** process. The lactic acid fermentation occurs not only in certain bacteria but also in our own muscles under conditions of extremely vigorous exercise. It also occurs continuously in some tissues, e.g., the transparent lens and cornea of the eye.

In the well-known fermentation of sugar to ethanol by yeast, the pyruvate generated by glycolysis is first decarboxylated to acetaldehyde (Fig. 10-3, step *i*). This decarboxylation of a 2-oxo acid is chemically difficult and the enzyme catalyzing it makes use of a special reagent known as a **coenzyme**. For this type of reaction, the coenzyme is the diphosphate (pyrophosphate) ester of **thiamin** (**vitamin B_1**). Its mode of action is discussed in Chapter 14. It is usually needed when a 2-oxo acid is decarboxylated. The alcoholic fermentation by yeast is completed by reduction of acetaldehyde to ethanol (Fig. 10-3, step *j*), again using the NADH produced in the oxidation of glyceraldehyde 3-phosphate. The conversion of glucose to lactic acid or to ethanol and CO_2 are just two of many fermentation reactions, most of which are carried out by bacteria and which are dealt with further in Chapter 17. An important requirement is that the Gibbs energy change for the overall fermentation reaction be sufficiently negative that ATP synthesis can be coupled to it. Thus, using data from Table 6-4:

$$C_6H_{12}O_6 \text{ (glucose)} \rightarrow 2\ C_3H_5O_3 \text{ (lactate)} + 2H^+$$
$$\Delta G' \text{ (pH 7)} = -196 \text{ kJ/mol } (-46.8 \text{ kcal/mol}) \qquad (10\text{-}3)$$

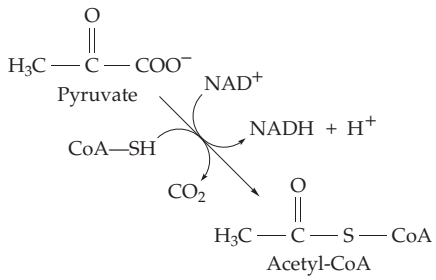
$$2ADP^{3-} + 2P_i^{2-} + H^+ \rightarrow H_2O + ATP^{4-}$$
$$\Delta G' \text{ (pH 7)} = +69 \text{ kJ/mol} \qquad (10\text{-}4)$$

$$\text{Sum: } C_6H_{12}O_6 + 2ADP^{3-} + 2P_i^{2-} \rightarrow H_2O + 2\ C_3H_5O_3 + 2H^+ + 2\ ATP^{4-}$$
$$\Delta G' \text{ (pH 7)} = -127 \text{ kJ/mol} \qquad (10\text{-}5)$$

A requirement for all fermentations is the existence of a mechanism for coupling ATP synthesis to the fermentation reactions. In the lactic acid and ethanol fermentations this coupling mechanism consists of the formation of the intermediate 1,3-bisphosphoglycerate by the glyceraldehyde 3-phosphate dehydrogenase (Fig. 10-3, step *a*). This intermediate contains parts of both the products ATP and lactate or ethanol.

4. Pyruvate Dehydrogenase

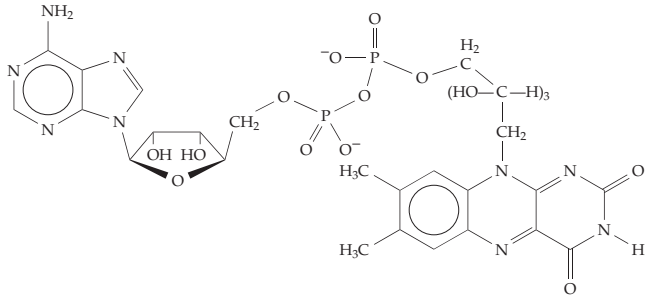
In most organisms undergoing aerobic metabolism, pyruvate is oxidized to acetyl-CoA in a complex process involving its decarboxylation (Eq. 10-6). This **oxidative decarboxylation**, like the decarboxylation of pyruvate to acetaldehyde, requires thiamin diphosphate. In addition, an array of other catalysts participate in the process (see Fig. 15-15). Among these are the electron carrier **flavin adenine diphosphate** (**FAD**), which is derived from the vitamin **riboflavin**. Like NAD^+, this



(10-6)

compound can accept two electrons (plus two protons) to form $FADH_2$. However, it sometimes serves as a one-electron carrier.

Acetyl-CoA is another major metabolic intermediate.



The coenzyme flavin adenine diphosphate (FAD)

It is an acyl-CoA of the type mentioned in Section 1 and can also be formed from acetate, ATP, and coenzyme A. Although the human diet contains some acetic acid, the two major sources of acetyl-CoA in our bodies are the oxidative decarboxylation of pyruvate (Eq. 10-6) and the breakdown of fatty acid chains. Let us consider the latter process before examining the further metabolism of acetyl-CoA.

5. Beta Oxidation

Whether fatty acids are oxidized to obtain energy or are utilized for biosynthesis, they are first converted to their acyl-CoA forms and are then cleaved to the two-carbon units represented by the acetyl groups of acetyl-CoA. The **beta oxidation** sequence, by which this occurs, is represented by the solid vertical arrow on the right side of Fig. 10-1 and is shown in greater detail in Fig. 10-4. We see from the latter figure that there are two dehydrogenation steps. The first (step *b*) removes hydrogen atoms from the α- and β-carbon atoms to produce a *trans* α, β unsaturated fatty acyl-CoA (Enoyl-CoA). The hydrogen acceptor is FAD. It is needed because this reaction requires a more powerful oxidant than does the dehydrogenation of an alcohol, for which NAD^+ is adequate. Addition of water to the double bond of the unsaturated acyl-CoA (step *c*)

generates an alcohol which is then dehydrogenated (step *d*) by NAD^+ to form a β-oxoacyl-CoA. This is cleaved (step *e*) by a reaction (thiolysis) with another molecule of coenzyme A to form acetyl-CoA and a new acyl-CoA with a shortened chain. The latter is "recycled" by passage through the β oxidation sequence repeatedly until the chain is shortened to a 2- or 3-carbon fragment, acetyl-CoA, or propionyl-CoA. Since most dietary fatty acids contain an even number of carbon atoms, acetyl-CoA is the predominant product.

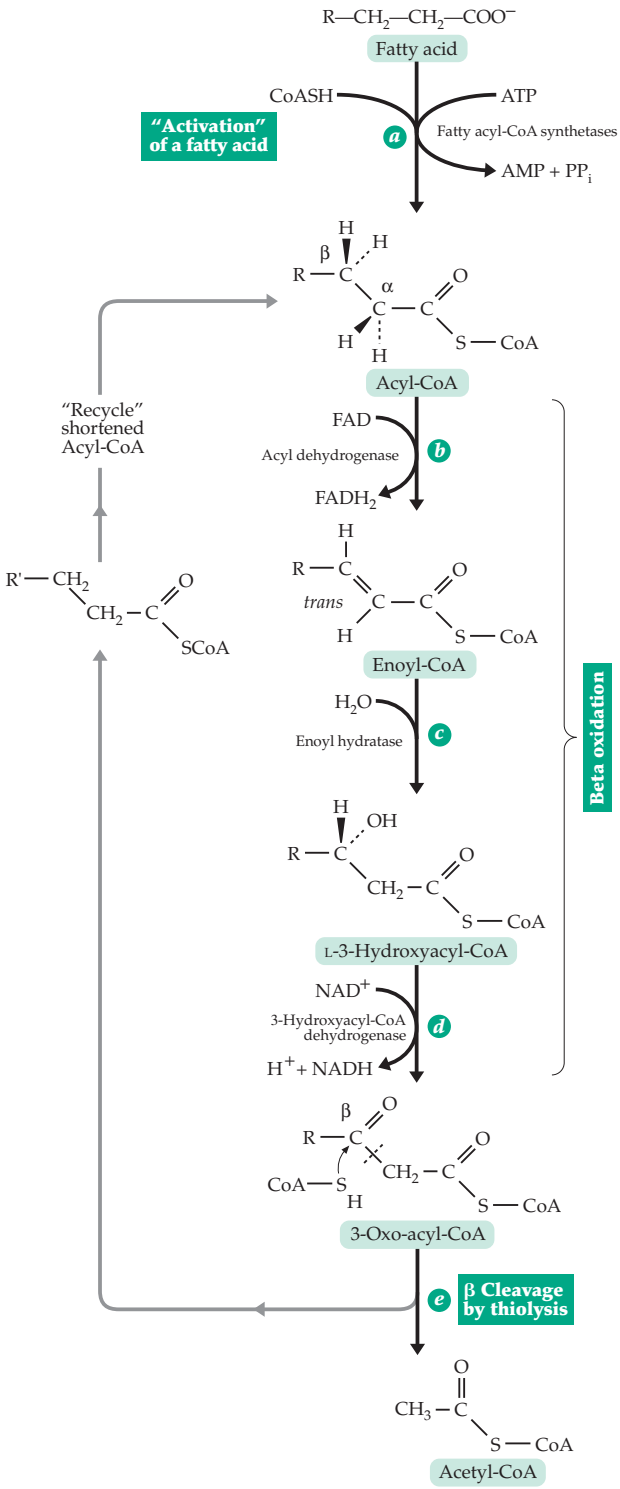


Figure 10-4 Reactions of fatty acid activation and of breakdown by β oxidation.

The beta oxidation of fatty acids, like the dehydrogenation of pyruvate to acetyl-CoA, takes place within the inner **matrix** of the mitochondria in eukaryotic organisms. The reduced hydrogen carriers $FADH_2$ and NADH transfer their electrons to other carriers located within the **inner membrane** of the mitochondria. In bacteria the corresponding reactions occur with electron carriers present in the plasma membrane. Both FAD and NAD^+ are regenerated in this way and are able to again accept hydrogen from the beta oxidation reactions. This transfer of hydrogen atoms from substrates to hydrogen carriers is typical of biological oxidation processes.

6. The Electron Transport Chain, Oxidative Phosphorylation

Reoxidation of the reduced carriers NADH and $FADH_2$ actually involves a sequence of electron carriers, the **electron transport chain**, whose function is indicated below the circle near the center of Fig. 10-1. The oxidation of reduced NADH by O_2 (Eq. 10-7) is a highly exergonic process and is accompanied by the

$$\text{NADH} + \text{H}^+ + 1/2\ \text{O}_2 \rightarrow \text{NAD}^+ + \text{H}_2\text{O}$$
$$\Delta G' \text{ (pH 7)} = -219 \text{ kJ/mol} \qquad (10\text{-}7)$$

generation of about three molecules of ATP (from ADP and inorganic phosphate). This process, termed **oxidative phosphorylation**, is the principal source of usable energy (in the form of ATP) provided by breakdown of both carbohydrates and fats in the human body.

The mechanism of oxidation of NADH in the electron transport chain appears to occur by transfer of a hydrogen atom together with two electrons (a hydride ion H^-). Oxidation of $FADH_2$ to FAD might occur by transfer of two hydrogen atoms or by transfer of $H^- + H^+$. However, it is useful to talk about all of these compounds as **electron carriers** with the understanding that movement of one or both of the electrons may be accompanied by transfer of H^+. The electron transport complex is pictured in a very simplified form in Fig. 10-5.

The electrons donated from NADH or other reductants, upon entering this complex, travel from one carrier to the next, with each carrier being a somewhat more powerful oxidant than the previous one. The

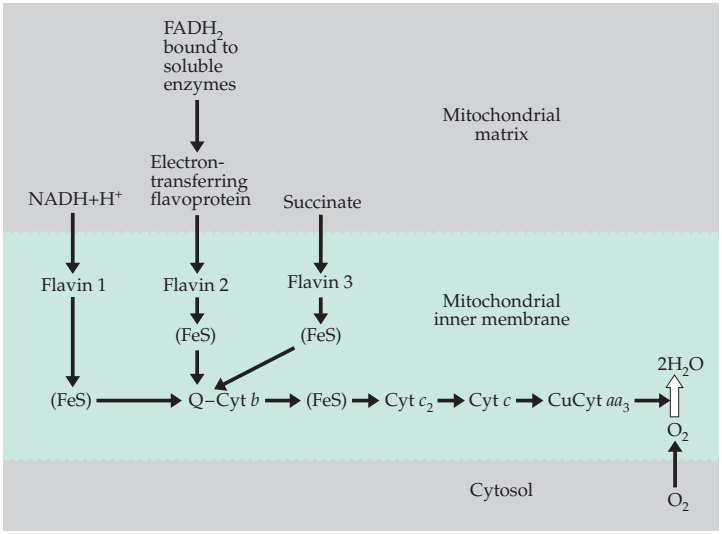


Figure 10-5 An abbreviated version of the electron transport chain of mitochondria. Four electrons reduce O_2 to 2 H_2O. For details see Figs. 18-5 and 18-6.

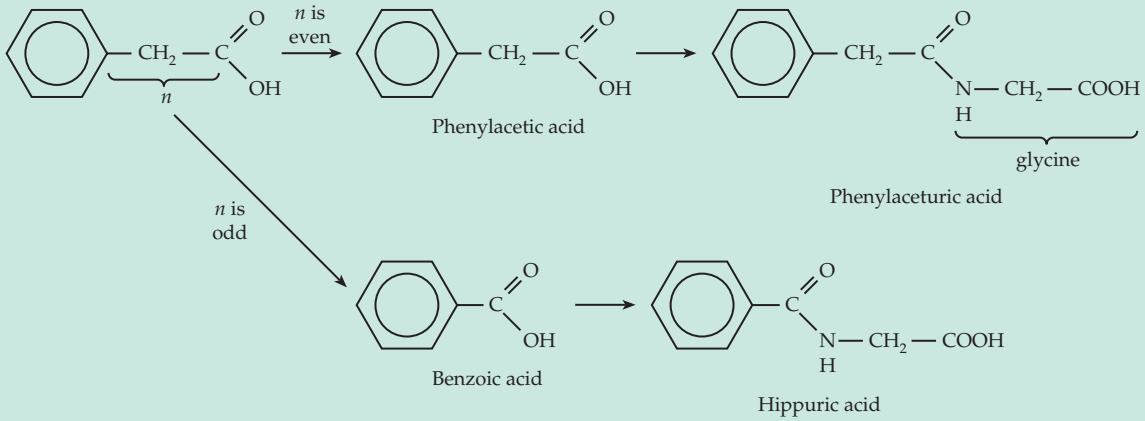
final carrier, known as cytochrome aa_3 or **cytochrome oxidase**, reacts with molecular oxygen. Each molecule of O_2, together with 4 H^+, is converted to two molecules of water. This stoichiometry requires that two molecules of NADH pass four electrons down the chain for each O_2 reduced.

The chemical structures of the components of the mitochondrial electron transport chain are varied but fall into several distinct classes. Most are proteins but these proteins always contain special coenzymes or **prosthetic groups** able to engage in oxidation–reduction reactions. At the substrate end of the chain NADH passes a hydrogen atom together with its bonding electron pair to a riboflavin-containing coenzyme **riboflavin 5'-phosphate** (FMN), which is tightly bound into a protein (designated Flavin 1 in Fig. 10-5). Similar **flavoproteins**, Flavin 2, and Flavin 3, act as oxidants for $FADH_2$ arising during beta

BOX 10-A AN EARLY LABELING EXPERIMENT

In 1904, long before the advent of radioactive tracers, Knoop synthesized fatty acids labeled by chemical attachment of a benzene ring at the end opposite the carboxyl group. He prepared these compounds with both odd and even numbers of carbon atoms in straight chains and fed them to dogs. From the dogs' urine he isolated **hippuric acid** and **phenylaceturic acid**, which are the amides of glycine with benzoic acid and phenylacetic acid, respectively. Knoop showed that the phenylacetic acid was produced from those fatty acids with an even number of carbon atoms, while the benzoic acid was formed from those with an odd number. From these experimental results, Knoop deduced that *fatty acid degradation occurs two carbon atoms at a time* and proposed his famous **β oxidation theory**.

Later experiments using isotopic labeling with ^{13}C verified Knoop's proposals,[a] but study of isolated enzymes was not possible until after the discovery of coenzyme A in 1950. Then, studies of fatty acid oxidation by extracts from isolated mitochondria established the details of the pathway.[b]



[a] Weinhouse, S. (1995) *FASEB J.* **9**, 820–821
[b] Quastel, J. H. (1984) *Trends Biochem. Sci.* **9**, 117–118

oxidation and oxidation of succinate, whose significance to metabolism is discussed in the next section.

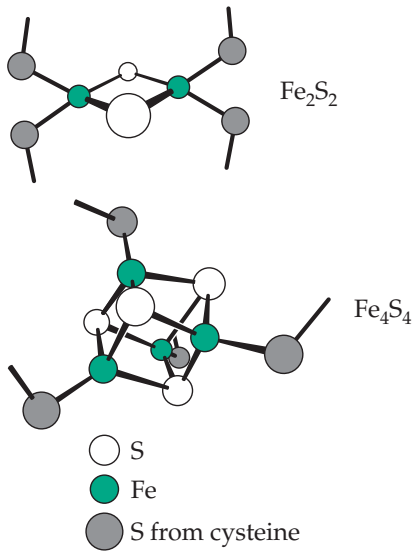
A significant difference between NADH and $FADH_2$ is that the former diffuses freely between the **dehydrogenases** that transfer hydrogen to it and the flavoprotein NADH dehydrogenase (Flavin 1) that reoxidizes it. However, FAD, whether in its oxidized state or as $FADH_2$, stays tightly bound to proteins at all times. Only hydrogen atoms or electrons are transferred into or out of these proteins. During beta oxidation the $FADH_2$ generated remains tied to the fatty acyl-CoA dehydrogenase protein. However, the two hydrogen atoms of this $FADH_2$ are transferred to another molecule of FAD, which is bound to an **electron-transferring flavoprotein.** This protein carries electrons one at a time, as FADH, by diffusion to the inner surface of the inner mitochondrial membrane. There it transfers the hydrogen atoms that it carries to the flavoprotein designated Flavin 2 in Fig. 10-5. The electron-transferring flavoprotein can also be viewed as a carrier of single electrons, each accompanied by H^+.

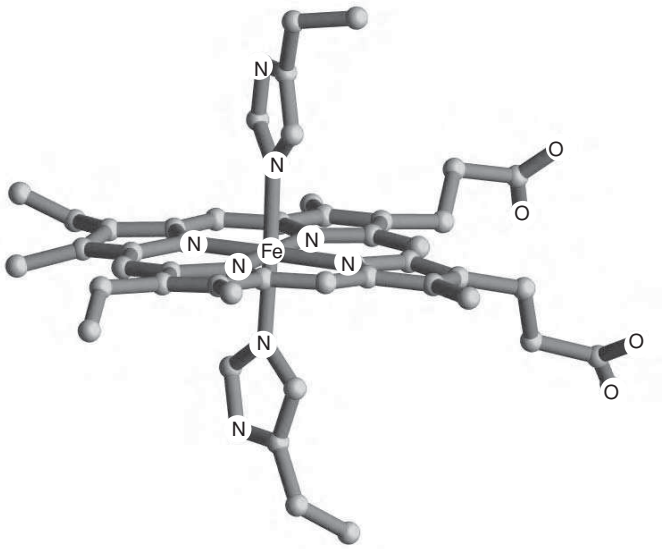
A second group of electron carriers in mitochondrial membranes are the **iron–sulfur [Fe–S] clusters** which are also bound to proteins. Iron–sulfur proteins release Fe^{3+} or Fe^{2+} plus H_2S when acidified. The "inorganic clusters" bound into the proteins have characteristic compositions such as Fe_2S_2 and Fe_4S_4. The sulfur atoms of the clusters can be regarded as sulfide ions bound to the iron ions. The iron atoms are also attached to other sulfur atoms from cysteine side chains from the proteins. The Fe–S proteins are often tightly associated with other components of the electron transport chain. For example, the flavoproteins Flavin 1, Flavin 2, and Flavin 3 shown in Fig. 10-5 all contain Fe–S clusters as does the Q-cytochrome *b* complex. All of these Fe–S clusters seem to be one-electron carriers.

A third hydrogen carrier of mitochondrial membranes, and the only one that is not unequivocally associated with a specific protein, is the isoprenoid quinone **ubiquinone** or **coenzyme Q** (Q in Fig. 10-5). Ubiquinone apparently serves as a common carrier, collecting electrons from three or more separate input ends of the chain and directing them along a single pathway to O_2.

The final group of mitochondrial redox components are one-electron carriers, small proteins (**cytochromes**) that contain iron in the form of the porphyrin complex known as **heme**. These carriers, which are discussed in Chapter 16, exist as several chemically distinct types: *a*, *b*, and *c*. Two or more components of each type are present in mitochondria. The complex cytochrome aa_3 deserves special comment. Although cytochromes are single-electron carriers, the cytochrome aa_3 complex must deliver four electrons to a single O_2 molecule. This may explain why the monomeric complex contains two hemes and two **copper** atoms which are also able to undergo redox reactions.[1,2]

Although the components of the electron transport chain have been studied intensively, there is still some mystery associated with the process by which ATP synthesis is coupled to electron transport (oxidative phosphorylation). A theory originally proposed by Mitchell[3] and now generally accepted[4,5] is that passage of electrons through the chain "pumps" protons from the inside to the outside of the tight inner mitochondrial membrane. As a result, protons accumulate along the outside of this membrane, as do negative counterions along the inside. The membrane becomes charged like a miniature electrical condenser. The synthesis of ATP takes place in the small knoblike **ATP synthase** which is partially embedded in the same membranes. The Gibbs energy for the formation of ATP from ADP and inorganic phosphate is apparently supplied by the flow of protons back to the inside of the membrane through the ATP synthase. Possible





Iron in a heme in a *b* type cytochrome

ways in which this may occur as well as many other aspects of electron transport are dealt with in Chapter 18.

7. The Citric Acid Cycle

The 2-carbon acetyl units removed from fatty acid chains or generated from sugars by glycolysis and the action of pyruvate dehydrogenase must be completely oxidized to carbon dioxide to provide cells with the maximum amount of energy. The oxidation of an acetyl group is a difficult chemical process, and probably for this reason nature has devised an elegant catalytic cycle, the **citric acid cycle**, which is indicated at the lower right in Fig. 10-1 and is shown in detail in Fig. 10-6. The cycle begins when the 4-carbon **oxaloacetate** (also spelled oxalacetate) is condensed with an acetyl group of acetyl-CoA to form the 6-carbon **citrate**. Then, in the remaining reactions of the cycle, two carbon atoms are removed as CO_2 and oxaloacetate is regenerated. Several oxidation steps are involved, each of which feeds additional **reducing equivalents** (i.e., hydrogen atoms removed from substrates) into the pool of hydrogen carriers and allows for more synthesis of ATP via the electron transport chain. The importance of this pathway to an organism can be understood from the fact that when glucose is oxidized completely to CO_2 and water via the citric acid cycle, about 38 molecules of ATP are formed, 19 times as much as by fermentation.

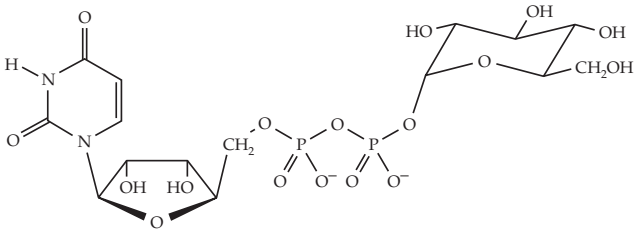
Although oxaloacetate is regenerated and therefore not consumed during the operation of the citric acid cycle, it also enters other metabolic pathways. To replace losses, oxaloacetate can be synthesized from pyruvate and CO_2 in a reaction that uses ATP as an energy source. This is indicated by the heavy gray line leading downward to the right from pyruvate in Fig. 10-1 and at the top center of Fig. 10-6. This reaction depends upon yet another coenzyme, a bound form of the vitamin **biotin**. Pyruvate is formed from breakdown of carbohydrates such as glucose, and the need for oxaloacetate in the citric acid cycle makes the oxidation of fats in the human body dependent on the concurrent metabolism of carbohydrates.

C. Biosynthesis

At the same time that cells break down foodstuffs to obtain energy, they are continuously creating new materials. The green lines in Fig. 10-1 indicate some pathways by which such biosynthesis takes place. Examining the left side of the figure, we see that either pyruvate or 3-phosphoglycerate can be converted back to glucose 6-phosphate and that the latter can be used to synthesize glycogen or other sugars or polysaccharides.

1. Reversing Catabolic Pathways

Breakdown of carbohydrates is thermodynamically spontaneous. Therefore, *cells cannot simply use catabolic pathways operating in reverse without finding ways of coupling the cleavage of ATP to synthesis.* In the formation of glucose from pyruvate in the liver, a process known as **gluconeogenesis**, there are three distinct points at which the enzymes used differ from those used in catabolism: (1) Pyruvate is converted to phosphoenolpyruvate by a mechanism utilizing more than one molecule of ATP, a pathway that is discussed in detail in Chapter 17; (2) as is shown in Fig. 10-2, fructose 1,6-bisphosphate is hydrolyzed to fructose 6-phosphate and inorganic phosphate by a **phosphatase** rather than through reversal of step *d*, which would form ATP; and (3) glucose-6-phosphate is hydrolyzed by a phosphatase rather than following the reverse of step *a* in Fig. 10-2. Furthermore, glycogen is synthesized from glucose 6-phosphate, not by reversal of the phosphorylase reaction (Fig. 10-2, step *g*), but via a new intermediate, **uridine diphosphate glucose** (UDPG), whose formation involves cleavage of UTP. The latter is generated by phospho transfer from ATP. Inorganic **pyrophosphate** (PP_i) is a product of UDPG formation and is removed by hydrolytic cleavage to two molecules of inorganic phosphate by the enzyme **pyrophosphatase**. This reaction helps to make the biosynthesis thermodynamically spontaneous by removing one of the reaction products.



Uridine diphosphate glucose (UDPG)

A similar situation exists in the case of fatty acid synthesis, which proceeds from acetyl-CoA and reverses fatty acid breakdown. However, both carbon dioxide and ATP, a source of energy, are needed in the synthetic pathway. Furthermore, while oxidation of fatty acids requires NAD^+ as one of the oxidants, and generates NADH, the biosynthetic process often requires the related NADPH. These patterns seen in biosynthesis of sugars and fatty acids are typical. *Synthetic reactions resemble the catabolic sequences in reverse, but distinct differences are evident.* These can usually be related to the requirement for energy and often also to control mechanisms.

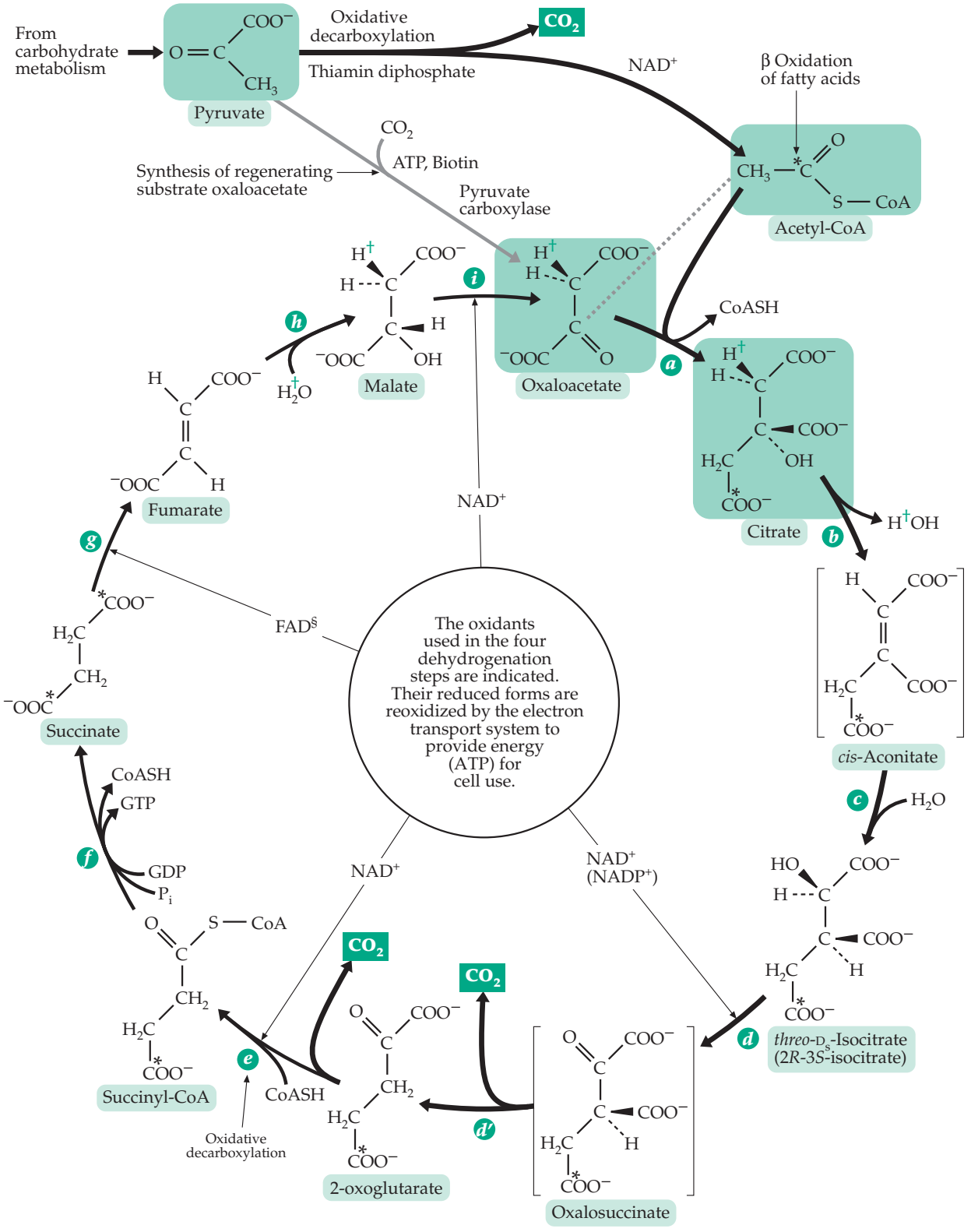


Figure 10-6 Reactions of the citric acid cycle (Krebs' tricarboxylic acid cycle). Asterisks designate positions of isotopic label from entrance of carboxyl-labeled acetate into the cycle. Note that it is *not* the two carbon atoms from acetyl-CoA that are immediately removed as CO_2 but two atoms from oxaloacetate. Only after several turns of the cycle are the carbon atoms of the acetyl-CoA completely converted to CO_2. Nevertheless, the cycle can properly be regarded as a mechanism of oxidation of acetyl groups to CO_2. Green daggers (†) designate the position of ^2H introduced into malate as $^2H^+$ from the medium. FAD§ designates covalently bound 8-histidyl-FAD (see Chapter 15).

BOX 10-B DISCOVERY OF THE CITRIC ACID CYCLE (KREBS' TRICARBOXYLIC ACID CYCLE)

One of the first persons to study the oxidation of organic compounds by animal tissues was T. Thunberg, who between 1911 and 1920 discovered about 40 organic compounds that could be oxidized by animal tissues. Salts of succinate, fumarate, malate, and citrate were oxidized the fastest. Well aware of Knoop's β oxidation theory, Thunberg proposed a cyclic mechanism for oxidation of acetate. Two molecules of this two-carbon compound were supposed to condense (with reduction) to succinate, which was then oxidized as in the citric acid cycle to oxaloacetate. The latter was decarboxylated to pyruvate, which was oxidatively decarboxylated to acetate to complete the cycle. *One of the reactions essential for this cycle could not be verified experimentally.* It is left to the reader to recognize which one.

In 1935, A. Szent-Györgyi discovered that all of the carboxylic acids that we now recognize as members of the citric acid cycle stimulated respiration of animal tissues that were oxidizing other substrates such as glucose. Drawing on this knowledge, Krebs and Johnson in 1937 proposed the citric acid cycle.[a,b,c] Krebs provided further confirmation in 1940 by the observation that malonate, a close structural analog and competitive inhibitor of succinate, in concentrations as low as 0.01 M blocked the respiration of tissues by stopping the oxidation of succinate to fumarate.[c]

$^{-}OOC—CH_2—COO^{-}$
Malonate

$^{-}OOC—CH_2—CH_2—COO^{-}$
Succinate

In muscle, 90% of all respiration was inhibited and succinate was shown to accumulate, powerful proof of the importance of the citric acid cycle in the respiration of animal tissues.

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[a] Krebs, H. A., and Johnson, W. A. (1937) *Enzymologia* **4**, 148–156
[b] Krebs, H. A. (1981) *Reminiscences and Reflections* Clarendon Press, Oxford
[c] Fruton, J. S. (1999) *Proteins, Enzymes, Genes: the Interplay of Chemistry and Biology* Yale Univ. Press, New Haven, CT
[d] Quastel, J. H. (1978) *Trends Biochem. Sci.* **3**, 68–69

2. Photosynthesis

The principal means of formation of glucose in nature is through photosynthesis in green plants (Fig. 10-1, lower left). Light energy is captured by chlorophyll and is used to transfer electrons from chlorophyll to other electron carriers, the most important of which is $NADP^+$. It is reduced to NADPH which is used to reduce carbon dioxide to sugar phosphates in a complex series of reactions known as the **reductive pentose phosphate pathway**, which is described in Chapter 17. ATP is also required for photosynthetic reduction of CO_2. It is generated by allowing some of the electrons to flow back through an electron transport chain in the membranes of the chloroplasts. This chain closely resembles that from Q to cytochrome *c* in mitochondria (Fig. 10-5), and the generation of ATP in this **photosynthetic phosphorylation** occurs in a manner analogous to that in the electron transport chain of mitochondria. In green plants the electrons removed from chlorophyll in one light-requiring reaction are replaced by electrons formed during the cleavage of water in a second light-dependent reaction, a reaction that also releases oxygen, O_2, and generates hydrogen ions (H^+). The first stable product from reduction of CO_2 in photosynthesis is 3-phosphoglycerate. It can be converted to sugars by pathways analogous to those employed by animals in gluconeogenesis. One interesting difference is the use in chloroplasts of NADPH + H^+ in reduction of 3-phosphoglycerate (reverse of step *a* in Fig. 10-3).

A small number of other biosynthetic pathways, which are used by both photosynthetic and nonphotosynthetic organisms, are indicated in Fig. 10-1. For example, pyruvate is converted readily to the amino acid **L-alanine** and oxaloacetate to **L-aspartic acid**; the latter, in turn, may be utilized in the biosynthesis of pyrimidines. Other amino acids, purines, and additional compounds needed for construction of cells are formed in pathways, most of which branch from some compound shown in Fig. 10-1 or from a point on one of the pathways shown in the figure. In virtually every instance biosynthesis is dependent upon a supply of energy furnished by the cleavage to ATP. In many cases it also requires one of the hydrogen carriers in a reduced form. While Fig. 10-1 outlines in briefest form a minute fraction of the metabolic pathways known, the ones shown are of central importance.

D. Synthesis and Turnover of Macromolecules

Proteins make up the bulk of the catalytic machinery of cells and together with other macromolecules most of the structure. Therefore, the synthesis and degradation of proteins and the control of those processes are of great importance to cells. Although the emphasis

in this section is on proteins, similar considerations apply to nucleic acids, polysaccharides, and other macromolecules.

With the exception of some antibiotics and other short-chain molecules, all polypeptides are formed on ribosomes, which assemble proteins according to the sequences of nucleotides in the messenger RNA (mRNA) molecules. The basic chemistry is simple. The carboxyl groups of the amino acids are converted to reactive acyl adenylates by reaction with ATP, just as in Eq. 10-1. Each "activated" amino acid is carried on a molecule of transfer RNA (tRNA) and is placed in the reactive site of a ribosome when the appropriate codon of the mRNA has moved into the site. The growing peptide chain is then transferred by a displacement reaction onto the amino group of the activated amino acid that is being added to the peptide chain. In this manner, new amino acids are added one at a time to the carboxyl end of the chain, which always remains attached to a tRNA molecule. The process continues until a stop signal in the mRNA ends the process and the completed protein chain is released from the ribosome. Details are given in Chapter 29.

1. Folding and Maturation of Proteins

A newly synthesized peptide chain probably folds quickly. However, the cytosol provides an environment rich in other proteins and other macromolecules that can interact with the new peptide and may catalyze or inhibit folding. Among the most abundant proteins of bacteria or eukaryotes are proteins known as **chaperonins**. They apparently help polypeptide chains to fold correctly, partly by "chaperoning" them through the cytoplasm and across cell membranes, protecting them from becoming entangled with other proteins and macromolecules while they fold.[6–9] There are several classes of chaperonins. Most are oligomers made up of 10- to 90-kDa subunits. They have a variety of names, which may be somewhat confusing. The first chaperonins were identified only as **heat shock proteins** (Hsp). They are produced in large amounts by bacteria or other cells when the temperature is raised quickly, and are designated Hsp70, Hsp90, etc., where the number is the subunit mass in kDa. Other chaperonins were recognized as products of genes needed for replication of the DNA of bacteriophage λ in *E. coli.* Consequently, one major chaperonin of the Hsp70 class is designated **DnaK**. The Hsp70 protein of mitochondria was named "binding protein" or BiP.[10] Other workers have abbreviated chaperonins as Cpn70, Cpn60, etc.

The abbreviations Hsp70, Cpn70, DnaK, and BiP all refer to a group of similar 70-kDa proteins that are apparently found in all organisms. Their role seems to be to stabilize unfolded proteins prior to final folding in the cytosol or after translocation into the endoplasmic reticulum (ER) or into mitochondria or other organelles. Each of these proteins consists of two functional domains. A 52-kDa domain at the C terminus binds 7- or 8-residue segments of unfolded peptide chains in an elongated conformation.[9] At some point in its reaction cycle the N-terminal 40-kDa domain, which binds ATP tightly, causes the ATP to be hydrolyzed to ADP and inorganic phosphate: $ATP + H_2O \rightarrow ADP + P_i$. Binding and release of a polypeptide by the *E. coli* DnaK protein is coupled tightly to this exergonic **ATPase** reaction.[10a,10b] The reaction is dependent upon potassium ions[11] and is regulated by two **co-chaperones, DnaJ** (Hsp40)[11a-11c] and **GrpE**.[10a] Both ATP and extended polypeptides bind weakly to DnaK, and the ATP in the DnaK•polypeptide•ATP complex is hydrolyzed slowly to ADP and inorganic phosphate.[10a,10b,12] Co-chaperone DnaJ, which shares a largely α-helical structural motif with J-domains in various other proteins, binds to the complex.[11a, 11b, 11c] It probably induces a conformational change that leads to rapid hydrolysis of ATP. In the resulting complex both ADP and the polypeptide are bound tightly to the DnaK protein and dissociate from it very slowly. The *E. coli* co-chaperone GrpE acts as a **nucleotide exchange factor** that catalyzes rapid loss of ADP from the complex. If ATP binds to this DnaK•polypeptide complex the polypeptide is released.[10a] This cycle, which can be repeated, accomplishes the function of DnaK in protecting extended polypeptides and releasing them under appropriate conditions. There is also evidence that DnaK participates in refolding of misfolded proteins.[11d] It cooperates with a ribosome-associated prolylisomerase in bacteria.[11e] The three-dimensional structure of the ATPase domain of Hsp70 is strikingly similar to that of the enzyme **hexokinase** (Chapter 12) and to that of the muscle protein **actin**, Fig. 7-10.[13,14] Archaeal chaperonins lack the Cpn10 ring but have lid-like extensions at the cylinder ends.[15a]

The Cpn60 class of chaperonins are amazing cage-like structures. Each oligomer is composed of two rings, each made up of seven 60-kDa subunits stacked back-to-back. Cpn60 structures from archaeobacteria are similar but may have 8- or 9-subunit rings.[15,15a] The best known member of this group is the *E. coli* protein known as **GroEL** whose three-dimensional structure is depicted in Box 7-A.[16,17] This 14-subunit oligomer of GroEL is a cylinder which is capped by a smaller ring composed of seven 10-kDa subunits of **GroES**, a Cpn10 protein.[17,18] Like Hsp70, GroEL has ATPase activity and an ATP binding site in its large equatorial domain.[16] Archaeal chaperonins lack the Cpn10 ring but have lid-like extensions at the cylinder ends.[15a]

Within the GroEL–GroES cage polypeptide chains can fold without becoming entangled with other proteins or being cleaved by protein-hydrolyzing enzymes

of the cytoplasm. An unfolded or partially folded protein may diffuse into the open end of the complex and bind temporarily via noncovalent interactions. The hydrophobic inner surface of the complex may favor the formation of helices within the folding protein. Binding of the polypeptide substrate and capping with GroES causes a major cooperative conformational change with doubling of the internal volume (Box 7-A). The character of the inner wall also changes as a result of exposure of hydrophilic groups.[18a,18b] Perhaps the expansion also stretches segments of the unfolded polypeptide. Subsequent hydrolysis of ATP may be coupled to release of the bound polypeptide, which may then leave the complex or bind again for another chance to fold correctly.[17,19] The two ends of the GroEL•GroES complex may function alternately, with each end in turn receiving the GroES cap.[17]

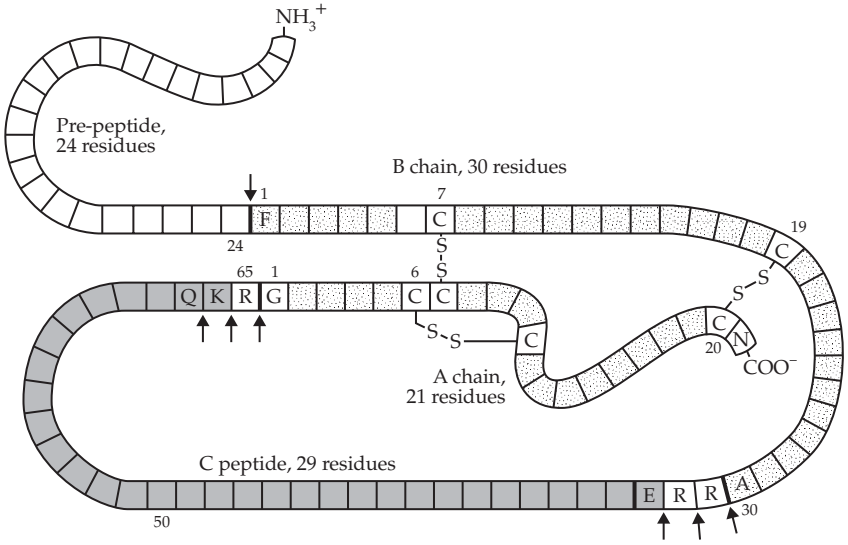


Figure 10-7 Schematic diagram of the structure of human preproinsulin. The 24-residue prepeptide, the 29-residue C-peptide and basic residues 31, 32, 64, and 65 are cut from the peptide upon conversion to insulin as indicated by the small arrows. Some amino acid residues are identified using the one-letter codes. See Fig. 7-17 for details of insulin structure.

Hsp90 is one of the most abundant cytosolic proteins in eukaryotic cells, but it seems to chaperone only a few proteins, among which are steroid-hormone receptors.[20,20a] Another group of over 30 different chaperone proteins participate in assembly of external bacterial cell-surface structures such as pili (Fig. 7-9). The chaperone PapD consists of two domains, both having an immunoglobulin-like fold.[20b] PapD binds to the pilin subunits, escorting them to the site of pilus assembly. Folding of integral membrane proteins may be facilitated by a chaperone function of membrane lipids.[20c]

For most proteins the initial synthesis is followed by a sequence of **processing** or **maturation** reactions. These reactions sometimes involve alteration of amino acid side chains and very often include hydrolytic cleavages by which pieces of the peptide chain are cut off. The initially synthesized polypeptides are often not functional and gain biological activity only after one or more pieces have been removed. For example, digestive enzymes are usually secreted as **zymogens** or **proenzymes** which are activated by hydrolytic cleavages only after secretion into the digestive tract. Many proenzymes are components of the extremely complex cascades of activating reactions involved in **blood clotting** (Chapter 12) and in the defensive **complement system** (Chapter 31).

Most peptide hormones are cut out from larger proteins. For example, human insulin is synthesized as a 110-residue **preproinsulin** which is converted in stages to the active two-chain, 51-residue hormone (Eq. 10-8 and Fig. 10-7).[20d,20e]

$$\underset{(110\ \text{residues})}{\text{preproinsulin}} \rightarrow \underset{\substack{(86\ \text{residues,}\\ \text{one chain})}}{\text{proinsulin}} \rightarrow \underset{\substack{(51\ \text{residues, two}\\ \text{disulfide-linked}\\ \text{chains})}}{\text{insulin}} \qquad (10\text{-}8)$$

The 24-residue prepeptide is cleaved from preproinsulin within a few minutes of synthesis. Then, over a period of about an hour additional cleavages occur to give the final product.[21] This crystallizes as the zinc hexamer (Fig. 7-18) within the "dense cores" of storage vesicles from which it is released into the bloodstream as needed.[22] Other examples are described in Section 3.

2. Transport of Proteins to Their Destinations within a Cell

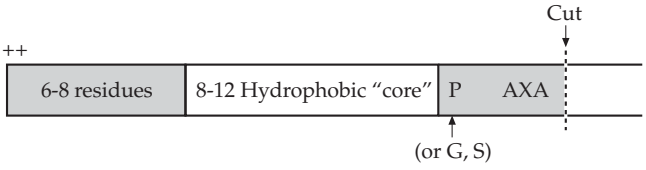
The synthesis of peproinsulin on ribosomes in the cytosol and the release of mature insulin from secretion vesicles involve not only the chain cleavages but also transport of the polypeptide across membranes of the ER, Golgi compartments, and secretion vesicles.

Signal sequences and translocation. In 1971, Blobel and Sabatini postulated that for some proteins an N-terminal segment of newly synthesized polypeptide chains contains a signal sequence or leader peptide of 15–30 amino acids which carries information concerning the location of the mature protein in the cell.[23–28b] The rather nonpolar signal sequences of proteins destined for secretion from cells would interact

with and pass through the membrane of the ER. On the other side of the membrane, a **signal peptidase** would cut off the signal peptide. In many cases, glycosyltransferases would add sugar residues near the N terminus to create a hydrophilic end which would help to pull the rest of the peptide chain through the membrane. A similar situation would hold for secretion of proteins by bacteria.

The signal hypothesis has been proven correct but with considerable added complexity. It now appears that when the N terminus of a new polypeptide chain carrying the proper signal sequence emerges from a ribosome it is intercepted by a **signal recognition particle (SRP)**, a 250-kDa ribonucleoprotein consisting of six polypeptide chains and a small 300-residue 7S RNA chain.[29–33] Binding to the ribosome, the SRP temporarily blocks further elongation of the peptide chain until it bumps against and binds to a 72-kDa membrane protein called the **SRP receptor** or **docking protein**. Then translation begins again and the protein moves into the ER, where it is cleaved by the signal peptidase and is modified further. Although the bacterial SRP is a single protein called **Ffh** (for 54 homolog),[33a] the basic machinery for signal recognition and secretion is remarkably conserved from *E. coli* to humans.[33b] Bacterial proteins that are destined for secretion or for a function in the periplasmic space or in external membranes also contain signal sequences which are cut off by a signal peptidase embedded in the plasma membrane.[33c]

Signal sequences vary in structure but usually have a net positive charge within the first 5–8 residues at the N terminus. This region is followed by a "hydrophobic core" made up of 8–10 residues with a strong tendency toward α helix formation. This sequence is often followed by one or a few proline, glycine, or serine residues and then a sequence AXA that immediately precedes the cleavage site. Here, A is usually alanine in prokaryotes but may also be glycine, serine, or threonine in eukaryotes. Residue X is any amino acid.[25,27,28a,28b]



It had often been assumed that a hydrophobic signal sequence, perhaps folded into a hairpin loop, spontaneously inserts itself into an ER membrane to initiate translocation. However, study of the genetics of protein transport suggests otherwise. Over 50 different genetic loci affect the translocation of proteins in yeast.[31,32,33d] Products of these **secretory genes**, which are named *SEC* 61, *SEC* 62, *SEC* 63, etc., are proteins with corresponding names, e.g., Sec 61 protein (or Sec 61p). Study of these proteins suggested that the Sec 61, Sec 62, and Sec 63 proteins are all directly involved in transport into the ER.[31,34,35] Additional proteins are needed for movement of vesicles out from the Golgi and for delivery of secretory vesicles to the plasma membrane.[33d] The protein Sec 61p has been identified as homologous to the α subunit of a similar trimeric αβγ Sec 61p complex of mammalian tissues.[32] It also appears to correspond to Sec Y, a protein required for secretion of proteins through the plasma membrane of *E. coli* into the periplasmic space. Proteins corresponding to the β and γ subunits of mammalian related proteins have been identified in yeast and *E. coli*[36] and in *Arabidopsis*, where it is necessary to bring proteins into the thylakoid membrane of the chloroplasts.[37]

In *E. coli* the products of genes *SecY, SecE,* and *SecG* are integral membrane proteins that, together with additional proteins, form a proteinaceous pore through which proteins pass.[38–40a] Additional proteins required for translocation of some bacterial proteins are the chaperonin **SecB**, a tetramer of 17.3-kDa subunits,[41] and **SecA**, a soluble ATPase that may participate in docking and serve as an engine for transport throught the pore.[38,40a,42] In eukaryotic cells a membrane-bound Ca^{2+}-dependent chaperonin called **calnexin** assists in bringing the protein into the ER in a properly folded state.[43,44]

Unlike transport across the membranes of the ER, transport across plasma membranes of bacteria often requires both hydrolysis of ATP and energy provided by the membrane electrical potential.[33,38,44–48] Secretion into the periplasmic space has been well characterized but less is known about transport of proteins into the external membranes of *E. coli*.[48] A 16 kDa periplasmic chaperone may be required.[48a]

Many bacteria have a second complete secretion system.[48b] This multi-gene type II system is present but usually inactive in *E. coli*. A third (type III) system is present in many pathogenic bacteria, and has evolved for delivery of specialized structural and regulatory proteins into host cells.[48c,48d]

Ticketing destinations. We have seen that proteins that contain suitable signal sequences are exported from the cytoplasm while other proteins remain. Some proteins are secreted while others take up residence as integral or peripheral membrane proteins or as soluble proteins within an organelle. All of the available evidence indicates that it is the sequence of a protein that determines its destination. Proteins targeted to pass through the inner mitochondrial or chloroplast membrane have 20- to 70-residue presequences that are rich in arginine and lysine and which are removed when the protein reaches its

destination.[49–53] Proteins meant to go to the intermembrane space or the outer membrane of mitochondria have a sequence containing basic amino acids followed by a long stretch of uncharged residues.[54] Peroxisomal preproteins may have signal sequences such as SKL at the C terminus[54–56] and proteins destined to become attached to phosphatidylinositol glycan anchors (Fig. 8-13)[57] as well as some bacterial surface proteins[57a] have signal peptides at both N and C termini. Some lysosomal membrane glycoproteins have an LE pair in the N-terminal cytoplasmic tail[58] and proteins with suitable dileucine and related pairs are often taken up in lysosomes.[59] Soluble proteins that are resident in the cisternae of the ER often have the C-terminal sequence KDEL or HDEL.[29,60–64] It may serve both as a **retention signal** and as a **retrieval signal** for return of the protein if it passes on into the Golgi vesicles.[65,66]

Transport of proteins into mitochondria is dependent upon both cytosolic chaperonins and mitochondrial chaperonins of the Hsp70 and Hsp60 classes.[67–70] Entrance into mitochondria[71–73] resembles passage through membranes of the ER. However, entry to the nucleus through the nuclear pore complex requires other localization signals[73a] as well as specialized proteins.[74,74a] The sorting of proteins into six different compartments within a chloroplast requires a whole series of recognition signals as well as chaperonins and channel proteins.[75–79] The various signal sequences that determine a protein's interactions with chaperonins, docking proteins, and proteinaceous pore complexes may be complex and overlapping but evolution has selected sequences that allow cells to live and function. Computer programs for detecting sorting signals are being developed.[79a] Not only proteins but also other macromolecules are automatically sorted to their correct destination.

Vesicular transport and the Golgi system. Movement of secreted proteins through the cytosol from the ER to the external surface occurs through the formation and opening up of small vesicles about 70 nm in diameter. It occurs in steps that involve passage from the ER to the various Golgi membranes. Figure 10-8 provides a sketch of the system of ER, Golgi, secretion vesicles, and lysosomes.[63] Newly formed secretory proteins flow from the ER into an intermediate compartment where vesicles are formed and are carried to the *cis* Golgi network (CGN). They move step-by-step through the Golgi stack (GS) and into the *trans* Golgi network (TGN). New vesicles are formed to carry proteins between each pair of membranous compartments. In each compartment new glycosylation reactions or other modifications may occur. Finally, vesicles carry the mature proteins to the plasma membrane, lysosomes, or vacuoles.[79b] A related process is *uptake* of proteins by endocytosis to form, consecutively, early and late endosomes which fuse with lysosomes (Chapter 8, Section C,6, Fig. 10-8).

At every step in these processes vesicles are formed and are carried to the next destination where the vesicles fuse with the new membrane and discharge their contents.[80–85a] This remarkable process is complex and highly specific. Rothman proposed that the vesicles to be transported are "docked" on appropriate receptor molecules (called SNARES) on the destination membrane. This is accomplished with the aid of specific soluble marker proteins (called SNAPS) with surfaces complementary to those of the receptors.[80] Proteins are sorted in this way according to their destination signals. The Golgi system is considered further in Chapter 20.

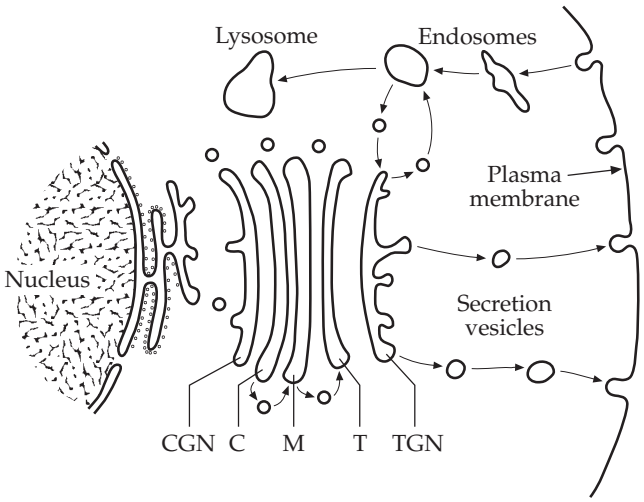
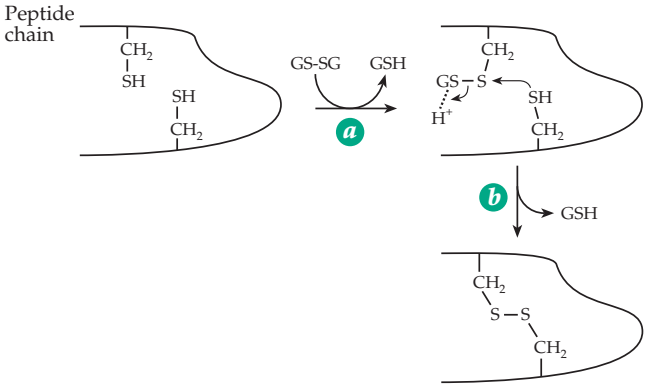


Figure 10-8 Current version of protein synthesis and processing via ER, Golgi, and secretory vesicles. CGN, *cis*-Golgi network: C, T, M are the *cis*, medial, and *trans* compartments of the Golgi stack; TGN, *trans* Golgi network. Arrows indicate some of the movements of transport vesicles.

3. Posttranslational Alterations

Chemical alterations to a protein begins as soon as the peptide chain is formed. Proteins translocated into the periplasmic space of bacteria or into the cisternae of the ER of eukaryotic cells meet several important enzymes. Peptidylprolyl isomerase (discussed in Box 9-F) assists the folding of the peptide chain and protein disulfide isomerases help to form disulfide linkages. The cytosol provides a reducing environment in which many proteins that have cysteine side chains carry free –SH groups. In contrast, the periplasmic space and the ER have more oxidizing environments in which disulfide bridges may form. As shown in Eq. 10-9, the oxidizing agent is often assumed to be the disulfide form of the tripeptide **glutathione** (see Box 11-B),



(10-9)

which is commonly abbreviated GS–SG. It can react with proteins by disulfide exchange reactions to form mixed disulfide groups with release of reduced glutathione, GSH (Eq. 10-9, step *a*). The mixed disulfides can be converted to disulfide bridges by a second exchange reaction (Eq. 10-9, step *b*). However, in the bacterial periplasm the dithiol protein DsbA appears to be the major oxidant (see Box 15-C). It functions together with other proteins, some of which carry electrons from the electron transport chains of the bacteria.[85a,85b] The rates of these reactions are greatly increased by the protein disulfide isomerases.[85a–85e,86–91] These enzymes tend to promote formation of disulfide linkages between the correct pairs of SH groups. If there are three or more –SH groups in a chain some incorrect pairing may, and often does, occur. The protein disulfide isomerases break these bonds and allow new ones to form.[92] The active sites of these isomerases contain pairs of –SH groups which can be oxidized to internal –S–S– bridges by NAD^+-dependent enzymes. These enzymes and their relatives **thioredoxin** and **glutaredoxin** are discussed further in Box 15-C. Glutathione and oxidation–reduction buffering are considered in Box 11-B.

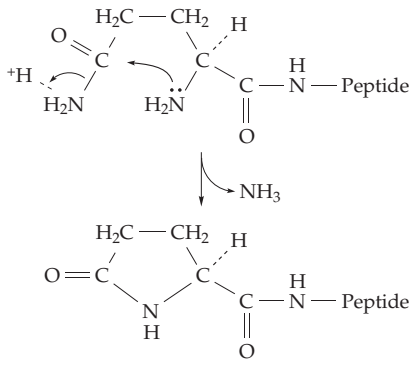
Proteolytic processing. For proteins exported from the cytosol, a signal peptidase (or leader peptidase) is usually waiting in the bacterial plasma membrane or in membranes of the ER. The *E. coli* leader peptidase is an integral membrane protein with its catalytic domain in the periplasm.[45,93] For insulin the 24-residue prepiece serves as the signal sequence which is hydrolyzed off in the ER. However, the other cleavages shown in Fig. 10-7 appear to take place in immature secretory granules.[21,22] For many proteins proteolytic processing begins earlier than this and may occur in more than one location. Eukaryotic processing proteases often resemble the bacterial protease subtilisin[20e] but cut peptide chains preferentially after dibasic amino acid pairs, e.g., Arg-Arg, Arg-Lys.[94–96] There are also other processing enzymes with differing specificities.[97]

Although most polypeptides leave ribosomes as single proteins, some are **polyproteins**, which give rise to two or more functional peptides. Polyproteins occur in all cells but are especially prevalent among virally encoded peptides. For example, the polio virus polyprotein is cut into at least ten pieces by proteases, some present in the host cell normally and some encoded by the virus.[98] Many neurohormones arise from polyproteins that undergo processing as they travel down an axon from the cell body before being secreted into a synapse (Chapter 30). Within our own brains the peptide **prepro-opiomelanocortin** undergoes numerous cleavages to give rise to at least seven different neurohormones (see Fig. 30-2).[96,99–101]

Altered ends. Proteins often contain "blocked" end groups in place of free $-NH_3^+$ or $-COO^-$. For many of the cytosolic proteins, which do not carry leader sequences, the N-terminal methionine that is always used to initiate the ribosomal synthesis of proteins in eukaryotes is removed. If the next residue after the N-terminal methionine is small and uncharged, the methionine is usually hydrolyzed off enzymatically after 30–40 residues have been added to the growing peptide chain.[102–105] An acetyl group is transferred onto the NH_2 termini of about 85% of all cytosolic proteins, whether or not the initiator methionine has been removed.[106] In other cases fatty acyl groups may be transferred to the terminal $-NH_2$ group or to side chain SH groups. For example, a **myristoyl** (tetradecanoyl) group is frequently combined in amide linkage at the N terminus of cellular and virally encoded proteins. A palmitoyl group is joined in thioester linkage to a cysteine side chain near the N terminus of the *E. coli* periplasmic lipoprotein (Fig. 8-28).[107] Together with the N-terminal myristoyl group it forms a membrane anchor for this protein. Polyprenyl groups are transferred onto cysteine –SH groups at or near the C termini of many eukaryotic proteins to form a different membrane anchor.[108–110] Some of the enzymes act on the sequence CAAX, where X = M, S, Q, C but not L or I. After transfer of a farnesyl or geranylgeranyl group onto the cysteine the AAX is removed proteolytically and the new terminal cysteinyl carboxyl group is methylated. Surface proteins of gram-positive bacteria are joined by amide linkage from their C termini to a pentaglycine chain of the peptidoglycan layer (Fig. 8-28)[57a]

A pyroglutamyl N terminus is found in the thyrotropin-releasing hormone (Fig. 2-4) and in many other peptide hormones and proteins. It presumably arises by attack of the α-NH_2 group of an N-terminal glutamine on the side chain amide group with release of NH_3 (Eq. 10-10).[111,112]

Another frequent modification at the C terminus of peptide hormones and of other proteins is **amidation**. In this reaction a C-terminal glycine is oxidatively removed as glyoxylate in an O_2^-, copper- and ascorbate



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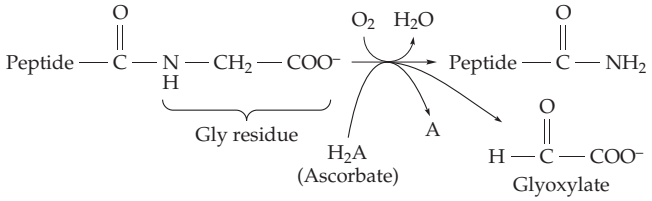


(10-11)

(vitamin C)-dependent process (Eq. 10-11).[113–117] See also Chapter 18, Section F,2.

An example of both of these modifications is formation of thyrotropin-releasing hormone shown in Fig. 2-4 from its immediate precursor:

$$\text{Gln–His–Pro–Gly} \rightarrow \text{pyroglutamyl–His–Pro–NH}_2 \qquad (10\text{-}12)$$

Proteins with long C-terminal hydrophobic signal sequences may become attached to phosphatidylinositol-glycan anchors embedded in the plasma membrane (Fig. 8-13). An example is a human alkaline phosphatase in which the α carboxyl of the terminal aspartate residue forms an amide linkage with the ethanolamine part of the anchor. Attachment may occur by a direct attack of the $–NH_2$ group of the ethanolanine on a peptide linkage in a transacylation reaction that releases a 29-residue peptide from the C terminus.[118,119] (See Chapter 29).

Many other covalent modifications of proteins are dealt with in other sections of the book.[120–122] A few are described in Chapter 2, Eqs. 2-14 to 2-22. Reversible alterations used to regulate enzymes are considered in Chapter 11. Of these, the phosphorylation of –OH groups of serine, threonine, and tyrosine is the most important. A large fraction of all cellular proteins appear to be modified in this way. Protein glycosylation, the transfer of glycosyl groups onto –OH side chain groups of serine and threonine (Chapter 4, and Chapter 20) and nonenzymatic glycation (Eq. 4-8) also affect many proteins, often at turns in the peptide chain. Hydroxylation, glycosylation, and other modifications of collagen are described in Chapter 8. Another common reversible alteration is formation of sulfate esters of tyrosine –OH groups.[99,123] Reactions by which cofactors become attached to proteins[124] are described in Chapters 15–17.

Methylation,[125] hydroxylation, and other irreversible modifications often affect specific residues in a protein. Oxidative alterations occur during aging of proteins (Chapter 18).[126] A few proteins even undergo "splicing" that alters the amino acid sequence (Box 29-E).[127] All of these reactions not only affect the properties of the proteins but also participate in driving the turnover of these macromolecules.

4. Intracellular Degradation of Proteins

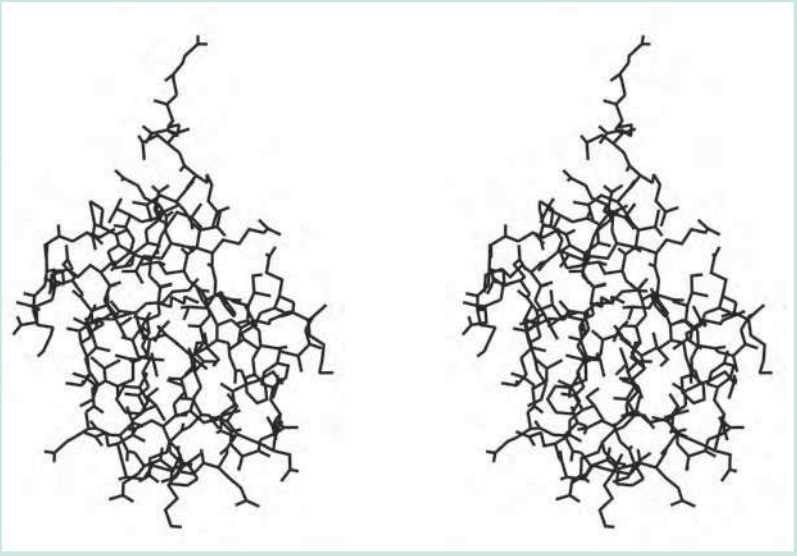
Once a protein has reached its correct location and has acquired its proper function, it usually has a limited lifetime, which may average only a few hours or a few days. The protein is then hydrolytically degraded back to its constituent amino acids.[128] Defective and damaged proteins are usually degraded much more rapidly than are intact proteins.[129,130] Under conditions of starvation, proteins are broken down more rapidly than usual to supply the cell with energy. Rapid degradation of proteins is often induced at certain stages of differentiation. For example, spore-forming bacteria contain a protease that becomes activated upon germination of the spore.[131] Within minutes this enzyme digests stored proteins to provide amino acids for the synthesis of new proteins during growth.

Eukaryotic cells degrade proteins within both the cytosol and lysosomes. Lysosomes apparently take up many proteins but have a preference for N-terminal KFERQ[132] and also for particular types of glycosylation (Chapter 20). Lysosomes act on many long-lived proteins.[133,133a] Once within the lysosomes, the proteins are broken down into amino acids with a half-life of ~8 minutes. During nutritional deprivation, the rate of uptake of proteins by lysosomes increases markedly. The same is true during certain developmental changes, for example, when a tadpole loses its tail.

Many short-lived proteins are degraded within the cytosol in ATP-dependent processes. A major process involves the small protein **ubiquitin** (Box 10-C).[134] Once "labeled" by formation of an isopeptide linkage to ubiquitin, a peptide is attacked by proteases in the **proteasome** complexes (Box 7-A, Chapter 12). There it is quickly degraded. Other proteases, most of which do not require ATP, are also present in the cytoplasm (Chapter 12). How do these enzymes as well as those within the lysosomes work together to produce a harmonious turnover of the very substance of our tissues? How is it possible that one protein has a long half life of many days while another lasts only an hour or two in the same cell? The answer seems to be that

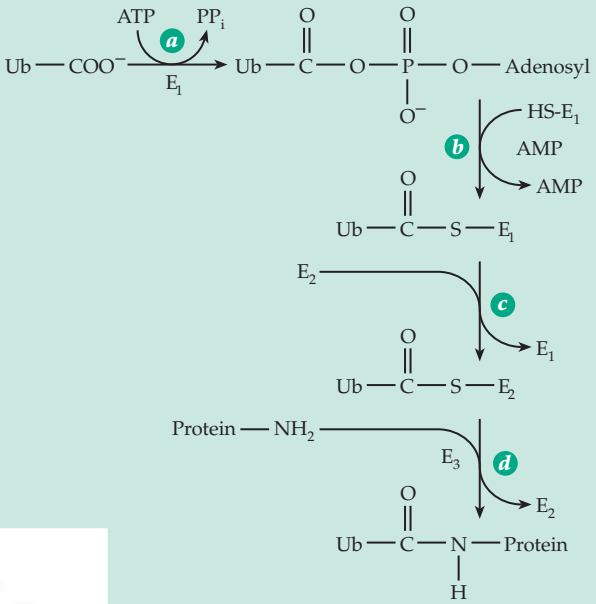
BOX 10-C UBIQUITIN

The small 76-residue protein called ubiquitin[a-c] is probably present in all eukaryotic cells. Found in the nucleus, cytoplasm, cell surface membranes, and extracellular fluids, ubiquitin is often joined by isopeptide linkages from its C-terminal glycine to the ε-amino groups of lysine side chains of other proteins. Ubiquitin has one of the most conserved of all known amino acid sequences. No amino acid substitutions have been found among animal species and only three differences distinguish plant ubiquitins from that of humans. In its three-dimensional structure ubiquitin is compact, tightly hydrogen bonded, and roughly spherical. It contains an α helix, a mixed β sheet, and a distinct hydrophobic core.



Stereoscopic drawing of human ubiquitin. The C terminus is at the top. From Vijay-Kumar *et al.*[c] Courtesy of William J. Cook

Linkage of ubiquitin to other proteins occurs through the action of a ligase system which catalyzes four sequential reactions as shown in the accompanying equation. In step *a* a **ubiquitin activating enzyme** (E_1) forms a C-terminal acyl adenylate by reaction of ubiquitin (Ub) and ATP.[d,e] In step *b* a sulfhydryl group of the same enzyme then displaces the AMP part to form a thioester linkage to ubiquitin. The chemistry of the reaction is the same as that in Eq. 10-1. The activated ubiquitin is next transferred (step *c*) by transacylation to several **ubiquitin-conjugating enzymes** (E_2), also called ubiquitin-carrier proteins.[f-i] These in turn (step *d*) transfer the ubiquitin to amino groups of lysine side chains of target proteins (Prot-NH_2). A third protein, **ubiquitin-protein ligase** (E_3), is sometimes required for this



last step. Most E_2 enzymes, acting without E_3, couple ubiquitin with amines or with small basic proteins whose cellular functions are still unclear. For example, an E_2 which is the product of the yeast gene CDC34 appears to function in the **cell cycle** (Fig. 11-15). Its absence from yeast is lethal[f,i,j] and it is clear that ubiquitin-mediated hydrolysis of the specialized proteins called **cyclins** is essential to operation of the cycle.[k,l]

The best understood function of ubiquitin is in nonlysosomal degradation of proteins.[j,m,n] Protein E_3 appears to select the proteins for degradation, binding them and catalyzing the formation of the isopeptide linkage to substrate. After one ubiquitin molecule has been attached, and while still held by E_3, a second activated ubiquitin is coupled to Lys 48 of the first ubiquitin. This process may continue until several molecules of ubiquitin are joined by Gly-Lys isopeptide linkages to form a **polyubiquitin** chain. Sometimes more than one lysine of the substrate becomes polyubiquitinated to form branched chains. A free α-NH_3^+ group on the protein being degraded is essential for rapid conjugation with ubiquitin and certain N-terminal residues such as arginine favor the conjugation and subsequent hydrolytic breakdown. Some proteins become attached to ubiquitin only after arginine is transferred

BOX 10-C (continued)

onto their N-termini from an aminoacyl-tRNA.[o]

Polyubiquitin chains serve as recognition markers that induce rapid hydrolysis of the marked proteins in the 2000 kDa 26S proteasome or **multicatalytic** protease.[p–s] This complex is discussed in Box 7-A and again in Chapter 12. During hydrolytic destruction of the protein the ubiquitin is released for reuse by **ubiquitin carboxyl-terminal hydrolases** or **isopeptidases** which cleave the thioester or isopeptide linkages that tie ubiquitin to proteins.[t,u]

While proteins may be modified to favor rapid ubiquitination, others may be altered to protect them from ubiquitination. For example, calmodulin produced from a cloned gene in bacteria is a good substrate for ubiquitination but within cells it appears to be protected by the posttranslational conversion of Lys 115 to trimethyllysine.[v]

About 10% of the histone H2A present in higher eukaryotes is ubiquitinated at Lys 119.[w] In the slime mold *Physarum* the content of ubiquitinated histones H2A and H2B changes rapidly during the various stages of mitosis. Apparently, ubiquitin must be cleaved from the histones to permit packaging of DNA into metaphase chromosomes and must become attached to the histones in some regions of the chromosomes to allow unfolding of the highly packed nucleosomes. A yeast enzyme that attaches ubiquitin to histones is encoded by the gene *RAD6*, which is required for DNA repair, sporulation, and other cellular processes. Terminal differentiation of reticulocytes to form erythrocytes involves loss of specific enzymes as well as of entire mitochondria. These processes also depend upon ubiquitin. Ubiquitin is one of the components of the paired helical filaments present in brains of persons with Alzheimer's disease.[x]

In most organisms there are two arrangements for ubiquitin genes. There is a cluster of up to 100 tandomly repeated genes whose transcription gives rise to polyubiquitin, a chain of ubiquitin molecules joined by Gly-Met linkages. These must be cleaved, perhaps by the same ubiquitin C-terminal hydrolase that releases ubiquitin from its conjugates.[y] Other ubiquitin genes are fused to genes encoding ribosomal proteins. The resulting polyproteins have the ribosomal peptides fused to the C termini of the ubiquitin sequences and must be proteolytically cleaved to give mature proteins.[z]

Recently a variety of modifiers of ubiquitin ligases have been discovered[aa,bb] as have ubiquitin-like domains in other proteins.[cc] These findings elucidate the complexity of the sorting of proteins and removal of improperly folded and otherwise defective proteins from the secretory pathway and return to the proteasomes in the cytosol.[dd,ee] They also suggest important roles for ubiquitination in a broad range of metabolic controls.

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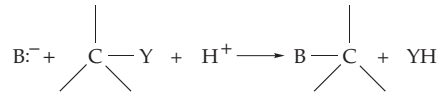
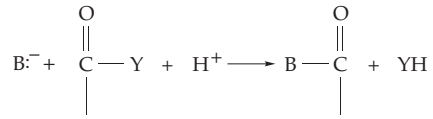
TABLE 10-1
Types of Biochemical Reactions with Ionic Mechanisms

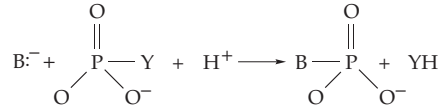
1. Nucleophilic displacement, often via an addition–elimination sequence

A. 

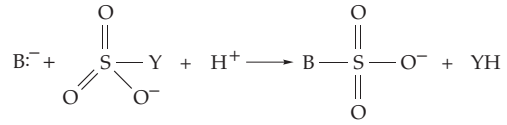
(Examples: methyl-, glycosyl-transferases)

B. 

(Examples: esterases, peptidases)

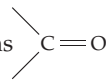
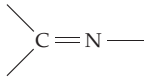
C. 

(Examples: phosphatases, kinases, phosphomutases, ribozymes)

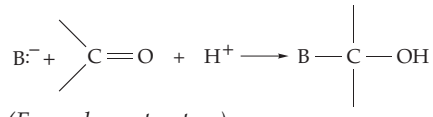
D. 

(Examples: sulfatases, sulfotransferases)

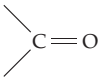
2. Addition

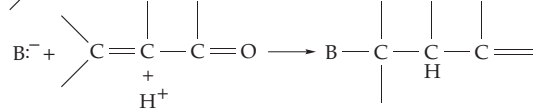
A. To polarized double bonds such as  and 

(This reaction most often occurs as a step in an enzymatic process e.g., formation of hemiacetals, hemiketals, hemimercaptals, carbinolamines)



(Example: mutarotase)

B. To double bond conjugated with  or 



(Examples: acyl-CoA, fumarate hydratases, aspartase)

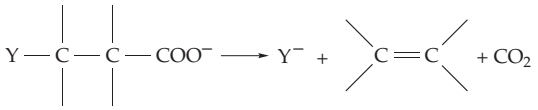
C. To isolated double bonds *(Example: oleate hydratase)* *(see p. 689)*

3. Elimination

A. and B. Precisely the opposite of *addition*

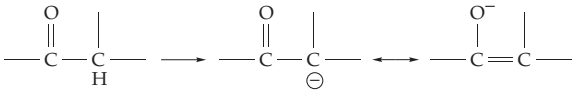
(Eliminations that are the reverse of type 2A are frequent steps in more complex enzyme mechanisms)

C. Decarboxylative elimination

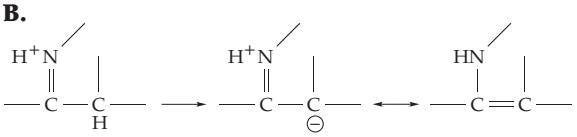


4. Formation of stabilized enolate anions and enamines

A.

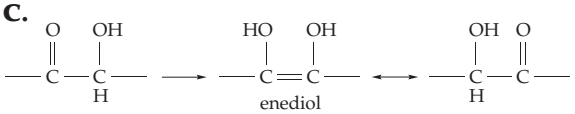


B.



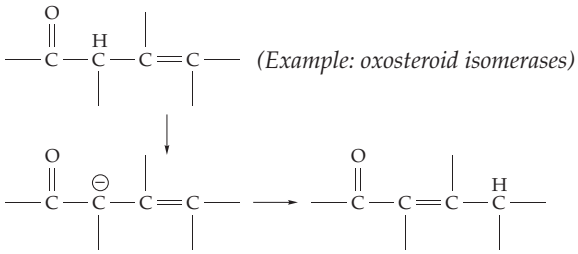
Isomerization reactions

C.



(Example: sugar isomerases)

D.

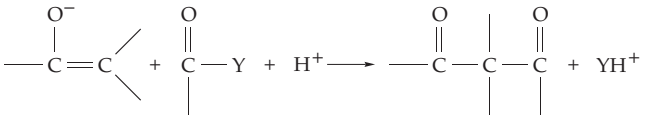


(Example: oxosteroid isomerases)

TABLE 10-1
(continued)

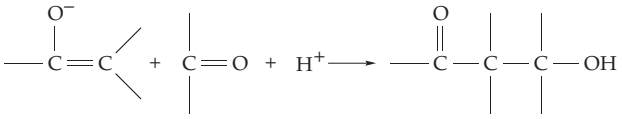
5. Stabilized enolate anions as nucleophiles: formation of carbon–carbon bonds (β condensation)

A. Displacement on a carbonyl group



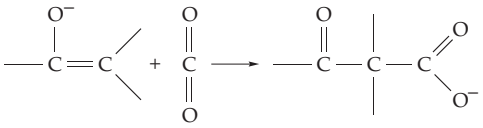
(Example: 3-Oxoacyl–CoA transferase (Y = —S—CoA)

B. Addition to a carbonyl group: aldol condensation



(Examples: aldolases, citrate synthases)

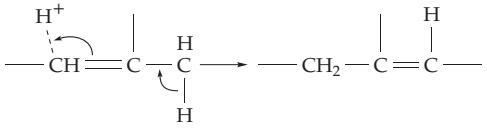
C. Addition to a carbon dioxide (β carboxylation); decarboxylation



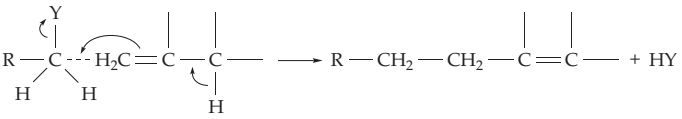
(Examples: phosphoenolpyruvate carboxylase, oxaloacetate decarboxylases)

6. Some isomerization and rearrangement reactions

A. Allylic rearrangement (1,3-proton shift)

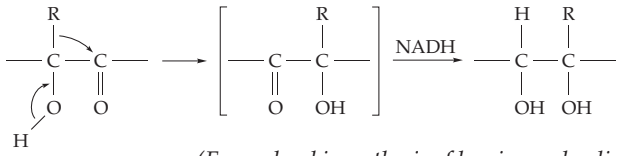


B. Allylic rearrangement with condensation



(Example: condensation of dimethylallyl pyrophosphate with isopentenyl pyrophosphate)

C. Rearrangements with alkyl or hydride ion shift



Glyoxalase

(Examples: biosynthesis of leucine and valine, xylose isomerase, glyoxalase)

the turnover rate of a protein is determined in large part by its sequence. Some proteins are tightly folded and have few bends on the outside that have sequences meeting the specificity requirements of intracellular proteolytic enzymes. These have long half lives. Other proteins may have external loops with sequences susceptible to attack or sequences that favor rapid reaction with the ubiquitin system or uptake into lysosomes. Prematurely terminated proteins and peptide fragments from partial degradation of proteins may tend to be unfolded at the N terminus and to be attacked rapidly.[128,135] Proteins that have undergone covalent modifications or oxidative damage also seem to be hydrolyzed rapidly.[136]

Regions rich in proline, glutamate, serine, and threonine (PEST regions) may be good substrates for Ca^{2+}-activated cytosolic proteases.[137] The ubiquitin system appears to act most slowly on a protein when the normal initiation amino acid methionine is present at the N terminus. For example, the half-life of β-galactosidase in yeast is over 20 hours. Replacement of the methionine with S, A, T, V, or G has little effect. However, replacement with other amino acids shortens the half-life as follows: I and E, 30 min.; Y and Q, 10 min.; F, L, D, and K, 3 min.; and R, 2 min.[138–140a]

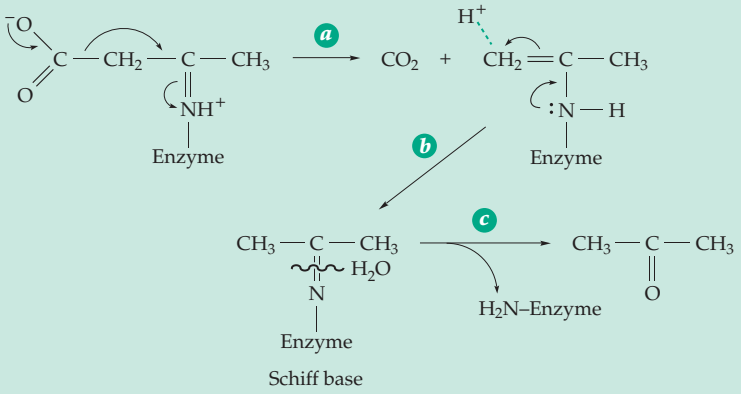
5. Turnover of Nucleic Acids

Because of its special role in carrying genetic information, DNA is relatively stable. An elaborate system of repair enzymes (Chapter 27) act to correct errors and to help DNA to preserve its genetic information. However, in some specialized cells such as those forming immunoglobulin, the DNA too undergoes major rearrangements (Chapter 31). RNA molecules are subject to extensive processing. This includes the conversion of RNA bases to modified forms, chain cleavages during maturation of ribosomal and transfer RNAs, cutting and splicing of gene transcripts to form mRNAs and finally degradation of the mRNA (Chapter 28). Proteins that serve as **RNA chaperones** assist in folding these molecules.[141]

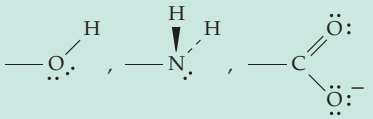
BOX 10-D DRAWING THOSE LITTLE ARROWS

Organic mechanisms are often indicated by arrows that show the flow of electrons in individual steps of a reaction. Many errors are made by students on exams and even in published research papers. The arrows are often drawn backward, are too numerous, or do not clearly indicate electron flow. Here are some tips.

1 Always write a mechanism step-by-step. Never combine two steps (e.g., *a* and *b* at right) *in which electron flow occurs in opposite directions*. Notice that step *c*, the hydrolysis of a Schiff base, is also a two-step process. The reaction is a familiar one that is commonly indicated as shown here. However, this scheme does not show a detailed mechanism for step *c*.



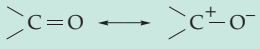
2 Identify the **nucleophilic** and **electrophilic** centers before starting to write a mechanism. Oxygen, sulfur, and nitrogen atoms are usually nucleophilic, e.g., those in —OH, —NH_2, —COO^-, —SH, —OPO_3^{2-}, and enolate anions. The weak nucleophiles —OH and —SH may be converted into strong nucleophiles —O^- —S^- by removal of a proton by a basic group of an enzyme. The nonnucleophilic —NH_3^+ becomes the good nucleophile —NH_2 by loss of a proton. Nucleophilic centers contain unshared electron pairs. Nucleophiles are basic but the basicity, as indicated by proton binding (by the pK_a), is not necessarily proportional to nucleophilic strength (nucleophilicity). Enolate anions and enamines provide nucleophilic centers on carbon atoms, important in formation and cleavage of C—C bonds.



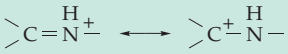
Enolate anion

Enamine

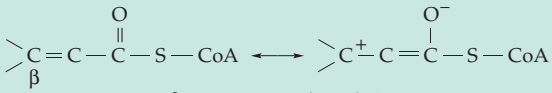
Groups with nucleophilic centers indicated by unshared electron pairs and/or negative charge.



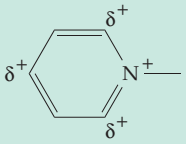
Carbonyl



Protonated imine



α,β-Unsaturated acyl-CoA

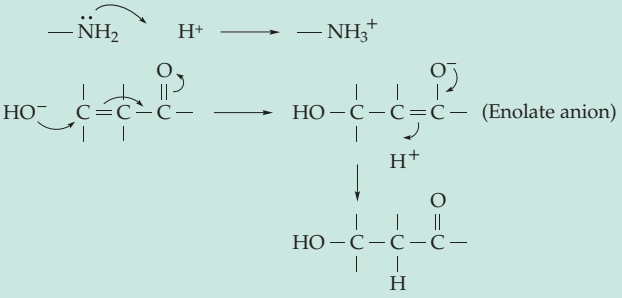


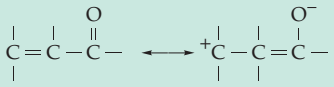
Some electrophilic centers are indicated by + or δ^+

Electrophilic centers include acidic hydrogen atoms, metal ions, the carbon atoms of carbonyl groups, and the β-carbon atoms of α, β unsaturated acids, ketones, or acyl-CoA derivatives. Highly polarized groups such as carbonyl and enamine generate electrophilic centers as indicated by the positive charges. They also affect more distant positions in conjugated systems, e.g., in α, β-unsaturated acyl-CoA derivatives, and in intermediates formed from thiamin diphosphate and pyridoxal phosphate.

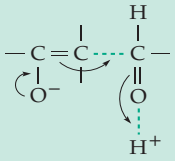
BOX 10-D (continued)

3 When an ionic organic reaction (the kind catalyzed by most enzymes) occurs a nucleophilic center joins with an electrophilic center. We use arrows to show the movement of pairs of electrons. The movement is always *away* from the nucleophile which can be thought of as "attacking" an electrophilic center. Notice the first step in the second example at right. The unsaturated ketone is polarized initially. However, this is not shown as a separate step. Rather, the flow of electrons from the double bond, between the α- and β-carbons into the electron-accepting C=O groups, is coordinated with the attack by the nucleophile. Dotted lines are often used to indicate bonds that will be formed in a reaction step, e.g., in an aldol condensation (right). Dashed or dotted lines are often used to indicate partially formed and partially broken bonds in a transition state, e.g., for the aldol condensation (with prior protonation of the aldehyde). However, *do not put arrows on transition state structures.*

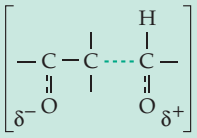




Polarized unsaturated ketone

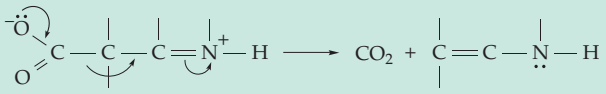


Aldol condensation



Transition state

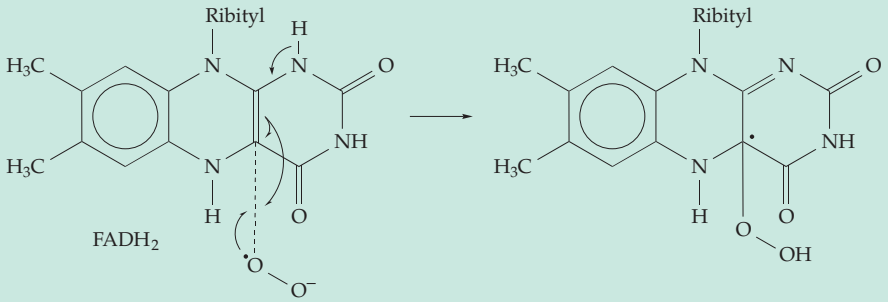
4 In a given reaction step all of the arrows must point the same way. *The arrows point into bonds that are forming* or toward atoms that will carry an unshared electron pair in the product. *Arrows originate from unshared electron pairs or from bonds that are breaking.*



All arrows point the same way

5 Never start an arrow from the same bond that another arrow is forming; i.e., electrons flow out of *alternate* bonds.

6 For reactions of radicals (homolytic reactions) arrows are used to indicate motion of single electrons rather than of electron pairs. It is desirable to use arrows with *half-heads*. For example, reaction of a superoxide radical ($\bullet O_2^-$) with $FADH_2$ could occur as follows:



Half-heads on arrows indicate one-electron movement

E. Classifying Enzymatic Reactions

The majority of enzymes appear to contain, in their active centers, only the side chains of amino acids. Most of the reactions catalyzed by these enzymes can be classified into a small number of types as is indicated in Table 10-1: (Type 1) **displacement** or **substitution** reactions in which one base or nucleophile replaces another, (Type 2) **addition** reactions in which a reagent adds to a double bond, and (Type 3) **elimination** reactions by which groups are removed from a substrate to create double bonds. Note that the latter are the reverse of addition reactions. Two other groups of reactions depend upon formation of transient **enolic forms**. These include (Type 4) **isomerases** and (Type 5) **lyases**, a large and important group of reactions that form or cleave carbon–carbon bonds. Finally, there is a group (Type 6) of **isomerization** and **rearrangement** reactions that do not appear to fit any of the foregoing categories. Another quite different group of enzyme-catalyzed reactions, which are considered in Chapter 16, function with the participation of free radical intermediates.

Biochemical displacement reactions include all of the hydrolytic reactions by which biopolymers are broken down to monomers as well as most of the reactions by which the monomers are linked together to form polymers. Addition reactions are used to introduce oxygen, nitrogen, and sulfur atoms into biochemical compounds and elimination reactions often provide the driving force for biosynthetic sequences. Complex enzymatic processes are often combinations of several steps involving displacement, addition, or elimination. The reactions involving formation or cleavage of C–C bonds are essential to biosynthesis and degradation of the various carbon skeletons found in biomolecules, while the isomerization reactions provide connecting links between the other kinds of reactions in the establishment of metabolic pathways.

In Chapters 12 and 13 the individual reactions of metabolism are classified into these types and the enzymes that catalyze them are described in some detail. The chemistry of coenzymes and metalloenzymes are presented systematically in Chapters 14 to 16, and in Chapter 17 the logic of the combining of individual reactions into metabolic sequences is considered. It is not necessary to read Chapters 12–16 in their entirety since much of their content is reference material. In the later chapters on metabolism, cross-references point out the discussions of individual enzymes in Chapters 12–16.

The following are topics that may be especially valuable to the student and which might be read initially: in Chapter 12, lysozyme (Section B,5), chymotrypsin (Section C,1), kinases (Section D,9), multiple displacement, reactions (Section G); in Chapter 13, imines (Section A,2), addition to C=C bonds (Section A, 4,5), beta cleavage and condensation (Section C); in Chapter 14, thiamin diphosphate (Section D), pyridoxal phosphate (Section E); in Chapter 15, NAD (Section A).

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Study Questions

1. Outline in detail, using structural formulas, the enzyme-catalyzed reactions by which cells in the human body convert glyceraldehyde 3-phosphate into pyruvate.

2. Describe, using chemical structural formulas, the reactions involved in the breakdown of glycogen to glucose 1-phosphate and the synthesis of glycogen from glucose 1-phosphate.

3. Describe the reaction steps in gluconeogenesis by which pyruvate is converted into glyceraldehyde 3-phosphate.

4. Compare the reactions of pyruvate that give rise to the following three compounds. List coenzymes or electron-carriers involved in each case and indicate any intermediate compounds.
   a) Ethanol
   b) Lactic acid
   c) Acetyl-Coenzyme A

5. Mammalian sperm cells metabolize D-fructose preferentially as a source of energy. Fructose is formed in cells of the seminal vesicle from D-glucose via reduction to the sugar alcohol sorbitol using NADPH, followed by oxidation of sorbitol to fructose using NAD^+. The fructose concentration in human semen is about 12 mM, whereas the glucose concentration within cells is usually less than 1 mM. If the ratio $[NADPH]/[NADP^+]$ is 10^4 times higher than the ratio $[NADH]/[NAD^+]$, what is the minimum glucose concentration in cells that could allow formation of 12 mM fructose? The standard Gibbs energies of formation from the elements G^o_f in kJ/mol at 25°C are: D-glucose –917, D-fructose –915.

6. Outline the reactions by which glyceraldehyde 3-phosphate is converted to 3-phosphoglycerate with coupled synthesis of ATP in the glycolysis pathway. Show important mechanistic details.

7. Why can't acetyl CoA be converted to glucose in animals?

8. Describe the parallel reaction sequences between the citric acid cycle and the β oxidation pathway.

9. Contrary to legend, camels do not store water in their humps, which actually consist of a large fat deposit. How can these fat deposits serve as a source of water? Calculate the amount of water (liters) that can be produced by the camel from 500 g of fat. Assume for simplicity that the fat consists entirely of tripalmitin.

Study Questions

10. A little-known microorganism carries out the following net reaction in a series of enzymatic steps:

$$\text{heptanoyl CoA} + \text{CoASH} + \text{NAD}^+ + \text{FAD} + \text{H}_2\text{O} \rightarrow \text{pentanoyl CoA} + \text{acetyl CoA} + \text{NADH} + \text{FADH}_2 + \text{H}^+$$

The net result is exactly the same as that of the β oxidation pathway, but for this microorganism, the pathway is demonstrably different in that pentanal, $CH_3(CH_2)_3CHO$, is an intermediate in the sequence. Which enzyme(s) of the β oxidation pathway does this microorganism lack? Propose an enzymatic reaction to account for the formation of this intermediate and a series for its conversion to the acyl CoA. ATP is not required.

11. Most natural fatty acids are even-numbered. What is the product of the *final* thiolase reaction to form an odd-chain fatty acid? Give a brief explanation.

12. It has been calculated that an average man takes in 1.5 kg of solid food and 1.4 kg of water per day. He gives off 3.5 kg of waste and 0.75 kg of sweat. It would thus appear that he should be losing over 1 kg per day through normal activites. How do you account for the fact that his weight remains relatively constant?

13. Suppose that fatty acids, instead of being broken down in two carbon fragments, were metabolized in three carbon units. What product(s) would Dr. Knoop have observed in the urine of his experimental dogs?

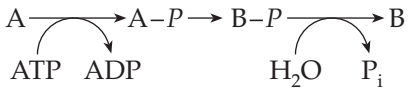
14. A renowned pharmacologist has announced the discovery of a new drug that specifically inhibits the fatty acid oxidation pathway within minutes of ingestion. The effects last for several hours only. The drug has no other effects on the subjects. The pharmacologist argues that this will increase athletic performance by shifting oxidative metabolism entirely to the more rapidly mobilized carbohydrate degradation pathway. Assume that the drug does work exactly as he suggests. Explain in a sentence or two how the drug would affect the performance of
    a) a sprinter in the 100 meter dash
    b) a long-distance runner in a marathon

15. Some bacteria use a "dicarboxylic acid cycle" to oxidize glyoxylate $OHC{-}COO^-$ to CO_2. The regenerating substrate for this cycle is acetyl-CoA. It is synthesized from glyoxylate by a complex pathway that begins with conversion of two molecules of glyoxylate to tartronic semialdehyde: $^-OOC{-}CHOH{-}CHO$. The latter is then dehydrogenated to D-glycerate. Write out a detailed scheme for the dicarboxylate cycle. Also indicate how glucose and other cell constituents can be formed from intermediates created in this biosynthetic pathway.

16. Write a balanced fermentation sequence by which glycogen can be converted rapidly to *sn*-glycerol 3-phosphate and pyruvate in insect flight muscle. How many molecules of ATP per glucose unit of glycogen will be formed?

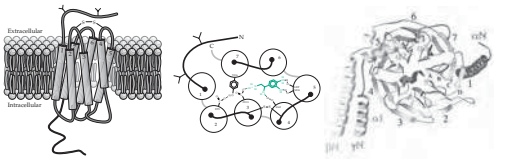
17. Show which parts (if any) of the citric acid cycle are utilized in each of the following reactions and what if any additional enzymes are needed in each case.
    a) Oxidation of acetyl-CoA to CO_2
    b) Catabolism of glutamate to CO_2
    c) Biosynthesis of glutamate from pyruvate
    d) Formation of propionate from pyruvate

18. a) If the Gibbs energy change $\Delta G'$ (pH 7) for the reaction A → B is +25 kJ/mol at 25°C, what would the ratio of [B]/[A] be at equilibrium?
    b) Suppose that the reaction were coupled to the cleavage of ATP as follows:



Suppose further that the group transfer potential ($-\Delta G'$) for the phospho group of A–*P* at 25°C, pH 7 is 12 kJ/mol and that the equilibrium constant for conversion of A–*P* to B–*P* is the same as that for conversion of A to B. Calculate the concentrations of A, B, A–*P* and B–*P* at equilibrium if the phosphorylation state ratio R_p is $10^4\ M^{-1}$.

19. This problem refers to the 13 meter climb described in Chapter 6, Study Question 14.
    a) Assuming that muscle accounts for 30% of total body mass, estimate the amounts (mmol/kg, total mmol, and total grams) of each of the following in their coenzyme forms: nicotinamide, pantothenic acid, thiamin. You may be able to obtain rough estimates from the vitamin content of pork.
    b) Calculate how many times, on the average, each molecule of nicotinamide would undergo reduction and re-oxidation (i.e., turn over) during the climb. Do the same for pantothenic acid (cycling between acyl-CoA and free CoA forms) and for thiamin through its catalytic cycles.

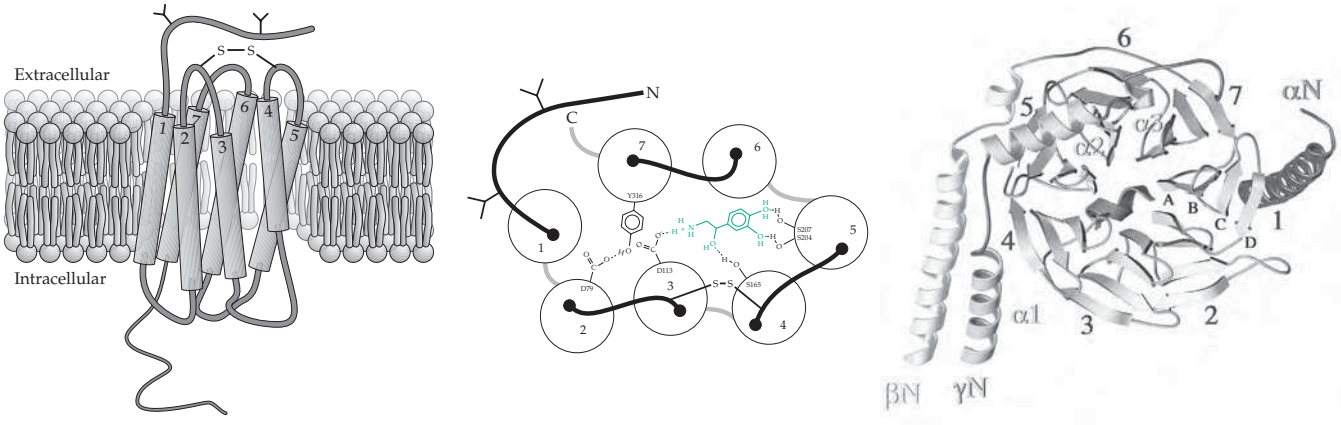


Much of the control of cellular metabolism is accomplished by hormones and other molecules that bind to receptor proteins embedded in the plasma membrane. Left: Many receptors are seven-helix transmembrane proteins, the best known being the light receptors of the rhodopsin family (Chapter 23). Center: A related adrenergic receptor, viewed here from the extracellular side, has a molecule of the hormone adrenaline (green) bound in the center (Fig. 11-6). Right: These receptors interact with GTP-hydrolyzing "G proteins," which pass signals, often for short periods of time, to enzymes and other proteins. One structural domain of a G protein (Fig. 11-7C) shown here contains a β propeller domain which binds to the Ras-like domain of the α subunit. A few residues of the latter are visible in the center of this image.

Contents

The Regulation of Enzymatic Activity and Metabolism

11



Living cells must operate with controls that provide a stable environment and a relatively constant supply of materials needed for biosynthesis and for meeting the energy needs of cells. They must also be responsive to changes in their environment and must be able to undergo mitosis and reproduce when appropriate. The necessary control of metabolism and of growth is accomplished largely through mechanisms that regulate the locations, the amounts, and the catalytic activities of enzymes. The purpose of this chapter is to summarize these control mechanisms and to introduce terminology and shorthand notations that will be used throughout this book. Many of the **control elements** considered are summarized in Fig. 11-1.

A. Pacemakers and the Control of Metabolic Flux

Metabolic control can be understood to some extent by focusing attention on those enzymes that catalyze rate-limiting steps in a reaction sequence. Such **pacemaker enzymes**[1–4] are often involved in reactions that determine the overall respiration rate of a cell, reactions that initiate major metabolic sequences, or reactions that initiate branch pathways in metabolism. Often the first step in a unique biosynthetic pathway for a compound acts as the pacemaker reaction. Such a reaction may be described as the **committed step** of the pathway. It usually proceeds with a large decrease in Gibbs energy and tends to be tightly controlled. Both the rate of synthesis of the enzyme protein and the activity of the enzyme, once it is formed, may be inhibited by **feedback inhibition** which occurs when an end product of a biosynthetic pathway accumulates and inhibits the enzyme. Enzyme activity may also be turned on or off by the effect of a hormone, by some other external stimulus, or by internal mechanisms that sense the metabolic state of the cell. Enzymes, other than the pacemaker, that catalyze reactions in a pathway may not be regulated and may operate at a steady state close to equilibrium.

If conditions within a cell change, a pacemaker reaction may cease to be rate limiting. A reactant plentiful in one circumstance may, in another, be depleted to the point that the rate of its formation from a preceding reaction determines the overall rate. Thus, as we have seen in Chapter 10, metabolism of glucose in our bodies occurs through the rapidly interconvertible phosphate esters glucose 6-phosphate and fructose 6-phosphate. The pacemaker enzyme in utilization of glucose or of glycogen is often **phosphofructokinase** (Fig. 11-2, step *b*) which catalyzes further metabolism of fructose 6-phosphate. However, if metabolism by this route is sufficiently rapid, the rate of formation of glucose 6-phosphate from glucose catalyzed by **hexokinase (**Fig. 11-2, step *a*) may become rate limiting.

Some catabolic reactions depend upon ADP, but under most conditions its concentration is very low because it is nearly all phosphorylated to ATP. Reactions utilizing ADP may then become the rate-limiting pacemakers in reaction sequences. Depletion of a reactant sometimes has the effect of changing the whole pattern of metabolism. Thus, if oxygen is unavailable to a yeast, the reduced coenzyme NADH accumulates and reduces pyruvate to ethanol plus CO_2 (Fig. 10-3). The result is a shift from oxidative metabolism to fermentation.

Pacemaker enzymes are often identified by the fact that the measured **mass action ratio**, e.g., for the reaction A + B → P + Q ,the ratio [P][Q]/[A][B], is far

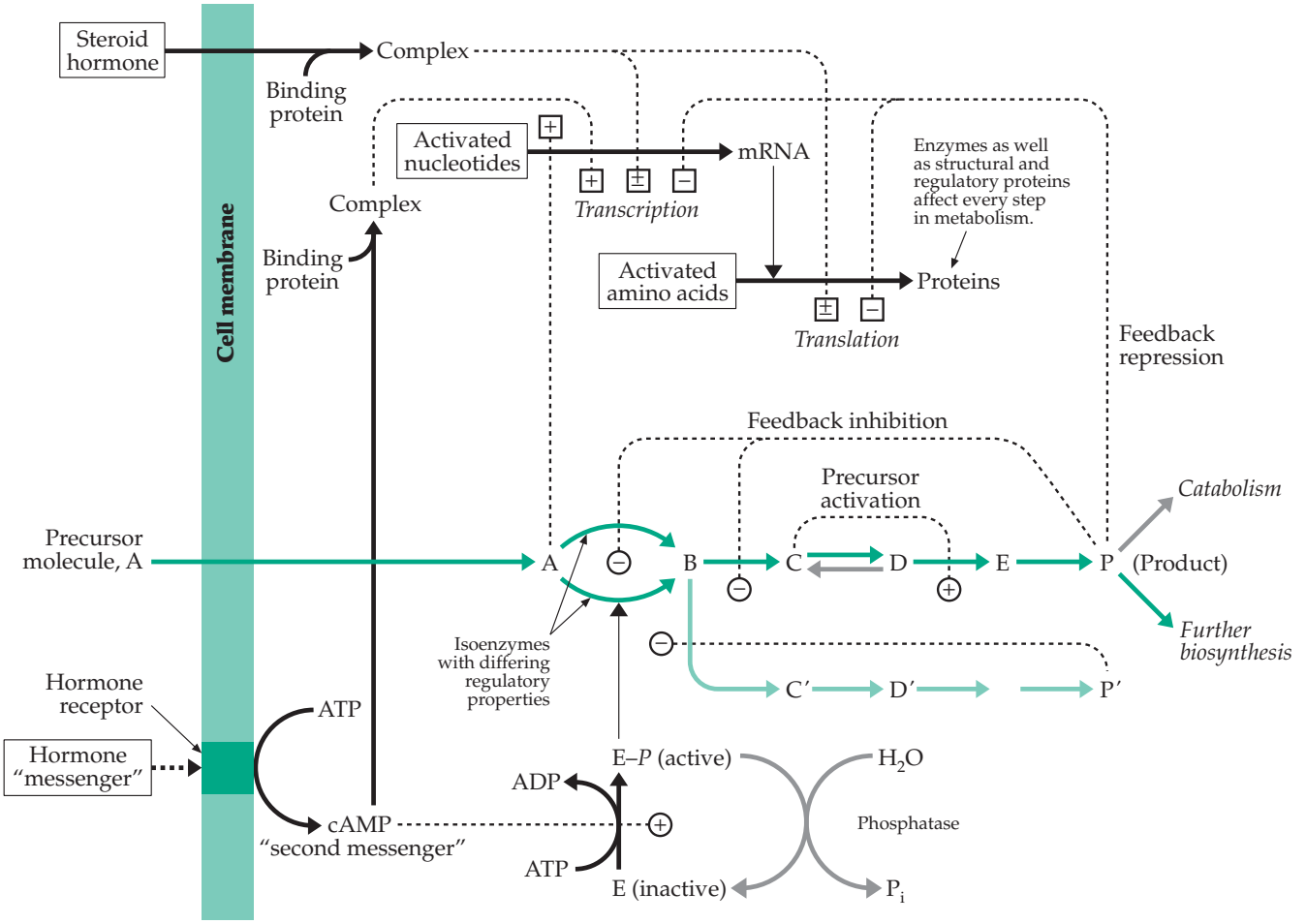


Figure 11-1 Some control elements for metabolic reactions. *Throughout the book modulation of the activity of an enzyme by allosteric effectors or of transcription and translation of genes is indicated by dotted lines from the appropriate metabolite.* The lines terminate in a minus sign for inhibition or repression and in a plus sign for activation or derepression. Circles indicate direct effects on enzymes, while boxes indicate repression or induction of enzyme synthesis.

from that predicted by the known equilibrium constant for the reaction. Another approach to identifying a pacemaker enzyme is to inhibit it and observe resulting changes in steady-state metabolite concentrations.[5] If the pacemaker enzyme is inhibited, its substrate and other compounds preceding the step catalyzed by this enzyme will accumulate. At the same time the concentrations of products of the pacemaker reaction will drop as a result of their relatively more rapid removal by enzymes catalyzing subsequent steps. However, conclusions drawn by this approach may sometimes be erroneous.[6]

In spite of its usefulness, the pacemaker concept is oversimplified. It is often impossible to identify a specific pacemaker enzyme. When both catabolism and biosynthesis occur (e.g., as in the scheme in Fig. 11-2) it may be more useful to model the entire system with a computer than to try to identify pacemakers.[7,8] It is also important to realize that reaction rates may be determined by the rate of diffusion of a compound through a membrane. Thus, membrane transport processes can serve as pacemakers.

A general approach to analysis of complex metabolic pathways or to cell growth was introduced by Savageau,[9–11] and similar approaches have been followed by others.[3,12–19] They emphasize the **flux** of material through a pathway under steady-state conditions and recognize that every enzyme in a sequence can have some effect on the overall rate. Consider a chain of enzymes E_1 to E_n acting on substrate S_0, which is converted via substrates S_1, S_2 ... S_{n-1} into product S_n:

$$S_0 \xrightarrow{E_1} S_1 \xrightarrow{E_2} S_2 \xrightarrow{E_3} S_3 \cdots \longrightarrow S_{n-1} \xrightarrow{E_n} S_n \qquad (11\text{-}1)$$

The flux $F = d[S_n]/dt$ is a constant. If the concentration of E_i were to change by an infinitesimal amount a corresponding change in F might be observed. The

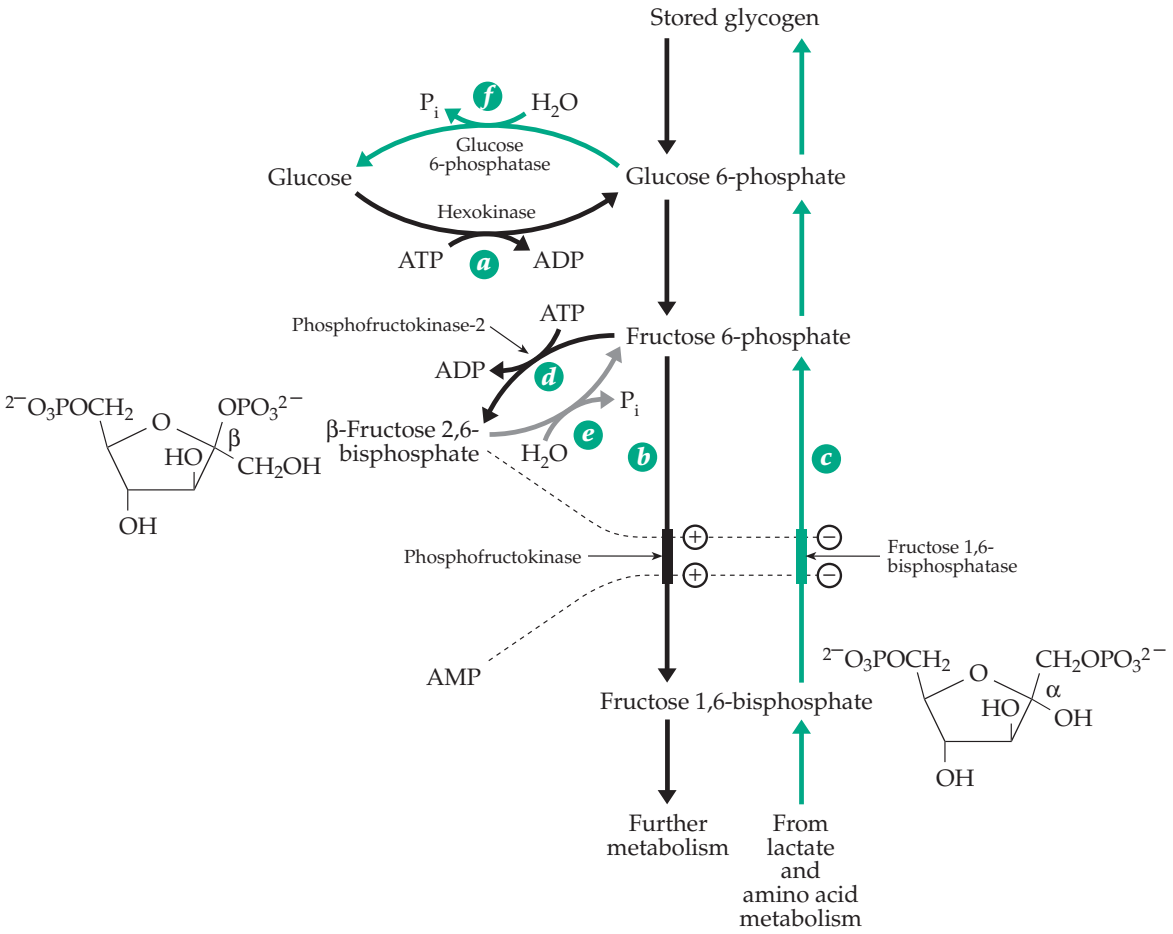


Figure 11-2 Roles of phosphofructose kinase and fructose 1,6-bisphosphatase in the control of the breakdown (⟶) and storage (⟶) of glycogen in muscle. The uptake of glucose from blood and its release from tissues is also illustrated. The allosteric effector fructose 2,6-bisphosphate (Fru-2,6-P_2) regulates both phosphofructokinase and fructose 2,6-bisphosphatase. These enzymes are also regulated by AMP if it accumulates. The activity of phosphofructokinase-2 (which synthesizes Fru-2,6-P_2) is controlled by a cyclic AMP-dependent kinase and by dephosphorylation by a phosphatase.

ratio of the change in flux to the change in $[E_i]$ is the sensitivity coefficient Z_i:

$$Z_i = \frac{dF/F}{d[E_i]/[E_i]} = \frac{d \ln F}{d \ln[E_i]} \tag{11-2}$$

The sensitivity coefficient can range from 1 for a pacemaker enzyme to almost zero for a very active enzyme that does not significantly limit the flux. The sum of Z_1 to Z_n is equal to 1:

$$\sum_{n}^{i=1} Z_i = 1 \tag{11-3}$$

Not only can the concentration $[E_i]$ change but also allosteric effectors can alter the activity. Kacser and Burns defined this in terms of a **controllability coefficient κ**, the ratio of the logarithmic change in velocity to the change in the concentration of an effector:

$$\kappa = d \ln v / d \ln [P] \tag{11-4}$$

It follows that the logarithmic change in flux $d \ln F$ can be related to the change in effector concentration [P] in the following way:

$$d \ln F = Z_i \kappa d \ln [P] \tag{11-5}$$

From this equation we see that for the flux to be sensitively dependent upon [P], both Z_i and κ must be reasonably large. This is exactly the case for many of the enzymes that have been identified as pacemakers. This approach can be applied to many aspects of metabolic control including cell growth.[20]

B. Genetic Control of Enzyme Synthesis

All cellular regulatory mechanisms depend upon the synthesis of proteins, that is, upon the expression of genetic information. Within a given cell many genes are transcribed continuously but others may remain unexpressed. The rates of both transcription and degradation of mRNA are regulated, as are the rates of synthesis of enzymes on the ribosomes in the cytoplasm and the rates of protein turnover. Although a single copy of a gene in each member of a pair of chromosomes is often adequate, there are situations in which extra copies of part of the DNA of a cell are formed. Such **gene amplification**, which is dealt with further in Chapter 27, provides the possibility for very rapid synthesis of an enzyme or other protein. It often happens in highly specialized cells such as those of the silkworm's silk glands which must make enormous amounts of a small number of proteins.[21]

1. One Gene or Many?

Many enzymes exist within a cell as two or more **isoenzymes**, enzymes that catalyze the same chemical reaction and have similar substrate specificities. They are not isomers but are distinctly different proteins which are usually encoded by different genes.[22,23] An example is provided by aspartate aminotransferase (Fig. 2-6) which occurs in eukaryotes as a pair of cytosolic and mitochondrial isoenzymes with different amino acid sequences and different isoelectric points. Although these isoenzymes share less than 50% sequence identity, their internal structures are nearly identical.[24–27] The two isoenzymes, which also share structural homology with that of *E. coli*,[28] may have evolved separately in the cytosol and mitochondria, respectively, from an ancient common precursor. The differences between them are concentrated on the external surface and may be important to as yet unknown interactions with other protein molecules.

Isoenzymes are designated in various ways. They are often *numbered in the order of decreasing electrophoretic mobility* at pH 7 to 9: Most enzymes are negatively charged in this pH range and the one that migrates most rapidly toward the anode is numbered one. This is the same convention used in the electrophoresis of blood proteins (e.g. see Box 2-A).

Lactate dehydrogenase exists in the cytoplasm of humans and most animals as *five forms* which are easily separable by electrophoresis and are evenly spaced on electropherograms.[8] This enzyme is a tetramer made of two kinds of subunits. Isoenzyme 1, which has the highest electrophoretic mobility, consists of four identical type B subunits. The slowest moving tetramer (isoenzyme 5) consists of four type A subunits, while the other three forms, AB_3, A_2B_2, and A_3B, contain both subunits in different proportions. The two subunits are encoded by separate genes which are expressed to different extents in different tissues. Thus, heart muscle and liver produce mainly subunit B, while skeletal muscle produces principally subunit A. A third subunit type (C) is found only in the testes.[29]

Why do cells produce isoenzymes? One reason may be that enzymes with differing kinetic parameters are needed.[30] Substrate concentrations may vary greatly between different tissues; between different subcellular compartments; and at different developmental stages of an organism. While the need for various isoenzymes of lactate dehydrogenase is not well understood,[31] it is easier to understand the roles of the multiple forms of hexokinase, the enzyme that catalyzes the reaction of step *a* in Fig. 11-2. The brain enzyme has a high affinity for glucose (K_m = 0.05 mM). Thus, it is able to phosphorylate glucose and to make that substrate available to the brain for metabolism, even if the glucose concentration in the tissues falls to low values.[31a] On the other hand, **glucokinase**, the hexokinase isozyme found in liver, has a much higher K_m of ~10 mM. It functions to remove excess glucose from blood, whose normal glucose content is ~5.5 mM. Glucokinase reaches its maximal activity only when the glucose concentration becomes much higher.[32] This happens after a meal when the absorbed glucose passes through the portal circulation directly to the liver.

Another important source of variation in enzymes as well as in other proteins is **alternative splicing** of mRNA.[33] For example, transcription of the mouse α-amylase gene in the salivary gland starts at a different site (promoter) than does transcription in the liver. The two common isoforms of the insulin receptor (Fig. 11-11) arise because a 36-nucleotide (12-amino acid) exon is spliced out of the mRNA for the shorter protein. Isoenzymes of aldolase[34] and of many other proteins are formed in a similar manner. **Frame-shifting** during protein synthesis (Chapter 29) and also post-translational alterations may give rise to additional modified forms. They are often synthesized in relatively small amounts but may be essential to the life of the cell. In addition, genetic variants of almost any protein will be found in any population. These often differ in sequence by a single amino acid.

2. Repression, Induction, and Turnover

The synthesis of some enzymes is referred to as **constitutive**, implying that the enzyme is formed no matter what the environmental conditions of the cell. For example, many bacteria synthesize the enzymes required to catabolize glucose under all conditions of growth. Other enzymes, known as **inducible**, are often produced only in small amounts. However, if

cells are grown in specific inducing conditions for these enzymes, they are synthesized in larger quantities. For example, when *E. coli* is cultivated in the presence of lactose, several of the enzymes required for the catabolism of that disaccharide are formed. Synthesis of these enzymes is normally **repressed**. The genes which code for them are kept turned off through the action of protein **repressors** which bind to specific sites on the DNA and block transcription of the genes that they control (Fig. 11-1). Repressors have allosteric properties; in one conformation they bind tightly to DNA but in another they do not bind. For example, the free tryptophan repressor binds to DNA only weakly, but if a high concentration of tryptophan develops within the cell the tryptophan binds to an allosteric site on the repressor protein (Fig. 5-35). This changes the conformation to one that binds tightly to the appropriate control sequence in the DNA. In the case of lactose catabolism the free repressor binds to control sequences in the DNA until the **inducer**, allolactose (Chapters 4 and 28), binds at an allosteric site. This decreases the affinity of the repressor for DNA and the controlled genes are **derepressed**. There are also many protein **transcription factors** that have a positive effect, binding to DNA and promoting transcription of specific genes.

Synthesis of many enzymes is repressed most of the time. The appearance of an enzyme at a particular stage in the life of an organism as well as the differing distributions of isoenzymes within differentiated tissue result from derepression. The control of enzyme synthesis may also be exerted during the splicing of transcripts and at the translational level as well. These control mechanisms are often relatively slow, with response times of hours or even days. However, effects on the synthesis of some hormones, such as insulin (Section G), may be observed within a few minutes.

Genetic factors influence the rate of not only synthesis of proteins but also their breakdown, i.e., the rate of turnover. As we have seen in Chapter 10, some enzymes are synthesized as inactive proenzymes which are later modified to active forms, and active enzymes are destroyed, both by accident and via deliberate hydrolytic pathways. Protein **antienzymes** may not only inhibit enzymes but may promote their breakdown.[35] An example is the antienzyme that controls ornithine decarboxylase, a key enzyme in the synthesis of the polyamines that are essential to growth.[36,37] As with all cell constituents, the synthesis of enzymes and other proteins is balanced by degradation.

3. Differences among Species

Catalytic mechanisms of enzymes have usually been conserved throughout evolution, and certain residues in an enzyme may be invariant among many species. However, there are usually many differences in the distribution of amino acid residues on the surface of the proteins. Since changes in the surface shape of a protein molecule may alter the sensitivity to a potential allosteric effector, very different regulatory properties are sometimes found between species.

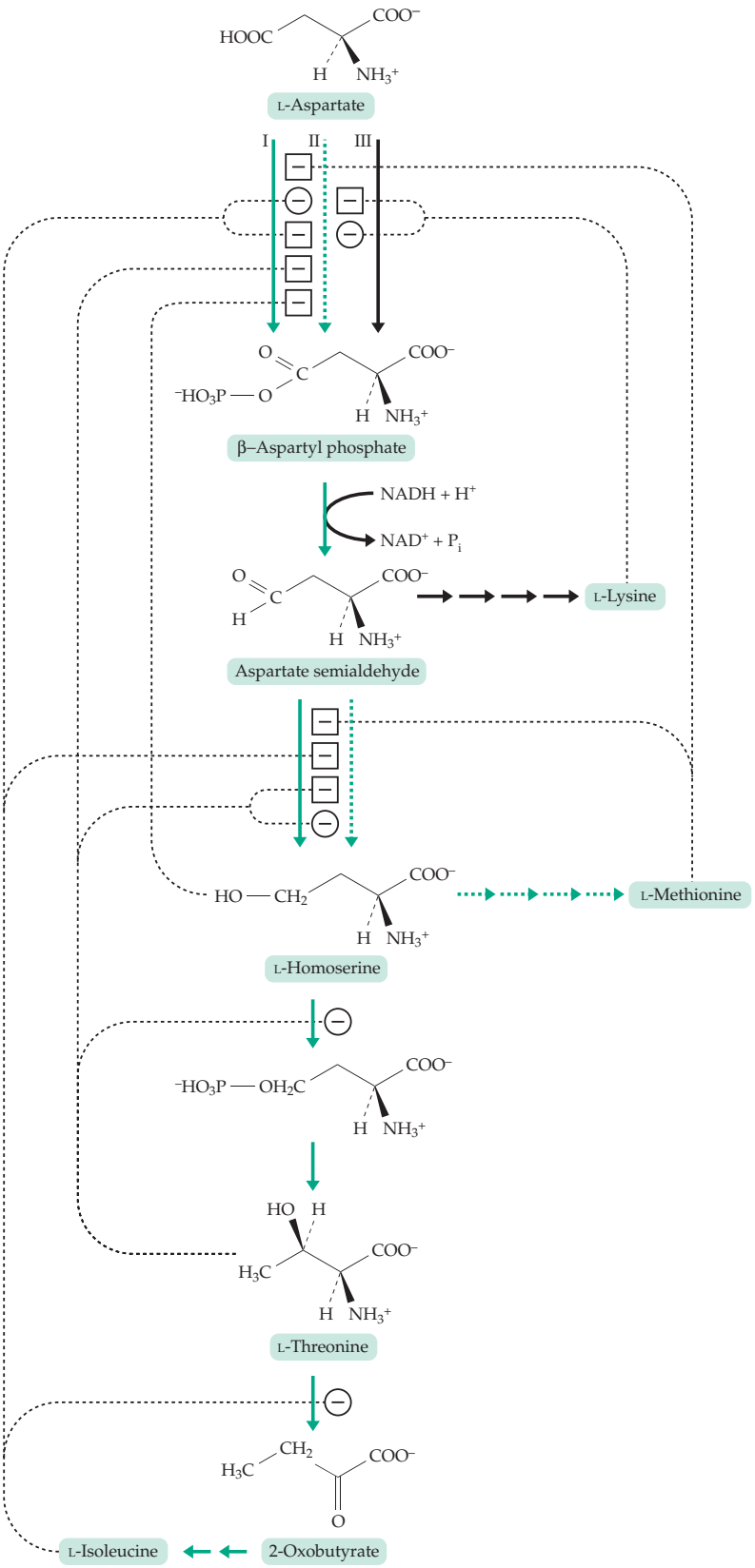
C. Regulation of the Activity of Enzymes

Some regulation of metabolism is provided by the kinetic properties of the enzymes. Thus, the value (mol/l) of the Michaelis constant K_m for an enzyme is usually low if the substrate normally occurs in very low concentration. It is likely to be higher if the enzyme acts on an abundant substrate. A tightly bound product of an enzymatic reaction will be released slowly from an enzyme if the product concentration within the cell is too high. However, while some regulation of metabolism is provided in such simple ways, the rapid changes that result from stimulation by hormones or by nerve impulses depend upon additional specific regulatory mechanisms that are discussed in the following sections.

1. Allosteric Control

Probably the most common and widespread control mechanisms in cells are **allosteric inhibition** and **allosteric activation**. These mechanisms are incorporated into metabolic pathways in many ways, the most frequent being **feedback inhibition**. This occurs when an end product of a metabolic sequence accumulates and turns off one or more enzymes needed for its own formation. It is often *the first enzyme unique to the specific biosynthetic pathway for the product that is inhibited.* When a cell makes two or more isoenzymes, only one of them may be inhibited by a particular product. For example, in Fig. 11-1 product P inhibits just one of the two isoenzymes that catalyzes conversion of A to B; the other is controlled by an enzyme modification reaction. In bacteria such as *E. coli*, three isoenzymes, which are labeled I, II, and III in Fig. 11-3, convert aspartate to β-aspartyl phosphate, the precursor to the end products threonine, isoleucine, methionine, and lysine. Each product inhibits only one of the isoenzymes as shown in the figure.

Feedback can also be positive. Since AMP is a product of the hydrolysis of ATP, its accumulation is a signal to *activate* key enzymes in metabolic pathways that generate ATP. For example, AMP causes allosteric activation of glycogen phosphorylase, which catalyzes the first step in the catabolism of glycogen. As is shown in Fig. 11-5, the allosteric site for AMP or IMP binding is more than 3 nm away from the active site. Only a



subtle conformational change accompanies binding of IMP.[38] However, ^{31}P NMR studies indicate that binding of the activator may induce a change in the state of protonation of the phosphate group of the coenzyme pyridoxal phosphate at the active site as explained in Chapter 12.

A second pattern of allosteric control may be referred to as **precursor activation** or feed**-**forward control. A metabolite acting as an allosteric effector turns on an enzyme that either acts directly on that metabolite or acts on a product that lies further ahead in the sequence. For example, in Fig. 11-1, metabolite C activates the enzyme that catalyzes an essentially irreversible reaction of compound D. An actual example is provided by glycogen synthase, whose inactive "dependent" or D form is activated allosterically by the glycogen precursor glucose 6-phosphate.[39] See also phosphorylase kinase (Section 2).

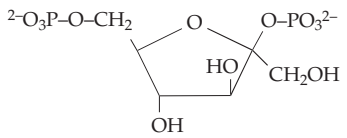
Regulatory subunits. Some enzymes consist not only of catalytic subunits, which contain the active sites, but also of **regulatory subunits**. The latter bind to the catalytic subunits and serve as allosteric modifiers. The binding of inhibitors or activators to specific sites on the regulatory subunits induces conformational changes in these subunits, altering their interaction with the catalytic subunits. A well-known example is aspartate carbamoyltransferase (ACTase) from *E. coli* (Fig. 7-20).[40–42] Its regulatory subunits carry binding sites for cytidine triphosphate (CTP), which acts as a specific allosteric inhibitor of the enzyme. The significance of this fact is that ACTase catalyzes the first reaction specific to the pathway of synthesis of pyrimidine nucleotides (Chapter 25). CTP is an end product of that pathway and exerts feedback inhibition on the enzyme that initiates its synthesis. ATP also binds to the regulatory subunits and causes inhibition of the enzyme.

Figure 11-3 Feedback inhibition of enzymes involved in the biosynthesis of threonine, isoleucine, methionine, and lysine in *E. coli*. These amino acids all arise from L-aspartate, which is formed from oxaloacetate generated by the biosynthetic reactions of the citric acid cycle (Fig. 10-6). ⊖ Allosteric inhibition. ⊟ Repression of transcription of the enzyme or of its synthesis on ribosomes.

In the presence of CTP the binding of the substrates is cooperative, as would be anticipated if there is a two-state conformational equilibrium involving the catalytic subunits. This would be similar to the case depicted in Fig. 9-13 except that trimers rather than dimers are involved and the inhibitor is a part of the regulatory subunit and is controlled by binding of CTP.

Binding of ATP to ACTase decreases the cooperativity in substrate binding, again as predicted for a two-state model. However, as suggested by its structure, this enzyme system is more complex, as also indicated by the observed anticooperative binding of the activator CTP. X-ray studies show that binding of ligands causes a movement along subunit interfaces as well as localized conformational changes within the subunits. These are reminiscent of the changes seen upon binding of oxygen to hemoglobin (Fig. 7-25).

Glycolysis and gluconeogenesis. The highly regulated enzymes phosphofructokinase and fructose 1,6-bisphosphatase catalyze steps *b* and *c* of the reactions in Fig. 11-2, reactions that control glucose metabolism in cells. These enzymes have been studied for many years but the important allosteric effector **β-fructose 2,6-bisphosphate** was not discovered until 1980.[43–46]



β-Fructose
2,6-bisphosphate

This compound, which is formed from fructose 6-phosphate by a new enzyme, **phosphofructokinase-2** (also called fructose 6-phosphate 2-kinase) in step *d*, Fig. 11-2 activates phosphofructokinase allosterically. At the same time it inhibits fructose 1,6-bisphosphatase, an enzyme required for reversal of glycogen breakdown, that is, for the conversion of various metabolites arising from amino acids into glycogen (Fig. 11-2, step *c*).[46a] These same two key regulated enzymes are also affected by many other metabolites. For example, ATP in excess inhibits phosphofructokinase, decreasing the overall rate of glucose metabolism and consequently of ATP production. Citrate, which is exported from mitochondria when carbohydrate metabolism is excessive, inhibits the same enzyme. On the other hand, AMP acts together with fructose 2,6-bisphosphate to activate the pathway for glycogen breakdown and to inhibit that for its synthesis (Fig. 11-2). The concentration of the regulator fructose 2,6-bisphosphate is controlled by mechanisms that are discussed in the following section.

2. Covalent Modification by Phosphorylation and Dephosphorylation

Rapid alteration of the activities of enzymes is often accomplished by **reversible covalent modification**.[39,47] Many different modification reactions are known (Table 11-1) and there are doubtless many more to be discovered. Probably the most widespread and certainly the most studied is **phosphorylation**, the transfer of a phospho group from ATP or other suitable donor to a side chain group on the enzyme. An example is the phosphorylation by ATP of hydroxyl groups of specific serine residues in the two enzymes glycogen phosphorylase and glycogen synthase. These modifications are accomplished through a series or **cascade** of reactions initiated by the binding of hormones to cell surface receptors or by nerve impulses as is shown in Fig. 11-4. In the absence of such a stimulus, glycogen phosphorylase is present in its unphosphorylated or *b* form. Although this form can be activated allosterically by AMP, it is normally nearly inactive. When an appropriate hormone binds to the cell surface a cascade of reactions, as described in Section D, leads to activation of an enzyme called **phosphorylase kinase**.[47a] This enzyme transfers a phospho group from ATP to the –OH group of the side chain of Ser 14 in each subunit (Fig. 11-4, left center), converting the enzyme into the active glycogen-degrading **phosphorylase *a***. This switches the cellular metabolism from that designed to deposit the storage polysaccharide glycogen to one that degrades glycogen to provide the cell with energy. Serine 14 of glycogen phosphorylase is located adjacent to the allosteric AMP binding site (Fig. 11-5) and is surrounded by positively charged arginine and lysine side chains. Phosphorylation induces a rearrangement of hydrogen bonds involving these residues and in some way sends an appropriate signal to the active site. It also increases the affinity for AMP in the allosteric sites.[48,48a] Phosphorylase kinase is allosterically activated by AMP, a product of its action – a feed-forward activator.

The control of glycogen phosphorylase by the phosphorylation–dephosphorylation cycle was discovered in 1955 by Edmond Fischer and Edwin Krebs[50] and was at first regarded as peculiar to glycogen breakdown. However, it is now abundantly clear that similar reactions control most aspects of metabolism.[51] Phosphorylation of proteins is involved in control of carbohydrate, lipid, and amino acid metabolism; in control of muscular contraction, regulation of photosynthesis in plants,[52] transcription of genes,[51] protein syntheses,[53] and cell division; and in mediating most effects of hormones.

Protein kinases and cyclic AMP. Phosphorylase kinase is one of hundreds of different protein kinases which differ in specificity toward their substrates, in

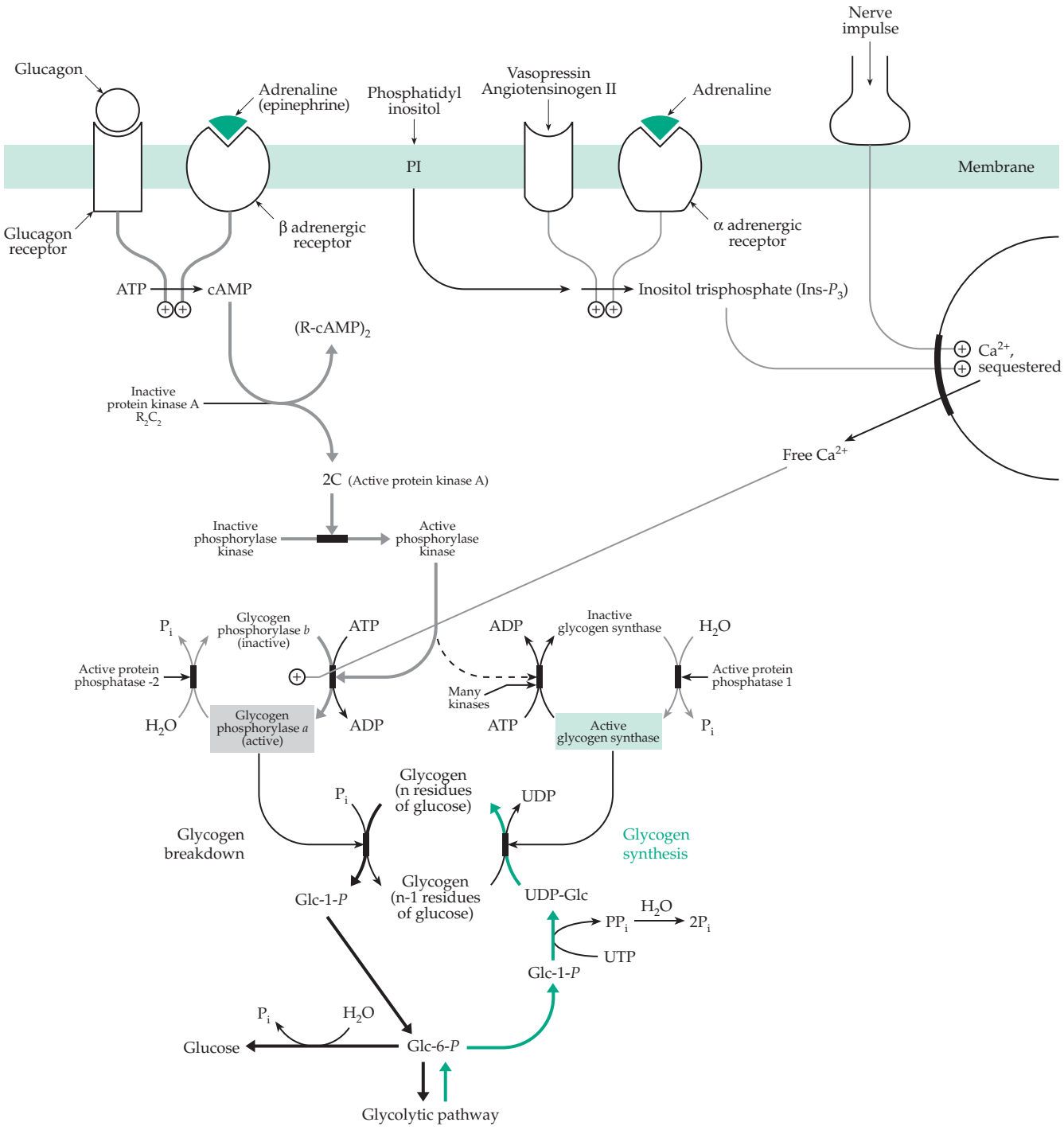


Figure 11-4 Cascades of phosphorylation and dephosphorylation reactions involved in the control of the metabolism of glycogen. Heavy arrows (➞) show pathways by which glucosyl units of glycogen are converted into free glucose or enter the glycolytic pathway. Green arrows (➞) trace the corresponding biosynthetic pathways. Gray arrows (➞) trace the pathway of activation of glycogen phosphorylase by a hormone such as adrenaline (epinephrine) or glucagon and by the action of protein kinases. A few of the related pathways in the network of reactions that affect glycogen metabolism are also shown. This includes protein phosphatases, which remove phospho groups from proteins and allow cells to relax to the state that preceded activation. One of these (protein phosphatase 1) is activated by phosphorylation by an insulin-stimulated protein kinase.[49] However, the significance is uncertain; control of glycogen synthase is complex.[49a]

TABLE 11-1
A Few Covalent Modification Reactions Utilized to Control Metabolism

Reaction	Example	Location of discussion
A. Phosphorylation–dephosphorylation		
Phosphorylation of Ser, Thr	Glycogen phosphorylase	This section
Phosphorylation of Tyr	Insulin receptor	Section G
Adenylylation, Uridylylation	Glutamine synthetase	Chapter 25
ADP-ribosylation		This section
B. Methylation of carboxyl groups	Bacterial glutamyl	Section D,5
	Aspartyl	Box 12-A
	Protein phosphatase 2A	Section C,3
	Ras	Section D,3
C. Formation of carbamino groups		
	In hemoglobin	Eq. 7-23
	In ribulose bisphosphate carboxylase	Chapters 13,23
D. Acylation		
Acetylation	Histones	Chapter 27
Palmitoylation	Ras	Section D,3
Prenylation	Ras	Chapter 22
E. Disulfide formation and cleavage		Chapters 10,15 Section C,4

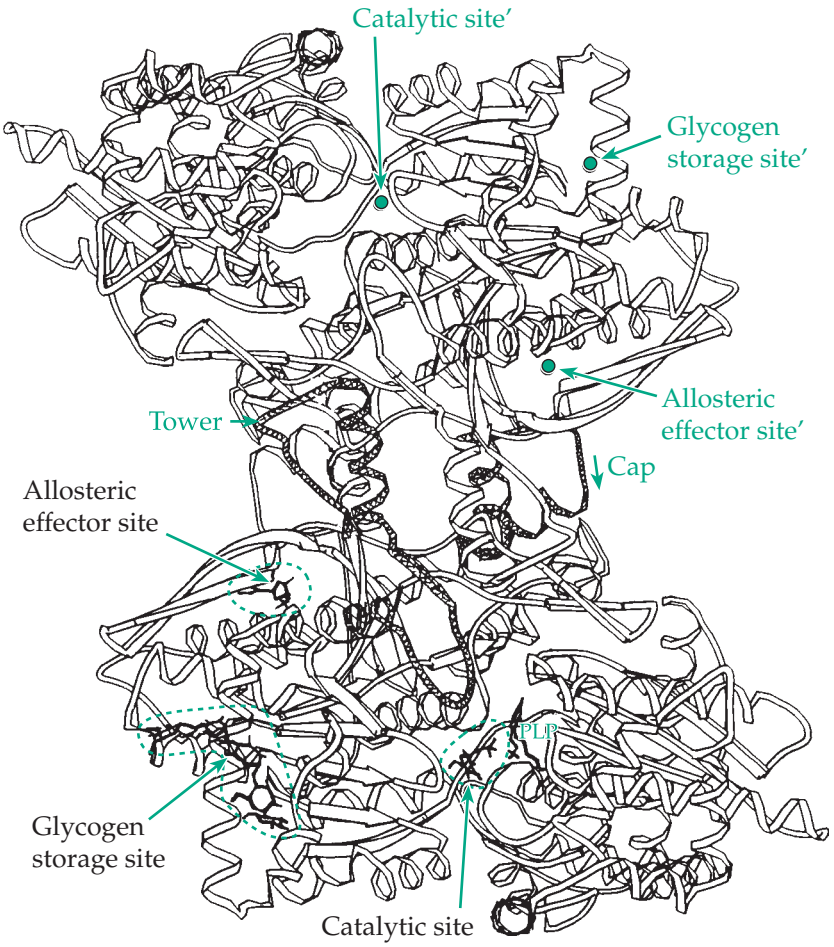
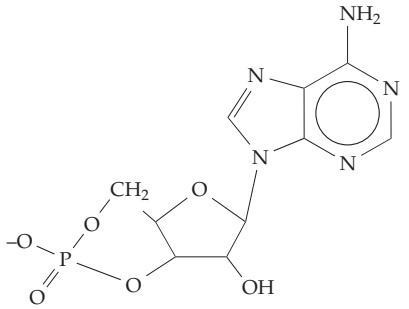


Figure 11-5 Schematic diagram of the glycogen phosphorylase dimer. The view is down the twofold axis of the dimer with the allosteric and Ser-*P* sites toward the viewer. Access to the catalytic site is from the far side of the molecule. The diagram shows the major change in conformation of the amino-terminal residues on phosphorylation. Residues 10–23 of glycogen phosphorylase *b* are shown as a thick solid line. These residues are not well ordered and make intrasubunit contacts. Upon phosphorylation, residues 10–23 change their conformation and are shown dark crosshatched with the position of Ser14-*P* indicated at the intersubunit interface. The fold of residues 24–80 through the α1 helix, the cap, and the α2 helix is shown lightly cross-hatched. The AMP allosteric effector site is located between the α2 helix and the cap region of the other subunit. The glycogen storage site is located on the surface of the subunit and is associated with a long α helix. The catalytic site is at the center of the subunit where the two domains come together. Courtesy of Louise N. Johnson.[48]

the functional groups phosphorylated, and in their allosteric activators.[39,51,54–56] There may be more than 1000 different protein kinase genes in vertebrate animals, accounting for ~2% of the genome.[57] Many cytosolic protein kinases transfer a phospho group from ATP to either a serine or threonine side chain at a β bend or other surface feature of the substrate protein. Some sites of phosphorylation in the substrate proteins contain lysine or arginine residues, separated from the serine or threonine by only one residue but many other sequences may also surround phosphorylation sites.[51,58] In the case of the 750-residue glycogen synthase, seven serine residues in different parts of the chain are phosphorylated by the action of at least five different kinases.[49,49a,59,60] The various kinases phosphorylate groups at different sites and their effects are roughly additive.

Some of the best known protein kinases (designated PKA or cAPK) are those that depend upon **3', 5'-cyclic AMP (cAMP)** as an allosteric effector. They are oligomeric proteins of composition R_2C_2, where R is a regulatory subunit and C is a catalytic subunit. Unless



3',5'-cyclic AMP

cAMP is present, the regulatory subunits interact with the catalytic subunits, keeping them in an inactive inhibited form. However, when cAMP is present it binds to the regulatory subunit dimer, releasing the two active catalytic units (Eq. 11-6). This reaction is

$$R_2C_2 + 4\ \text{cAMP} \rightarrow R_2\ (\text{cAMP})_4 + 2C \qquad (11\text{-}6)$$

reversible and as the concentration of cAMP is reduced by hydrolysis (see Eq. 11-8) the regulatory units recombine with the catalytic subunits and again inhibit them.

There are two prominent types of mammalian cAMP-dependent protein kinases.[51,61] The catalytic subunit is identical for both; the 41-kDa peptide as isolated from beef heart has 350 residues and an N terminus blocked by a myristoyl (tetradecanoyl) group.[62] One phosphoserine and one phosphothreonine are also present.[51] The ~50-kDa regulatory subunits vary in size and may also be subject to additional regulation by phosphorylation.[63] Three-dimensional structures are known for both the catalytic[62,64,65] and the regulatory[66] subunits. A **cyclic GMP** (cGMP)-activated protein kinase is present in some mammalian tissues[67,68] and is widespread in invertebrates.[51,67] The cyclic nucleotide-binding domains of these kinases have structures similar to that of the *E. coli* catabolite activator protein (Fig. 28-6)[51,68] and the catalytic domains are structurally similar to those of many other kinases.

Among the kinases that phosphorylate glycogen synthase is a **casein kinase**, named for the fact that the milk protein casein is also a good substrate. A family of casein kinases are found in the cytoplasm and nuclei of all eukaryotic cells. They phosphorylate serine and threonine side chains but have structures distinct from those of the cAMP-dependent kinases.[69–72] Casein kinase-2 (CK2) phosphorylates many proteins, including several that function in gene replication, transcription, and cell growth and division.[72a] **Theileriosis**, a parasitic disease of cattle in Africa, is caused by the tick-borne protist *Theileria parva*. The condition is often fatal as a result of a leukemia-like condition resulting from overexpression of the casein kinase-2 gene.[72]

Phosphorylase kinase is one of a large group of *specialized* protein kinases, each of which acts on a small number of proteins. It is regulated both by covalent modification and allosterically by **calcium ions**.[73–78] It contains four different kinds of subunits ranging in size from 17 kDa to about 145 kDa and has the composition $(\alpha\beta\gamma\delta)_4$. Phosphorylation of one serine on each of the 145-kDa α and 120-kDa β regulatory subunits is catalyzed by a cAMP-dependent protein kinase. The δ subunit is the Ca^{2+}-binding protein **calmodulin** (Fig. 6-8) and serves as a regulatory subunit sensitive to Ca^{2+}. The 45-kDa γ subunit contains the catalytic domain as well as a calmodulin-binding domain.[79] Other Ca^{2+}-dependent protein kinases include the **protein kinase C** family, which is discussed further in Section E, Ca^{2+}/calmodulin-dependent protein kinases,[80,81] and a plant kinase with a regulatory domain similiar to calmodulin.[82]

Protein tyrosine kinases (PTKs) place phospho groups on the phenolic oxygen atoms of tyrosyl residues of some proteins.[83–85] The resulting phosphotyrosine accounts for only about 1/3000 of the phospho groups in proteins but has aroused interest for two reasons. First, binding of growth hormones, such as **epidermal growth factor** (EGF), **platelet-derived growth factor** (PDGF), and **insulin**, to their receptors stimulates tyrosine-specific protein kinase activity of the receptor proteins (see Figs. 11-11 to 11-13).[69,83,86] Second, a number of cancer-causing **oncogenes** encode similar kinases (Section H). Tyrosine protein kinases are essential components of the cell division cycle (see Fig. 11-15).

Protein phosphatases. Most regulatory alterations in enzymatic activity are spontaneously reversible. The concentration of the allosteric effector soon drops and the covalent modifications are reversed so that the system relaxes to a state approximating the

original one. Among the enzymes required for this reversal are the protein phosphatases that remove the phospho groups placed on amino acid side chains by kinases.[54,86–91] For example, four different phosphatases act on the (inactive) phosphorylated glycogen synthase and on the phosphorylated forms of glycogen phosphorylase and phosphorylase kinase. At least one of these (a protein phosphatase 1; see Fig. 11-4) is regulated by phosphorylation that is stimulated by insulin.[49,49a] The matter is made yet more complex by the presence in tissues of small protein inhibitors which can prevent the action of these phosphatases.[88,92–94] Another naturally occurring inhibitor is the polyether fatty acid **okadaic acid**, a "shellfish poison," actually produced by dinoflagellates but which accumulates in sponges and in mussels and other bivalves.[94] See also Chapter 12, D,4.

Most protein phosphatases are specific toward phosphoserine and phosphothreonine[89,95] or toward phosphotyrosine residues.[90,96–98] Some have a dual specificity.[99] There are Ca^{2+}-activated phosphatases[100] and phosphatases with transmembrane segments connected to receptors on the external cell surface. Some phosphorylation–dephosphorylation regulatory systems utilize bifunctional enzymes consisting of a protein kinase fused to a protein phosphatase. For example, the same *E. coli* enzyme that phosphorylates isocitrate dehydrogenase also hydrolyzes off the phospho group that it has put on.[101] Another bifunctional kinase–phosphatase catalyzes both the formation (reaction *d* of Fig. 11-2) and breakdown (reaction *e*) of fructose 2,6–bisphosphate in eukaryotic tissues. This kinase–phosphatase enzyme is, in turn, controlled by phosphorylation by a cAMP-dependent protein kinase. In the liver, this latter phosphorylation strongly inhibits the kinase activity preventing buildup of fructose 2,6-bisphosphate, the allosteric activator of phosphofructokinase-1, which catalyzes reaction *b* of Fig. 11-2. Consequently, when a pulse of cAMP is generated within a hepatocyte, glycolysis is blocked but **glycogenolysis**, the breakdown of glycogen by phosphorylase, is stimulated. The glucose 6-phosphate that is formed is hydrolyzed by glucose-6-phosphatase releasing glucose into the bloodstream. On the other hand, in heart and probably in most other tissues, a different isoenzyme form of the bifunctional enzyme is present. Phosphorylation by cAMP activates the kinase and inhibits the phosphatase. Consequently, in these tissues cAMP induces the activation of glycogenolysis and glycolysis coordinately.[102–105] In brain a different allosteric activator, **ribose 1, 5-bisphosphate**, may regulate phosphofructokinase-1.[106]

Phosphorylation in bacteria. A bacterial enzyme whose activity is controlled by phosphorylation is **isocitrate dehydrogenase**. Transfer of a phospho group to the –OH of Ser 113 completely inactivates the *E. coli* enzyme,[107,108] causing isocitrate to build up in the citric acid cycle (Fig. 10-6) and to be diverted into the glyoxylate pathway, which is depicted in Fig. 17-16. In this instance, it appears likely that the negative charge of the added phospho group causes electrostatic repulsion of the substrate isocitrate. In agreement with this concept, mutation of Ser 113 of this enzyme to Asp (mutant S113 D) also inactivates the enzyme.[109]

In addition to kinase–phosphatase cycles, bacteria use at least two other ATP-dependent regulatory mechanisms.[110] In the **sensor kinase/response regulator** (or "two-component") systems[110-111] a sensor protein, upon being allosterically activated, phosphorylates itself (autophosphorylation) on a specific histidyl residue to form an *N*-phosphohistidine derivative. The phosphohistidine then transfers its phospho group onto a specific aspartyl group in the response regulator causing the regulator to bind to its target protein and to exert its regulatory effect. The best known example involves the control of the motion of bacterial flagella which is discussed briefly in Section D,5 and further in Chapter 19.

The third bacterial regulatory device is the phosphoenolpyruvate:sugar phosphotransferase system (Eq. 8-4). It is involved not only in transport but also in controlling a variety of physiological processes.[110,112,113]

3. Other Modification Reactions Involving Group Transfer

Nucleotidylation, the transfer of an entire nucleotidyl unit, rather than just a phospho group, to a protein is sometimes utilized for regulation. For example, glutamine synthetase of *E. coli* is modified by **adenylylation**, transfer of an adenylyl group from ATP to a tyrosine side chain.[114–116] Relaxation to the unmodified enzyme is catalyzed by a deadenylylating enzyme. A regulatory protein that undergoes reversible uridylylation[117] also functions in this system (see Fig. 24-7). Several mitochondrial and cytoplasmic proteins are modified by attachment of ADP-ribosyl groups to specific guanidino groups of arginine and other side chain groups.[114,118–123] This **ADP–ribosylation** requires the coenzyme NAD^+ as the ADP-ribosyl donor and is also catalyzed by bacterial toxins such as cholera toxin (Box 11-A) and by diphtheria toxin (Box 29-A). A second kind of ADP–ribosylation occurs within nuclei where the enzyme **poly(ADP–ribose) synthase** catalyzes both an initial ADP–ribosylation of an amino acid side chain group and also addition of more ADP-ribosyl units to form a polymer[124] (see Chapter 27).

Like inorganic phosphate, inorganic sulfate can be converted into an activated form in which the sulfate resembles the terminal phospho group of ATP (Eq. 17-38). The resulting **activated sulfo group** can be transferred to other compounds including enzymes. Formation of

BOX 11-A CHOLERA TOXIN AND OTHER DANGEROUS PROTEINS

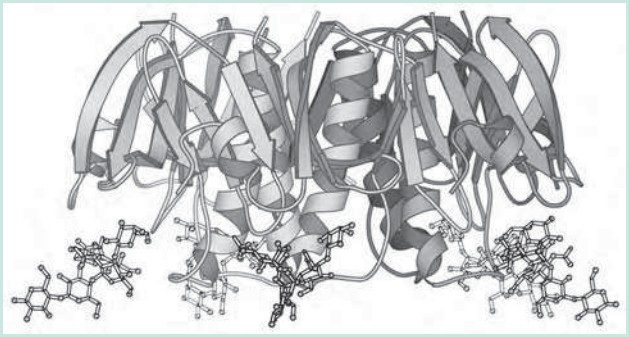
As recently as 1980 it was estimated that there were ~ 100 million cases of acute diarrhea in Asia, Africa, and Latin America;[a] in 1991 there were four million deaths among children under five years of age.[b] The causative agents are bacteria and one of the most dangerous is *Vibrio cholerae*, which multiplies in the small intestine and secretes an **exotoxin**. Cholera toxin causes such a rapid loss of fluid and salts from the body that death occurs very quickly, even in adults. There is little cellular damage and almost all deaths can be prevented by intravenous administration of water, salts, and the antibiotic tetracycline. Fluids can also be given orally if glucose, which promotes intestinal absorption, is included with the Na^+, K^+, Cl^-, and HCO_3^- salts.[b]

Cholera toxin consists of one 240-residue A subunit and a ring of five 103-residue B subunits.[c–e] The latter is a "targeting complex," a bacterial lectin each subunit of which binds to the galactose and sialic acid termini of a single molecule of ganglioside G_{M1}.

Galβ1→3Galβ1→4Galβ1→4Glcβ1–Ceramide
3
↑
NeuNAcβ2

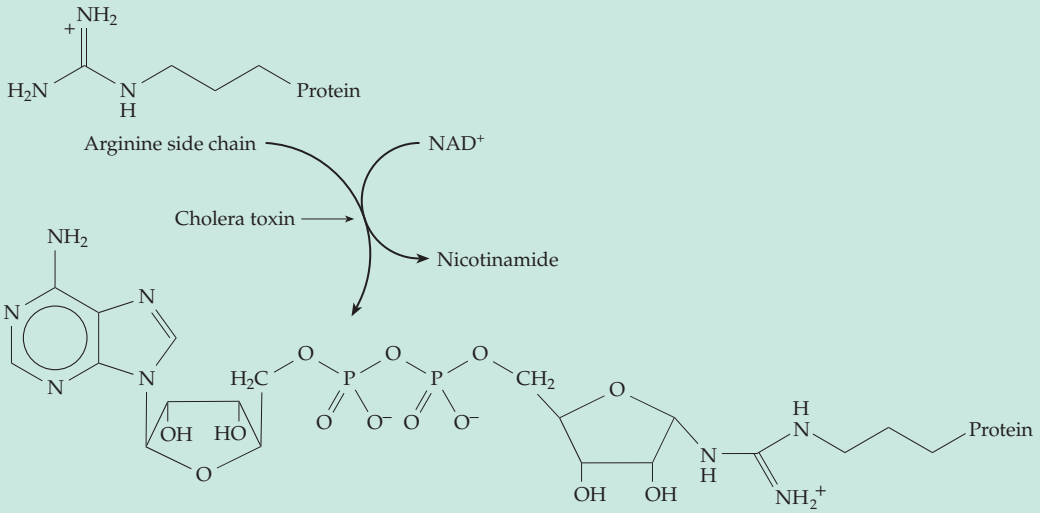
Ganglioside G_{M1} (See also Fig. 20-11)

The nonpolar ceramide chains of the ganglioside are buried in the membrane bilayer. The initial tight binding (K_f ~ 2 x 10^7 M^{-1}) of the toxin is followed by a poorly understood process in which the A subunit enters the cell. The peptide chain of this subunit is cut by bacterial proteases to give A_1 and A_2 fragments joined by an S–S bridge. Within the victim's cells the disulfide is reduced to generate the active toxin, the 192- to 194- residue A_1 fragment.[f,g]

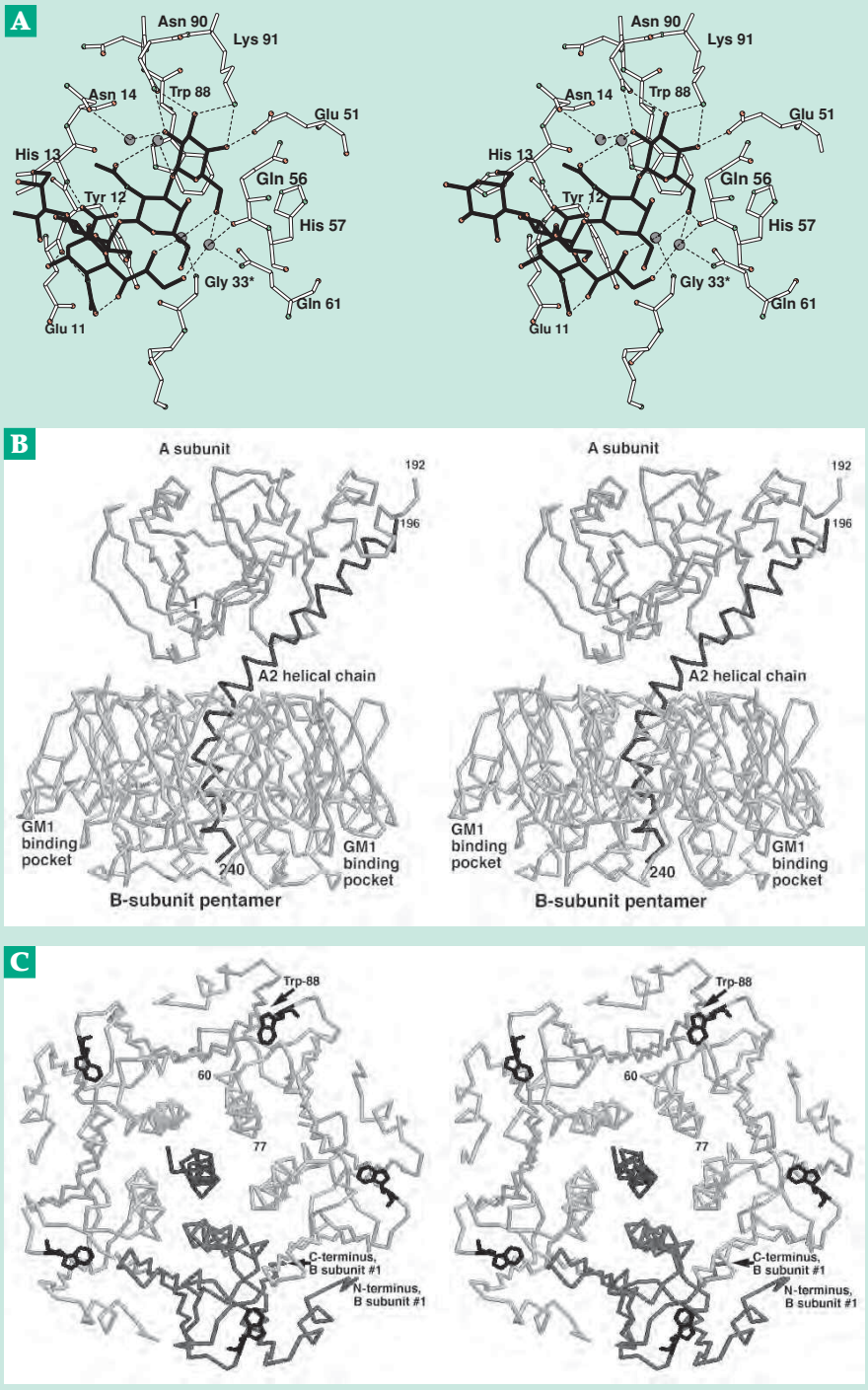


The three-dimensional structure of cholera toxin.[e] Side view of the β subunit pentamer as a ribbon drawing. Bound noncovalently to it are five molecules of the ganglioside G_{M1} (compare with the structure in Fig. 7-5). The diacyl glycerol parts of the gangliosides are buried in the membrane that lies below the toxin molecule. Courtesy of W. G. J. Hol.

This toxin subunit is an enzyme, an ADP-ribosyltransferase which catalyzes transfer of ADP-ribosyl units from the coenzyme NAD^+ to specific arginine side chains to form *N*-ADP-ribosyl derivatives of various proteins. Of the proteins modified by cholera toxin, the most significant is the guanyl nucleotide regulatory protein G_s of the adenylate cyclase system.[c,f,h] ADP ribosylation of arginine 201 of the α subunit of protein G_s inhibits the GTP hydrolysis that normally allows the protein to relax to an unactivated form.[e] The ADP-ribosylated G_s keeps adenylate cyclase activated continuously and



BOX 11-A (continued)



(A) Stereoscopic view of the G_{M1} pentasaccharide binding site, showing both direct and solvent-mediated hydrogen bonding interactions between sugar and protein residues. The viewpoint is from "underneath" the membrane binding surface of the B pentamer. Starred residues are from an adjacent monomer. The terminal galactose residue of the pentasaccharide (upper right) is most deeply inserted into the binding site and is involved in the greatest number of identifiable binding interactions. The sialic acid residue, near the bottom of the figure, is also involved in several hydrogen-bonding interactions. Hydrophobic interactions include the approach of the sialic acid acetyl group to the edge of the Tyr 12 phenyl ring and the positioning of the terminal galactose sugar ring parallel to the indole ring of Trp 88. Figures generated using program MolScript (Kraulis, 1991). From Merritt *et al.*[e] (B) Side view of the intact AB_5 toxin as an α-carbon trace. The nicked A chain is at the top. The nick can be seen in the upper right corner, as can a disulfide bridge connecting the A_1 and A_2 segments. A single α helix extends into the "pore" in the center of the B pentamer. The side chains of Trp 88 of the B subunits have been added to mark the ganglioside binding sites. These side chains are also seen in (A). (C) View of the AB_5 molecule from the bottom showing the helix from the A_2 fragment surrounded by a tight cage of five long helices from the B subunits. Residues 237–240 of the A_2 fragment have the KDEL sequence and may extend from the pore to contact the membrane. (B) and (C) courtesy of Edwin M. Westbrook. See Zhang *et al.*[c]

generating abnormally high concentrations of cAMP. It is the effect of the cAMP on proteins of the intestinal mucosal cell membranes that causes the disastrous excessive secretion of water and salts that are characteristic of cholera.

Most strains of *V. cholerae* are relatively harmless but they may suddenly be transformed into a virulent toxin-producing form by infection with a bacterial virus similar to M13 (Chapter 5), which carries the toxin gene. Entrance into the *Vibrio* cell occurs with the help of pili which are present in many strains.[i,j]

Many other bacterial toxins have AB_n structures similar to that of cholera toxin. For example, toxin-producing strains of *E. coli* are also important causes of diarrhea in humans and in domestic animals.[k–m]

BOX 11-A CHOLERA TOXIN AND OTHER DANGEROUS PROTEINS (continued)

The heat-labile *E. coli* enterotoxin, whose gene is carried on a plasmid, is a close relative of cholera toxin[n,o] and also catalyzes ADP ribosylation of arginine 201 of the $G_{s\alpha}$ subunit.[m] *Bordetella pertussis*, which causes whooping cough, forms a similar toxin that attacks the inhibitory regulatory protein G_i[p,q] as well as transducin and inactivates them by ADP ribosylation. Diphtheria toxin (Box 29-A), the exotoxin from *Pseudomonas aeruginosa*, and the toxin from *Clostridium botulinum* also catalyze ADP-ribosylation reactions.[k,r]

Some bacteria produce effects similar to those of cholera toxin in different ways. For example, among a variety of toxic proteins produced by *Bacillus anthracis*, the causative agent of anthrax, is an adenylate cyclase that is able to enter the host's cells.[s] Similarly, *B. pertussis*, in addition to its ADP-ribosylating toxin, produces a calmodulin-stimulated adenylate cyclase which, when taken up by phagocytic cells, disrupts their function in the body's defense system.[t–w] In addition to the heat-labile enterotoxin, some strains of *E. coli* produce *heat-stable* toxins, small 18-residue peptides related in structure to the intestinal peptide **guanylin**.[l,x] These peptides bind to a membrane-bound guanylate cyclase activating fluid secretion into the intestine.

Why do our cells obligingly provide both initial receptors and means of uptake for these dangerous toxic proteins? Some ADP-ribosylation reactions are a natural part of cell function[y] and some hormones, for example thyrotropin, seem to stimulate their activity. It may be that the toxins use mechanisms designed to respond to normal hormonal stimulation.

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tyrosine-*O*-sulfate residues may be a widespread regulatory mechanism.[125–127] One of the proteins known to contain a tyrosine sulfate residue is the blood protein fibrinogen. Many polysaccharides and oligosaccharides on glycoproteins exist in part as sulfate esters (Fig. 4-11).[128,129]

Carboxyl groups of certain glutamate side chains in proteins that control bacterial **chemotaxis** are methylated reversibly to form methyl esters.[130] This **carboxylmethylation** occurs as part of a reaction sequence by which the bacteria sense compounds that can serve as food or that indicate the presence of food (Section D,5, Fig. 11-8, and Chapter 19). Demethylation occurs by hydrolysis, which is catalyzed by esterases. Carboxylmethylation also occurs in eukaryotic cells but is often substoichiometric and part of a mechanism for repair of isomerized or racemized aspartyl residues in aged proteins (Box 12-A). However, the major eukaryotic protein phosphatase 2A is carboxylmethylated at its C terminus,[131] as are the Ras proteins discussed in Section D,3.

It was pointed out in Chapter 4 that many proteins, especially those secreted from cells or taking up residence within membranes, are **glycosylated**. Specific

glycosyl groups may be removed or added to a protein. Such alterations may all be regarded as parts of control mechanisms that direct the proteins to their proper locations or determine the length of time that they remain active. The nuclear histones undergo extensive and reversible **acetylation**[132,133] which is thought to be important to replication of DNA and to transcription (Chapters 27 and 28).

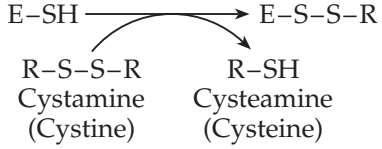
4. Thiol-Disulfide Equilibria

The activity of enzymes is sometimes controlled by the formation or the reductive cleavage of disulfide linkages between cysteine residues within the protein. An example is provided by the effects of light on enzymes of chloroplasts. Light absorbed by the photosynthetic reaction centers generates NADPH, which in turn reduces **thioredoxin**. This is a small protein containing a readily accessible S–S bridge that can be reduced to a pair of SH groups (Box 15-C). Within illuminated chloroplasts, these newly formed SH groups reduce disulfide bridges to activate a series of enzymes including fructose 1,6-bisphosphatase that participate in the photosynthetic incorporation of CO_2 into sugars[134,135] (see Chapter 23). Thioredoxins also function within bacteria, fungi, and animals, serving as electron carriers for processes, some of which are involved in metabolic control. A number of known enzymes and other proteins, including insulin, contain reducible S–S bridges within peptide loops as in the thioredoxins. Another possible control mechanism for SH-containing enzymes depends upon formation of **mixed disulfides** with small SH-containing metabolites. For example, **cysteamine disulfide**, a minor constituent of cells, cysteine, or some small disulfide, could be released

$$H_3^+N{-}CH_2{-}CH_2{-}S{-}S{-}CH_2{-}CH_2{-}NH_3^+$$

Cysteamine disulfide (cystamine)

following hormonal or other stimulation and could react by disulfide exchange (Eq. 11-7) to either inactivate or activate an enzyme.



(11-7)

Almost all cells contain a high concentration (3–9 mM) of the thiol-containing tripeptide **glutathione** (G–SH, Box 11-B). In its disulfide form it participates in forming disulfide bridges in secreted extracellular proteins (Eq. 10-9) via intermediate mixed disulfides. Mixed disulfides with glutathione as well as with other thiols can also be formed within cells by oxidative reactions. Reduction of these disulfides by reduced glutathione will return the enzymes to their reduced states. The small protein **glutaredoxin**, whose eukaryotic forms are also called **thioltransferase**, resembles thioredoxins. It undergoes reduction by glutathione and, in turn, reduces S–S linkages in a different set of proteins than those reduced by thioredoxin.

Low-molecular-mass thiols such as coenzyme A and protein-bound thiol cofactors such as phosphopantetheine are present in all cells. Their SH groups can also be oxidized to disulfides and it is of interest that in resting bacterial spores these compounds exist largely as disulfides or mixed disulfides. Upon germination of the spores special enzymes reduce the disulfides.[136] Some proteins involved in control of protein synthesis contain SH groups that add covalently to C-6 atoms of a uracil ring in specific mRNA molecules. Control of their state of reduction may also be important.[137]

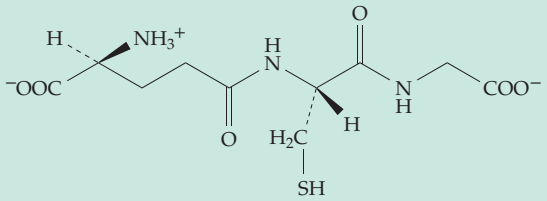
5. Regulatory Effects of H^+, Ca^{2+}, and Other Specific Ions

The pH of the cytoplasm is controlled tightly. Yet transient changes can occur and may affect many aspects of metabolism. For example, rapid glycolysis leads to lactic acid formation with an associated drop in internal pH. Both increases and decreases in pH have been associated with successive stages in embryonic or larval development.[138] Cytoplasmic pH changes may serve as regulatory signals. A well-understood example is the Bohr effect on oxygenation of hemoglobin (Chapter 7). Another is the protein kinase C-stimulated H^+/Na^+ exchange through membranes. Because the Na^+ concentration is high outside cells and low inside, the exchange leads to an increase in cytosolic pH with many resultant effects on metabolism.[139,140] Exchange of external Cl^- for internal HCO_3^- also affects pH.[141]

Uptake of Ca^{2+} into cells, or release of this ion from intracellular stores, is a major regulatory mechanism in many if not all cells (see Section E). Mn^{2+} activates **phosphoenolpyruvate carboxykinase** (Eq. 13-46) and may be a regulator of gluconeogenesis.[142] Iron controls the synthesis of ferritin and of transferrin receptors[137] (Chapter 16). The specific metal ions present in many biological macromolecules are likely to participate in additional regulatory processes.

Phosphate and bicarbonate ions are important substrates for many enzymatic processes and as such have regulatory functions. Bicarbonate controls the key enzyme of photosynthesis, **ribulose bisphosphate carboxylase**, by carbamate formation (Fig. 13-12). Chloride ions activate amylases and may affect the action of "G proteins" that mediate hormone actions. Other observed effects of ions are too numerous to mention.

BOX 11-B GLUTATHIONE, INTRACELLULAR OXIDATION–REDUCTION BUFFER



Glutathione (γ-L-Glutamyl-L-cysteinylglycine)

In 1888, de Rey-Pailhade discovered the sulfur-containing tripeptide that we now know as glutathione (G–SH).[a] By 1929 it had been characterized by F. G. Hopkins and others as an unusual tripeptide present in most, if not all, eukaryotic cells. Within animal cells the concentration is typically 1–5 mM. Lower levels are found in many bacteria.[b–g]

The most interesting chemical characteristics of glutathione are the γ-glutamyl linkage and the presence of a free SH group. The latter can be oxidized to form a disulfide bridge linking two glutathione molecules.

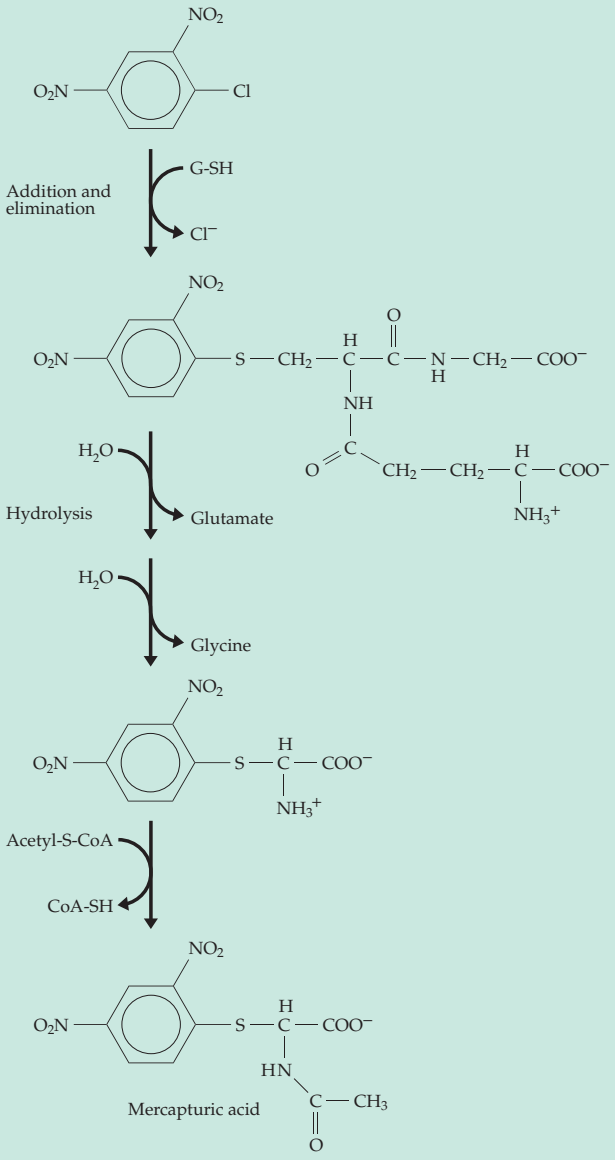
$$2G\text{—}SH - 2[H] \rightarrow G\text{—}S\text{—}S\text{—}G$$
$$E^{\circ\prime}\ (pH\ 7) = -0.25\ V$$

It is this redox reaction that has focused attention on glutathione as an intracellular reducing agent whose primary function may be to keep the SH groups of proteins reduced (see Section C). Glutathione is usually maintained in its reduced form by the flavoprotein glutathione reductase (Fig. 15-12).

Although it is primarily an intracellular compound, glutathione is secreted by epithelial and other cells. It may regulate the redox state of proteins in plasma and other extracellular fluids as well as within cells. In addition, glutathione released from the liver may be an important source of cysteine for other tissues. In the endoplasmic reticulum and the periplasm of bacteria glutathione functions in crosslinking thiol groups in newly formed proteins (Eq. 10-9).

Glutathione also has a series of **protective functions**. It reduces peroxides via the selenium-containing **glutathione peroxidase** (Box 15-H) and is part of a system for trapping and detoxifying harmful free radicals. The importance of this function is suggested by the fact that *Entamoeba histolytica*, an organism that lacks both mitochondria and aerobic respiration, produces no glutathione.[h] It may be that the primary function of glutathione in eukaryotes is to protect cells against oxygen toxicity associated with their mitochondria.[i] The renewal of free radicals and regeneration of protein –SH groups may involve the cooperation of glutathione, glutaredoxin (Box 15 - C), and ascorbic acid.[j]

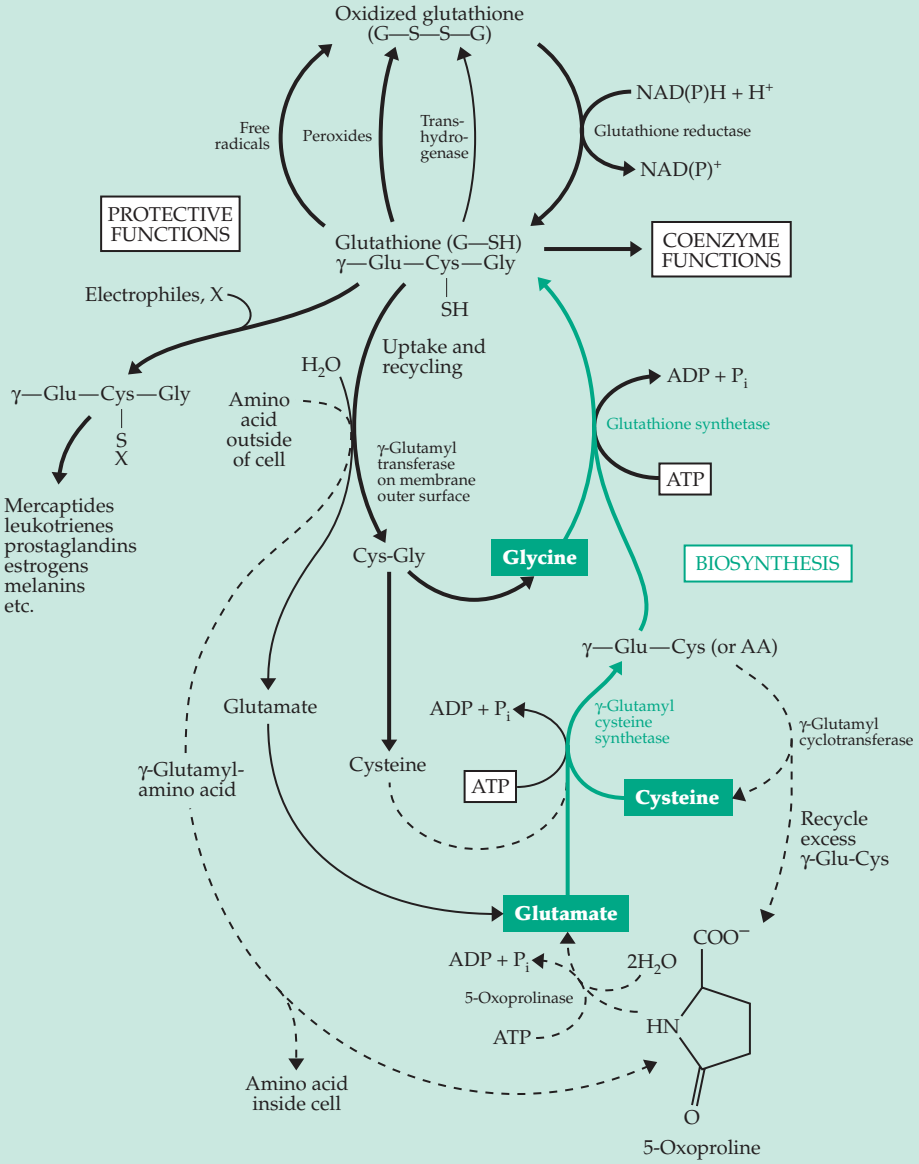
Another protective function is fulfilled by the formation of soluble **mercaptides** and other "conjugates" of glutathione with many foreign substances (**xenobiotics**). These conjugates are made by the action of a large family of **glutathione transferases**[e,k,l] (see also Chapter 13) which catalyze addition reactions of the thiolate group of glutathione with epoxides, alkylating compounds, and chlorinated aromatic hydrocarbons. The addition step is often followed by elimination, e.g., of chloride ion, as in the following example. Two steps of hydrolysis and an acetylation by acetyl-CoA form a mercapturic acid:



BOX 11-B (continued)

The mercapturic acids and related compounds can then be exported from cells by an ATP-dependent export pump.[m] Glutathione is a coenzyme for **glyoxalase** (Eq. 13-33), **maleylacetoacetate isomerase** (Eq. 13-20), and **DDT dehydrochlorinase**. The latter enzyme catalyzes elimination of HCl from molecules of the insecticide and is especially active in DDT-resistant flies.[a] Glutathione is said to be the specific factor eliciting the feeding reaction of *Hydra*; that is, the release of glutathione from injured cells causes the little animal to engulf food.

Synthesis of glutathione occurs within cells via the ATP-dependent reactions in the following scheme.[n,o] Much more γ-glutamylcysteine is synthesized than is converted to glutathione and the excess is degraded by γ-glutamyl cyclotransferase to form the cyclic amide 5-oxoproline. Cleavage of ATP is required to reopen the ring to form glutamate. Although biosynthesis is exclusively intracellular, most glutathione appears to be secreted and degraded by extracellular enzymes. The membrane-bound γ-glutamyl transferase catalyzes hydrolysis of



Scheme illustrating interrelationships of the biosynthesis and protective, coenzymatic, and transport functions of glutathione. See also Meister.[c]

BOX 11-B GLUTATHIONE, INTRACELLULAR OXIDATION–REDUCTION BUFFER (continued)

glutathione to cysteinylglycine which is further cleaved by a peptidase. The activity of γ-glutamyltransferase varies among tissues and is especially high in cells of the kidney tubules. The cysteine and glycine released by the peptidase may reenter the cells by a Na^+-dependent process. Meister proposed that the γ-glutamyltransferase, acting on amino acids, forms γ-glutamyl amino acids (see scheme) which are released *within* cells and are cleaved by γ-glutamyl cyclotransferase.[c] The cleavage of glutathione would provide the driving force for amino acid uptake. However, this is probably a minor pathway.[p]

Trypanosomes contain little glutathione but a large amount of **trypanothione** [N^1,N^8-bis (glutathionyl) spermidine].[q] This diamide of spermidine (Chapter 24) is in equilibrium with its disulfide, a 24-membered macrocyclic structure, and appears to have functions similar to those of glutathione. Trypanothione reductase, which is unique to trypanosomes, is a potential target for antitrypanosomal drugs,[q–s] as is trypanothione synthetase.[t] Bacteria that do not synthesize glutathione usually accumulate some other thiol, e.g., α-glutamylcysteine or coenzyme A, in high concentrations.[u,v]

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6. Compartments and Organized Assemblies

The geometry of cell construction provides another important aspect of metabolic control. In a bacterium, the periplasmic space (Fig. 8-28) provides a compartment that is separate from the cytosol. Some enzymes are localized in this space and do not mix with those within the cell. Other enzymes are fixed within or attached to the membrane. Eukaryotic cells have more compartments: nuclei, mitochondria (containing both matrix and intermembrane spaces), lysosomes, microbodies, plastids, and vacuoles. Within the cytosol the tubules and vesicles of the endoplasmic reticulum (ER) separate off other membrane-bounded compartments. The rate of transport of metabolites through the membranes between compartments is limited and often is controlled tightly.

While many enzymes appear to be dissolved in the cytosol and have no long-term association with other proteins, enzymes that catalyze a series of consecutive reactions may form complexes within which substrates are **channeled**.[143–147] Many enzymes are attached to membranes where they may be held close together as organized assemblies.[146] This appears to be the case for oxidative enzymes of mitochondria (Chapter 18) and for the cytoplasmic fatty acid synthetases (Chapter 21). In bacterial fatty acid synthesis, the product of the first enzyme is covalently attached to a "carrier" and, while so attached, is subjected to the action of a series of other enzymes. In eukaryotes several enzymes form domains of a single fatty acid synthase. Efficient substrate channeling results. Tryptophan synthase (Fig. 25-3) passes indole through a tunnel between subunits.[146a] Both NH_3 and carbamate ions pass through tunnels between subunits of *E. coli* carbamoyl phosphate synthetase (Eq. 24-22). The product carbamoyl phosphate may then be passed directly to aspartate carbamoyltransferase for synthesis of carbamoylaspartate in the pyrimidine biosynthetic pathway.[146b] Channeling is sometimes difficult to

prove. Geck and Kirsch have provided a generally useful technique for testing. A large amount of a genetically modified, inactive form of an enzyme is added. Unless channeling occurs, this will decrease the rate of a reaction whose rate is limited by diffusion or by instability of an intermediate.[146c]

Proteasomes (Box 7-A) have enzymatic sites within a protected box which limits the escape of long peptide fragments. Membrane anchors (Chapter 8), often consisting of acyl or polyprenyl groups, hold many proteins to cell surfaces and strong protein–protein bonds hold many others.[146d] Enzymes involved in cell signaling, and discussed in the following section, are often anchored close together on membrane surfaces.[148,149]

D. Hormones and Their Receptors

A major element in the control of the metabolism of a cell is provided by chemical messages sent from *other cells* and sensed by receptors on the cell membrane or in the cytoplasm. Hormones such as insulin, adrenaline, and the sex hormones are released from an organ and travel through the blood, affecting tissues throughout the body. There are many such hormones. A large number of other hormones have more local effects, influencing mostly adjacent cells. When released at nerve endings these substances are called **neurotransmitters**. Chemical messages are probably sent from virtually all cells to their immediate neighbors, affecting both their growth and their behavior. In recent years the very reactive and toxic compounds **nitric oxide (NO)** and **carbon monoxide (CO)** have been identified as important hormones. These compounds can diffuse rapidly and react with many compounds within cells. Their actions are more rapid, more rapidly ended, and probably less specific than those of most hormones.

Hormones bind to **receptors** on their "target" cells. The receptors are often integral membrane proteins on cell surfaces. Binding of the hormone often causes a conformational alteration that "activates" the receptor. In some cases the receptor is an enzyme and the hormone an allosteric activator. In others, the activated receptor interacts with an enzyme in the cytoplasm or on the membrane facing the cytoplasm. This enzyme may generate a **second messenger**, a substance that can diffuse throughout the cell and alter metabolism by exerting allosteric effects on various other enzymes. The best known second messenger is cyclic AMP but there are many others, a few of which are listed in Table 11-2.

Some hormone–receptor complexes enter the cell via endocytosis in coated vesicles. Within the cell both the receptor and the bound hormone, if a peptide, may be degraded by proteases. The initial binding of the hormone may induce the release of a second messenger, while the degradation of the receptor complex at a later time releases peptides that may be additional second messengers. This is one way in which hormones may elicit a rapid response followed by delayed responses.

While many hormones bind to surface receptors the steroid hormones, which are lipid in nature, pass through the cell membrane and bind to receptor proteins in the nucleus. The resulting hormone–protein complexes induce changes in gene expression through regulation of transcription (Fig. 11-1, top). These receptors are considered in Chapter 22 and hormones are considered further in Chapter 30.

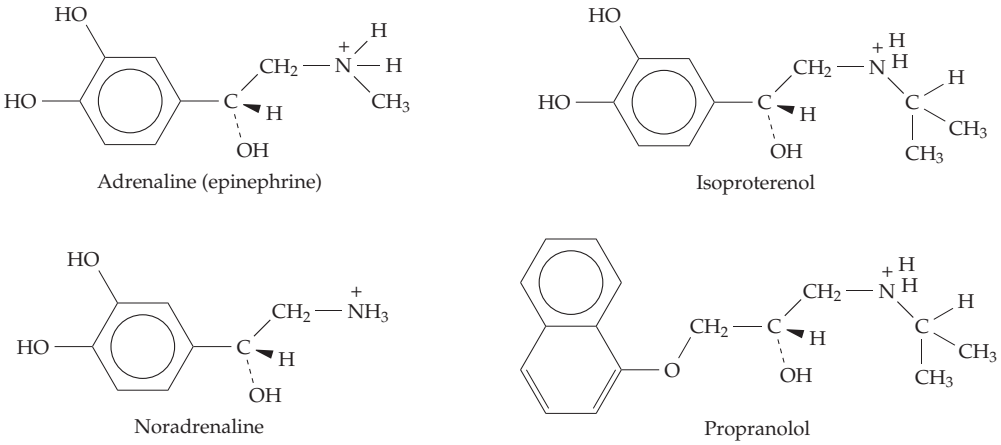
1. Beta Adrenergic Receptors and Related Seven-Helix Proteins

Sites that bind **adrenaline** (epinephrine), **noradrenaline** (norepinephrine), and related **catecholamines** (see Chapter 30) to almost all cell surfaces are classified as either **α adrenergic** or **β adrenergic receptors**. The β receptors, which have been studied the most,[150] occur as two major types. The β_1 receptors have approximately equal affinity for adrenaline and noradrenaline, whereas the β_2 receptors, the most common type, are more nearly specific for adrenaline. Binding of the hormone or other agonist such as **isoproterenol** to any of the β receptors stimulates cAMP formation within the cell.[153] The receptors belong to a family of integral membrane proteins related in sequence to the **opsins** of the retina and the light-operated proton pump bacteriorhodopsin (Fig. 23-45) and probably have a very similar three-dimensional structure. Based upon the known three-dimensional structures of rhodopsin and bacteriorhodopsin (Chapter 23), sequence comparisons, and much other evidence, all of these receptors are probably folded into groups of seven hydrophobic membrane-spanning helices arranged as closely packed bundles with folded loops protruding into the cytoplasm and into the extracellular space. The N termini of the proteins are thought to be in the extracellular space and the C termini in the cytoplasm as shown in Fig. 11-6.[153a,b,c] Defects in structure or functioning of human β receptors have been associated with both asthma[154] and heart failure.[155,155a,b]

Among the proteins phosphorylated in response to formation of cAMP are the β adrenergic receptors themselves. Phosphorylation occurs within a cluster of serine and threonine residues near the C terminus as is indicated in Fig. 11-6A by the action of various kinases including a **receptor kinase**[156,156a] which may be anchored nearby.[157] The effect of this C-terminal phosphorylation is to decrease the sensitivity of the receptor so that after a few minutes it conveys a diminished response. Thus, cAMP exerts feedback inhibition of its own synthesis. *Desensitization upon continuous occupancy*

TABLE 11-2
Some Molecules (Second Messengers) That Carry Intracellular Signals

Compound	Metabolic state	Response	Location of discussion
Cyclic AMP	Stimulation of β adrenergic receptors	Increased glycogenolysis, glycolysis	Sections C,2 and D,2;
Cyclic GMP	Visual stimulation	Neuronal signal	Chapter 23, Section D
Ca^{2+}	Ca^{2+} channels open	Muscular contraction, others	Box 6-D, Section E Chapter 19, Section B,4
Inositol-1,4,5-trisphosphate and related compounds	Stimulation of α adrenergic receptors, various other stimuli	Opens Ca^{2+} channels in ER	Section E
Diadenosine 5'-tetraphosphate (Ap_4A)	Oxidative or heat stress		Chapters 28, 29
Guanosine 5'-diphosphate, 3-diphosphate (ppGpp)	Nutritional stress		Chapter 29
Mn^{2+}	Low glucose	Increased PECK activity	Chapter 17, Section L
		Increased arginase activity	Chapter 16, Section E
Cyclic ADP-ribose (cADPR) and 2'-P-cADPR			Chapter 15, Section E



by an agonist is a property of many other receptors as well. When a receptor is no longer occupied, phosphatases remove the phospho groups added by the receptor kinase permitting the sensitivity to rise again. This phosphorylation–dephosphorylation cycle appears to be only one of several mechanisms by which cells regulate receptor sensitivity.[155b,158,158a,158b]

Many other receptors also have a seven-helix structure similar to that of the adrenergic receptors. These include receptors for the following: **glucagon**, one of the pancreatic hormones regulating glucose metabolism;[159,160] **vasopressin** (Fig. 2-4);[161,162] **lutropin**, another pituitary hormone;[163] other gonadotropins;[164] the **thyrotropin-releasing factor** (TRF; Fig. 2-4);[165] a receptor for **KDEL** peptide sequences. The KDEL receptor functions in the return of soluble proteins containing the KDEL motif from the Golgi to the endoplasmic reticulum.[152]

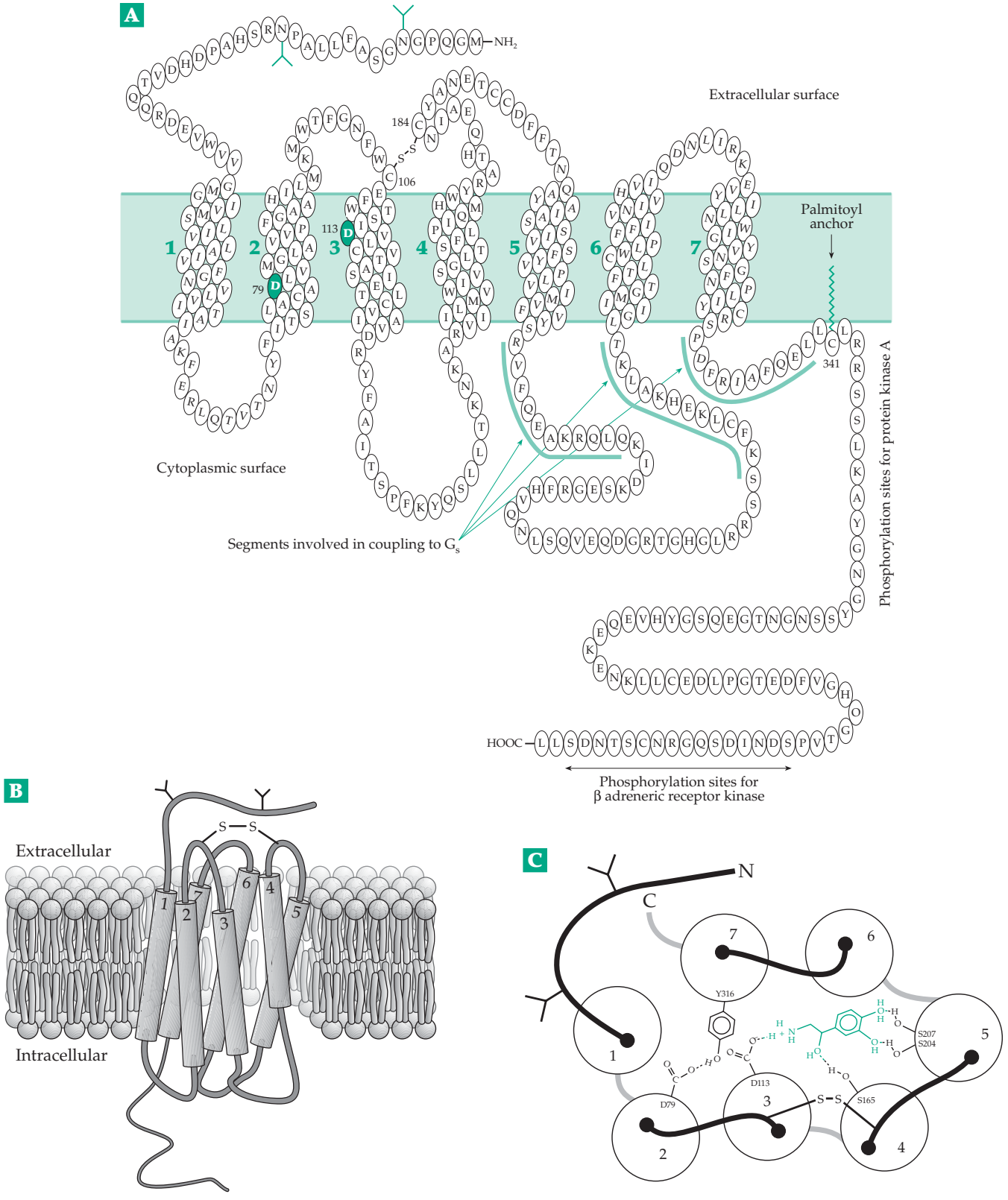


Figure 11-6 (A) Proposed organization of the human β_2 adrenergic receptor. The 413-residue polypeptide chain is arranged according to the model for rhodopsin as seven transmembrane helices. The two N-glycosylation sites, which may carry very large oligosaccharides, are indicated by Y. The palmitoylated cysteine 341 is shown with its alkyl side chain embedded in the membrane. Aspartates 79 and 113, also shown in (C), are shaded. After Strosberg[151] and Scheel and Pelham.[152] (B) Arrangement of the seven helices suggested by the rhodopsin structure. (C) Hypothetical view of the receptor from the external membrane surface showing a molecule of noradrenaline bound to hydrophilic residues deep in the cleft between the helices. From Strosberg.[151]

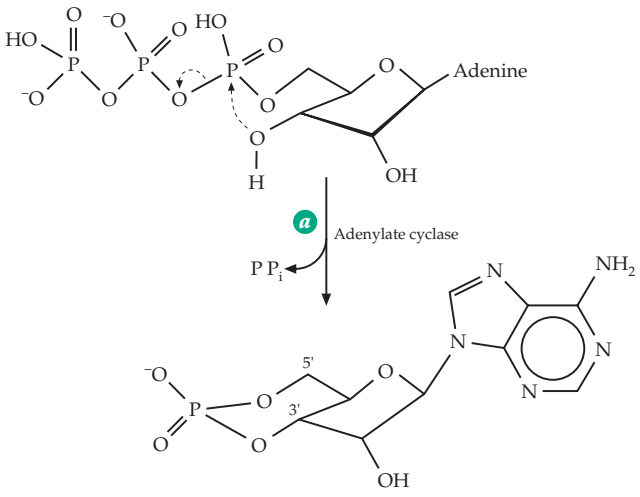
2. Adenylate Cyclases (Adenylyl Cyclases)

All of the effects of the catecholamines bound to β adrenergic receptors and of **glucagon**, **ACTH**, and many other hormones appear to be mediated by **adenylate cyclase**. This integral membrane protein catalyzes the formation of cAMP from ATP (Eq. 11-8, step *a*). The reaction, whose mechanism is considered in Chapter 12, also produces inorganic pyrophosphate. The released cAMP acts as the second messenger and diffuses rapidly throughout the cell to activate the cAMP-dependent protein kinases and thereby to stimulate phosphorylation of a selected group of proteins (Fig. 11-4). Subsequent relaxation to a low level of cytosolic cAMP is accomplished by hydrolysis of the cAMP by a phosphodiesterase (Eq. 11-8, step *b*).[166,167] In the absence of phosphodiesterase cAMP is extremely stable kinetically. However, it is thermodynamically unstable with respect to hydrolysis.

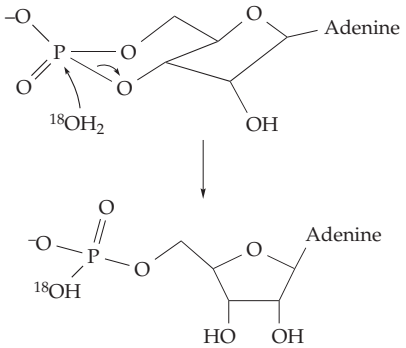
The existence of cAMP as a compound mediating the action of adrenaline and glucagon on glycogen phosphorylase was first recognized in 1956 by Sutherland.[168,169] However, for many years most biochemists regarded cAMP as a curiosity and the regulatory chemistry of phosphorylase as an unusual specialization. That view was altered drastically when cAMP was found to function as a second messenger in the action of over 20 different hormones. Phosphorylation by cAMP-dependent protein kinases regulates several enzymes concerned with energy metabolism or with the control of cell division. Phosphorylation reactions can lead to different responses in different specialized cells. Proteins of membranes, microtubules, and ribosomes are phosphorylated, as are nuclear histones. Cyclic AMP mediates the action of some neurotransmitters released at synapses and functions as a signal between cells of some slime molds (Box 11-C). Transcription of genes can be either stimulated or inhibited by cAMP.[170] Even in *E. coli* cAMP is generated and acts as a positive effector for transcription of some genes (Chapter 28). Cyclic AMP may function in signaling *stress* in plants.[171]

The isolation and characterization of adenylate cyclases have been difficult because of their location within membranes and because there is so little (usually only 0.001– 0.01%) of the membrane protein.[172] The 1060- to 1250-residue, 120 kDa proteins are also easily denatured. However, the sequences of cloned adenylate cyclase genes and the observed patterns of synthesis of the corresponding mRNAs have been revealing. There are at least eight mammalian adenylate cyclase genes with complex regulatory properties.[173 – 177] The sequences suggest that most of the isoenzymes are integral membrane proteins, *each with 12 transmembrane helices* organized as two sets of six with a large ~40-kDa cytoplasmic domain between the sets. This is similar to the organization of the cystic fibrosis transmembrane conductance regulator (Box 26-A) and some other membrane transporters. However, there is no firm evidence that adenylate cyclases contain ion channels. These enzymes are also discussed in Chapter 12, Section D,9.

A quantitative indication of the importance of the cAMP system within cells can be derived from measurement of the kinetics of the incorporation of ^{18}O from water into the α-phospho groups of AMP, ADP, and ATP. This incorporation will result from hydrolysis of cAMP by the phosphodiesterases that allow relaxation to a low cAMP level (Eq. 11-8). It is thought that in human blood platelets this represents the major pathway of this labeling (Eq. 11-9), which occurs at a rate of about 1.1 μmol of ^{18}O $kg^{-1}s^{-1}$.



(11-8)



(11-9)

Stimulation of the platelets by prostacyclin (prostaglandin I_2; see Chapter 22) leads to a 10- to 40- fold increase in cAMP concentration and a 4- to 5-fold increase in the rate of ^{18}O incorporation.[178] A quite different *soluble* adenylate cyclase is present in spermatozoa. It is stimulated directly by bicarbonate ions. It may be a **bicarbonate sensor** in sperm cells and in some other bicarbonate-responsive tissues as well as in cyanobacteria.[178a,178b]

3. Guanine Nucleotide-Binding Proteins (G Proteins)

The β adrenergic receptors are not coupled directly to adenylate cyclase but interact through an intermediary stimulatory protein $\mathbf{G_s}$, which contains three subunits, α, β, and γ.[179–182] We know that the G_s protein associated with β adrenergic stimulation is only one of a very large number of related **G proteins**, so named because of their property of binding and hydrolyzing GTP. In its unactivated state the α subunit of a $G_{\alpha\beta\gamma}$ heterotrimer carries a molecule of bound GDP. Apparently, the G_s proteins and the hormone receptors, which are activated by the binding of hormones, diffuse within the membrane until they make contact and form molecules of the G_s•GDP complex (Eq. 11-10, step *b*). The complex then undergoes a rapid exchange of the bound GDP for GTP, after which the hormone and receptor dissociate. The G_α•GTP complex may also dissociate from the complex, perhaps entering the cytosol as a soluble protein (Eq. 11-10, step *c*). The G_α•GTP complex combines with adenylate cyclase (step *d*) and activates it to generate cAMP. However, the activation is transient. G_α also contains GTP-hydrolyzing (GTPase) activity and within a few minutes G_α•GTP is completely converted to G_α•GDP and the adenylate cyclase dissociates (Eq. 11-10, step *e*). The G_α•GDP recombines with the βγ complex which may serve as a membrane anchor, to complete the regulatory cycle. The overall effect is for hormone binding to cause a rapid release of cAMP in a short burst that may last only about 15 s.

It is not the binding of GTP but a slow subsequent conformational alteration that activates the G_α•GTP adenylate cyclase complex. This was deduced by study of analogs of GTP, such as guanosine 5'-(β, γ-imido) triphosphate (GMP-*P*-(NH)-*P* or GppNp), which are

BOX 11-C THE ATTRACTION OF *DICTYOSTELIUM* TO CYCLIC AMP

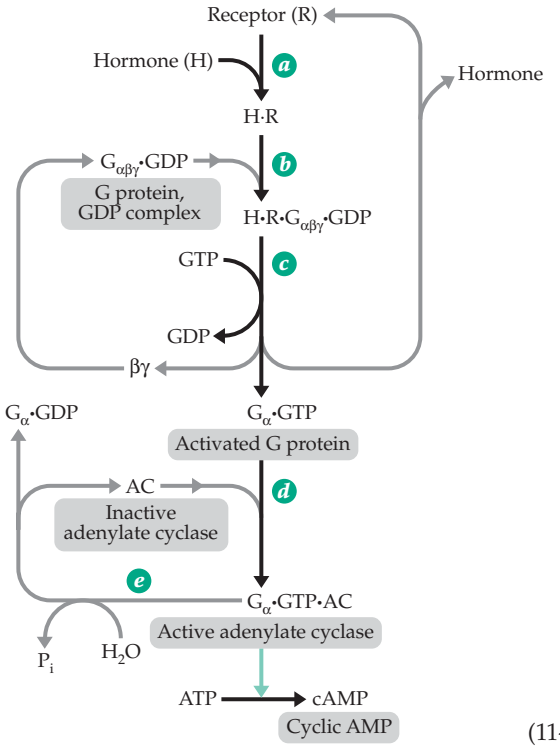
In higher animals cyclic AMP (cAMP) is an intracellular second messenger, but in the cellular slime mold *Dictyostelium discoideum* it serves to convey signals *between* cells.[a–c] As was mentioned on p. 20, the organism exists as individual amebas until the food supply is exhausted. Then the cells begin to signal their lack of food by secreting pulses of cAMP. Because they also secrete a phosphodiesterase, the cAMP is short-lived.[d] However, it is present long enough for any other nearby ameba to sense the gradient of cAMP concentration from one end of the cell to the other. As little as a 2% difference in concentration can induce chemotaxis.[e] The amebas move toward the source of cAMP and emit pulses of the compound. This results in the formation of aggregation centers in which the concentration of cAMP oscillates spontaneously as the cAMP moves outward in waves. The cells move up the concentration gradient until the peak of a wave reaches them. Then they move in a random direction until the next wave reaches them and again orients their motion.

After ~50 movement steps an aggregation center contains ~10^5 cells which now follow a "development program." The amebas adhere to each other to form 1- to 2 mm-long multicellular "slugs" in which all of the cells move forward together. About 30 h after aggregation begins the slugs stop and form stalks with spore-containing fruiting bodies on top. The lead cells in a slug become the stalk.[a,c]

Two types of G protein-coupled cAMP receptors in the cell membranes of the amebas have been identified. One leads to activation of adenylate cyclase and the release of new pulses of cAMP and the other to activation of guanylate cyclase.[e] This enzyme causes a rapid 7- to 10-fold increase in intracellular cGMP which plays an important role in controlling chemotaxis.

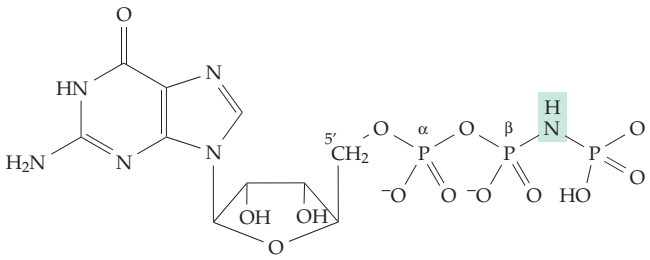
Some species of cellular slime molds use other chemical attractants (**acrasins**). For example, *D. minutum* secretes an analog of folic acid[f] and cells of *Polysphondylium violaceum* are attracted by the ethyl ester of *N*-propionyl-γ-L-glutamyl-L-ornithine-δ-lactam.[g]

[a] Gerisch, G. (1987) *Ann. Rev. Biochem.* **56**, 853–879
[b] Devreotes, P. (1989) *Science* **245**, 1054–1058
[c] Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1994) *Molecular Biology of the Cell*, 3rd ed., Garland, New York
[d] Levine, H., Aranson, I., Tsimring, L., and Truong, T. V. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6382–6386
[e] Kuwayama, H., and Van Haastert, P. J. M. (1996) *J. Biol. Chem.* **271**, 23718–23724
[f] Schapp, P., Konijn, T. M., and van Haastert, P. J. M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2122–2126
[g] Shimomura, O., Suthers, H. L. B., and Bonner, J. T. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7376–7379



(11-10)

not hydrolyzed to GDP. These compounds may require many minutes to activate the complex, but because the GppNp is not hydrolyzed, the complex remains activated and able to catalyze cAMP formation continuously.



Guanosine 5'-(β,γ-imido) triphosphate (GppNp); in place of the NH, O is present in GTP, S is present is GTPγS

Occupied receptors for adrenaline, glucagon, ACTH, and histamine activate adenylate cyclase via G_s proteins. Other G_s proteins, which contain subunits designated α_{olf} and which exist as a number of subtypes, mediate **olfactory responses**. Subunit α_o is another specialized polypeptide which is located primarily in neural tissues. A variety of additional G proteins have been discovered in organisms ranging from bacteria to mammals.[179,183–186] All have similar structures with 39- to 45-kDa α subunits, 35- to 36-kDa β subunits and 5- to 8-kDa γ subunits. Whereas the α subunits are unique to each G protein, β and γ subunits may be shared among several G proteins. These proteins appear to function with many kinds of hormone receptors and in nerve transmission, secretion, and endocytosis.[187] Mammals can form at least 23 different α_s subunits, which are coded for by 16 genes. Some of the forms arise by alternative splicing of mRNA transcribed from a single gene.[180] There are at least 6 β and 12 γ subunits.[179,180,182,188,189] Other G proteins, found in heart muscle, induce the opening of K^+ or Ca^{2+} channels in cell membranes.[185,190] It has often been assumed that all of the effects of G proteins are mediated by activated α subunits. However, the βγ complex, which sticks together very tightly, can also have signaling functions.[191] For example, an auxiliary protein called **phosducin** binds to the βγ complex of G_s, slowing the reconversion to the αβγ trimer.[192] Phosducin was discovered in rod cells of the retina but has been found to be distributed broadly in other tissues as well.[192,193] In yeast the βγ subunit of a G protein linked to the receptor for the yeast mating factor initiates the pheromone response of a cell.[193a]

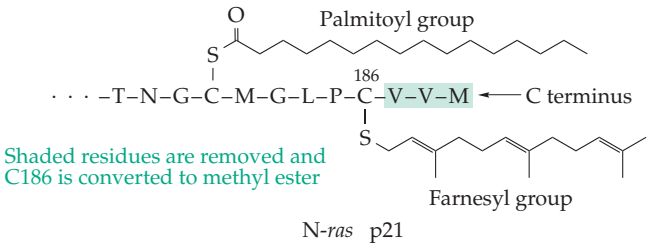
There is another large class of receptors whose occupancy by an agonist leads to *inhibition* of adenylate cyclase. These include the α_2 adrenergic receptors, receptors for acetylcholine, adenosine, prostaglandin E_2 (Chapter 21), somatostatin, and some receptors for dopamine. Their responses are mediated by inhibitory proteins G_i, which closely resemble G_s in their sizes, amino acid sequences, and heterotrimeric structures, but which inhibit adenylate cyclase when activated.[180] A clear distinction between the G_s and G_i proteins is evident in the fact that G_s is irreversibly activated by the action of cholera toxin, while G_i loses its ability to respond to occupied receptors when modified by the action of *Pertussis* toxin (Box 11-A). A specialized heterotrimeric G_i protein known as **transducin** mediates the light-induced activation of a **cyclic GMP phosphodiesterase** in the retina[194,195] (see Chapter 23). Its α subunit is designated α_t. The related **gustducin** is found in taste buds.[196]

Monomeric G proteins. An entirely different class of G proteins was discovered when it was found that the small 189-residue, 21-kDa protein products of the human oncogenes and proto-oncogenes known as **ras** are monomeric G proteins.[186,197–200] There are over 80 related proteins of this group of nine families.[200a] They include the much larger **elongation factor** EF-Tu, which functions in protein synthesis (Chapter 29). Even though there is a large difference in size, the three-dimensional structures of the GTP-binding domains of EF-Tu[201–204] and of the 21-kDa Ras p21 proteins[205–209] are very similar (Fig. 11-7). They also resemble that of adenylate kinase (Fig. 12-30).[205] The human genome contains four "true" *ras* genes: H-*ras*, N-*ras*, K-*ras*, and K-*rasB*. The occurrence of mutations in these genes in human cancer is discussed in Section H, as are the complex signaling functions of r*as* genes, (see also Fig. 11-13).[210]

Other proteins in the Ras superfamily include some members of the **Rho** family, which function in the cytoskeleton.[200a,211,212,212a,b,c,213] These include at least 14 mammalian proteins, among them RhoA, RhoB, etc., Rac1, Rac2, etc., and Cdc42. The latter is associated with the formation of fingerlike extensions of the cell membrane containing actin bundles.[200a,212d,212e] Genes *rac1* and *rac2* encode proteins involved in the initiation of superoxide radical formation by activated phagocytes (Chapter 18).[214] The **Rab** family of proteins are involved in exocytosis and endocytosis and vesicular transport. There are 24 or more human *rab* genes.[212,215,216] Yeast cells also contain Ras proteins. One *RAS* gene must be present for yeast to form spores.[217] **ADP-ribosylation factors** (ARFs), which stimulate the action of cholera toxin A subunit (Box 11-A) are also members of the Ras superfamily.[120,217a]

Like the trimeric G proteins, the monomeric proteins also serve as "timed switches" that are turned on by GDP–GTP exchange and are turned off by the hydrolysis of the bound GTP. In the absence of other regulatory proteins both the exchange of bound GDP for GTP and the hydrolysis of bound GTP are slow. While the receptor-associated trimeric G proteins are activated by the hormone receptors, the monomeric G proteins are activated toward GDP–GTP exchange by binding to proteins called **guanine nucleotide dissociation stimulators** (**GDSs**).[217b] They speed the release of GDP from the G protein allowing the active GTP complex to be formed. The velocity of hydrolysis of the bound GTP in the activated G protein is greatly increased by **GTPase activating proteins** (**GAPs**). These auxiliary proteins can be thought of as signaling molecules that pass their messages to the G protein, controlling the extent of its activation and therefore the strength of a signal that it sends on to the processes that it is controlling.[200,210,218] One group of GAP proteins are known as **regulators of G-protein signaling** (RGS).[218-218b] These proteins, which contain a characteristic 12-residue core, were first recognized as negative regulators of signaling in yeast cells called **GDP-dissociation inhibitors** (GDIs).[219] They can also be regarded as a family of GAPs.

Acylation and prenylation. The amino terminus (usually glycine) of the α subunit of any G protein is nearly always converted to an ***N*-myristoyl group**.[220–223] This modification occurs in a **cotranslational process** after removal of the initiating methionine (Chapter 29) and can be described as an acyl transfer from coenzyme A.[224] The C termini of the γ subunits of heterotrimeric G proteins, and also of the monomeric proteins of the Ras family, also undergo processing. For example, the C-terminal end of an intact Ras protein contains 18 residues which probably assume a largely α-helical conformation. A cysteine side chain near the terminus and having the sequence CAAX is converted within cells to a **thioether** with all-*trans*-farnesol or longer polyprenyl alcohols.[225] Another nearby cysteine may form a **thioester** with palmitic acid,[221,226,227] for example:



The farnesyl (or longer) polyprenyl and palmitoyl groups, together with nearby nonpolar side chains, serve as a membrane anchor. After the prenylation the C-terminal three amino acids (VVM in N-*ras* p21) are removed proteolytically and the new terminal carboxyl group of the farnesylated Cys 186 is converted to a methyl ester.[212c,225,228] N-terminal myristoylation is usually regarded as irreversible. However, the thioester linkages by which palmitoyl groups are attached to proteins are labile and may be cleaved rapidly by hydrolases. It follows that palmitoylation can be a rapid, regulated modification of proteins,[229–230a,b] strongly affecting the location and extent of adherance to membrane surfaces.

Three-dimensional structures. The structure of the GTP-binding domain of elongation factor EF-Tu was determined by Jurnak in 1985[201] and that of the complete three-domain structure later.[202,203] When the structure of the *catalytic domain* of the first Ras protein was determined (Fig. 11-7A) it was clear that it was similar to that of EF-Tu.[205,207] The same was true for the transducin α_t,[194,231,232] for the inhibitory $G_{i\alpha 1}$,[218b,233,234] and for other G proteins.[235] In every case the differences in structure of the enzyme with GDP or with analogs of GTP were small and limited to a region close to the γ-phospho group of the bound GTP. This group can be seen clearly in Fig. 11-7A adjacent to residue 60 of the protein. See also Fig. 12-36.

The β strands and helices in the GTP-binding domain of the G proteins are connected by a series of loops. The first, second, and fourth loops (residues 10–17, 32–40, and 57–65, respectively) in the Ras structure of Fig. 11-7A form the catalytic site in which the hydrolysis to GDP takes place. The first loop, also called the P loop, is conserved in all GTP-binding proteins. In the Ras proteins it has the following sequence:

10	17	
G–A–G–G–V–G–K–S		Ras
G–X–X–X–X–G–K–S(T)		Consensus

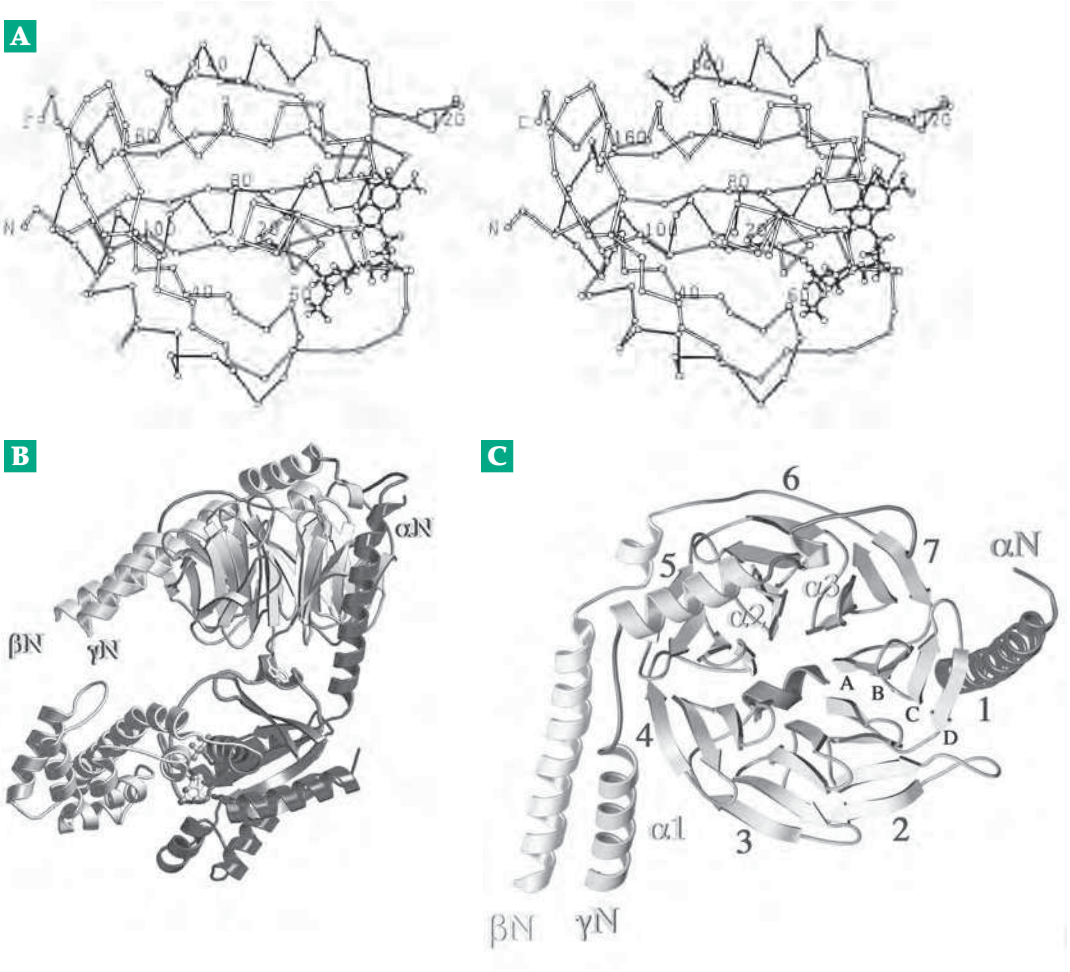


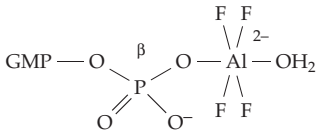
Figure 11-7 (A) Stereoscopic drawing showing α-carbon positions in the 166-residue "catalytic domain" of the human c-H-*ras* gene product and the bound GTP analog GppNp (solid bond lines). The intact protein contains an additional 23-residue C-terminal extension. From Pai *et al.*[241] (B), (C) Ribbon drawings showing two nearly orthogonal views of a hetero-trimeric inhibitory G_i brain protein produced using a cloned bovine gene. (B) The amino termini (N) of the three subunits are seen in the left-to-right order: γ, β, α. A side view of the β propeller domain of the β subunit is seen at top center. The Ras-like domain and the additional large helical domain of the α subunit are marked. (C) View from the flared end of the β propeller looking toward the α subunit. The strands of each propeller blade are labeled A, B, C, and D and the seven blades are numbered around the periphery of the propeller. From Wall *et al.*[242] (B) and (C) courtesy of Stephen Sprang.

Also shown here is the "consensus" sequence for all GTP-binding proteins. Cancer cells frequently have mutations in Ras at Gly12, which may be substituted by Asp, Lys, Val, or Arg, and at Gly 13 and Gln 61. The latter is in loop 4, a part of the sequence that appears especially mobile but is highly conserved. These mutations all activate the protein, i.e., they decrease the GTPase activity, allowing the G protein to remain in its active conformation longer than normal.

Ras proteins fulfill their functions by interacting closely with two or more proteins in signaling pathways as described in Section H. Other G proteins have additional domains. The 405-residue EF-Tu from *Thermus thermophilus* has three domains: the C-terminal nucleotide-binding domain and two β-barrel domains following it. A major difference in conformation is observed between forms of the protein with bound GTP or bound GDP, the GDP form being opened up by a hinging motion of ~ 90° between domains I and II.[202,203] The conformational change is triggered by the GDP–GTP exchange. An enhanced rate of GTP hydrolysis, which results from binding of a transfer RNA and binding to a ribosome, is apparently accomplished by interaction with loop 2 of the catalytic domain (corresponding to residues 32 – 40 in Fig. 11-7A). The function of this protein is considered further in Chapter 29.

Transducin has a 113-residue domain inserted into loop 2 of the catalytic domain. In this case, too, a large hinging movement is associated with the GDP–GTP exchange. The light-activated receptor rhodopsin induces the conformational change.[194,231] Again, structural changes resulting from the presence or absence of interactions of the protein with the γ-phospho group

of GTP are involved. The GTPase activity of heterotrimeric G proteins can also be activated by aluminum fluoride AlF_4.[232,236] From the X-ray structure of the transducin α•GDP•AlF_4^- complex it is seen that hydrated AlF_4^- is covalently bonded in the position of the γ-phospho group and makes hydrogen bonds to active site groups, mimicking a possible transition state for the GTPase reaction[232] (see Chapter 12, Section D).



The β subunit of the βγ complex of transducin is a **seven-bladed β propeller** (Fig.11-7C). It is composed of seven GH–WD repeat units: $-[GH-X_n-WD]_{4-8}-$ where GH = Gly – His, WD = Trp–Asp, and X_n is a core repeating sequence, usually 32–42 residues in length. This motif is also found in at least 40 other eukaryotic proteins.[188,237–240] In the βγ complex (Fig. 11-7B) the γ subunit assumes an elongated, largely α-helical structure. It is often anchored at its C terminus by a farnesyl or geranylgeranyl chain, while G_α may be myristoylated or palmitoylated.[195,230,243]

The three-dimensional structure of the GDP complex of the intact transducin heterotrimer[195] also shows a tight interaction between α and β subunits. The major interaction is probably disrupted by replacement of the bound GDP by GTP and the conformational change that occurs around the γ - phospho group. This explains the dissociation of the α subunit from βγ upon activation. An entirely similar picture has been obtained for the action of the inhibitory G protein, G_{i2}, for which structures of the α subunit and of the αβγ heterotrimer (Fig. 11-7,B,C) have been determined.[188,233,234,242] The structures resemble those of transducin, but differ in details.

4. Guanylate Cyclase (Guanylyl Cyclase), Nitric Oxide, and the Sensing of Light

Formation of the less abundant cyclic guanosine monophosphate (cGMP) is catalyzed by guanylate cyclases found in both soluble and particulate fractions of tissue homogenates.[244–247] However, its significance in metabolism is only now becoming well understood. There are cGMP-dependent protein kinases,[247] but, until recently cGMP could not be regarded as a second messenger for any mammalian hormone. Now we know that cGMP has several essential functions. It mediates the effects of the **atrial natriuretic factor**, a peptide hormone causing dilation of blood vessels (Box 23-D),[245,248–250] and also of a peptide called **guanylin**, which is formed in the intestinal epithelium.[245,251] The receptors for both of these peptides are *transmembrane proteins with cytoplasmic domains that have guanylate cyclase activity*. The released cGMP appears to induce relaxation of smooth muscles of the blood vessel walls. Another mediator of smooth muscle relaxation is the **endothelial cell-derived relaxing factor**, which is evidently **nitric oxide**, **NO**[252] (see Chapter 18). A soluble guanylate cyclase appears to be an NO receptor and is activated by binding of the NO to a heme group in one of the two subunits of the cyclase.[253–255] The cyclase can also be activated by carbon monoxide, CO, in a similar fashion.[256,257] Carbon monoxide is produced in the body by degradation of heme (Fig. 24-24) and is thought to be a neurohormone.[258]

Cyclic GMP also plays an important role in vision. Specific phosphodiesterases hydrolyze cGMP to GMP. The cGMP phosphodiesterases of the rod and cone cells of the retina are activated by the G protein transducin, which has been activated by light absorbed by the visual pigments.[194] The resulting decrease in the cGMP concentration is thought to cause the closing of cation channels and thereby to initiate a nerve impulse (see Chapter 23). Cyclic nucleotide forms of the pyrimidine nucleotides are present only in very small amounts in cells.

5. Bacterial Chemoreceptors

Bacteria are attracted to foods with the aid of chemoreceptors that bind certain amino acids such as aspartate. The receptors send signals to the mechanisms that ensure that the bacterium is swimming toward the food[113,259,259a] (more details are given in Chapter 19). The aspartate and serine chemoreceptors found in membranes of *E. coli* and *Salmonella typhimurium* are among the most carefully studied of all receptors. Like the seven-helix receptors, they have an extracellular sensory domain to which the signaling molecule, aspartate or serine, binds, and a transmembrane helical bundle. In these receptors the transmembrane part consists of four helical segments coming from the two subunits of the dimeric protein. For the aspartate receptor there is a high-resolution X-ray structure for the cytoplasmic domain[260,261] including the bound aspartate[262] as is shown in Fig. 11-8A,B.[260,263] The structures of the receptors for aspartate, serine, and many other signaling molecules are evidently very similar. The structure of the cytosolic domain of the serine receptor has been determined and a model for the complete receptor has been proposed.[259a] The molecular mass of the 188-residue extracellular domain is ~18 kDa, while that of the larger cytoplasmic domains is ~36 kDa. The latter includes a linker region as well as a long four-helical bundle domain which can be divided into a methylation domain and a signaling domain as shown in (Fig. 11-8C).

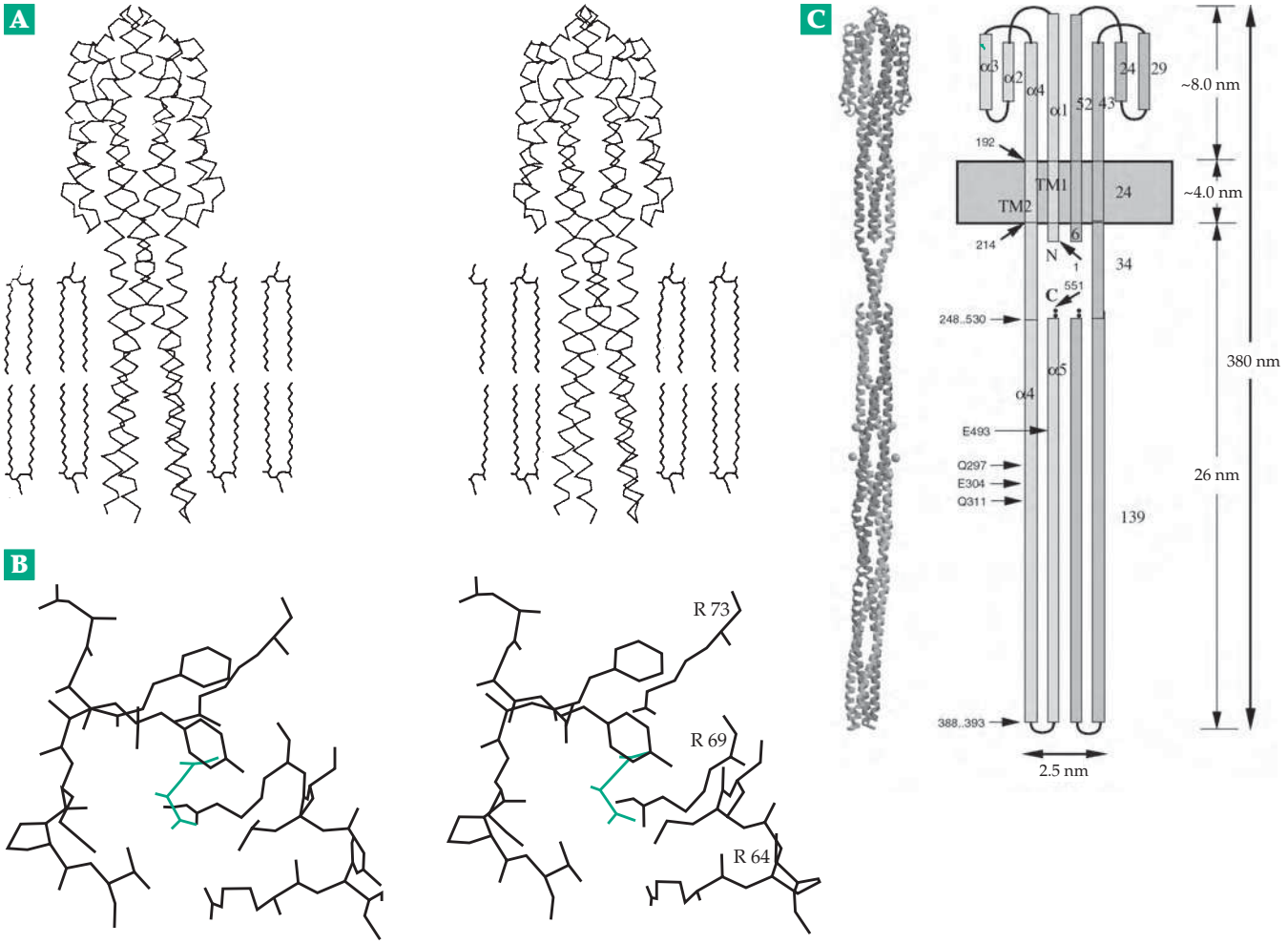


Figure 11-8 (A) Stereoscopic view of the sensory domain of the dimer with modeled transmembrane region. Each monomer contains a four α-helix bundle with two helices continuing through the membrane. From Scott *et al.*[260] (B) Stereoscopic image of aspartate in the major binding site of the receptor. The atomic model of aspartate (green) has been fitted into the observed difference electron density map. From Yeh *et al.*[262] (C) Model of an intact *E. coli* serine chemoreceptor. Left. Ribbon drawing viewed perpendicular to the molecular twofold axis. Methylation sites are represented by the dark balls by the cytoplasmic domain. The bound serine is drawn as a partially hidden green ball at the upper left in the extracellular domain. Right. Diagram of the receptor. The presumed membrane bilayer is represented by the gray band. Positions of some residues are marked on the left side. On the right, the numbers of residues in various peptide segments are indicated. From Kim *et al.*[259a] Courtesy of Sung-Hou Kim.

What happens chemically when aspartate binds into a deep pocket in the center of the sensory domain? What kind of signal can be passed from the aspartate to the "signaling domain"? Several possibilities were discussed by Kim,[264] who suggested that the binding of aspartate causes the rotation of the sensory domain of one subunit relative to that of the other and that this rotation is transmitted through the membrane to the signaling domain. The signaling domain of the receptor forms a complex with a multimeric **protein histidine kinase** called **CheA** (chemotaxis protein A) together with an auxiliary protein **CheW**. A more likely possibility is that some kind of "piston" action occurs between the transmembrane helices of the two subunits.[264a] Binding of aspartate to the receptor, and the associated alterations in the receptor•CheA•CheW complex, causes a strong *decrease* in the catalytic activity of CheA.

In its active form CheA undergoes **autophosphorylation**, that is, the phosphorylation of a histidine imidazole group in one of its subunits by the protein kinase active site of an adjacent subunit. The phospho group is then transferred from phospho-CheA to another protein, **CheY**. Phospho-CheY interacts with the flagellar motor proteins (Chapter 19) periodically causing a reversal of direction of the bacterial flagella. As a result the bacteria tumble and then usually move

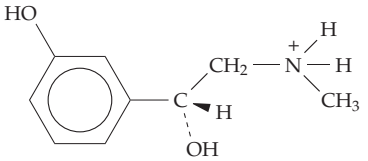
in a new direction. If the attractant molecule aspartate is present on the receptor the signal is weakened and the bacteria continue to swim in the same direction–toward food. Phospho-CheY is spontaneously hydrolyzed to remove the phospho group, but the dephosphorylation is promoted by an additional protein **CheZ**. At least two other auxiliary proteins also function in this control system. **CheR** is a **methyltransferase** that methylates the glutamate side chain carboxylates in the methylation domain of the coiled coil region and **CheB** is a **methylesterase** that removes the methyl groups. These two proteins control the methylation level in the coiled coil region of the receptor (Fig. 11-8A). Increased methylation increases the strength of the signal sent from the receptor, perhaps because methylation removes negative carboxylate charges that repell each other and weaken the structure, interfering with signaling.[259] See also Fig. 19-3.

E. Calcium, Inositol Polyphosphates, and Diacylglycerols

Calcium ions entering cells from the outside or released from internal stores trigger many biological responses (see Box 6-D). Within cells Ca^{2+} often accumulates in mitochondria, in the ER, or in vesicles called **calciosomes**.[265] Release of the stored Ca^{2+} is induced by hormones or by nerve impulses. For example, impulses flow from the nerve endings into the muscle fibers and along the invaginations of the plasma membrane called transverse tubules (Chapter 19). There they induce release of Ca^{2+} from the ER. The released ions activate enzymes[266] and induce contraction of the muscle fibers. In many cells, Ca^{2+} causes release of secreted materials, for example, neurotransmitters in the brain.[267,268]

1. Alpha Adrenergic Receptors

The release of stored Ca^{2+} is often triggered by α adrenergic receptors.[269] Like β adrenergic receptors, the α receptors are activated by adrenaline. Specific inhibitors distinguish them. For example, the β receptors are inhibited by propranolol, while the α receptors are blocked by phenoxybenzamine. The synthetic agonist **phenylephrine** activates only α receptors and no increases in cAMP or in protein kinase activity are observed.



Phenylephrine

There are two major α adrenergic receptor subtypes.[151,269] Activation of the α_2 receptors, which are present in various tissues including blood platelets, causes *inhibition* of adenylate cyclase. This inhibition is evidently mediated by the G_i protein considered in Section D,3. The nucleotide sequences of cloned α_2 receptor genes and other properties suggest close structural similarity to the β receptors.[270–273] Subtle differences in hydrogen bonding to the serine side chains shown in Fig. 11-6C may distinguish α_2 from β_2 receptors.[273] A characteristic of α_2 receptors is that pertussis toxin (Box 11-A) abolishes the inhibition of adenylate cyclase, which they mediate. In contrast, the action of α_1 adrenergic receptors is not affected by pertussis toxin.

The α_1 receptors are activated not only by catecholamines but also by the hormones **vasopressin** and **angiotensin II**. Binding of these hormones to α_1 receptors induces a complex response that involves rapid hydrolysis of **phosphatidylinositol** derivatives and release of Ca^{2+} into the cytoplasm, and of diacylglycerols into the lipid bilayer of the membrane. The response is mediated by another G protein called G_q.[274,275] When this G protein is activated it induces the hydrolysis of **phosphatidylinositol 4,5-bisphosphate** (PtdInsP_2), a normal minor component of the lipid bilayer, by **phospholipase C** (phosphoinositidase C).[265,276–281]

$$\text{Receptor} \rightarrow G_q \rightarrow \text{phospholipase C} \rightarrow \begin{cases} \text{inositol phosphates} \\ + \text{ diacylglycerol (DAG)} \end{cases} \rightarrow \text{increased } [Ca^{2+}] \quad (11\text{-}11)$$

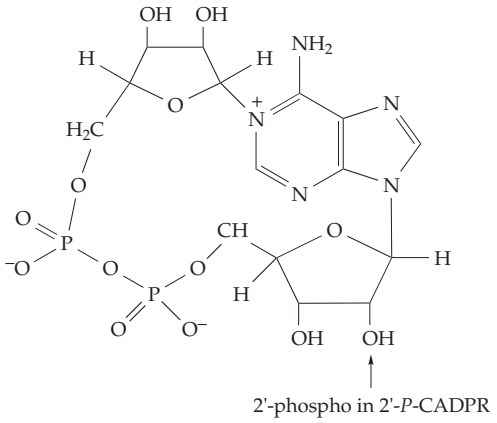
The products, **inositol 1,4,5-trisphosphate** [abbreviated InsP_3 or Ins(1,4,5)P_3], and **diacylglycerol** (DAG) are both regarded as second messengers for the catecholamines acting on α_1 receptors and for about 20 other hormones, neurotransmitters, and growth factors upon binding to their specific receptors.[269,282] In addition to vasopressin, the gonadotropin-releasing hormones, histamine, thrombin (upon binding to platelet surfaces), and acetylcholine (upon binding to its "muscarinic" receptors; Chapter 30) all stimulate inositol phosphate release.

2. Phosphatidylinositol and the Release of Calcium Ions

The phosphoinositides constitute ~2–8% of the lipid of eukaryotic cell membranes but are metabolized more rapidly than are other lipids.[265,278,279,283–285] A simplified picture of this metabolism is presented in Fig. 11-9. Phosphatidylinositol is converted by the consecutive action of two kinases into phosphatidylinositol 4,5-bisphosphate.[286,287] The InsP_3 released from this precursor molecule by receptor-stimulated phospholipase C is thought to mobilize calcium ions by

opening "gates" of calcium channels in membranes of the ER or of calciosomes.[282,288–289a] It diffuses across the peripheral cytoplasm to **Ins*P*$_3$ receptors** which are embedded in the membranes of the ER.[282,290] One of the several isotypes of InsP_3 receptors is a 2749-residue protein thought to contain a calcium ion channel.[290] Similar receptors are also found in inner membranes of the nucleus.[291,292]

Several uncertainties have complicated our understanding of the role of Ca^{2+} in signaling. What is the source of Ca^{2+}? How much of it enters cells from the outside and how much is released from internal stores? Where are the internal stores? What other kinds of ion channels are present and what second messengers regulate them? The sarcoplasmic reticulum of skeletal muscle and also membranes in many other cells contain **ryanodine receptors** as well as InsP_3 receptors.[282,293] Both of these receptors have similar structures and contain Ca^{2+} channels. However, the ryanodine receptors are activated by **cyclic ADP ribose** (cADPR),[294,295] which was first discovered as a compound inducing the release of Ca^{2+} in sea urchin eggs.[296] The 2-phospho derivative of cADPR may also have a similar function.[297]



Cyclic ADP-ribose (cADPR)

Phospholipase C, which initiates the release of phosphatidylinositol derivatives, also requires Ca^{2+} for activity. It is difficult to determine whether release of Ca^{2+} is a primary or secondary response. There are three isoenzyme types of phospholipase C–β, γ, and δ– and several subforms of each with a variety of regulatory mechanisms.[298–300a] For example, the γ isoenzymes are activated by binding to the tyrosine kinase domain of receptors such as that for epidermal growth factor (see Fig. 11-13). In contrast, the β forms are often activated by inhibitory G_i proteins and also by G_q, which is specific for inositol phosphate release.

Calcium ions are usually released in distinct pulses or "quanta." The kinetic characteristics of the system of receptors, diffusing InsP_3, calcium buffers, and calcium pumps in the cell membrane, and the membrane potential may account for this behavior.[174,301–306] Since the phosphoinositols are chelators of Ca^{2+}, the equilibria involved in this control system are complex.[307]

In addition to InsP_3, several other inositol derivatives are released by adrenergic stimulation. **Inositol 1,3,4,5-tetrakisphosphate** (InsP_4) is formed from InsP_3 by action of a soluble kinase.[308,309,309a,b] A controversial suggestion is that InsP_4 may induce opening of Ca^{2+} channels through the outer membrane of the cell[310–312] and may also function to promote storage of Ca^{2+}. It also acts as a transcriptional regulator. Other possible second messengers are inositol 1,2-cyclic-3,4-trisphosphate and related metabolites that arise by the action of phospholipase C on PtdIns 4-*P* followed by additional actions of kinases and phosphatases.[278,309,313–315] A different inositol tetrakisphosphate, Ins(3,4,5,6)P_4, may control chloride ion channels.[316,317] Both inositol 1,3,4,5,6-pentakisphosphate (**Ins*P*$_5$**) and inositol hexakisphosphate (**Ins*P*$_6$**) are also found in plants[318] and animals.[319] InsP_5 serves as an allosteric effector regulating hemoglobin in avian erythrocytes (Chapter 7). InsP_6, also known as **phytate**, is present in large amounts in cereals and recently has been found to play a role in regulating the export of mRNA from the nucleus.[319a] Additional phospho groups can be added to InsP_6 to form pyrophosphates[320,320a] and other complex polyphosphates.[321] Both InsP_5 and InsP_6 can also serve as precursors to Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 by hydrolytic dephosphorylation.[322]

Following stimulation of a cell the induced metabolism of phosphoinositides decays rapidly. The diacylglycerols are converted into phosphatidic acid and resynthesized into phospholipids (Chapter 21). The InsP_4, InsP_3, and other inositol metabolites are hydrolyzed by phosphatases.[278,309,323–327] Two of these phosphatases are inhibited by Li^+ as indicated in Fig. 11-9. They may represent one site of action of **lithium ions** in the brain.[324,327,328] Lithium salts are one of the most important drugs for treatment of **bipolar (manic-depressive) illness**. By blocking the release of free inositol, which can be resynthesized into PtdInsP_2, Li^+ may prevent neuronal receptors from becoming too active. However, the basis for the therapeutic effect of lithium ions remains uncertain.

The diacylglycerols released by phospholipase C diffuse laterally through the bilayer and, together with the incoming Ca^{2+}, activate **protein kinases C**. These kinases also require **phosphatidylserine** for their activity and phosphorylate serine and threonine side chains in a variety of proteins.[329–330b] They are stimulated by the released unsaturated diacylglycerols. In addition protein kinases C can be activated by **phorbol esters**, which are the best known tumor promoters (Box 11-D). The diacylglycerol requirement favors a function for these protein kinases in membranes. They also appear to cooperate with calmodulin to activate the Ca^{2+}-dependent contraction of smooth muscle.[330]

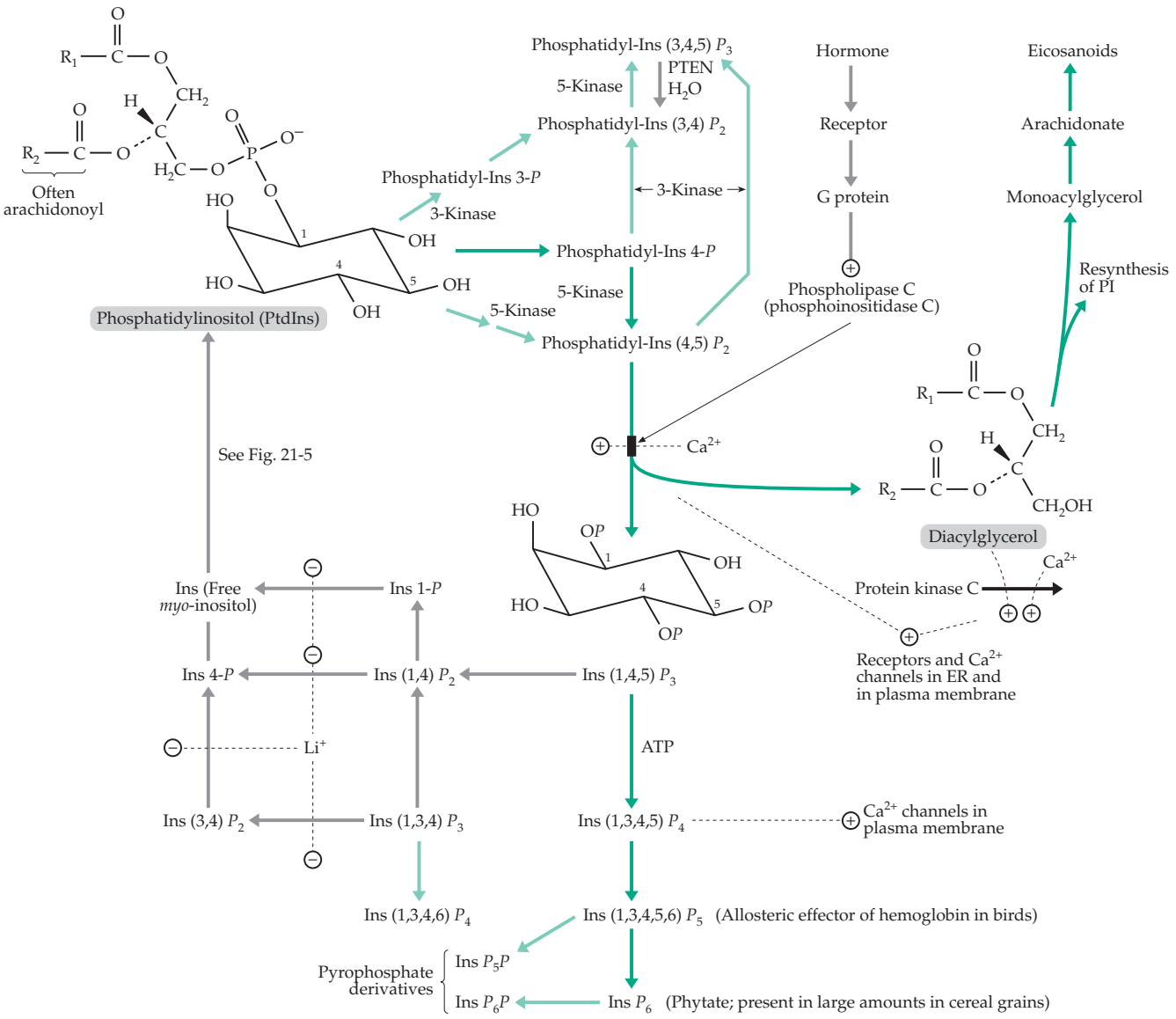
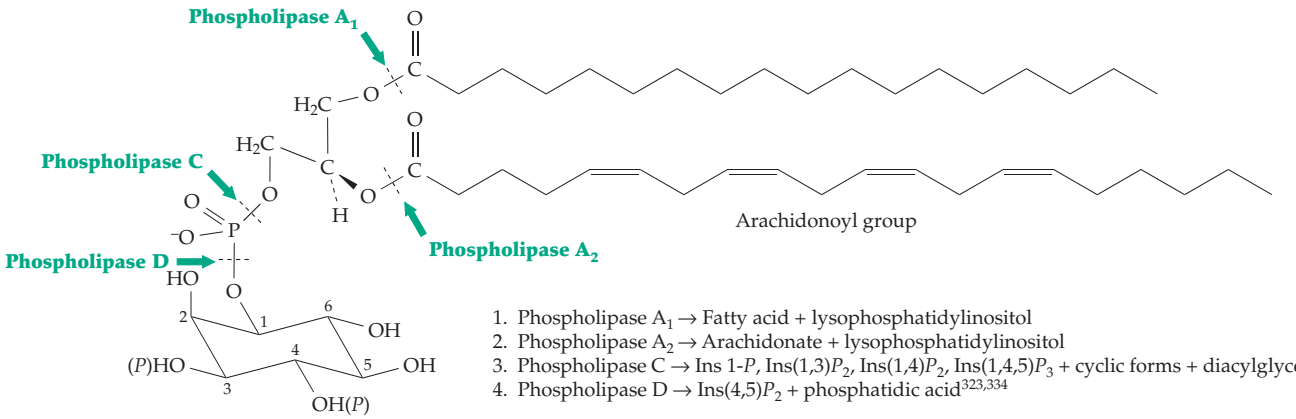


Figure 11-9 Scheme showing synthesis and release of diacylglycerol and inositol phosphates and their regulation of calcium concentration in response to hormonal stimulation.

Diacylglycerols released by phospholipase C usually contain **arachidonic acid** in the 2 position. Hydrolytic cleavage of this linkage[284,331] is a major source of arachidonate for synthesis of **eicosanoids** such as the **prostaglandins** whose functions are discussed in Chapter 21. Arachidonate can also be formed directly by the action of phospholipase A_2 on membrane phospholipids[332,332a] (see the structure on p. 566). It has been suggested that protein G_q, which activates the hydrolysis of phosphoinositides, may also regulate phospholipase A_2 directly. Released diacylglycerols may not only activate protein kinases C but also have a direct role in promoting the membrane fusion required in exocytosis and endocytosis.[332] Other breakdown pathways of phosphatidylinositols are also indicated in the following structure.[298,333]

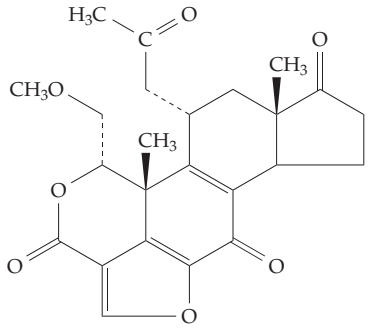
Initially most attention was paid to the water-soluble inositol phosphates that are released from phosphoinositides. However, phosphoinositol derivatives that retain the diacylglycerol part of the molecule have regulatory functions while remaining in membranes. A phosphatidylinositol 3-kinase phosphorylates the 3-OH of inositol in PtdIns, PtdIns 4-*P* and PtdIns (4,5)P_2 to give PtdIns 3-*P*, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_2, respectively.[335–337d] However, they are ideally suited to function as spatially restricted membrane signals.[337a] They affect protein kinase C as well as the Ser / Thr protein kinase Akt (discussed in Section F,3),[326b] and have several functions in vesicular membrane transport and in regulation of the cytoskeleton.[337e,f]



1. Phospholipase A_1 → Fatty acid + lysophosphatidylinositol
2. Phospholipase A_2 → Arachidonate + lysophosphatidylinositol
3. Phospholipase C → Ins 1-*P*, Ins(1,3)P_2, Ins(1,4)P_2, Ins(1,4,5)P_3 + cyclic forms + diacylglycerol
4. Phospholipase D → Ins(4,5)P_2 + phosphatidic acid[323,334]

Phosphatidylinositol

Characteristic of many of the phosphoinositide-regulated proteins is a proline-rich PH domain (Table 7-3) which can transmit regulatory signals to additional proteins in a cascade. Their importance is emphasized by the finding that a 405-residue phosphatase **PTEN** (named after its gene symbol) which catalyses hydrolytic removal of one phospho group from PtdIns(3,4,5) P_3 to form PtdIns(3,4) P_2, (Fig. 11-9) is a major human tumor suppressor (see Box 11-D).[338,338a,b] These lipids are also important in insulin action (Section G,3). The steroid-like fungal metabolite wortmannin is a specific inhibitor of the 3-kinase.[339,339a]



Wortmannin

F. Regulatory Cascades

The effect of a regulated change in the activity of an enzyme is often amplified through a cascade mechanism. The first enzyme acts on a second enzyme, the second on a third, etc. The effect is to rapidly create a large amount of the active form of the last enzyme in the series.

We have already considered regulatory cascades initiated respectively by the β and α_2 adrenergic receptors. The effect of these cascades on glycogen phosphorylase is outlined on the left side of Fig. 11-4. One branch of the cascade sequence begins with release of adrenaline under control of the autonomic nervous system. In muscle, binding of this hormone to the β receptors on the cell membrane releases cAMP which activates a protein kinase. The kinase then phosphorylates phosphorylase kinase. At this point the muscles are prepared for the rapid breakdown of glycogen. However, an additional initiating signal is the release of Ca^{2+} into the cytoplasm in response to impulses to specific muscles via the motor neurons. Calcium ions can also be released in the liver by α adrenergic stimulation. Phosphorylase kinase is activated by the calcium ions, and in their presence it converts inactive phosphorylase *b* to the active phosphorylase *a*. Both protein kinases and phosphorylase kinase also act on glycogen synthase, phosphorylating it and converting it to an inactive form. This turns off the biosynthetic pathway at the same time that glycogenolysis (glycogen breakdown) is turned on. Spontaneous reversion of the enzymes to their resting states occurs through the action of phosphatases that cut off the phospho groups placed on the protein by the kinases. Also essential are phosphodiesterases, which destroy the cAMP, and the calcium ion pump, which reduces the concentration of the activating calcium ion to a low level.

Elaborate cascades initiate the clotting of blood (Chapter 12) and the action of the protective complement system (Chapter 31). Cascades considered later in the book are involved in controlling transcription (Fig. 11-13) and in the regulation of mammalian pyruvate dehydrogenase (Eq. 17-9), 3-hydroxy-3-methylglutaryl-CoA reductase and eicosanoids (Chapter 21), and glutamine synthetase (Chapter 24).

1. Advantages of Regulatory Cascades

Computer simulations as well as studies of experimental models have led to the following conclusions.[340,341] Even simple cascade mechanisms, such as the one shown in Fig. 11-10, can provide a more flexible response

to allosteric effectors (such as e_1 in the figure) than if the effector acted directly on the enzyme rather than on the protein kinase. The cascade also provides **amplification**. This is especially true if additional cycles are added. A response can result from the binding of only a small number of hormone molecules to a receptor in a cell membrane or from activation of only a few molecules of a protease in the initiation of blood clotting. A striking amplification occurs in visual responses. Under appropriate conditions a single quantum of light falling on a receptor cell in the retina of an eye can initiate a nerve impulse (Chapter 23). The latter requires the flow of a large number of Na^+ ions across the plasma membrane. It would be hard to imagine how absorption of one quantum could initiate a photochemical reaction leading to that much sodium transport without intermediate amplification stages. Another advantage of cascades is that they may provide **ultrasensitive responses**. Not only can a response be sensitive to a higher power than the first of the concentration of a signaling molecule but also the amplification provided by the cascade confers a high sensitivity to the response.[342]

Cascade systems also provide for response to more than one allosteric stimulus in a single pathway. Thus, as shown in Fig. 11-4, glycogen catabolism can be initiated in more than one way. Two pathways are known for initiation of both blood clotting and activation of the complement system. Many pathways activate the MAP kinase pathway shown in Fig. 11-13.

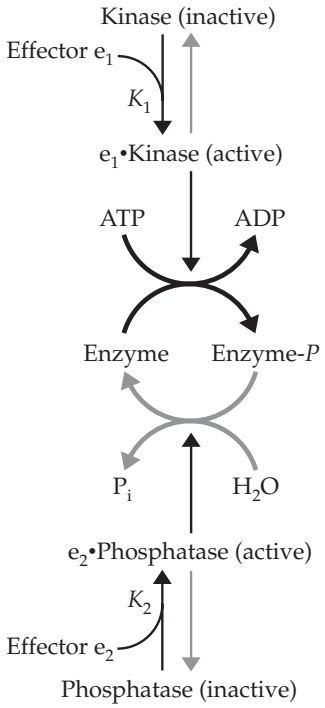


Figure 11-10 A "monocyclic" regulatory cascade involving phosphorylation and dephosphorylation of an enzyme. After Stadtman and Chock.[341]

2. Substrate Cycles

Although cascade systems offer advantage to cells, there is a distinct energy expenditure associated with the controls.[343] As can be seen in Fig. 11-10, in addition to providing for turning on and turning off the regulated enzyme, the kinase and the phosphatase together catalyze hydrolysis of ATP to ADP and inorganic phosphate. Thus, the regulated enzyme is continually cycling between active and inactive forms. The relative amounts of each are determined by the amounts of the effectors e_1 and e_2, the concentrations of modifying enzymes, and the kinetic constants. Such cycles have sometimes been called "futile cycles" because they seemingly waste ATP. This is particularly true of cycles that involve major metabolites, sometimes called **substrate cycles**. An example is the conversion of fructose 6-*P* to fructose 1,6-bisphosphate by phosphofructokinase and hydrolysis of the bisphosphate back to fructose 6-*P* by a phosphatase (Fig. 11-2). However, *the cycles are not futile* because the hydrolysis of the ATP provides the energy required to maintain the concentrations of modified (phosphorylated) enzyme or enzymes at steady-state levels that are required for efficient catalysis.[341,344] The energy utilized in this way in regulation of enzymes is often small.[345] Substrate cycles are not only an unavoidable consequence of the need to regulate enzymes but also provide for improved sensitivity in metabolic control. Consider Fig. 11-2 again. If the enzymes are set for conversion of fructose 1,6-P_2 to glycogen (backwards direction), the flux of materials flowing in the forward direction (glycolysis) will be efficiently curtailed because any fructose 1,6-P_2 formed will be hydrolyzed rapidly by the active phosphatase. Under these circumstances the flux in the forward direction will be ultrasensitive to the activation of phosphofructokinase and to the inhibition of fructose 1,6-bisphosphatase.[346,347]

G. Insulin and Related Growth-Regulating Hormones

Since its isolation in 1921, insulin has been the object of an enormous amount of experimentation aimed at clarifying its mode of action. It is produced by the β cells of the pancreatic islets of Langerhans and released into the bloodstream in response to elevated glucose levels. The absence of insulin or of a normal response to insulin results in the condition of **diabetes mellitus**, which is the most prevalent human metabolic disorder (see Box 17-G).[348]

Insulin or insulin-like material is also produced in ciliated protozoa, vertebrate and invertebrate animals, fungi, green plants,[349,350] and even in *E. coli*.[351]

1. Metabolic Effects of Insulin

Insulin has many effects on metabolism.[348,352,353] (Some of these are listed in Table 17-3). They can be summarized by saying that: (1) In most tissues insulin stimulates synthesis of proteins, glycogen, and lipids. (2) It affects the permeability of membranes, promoting the uptake and utilization of glucose and amino acids and of various ions from the blood. (3) It promotes both synthesis of glycogen and the breakdown of glucose by glycolysis. At the same time, it inhibits synthesis of glucose from amino acids by the gluconeogenesis pathway. The effects are not uniformly the same in all tissues. Some can be observed within a few minutes after administration, presumably as a result of regulation of enzymes. Effects on mRNA metabolism, protein synthesis, and cell growth are seen at later times.

The uptake of glucose by brain, liver, kidneys, erythrocytes, and the islets of Langerhans is unaffected by insulin. However, in muscle and adipose tissues insulin stimulates glucose uptake. Part of this effect results from insulin-induced translocation of molecules of the 509-residue glucose transport protein GLUT4 (Chapter 8) from the cytosol into the plasma membrane where it can function.[354–356a] Insulin apparently also increases the rate of synthesis of the transporters.

Insulin stimulates the phosphorylation of serine side chains of many proteins, including ATP citratelyase, acetyl-CoA carboxylase, and ribosomal subunit S6. At the same time it stimulates the dephosphorylation of other proteins, including acetyl-CoA carboxylase, glycogen synthase in skeletal muscle (Fig. 11-4),[49] pyruvate dehydrogenase, and hormone-sensitive lipase in adipose tissue. Yet another effect of insulin is to alter the amounts of specific messenger RNA molecules. For example, the transcription of the gene for phosphoenolpyruvate carboxykinase (PEPCK; Eq. 13-46) a key enzyme in gluconeogenesis, is inhibited by insulin within seconds after binding. The hexokinase isoenzyme called **glucokinase** phosphorylates glucose to glucose-6-*P* in liver and in pancreatic β cells. Its synthesis in liver is induced by insulin. (However, in the β cells the synthesis of glucokinase is induced by glucose.)

2. Insulin Receptors

All of the effects of insulin appear to result from its binding to insulin receptors, of which ~10^2 to 10^5 are present in the plasma membranes of most animal cells. First isolated in 1972,[357] insulin receptors and their cloned genes have been studied intensively. The receptors are $\alpha_2\beta_2$ disulfide crosslinked oligomers composed of pairs of identical 120- to 135-kDa α subunits and 95-kDa β subunits. An α and a β subunit are cut from a single precursor chain. The human insulin receptor precursor exists as two isoforms, A and B, which arise as a result of a difference in splicing of the mRNA.[358] Twenty-one introns are removed by splicing to form the mRNA for the 1355-residue B precursor.[348,358a] The mRNA for the A form lacks a 36-nucleotide segment (exon 11) that is discarded during splicing. The α chains come from the N-terminal part of the precursor and the β chains from the C-terminal part. The receptor sequence is numbered as in the longer B form precursor. (However, many authors number the chains of the A form receptor as in its precursor, 12 less than the numbers given here for residues 719 or higher[359]). The B form receptor has 731-residue α chains while the A form has 719-residue α chains as a result of the missing sequence from exon 11 (see Fig. 11-11A). Four residues (732–735 in the B form) are cut out and discarded, leaving 620-residue β chains for both isoforms. The chains become linked by three or more disulfide crossbridges.[360]

The two α subunits, which contain the insulin binding sites, are apparently present entirely on the outer surface of the plasma membrane. The β subunits pass through the membrane with their C termini in the cytoplasm.[361–363] Both α and β subunits are glycoproteins. Study of the amino acid sequences suggests that only one 23-residue segment (residues 930–952 of the B isoform) of hydrophobic amino acid residues in each β subunit is likely to exist as an α helix that spans the membrane. This raises several questions. How can a signal be sent from the cell surface into the cytoplasm through a single pair of α helices? Are there additional parts of the receptor that span the bilayer of the membrane? To make it easier to visualize these questions refer to Fig. 11-11B, which is a more realistic, although fanciful, drawing than that in Fig.11-11A. Electron microscopy and crystallography are now providing the first direct images of the receptor.[363a, b, c]

One possible way in which a signal could be sent through the membrane is for binding of insulin to promote aggregation of two or more receptors.[364] If insulin induces the receptors to stick together on the outside of the cell, the parts protruding into the cytoplasm would also tend to aggregate. This could induce a response. A second possibility is that a conformational change in the α subunits pulls or pushes on the β subunit allowing the latter to be exposed less or more on the cytoplasmic side. This difference might be sufficient to cause a conformational change in the cytoplasmic domain of the subunit. A third possibility involves activation by a twisting mechanism as proposed for the aspartate receptor (Fig. 11-8).

A large part of the cytoplasmic domain of the insulin

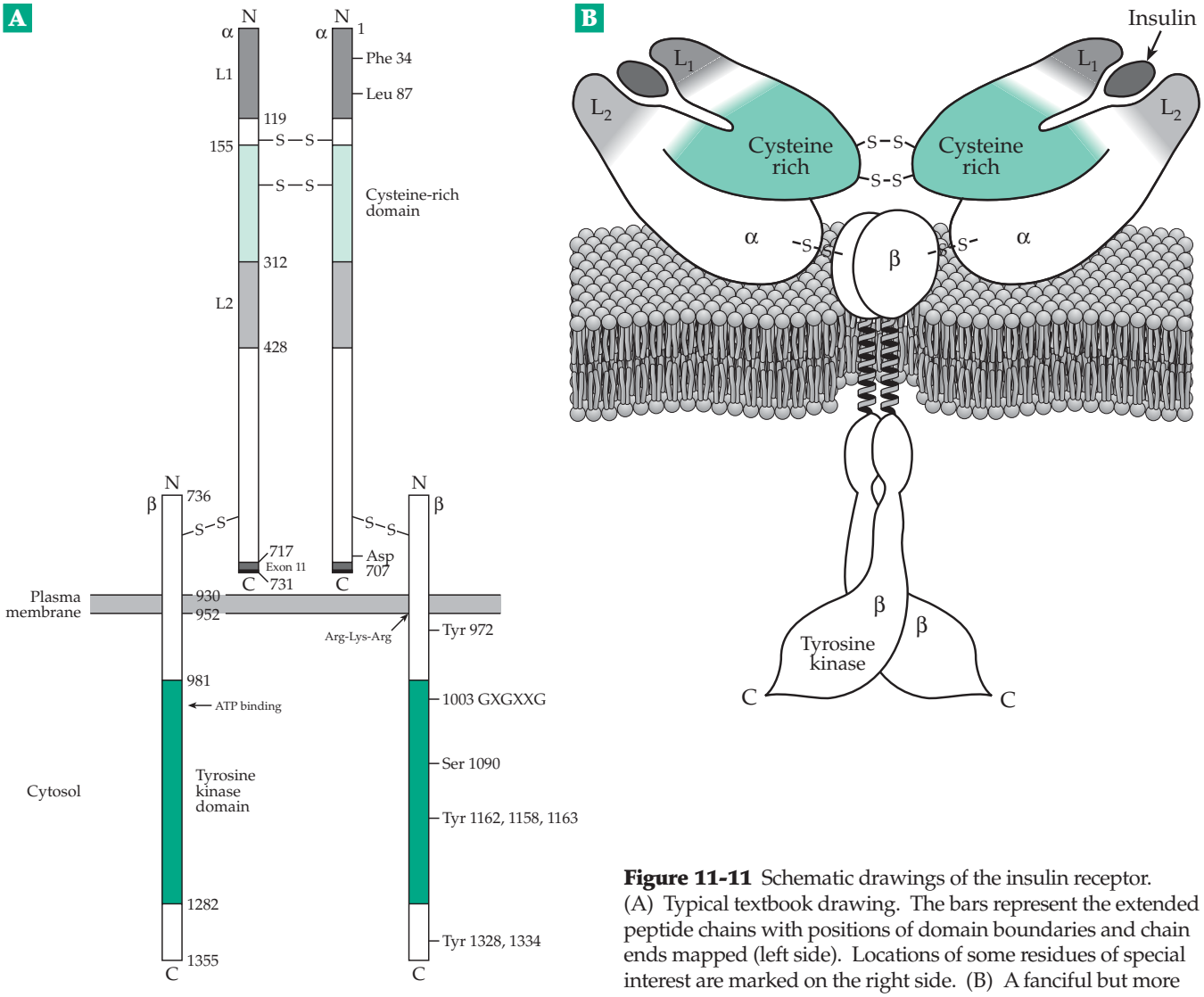


Figure 11-11 Schematic drawings of the insulin receptor. (A) Typical textbook drawing. The bars represent the extended peptide chains with positions of domain boundaries and chain ends mapped (left side). Locations of some residues of special interest are marked on the right side. (B) A fanciful but more realistic picture of the receptor. See Fabry *et al.*[367]

receptor consists of a tyrosine-specific protein kinase whose three-dimensional structure is depicted in Fig. 11-12.[365,365a] Not only can it phosphorylate –OH groups on tyrosine side chains of other proteins[362] but also it catalyzes ATP-dependent **autophosphorylation** of several residues in the C-terminal region. The most readily phosphorylated residue is Tyr 1158.[366] Some diabetic individuals have receptors with impaired tyrosine kinase activity.[362]

Using directed mutation of the cloned receptor gene, Lys 1018 in the ATP-binding part of the tyrosine kinase domain was replaced by alanine. This caused a loss both of kinase activity and of biologic response to insulin.[362] Thus, both the tyrosine kinase activity and autophosphorylation appear essential. If so, aggregation of two or more receptors may increase the extent of autophosphorylation and initiate a response.

Studies of many mutant proteins indicate that insulin binds to the α chains of the receptor between the two domains labeled L1 and L2 in Fig. 11-11.[368] Among essential residues is Phe 39 (marked).[369] Insulin contains three disulfide linkages (Fig. 7-16) and might undergo a thiol–disulfide exchange reaction (Eq. 11-7) with SH groups present in a cytoplasmic cysteine-rich domain of the α subunit of the receptor or with an external thiol compound.[369a,b] Such an exchange may also be essential for activation of the receptor.[370]

3. A Second Messenger for Insulin?

The known regulatory effects of insulin (Table 17-3) often involve phosphorylation of serine or threonine side chains on specific proteins. The tyrosine kinase of the activated insulin receptors does not catalyze such phosphorylation. Therefore, it seems likely that one or more second messengers or mediator substances are needed. Much effort has gone into searching for

Figure 11-12 (A) Stereoscopic view of an α-carbon trace of the insulin receptor kinase domain. Every tenth residue is marked with a filled circle and every twentieth residue is labeled. (B) Locations of missense mutations in noninsulin-dependent diabetes mellitus patients mapped onto the receptor kinase structure. The mutations are R993Q, G1008V, A1048D, R1164Q, R1174Q, P1178L, W1193L, and W1200S. Here, R993Q is the mutant in which arginine 993 is replaced with glutamine, etc. From Hubbard *et al*.[365]

substrates for the tyrosine kinase,[353,362,371,372] whose activity on external protein substrates reaches a maximum after receptor tyrosines 1158, 1162, and 1163 have been autophosphorylated. Phosphorylation of such **insulin receptor substrates (IRSs)** as well as of some smaller "adapter" proteins is thought to initiate a series of complex cascades that serve to pass the insulin signal on to a variety of sites of action within a cell.[373–376] The large 185-kDA **insulin receptor substrate-1 (IRS-1)**, which is present in most cells, is phosphorylated on several tyrosines, usually within the sequences YXXM or YMXM. Phosphotyrosine within such sequences is known to be a ligand for the recognition domain SH2, which is present in many proteins. Binding of SH2 domains of other proteins to IRS-1 allows the insulin signal to be passed to several different proteins in the branched signaling pathways. Other insulin receptor substrates include **IRS-2**,[376a,b] protein Gab-1[376c], and the smaller protein called **Shc**, which occurs as 46-, 52-, and 66-kDa isoforms and is discussed further on p. 568.[376,377] Shc is an adapter protein which forms a complex that triggers the activation of the G protein Ras and the MAP kinase cascade shown in Fig. 11-13.[377,378] This pathway (see Section H,2) is thought to mediate the mitogenic (growth promoting) effects of insulin and also to promote phosphorylation of serine and threonine side chains of many proteins in the cytoplasm, the cytoskeleton, ribosomes, membranes, and the nucleus.[379,380] One serine kinase specifically phosphorylates the insulin receptor on serine 1078.[381]

In addition to IRS-1, IRS-2, and Shc, there are additional adapter proteins that interact with the phosphorylated tyrosine kinase domain of the insulin receptor.[382] Furthermore, the receptor tyrosine kinase may catalyze *direct* phosphorylation of some proteins, e.g. a cytoplasmic loop of the β_2 adrenergic receptor (Fig. 11-6), without intervention of an adapter.[383]

Phosphorylated IRS-1 activates a second signaling pathway by interacting with an 85-kDa SH2-containing protein that is a subunit of phophatidylinositol 3-kinase.[384–386] This activates the 110-kDa catalytic subunit of the 3-kinase, which catalyzes formation of phosphatidylinositol 3-phosphate as well as PtdIns (3,4)P_2 and PtdIns (3,4,5)P_3.[387,387a] These compounds, which remain within membranes, activate other branches of the signaling cascade, some of which may converge with those of the MAP kinase cascade. However, there appears to be specific activation of a ribosomal Ser/Thr kinase that, among other activities, phosphorylates ribosomal protein S6, a component of the small ribosomal subunit.[388] It also phosphorylates some isoforms of protein kinase C and other enzymes. PtdIns 3-kinase may also activate 6-phosphofructo-2-kinase (Fig. 11-2, step *d*).[384,388]

One of the most important effects of insulin is to increase glucose uptake by cells.[373,389] The mechanism is thought to depend upon the transporter protein GLUT4, which is stored within the membranes of small cytoplasmic vesicles. Binding of insulin to its receptors induces movement of these vesicles to the plasma membrane where fusion with the plasma membrane makes the GLUT4 molecules available for glucose transport.[390] Phosphatidylinositol 3-kinase also plays an important role. The PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 generated by this enzyme (Fig. 11-9)

remain in the membrane and provide sites to which various proteins, e.g., those containing PH domains (Chapter 7) may bind. Among the proteins "recruited" to the inner membrane surface in this way is a Ser/Thr protein kinase known as PKB/Akt. The Akt abbreviation refers to its relationship to a particular viral oncogene.[390a–390e] and the phosphatidyl-inositol derivatives generated by the 3-kinase may have a direct effect upon the exocytosis of the GLUT-4 containing vesicles as may Gq-coupled receptors.[391]

Although insulin unquestionably stimulates phosphorylation of many proteins, the major metabolic effect appears to result from *dephosphorylation* by phosphatases of phosphorylated forms of several enzymes.[379,392] These include **glycogen synthase** and **pyruvate dehydrogenase**, which are both activated by dephosphorylation, and **glycogen phosphorylase**[393] and **hormone-sensitive lipase**, which are deactivated by dephosphorylation. These changes result in increased synthesis and storage of both glycogen and triacyglycerols. Insulin also stimulates a membrane-bound cAMP phosphodiesterase causing a reduction in cAMP concentrations.[394]

Insulin injected into the human body has a half-life of only 5–10 minutes. Much of the hormone is destroyed by hydrolytic cleavage of the peptide chain by a non-lysosomal protease.[395–397] The receptors together with the hormone are taken into cells by endocytosis from clathrin-coated pits and both may be degraded in the lysosomes.[396,398] Some signaling may arise from insulin receptors in endosomes.[399] Some of the hormone enters the nucleus, perhaps still bound to the receptors,[400] and may have a direct effect upon gene transcription. Some receptors are recycled to the cell surface.[398] This latter process, together with that of new receptor synthesis, controls the number of receptors on the surface and, in turn, the sensitivity of the cell to insulin. This is only one mechanism used for desensitization or **downregulation** of receptor sensitivity and numbers, something that happens normally for any hormone when its concentration is high.[401,402]

H. Growth Factors, Oncogenes, and the Cell Cycle

Insulin is just one of a large number of proteins that are secreted by cells and which influence the growth of nearby cells.[403–405] For example, blood platelets, which aggregate at the site of an injury to a blood vessel, contain granules (α granules) which, when the platelet is "activated" (see Box 8-A) release **platelet-derived growth factor** (PDGF), a 28- to 31-kDa glycosylated peptide which stimulates growth and tissue repair in the injured region.[406,407] There are many other protein growth factors, a few of which are considered here. Others are discussed in Chapters 30–32.

1. Oncogenes and Proto-oncogenes

Studies of cancer-causing cellular oncogenes and **proto-oncogenes** (**c-*onc***) identified in the human genome[408–411] have contributed greatly to our understanding of the action of growth factors. Proto-oncogenes are segments of DNA that code for proteins that have a normal function but which may become "activated" by a mutation or by chromosomal rearrangement to become cancer-causing oncogenes (Box 11-D). Oncogenes were discovered first in oncogenic (cancer-causing) retroviruses,[412] where they are designated v-*onc*. The v-*onc* genes are usually very similar but not identical to the corresponding c-*onc* genes and are thought to have arisen from them. The v-*onc* genes are often incomplete or have become fused to other genes by genetic recombination. Considerable excitement has attended the discovery that *many solid human tumors contain activated oncogenes*,[413] several of which are listed in Table 11-3.

In 1983 it was found that the oncogene *sis*, which is carried by the simian sarcoma virus, has a 104-residue sequence nearly identical to that of human PDGF.[404,414] The PDGF receptor has a large extracellular domain consisting of five immunoglobulin-like domains. Like the insulin receptor, it has tyrosine kinase activity which resides in a C-terminal cytoplasmic domain.[406,407,415] This suggests that the malignant transformation of cells by the viral v-*sis* gene leads to an excessive production of normal PDGF and, consequently, to excessive growth. The sequences related to v-*sis* are often found in human tumors and are located on chromosome 22.[416] Rearrangements by which part of this chromosome is moved to another location are well known and sometimes lead to conversion of the proto-oncogene to an active cancer-inducing oncogene.

The avian v-*erbB* oncogene and oncogene *neu*[417-419] both have sequences homologous to that of the gene for the receptor for the 53-residue **epidermal growth factor** EGF.[420–423a] The corresponding cellular c-*erbB* is the gene for the EGF receptor, a large 170-kDa 1186-residue protein. It also resembles the insulin receptor in having an N-terminal domain outside the cell, a single hydrophobic helix that spans the membrane, and a cytoplasmic domain with tyrosine-specific protein kinase activity.[403,419,424,425] It differs from the insulin receptor in being a single peptide chain. However, when EGF binds, the receptor dimerizes and the protein kinase is activated.[426] It phosphorylates its own Tyr 1173 near the C terminus, as well as tyrosines in the **lipocortins**, 36- to 38-kDa calcium-binding proteins located on the cytosolic face of plasma membranes.[427] The activated receptor also stimulates phospholipase C with a resultant increase in concentrations of inositol trisphosphate and Ca^{2+} (see Fig. 11-13).[428] The active oncogene v-*erbB* encodes a major fragment of the EGF receptor. However, most of the N-terminal domain

TABLE 11-3
A Few Oncogenes That Have Interested Biochemists

Oncogene Symbol	Source	Properties
v-*sis*	Simian sarcoma virus	Gene product is closely related to the B-chain of platelet-derived growth factor
v-*erbB*	Avian erythroblastosis virus	Gene product is shortened version of the EGF receptor, a tyrosine kinase
neu	(Her-2) Rat neuroblastomas A similar gene is found in human breast cancers and adenocarcinomas	Homologous to EGF receptor; has tyrosine kinase activity; may control phosphotidylinositol 3-kinase
v-*src*	Rous avian sarcoma virus	Gene product is another tyrosine-specific protein kinase
abl	Chronic myelogenous leukemia	Similar to *src*; characteristic chromosomal translocations yield cancer
ras, *has*, v-H-*ras* v-K-*ras* N-*ras*	Human bladder, colon, lung carcinomas	Homology with G_s and G_i regulatory proteins; several human proto-oncogenes exist; a single mutation in H-*ras* may lead to cancer
bas, *kis*	Murine sarcoma viruses	
v-*myc*	Burkitt's lymphoma, mouse plasmacytomas, avian retrovirus MC29	Nuclear location for gene product; which forms complex with protein Jun
mos	Burkitt's lymphoma, mouse plasmacytomas	
v-*fos*	Osteosarcoma virus (mouse)	Nuclear location for gene product, which forms complex with protein Jun
v-*jun*		Protein product is subunit of transcription factor AP-1
*bcl*1		Cyclin D1

including the EGF binding site is missing and the C-terminal end has been shortened. The tyrosine kinase domain is intact but Tyr 1173 is missing.

The rat *neu* oncogene (also called *erbB*-2 and *HER*-2), which apparently is derived from the gene for another growth factor receptor, differs from normal *neu* by a single nucleotide. This change causes valine to be substituted for glutamic acid at position 664 in the membrane-spanning domain of the 185-kDa protein.[418] This evidently gives an overactive and perhaps uncontrolled tyrosine-specific protein kinase. The c-*erbA* proto-oncogene appears to be the nuclear receptor for the thyroid hromone **triiodothyronine** (Chapter 25).

The Rous sarcoma virus oncogene v-*src* and a family of related oncogenes are derived from protein tyrosine kinases that are attached with the aid of a myristoyl anchor to the inner, cytoplasmic surfaces of membranes.[429–431] They may be activated by interaction with an occupied surface receptor. It has been difficult to understand the functioning of the normal Src protein. However, inactivation of c-*src* in mice caused the serious bone disease **osteopetrosis** in which the osteoclasts fail to function properly in resorbing the bone matrix, thereby allowing excessive accumulation of calcium phosphate.[432,433] A c-*src* deficiency also decreases formation of the bone adhesion protein **osteopontin**, an RGD protein.[434] There is actually a *family* of src proteins, some of which have important functions in lymphocytes.[435–438] The gene for one of these is mutated in the β cell disorder **agammaglobulinemia**.[437]

Another oncogene derived from a tyrosine kinase

BOX 11-D CANCER

Although cancer occurs in about 200 clinically distinct types, most cancers can be classified into four categories. In **leukemias**, which account for 3% of the ~700,000 cases of cancer diagnosed per year in the United States, an abnormal number of leukocytes are produced by the bone marrow. **Lymphomas**, such as Hodgkin's disease and Burkitt's lymphoma, arise from lymphocytes. They account for ~5% of human cancers. In these diseases malignant cells are produced in the spleen and lymph nodes and sometimes aggregate in lymphoid tissues. **Sarcomas**, solid tumors of bone or other connective tissue, contribute ~2% to the total of human cancers, while **carcinomas**, cancers of epithelial tissue, account for 85%. Carcinomas may develop from either the external or the internal epithelia, including the glands, lungs, and nerves.[a–d] More than one-third of all cancers in the United States are nonmelanoma carcinomas of the skin;[e] for these the mortality rate is low. Lung, colorectal, breast, and prostate tumors account for 55% of cancer deaths.[c] Carcinomas predominate in humans, but lymphomas, leukemias, and sarcomas are much more prevalent in laboratory animals and fowl.

An important characteristic of cancer cells is their uncontrolled proliferation. They don't respond to the normal signals from adjacent cells that indicate that cell division should stop. Cancer cells also differ dramatically from those present in warts and other benign tumors and in psoriasis. These conditions also result in excessive proliferation of cells and partial derangement of normal regulatory processes.

A second characteristic of cancer cells is that they usually appear less differentiated than the tissues from which they arise and are more like embryonic cells. Many cancers produce **ectopic proteins**, proteins inappropriate to the tissue involved and often identical to proteins synthesized by embryonic or fetal cells. A well-known example is **α-fetoprotein**, a 72-kDa glycoprotein normally present in serum in almost undetectable amounts but present in large amounts when some types of cancer are present.[f] A third property of cancers is the tendency toward **metastasis**, the detachment of cells from the cancer and their development in distant parts of the body.[g–i]

Cancer cells don't grow any faster than normal cells but they continue to divide when normal cells would not. For this reason, a cancer can grow rapidly and its demands for nutrients can literally starve the host. Cancer tends to weaken the immune system, making the host more susceptible to infections. In addition, cancers often interfere directly with the functioning of various organs and may cause death in this way.

What initiates a cancer? We know that cancers can arise from only one or a very small number of cells and that cancer can be induced by carcinogenic chemical compounds, by certain viruses, and by radiation. Use of tobacco appears to be responsible for about 30% of all human tumors.[c,j] Diet also affects the likelihood of developing cancer. For example, diets containing less animal fat and more fruits and vegetables are associated with lower levels of colon cancer.[c,k] Many carcinogenic compounds are naturally present in foods. Genetic factors help to determine susceptibility to cancer[b,l] and characteristic chromosomal aberrations are usually associated with cancer.[m–o] The incidence of cancer increases markedly with age.

A common feature of all the agents that induce cancer is the production of mutations and cancer probably always involves some alteration in the cell's DNA. The long lag between exposure to carcinogenic materials and development of cancers, often 20 years or more, suggested that more than one mutation or chromosomal rearrangement is required for production of a cancer. Recent evidence confirms that several mutations are required.[b,p] Relevant to this conclusion is the fact that carcinogenic compounds can be applied to the skin in amounts sufficient to cause a number of mutations in the epithelial cells but insufficient to actually induce cancer. Then, even many years later, irritant compounds known as **cancer promoters** can be applied and cancer will develop promptly. The promoters apparently induce cell proliferation which leads to more errors in DNA replication, converting an initially mutated cell to a cancerous cell. The most studied promoters are the phorbol esters, which are known to activate the protein kinase C isoenzymes (Section E,2). Any factors that increase rates of cell division such as some hormones, excess calories, or chronic inflammation cause increased cancer.[c] Cell divisions are accompanied by errors in replication of DNA and sometimes by translocation or deletion of parts of chromosomes. Chronic infection by bacteria, viruses, or other organisms may cause cancer as a result of continuing inflammation. Other cancers arise from integration of viral DNA into the host's DNA.

Virally induced cancers are often epidemic among poultry and rodents. When infected with cancer-causing viruses from these animals, cells in culture often become **transformed**. Whereas normal cells tend to respond to **contact inhibition** and grow as a monolayer, transformed cells continue to divide after the monolayer is complete. In laboratory studies, transformation of cells is often taken as the equivalent to an early step in cancer production in an animal. Studies of transformation led to the identification and characterization of several **viral oncogenes** which cause the transformation. These are designated by abbreviations such as v-*src* (the oncogene of Rous sarcoma virus, which induces cancer in chickens) and v-*sis* (the oncogene of simian sarcoma virus, which causes cancers in monkeys). Some other oncogenes are described in the main text. Naturally occurring gene

BOX 11-D CANCER (continued)

sequences closely homologous to those of the viral oncogenes have been found in many solid human tumors. Study of oncogenes has shown that they are related to and derived from **proto-oncogenes**, normal cellular genes that are involved in control of growth and differentiation.

Oncogenes are often "amplified" in tumor cells so that their copy number is greater than that of the corresponding genes in normal cells. For example an oncogene related to the viral oncogene *neu* is amplified in many human breast and ovarian cancers[q] and oncogene *src* in many colon cancers.[r] Mutated *ras* genes have been found in over one-third of human colorectal cancers.[s] Amplified oncogenes *ras*, *myc*, and *myb* have been observed in other cancers.[t] Oncogenes are often overexpressed or are responsible for over-expression of other genes. One idea that developed from these observations is that cancer cells may secrete new or mutated growth factors that stimulate their own receptors (**autocrine** stimulation) in a way that promotes uncontrolled growth.[u]

Cancer develops in stages and in many cases defective proto-oncogenes appear before truly malignant cells appear. The latter must result from additional mutations that often involve *loss* of parts of chromosomes. A major breakthrough in our understanding of cancer and how it is induced by loss of genes has come from studies of some rare cancers that are inherited in a Medelian fashion. One of these is **retinoblastoma**, an intraocular tumor which affects 1 child in 20,000. Homozygotes always develop the disease between the ages of 1 and 5. The hereditary defect has been traced to the absence of a functional retinoblastoma gene *RB1*, which is found in band q14 of chromosome 13.[v] Additional mutational events are required to induce cancer. The *RB1* gene was the first **tumor-suppressor** gene (anti-oncogene) identified. These suppressor genes encode proteins that inhibit growth.[w–z] The retinoblastoma gene encodes a 105-kDa DNA-binding phosphoprotein (Rb-*P*).[aa–bb] The Rb protein is phosphorylated and dephosphorylated in a cyclic fashion that is synchronized with the cell replication cycle (Fig. 11-15). It forms a complex with a transcription factor E2F that functions in transcription of the adenovirus genes and is also involved in control of the cell replication cycle.[cc] Deletion of the Rb gene from mice leads to death of embryos homozygous for the mutation.[dd]

Study of other rare hereditary cancers has led to the location of 20 or more additional probable tumor-suppressor genes. One of these, ***p53***, is inactive in over 50% of all human cancers and over 90% of sqamous cell carcinomas of the skin.[ee] In small-cell lung cancers and osteosarcoma *both RB* and *p53* are inactive.[z] Protein p53 is a stronger tumor suppressor than protein Rb. Results of a variety of experiments have suggested that p53, a DNA-binding protein of known structure,[ff] plays a key role in checking DNA for damage at the G_1 to S-phase checkpoint in the cell cycle. If the DNA has too many defects the cycle is stopped in the G_1 stage and the cell may be killed by the process known as **apoptosis**.[gg–jj] Protein p53 has been called the "guardian of the genome." The mechanisms by which it functions are complex and poorly understood. It may act with the assistance of Rb and many other proteins. Mutations in DNA and their repair are discussed in Chapter 27 and the cell cycle is discussed in this chapter and further in Chapters 26 and 32.

Many other cancer susceptibility genes also encode suppressors. Mutation in genes *BRACA1*[kk] and *BRACA2*[ll] are responsible for early onset ovarian and breast cancer. Gene *DPC4* may be a suppressor of pancreatic cancer.[mm] The gene *ptc* (patched), first studied as a developmental gene in *Drosophila*, may encode a suppressor of basal cell carcinoma, the commonest form of human skin cancer.[nn] Gene *p16* (also called *CDKN2*) may be a major suppressor that is mutated in many cancers including the dangerous skin melanoma.[oo] Mutations in the *NF* gene, which may be a cytoskeletal protein, are associated with **neurofibromatosis**,[pp] a relatively common hereditary disease causing tumors of the nervous system. The tumors are usually not malignant but are numerous and disfiguring. Several cancer susceptibility genes are associated with faulty mismatch repair of DNA. Among these is the *APC* gene, whose malfunction is associated with human **familial adenomatous polyposis** which causes thousands of benign tumors in the lining of the large intestine and often colorectal cancer.[qq,rr,ss] In the much more common nonpolyposis colon cancer a complex pattern of instability in several genes is associated with DNA repair,[tt] a topic dealt with further in Chapter 27. The *ATM* gene defective in **ataxia telangiectasia** (Chapter 27) may encode a phosphatidylinositol kinase that is in some way involved in repair of DNA.[ss,uu,vv] A transcription factor gene *nm23* may be a suppressor gene for metastasis[ww] and the cell–cell adhesion molecule **E-cadherin** may suppress tumor invasion in some kinds of breast cancer.[xx] The **VHL** (van Hippel-Lindau cancer syndrome) suppressor protein is defective in the majority of kidney cancers. It normally binds to the **elongin complex**, a DNA-binding complex that functions in control of transcription.[zz]

In addition to treatment by surgery there are numerous chemical approaches to combating cancer.[xy] They usually exploit the tendency of cancers to grow continuously. For example, a toxic analog of a metabolite needed for growth, such as methotrexate (Chapter 16) and 5-fluorouridine (Box 28-C) may be taken up more rapidly by tumor cells than by normal cells. A variety of DNA-binding compounds are useful in chemotherapy (Box 5-B). Alkylating agents such as the nitrogen mustards (Eq. 5-20) and certain antibiotic compounds are also used widely, as are intercalating compounds

BOX 11-D (continued)

such as adriamycin (14-hydroxydaunomycin; Fig. 5-22) and cisplatin (Box 5-B). A disadvantage to most present-day chemotherapy is that normal proliferation of cells, especially of glandular tissues, intestinal epithelium, hair, etc., is severely damaged. A possibility for circumventing this problem is to put normal growth "on hold" temporarily while cancer growth is being inhibited. Some inhibitors of **topoisomerase I** (see Chapter 27) have low toxicity and could be useful. Another therapeutic approach is based on the fact that one function of the immune system is to destroy cancerous or precancerous cells. The immune system tends to weaken with age, which may be one reason that the incidence of cancers rises rapidly in older age Are there ways of stimulating the immune system into increased activity against cancer cells? Another approach is to find ways of increasing the activity of tumor-suppressor genes.

Can drugs be developed to *prevent* cancer? Such "chemoprevention"[yz] may be appropriate for persons carrying genes that make them highly susceptible to cancer. The antiestrogenic drug tamoxifen (Chapter 22) is currently being tested on women with a high risk for breast cancer. Use of oral contraceptives appears to have cut the risk of endometrial cancer substantially.[yy] Newer approaches to contraception may help prevent breast cancer and chemoprevention may also be possible for prostate cancer.[yy]

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gene is the Abelson murine leukemia virus v-*abl*.[438a] In the mouse the c-*abl* gene is split into at least ten exons. However, v-*abl* contains all of these as a correctly spliced sequence suggesting that v-*abl* was derived from a c-*abl* messenger RNA. Part of one exon of the cellular gene is missing in v-*abl* and there is at least one base substitution mutation as well. Human c-*abl* is located on the long arm q of chromosome 9. This arm has long been known to be translocated to chromosome 22 (the "Philadelphia translocation") in patients with chronic myelogenous leukemia. There the c-*abl* gene is fused with another gene.[439] The human c-*sis* gene is also located in the region of chromosome 22 that is translocated to chromosome 9 in the same patients.

A group of human pituitary tumors have been shown to contain an oncogene that is apparently a mutated gene for the stimulatory protein G_s.[440] This is the protein that activates adenylate cyclase in response to hormonal activation. The oncogenic mutations inhibit the GTPase activity that normally turns off this activation. These tumors secrete growth hormone which binds to receptors on the tumor cells activating the defective G_s proteins and causing excessive synthesis of cAMP. This in turn promotes growth of the tumor.

The ras oncogenes. Activated *ras*-oncogenes have been found in at least 25% of all human tumors. The proto-oncogenes, which are designated c-H-*ras*, c-K-*ras*, and c-N-*ras*, are found on the short arms of chromosomes 11, 12, and 1, respectively. A single base substitution (G → T) at position 35 of any of the genes, resulting in a Ras protein containing valine instead of glycine at position 12 of the 21 kDa protein product (usually designated p21) produces an active oncogene. Substitutions at position 13 or at positions 59 and 61, which are adjacent to Gly 12 in the three-dimensional structure, can also activate the oncogenes.[441] From the drawing in Fig. 11-7A the locations of glycines 12 and 13 and of residues 59/61 are seen to be close to the β phospho group of bound GTP. Proteins encoded by activated *ras* oncogenes are less active in catalyzing GTP hydrolysis than are the corresponding normal proteins. In addition, the GTPase-activating protein (GAP) that binds to normal *ras* proteins and stimulates their GTPase activity does not affect the mutant oncogenic proteins.

A single-base alteration is capable of activating a *ras* gene with respect to cell transformation. However, initiation of a malignant tumor requires the additional presence of at least a second activated oncogene such as *myc*[442,443] or *fos*[444] or previous transformation of a fibroblast into a nonmalignant but "immortalized" form by treatment with carcinogens. The c-*myc* gene, which is found in active form in plasmacytomas (tumors of B lymphocytes) of mice as well as in the human Burkitt's lymphoma, is normally located on human chromosome 8. In most Burkitt's lymphomas a translocation has brought the c-*myc* gene into the locus of the immunoglobulin heavy chains on chromosome 14. There its transcription may be subject to different controls than in its original location.[445,446] During the translocation process the c-*myc* gene is often broken within the first intron. Thus, the activated gene lacks the first exon and is placed after a new controlling sequence that may drastically alter its transcription rate. Viral *myc* genes are found in at least one human virus, **cytomegalovirus**, which has been associated with carcinomas.

Transcription factors. The proto-oncogenes c-*myc*[447–451a], c-*myb*,[452–454] c-*fos*, c-*jun* and c-*ets*[455] all encode nuclear proteins involved in regulation of transcription. The 39 kDa protein Jun, which is encoded by c-*jun*, is a major component of the **transcriptional activator** called **AP-1**.[456–459] It binds to palindromic **enhancer** sites (Chapter 28) in DNA promoters to increase the transcription rate for a group of genes. Jun is actually a multigene family whose encoded proteins bind to DNA as complexes formed with the 62 kDa phosphoprotein Fos, the product of the c-*fos* gene.[460] The heterodimeric Fos/Jun complex is held together, at least in part, by interactions between leucine side chains lying along a pair of parallel α-helices in a "leucine zipper" (Fig. 5-36; Fig. 2-21).[461,462]

Regulation of the synthesis of Jun is complex, but growth factors such as Neu, EGF,[463] and PDGF stimulate transcription of c-*jun* in cultured cells. Messenger RNA for synthesis of Fos appears within a few minutes of stimulation of PDGF receptors.[464,465] This is one of the earliest known nuclear reactions to a mitogenic stimulus and suggested that a Ras p21 protein is involved in stimulating transcription of c-*fos* in the signaling pathway from PDGF.[466] Synthesis of Fos is also induced by a variety of other stimuli.[456] Upon translocation into the nucleus Fos combines with preexisting Jun to form AP-1, which binds to sites on DNA and induces the transcription of a large number of proteins (Chapter 28). Deletion of the c-*fos* gene in mice leads to defects in developing bone, teeth, and blood cells,[467] while excessive synthesis of Fos has been associated with the human bone disease **fibrous dysplasia**.[468]

2. The MAP Kinase Cascade

Insulin, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and many other proteins have **mitogenic activity**, that is, they induce cells to transcribe genes, to grow, and to divide. How is this accomplished? Study of such oncogenes as *src* and *ras* suggested that the proteins that they encode also participate in the process, as do transcription factors, including those encoded by the proto-oncogenes

Table 11-4
A Few Abbreviations Used in Discussions of Cell Signaling

AP-1	A major transcriptional activator protein
βARK	Beta adrenergic receptor kinase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK-1, ERK-2	Extracellular signal-regulated protein kinases (proline-directed protein kinases)
Fos	Protein encoded by proto-oncogene *fos* (Table 11-3)
Grb2	Adapter protein containing one SH2 and two SH3 domains
IGF-1	Insulin-like growth factor 1
InsRTK	Insulin receptor tyrosine kinase
InsP_3 or IP_3	Inositol (1,3,5)-trisphosphate
Jun	Protein encoded by proto-oncogene *jun*, an AP-1 gene
MAPK	Mitogen-activated protein kinase (also designated ERK)
MAPKK	A kinase acting on MAPK
MEK-1,MEK-2	Mitogen-activated ERK-activating kinases; dual function Ser/Thr and Tyr protein kinases; also called MAP kinase kinases
c-*myc*	A cellular proto-oncogene
PDGF	Platelet-derived growth factor
PtdIns (or PI) 3-kinase	Phosphatidylinositol 3-kinase
PKA (cAPK)	cyclic AMP-dependent protein kinase
PKC, PKCα, β, γ	Protein kinase C,α, β, γ subforms
PKR	dsRNA-activated protein kinase
PLA	Phospholipase A
PLC	Phospholipase C
PP2A	Protein phosphatase, type 2A
PTK	Protein tyrosine kinase
PTP (PTPase, PTP 1B, etc.)	Protein tyrosine phosphatases
pY	Phosphotyrosine residue
Raf-1	A cytoplasmic serine/threonine protein kinase; also called MAP kinase kinase kinase (MAP3K)
Ras	A monomeric G protein encoded by the proto-oncogene *ras*
RSK	Ribosomal protein S6 kinase
RTK	Receptor tyrosine kinase
RTK-*P*	Phosphorylated receptor tyrosine kinase
SH2, SH3	Src homology domains 2 and 3
Sos	"son of sevenless," a GDP – GTP exchange factor named for a similarity to the protein encoded by the *Drosophila* sevenless gene
Src	Protein tyrosine kinase encoded by oncogene *src* (Table 11-3); contains recognition domains SH2 and SH3
V2R	Type 2 vasopressin receptor
Shc	A proline-rich adapter protein containing SH2 and PH domains

myc, *fos*, and *jun*. The Src and Ras proteins are anchored on the inner surfaces of cytoplasmic membranes. Although the exact functional relationships of all of the components, one to another, has not been completely established, a general picture, usually described as the **mitogen-activated protein kinase** (MAP kinase) **cascade**, has emerged.[69,380,469-470b] This is sketched in simplified form in Fig. 11-13. The many amplification steps provide for ultrasensitive responses.[342]

The binding of a hormone or growth factor (a ligand) to a dimeric receptor activates the protein kinase domain of the receptor which phosphorylates a number of tyrosine hydroxyl groups of the receptor itself. This autophosphorylation is followed by a variety of events, which include phosphorylation of tyrosine side chains of various other proteins.[426] Another major event is the *binding of a variety of different protein molecules containing recognition domains to the phosphotyrosyl groups of the activated receptors.*[471] The major recognition motif is the SH2 domain. See Figs. 7-30 and 11-14.[472–475] Proteins containing SH2 domains can bind to the phosphotyrosyl groups of the activated receptors and while bound become phosphorylated by the receptor tyrosine kinase action and/or be activated allosterically.

Other proteins interact with the receptors indirectly through adapter molecules which have no catalytic activity. Two well-known adapter proteins are **Grb**2[476] and **Shc**.[477,478] The 25-kDa protein Grb2 consists entirely of recognition domains, one SH2 and two SH3 domains (Fig. 11-14). The larger Shc, which is found in all mammalian tissues, contains a 200-residue phosphotyrosyl-binding PH domain (Chapter 7) at the N terminus, a

collagen-like domain that binds to Grb2 and an SH2 domain at the C terminus.[376,377,477]

Adapter Grb2 binds to a phosphotyrosine side chain of an activated receptor, such as that for EGF, and simultaneously binds to the GDP-GTP exchange protein called **Sos** (Table 11-4). This signals Sos to activate the membrane-bound Ras protein by converting it into the GTP form. The second adapter Shc may also participate in formation of the receptor kinase - Grb2–Sos complex,[479] perhaps permitting formation of a more robust complex that may receive signals from more than one kind of receptor. Functions of other members of the Grb adapter family are being discovered.[479a]

Activated Ras binds to and activates the cytoplasmic serine/threonine protein kinase called **Raf-1**.[380,480,481] This kinase becomes transiently activated within 2–3 min of the binding of a mitogen to a receptor. Raf-1 initiates a cascade of other protein kinases by acting on the dual-function Ser/Thr and tyrosine protein kinases called **MEK-1** and **MEK-2**. The phosphorylated, active MEK proteins phosphorylate the mitogen-activated protein kinases **MAPK** which act on a variety of other proteins. Two of the best known MAPK proteins are designated **ERK-1** and **ERK-2**. These are *proline-directed* kinases which phosphorylate serines and threonines that are neighbors to prolines, e.g. in the sequence PLS/TP.[380] The activated ERKs are able to phosphorylate a large number of different proteins including nuclear proteins that control the transcription of such protein transcription factors as AP-1 and Myc. A protein known as the **serum response factor** binds to nucleotide sequences $CC(A/T)_6GC$ in the DNA to locate initiation sites for transcription. Other proteins that have been phosphorylated by the MAP kinase cascade then induce transcription.[482] The induction of c-*fos* mRNA is one of the earliest identified responses to growth factors.[375,456,483] Protein Jun, whose synthesis is induced independently by almost all growth factors, is usually present in excess.

The Fos/Jun complex is transcription factor AP-1, which induces transcription of many genes needed for cell growth. However, transcription of specific genes often depends upon additional nucleotide sequences. For example, the sequence CGGAAA is present in an **insulin response element** found in the promoter sequences of genes encoding such proteins as phosphoenolpyruvate carboxykinase, glyceraldehyde phosphate dehydrogenase, and prolactin–proteins whose synthesis is induced by insulin.[484]

The MAPK cascade also has direct effects upon protein synthesis, i.e., on the translation of mRNA messages. For example, insulin stimulates phosphorylation of proteins that regulate a translation initiation factor, a protein called eIF-4E (see Chapter 29). Phosphorylation of inhibitory proteins allows them to dissociate from the initiation factor so that protein synthesis can proceed.[485,486]

The scheme in Fig. 11-13 is complex, but in reality it is *much* more complex than is shown. Each protein kinase (receptor kinase, Raf-1, MEK, and MAPK) will phosphorylate not only the proteins indicated in this scheme but also any others that meet the specificity requirements of the kinases. Thus, there will be branches diverging from the pathways shown.[486a,b,c] There are isoenzymes that provide further divergence and interaction.[487] Not only do pathways diverge but also others *converge*. Thus, binding of many different ligands to their receptors activates the same MAPK cascade. For example, seven-helix G protein-coupled receptors release their βγ subunits which may also activate Ras as indicated on the right edge of Fig. 11-13A. At the same time the α subunits of the heterotrimer G proteins can affect not only adenylate cyclase but also phospholipases C which can, in some cases, also activate the MAP kinase pathway.[488,489] Sphingosine 1-phosphate may be released from membrane spingolipids and activate the same cascade.[490] However, hormones do not all affect cells in the same way. In view of all the converging pathways, how is this possible? Part of the answer lies in the proximity or spatial separation of components of the pathway. The kinases exist in complexes with other signaling proteins and may phosphorylate them, sending a signal back, as well as forward via other protein substrates. There are also unknown kinetic considerations. Hormones, neurotransmitters, and calcium ions are often released in pulses. The signaling system must integrate effects of all the stimuli that arise from different parts of the cell, at different times, and from differing receptors. At the same time all of the phosphorylated proteins are acted upon by phosphatases that may either activate or deactivate the proteins and by proteases that process newly formed peptides and modify or destroy mature proteins. The various modifying enzymes act on cytosolic proteins, proteins of membranes, of the cytoskeleton, and of the nucleus. The regulatory processes that we discuss in such minute detail involve the very substance of living cytoplasm which is ever-changing and responding to its surroundings. The flow of energy, provided by synthesis of ATP and by the use of ATP by kinases, phosphatases, and the protein synthetic machinery, goes along with the flow of information and drives the signaling network. Evolution has shaped this system to allow it to respond appropriately for every species.

3. The Cell Cycle and Control of Growth

When a cell divides it is of utmost importance that the DNA be replicated reliably. This requires that the dividing cell be large enough and contain enough biosynthetic precursor materials to complete the elaborate process of DNA synthesis and of mitosis. The **cell**

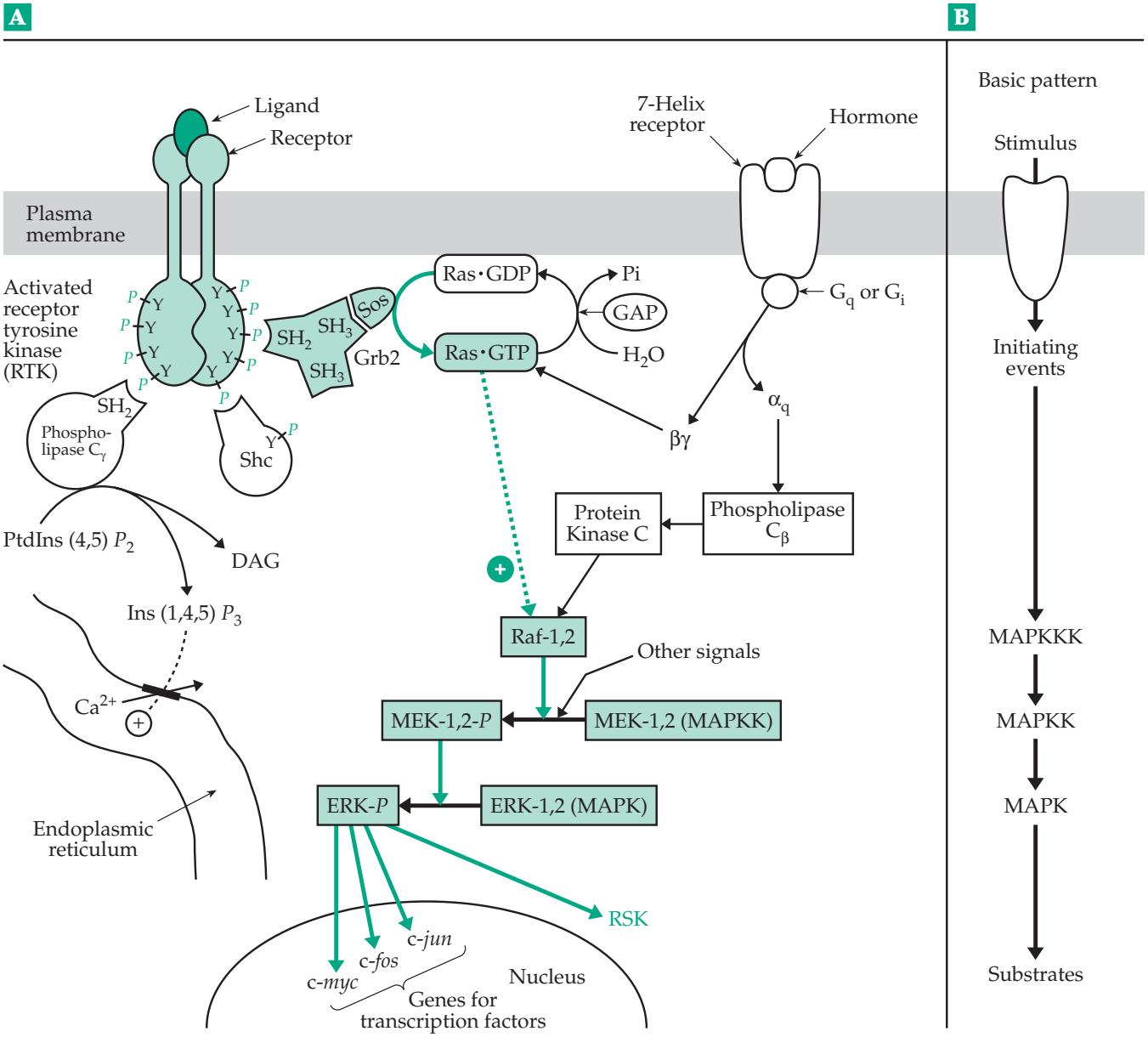

Figure 11-13 (A) A simplified version of the mitogen-activated kinase (MAPK) signaling cascade. At left is shown a hormone receptor, e.g., that for the epidermal growth factor (EGF). The receptor tyrosine kinase undergoes autophosphorylation on numerous tyrosines. The resulting phosphotyrosyl (Y-P) groups bind to SH2 domains of adapters such as Grb2 and Shc. Two pathways from the activated receptor are shown. At the left is activation of phospholipase Cγ and formation, at a membrane-bound site, of inositol trisphosphate and diacylglycerol (DAG). The main pathway, in the center, activates Ras with the aid of the G protein Sos. Activated Ras, in turn, activates Raf and successive components of the MAPK cascade. At the right a seven-helix receptor activates both phospholipase Cβ and Ras via interaction with a βγ subunit. (B) A generalized scheme for the MAP kinase pathway. See Seger and Krebs.[380]

replication cycle (or simply cell cycle) is commonly shown as a circle in which the time from one cell division to the next, in a rapidly growing organism or tissue, is represented by the circumference. The time required for DNA synthesis is the **S-phase** and the time required for mitosis the mitotic or **M-phase** (Fig. 11-15). After metaphase there is a **gap** in time denoted $\mathbf{G_1}$. A second gap $\mathbf{G_2}$ separates the synthetic S-phase and the M-phase. The total time required for one cycle varies with conditions. It may be as short as 8–60 min in an early embryo but is usually two hours or more. A slowly growing cell may pause before the G_1 phase in a nongrowing **G_0 phase**.[491,492]

What controls the cell replication cycle? Signals

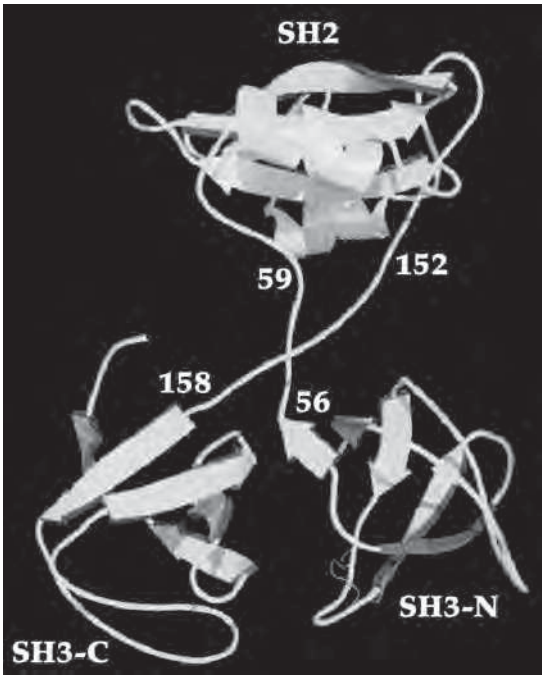

Figure 11-14 Ribbon drawing of the three-dimensional structure of adapter protein Grb2. The two SH3 domains at the N and C termini are labeled, as is the central SH2 domain. Produced with programs MolScript and Raster3D. From Maignan *et al.*[476] Courtesy of Arnaud Ducruix.

from growth factors can stimulate a cell to leave G_0 and enter G_1. However, to go further the cell depends upon a large number of proteins of which two types are prominent. The **cyclins** are labile 45- to 60-kDa proteins which are degraded by the ubiquitin system as part of the cycle. The cyclins associate with a series of protein Ser/Thr kinases known as **cyclin-dependent kinases** (**CDK**s). It is the cyclin–CDK complexes that signal the start of the next step in the cycle.[493–495]

The number of different cyclins and CDK enzymes needed varies with the organism, often being greater for more complex species. As a rule, there are at least two types of cyclins. The **G_1** or **start cyclins** initiate the passage through the start (G_1 checkpoint) into the S-phase, while **mitotic cyclins** initiate the passage from the S-phase into the M-phase. Both vertebrates and *Drosophila* utilize at least four different types of cyclin (A, B, D, and E) and also four or more CDKs (CDK1, CDK2, CDK4, and CDK6).[492] A somewhat different set of these proteins are found in yeast.

Control of cell growth is directly related to the cell cycle. Mitogenic signals from growth factors act to initiate progression through G_1, apparently by stimulating transcription of D-type cyclins.[496] They may affect other steps as well. Oncogenic signals can arise from such oncogenes as *ras* and *abl*.[497,498] As pointed out in Box 11-D, the tumor-suppressor protein Rb becomes phosphorylated in synchrony with the cell

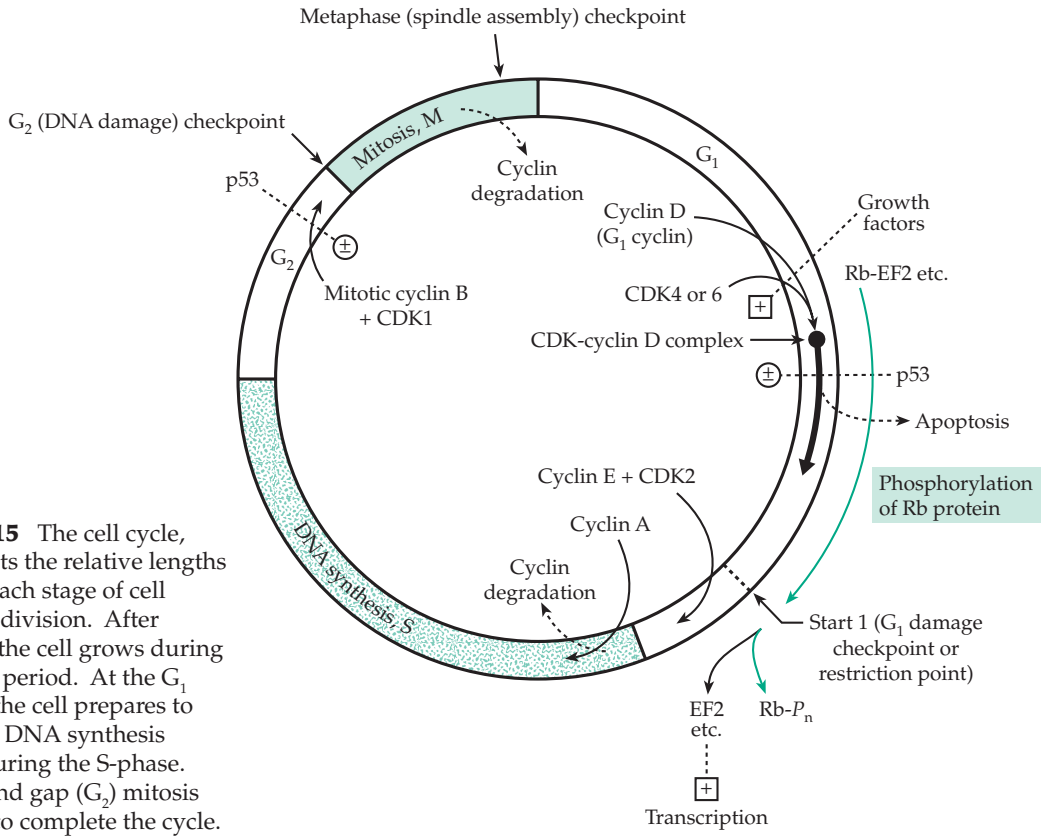

Figure 11-15 The cell cycle, which depicts the relative lengths of time for each stage of cell growth and division. After mitosis (M) the cell grows during the G_1 (gap) period. At the G_1 checkpoint the cell prepares to divide, with DNA synthesis occurring during the S-phase. After a second gap (G_2) mitosis takes place to complete the cycle.

cycle. This seems to be an essential process for passage through the G_1 "checkpoint." The checkpoints in the cycle should be viewed not as points but as essential interlocking processes that must be accomplished before transcription of genes essential for the next step in the cycle can take place.[499] A possibility is that the cyclin-dependent kinase in the cyclin D•CDK4 (or CDK6) complex phosphorylates the Rb protein which in its unphosphorylated state forms a complex with transcriptional regulators of the E2F family. Phosphorylation of Rb allows this complex to dissociate and permits E2F to induce transcription of genes encoding essential proteins for the next step in the cycle.[499a,b,c] Each CDK may also phosphorylate its associated cyclin, a modification that may be essential to the proteolysis of the cyclin and progression to the next stage of the cycle.

The powerful cancer suppressor, p53, which is also described in Box 11-D, in some way senses DNA damage. It prevents passage through the G_1 checkpoint and also through the G_2 checkpoint if the DNA has not been adequately repaired.[496,497] Protein p53, whose three-dimensional structure is known,[500,501] binds to DNA and also induces transcription of genes that cause arrest of the cell cycle. It may also induce cell death (apoptosis), a process that may also require the protein product of protooncogene c-*myc*.[502-505] A variety of protein kinases and phosphatases act on p53 and influence its activity.[506]

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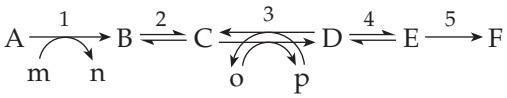
Study Questions

1. Illustrated is a generalized metabolic pathway in which capital letters indicate major metabolites in the pathway, small letters indicate cofactors and numbers indicate enzymes catalyzing the reactions.



   List and describe four different ways in which the pathway might be regulated, referring to the specific enzymes, reactants, and cofactors indicated in the diagram. NOTE: Do not just refer to four different reactions as possible sites of regulation, but give four different general methods for regulation.

2. Describe the role of β-fructose-2,6-bisphosphate in the control of the further breakdown and resynthesis of glucose 1-phosphate.

3. It has been proposed that the substrate cycle involving phosphofructokinase and fructose bisphosphatase is used by bumblebees to warm their flight muscles to 30°C before flight begins. Clark *et al.* (1973) *Biochem. J.* **134**, 589–597 found maximal rates of catalytic activity for both enzymes to be about 44 μ mol / min / g of fresh tissue. In flying bees glycolysis occurred at a rate of about 20 μ mol / min / g of tissue with no substrate cycling. In resting bees at 27°C no cycling was detected, but at 5°C substrate cycling occurred at the rate of 10.4 μ mol / min / g while glycolysis had slowed to 5.8 μ mol / min / g. If the cycling provides heat to warm the insect, estimate how long it would take to reach 30°C if a cold (5°C) bee could carry out cycling at the maximum rate of 40 μ mol / min / g and if no heat were lost to the surroundings.

4. a) Compare the reaction cycle of a small GTPase (G protein) that is regulated by the actions of a GTPase-activating protein (GAP) and a guanine-nucleotide exchange factor (GEF) with that of a G protein linked to a receptor.
   b) Some G proteins have been described as "timed switches" and others as "triggered switches." In what ways might these two groups differ? See Kjeldgaard *et al.* (1996) *FASEB J.* **10**, 1347–1368.

View of a modified bovine fibrinogen molecule. The 45-nm-long disulfide-linked dimer is composed of three nonidentical polypeptide chains. The N termini of the six chains from the two halves come together in the center in a small globular "disulfide knot." The C termini form globular domains at the ends. The 340-kDa molecule has been treated with a lysine-specific protease which has removed portions of two chains to give the ~285-kDa molecule whose crystal structure is shown. Arrows point to attached oligosaccharides. From Brown *et al.* (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 85–90. Courtesy of Carolyn Cohen.

Contents

Transferring Groups by Displacement Reactions

12

The majority of enzymes that are apt to be mentioned in any discussion of metabolism catalyze nucleophilic displacement reactions (Type 1, Table 10-1). These include most of the reactions by which the energy of ATP cleavage is harnessed and by which polymers are assembled from monomers. They include reactions by which pieces, large or small, are transferred onto or off of polymers as well as the reactions by which polymers are cleaved into pieces.[1–3d]

In these reactions a **nucleophilic group** (a base), designated B^- in Table 10-1, approaches an **electrophilic center,** often an electron-deficient carbon or phosphorus atom. It forms a bond with this atom, at the same time displacing some other atom, usually O, N, S, or C. The displaced atom leaves with its bonding electron pair and with whatever other chemical group is attached, the entire unit being called the **leaving group**. This is designated YH in Table 10-1. Simultaneous or subsequent donation of a proton from an acidic group of the enzyme, or from water, to the O, N, or S atom of the leaving group is usually required to complete the reaction. The entering base B, which may or may not carry a negative charge, must often be generated by enzyme-catalyzed removal of a proton from the conjugate acid BH. If BH is water, the enzyme is a **hydrolase**; otherwise, it is called a **transferase**.

For purposes of classifying the reactions of metabolism, in this book the nucleophilic displacements are grouped into four subtypes (Table 10-1). These are displacements on (A) a saturated carbon atom, often from a methyl group or a glycosyl group; (B) a carbonyl group of an ester, thioester, or amide; (C) a phospho group; or (D) a sulfur atom. In addition, many enzymes employ in sequence a displacement on a carbon atom followed by a second displacement on a phosphorus atom (or vice versa).

A. Factors Affecting Rates of a Displacement Reaction

In Chapter 9, the displacement of an iodide ion from methyl iodide by a hydroxide ion (Eq. 9-76) was considered. Can we similarly displace a methyl group from ethane, $CH_3–CH_3$, to break the C–C bond and form CH_3OH? The answer is no. Ethane is perfectly stable in sodium hydroxide and is not cleaved by a simple displacement process within our bodies. Likewise, long hydrocarbon chains such as those in the fatty acids cannot be broken by a corresponding process during metabolism of fatty acids. Not every structure will allow a nucleophilic displacement reaction to occur and not every anion or neutral base can act to displace another group.

At least four factors affect the likelihood of a displacement reaction:[1–3d]

(1) *The position of the equilibrium in the overall reaction*. An example is provided by the hydrolases that catalyze cleavage of amide, ester, and phosphodiester linkages using water as the entering nucleophile. Because enzymes usually act in an environment of high water content, the equilibrium almost always favors hydrolysis rather than the reverse reactions of synthesis. However, in a nonaqueous solvent the same enzyme will catalyze synthetic reactions.

(2) *The reactivity of the entering nucleophile*. Nucleophilic reactivity or **nucleophilicity** is partly determined by basicity. Compounds that are strong bases tend to react more rapidly in nonenzymatic displacement reactions than do weaker bases; the hydroxyl ion, HO^-, is a better nucleophile than $–COO^-$. However, enzymes usually act optimally near a neutral pH. Under these conditions the $–COO^-$ group may be

more reactive than strongly basic groups such as $-NH_2$ or HO^- because at pH 7 the latter groups will be almost completely protonated and the active nucleophile will be present in low concentrations.

A second factor affecting nucleophilic reactivity is **polarizability**, which is the ease with which the electronic distribution around an atom or within a chemical group can be distorted. A large atomic radius and the presence of double bonds in a group both tend to increase polarizability. In most cases, the more highly polarizable a group, the more rapidly it will react in a nucleophilic displacement, apparently because a polarizable group is able to form a partial bond at a greater distance than can a nonpolarizable group. Thus, I^- is more reactive than Br^-, which is more reactive than Cl^-. Polarizable bases such as imidazole are often much more reactive than nonpolarizable ones such as $-NH_2$. Sulfur compounds also tend to have a high nucleophilic reactivity. In displacement reactions on carbonyl groups (reaction type 1B), the less polarizable "hard" nucleophiles are more reactive than polarizable ones such as I^-. Attempts have been made to relate quantitatively nucleophilic reactivity to basicity plus polarizability.[1]

Certain chemical groups, e.g., those in which an atom with unpaired electrons is directly bonded to the nucleophilic center undergoing reaction, are more reactive than others of similar basicity. This **α effect** has been invoked to explain the high reactivity of the poisons hydroxylamine (NH_2OH) and cyanide ion[4] and other puzzling results.[1]

(3) *The chemical nature of the leaving group that is displaced*. The chemistry of the leaving group affects both the rate and the equilibrium position in nucleophilic displacements. The leaving group must accommodate a pair of electrons and often must bear a negative charge. A methyl group displaced from ethane or methane as CH_3^- would be an extremely poor leaving group; the pK_a of methane as an acid[5] has been estimated as 47. Iodide ion is a good leaving group, but F^- is over 10^4 times poorer.[5] In an aqueous medium, phosphate is a much better leaving group than OH^-, and pyrophosphate and tripolyphosphate are even better.

(4) *Special structural features present in the substrate*. Enzymes are usually constructed so that they recognize unique features in substrates. As a consequence, they have many ways of lowering the energy of the transition state and increasing the apparent nucleophilic reactivity.

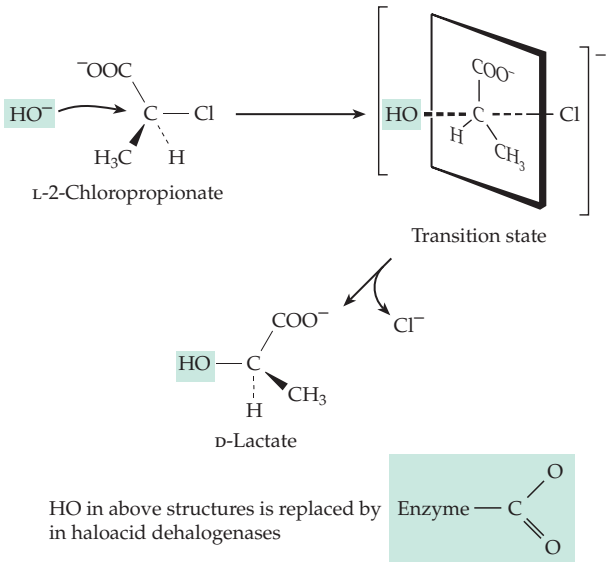
B. Nucleophilic Displacements on Singly Bonded Carbon Atoms

The enzymatic counterpart of Eq. 9-76, the displacement of I^- from a saturated carbon atom by HO^-, is catalyzed by the **haloacid dehalogenases** of soil pseudomonads (Eq. 12-1).[6–8] Even the poor leaving group F^- can be displaced by OH^- in the active site of these enzymes.

$$HO^- + F{-}CH_2COO^- \rightarrow HO{-}CH_2{-}COO^- + F^- \qquad (12\text{-}1)$$

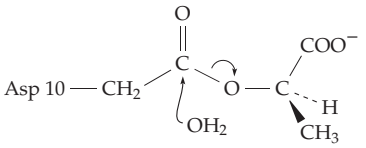
1. Inversion as a Criterion of Mechanism

An interesting result was obtained when a haloacid dehalogenase was tested with a substrate containing a chiral center.[6] Reaction of L-2-chloropropionate with hydroxyl ion gave only D-lactate, a compound with a chirality opposite to that of the reactant. A plausible explanation is that the hydroxyl ion attacks the central carbon from behind the chlorine atom (Eq. 12-2). The resulting five-bonded transition state (center) loses a chloride ion to form the product D-lactate in which inversion of configuration has occurred. *Inversion always accompanies single displacement reactions in which bond breaking and bond formation occur synchronously, as in Eq. 12-2*. This is true for both enzymatic and nonenzymatic reactions. However, the occurrence of inversion does not rule out more complex mechanisms. Indeed, in this case the displacing group is evidently not HO^- but a carboxylate group from the enzyme.



(12-2)

In one of the haloacid dehalogenases, a 232-residue protein for which the three-dimensional structure is known,[8,9] Asp 10 is in a position to carry out the initial attack which would give an enzyme-bound intermediate with an ester linkage:



Hydrolytic cleavage, as indicated in the diagram, yields the product D-lactate.[7] Thus, we have a direct displacement with inversion followed by an additional hydrolytic step. This is an example of **covalent catalysis**, the enzyme providing a well-oriented reactive group instead of generating a hydroxyl group from a bound water molecule.

Related **haloalkane dehalogenases** as well as **epoxide hydrolases** and a large superfamily of other enzymes utilize similar mechanisms.[10,11,11a] In the active site of a haloalkane dehalogenase from *Xanthobacter* (Fig. 12-1) the carboxylate of Asp 124 acts as the attacking nucleophile that displaces Cl^- from the substrate dichloroethane, which is held in a small cavity with the aid of two tryptophan indole rings.[12–14] However, the histidine–aspartate pair and the bound water molecule shown in Fig. 12-1 are essential for the subsequent hydrolysis of the intermediate ester. (see also Fig. 12-11).[15] The substrate shown in Fig. 12-1 is 1,2-dichloroethane, a widespread environmental pollutant that is not known to occur naturally. An interesting question considered by Pries *et al.*[16] is how this dehalogenase has evolved in the years since 1922 when industrial production of dichloroethane began.

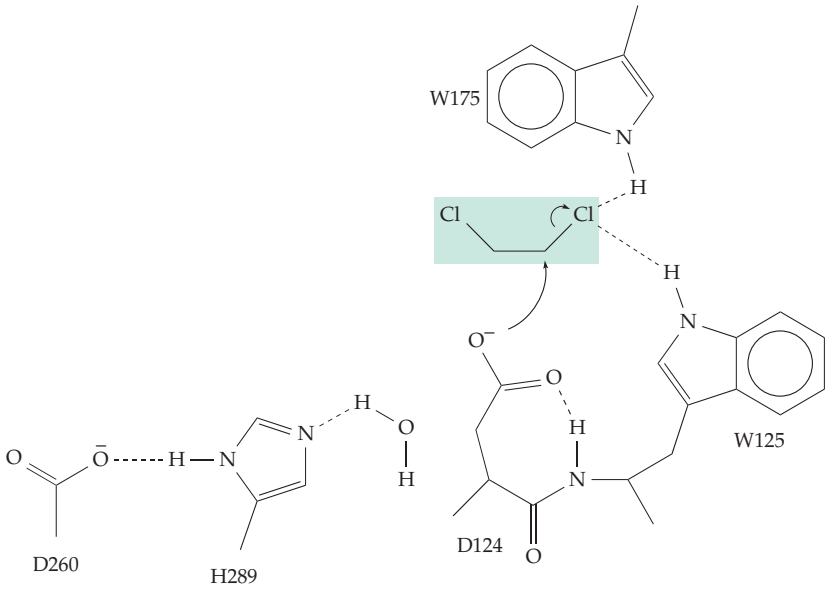


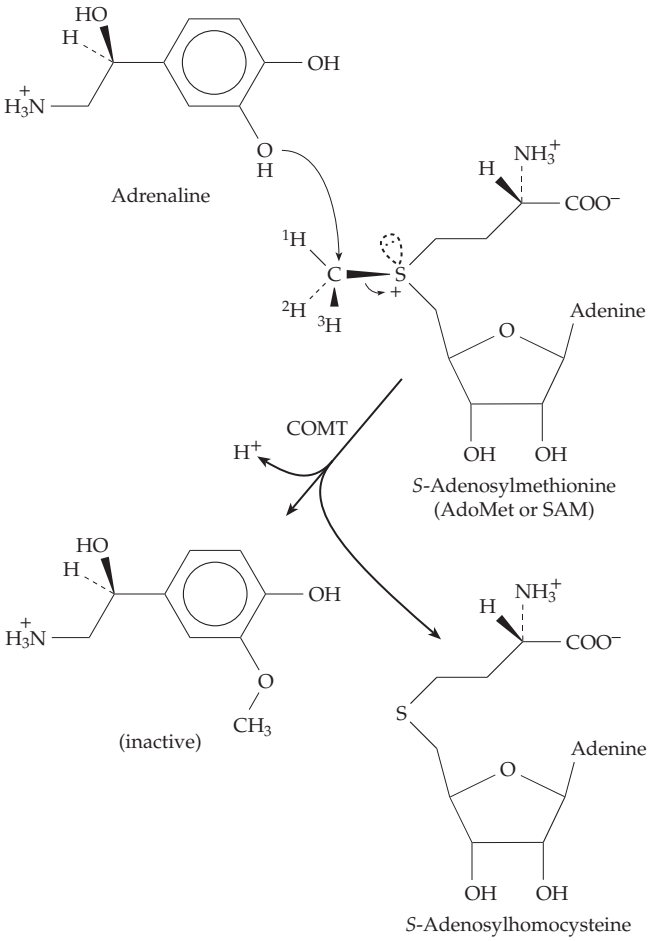
Figure 12-1 The active site structure of haloalkane dehalogenase from *Xanthobacter autotrophicus* with a molecule of bound dichloroethane. See Pries *et al.*[13] The arrows illustrate the initial nucleophilic displacement. The D260 – H289 pair is essential for the subsequent hydrolysis of the intermediate ester formed in the initial step.

2. Transmethylation

Nucleophilic attack on a methyl group or other alkyl group occurs most readily if the carbon is attached to an atom bearing a positive charge, for example, the sulfur atom of a sulfonium ion such as that present in ***S*-adenosylmethionine** (abbreviated **AdoMet** or SAM). The ensuing **transmethylation** reaction results in the transfer of the methyl group from the sulfur to the attacking nucleophile (Eq. 12-3). Transmethylation is an important metabolic process by which various oxygen, nitrogen, and other atoms at precise positions in proteins, nucleic acids, phospholipids, and other small molecules are methylated.[17] The methyl group donor is usually *S*-adenosylmethionine. This compound has two chiral centers, one at the α-carbon of the amino acid and one at the sulfur atom, with an unshared electron pair serving as the fourth group around the S atom. In the naturally occurring AdoMet both centers have the *S* configuration.[18] The reaction of Eq. 12-3, which is catalyzed by **catechol *O*-methyltransferase** (COMT), inactivates the neurotransmitters adrenaline, dopamine, and related compounds by methylation. When the substrate contains a chiral methyl group (– $C^1H^2H^3H$; see also Chapter 13), the inversion of the methyl group expected for a simple S_N2-like reaction is observed.[19]

Structural features similar to those of COMT[20] are found in glycine *N*-methyltransferase[21] and guanidinoacetate methyltransferase[22] from liver, and transferases that place methyl groups on N-6 of adenine[23–25a] and N-4 of cytosine or on C-5 of cytosine[26] in nucleic acids.[27] A stereoscopic view of the active site of glycine *N*-methyltransferase is shown in Fig. 12-2. An acetate ion present in the active site in the crystal has been converted into glycine by computer-assisted modeling. The amino group of the bound glycine is adjacent to the methyl group of AdoMet. A nearby glutamic acid side chain (E15) may have removed a proton from the glycine dipolar ion to create the free $–NH_2$ group required for the reaction. In COMT a magnesium Mg^{2+} ion binds to the two aromatic hydroxyl groups of the substrate and helps to hold it correctly in the active site.[28–29a]

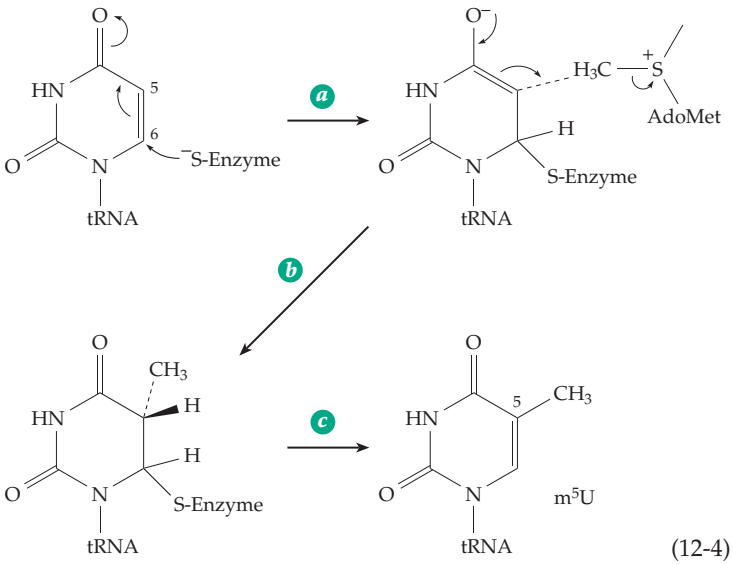
In the case of the more difficult C-methylation of uracil 54 in transfer RNA, an –SH group from Cys 324 of the methyltransferase adds to the C-6 position of U54 of the substrate, which may be any of the *E. coli* tRNAs (Eq. 12-4, step *a*) .[30] In the



(12-3)

adduct carbon atom C-5 acquires substantial nucleophilic character which permits the transfer of the methyl group in step *b*. The adduct breaks up (step *c*) and the product is released.

Methylation of nucleic acids is considered further in Chapers 27 and 28. Methylation of carboxyl groups of certain proteins regulates motion of bacterial flagella (Chapter 19) and other aspects of metabolism[31] while methylation of isomerized aspartyl residues is part of a protein repair process (Box 12-A). Methyl groups are usually transferred from *S*-adenosylmethionine, but sometimes from a folic acid derivative (Chapter 15) or from a cobalt atom of a corrin ring (Chapter 16).



(12-4)

3. Kinetic Isotope Effects

An S–^{12}C bond breaks a little faster than an S–^{13}C bond in a nucleophilic displacement reaction. This **primary kinetic isotope effect** (KIE)[3a,3b,31a–d] is usually discussed first for breakage of a C–H bond. In a linear transition state, in which the C–H bond is being stretched, then cleaved, the difference between the transition state barrier for a C–^{1}H bond and a C–^{2}H bond is thought to arise principally from a difference in the energies of the C–H stretching vibration.[3a] This vibrational energy, in the ground state of a molecule (the zero-point energy) is equal to $1/2\,h\nu_0$ where ν_0 may be observed in the infrared absorption spectrum (see Fig. 23-2). For a C–^{2}H bond, with a stretching vibration at a wave number of ~2900 cm^{-1}, the zero point energy is about + 17.4 kJ mol^{-1}. For a C–^{2}H bond, with a stretching wave number of ~2200 cm^{-1}, it is about +17.4 kJ mol^{-1}. This difference is a result of the difference in mass of ^{1}H (1.67 x 10^{-24} g) and ^{2}H (2.34 x 10^{-24} g). The isotope effect arises because the vibration occurs along the axis of the bond being broken, and the vibrational energy is converted into translational motion along the reaction coordinate, in effect lowering the transition state barrier by $\Delta G^{0\ddagger}$. The difference in this effect between ^{1}H and ^{2}H ($\Delta\Delta G^{0\ddagger}$) gives the ratio of expected rate constants as follows:

$$\frac{k^{C-H}}{k^{C-D}} = e^{-\Delta\Delta G^{0\ddagger}/RT} = 7$$

For heavier nuclei, such as C, N, O the kinetic isotope effect is much smaller.

When ^{13}C was introduced into the methyl group of *S*-adenosylmethionine and its rate of reaction with catechol *O*-methyltransferase was compared with that of the normal ^{12}C-containing substrate, the expected effect on V_{max}, expressed as a first-order rate constant, was seen: $k_{12}/k_{13} = 1.09 \pm 0.05$. This effect is small but it can be measured reliably and establishes that the methyl transfer step rather than substrate binding, product release, or a conformational change in the protein is rate limiting.[32]

Substitution of ^{1}H by ^{2}H in the CH_3 group has a larger effect. This **secondary kinetic isotope effect** (or α-deuterium

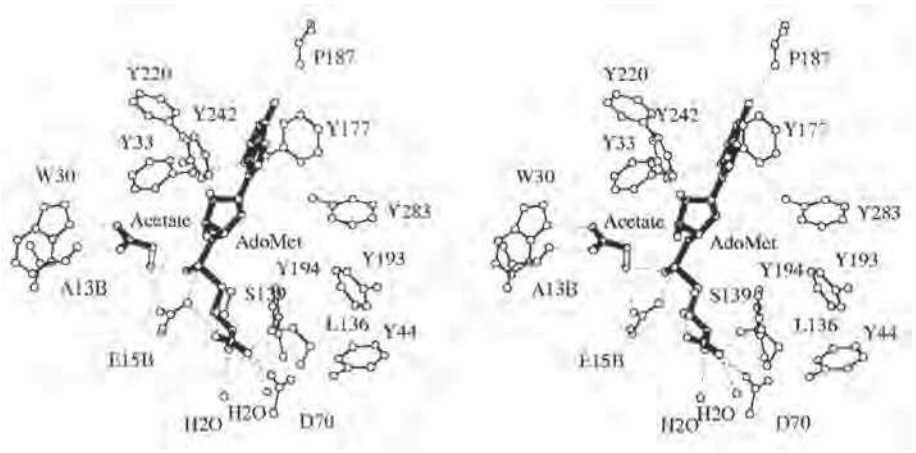


Fig. 12-2 *S*-Adenosylmethionine (AdoMet, solid bonds) bound in the active site of glycine *N*-methyltransferase together with an acetate ion bound in the glycine site. Glycine was built by attaching an amino group (open bond) to the acetate (solid bonds). Possible polar interactions (O–O and O–N < 0.31 nm and O–S < 0.4 nm) are indicated by dotted lines. Tyrosine residues located at the inner surface of the active site are also shown. From Fu *et al.*[21]

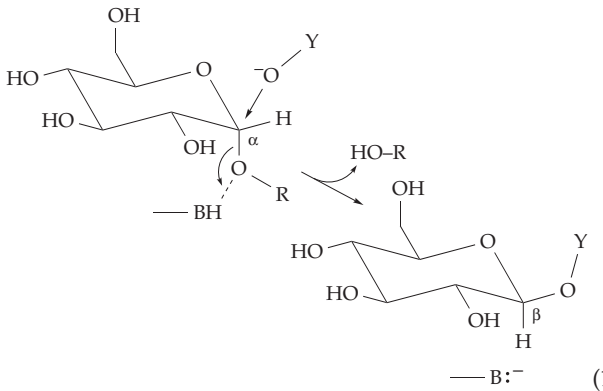
effect) arises because of small differences in the vibrational energies of the methyl group resulting from the differences in mass of ^{1}H and ^{2}H. It often leads to a more rapid reaction for the ^{1}H-containing substrates. In a model nonenzymatic displacement reaction of a similar type[32] the ratio of rate constants was $k_{1_H} / k_{2_H} = 1.17 \pm 0.02$. However, for COMT an inverse α-deuterium effect was seen: $k_{(c1_{H_3})} / k_{(c2_{H_3})} = 0.83 \pm 0.05$. Theoretical calculations suggested that such an effect might be observed if the enzyme compresses an S_N2-like transition state, shortening the bonds from the central carbon atom to both the oxygen atom of the entering nucleophile and the sulfur atom of the leaving group.[33,34] However, more recent calculations suggest that it is difficult to draw such conclusions from secondary isotope effects.[35,36] Calculations by Zheng, Kahn, and Bruice predict that in the gas phase the two substrates, upon collision, will react with a very low energy barrier.[29,29a] For this enzyme, as for the haloalkane dehalogenase (Fig. 12-1), it has been concluded that the enzyme excludes water from the active site and binds the two substrates very close together and in a correct orientation for reaction. The computations predicted secondary isotope effects for the enzymatic reaction similar to those measured by Hegazi *et al.*[32] suggesting that the transition states for the enzymatic reactions closely resemble those for the gas phase.

4. Glycosyltransferases

The polarization of a single C–O bond in an ether is quantitatively much less than that of the C–S^+ bond of *S*-adenosylmethionine, and simple ethers are not readily cleaved by displacement reactions. However, glycosides, which contain a carbon atom attached to *two* oxygen atoms, do undergo displacement reactions which lead to **hydrolysis**, **phosphorolysis**, or **glycosyl exchange**. The corresponding enzymes are glycosylhydrolases, phosphorylases, and glycosyltransferases. Two characteristics are commonly used to classify glycosylhydrolases that act on polysaccharides: **Endoglycanases** cut at random locations within the chains of sugar units, while **exoglycanases** cut only at one end or another, usually at the nonreducing end. **Inverting enzymes** invert the configuration at the anomeric carbon atom which they attack, while **retaining enzymes** do not. Although simple glycosides undergo acid-catalyzed hydrolysis readily, uncatalyzed hydrolysis is extremely slow, the estimated first order rate constant[37] at 25°C being about 2×10^{-15} s^{-1}. Polysaccharides may be the most stable of all bioploymers.

Glycosyltransferases are numerous. For example, the amino acid sequences for about 500 glucosidases, which hydrolyze linkages between glucose residues, have been determined and this one group of enzymes has been classified into over 60 families[38,39] and eight larger groups.[40] Many three-dimensional structures have been reported and numerous studies of the reaction mechanism for both enzymatic and nonenzymatic hydrolysis of glycosides have been conducted. Most of the experimental results have been carefully verified. Nevertheless, they serve to illustrate how difficult it is to understand how enzymes catalyze this simple type of reaction.[41]

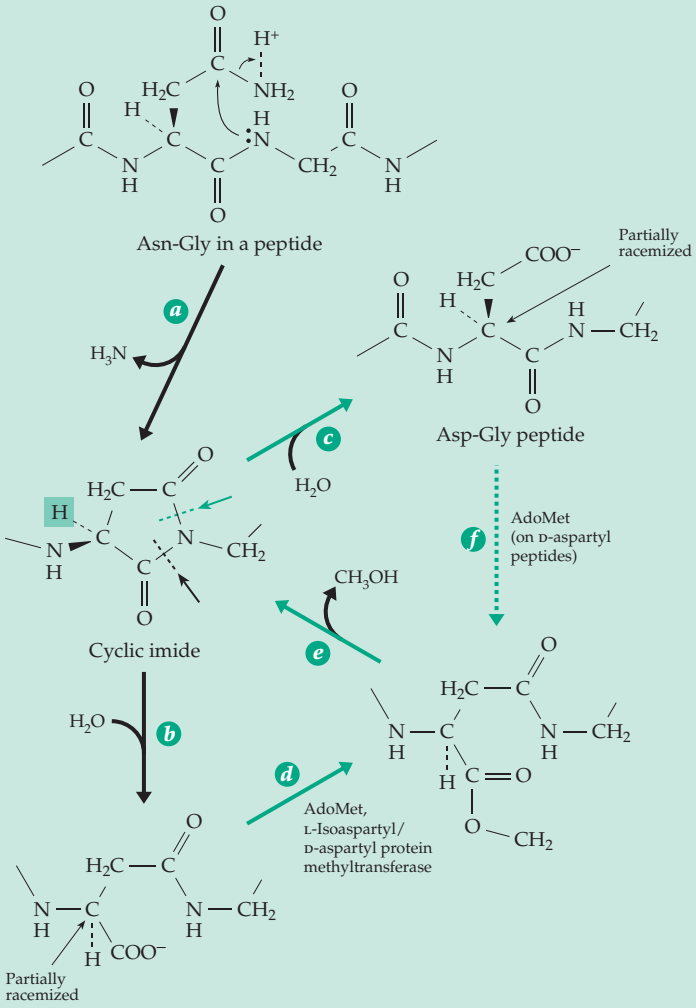
Inversion or retention? Equation 12-5 pictures the reaction of a glycoside (such as a glucose unit at



(12-5)

BOX 12-A CARBOXYMETHYLATION OF PROTEINS

Methylation and demethylation of the carboxyl groups of the side chains of specific glutamyl residues play a role in bacterial chemotaxis (Chapter 19, Section A). However, similar functions in eukaryotes have not been found. There is a specific methyltransferase that methylates C-terminal carboxyl groups on prenylated and sometimes palmitoylated peptides.[a] Another type of carboxylmethyltransferase acts on only a small percentage of many proteins and forms labile methyl esters. These methyltransferases have a dual specificity, acting on L-**isospartyl** residues and usually also on D-**aspartyl** residues. Both of these amino acids can arise from deamidation of asparagine, especially in Asn-Gly sequences (Eq. 2-24 and steps *a* and *b* of following scheme).[b–g] A similar isomerization of aspartyl residues occurs more slowly. Asn-Gly sequences are present in many proteins and provide weak linkages whose isomerization is an inevitable aspect of aging.[e,h] Furthermore, the α proton of the cyclic imide (green box) is more readily dissociated than in a standard peptide, leading to racemization (step *c*). The isoaspartyl and D-aspartyl residues provide kinks in the peptide chain which may interfere with normal function and turnover.



The L-isoaspartyl / D-aspartyl methyltransferase (step *d*), which is especially abundant in the brain,[i,j] provides for partial repair of these defects.[k–o] The methyl esters of isoaspartyl residues readily undergo demethylation with a return to a cyclic imide (step *e*). The cyclic imide is opened hydrolytically (step *c*) in part to an isoaspartyl residue, but in part to a normal aspartyl form. The combined action of the carboxylmethyltransferase and the demethylation reaction tends to repair the isomerized linkages. Methylation of D- aspartyl residues returns them to the cyclic imide (steps *f*, *e*), allowing them to also return to the normal L configuration. Nevertheless, the protein will have a different net charge than it did originally and must be considered a new modified form.

[a] Dai, Q., Choy, E., Chiu, V., Romano, J., Slivka, S. R., Steitz, S. A., Michaelis, S., and Philips, M. R. (1998) *J. Biol. Chem.* **273**, 15030–15034

[b] Geiger, T., and Clarke, S. (1987) *J. Biol. Chem.* **262**, 785–794

[c] Tyler-Cross, R., and Schirch, V. (1991) *J. Biol. Chem.* **266**, 22549–22556

[d] Brennan, T. V., and Clarke, S. (1993) *Protein Sci.* **2**, 331–338

[e] Paranandi, M. V., Guzzetta, A. W., Hancock, W. S., and Aswad, D. W. (1994) *J. Biol. Chem.* **269**, 243–253

[f] Donato, A. D., Ciardiello, M. A., de Nigris, M., Piccoli, R., Mazzarella, L., and D'Alessio, G. (1993) *J. Biol. Chem.* **268**, 4745–4751

[g] Tomizawa, H., Yamada, H., Ueda, T., and Imoto, T. (1994) *Biochemistry* **33**, 8770–8774

[h] Man, E. H., Sandhouse, M. E., Burg, J., and Fisher, G. H. (1983) *Science* **220**, 1407–1408

[i] Orpiszewski, J., and Aswad, D. W. (1996) *J. Biol. Chem.* **271**, 22965–22968

[j] Najbauer, J., Orpiszewski, J., and Aswad, D. W. (1996) *Biochemistry* **35**, 5183–5190

[k] Johnson, B. A., Murray, E. D. J., Clarke, S., Glass, D. B., and Aswad, D. W. (1987) *J. Biol. Chem.* **262**, 5622–5629

[l] Brennan, T. V., Anderson, J. W., Jia, Z., Waygood, E. B., and Clarke, S. (1994) *J. Biol. Chem.* **269**, 24586–24595

[m] Mudgett, M. B., and Clarke, S. (1993) *Biochemistry* **32**, 11100–11111

[n] Mudgett, M. B., and Clarke, S. (1994) *J. Biol. Chem.* **269**, 25605–25612

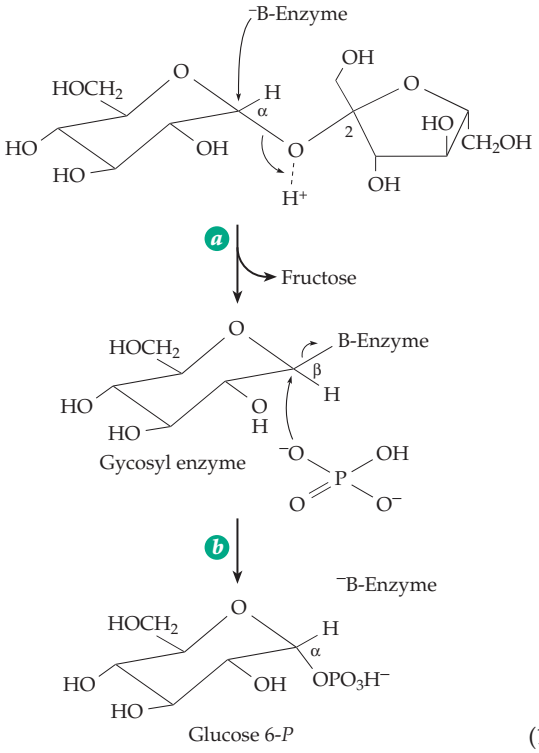
[o] Aswad, D. W., ed. (1995) *Deamidation and Isoaspartate Formation in Peptides and Proteins*, CRC Press, Boca Raton, Florida

the end of a starch chain) with a nucleophile Y–O$^-$ as the displacing group. An enzyme-bound acidic group –BH is shown assisting the process. Inversion of configuration with formation of a product of the β configuration at the anomeric carbon atom would be predicted and is observed for many of these enzymes. However, many others do *not* cause inversion.[41,42] An example is the reaction catalyzed by **sucrose phosphorylase** from *Pseudomonas saccharophila*:

Sucrose (an α-glucoside of fructose) + inorganic phosphate (P_i) → α-D-glucose 1-phosphate + fructose (12-6)

Two possible explanations for the lack of inversion during this reaction are that the enzyme acts by a **double-displacement** reaction or through a stabilized **carbocationic intermediate**. Let us consider these possibilities in turn.

Double-displacement mechanisms. In a double-displacement mechanism sucrose phosphorylase would catalyze two consecutive single displacements, each with inversion. A nucleophilic group of the enzyme would react in Eq. 12-7, step *a*. In step *b*, a phosphate would react to regenerate the enzyme with its free nucleophilic group –B$^-$.



(12-7)

Four kinds of experiments were used to identify this double-displacement mechanism.

(1) *Kinetics*. In a double-displacement mechanism the enzyme shuttles between free enzyme and the intermediate carrying the substrate fragment (here, the glycosyl enzyme). With sucrose phosphorylase the maximum velocity varies with the concentrations of sucrose and HPO_4^{2-} in the characteristic fashion expected for this "ping-pong" mechanism (Eq. 9-47).[43]

(2) *Exchange reactions*. In a double-displacement mechanism sucrose containing ^{14}C in the fructose portion of the molecule should react with free enzyme E to form glycosyl enzyme and free radioactive fructose (Eq. 12-8). The ^{14}C-containing groups are designated here by the asterisks.

D-Glucosyl-fructose* + E ⇌ glucosyl-E + fructose* (Sucrose) (12-8)

If a very low molar concentration of enzyme is present, and a large excess of nonradioactive fructose is added, the enzyme will catalyze no net reaction but will change back and forth repeatedly between the free enzyme and glucosyl enzyme. Each time, in the reverse reaction, it will make use primarily of unlabeled fructose. The net effect will be catalysis of a sucrose–fructose exchange:

Sucrose* + fructose ⇌ sucrose + fructose* (12-9)

This exchange reaction, as well as other predicted exchanges, has been observed.[44] Although the exchange criterion of the mechanism is often applied to enzymatic processes, the observation of exchange reactions does not *prove* the existence of a covalently bound intermediate. Furthermore, enzymes using double-displacement mechanisms may not always catalyze the expected exchanges.

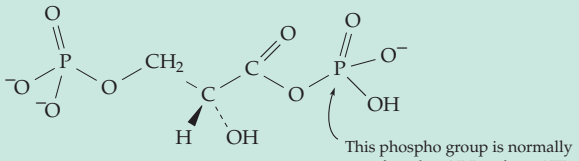
(3) *Arsenolysis*. Sucrose phosphorylase also catalyzes the cleavage of sucrose by arsenate and promotes a rapid cleavage (**arsenolysis**) of glucose 1-phosphate to free glucose. This reaction is evidently a result of a displacement by arsenate on a glycosyl enzyme intermediate. The resulting unstable glucose 1-arsenate (see Box 12-B) is hydrolyzed rapidly. Arsenolysis is a general way of trapping reactive enzyme-bound intermediates that normally react with phosphate groups. Arsenate is one of many substrate analogs that can be used to siphon off reactive enzyme-bound intermediates into nonproductive side paths.

(4) *Identification of glycosyl-enzyme intermediates*. Studies with pure enzymes often make it possible to confirm directly the existence of enzyme-bound intermediates. The intermediates detected are frequently **glycosyl esters** of glutamate or aspartate side chain

BOX 12-B ARSENIC

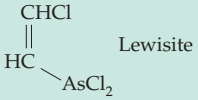
Arsenate, AsO_4^{3-}, is chemically similar to phosphate in size and geometry and in its ability to enter into biochemical reactions. However, arsenate esters are far less stable than phosphate esters. If formed in the active site of an enzyme, they are quickly hydrolyzed upon dissociation from the enzyme. This fact accounts for some of the toxicity of arsenic compounds.[a]

Arsenate will replace phosphate in all phosphorolytic reactions, e.g., in the cleavage of glycogen by glycogen phosphorylase, of sucrose by sucrose phosphorylase, and in the action of purine nucleoside phosphorylase.[b] Glucose 1-arsenate or ribose-1-arsenate is presumably a transient intermediate which is hydrolyzed to glucose. The overall process is called **arsenolysis**. Another reaction in which arsenate can replace phosphate is the oxidation of glyceraldehyde 3-phosphate in the presence of P_i to form 1,3-bisphosphoglycerate:



1,3-Biphosphoglycerate

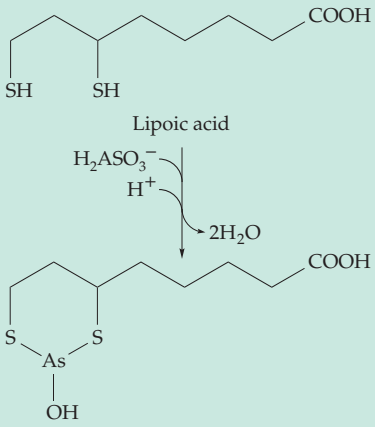
The subsequent transfer of the 1-phospho group to ADP is an important energy-yielding step in metabolism (Chapter 12). When arsenate substitutes for phosphate the acyl arsenate (1-arseno-3-phosphoglycerate) is hydrolyzed to 3-phosphoglycerate. As a consequence, in the presence of arsenate oxidation of 3-phosphoglyceraldehyde continues but ATP synthesis ceases. Arsenate is said to **uncouple** phosphorylation from oxidation. Arsenate can also partially replace phosphate in stimulating the respiration of mitochondria and is an uncoupler of oxidative phosphorylation (Chapter 18). Enzymes that normally act on a phosphorylated substrate will usually catalyze a slow reaction of the corresponding unphosphorylated substrate in the presence of arsenate. Apparently, the arsenate ester of the substrate forms transiently on the enzyme surface, permitting the reaction to occur.



Lewisite

Arsenite is noted for its tendency to react rapidly with thiol groups,[c] especially with pairs of adjacent (vicinal) or closely spaced –SH groups[d] as in lipoic acid. By blocking oxidative enzymes requiring lipoic acid (Chapter 15), arsenite causes the accumulation of pyruvate and of other 2-oxo acids. Similar chemistry underlies the action of the mustard gas Lewisite, which also attacks lipoic acid.[de]

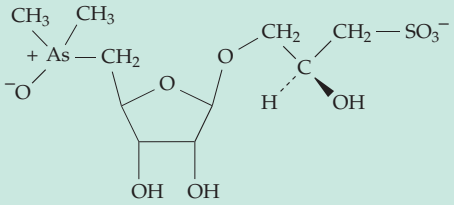
Many, perhaps all, organisms have enzyme systems for protection against arsenic compounds.



Lipoic acid

In *E. coli* arsenate is reduced to arsenite by a glutaredoxin- and NADH-dependent system.[e–g] The arsenite as well as antimonite and tellurite are pumped out by an ATP-dependent transporter. The genes for reductase, periplasmic-binding protein, and transporter components are encoded in a conjugative plasmid.[h,i] A quite similar system functions in yeast.[j]

Marine algae as well as some higher aquatic plants detoxify and excrete arsenate by conversion to various water-soluble organic forms such as trimethyarsonium lactic acid (Chapter 8) and the following ribofuranoside.[a]



2-Hydroxy-3-sulfopropyl-5-deoxy-5-(dimethylarseno)-ribofuranoside

Arsenic-containing phospholipids are also formed (Chapter 8).[k]

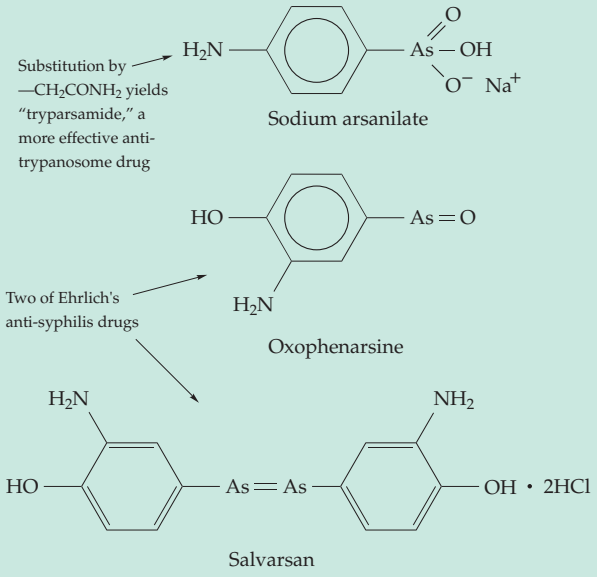
Arsenic is present at high levels in some soils and contamination of drinking water with arsenic is a major problem is some areas of the world. In West Bengal, India, millions of people drink contaminated well water. As a result hundreds of thousands have developed debilitating nodular keratoses on their feet.[l,m] The problem is made worse by the increasing

BOX 12-B (continued)

use of fresh water for irrigation and the difficulty of removing arsenic contamination.

Although it is most known for its toxicity, arsenic may be an essential nutrient. Data from feeding of chicks, goats, rats, and miniature pigs suggest a probable human need for arsenic of ~12 μg / day.[n]

Compounds of arsenic have been used in medicine for over 2000 years, but only in the past century have specific arsenicals been created as drugs. In 1905, it was discovered that sodium arsanilate is toxic to trypanosomes. The development by P. Ehrlich of **arsenicals** for the treatment of syphilis (in 1909) first focused attention on the possibility of effective chemotherapy against bacterial infections.



[a] Knowles, F. C., and Benson, A. A. (1983) *Trends Biochem. Sci.* **8**, 178–180
[b] Kline, P. C., and Schramm, V. L. (1995) *Biochemistry* **34**, 1153–1162
[c] Lam, W.-C., Tsao, D. H. H., Maki, A. H., Maegley, K. A., and Reich, N. O. (1992) *Biochemistry* **31**, 10438–10442
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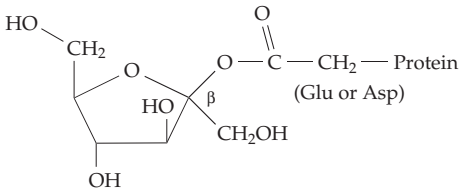
[e] Silver, S., Nucifora, G., Chu, L., and Misra, T. K. (1989) *Trends Biochem. Sci.* **14**, 76–80
[f] Ji, G., Garber, E. A. E., Armes, L. G., Chen, C.-M., Fuchs, J. A., and Silver, S. (1994) *Biochemistry* **33**, 7294–7299
[g] Rosen, B. P., Weigel, U., Karkaria, C., and Gangola, P. (1988) *J. Biol. Chem.* **263**, 3067–3070
[h] Gladysheva, T. B., Oden, K. L., and Rosen, B. P. (1994) *Biochemistry* **33**, 7288–7293
[i] Chen, Y., and Rosen, B. P. (1997) *J. Biol. Chem.* **272**, 14257–14262
[j] Wysocki, R., Bobrowicz, P., and Ulaszewski, S. (1997) *J. Biol. Chem.* **272**, 30061–30066
[k] Cooney, R. V., Mumma, R. O., and Benson, A. A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4262–4264
[l] Bagla, P., and Kaiser, J. (1996) *Science* **274**, 174–175
[m] Saha, D. P., and Subramanian, K. S. (1996) *Science* **274**, 1287–1288
[n] Nielsen, F. H. (1991) *FASEB J.* **5**, 2661–2667

carboxyl groups. Such an intermediate, fructofuranosyl-enzyme, was identified tentatively for sucrose phosphorylase and also for a related levan sucrase.[45] Recently, identification of glycosyl-enzyme intermediates has been accomplished for many other glycosyltransferases. Among these are human pancreatic and salivary α-amylases,[42,46] α-glucosidases, and some cellulases and xylanases.

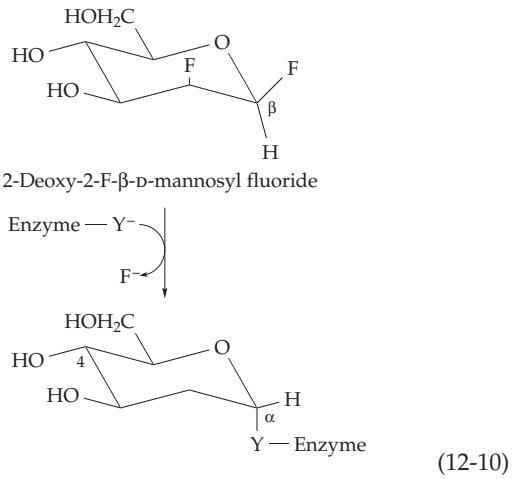


Fructofuranosyl - enzyme

For these hydrolytic enzymes the glycosyl-enzyme would be attacked by a hydroxyl ion derived from H_2O, whose deprotonation would presumably be assisted by the conjugate base (—B: in Eq. 12-5) of the catalytic acidic group.

A convenient way to form and identify covalently linked glucosyl-enzyme intermediates, developed by Withers and coworkers, employs enzyme-activated inhibitors such as 2-deoxy-2-fluoroglycosyl fluorides (Eq. 12-10).[42,47,48] The 2-F substituent greatly decreases both the rate of formation of a glycosyl-enzyme and its rate of breakdown by hydrolysis or transglycosylation. This may be in part because these compounds lack the 2–OH group which helps to stabilize, by hydrogen bonding, the complexes of normal substrates. A second factor is the high electronegativity of –F versus –OH, which leads to significant loss of stability for a transition state in which the anomeric carbon atom carries a significant amount of positive charge (see next section). Having a good leaving group, such as fluorine or 2,4-dinitrophenyl, the compounds react rapidly to give stable glucosyl-enzymes which can be characterized. In the example shown in Eq. 12-10, the mannosyl-enzyme was identified by the chemical shifts and linewidths of the ^{19}F resonances of the intermediates and the anomeric configuration was established. More recently, mass spectrometry has been used.[49] For example, a maltotriosyl-enzyme intermediate in the

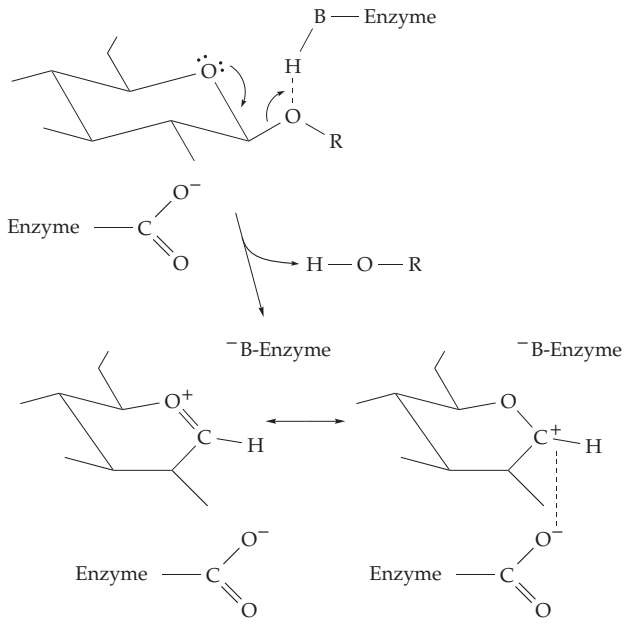
action of glycogen-debranching enzyme was identified by separation of an active site peptide by HPLC followed by mass spectrometry (Chapter 3).[50]



2-Deoxy-2-F-β-D-mannosyl fluoride

(12-10)

Carbocationic intermediates. In a second mechanism of nucleophilic displacement the leaving group departs (often in a protonated form) before the entering nucleophile reacts.

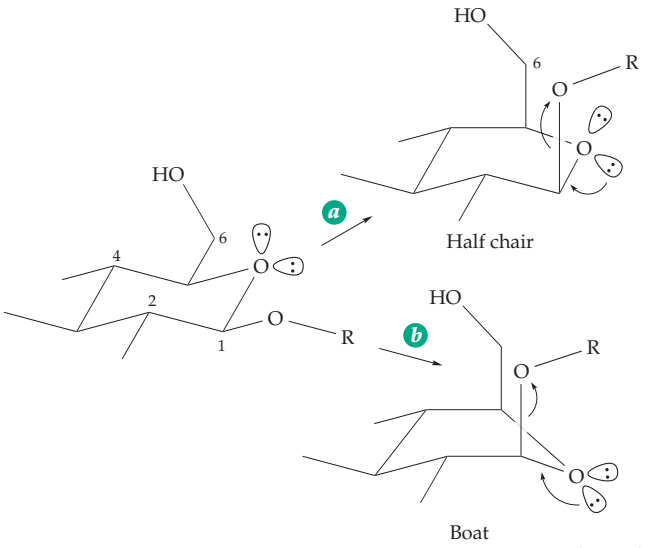


Oxocarbenium ion, a stabilized carbocation

(12-11)

A carbocation is formed as shown in Eq. 12-11, which represents just one-half of the overall displacement reaction. In the common terminology of physical organic chemistry this is an S_N1 reaction rather than an S_N2 reaction of the kind shown in Eqs. 12-3 and 12-5. This terminology is not quite appropriate for enzymes because the breakdown of ES complexes to product is usually a zero-order process and the numbers 1 and 2 in the symbols S_N1 and S_N2 refer to the order or molecularity of the reaction. It is better to speak of S_N1-like or S_N2-like reactions.

The carbocation in Eq. 12-11 is depicted as a resonance hybrid of two forms. One of these is an **oxocarbenium** (or oxonium) ion which contains a double bond between carbon and oxygen.[50a] This double-bonded structure can be visualized as arising from the original structure by an internal displacement or elimination by the unshared electrons on oxygen, as indicated by the small arrows. In the oxocarbenium ion, the ring atoms C-2, C-1, and C-5 and the oxygen atom are almost coplanar and the ring conformation is described as **half-chair** or **sofa**. As pointed out in Chapter 9, the theory of stereoelectronic control predicts that elimination of the group –OR of Eq. 12-11 should occur most easily if a lone pair of electrons on the ring oxygen atom are antiperiplanar to the O–R bond. This is impossible for a β-glycoside with a ring in the chair conformation shown in Eq. 12-11. This fact suggested that enzymes cleaving β-glycosides may preferentially bind substrate with the appropriate sugar ring in a less stable half-chair or flexible boat conformation prior to bond cleavage (Eq. 12-12).[51,52] This would allow an unshared pair of electrons on oxygen antiperiplanar to the C-1 to O – R bond to participate in the elimination reaction as is indicated in the following diagrams.



(12-12)

On the other hand, Bennet and Sinnott provided evidence that an antiperiplanar lone electron pair is *not* needed in acid-catalyzed cleavage of glycosides via a carbocationic intermediate.[53,54] Theories of stereoelectronic control must be applied to enzymes with caution!

5. Lysozymes and Chitinases

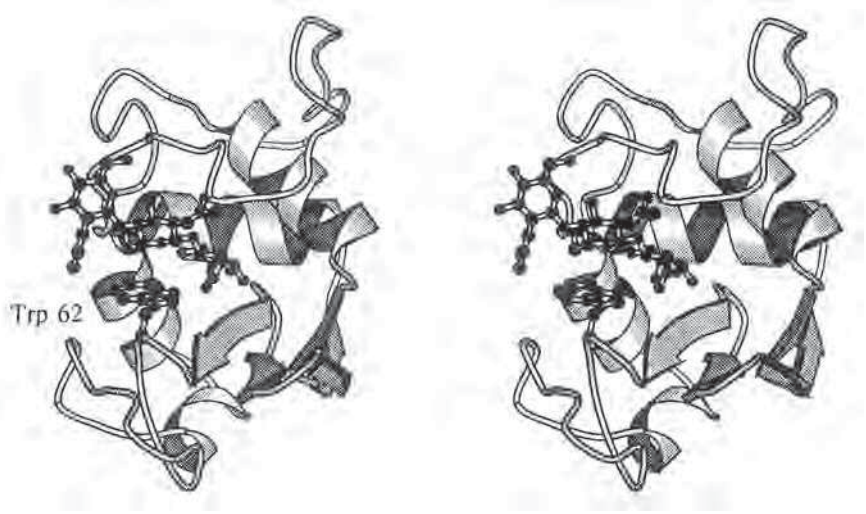


Figure 12-3 Stereoscopic MolScript view of hen egg white lysozyme complexed with a trisaccharide of *N*-acetylglucosamine $(GlcNAc)_3$ in binding subsites A, B, and C. The side chain of Trp 62 and the trisaccharide above it are shown in ball-and-stick form. From Maenaka *et al.*[65] Courtesy of Izumi Kumagai.

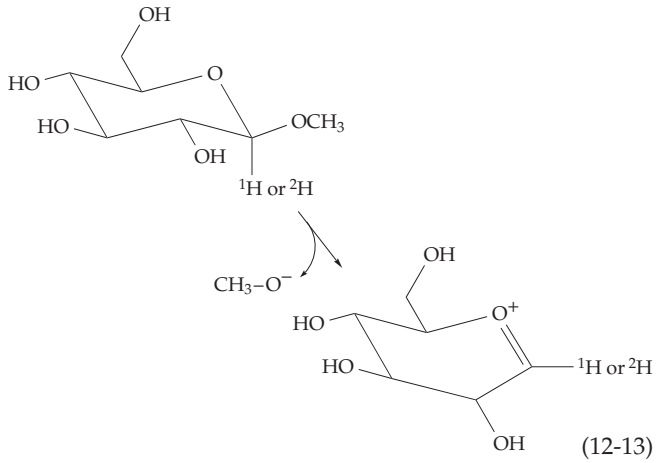
Polysaccharide chains in the peptidoglycan layer (Fig. 8-29) of the cell walls of bacteria are attacked and cleaved by lysozymes,[55] enzymes that occur in tears and other body secretions and in large amounts in egg white. Some bacteria and fungi, and even viruses, contain lysozymes.[56] Their function is usually to protect against bacteria, but lysozyme of phage T4 is a component of the baseplate of the virus tail (Box 7-C). Its role is to cut a hole in the bacterial cell wall to permit injection of the virus' own DNA. Egg white lysozyme, the first enzyme for which a complete three-dimensional structure was determined by X-ray diffraction,[55] is a 129-residue protein. The active site is in a cleft between a large domain with a nonpolar core and a smaller β-sheet domain that contains many hydrogen-bonded polar side chains (Figs. 12-3, 12-4). Human lysozyme has a similar structure and properties.[57–59] The T4 lysozyme has an additional C-terminal domain whose function may be to bind the crosslinking peptide of the *E. coli* peptidoglycan. Goose lysozyme is similar in part to both hen lysozyme and T4 lysozyme. All three enzymes, as well as that of our own tears, may have evolved from a common ancestral protein.[60] On the other hand, *Streptomyces erythaeus* has developed its own lysozyme with a completely different structure.[61] An extensive series of T4 lysozyme mutants have been studied in efforts to understand protein folding and stability.[61–63]

Catalytic side chain groups. Six *N*-acetylglucosamine (GlcNAc) or *N*-acetylmuramic acid (MurNAc) rings of the polysaccharide substrate are able to fit precisely into six subsites (designated A to F) in a groove between the two structural domains of egg white lysozyme (Fig. 12-4). The bond between the fourth and fifth rings (subsites D and E) is then cleaved. At the active site, the side chain carboxyl group of Glu 35 is positioned correctly to serve as the proton donor BH of Eq. 12-11, while the carboxyl of Asp 52 lies on the opposite side of the groove. Both Glu 35 and Asp 52 have relatively high pK_a values for carboxyl (microscopic pK_a's are ~5.3 and 4.6, respectively, in the fully protonated active site when the ionic strength is ~ 0.2)[64] as a result of the hydrophobic environment and hydrogen bonding to other groups. If the pH is raised from an initially low value, a proton from Asp 52 usually dissociates first and the electrostatic field of the resulting anion keeps Glu 35 largely protonated until the pH approaches 6. Positively charged basic groups nearby affect the pK_a values; hence, the behavior of the enzyme is sensitive to the ionic strength of the medium.[63] The Asp 52 anion lies only ~ 0.3 nm from the center of positive charge expected for an oxocarbenium ion intermediate and presumably stabilizes it. Replacement of Glu 35 by Gln destroys all catalytic activity and replacement of Asp 52 by Ala or Asn decreases activity to ~4–5% of the original.[66–68] Less than 1% activity remained for the D52S mutant.[69]

Nevertheless, Asp 52 is not absolutely essential for lysozyme activity. Goose lysozyme lacks this catalytic aspartate.[70] Matsumura and Kirsch suggested that carboxyl groups of glycine residues covalently attached to *N*-acetylmuramic acid rings in the natural substrates may participate in catalysis.[66] Many mutant forms of lysozyme have been studied. Of special interest is the D52E mutant. In this enzyme the carboxylate of the longer glutamate side chain reacts with the oxocarbenium ion intermediate to form a covalent adduct and apparently alter the basic mechanism.[71] Replacement of asparagine 46, which can hydrogen bond to Asp 52, also decreases k_{cat} greatly, suggesting a role in catalysis.[72]

The lysozyme-catalyzed reaction is completed by stereospecific addition of a hydroxyl ion to the oxocarbenium ion with the original β configuration being retained in the product. Such stereospecificity for reactions of enzyme-bound carbocations is not surprising because the enzyme probably assists in generation, on the appropriate side of the sugar ring, of the attacking hydroxyl ion.

Kinetic isotope effect for lysozyme. A secondary kinetic isotope effect is expected because a molecule with ^1H in the number 1 position can be converted to the corresponding oxocarbenium ion somewhat more easily than the molecule with ^2H in the same position (Eq. 12-13). For example, in the nonenzymatic acid-catalyzed hydrolysis of a methyl- glucoside, a reaction also believed to proceed through a carbocation intermediate,[41,75] the ratio k_{1_H} / k_{2_H} is 1.14 for the α anomer and 1.09 for the β anomer.[53] In the base-catalyzed hydrolysis of the same compound, which is believed to occur by a double-displacement reaction involving participation of the neighboring OH group on C-2, the ratio k_{1_H} / k_{2_H} is 1.03. The corresponding ratio measured



(12-13)

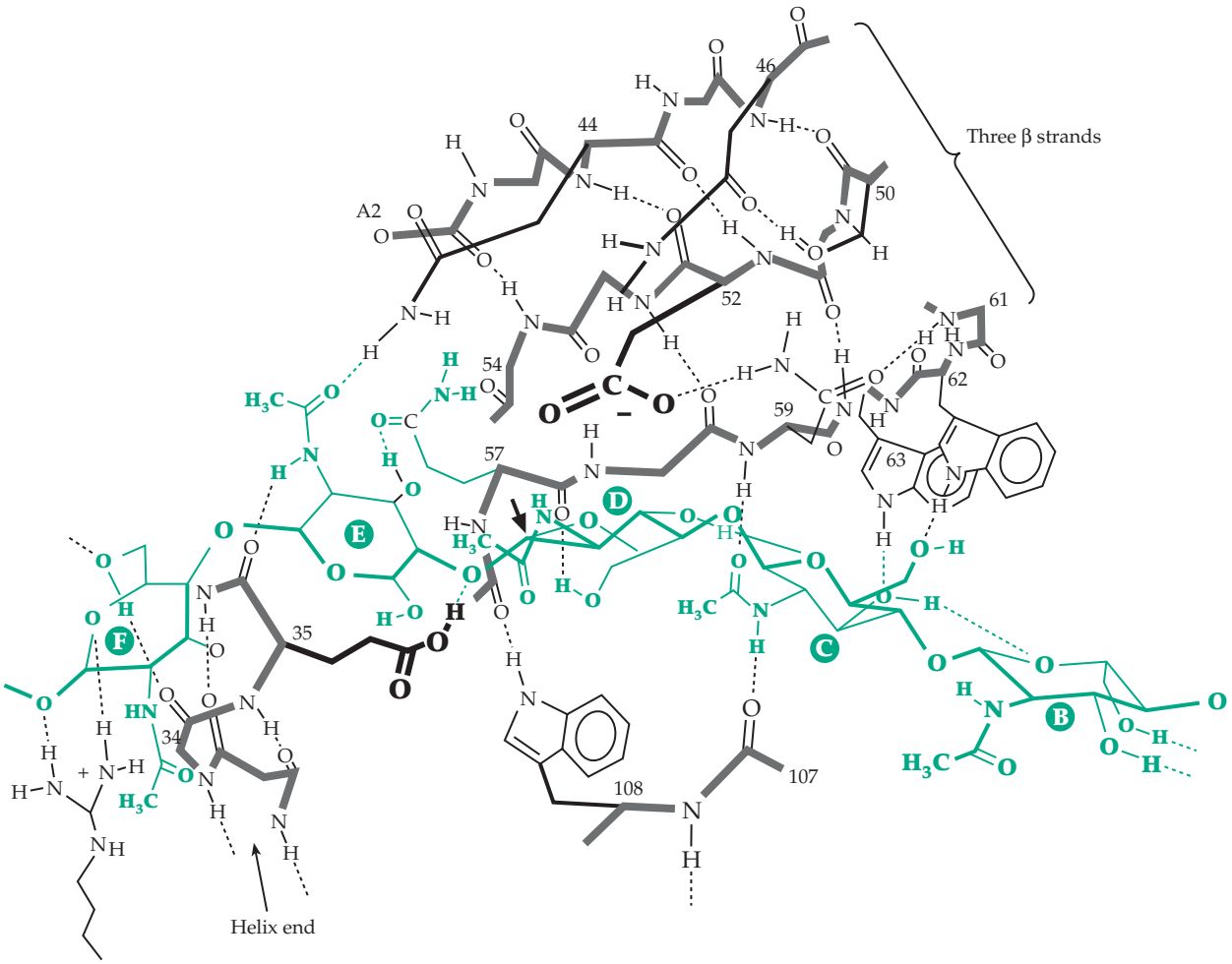


Figure 12-4 Schematic drawing of the active site of egg white lysozyme with substrate in place and about to be cleaved. Three strands of the small β-sheet domain, which contains an extensive hydrogen bond network, are seen at the top. Notice that this view into the active site is different from that in Fig. 12-3. A 180° rotation of either figure will make the views more similar. The three-strand β sheet can be seen (in stereo) at the lower right in Fig. 12-3 and the helix end carrying Glu 35 in the right center foreground. A segment of a chitin oligosaccharide (green), whose reducing end is to the left, is bound into subsites B –F. Cleavage occurs between rings D and E as indicated by the heavy arrow which points to the anomeric carbon atom of ring D. The side chain of Glu 35 is shown protonating the bridge oxygen. Ring D has been distorted into a twist conformation to facilitate cleavage. A larger domain, which contains one long α helix, is at the bottom of the drawing. The active site lies in a cleft between the domains. Notice the chain of hydrogen bonds between the carbonyl of residue 107 in the large domain and the peptide NH of residue 59 in the small domain. Also notice the aromatic side chains which are usually present in carbohydrase active sites. Drawing is based in part on those of Irving Geis[73] and Levitt[74] and on a sketch by author from a three-dimensional model.

for the action of lysozyme is 1.11, much closer to that of the oxocarbenium ion mechanism than to that of the double-displacement mechanism. Similar observations have been made with amylases.[76] Kinetic isotope effects have also been measured for ^{12}C vs ^{13}C in the anomeric position, ^{16}O vs ^{18}O in the leaving group ($-OCH_3$) of the methyl glucosides, and for other locations[53] as well as for hydrolysis of glucosyl fluorides.[77–79] The results are generally supportive of the oxocarbenium ion mechanism for lysozyme. However, as mentioned in Section B,3, the interpretation of secondary isotope effects is difficult. Such effects cannot reliably identify a carbohydrase mechanism.[80,81]

For acid-catalyzed hydrolysis of methyl glucosides[53] the kinetic isotope effect observed for the oxygen of the leaving group was $k_{16_O}/k_{18_O} = 1.024-1.026$. Observation of similar effects for enzymes supports the participation of an acidic group of the protein (Glu 35 of lysozyme) in catalysis but does not eliminate the possibility of concerted involvement of a nucleophilic group, e.g., Asp 52 in lysozyme.[81,82]

Does lysozyme distort its substrate? An early study of models indicated that for six sugar rings of a substrate to bind tightly into the active site of lysozyme, the ring in subsite D, which contains the carbon atom on which the displacement occurs, had to be distorted from its normal chair conformation into the half-chair conformation.[83] This is illustrated in Fig. 12-4. It was suggested that by binding the substrate chain at six different sites the enzyme provides leverage to distort the ring in subsite D into a conformation similar to that of the transition state. This idea was criticized on the basis that an enzyme would be too flexible to act in this manner.[74] Furthermore, the non-hydrolyzed trisaccharide MurNAc–GlcNac–MurNAc was shown to fit into subsites B, C, and D of the active site groove without distortion.[84] However, it is bound weakly.

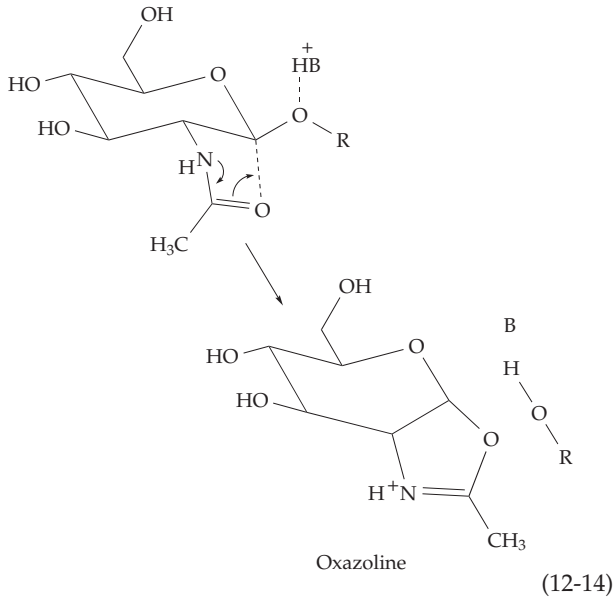
Can electrical forces acting within the active site help to distort the substrate and assist in formation of the carbocation intermediate? Levitt and Warshel suggested this possibility and proposed that the necessary electrostatic force arises from the arrangement of dipoles within the peptide backbone of the protein and in the amino acid side chains.[74] As can be seen from Fig. 12-4, the enzyme forms many hydrogen bonds with the substrates. Of special interest is a chain of hydrogen bonds that passes from the backbone carbonyl of Ala 107 through the 2-acetamido group of the substrate in subsite C and into the edge of the β sheet at the backbone NH of Asn 59. This interaction provides specificity toward GlcNac-containing substrates. Perhaps oscillation of charge within such polarizable chains of hydrogen bonds can also help a substrate to move toward its transition state structure.[85,86]

From simulations of lysozyme action by the methods of molecular dynamics, Post and Karplus observed that Glu 35 tended not to hydrogen bond to the exocyclic bridge oxygen, even though that would be a logical step in the protonation (by –BH) shown in Eq. 12-11. They suggested that Glu 35 may protonate the ring oxygen.[87] The ring could then open to form an exocyclic oxonium ion, which could hydrolyze and cyclize to the final product. The initial elimination could receive stereoelectronic assistance from the antiperiplanar lone pair of electrons on the bridge oxygen. This proposal has been criticized.[81,88] For example, it is hard to explain the rapid cleavage of glycosyl fluorides by this exocyclic oxonium mechanism.

As attractive as the arguments for a oxocarbenium mechanism in lysozyme are, is it still possible to explain the experimental observations by a double displacement in which Asp 52 serves as the nucleophile to form a glycosyl enzyme?[38,82,82a] Sucrose phosphorylase and other glucosyl transferases, like lysozyme, apparently have a carboxylate ion at the active site. In some enzymes, the carboxylate ion forms a covalent glucosyl enzyme, whereas in lysozyme it apparently only stabilizes a carbocation. Is there really a difference in mechanism? Do glucosyl enzymes form only with certain substrates or upon denaturation of the enzymes? Nature hides her secrets well. The difficulty in pinning down the fine mechanistic details of enzymatic action makes it essential to be skeptical, to examine data critically, and to try to imagine all possible alternatives –even when things seem to be proven beautifully.

Help from a neighboring group. In the 1960s it was suggested that the acetamido group of *N*-acetylglucosamine residues might participate as a nucleophile, either stabilizing an oxocarbenium ion or forming an oxazoline intermediate.[89–91] The proposal received little support, as applied to lysozyme, until sequences and structures of many larger carbohydrases were determined. Among these are **chitinases**, enzymes that act on the same substrates as the small lysozymes. One group of plant chitinases have a structure similar to that of egg white lysozyme. The 243-residue enzyme from barley seeds apparently has Glu 67 as a proton donor and Glu 89 as a possible stabilizing nucleophile.[92] Another group of chitinases from both plants and bacteria have active sites at ends of $(\alpha\beta)_6$ barrels.[93–95] They all have a proton-donating glutamate in a conserved position but *no aspartate* that could serve as a nucleophile. Study of complexes with substrates and inhibitors with these enzymes has provided direct evidence of ring distortion and of the probable role of the acetamido group as indicated in Eq. 12-14.[94,95] The distortion is beyond that in a sofa conformation and allows for maximum stereoelectronic assistance as well as participation of the acetamido group.

In these relatively large enzymes the substrate is deeply buried and cannot reach a conformation



Oxazoline (12-14)

approaching that of the transition state without having a strained ring conformation. These enzymes may use binding forces exerted on many parts of the substrate to stabilize the transition state structure.

6. Cellulases and Other β-Glycosidases

Cellulose, the most abundant of all biopolymers, is extremely stable but is attacked by a host of bacterial and fungal β-glycanases.[96] Animals do not ordinarily produce cellulases but some termites do.[97] Cellulase structures are varied, being represented by 10 of 57 different glycosylhydrolase families.[98] Most, like lysozyme, retain the β configuration in their products but some invert.[98–100]

Among the cellulases are **endoglycanases**, which cleave chains at random positions, and **exoglycanases** (also called **cellobiohydrolases**) that cleave cellobiose units from ends of chains. Some act on the nonreducing ends and some on reducing ends; a mixture of enzymes is most effective.[99,101,102] In some bacteria a whole series of different cellulases, together with a large (197-kDa) organizing protein, form a **cellusome**, a complex with high catalytic activity for crystalline cellulose.[103–105] Cellulases usually have tightly packed catalytic domains which may vary in size from ~200 residues[98] to over 400.[106] In most cases the catalytic domain is connected by a flexible linker to one or more small, globular **cellulose-binding domains**. These vary in size from 36 to 200 residues and often have a β-barrel fold.[104,107–108a] Their function is to hold the enzymes to the cellulose surfaces. They may also facilitate disruption of the tightly hydrogen bonded cellulose structure (Fig. 4-5). As with other carbohydrases, carboxyl groups of amino acid side chains provide the major catalytic groups (Table 12-1). An extensive hydrogen-bond network which often includes imidazole groups may influence activity.[109,110]

A striking feature of a 411-residue endoglucanase from *Fusarium* was revealed by the binding of a nonhydrolyzable thiooligosaccharide substrate analog. The pyranose ring at the cleavage site was distorted in an identical manner to that mentioned in the preceding section for a chitinase thought to use the substrate's acetamido group as a nucleophile (Eq. 12-14).[106] The distortion observed is beyond that required for a sofa conformation and allows for the maximum stereoelectronic assistance (Eq. 12-12*b*). An oxygen atom of the E197 carboxylate, the catalytic nucleophile, occupies a position in the complex that is coincident with that of the C2 acetamido oxygen in the catalytic site of the chitinase discussed in the previous section.

The *inverting* β-glucanases also have two catalytic acid base groups but they are ~0 .9 – 1.0 nm apart rather than ~ 0.6 nm for retaining enzymes. This allows space for a water molecule whose $^{-}$OH is the nucleophilic reactant ($^{-}$OY in Eq. 12-5) and in which a carboxylate group assists in dissociating the water molecule.[98] (This mechanism is illustrated for glucoamylase in Fig. 12-7).

Structural features met in some cellulases include an α,α barrel[111] similar to that of glucoamylase (Fig. 2-29) and, in a cellobiohydrolase,[101] a 5-nm-long tunnel into which the cellulose chains must enter. Ten well-defined subsites for glycosyl units are present in the tunnel.[101] A feature associated with this tunnel is **processive** action, movement of the enzyme along the chain without dissociation,[105] a phenomenon observed long ago for amylases (see Section 9) and often observed for enzymes acting on nucleic acids.

Another group of **β-glucanases**, found in plants and their seeds, hydrolyze β-1,3-linked glucans[112] and, in some cases, also mixed 1,3- and 1,4-linked polysaccharides. A characteristic enzyme is found in barley (Table 12-1).[113,114] Similar enzymes are produced by some bacteria[113] and also by molluscs.[115]

Xylanases act on the β-1,4-linked xylan, the most abundant of the **hemicelluloses** that constitutes over 30% of the dry weight of terrestrial plants.[116] They resemble cellulases and cooperate with cellulases and **xylosidases**[117] in digestion of plant cell walls.[110,116,118–121] **Galactanase** digests the β-1,4-linked component of pectins.[122]

The final step in degradation of cellulose is hydrolysis of cellobiose to glucose. This is accomplished by **β-glucosidases**, enzymes that also hydrolyze lactose, phosphorylated disaccharides, and cyanogenic glycosides.[123 – 126] Lactose is also cleaved by **β-galactosidases**.[127] The large 1023-residue β-galactosidase from *E. coli*[128] is famous in the history of molecular biology as a component of the *lac* operon.[129 – 132] Its properties are also employed to assist in the cloning of genes (Chapter 26).

TABLE 12-1
Acidic and Basic Catalytic Groups in a Few Glycosyltransferases[a]

Enzyme	Number of residues	Inverting?	Glycosyl-enzyme identified?	Nucleophile (–COO^-)	Electrophile (proton donor) * Assisting group
Lysozyme					
Human, hen[b]	130	No		E35	D53(52)
Bacteriophage T4[c]		No		E11	D20
Chitinase					
Rubber plant[d]	273	No			E127
Cellulases					
E1 endocellulase, *Acidothermus*[e]	521				
catalytic domain	358	No		E282	E162, D252*
Endoglucanase I *Fusarium*[f]	411	No		E197	E202
Endoglucanase Cen A *Cellulomonas*[g]	>351	Yes		D392	D78
1,3-β-D-Glucanase					
Barley[h]		No		E231	E288
Xylanase					
B. circulans[i]		No	Yes	E78	E172
β-Glucosidase					
Agrobacterium[j]	458	No	Yes	E358	E170, Y298*
β-Galactosidase					
E. coli[k]	1023	No	Yes	E537	E461, Y503*
α-Amylase					
Human and pig[l]	496	No		D197	E233, D300*
Barley[m]		No		D179	E204, D289*
Aspergillus[n]		No		D206	E230
Cyclodextrin glucosyltransferase					
B. circulans[o]				D229	E257, D328
α-Glucosidase					
Saccharomyces[p]	No	Yes		D214	E233, D300
Glycogen debranching enzyme, rabbit[s]	No	Yes		D549	
Glucoamylase					
Aspergillus[q]	616	Yes		H_2O, E400	E179
β-Amylase					
Soybean[r]		Yes		E186	E380
Glucocerebrosidase					
Human[t]				E340	

[a] For classification of glycosyl hydrolases into families, see Henrissat, B., Callebaut, I., Fabrega, S., Lehn, P., Mornon, J.-P., and Davies, G. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7090–7094

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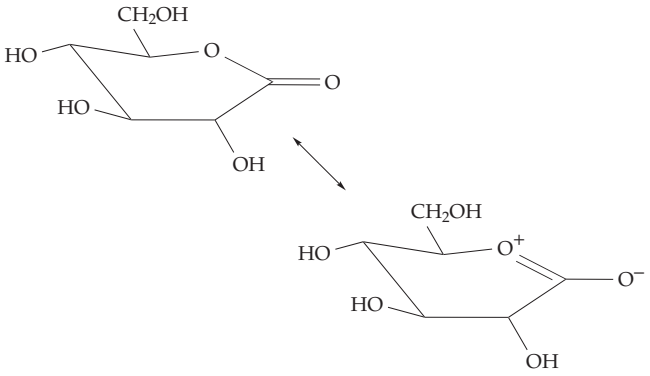
7. Glycogen Phosphorylase

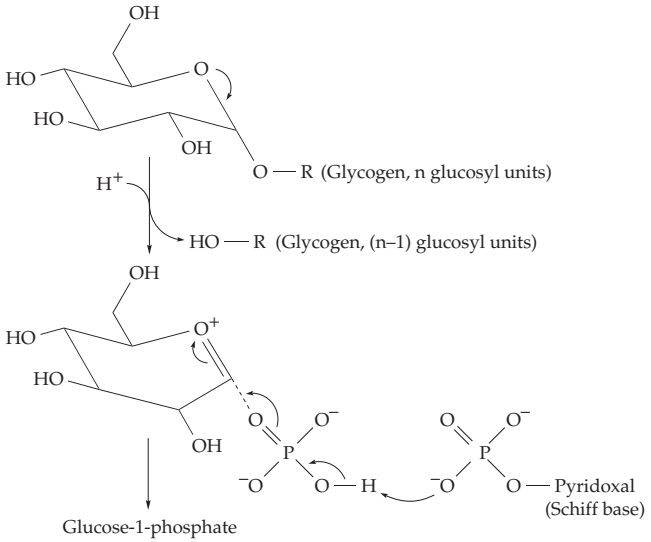
A large number of different glycosyltransferases act on the α1,4 linkages of glycogen, starch, and related polysaccharides. Among these, one of the most studied is glycogen phosphorylase. It is not a hydrolase, but it catalyzes cleavage of α1,4 linkages at the nonreducing ends of glycogen molecules by displacement with inorganic phosphate to give α-D-glucose-1-phosphate. It is a very large enzyme (841 residues in rabbit muscle) whose structure is shown in Fig. 11-5. Its complex regulatory mechanisms were discussed briefly in Chapter 11.

Neither partial exchange reactions nor inversion of configuration occur when glycogen phosphorylase acts on its substrates. The enzyme apparently does nothing until both substrates are present. These are glycogen + inorganic phosphate or, for the reverse reaction, glycogen (shifted over by one sugar binding subsite) + glucose-1-phosphate. The active site is in a deep groove in the enzyme. Evidently, the protein must close and fold around the substrates before it becomes active.[133] An oxocarbenium ion mechanism has also been proposed for this enzyme, partly on the basis of strong inhibition of phosphorylase by **5-gluconolactone**,[134,135] a compound having a half-chair conformation and perhaps acting as a transition state inhibitor (Chapter 9). This gluconolactone also inhibits many other carbohydrases.[123,136]



5-Gluconolactone

An unexpected discovery was that glycogen phosphorylase contains a molecule of the coenzyme pyridoxal 5'-phosphate (PLP) bound into the center of the protein behind the active site[134,135,137–139] with its phosphate group adjacent to the binding site of the phosphate of glucose-1-phosphate. It probably serves as a general base catalyst, e.g., assisting the attack of a phosphate ion on the oxocarbenium formed by cleavage of the glycogen chain (Eq. 12-15).[137,140–142] A key observation by Graves and associates was that pyridoxal alone does not activate apo-phosphorylase but that pyridoxal



(12-15)

plus phosphite, phosphate, or fluorophosphate does provide up to 20% of full activity.[143] X-ray crystallography confirmed that these activating anions are bound into the active site at the approximate position of the phosphate group of PLP.[139,143a] Glycogen phosphorylase is being studied by X-ray diffraction techniques that allow observation of structural changes in as short a time as a few milliseconds (see Chapter 3, Section H).[144]

The regulation of glycogen phosphorylase, like that of many other allosteric proteins, depends upon the existence of two distinct conformational states, whose structures have been established by crystallography.[137] It is not immediately evident how they can affect the active site. In the R-state the enzyme has a low affinity for both substrates and activators such as AMP. In the T-state the affinities are much higher. For example, that of inorganic phosphate is raised by a factor of fifteen.[137] As we have already seen (Figs. 11-4, 11-5) the relatively inactive phosphorylase *b* is converted to the active phosphorylase *a* by phosphorylation of the side chain of serine 14, a structural change which favors the R-state. In the inactive T-state of phosphorylase *b*, an 18-residue N-terminal segment of the polypeptide is not seen by X-ray diffraction, presumably because it does not assume a fixed conformation but projects into a solvent channel in the crystal and moves freely within it. However, in the active R-state of phosphorylase *a*, in which Ser 14 has been phosphorylated, the N-terminal segment is rigid. The phospho group on Ser 14 binds to Arg 69 and other arginine, lysine, and histidine side chains from both subunits.[137,145] The phosphorylation of Ser 14 occurs 1.5 nm from the active sites. The conformational changes induced by phosphorylation of Ser 14 cause a rotation of about 10° between subunits, somewhat reminiscent of the changes accompanying oxygenation of hemoglobin (Fig. 7-25).

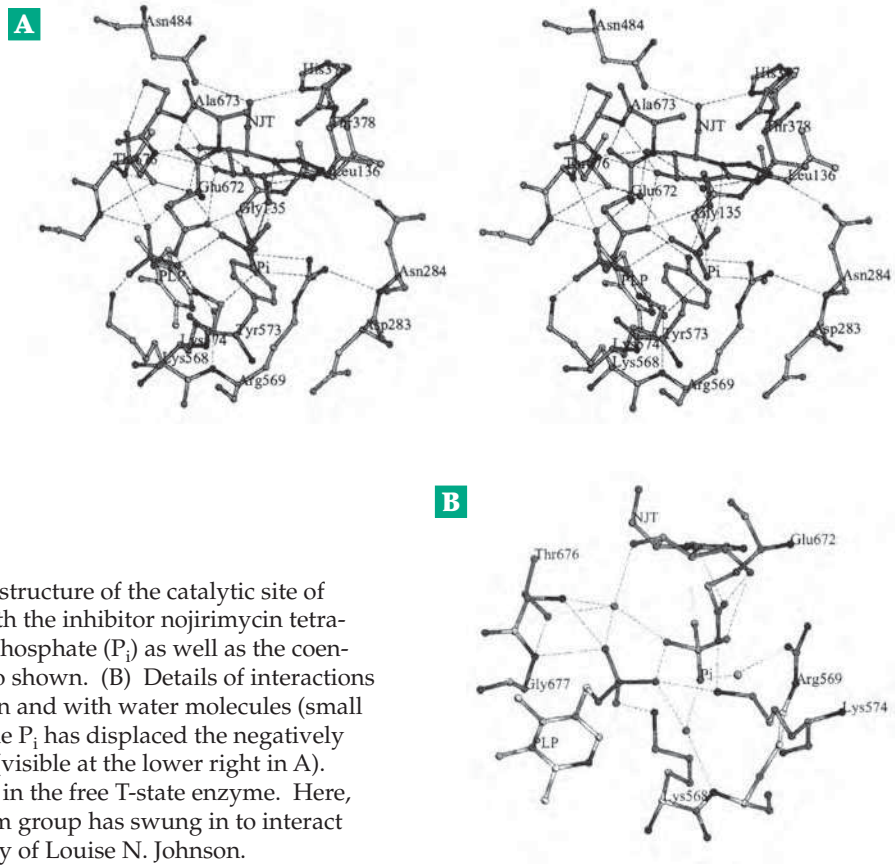
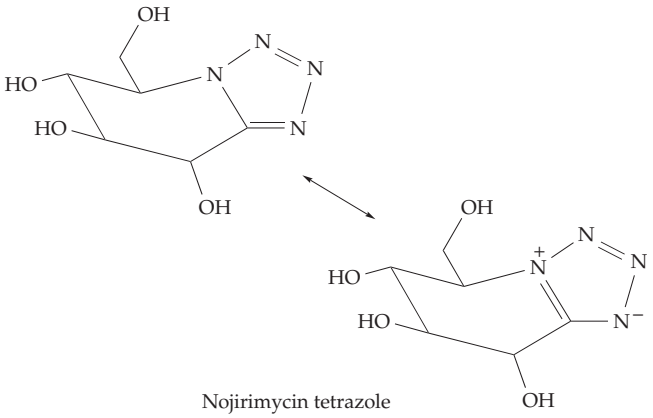


Figure 12-5 (A) Stereoscopic view of the structure of the catalytic site of phosphorylase *b* in the inhibited T-state with the inhibitor nojirimycin tetrazole bound into the active site. Inorganic phosphate (P_i) as well as the coenzyme pyridoxal 5'-phosphate (PLP) are also shown. (B) Details of interactions of the inhibitor, P_i, and PLP with the protein and with water molecules (small circles). This is a weak-binding state but the P_i has displaced the negatively charged side chain carboxylate of Asp 283 (visible at the lower right in A). This carboxylate blocks access to the P_i site in the free T-state enzyme. Here, the positively charged Arg 569 guanidinium group has swung in to interact with the P_i. From Mitchell *et al.*[133] Courtesy of Louise N. Johnson.

A related structural change accompanies binding of caffeine, adenosine, or AMP at high concentrations. These substances inhibit the enzyme by binding at a site next to the catalytic site (Fig. 11-5), stabilizing the T-state and causing a loop of protein to move into the catalytic site and to block it.[137] The catalytic site lies deeply buried in the protein between the two large structural domains (Fig. 11-5). The substrate is held by a network of hydrogen bonds, some of which are shown in Fig. 12-5, in which the active site contains an inhibitory substrate analog **nojirimycin tetrazole**, which is viewed toward the edges of the two rings.



Nojirimycin tetrazole

The enzyme is in the weak-binding T-state but inorganic phosphate (P_i) is bound below it and next to the phosphate group of PLP, as required by the mechanism of Eq. 12-15.

The difference in binding affinities for the T- and R-states lies in a flexible loop of residues 280–288, which in the T-state blocks access to the substrate-binding cleft. The universally conserved Asp 238 behaves as a substrate mimic, occupying the P_i site.[137,146,147] In the R-state this residue moves, allowing P_i to enter and bind (Fig. 12-5).

Most of the structure of mammalian phosphorylases is conserved in species as diverse as *E. coli* and the potato.[148] However, *E. coli* maltodextrin phosphorylase[143a,149–151] and potato phosphorylases have less sophisticated regulatory mechanisms than do the animal enzymes. Another feature of glycogen phosphorylase is a "glycogen storage site" about 2.5 nm from the active site (Fig. 11-5).[152] This provides a means for the enzyme to hold onto the giant glycogen molecule while "nibbling off" the outside ends of nearby branches.

8. Starch-Hydrolyzing Enzymes

Among the hydrolases are the widely distributed **α-amylases**, *endo*-glycosidases which hydrolyze

starch chains by random attack at points far from chain ends to form short polysaccharide chains known as **dextrins** as well as simpler sugars.[153] The catalyzed reactions proceed with retention of the original α configuration. Alpha-amylases are found in fungi, plants, and animals. One powerful enzyme of this class is present in the saliva of most humans and other isoenzymes are formed by the pancreas.[154] They are encoded by a family of genes, a fact that accounts for the existence of some healthy individuals completely lacking salivary amylase.[155]

Structures are known for human[154] and porcine[156–159] α-amylases as well as for corresponding enzymes from barley,[160] mealworms,[161] fungi, and bacteria.[162–164] The α-amylase from *Aspergillus oryzae* (Taka-amylase), widely used in laboratory work, was the first for which a structure was determined.[165,166] The α-amylases fold into three domains (Fig. 12-6), with the active site in the center of an $(\alpha/\beta)_8$ barrel. All of the α-amylases contain one or more bound Ca^{2+} ions. Some, including the human α-amylases, also require a **chloride ion**. The Cl^- is held by a pair of arginine guanidinium groups[154] and interacts with adjacent carboxyl groups, inducing pK_a shifts and allosteric activation.[163]

The sequences of α-amylases vary widely but a conserved cluster of one glutamate and two aspartates is usually present (Table 12-1).

Studies of the **action patterns**, i.e., the distribution of products formed by α-amylases when acting on a variety of α 1,4-linked oligosaccharides, suggested that the substrate binding region of the porcine pancreatic enzyme has five subsites, each binding one glucose residue.[153] The α-glucan chain is cleaved between the residues bound at the second and third subsites (numbered from the reducing end of the substrate) by a lysozyme-like mechanism. Endolytic enzymes, which cleave biopolymer chains internally, are usually thought to carry out random attack. However, after the initial catalytic reaction one of the polysaccharide products of porcine pancreatic amylase action often does not leave the enzyme. The polysaccharide simply "slides over" until it fully occupies all of the subsites of the substrate binding site and a second "attack" occurs. An average of seven catalytic events occur each time it forms a complex with amylase.[167] Is there a mechanism by which the enzyme deliberately promotes the "sliding" of the substrate in this **multiple attack** or **processive** mechanism[168,169] or does the dissociated product simply diffuse a short distance while enclosed in a solvent cage? The latter explanation may be adequate for some enzymes. In contrast to pancreatic α-amylase a bacterial "maltogenic" α-amylase produces principally maltose as a product.[169a,b]

Digestion of starch and glycogen by α-amylases produces a mixture of glucose, oligosaccharides, and dextrins, which in the human body are further degraded by **α-glucosidases** of the brush border membrane of the small intestine.[154] A lysosomal form of the enzyme is missing in Pompe's disease (Box 20-D).[170,171] The α-glucosidases are also members of the α-amylase family.[46,172] For digestion of the branched amylopectin and glycogen a **debranching enzyme** and **α-1,6-glucosidase** activity are required. In mammals these activities are found in a single polypeptide chain with separate but adjacent active sites.[50,173,174] The debranching enzyme catalyzes transfer of oligosaccharide chains from α1,6-linked branch positions to new locations at ends of chains with α1,4 linkages. A bacterial oligo-1,6-glucosidase has a catalytic site formed from

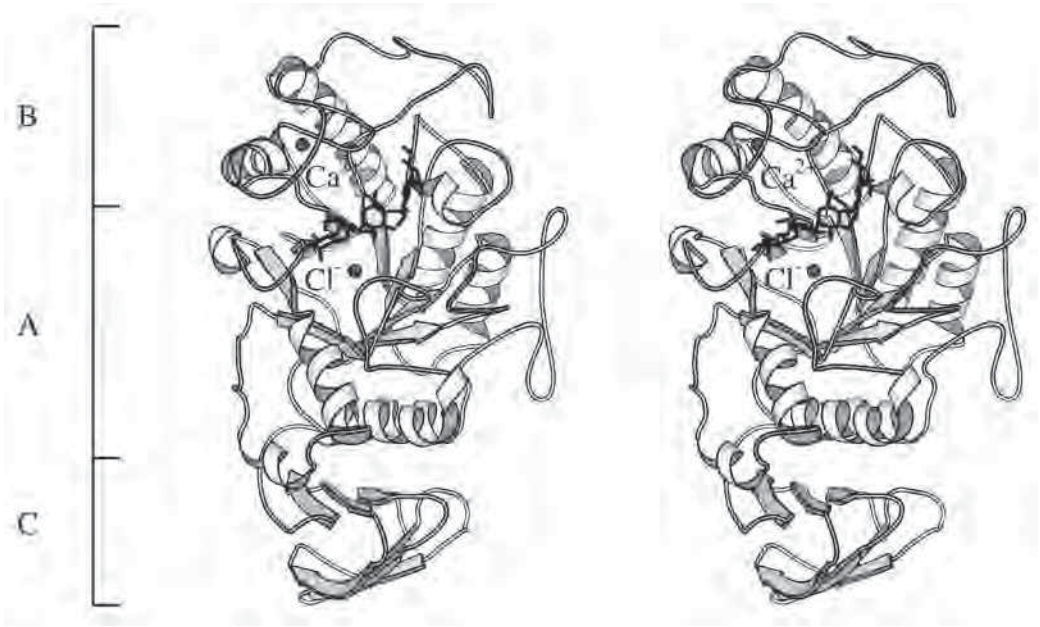
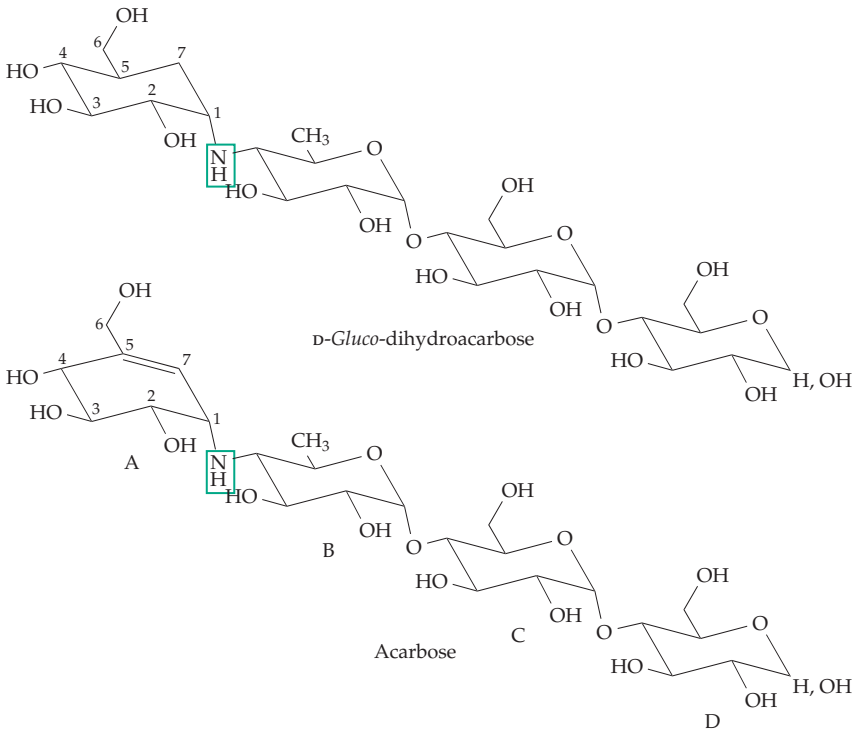


Figure 12-6 Drawing showing the overall polypeptide chain fold and relative positioning of the three structural domains of human pancreatic α-amylase. Also drawn are the locations of the calcium and chloride binding sites. Overlaid is the placement of a modified form of the inhibitor acarbose (p. 607) that binds in the active site cleft. MolScript drawing courtesy of G. Sidhu and G. Brayer.

D199, E255, and D329 similar to that of α-amylases.[175]

Another member of the α-amylase family has principally a glycosyltransferase activity. **Cyclodextrin glucanotransferase** forms cyclodextrins (Box 4-A) by a transferase reaction in which a 6- to 8- member oligo-saccharyl group is transferred from a straight amylase chain onto a protein side chain (Glu 257) and then joins the ends of the oligosaccharide to form the cyclo-dextrin rings (an overall double-displacement process).[176–177b] However, large circular dextrins are produced initially and are then converted into the smaller cyclodextrins.[178]

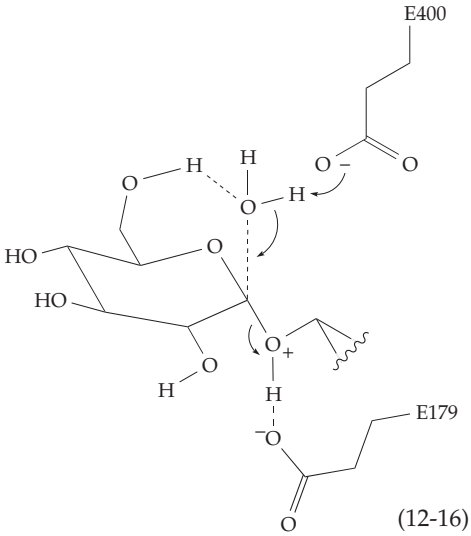
A starch-digesting enzyme of great industrial importance is **glucoamylase**, whose $(\alpha,\alpha)_6$-barrel structure is shown in Fig. 2-29. That figure also shows the tetrasaccharide inhibitor **acarbose** in the active site. The ring at the nonreducing end is deeply embedded in the protein and, as shown in Fig. 12-7 for the related D-*gluco*-dihydroacarbose, is held by many hydrogen bonds. This slow enzyme (k_{cat} ~50 s^{-1} at 45°C)[179] cuts off a single glucose unit, then releases



itself from the starch before releasing the glucose and rebinding to the starch. The catalytic acid has been identified as the carboxyl group of Glu 179. In Fig. 12-7 it is seen, presumably as a carboxylate group, tightly hydrogen bonded to what is doubtless the bridge $-NH_2^+-$ between rings A and B of the inhibitor D-*gluco*-dihydroacarbose. Thus, the complex mimics that of a true substrate protonated on the bridge oxygen, a possible first step in normal catalysis. In accord with this mechanism (Eq. 12-16), glucoamylase is an inverting enzyme.

Notice in Fig. 12-7 the abundance of polar groups, many of which are charged. Four carboxyl groups, from D55, E179, E180, and E400, are present in the active site region and participate in the hydrogen bonded network. Active sites of other carbohydrases differ from that of glucoamylase. However, the presence of many hydrogen-bonded polar groups, including two or more carboxyl groups, is characteristic. As illustrated in Figs. 9-8 and 9-9, the pH dependence of the maximum velocity v_{max} is often determined by these carboxyl groups. At least one of them must be protonated for maximum catalytic activity and if two are protonated activity may fall again. Under these circumstances a single proton bound to a molecule of enzyme may spend a fraction of its time on each of several different carboxylate, imidazole, or other groups.[181] In the case of glucoamylase it was concluded from the X-ray structure that the catalytic acid group, E179, is probably *unprotonated* most of the time.[181] However, it can still bind to starch. Then, after a fraction of a second a proton may be transferred onto the Glu 179 carboxylate and from there to the bridge oxygen of the substrate, inducing reaction according to Eq. 12-16. It may even be necessary to have enzyme protonated initially on a group other than E179 to allow small conformational changes to occur prior to formation of the final activated complex. Such essential conformational changes have often been invoked for glucoamylase.[182]

Just as most cellulases have special cellulose-binding domains, glucoamylase has a compact C-terminal starch-binding domain (residues 509–616) similar to the



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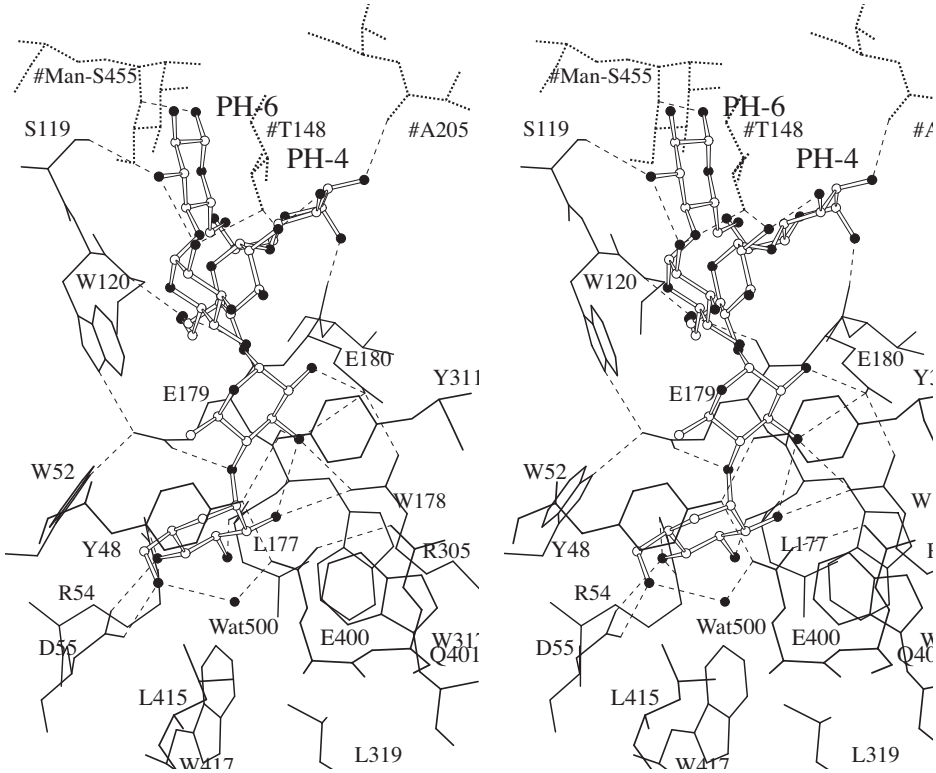


Figure 12-7 Stereoscopic view of the inhibitor D-*gluco*-dihydroacarbose in its complex with glucoamylase of *Aspergillus*. Residues from symmetry-related molecules of the enzyme are shown as dotted lines. Ring A at the nonreducing end of an amylase chain is thought to bind in a similar way (with ring A at the bottom in this figure). Cleavage is between the A and B rings E178 acting as a proton donor to the bridge oxygen (or NH for the inhibitor). The attacking nucleophile is thought to be a water molecule, which is labeled Wat 500 and is held by the assisting carboxylate of E400.[180] Courtesy of Alexander Aleshin.

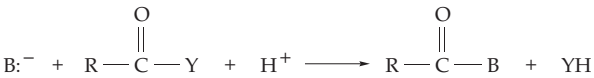
corresponding domain of cellulases.[183] It is connected by a glycosylated linker to the 470-residue catalytic subunit. Cyclodextrin glucanotransferase also has a starch-binding domain.[184]

Beta-amylases, characteristic plant and bacterial enzymes[184a], have an *exo* action, cutting off chain ends two sugar units at a time as *maltose*. The original α linkage is inverted, the product being β-maltose. The $(\alpha/\beta)_8$-barrel structure is unlike that of glucoamylase, but the spacing of active site carboxyl groups suggests that a water molecule is held and activated as in glucoamylase.[185,186] The multiple subsite structure of the active site may permit the substrate amylase to slip forward after a maltose product molecule leaves the site. This may account for the observed processive action.[186]

Many specialized glycosyl transferases synthesize glycogen, starch, cellulose, and other polysaccharides and add glycosyl groups to glycoproteins and glycolipids.[187,188] Often the glycosyl group is transferred from a carrier such as uridine diphosphate (UDP). An example is glycogen synthase (Fig. 11-4), which transfers glucosyl groups of UDP-glucose to the 4'–OH groups at the nonreducing ends of the bushlike glycogen molecules. Other similar synthetic reactions are considered in Chapter 20.

C. Displacement Reactions on Carbonyl Groups

A second major class of nucleophilic displacement reactions (Type 1B in Table 10-1) involve replacement of a group Y attached to a carbonyl carbon:

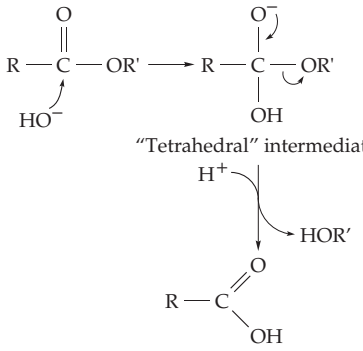


(12-17)

Group Y may be –OR (esters), –SR (thioesters) or –NHR (amides or peptides). If B^- is a hydroxyl ion formed from H_2O, the reaction is **hydrolysis**. If B^- is the anion of an alcohol, thiol, or amine, the reaction is **transacylation**. Transacylation is an essential process in biosynthesis of proteins and lipids, but it is the digestive enzymes, which catalyze hydrolysis, that have been studied most intensively.

Uncatalyzed hydrolysis of a peptide linkage is very slow with $t_{1/2}$ at neutral pH and 25°C of ~300–600 years.[189] Both acids and bases catalyze hydrolysis, but enzymes are needed for rapid digestion. The carbonyl group C=O is highly polarized, with the resonance form $C^+–O^-$ contributing substantially to its structure. An attack by a base will take place readily on the electrophilic carbon atom. While the reactivity of the carbonyl group in esters and amides is relatively low

because of the resonance stabilization of these groups, the carbon atom still maintains an electrophilic character and may combine with basic groups. Thus, in the base-catalyzed hydrolysis of esters a hydroxyl ion adds to the carbonyl group to form a transient single-bonded "tetrahedral" intermediate (Eq. 12-18). Similar intermediates are believed to form during the action of many enzymes. However, for purposes of classification, we can regard them as simple displacement reactions on a carbon atom with the understanding that there are probably transient single-bonded intermediates.



(12-18)

A large number of hydrolytic enzymes, the **proteases**, and **peptidases** act on peptide linkages of proteins.[190] At present the traditional name protease, which implies proteolysis, is most often used. However, the IUB encourages use of the name **proteinase**. Although this seems less specific in meaning, its use will probably increase. Some proteases trim newly formed peptide chains, others convert proteins from precursor forms into biologically active molecules, and others digest proteins. In addition to endopeptidases, such as trypsin and chymotrypsin, which cleave at positions *within* a long peptide chain, there are many enzymes that cleave amino acids from the *ends* of chains. These are usually called peptidases and are designated **aminopeptidases** if they cleave from the N terminus and **carboxypeptidases** if they cleave from the C terminus. Most of these enzymes can be classified into **serine** proteases, **cysteine** proteases, **aspartic** proteases, **metallo** proteases, or **N-terminal nucleophile** hydrolases, depending upon the chemical nature of the active site. These groups are further divided into families or "clans."[190]

1. The Serine Proteases

The digestive enzymes **trypsin**, **chymotrypsin**, **elastase**, and **proteinase E** are related serine proteases. All three are synthesized in the pancreas which secretes 5–10 g per day of proteins, mostly the inactive proenzymes (zymogens) of digestive enzymes.[191,192] These proenzymes are synthesized and "packaged" as **zymogen granules** which travel to the surfaces of the secretory cells. The contents of the granules are secreted into the extracellular medium and are discharged via the pancreatic duct into the small intestine. At their sites of action, the zymogens are converted into active enzymes by the cutting out of one or more pieces from the precursor. This occurs in a cascade-type process triggered by **enteropeptidase** (historically enterokinase), another serine protease which is secreted by the intestinal lining.[192a] Human enteropeptidase consists of a 235-residue catalytic subunit bonded through a disulfide bridge to a larger 784-residue membrane-anchoring subunit.[193–195] It attacks specifically **trypsinogen**, converting it to active trypsin.[196,197] Trypsin in turn activates the other zymogens, as is indicated in Fig. 12-8. Trypsin can also activate its own zymogen, trypsinogen, in an autocatalytic process.

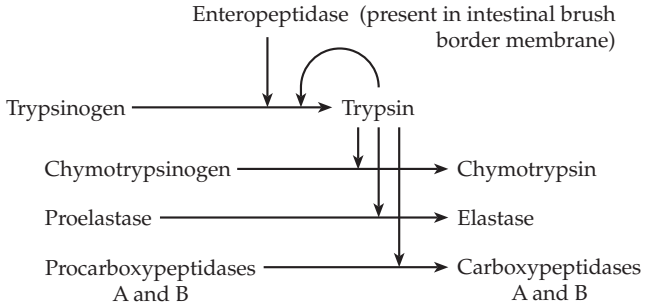
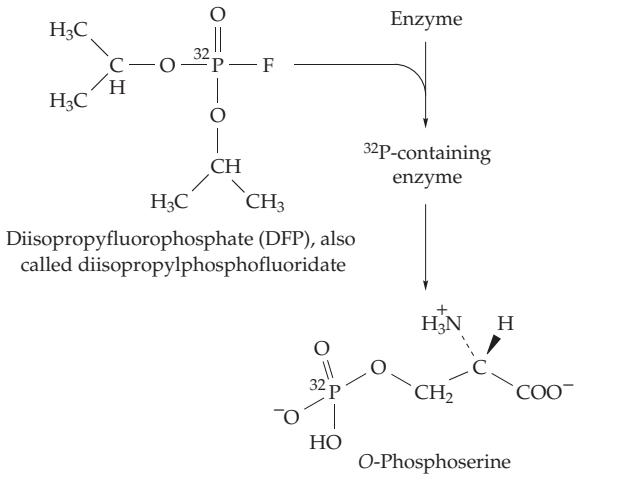


Figure 12-8 Cascade of reactions that activate pancreatic proteases. Enteropeptidase, or trypsin, cleaves the proenzyme (zymogen) at specific sites.

Chymotrypsinogen consists of a single 245-residue chain. The amino acid residues in chymotrypsin, trypsin, and elastase are usually all numbered according to their position in this zymogen. Inactive proenzymes are formed as precursors to enzymes of many different classes and are activated in a variety of ways. A part of the polypeptide chain of the proenzymes is often folded over the active site, interacting in a nonsubstrate-like fashion and blocking the site.[197a]

Serine as a nucleophile. An early clue to the mechanism of action of chymotrypsin came from investigation of the related **acetylcholinesterase**. This key enzyme of the nervous system is inactivated irreversibly by powerful phosphorus-containing poisons that had been developed as insecticides and as war gases (nerve gases, Box 12-C). Around 1949, the nerve gas diisopropylfluorophosphate (DFP) was shown also to inactivate chymotrypsin. When radioactive ^{32}P-containing DFP was allowed to react the ^{32}P became

covalently attached to the enzyme. When the labeled enzyme was denatured and subjected to acid hydrolysis the phosphorus stuck tightly; the radioactive fragment was identified as *O*-phosphoserine. It was evident that this product could be formed by an attack of the hydroxyl group of the serine side chain on the phosphorus with displacment of the fluoride ion. This is a nucleophilic displacement on phosphorus, occurring on an enzyme that normally catalyzes displacement on C=O. The DFP molecule acts as a pseudosubstrate which reacts with the enzyme in a manner analogous to that of a true substrate but which does not complete the reaction sequence normally.



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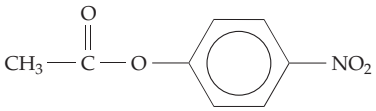
From study of peptides formed by partial hydrolysis of the ^{32}P-labeled chymotrypsin, the sequence of amino acids surrounding the reactive serine was established and serine 195 was identified as the residue whose side chain hydroxyl group became phosphorylated. The same sequence Gly-Asp-Ser-Gly was soon discovered around reactive serine residues in trypsin, thrombin, elastase, and in the trypsin-like **cocoonase** used by silkmoths to escape from their cocoons.[198] We know now that these are only a few of the enzymes in a very large family of serine proteases, most of which have related active site sequences.[199,200] Among these are **thrombin** and other enzymes of the blood-clotting cascade (Fig. 12-17), proteases of lysosomes, and secreted proteases.

Numerous serine proteases, including trypsin-like enzymes called **tryptases**[201–204a] and chymotrypsin-like chymases,[205–207] are found within tissues in which they are stored in granules of mast cells,[208] neutrophils, lymphocytes, and cytotoxic T cells.[205] Secretory granules of mast cells present in skin and other tissues contain high concentrations of tryptase and chymase precursors[202,206] which may be released as part of an inflammatory response. Tryptase may be involved in asthma and other allergic resposes.[201] **Cathepsin G**[209] (proteinase II), **neutrophil elastase**, and **proteinase III**[210] are found in granules of neutrophils and monocytes as well as in mast cells.[209] Cytoplasmic granules of cytotoxic T cells contain at least seven proteases called **granzymes** that can be released to attack target cells.[211–213a]

Many secreted proteins, as well as smaller peptide hormones, are acted upon in the endoplasmic reticulum by tryptases and other serine proteases. They often cut between pairs of basic residues such as KK, KR, or RR.[214–216] A substilisin-like protease cleaves adjacent to methionine.[217] Other classes of proteases (e.g., zinc-dependent carboxypeptidases) also participate in this processing. **Serine carboxypeptidases** are involved in processing human prohormones.[218] Among the serine carboxypeptidases of known structure is one from wheat[219] and **carboxypeptidase Y**, a vacuolar enzyme from yeast.[220] Like the pancreatic metallocarboxypeptidases discussed in Section 4, these enzymes remove one amino acid at a time, a property that has made carboxypeptidases valuable reagents for determination of amino acid sequences. Carboxypeptidases may also be used for modification of proteins by removal of one or a few amino acids from the ends.

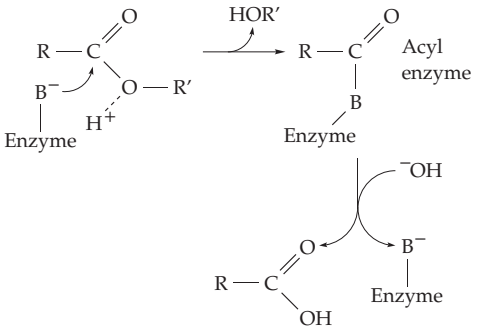
The variety of bacterial serine proteases known include the 198-residue **α-lytic protease** of *Myxobacter*,[221] a family of at least 80 **subtilisins** which are produced by various species of *Bacillus*[222–225a] as well as by many other organisms,[226] and a trypsinlike enzyme from *Streptomyces griseus*.[227,228] **Tripeptidyl peptidases**, subtilisin-like enzymes, cut tripeptides from the N-termini of proteins.[228a,b,c] One participates in lysosomal protein degradation and the other, an oligomer of 138 kDa subunits, cuts precursor proteins to form neuropeptides and other hormones (Chapter 30).

Acyl-enzyme intermediates. Serine proteases are probably the most studied of any group of enzymes.[229] Early work was focused on the digestive enzymes. The pseudosubstrate, *p*-nitrophenyl acetate, reacts with chymotrypsin at pH 4 (far below the optimum pH for hydrolysis) with rapid release of *p*-nitrophenol and formation of acetyl derivative of the enzyme.



p-Nitrophenyl acetate

This acetyl enzyme hydrolyzes very slowly at pH 4 but rapidly at higher pH. These experiments suggested a double displacement mechanism:



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Although the experiments with DFP suggested that the $-O^-$ group from Ser 195 might be the base $-B^-$ in this equation, there was reluctance to accept this deduction because of the very weak acidity of the $-CH_2OH$ group. Furthermore, the pH dependence of catalysis suggested an imidazole group of a histidine side chain as the attacking nucleophile B. Indeed, imidazoles catalyze the nonenzymatic hydrolysis of *p*-nitrophenyl acetate with formation of unstable *N*-acetyl imidazoles as intermediates. Thus, while the stable end products of reactions with pseudosubstrates were unquestionably derivatives of serine, the possibility remained that these were side products and that histidine was involved in transient, rapidly forming, and reacting intermediates. It remained for the results of the then newly developed science of X-ray crystallography to clarify this question.[230]

Three-dimensional structures. The structures of chymotrypsin,[199,230,231] trypsin,[232,233] elastase,[234,235] thrombin,[236] kallikrein,[237,238] and many other enzymes are similar, with the basic fold shown in Fig. 12-9.[199,228] Both Ser 195 and His 57 (or corresponding residues) are present in the active site (Figs. 12-9, 12-10). From the observed positions of competitive inhibitors occupying the active site, the modes of binding depicted in Fig. 12-10A for the chymotrypsin family and in Fig. 12-10B for the subtilisin family have been deduced. Bear in mind that the X-ray diffraction results do not

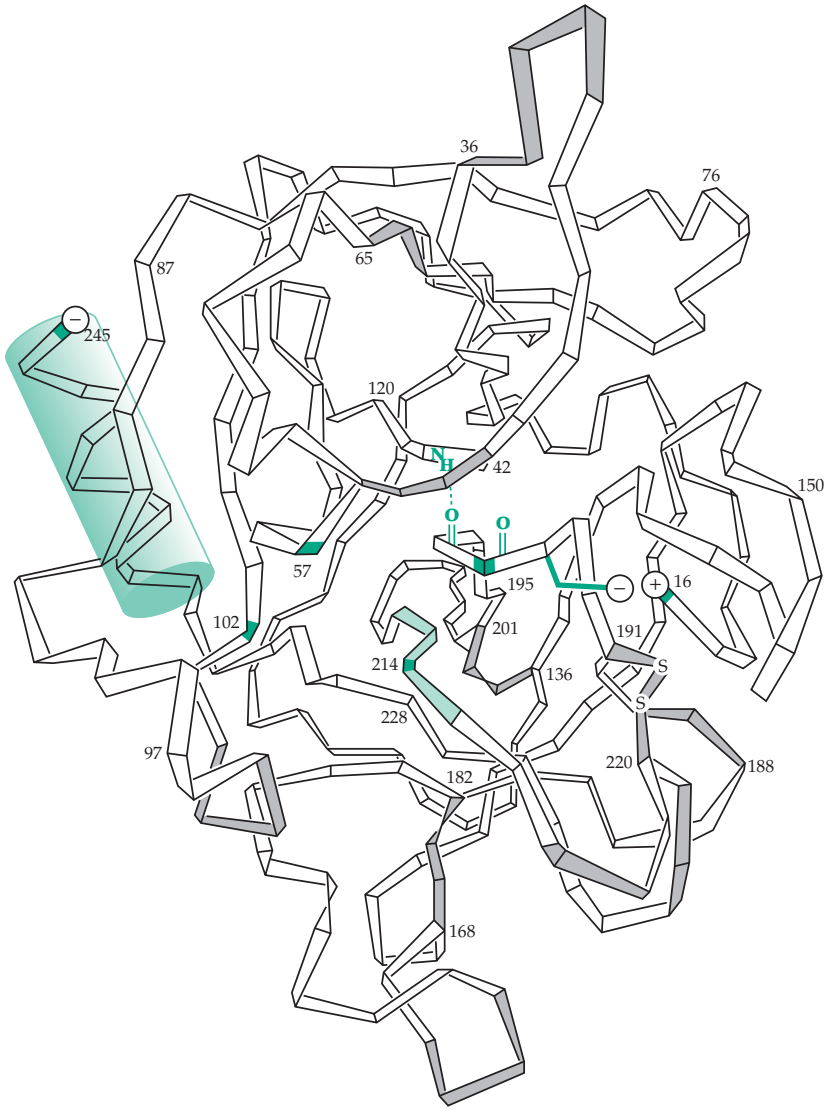


Figure 12-9 Alpha-carbon diagram of the three-dimensional structure of pancreatic elastase. A principal structural feature is a pair of β cylinders. One of these, at the top of the figure, is viewed end-on, while the other, at the bottom of the figure, is viewed from a side. The prominent interface between them is seen in the center. The α-carbon positions of the catalytic triad serine 195, histidine 57, and aspartate 102 are marked. As shown in Fig. 12-10 the catalytic triad is located across the interface between β cylinders, which may allow for easier conformational alterations during the action of the enzyme. The four disulfide bridges and the long C-terminal helix are also emphasized. The cross-hatched loop regions are residues present in elastase but not present in chymotrypsin. The green shaded strand (residues 214–216) is a segment that joins residues P_1 and P_2 of the substrate to the edge of the lower β cylinder in an extended β structure. The ion pair formed by aspartate 194 and isoleucine 16 during zymogen activation is also shown, as are two peptide carbonyl groups that protrude into the interface area, one forming a hydrogen bond across the interface. Modified from a drawing of Sawyer *et al.*[234] which was in turn based on the original chymotrypsin drawing of Annette Snazle.[230]

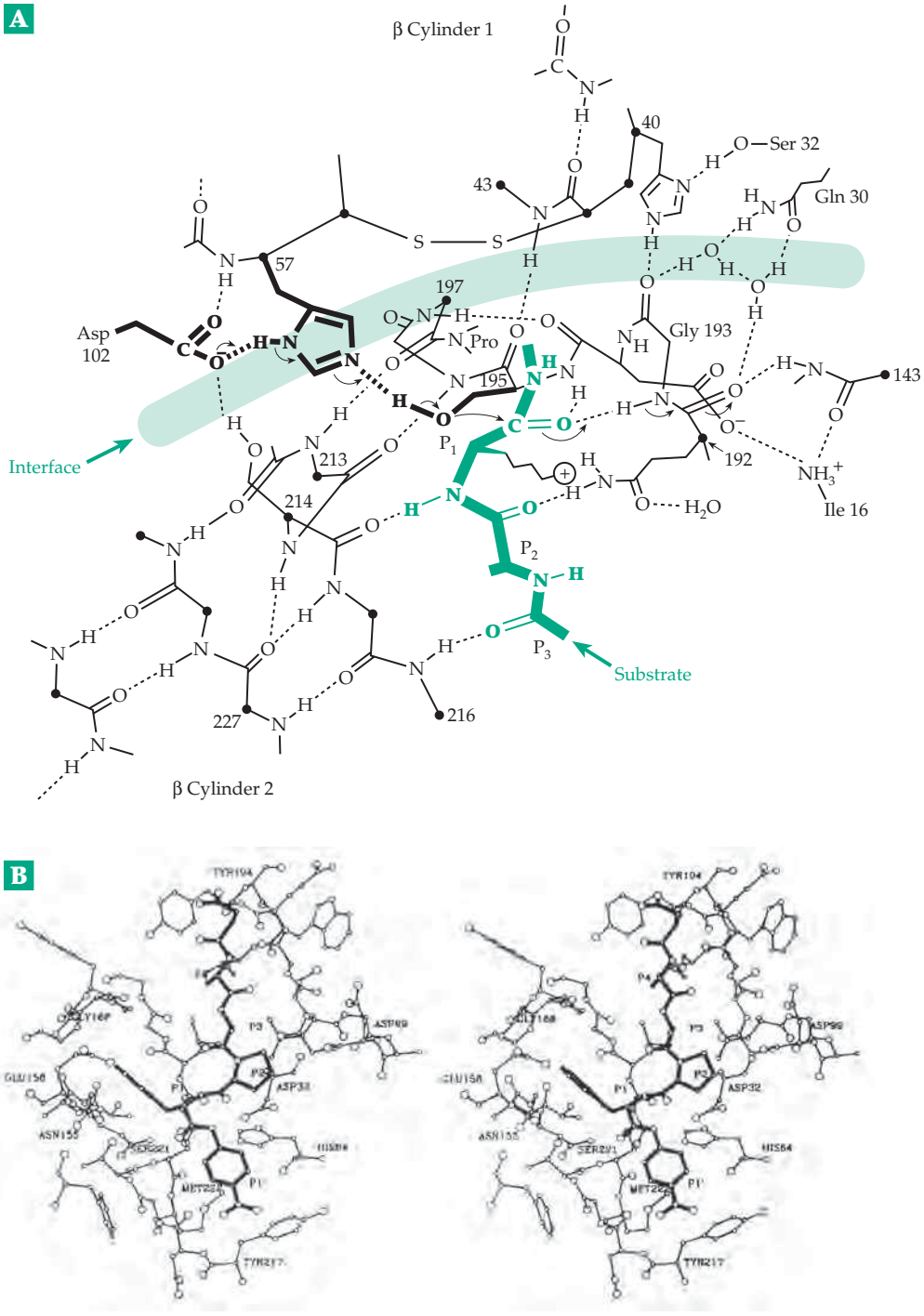
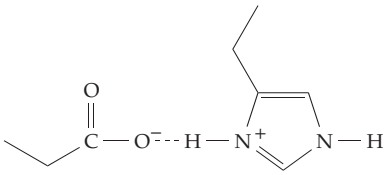


Figure 12-10 (A) Part of the hydrogen-bonding network of trypsin and other serine proteases with a bound trypsin substrate (green). Residues P_1 to P_3, as defined in Fig. 12-14, have been marked. The view is similar to that in Fig. 12-9 but some background lines have been omitted. Note the two competing hydrogen-bonded chains passing through C=O of residue 227 in β cylinder 2. One of these chains passes through the backbone of the substrate and the other through residues 214 and 195 across the interface between domains into β cylinder 2. Asp 102, His 57, and Ser 195 of the catalytic triad are emphasized. Arrows indicate probable movement of electrons from the negative charge of Asp 102 into the "oxyanion hole." After Metzler.[85] Based on papers of Sawyer *et al.*[234] and Huber and Bode.[240] (B) Stereoscopic view showing the model substrate *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide bound into the active site of subtilisin BPN′. Residues of the catalytic triad (Ser 221, His 64, Asp 32) and some others are labeled. Subsites P_1', P_1, P_2, P_3, and P_4 (see Fig. 12-14) are also labeled. Notice that site P_3, which is near the top in this drawing, is at the bottom in (A). Based on X-ray data of R. Bott and M. Ultsch at 0.2 nm resolution. From Wells and Estell.[223]

show where the hydrogen atoms are and that these have been added in Fig. 12-10. The imidazole group of His 57 is located next to the side chain –OH group of Ser 195 and is able to form an N---H–O hydrogen bond to it. The obvious conclusion is that His 57 acts as a general base catalyst that assists in removing the proton from the –OH of Ser 195, making that hydroxyl group more nucleophilic than it would be otherwise. This may happen after the –OH group has started to add to the substrate carbonyl.

The second nitrogen atom of His 57 is hydrogen bonded to the carboxylate group of Asp 102, which is in turn hydrogen bonded to two other groups. Aspartate 102 has one of the few carboxylate side chains that is buried inside the protein. To Blow,[230,231,239] the structure suggested a **charge-relay system** by which negative charges might move synchronously from Asp 102 to the imidazole which could then deprotonate the hydroxyl group of Ser 195, allowing the serine oxygen to add to the substrate carbonyl to form the tetrahedral

or **oxyanion** intermediate, which is depicted in step *b* of Fig. 12-11. The small arrows in Fig. 12-10 also indicate the movement of charge. Blow suggested that in the extreme case a *proton* might be transferred also from the His 57 imidazole to the Asp 102 carboxylate. However, a variety of experiments, including studies by ^{15}N NMR[241,242] and by neutron diffraction,[243] suggest that the imidazole does not transfer its proton to the carboxylate of Asp 102,[244,245] unless it does so transiently, e.g., in a transition state complex. Instead, the carboxylate and the imidazolium ions formed by protonation of His 57 probably exist as a tight ion pair:



Support for this concept is provided by ^1H NMR studies which have identified a downfield resonance of the hydrogen-bonded proton in this pair at ~18 ppm in chymotrypsinogen and chymotrypsin at low pH and at ~14.9–15.5 ppm at high pH values.[246,247] Similar resonances are seen in the α-lytic protease,[248] in subtilisin,[249] in adducts of serine proteases with boronic acids[250,251] or peptidyl trifluoromethyl ketones,[252] in alkylated derivative of the active site histidine,[253] and in molecular complexes that mimic the Asp-His pair in the active sites of serine proteases.[254]

The catalytic cycle. Figure 12-11 depicts the generally accepted sequence of reactions for a serine protease. If we consider both the formation and the subsequent hydrolysis of the acyl-enzyme intermediate with appropriate oxyanion intermediates, there are at least seven distinct steps. As indicated in this figure, His 57 not only accepts a proton from the hydroxyl

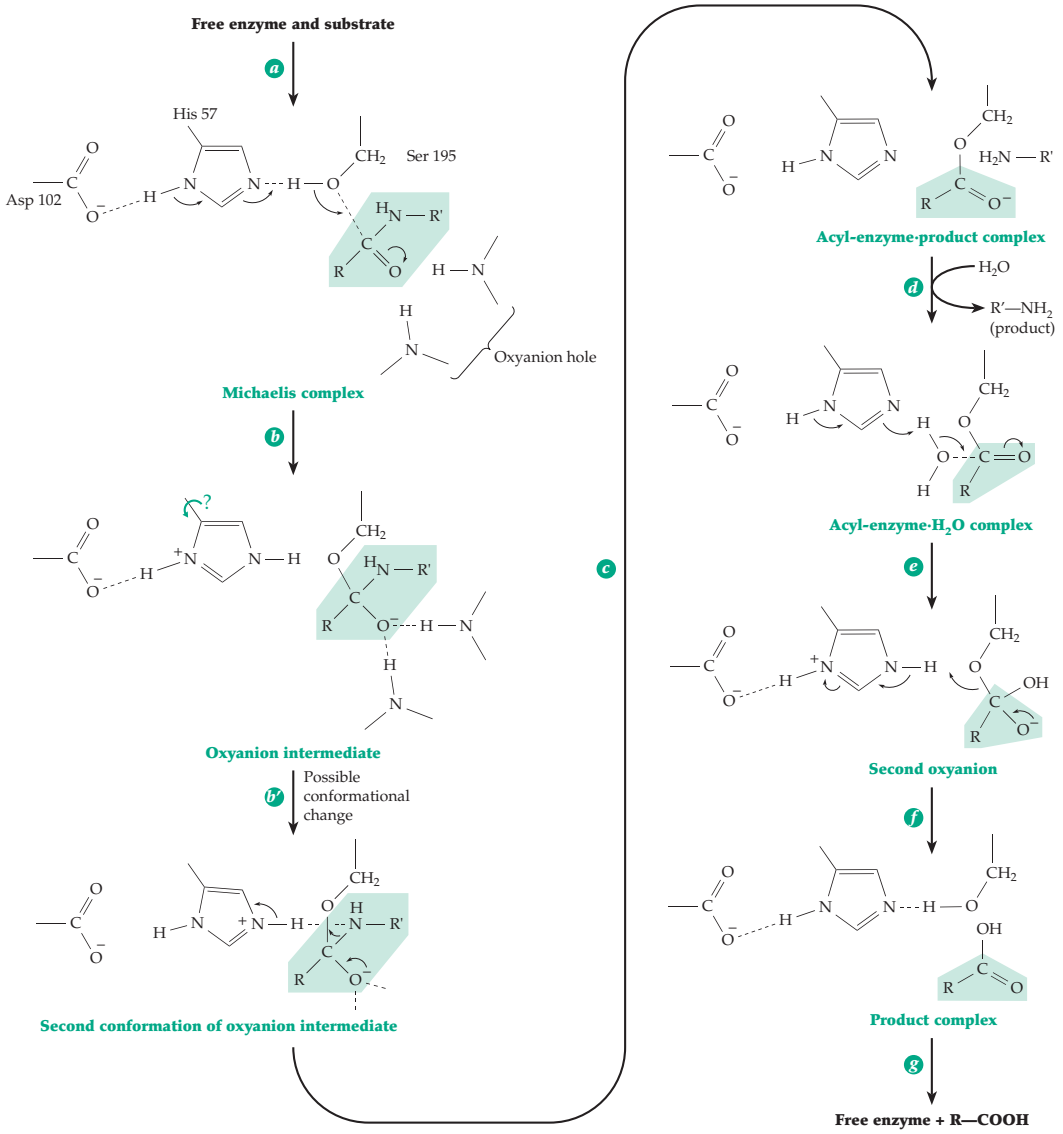


Figure 12-11 Sequence of chemical reactions involved in the action of a serine protease. The oxyanion hole structure has been omitted in the right-hand column. The imidazole ring may rotate, as indicated by the green arrow, to provide two different conformations.

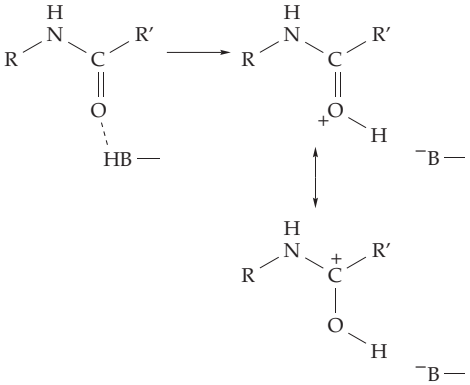
group of Ser 195 (step *b*) but probably also functions in protonation of the –NH–R′ leaving group (step *c*). An unprotonated –NH–R′ would be such a poor leaving group that the oxyanion intermediate would not go on to acyl-enzyme. However, donation of a proton to that leaving group from the protonated His 57 (general acid catalysis) permits elimination of H_2N–R′ (step *c*). The product of this step is the acyl-enzyme intermediate which must be hydrolyzed to complete the catalytic cycle. This is accomplished through steps *d*–*f* of Fig. 12-11. The product R′–NH_2 is replaced by H_2O, which, in steps paralleling steps *b* and *c*, is converted to an HO^- ion that serves as the attacking nucleophile to form (in step *e*) a second oxyanion intermediate which is cleaved to the second product R–COOH. The water molecule that enters in step *d*, and which participates in hydrolysis of the acyl enzyme, has apparently been observed directly by time-resolved Laue crystallography at low temperature.[255]

The catalytic triad. The significance of the charge-relay effect may not be fully understood but the importance of the Ser•His•Asp cluster, which has become known as the catalytic triad, cannot be doubted. It has evolved independently in several subfamilies of bacterial and plant proteases, a well-known example of "convergent evolution." This triad is also found throughout a broad range of many different kinds of enzymes and other proteins. In pancreatic enzymes the catalytic triad consists of Ser 195•His 57•Asp 102 but in the bacterial subtilisin it consists of Ser 221•His 64•Asp 32 and in a wheat serine carboxypeptidase it is Ser 146•His 397•Asp 338.[219] In all three cases the folding patterns of the polypeptides are entirely different but the geometry of the triad is the same. Another folding pattern is seen in a protease encoded by cytomegalovirus, which contains an active site Ser 132•His 63 pair.[256,257]

Investigation of a host of mutant proteins also demonstrates the importance of the catalytic triad. For example, if either the histidine or the serine of the triad of subtilisin was replaced by alanine the catalytic activity decreased by a factor of 2 x 10^6 and replacement of the aspartate of the triad by alanine decreased activity by a factor of 3 x 10^4.[229,258] When Asp 102 of trypsin is replaced by asparagine the catalytic activity falls by four orders of magnitude.[259] This may be in part because the histidine in this mutant is hydrogen bonded to Asn 102 as the tautomer with a proton on N^ε, the nitrogen that should serve as the catalytic base in step *b* (Fig. 12-11).[260] A mutant in which Ser 214 (see Fig. 12-10) was replaced with alanine is fully active but charged residues in this position interfere with catalysis.[261]

Does the "low-barrier hydrogen bond" in the catalytic triad play any special role in catalysis? Blow's suggestion of a charge relay from Asp 102 to Ser 195 of chymotrypsin is probably correct. Some theoretical calculations have indicated the possibility of synchronous movement of the two protons in the system during step *a* of the sequence shown in Fig. 12-11, with the proton in the strong hydrogen bond to Asp 102 moving away from His 57 and toward the midpoint distance of the hydrogen bond. Could the presumed high energy of the short hydrogen bond be harnessed to lower the transition state energy? A realistic possibility is that the hydrogen bond increases the polarizability of the catalytic triad, facilitating movement of the substrate to the transition state. Cassidy *et al.* suggested that the strong hydrogen bond may be formed by compression of the triad resulting from binding of substrate in the S_1 and S_2 subsites.[252] They suggested that this would raise the pK_a of His 57 from ~7 in the free enzyme to 10–12, high enough to enable it to remove the proton from the Ser 195 –OH group and low enough to allow the protonated form to be the proton donor to the leaving group (step *c*, Fig. 12-11).

The "oxyanion hole." A third mechanism by which an enzyme can assist in a displacement reaction on a carbonyl group is through protonation of the carbonyl oxygen atom by an acidic group of the enzyme (Eq. 12-21). This will greatly increase the positive charge on the carbon atom making attack by a nucleophile easier and will also stabilize the tetrahedral



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intermediate. Although the carbonyl oxygen is very weakly basic, it can interact with a suitably oriented acidic group of the enzyme. In many serine proteases this acidic function is apparently fulfilled by NH groups of two amide linkages. In chymotrypsin these are the backbone NH groups of Ser 195 and Gly 193 (Figs. 12-10,12-12). No actual transfer of a proton to the carbonyl oxygen of the substrate is expected. However, the NH groups are positive ends of amide dipoles and can interact electrostatically with the negative charge that develops on the oxyanion. The fit of substrate into the **oxyanion hole** between the two

NH groups is apparently good only for the tetrahedrally bonded oxyanion intermediate,[262] a structure thought to be close to that of the transition state. The importance of the oxyanion hole for catalysis has been supported by theoretical calculations[245] and by the study of mutant enzymes.[263] In subtilisin, in which a side chain of asparagine forms part of the oxyanion hole, replacement of Asn with the isosteric Leu causes k_{cat} to fall by a factor of about 200 while K_m is unaffected.[264] It is also of interest that thiono ester substrates, in which the C=O of an oxygen ester has been replaced by C=S, bind with normal affinity to chymotrypsin but are not hydrolyzed at significant rates.[265]

The formation of the oxyanion intermediate during serine protease action is also supported by the existence of tetrahedral forms of enzymes inhibited by substrate-like aldehydes. The –OH group of Ser 195 can add to the carbonyl group to form hemiacetals. For example, a ^{13}C-enriched aldehyde whose carbonyl carbon had a chemical shift of 204 ppm gave a 94 ppm resonance as it formed the tetrahedral hemiacetal with one of the inhibitory aldehydes, *N*-acetyl-L-Leu-L-Leu-L-arginal (**leupeptin**; Box 12-C). A natural product from a species of *Streptomyces*, this aldehyde inhibits trypsin and several other enzymes strongly.[266] Adducts of wheat serine carboxypeptidases with aldehyde inhibitors have also been observed.[267] While the carbonyl group of the substrate amide linkage that is to be cleaved apparently can't form strong hydrogen bonds to the NH groups of the oxyanion hole, that of the acyl-enzyme intermediate can, as judged by resonance Raman spectroscopy.[268,269] The strength of the hydrogen bonding, as judged by the stretching frequency of the C=O group, is correlated with the reactivity of the acyl group.[269]

Chymotrypsinogen and related proenzymes have extremely low catalytic activity even though a major part of the substrate binding site as well as the catalytic triad system are already in place. However, the oxyanion hole is created during activation of the proenzyme by a subtle conformational change[197,262,271] that involves the chain segment containing Gly 193 (Fig. 12-12). This is further evidence of the importance of this part of the active site structure.

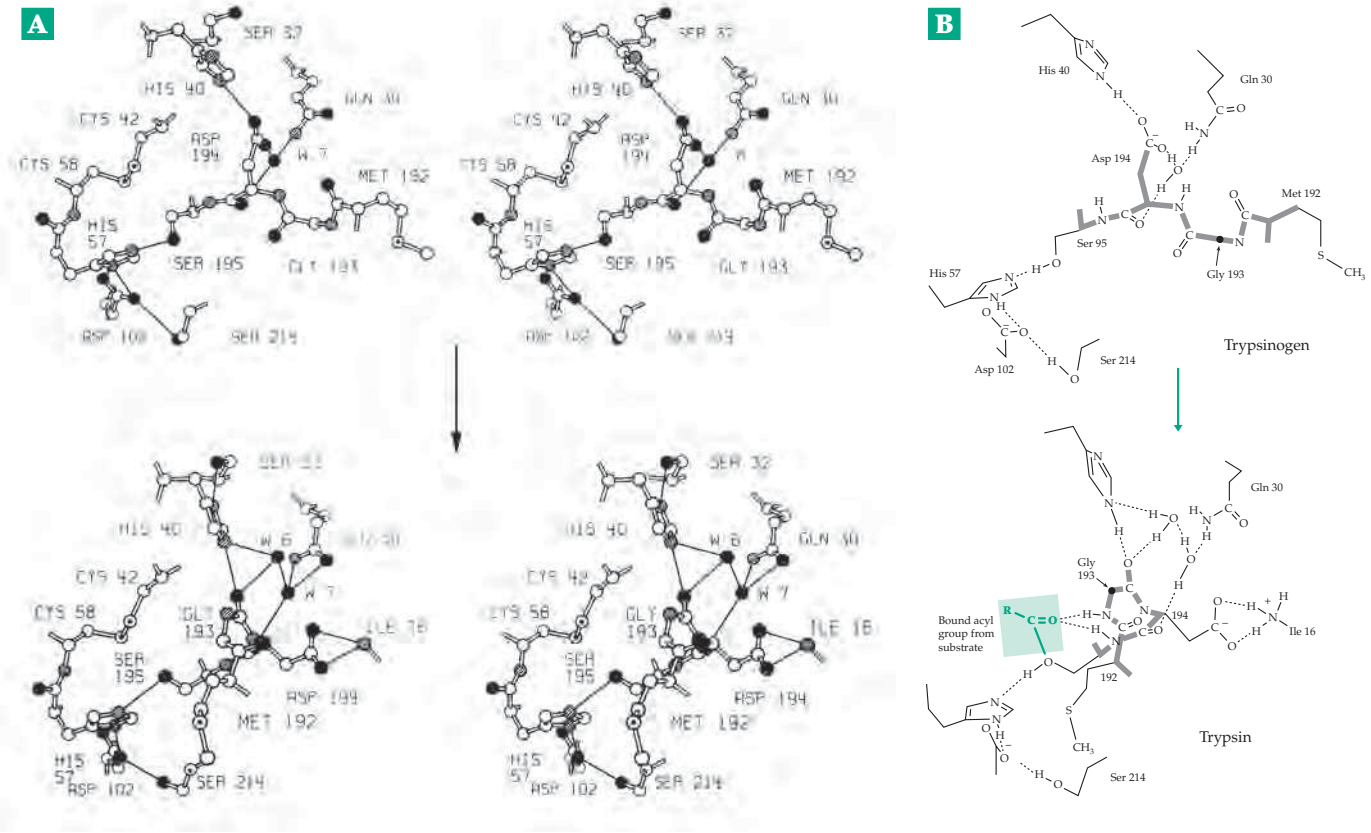


Figure 12-12 Formation of the oxyanion hole following cleavage of trypsinogen between Lys 15 and Ile 16. (A) Stereoscopic view. (B) Schematic representation. The newly created terminal $-NH_3^+$ of Ile 16 forms a hydrogen-bonded ion pair with the carboxylate of Asp 194. This breaks the hydrogen bond between Asp 194 and His 40 in trypsinogen, inducing the peptide segment 192–194 to shift from an extended conformation to a helical form in which the NH groups of Gly 193 and Ser 195 form the oxyanion hole. Notice that the positions and interactions of Asp 102, His 57, and Ser 195, the catalytic triad, are very little changed. From Birktoft *et al.*[270]

Stereoelectronic considerations. The amide group that is cleaved by a protease is a resonance hybrid of structures A and B of Fig. 12-13. The unshared pair of electrons on the nitrogen atom of structure A and the third unshared pair on the oxygen atoms of structure B (shaded orbitals) have been drawn in this figure in such a way that they are anti-periplanar to the entering serine oxygen. This is required in the transition state according to stereoelectronic theory. The newly created (shaded) electron pair on oxygen is one of those that hydrogen bonds to an NH group of the oxyanion hole. The tetrahedral intermediate has another unshared pair, which is not hydrogen bonded, and is antiperiplanar to the HN-R leaving group. Thus, it appears to be set up for easy elimination. Nevertheless, there is some doubt about the need for adherence to the stereoelectronic "rule" that two antiperiplanar lone pairs are necessary for elimination.

There is another complication. The leaving group –NH–R′ cannot be eliminated from the oxyanion until it is protonated, presumably by the imidazolium group of His 57. However, the proton on His 57 will be adjacent to the proton that is already on this nitrogen rather than to the unshared pair of electrons on the same nitrogen atom. A conformational change in which the tilt of the catalytic imidazole ring is altered (step *b′*, Fig. 12-11) or in which the ring rotates, may have to precede the protonation of the leaving group (step *c*, Fig. 12-11).[272–274b] This change may be assisted by the presence of positive charge on the protonated imidazole, but it still does not solve the problem. Bizzozero and Butler suggested that a rapid inversion of this chiral center on the –NH–R′ group may be required prior to protonation and elimination.[273] This would explain their observation that *N*-alkylated peptide linkages of otherwise fast substrates are not cleaved by chymotrypsin. A high-resolution structure determination demonstrated that a very slow elastase substrate that contains *N*-methylleucine at the cleavage site forms a normal ES complex.[275] However, inversion would be hindered by the *N*-methyl group. An alternative in inversion would be a torsional rearrangement of the substrate during binding.[276] The structure of an acyl-enzyme intermediate with elastase has been determined by crystallographic cryoenzymology. The intermediate was allowed to accumulate at –26°C, after which the temperature was lowered to –50°C and the structure determined. The structure shows the carbonyl group in the oxyanion hole as anticipated.[277]

pH dependence. A plot of k_{cat}/K_m for chymotrypsin is bell shaped with a maximum around pH 7.8 and pK_a values of 6.8 and 8.8. These represent pK_as of the free enzyme (Eq. 9-57). That of 6.8 has been shown to represent His 57. As the pH is lowered from the optimum the affinity for substrate falls off as His 57 becomes protonated. The high pK_a of 8.8 is thought to belong to the N-terminal amino group of Ile 16, a group that is generated during the conversion of the proenzyme to active enzyme.[278] The Ile 16 amino group forms an ion pair with Asp 194 (Fig. 12-12B) which is next to the serine at the active center. This salt linkage helps to hold the enzyme in the required conformation for reaction and its deprotonation at high pH causes a decrease in substrate affinity.[279]

The value of k_{cat} is also pH dependent and falls off at low pH around a pK_a value of from 6–7 depending upon the substrate. However, no higher pK_a affects k_{cat} in the experimentally accessible range. These results provide the basis for believing that an unprotonated His 57 is needed in the ES complex for catalysis to occur. Similar conclusions have been reached for other serine proteases.

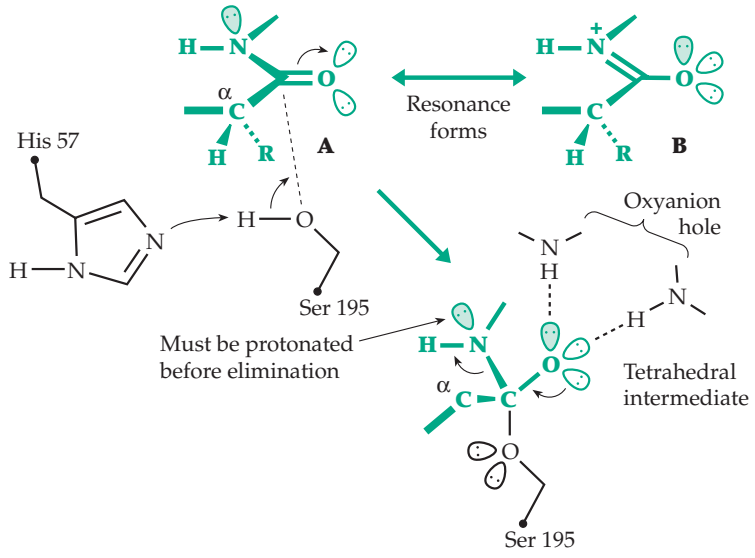
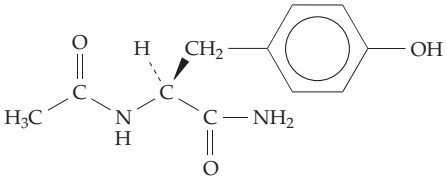


Figure 12-13 The stereochemistry of formation of a tetrahedral intermediate by a serine protease. The most probable orientation of groups as deduced by model building is shown. The shaded orbitals in A and B are antiperiplanar to the entering oxygen of Ser 195. See Polgár and Halász.[272]

Substrate specificity. Like most other enzymes, proteases display distinct preferences for certain substrates. These are often discussed using the nomenclature of Fig. 12-14. The substrate residue contributing the carbonyl of the amide group to be cleaved is designated P_1 and residues toward the N terminus as P_2, P_3, etc., as is shown in Fig. 12-14. Residues toward the C terminus from the peptide linkage to be cleaved are designated P_1', P_2', etc. Chymotrypsin acts

most rapidly if the P_1 residue is one of the aromatic amino acids.[280] Thus, the S_1 part of the substrate binding site must bind preferentially to large, flat aromatic rings. The crystal structure of chymotrypsin showed this site to be composed of nonpolar side chain groups. On the other hand, in trypsin the specificity portion of the S_1 site is a deep "specificity pocket" containing a fixed negative charge provided by the carboxylate side chain group of Asp 189. This explains why trypsin acts only upon peptide linkages containing the positively charged arginine or lysine residues in the P_1 position. In elastase the specificity pocket is partly filled by nonpolar side chains and the enzyme can accommodate only small P_1 side chains such as the methyl group of alanine. Replacement of Asp 189 of trypsin by lysine led to the predicted loss of specificity for basic side chains. However, the mutant enzyme did not become specific for negatively charged side chains.[281] This is presumably because the lysine $-NH_3^+$ group was not located in the same position as the Asp 189 carboxylate as a result of a different packing of side chains between native and mutant enzymes. Residues P_1' and P_2' also have major effects on substrate binding by serine proteases.[282–284] Now many mutant forms of subtilisin[225,285] and other serine proteases are being made and are yielding a more sophisticated understanding of the basis of the specificity of these enzymes. An important factor that has emerged is flexibility of surface loops in allowing an enzyme to adjust its structure to give a better fit to some substrates.[286,287] The specificity of the serine proteases is also being exploited in the design of specific inhibitors (Box 12-D).

Many serine proteases react with *p*-nitrophenylacetate to give acetyl enzymes. However, its rate of hydrolysis to give acetate is orders of magnitude slower than that of acyl-enzymes derived from small substrates such as the chymotrypsin substrate *N*-acetyltyrosine amide.



N-Acetyltyrosine amide

In addition to the specificity-determining P_1 aromatic side chain, the amide groups of this substrate can form specific hydrogen bonds to the protein (Fig. 12-10). These hydrogen bonds presumably help the enzyme to recognize the compound, which is bound with K_m of ~0.03 M and is hydrolyzed (with liberation of NH_3)[288,289] with k_{cat} ~0.17 s^{-1}.

What happens when the length of the substrate is extended in the direction of the N terminus? The tyrosyl residue in the foregoing compound may be designated P_1. For extended substrates which contain P_2, P_3, and additional residues (this includes most natural substrates) the K_m values decrease very little from that of short substrates despite the larger number of "subsites" to which the substrate is bound. However, the maximum velocity is often much greater for the extended substrates than for short ones. Thus, for *N*-acetyltyrosyl-glycine amide K_m is 0.017 M, only a little less than for *N*-acetyltyrosine amide, but k_{cat} is 7.5 s^{-1}, 440 times greater than for the shorter substrate.[229,288,289] Other examples have been tabulated by Fersht.[279]

These observations suggest that *the binding energy that would be expected to increase the tightness of binding is, instead, causing an increase in* V_{max}[279,290] that is, it is reducing the Gibbs energy of activation. How can this be? Imagine that as the extended substrate binds, for example, into the subsite S_2 (which binds residue P_2), it must compress a spring in the enzyme. Could not the compressed spring now provide a source of energy for assisting in peptide bond cleavage? If this is the case, we must ask "what are the springs?" Are there amide linkages of the peptide backbone that are distorted when the substrate hydrogen bonds into the site? How is the distortion transmitted to the active site and how does it stabilize the transition

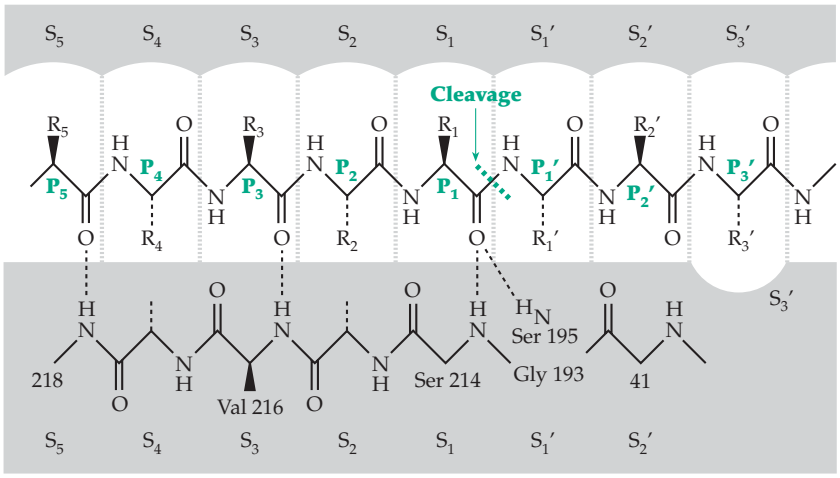


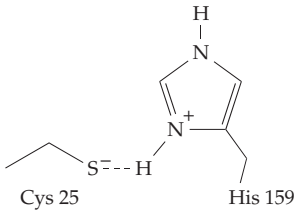
Figure 12-14 Standard nomenclature used to define the residues P_1, P_2 . . . toward the N terminus and P_1', P_2' . . . toward the C terminus of a peptide substrate for a protease. The corresponding subsites of the protease are designated S_1, S_2 . . . S_1', S_2'.

state? We are far from understanding the answers to these questions. However, it is of interest that one chain of H-bonds that passes through the amide between P_1 and P_2 also passes through a carbonyl group of a β bulge (Fig. 12-10). A second chain of H-bonds that connects to the same β bulge passes through the backbone amide of the active site Ser 195, across the interface between the two domains, and through the backbone of the second β cylinder.[85] Perhaps when substrate binds, the strengthening of the first H-bond chain weakens the second through competition at the β bulge, allowing some subtle rearrangement in the active site structure. We are talking here about the P_1–P_2 amide. What happens at P_2–P_3, and further along the chain in both directions? It is important to understand these effects; the phenomenon of increased reaction rate for longer, more specific substrates is observed with many proteases and other enzymes as well.

Another difference between small substrates and longer, more specific substrates has been found in studies of the effects of changing the solvent from pure H_2O to mixtures containing an increasing mole fraction of ^2H_2O. This is called a **proton inventory**.[291] For the serine proteases the rates are decreased as the ^2H content of the solvent increases, a fact that suggests that some step involving a proton transfer, for example, the deprotonation of Ser 195, is rate limiting. For simple substrates, the effect is directly proportional to the mole fraction of ^2H. However, for extended substrates, a quadratic dependence on the mole fraction of ^2H is observed. This suggests that a process involving synchronous transfer of two protons, as in the postulated charge relay system, may be more important in extended substrates than in simple ones and may account for the more rapid action on these substrates.[290,292] However, other interpretations of the data are possible, leaving this conclusion uncertain.[229,291]

2. The Cysteine Proteases (Thiol Proteases)

Papain from the papaya is one of a family of enzymes that includes **bromelain** of the pineapple, **ficin** of the fig, and **actinidin** of the kiwifruit.[293] Additional cysteine proteases[294] from the latex of the papaya tree *Carica papaya* are known as **caricain**, **chymopapain**,[294] and **glycyl endopeptidase**.[295] All are members of a large superfamily which includes at least 12 mammalian enzymes and many others from both eukaryotic and prokaryotic organisms.[296] All share with papain a characteristic structure which was determined by Drenth and coworkers in 1968.[297] The participating nucleophile in the active sites of these enzymes is an SH group, that of Cys 25 in papain. An adjacent imidazole from His 159 removes the proton from the SH group to form a thiolate-imidazolium pair.[298–300]



The close proximity of the imidazolium group greatly lowers the microscopic pK_a of the Cys 25 thiol group and the proximity of the resulting hydrogen-bonded S^- group greatly raises the microscopic pK_a of the imidazolium group.

Studies of the pH dependence of V_{max}/k_{cat} (Eq. 9-57) reveal a bell-shaped dependence on pH with pK_a values[301,302] of ~4 and ~9. However, the ion pair is formed at a pH below four with apparent pK_a values of 2.5, 2.9, and 3.3 for ficin, caricain, and papain, respectively.[302] These low values can be assigned principally to Cys 25 with only very small contributions from His 159 (see Eq. 6-75). A third pK_a, perhaps of a nearby carboxylate from Glu 50, affects the rate. For caricain the nearby Asp 158 (Fig. 12-15) has been implicated.[303,304]

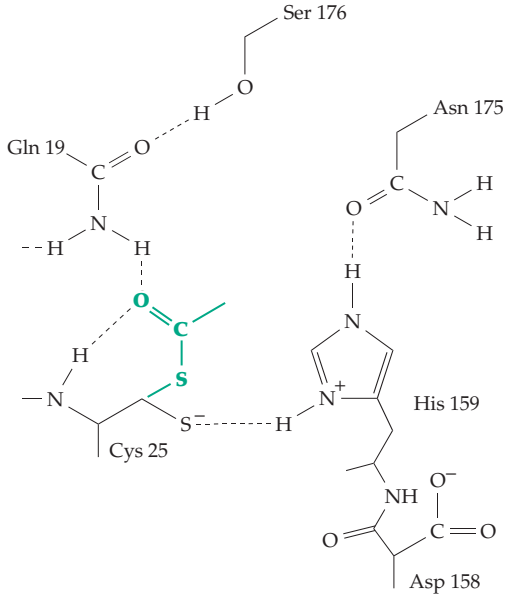


Figure 12-15 Schematic drawing of the active site of a cysteine protease of the papain family with a partial structure of an acyl-enzyme intermediate in green. The thiolate-imidazolium pair of Cys 25•His 159 lies deep in the substrate-binding cleft and bridges an interface between two major structural domains, just as the Ser•His pair does in serine proteases (Fig. 12-10). This may facilitate small conformational changes during the catalytic cycle. Asn 175 provides a polarizable acceptor for positive charge, helping to stabilize the preformed ion pair, and allows easy transfer of an imidazolium proton to the product of substrate cleavage. The peptide NH of Cys 25 and the side chain of Gln 19 form an oxyanion hole.

As shown in Fig. 12-15, the side chain of Asn 175 provides papain with a third member of a catalytic triad analogous to that of serine proteases.[305] Glutamine 19, together with the peptide backbone NH of Cys 25, provides an oxyanion hole.[306–308] Many studies, including structure determinations on bound aldehyde and other inhibitors, and observation by ^{13}C NMR[309] indicate that thiol proteases act by addition of the thiolate anion to the peptide carbonyl of the P_1 residue, just as in step *b* of Fig. 12-11 (see also Fig. 12-12) for serine proteases.[308,310] However, alternative sequences of proton transfer have been suggested.[311] A possible role for a strong hydrogen bond has also been proposed.[310] For a detailed discussion see Brocklehurst *et al.*[312]

Most of the lysosomal proteases called **cathepsins** are small 20- to 40-kDa glycoproteins found in all animal tissues.[313] Most are cysteine proteases which function best and are most stable in the low pH reducing environment of lysosomes. They resemble papain in size, amino acid sequence, and active site structures. Papain is nonspecific but most cathepsins have definite substrate preferences. Cathepsin B is the most abundant. There are smaller amounts of related cathepsins H (an aminopeptidase)[314] and L[315] and still less of cathepsins C, K, and others. Cathepsin B is both an endopeptidase and an exopeptidase.[316] It acts on peptides with arginine at either P_1 or P_2 but also accepts bulky hydrophobic residues in P_1 and prefers tyrosine at P_3.[317] Cathepsin S is less stable at higher pH than other cathepsins and has a more limited tissue distribution, being especially active in the immune system.[318,319]

Cathepsin K is especially abundant in the bone resorbing osteoclasts (Chapter 8). It is essential to normal bone structure and its absence is associated with the rare hereditary disease pycnodysostosis (pycno) which causes short stature, fragile bones, and skull and skeletal deformities.[320] It may also play a role in the very common bone condition osteoporosis. Cathepsin C is also called **dipeptidyl peptidase**. It removes N-terminal dipeptides from many intracellular proteins activating many enzymes, including some other cathepsins.[321,322] A **prohormone thiol protease** cleaves peptide chains between pairs of basic residues, e.g., in the brain peptide precursor proenkephalin (Chapter 30), and also on the N-terminal side of arginine residues.[323] **Pyroglutamate aminopeptidase** removes pyroglutamyl (5-oxoprolyl) groups from amino termini of some peptides and proteins (see Fig. 2-4).[324] Another cysteine protease cleaves **isopeptide** linkages such as those formed by transglutaminase or those involving ubiquitin (Box 10-C).[325] Another cysteine protease present in animal tissues was recognized by its ability to hydrolyze the anticancer drug bleomycin. This **bleomycin hydrolase** is a hexamer with a central channel lined with papainlike active sites as in the proteasome structure (Box 7-A)[326] The enzyme also binds to DNA.[327] Unfortunately, cancer tissues often contain high levels of the enzyme, whereas it is low in skin and lung tissues, which are damaged by bleomycin.

The Ca^{2+}-dependent neutral proteases called **calpains** are found within the cells of higher animals. The 705-residue multidomain peptide chain of a chicken calpain contains a papain-like domain as well as a calmodulin-like domain.[328] It presumably arose from fusion of the genes of these proteins. At least six calpains with similar properties are known.[329] Some have a preference for myofibrillar proteins or neurofilaments.[330] They presumably function in normal turnover of these proteins and may play a role in numerous calcium-activated cellular processes.[331–332a]

A group of **cysteinyl aspartate-specific proteases (caspases)** play an essential role in programmed cell death (apoptosis).[333–335] Recall that nematodes are cell-constant organisms. For maturation of *Caenorhabditis elegans* 131 programmed cell deaths must occur at specific stages of development. An essential gene for this process was identified and named ***CED-3***. Deletion of this gene completely blocked the death of these cells. *CED*-3 encodes a cysteine protease that is highly homologous to the mammalian **interleukin-1β-converting enzyme** (**ICE** or caspase 1) which cleaves the 31-kDa **pro-interleukin-1β** to form the active 175 kDa species of this cytokine (Chapter 30). At least ten caspases are known and many observations have confirmed their role in apoptosis (see also Chapter 32). In caspases 1 and 2 the side chains of Cys 285 and His 237 form the catalytic dyad and peptide NH groups of Cys 285 and Gly 238 form the oxyanion hole.[333]

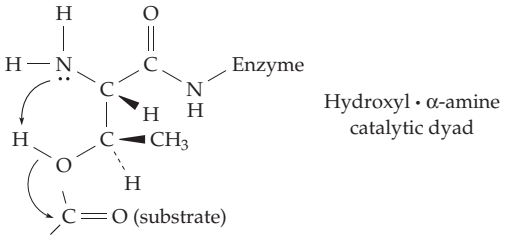
Parasites often use proteases in attacks on their hosts. The cysteine protease **cruzain** is secreted by the trypanosomes that cause Chagas' disease and is essential to their survival within the human body.[336,337] Cruzain is consequently an attractive target for development of drugs for treating this major disease.

Among cysteine proteases of bacteria is a papain-like enzyme from *Clostridium histolyticum* with a specificity similar to that of trypsin.[338] The anaerobic *Porphyromonas gingivalis*, which is implicated in periodental disease, produces both arginine- and lysine-specific cysteine proteases designated **gingipains**.[339,339a] Some virally encoded cysteine proteases, including one from the polio virus, have trypsin-like sequences with the serine of the catalytic triad replaced by cysteine.[340,341] A human adenovirus protease also has a Cys•His•Glu triad but a totally different protein fold.[342]

Zymogens of cysteine proteases usually have a long terminal extension which is removed, sometimes by autoactivation. Propapain has a 107-residue extension.[343] The 322-residue cathepsin B carries an unusually short 62-residue extension in its proenzyme form.[315,343,344] In every case the N-terminal extension folds into a domain, one of whose functions is to block the active site cleft.

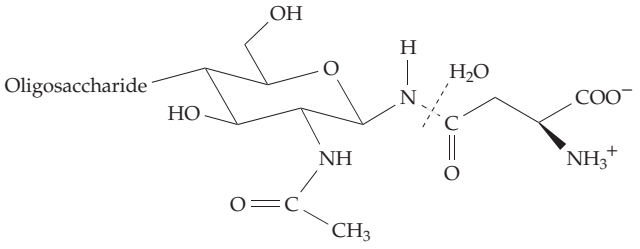
3. N-terminal Nucleophile Hydrolases and Related Enzymes

The most recently discovered group of proteases are the N-terminal threonine hydrolases of the **multicatalytic protease complex** (MPC) of proteasomes. The enzymes are arranged in a regular array inside proteasomal compartments as shown in Box 7-A. The active site is a catalytic dyad formed from the amino group at the N terminus of the β subunits.[345–346a]

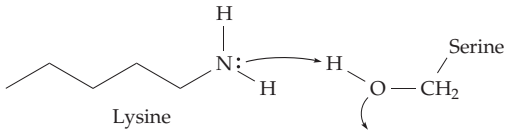


Proteasomes of *Thermoplasma* contain a single type of β subunit but eukaryotic proteasomes contain subunits with at least three distinct substrate preferences.[347–349c] They all appear to use the same hydrolytic mechanism but in their substrate specificities they are chymotrypsin-like, peptidylglutamyl-peptide hydrolyzing, branched chain amino acid preferring, and small neutral amino acid preferring based on the P_1 amino acid residue. In the spleen some of the β subunits of the proteasomes appear to have been replaced by proteins encoded by the major histocompatibility complex of the immune system (Chapter 31).[347] This may alter the properties of the proteasome to favor their function in antigen processing. Proteasomes are also ATP- and ubiquitin-dependent, as discussed in Section 6.

The enzyme glucosylasparaginase (aspartylglucosaminidase) is one of a group of other enzymes that use N-terminal threonyl groups as catalytic dyads.[346,350–353] It removes N-linked glycosyl groups from asparagine side chains of proteolytically degraded proteins and, as indicated on the accompanying structural formula, releases free aspartate and a 1-amino-*N*- acetylglucosamine-containing oligosaccharide. The amino group is then released as NH_3 from the product by acid catalysis in the lysosome.[350]

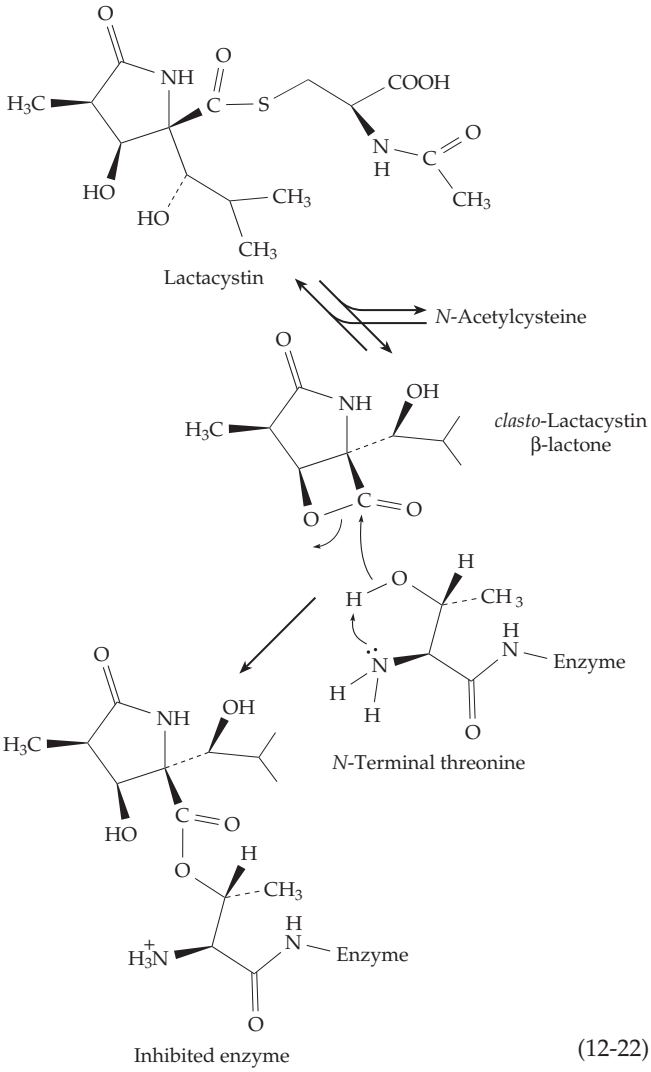


Glutamine PRPP amidotransferase (Fig. 25-15) and a penicillin acylase have similar active sites and overall structures.[354] The $-NH_2$ group is basic enough in the environment of the protein to remove the proton from the threonine –OH group, activating it as indicated below. Several serine proteases use an $-NH_2$ group of a lysine to form a serine•lysine dyad.[346]



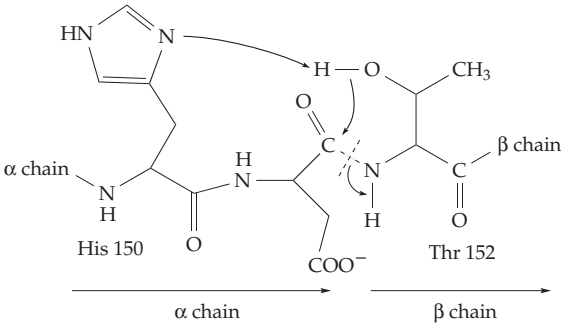
Among these are the well-known *E. coli* **leader peptidase**[355,356] and other signal peptidases.[357] These are integral membrane proteins that cleave N-terminal signal sequences from proteins incorporated into plasma membranes. Another enzyme of this class is the **lexA** repressor and protease discussed in Chapter 28.

A specific inhibitor of the major proteasomal activities is **lactacystin**, a compound formed by *Streptomyces*. Lactacystin is converted reversibly, by loss of *N*-acetylcysteine, into a β-lactone known as *clasto*-lactacystin. The N-terminal amino group attacks the reactive four-membered ring of the lactone (Eq. 12-22).[358,359]



(12-22)

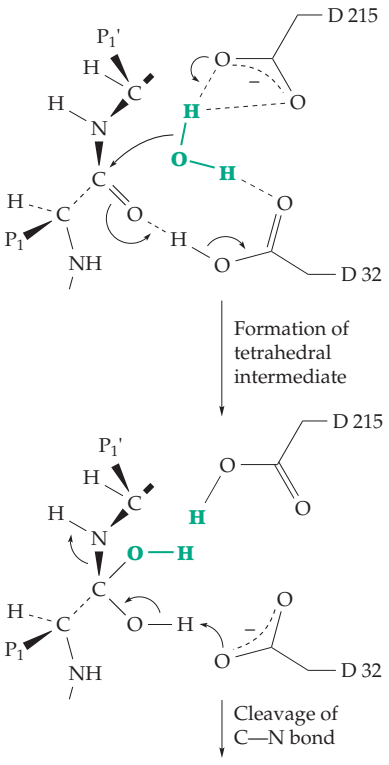
The active sites of the N-terminal nucleophile hydrolases are generated *autocatalytically*.[360–362] A single peptide chain is cleaved to form α and β chains as in the following diagram. An activating nucleophile such as histidine removes a proton from an adjacent threonine –OH and the resulting alkoxide ion attacks the adjacent peptide linkage, presumably via a tetrahedral intermediate, to form an ester linkage. Compare this sequence with reactions in Box 12-A and Eq. 14-41. Hydrolysis generates the N-terminal threonine as indicated:



4. The Aspartic Proteases

A fourth large group of protein-hydrolyzing enzymes consists of **pepsin** of the stomach[363] and related enzymes.[364] Each of these ~320-residue proteins is folded into two domains which associate with a pseudo-twofold axis of symmetry that passes through the active site.[365] A second human gastric aspartic proteinase is **gastricsin**.[365] The related **chymosin** (rennin),[366] which is obtained from the fourth stomach of the calf, causes a rapid clotting of milk and is widely used in manufacture of cheese. Pepsin has a broad specificity but cleaves preferentially between pairs of hydrophobic residues, converting proteins into soluble fragments. It is unusual in being able to cleave X-Pro peptide bonds.[367] Chymosin has a more restricted specificity, cutting the κ-casein of milk between a Phe–Met bond. This decreases the stability of the milk micelles, inducing clotting.[366] The serum protein **renin** (distinct from rennin), the lysosomal **cathepsins D** and **E**,[368–370] an **aspartyl aminopeptidase**,[371] and various fungal proteases are also closely related.[372–375] Renin,[376,377] which is synthesized largely in the kidneys, is involved in blood pressure regulation (Box 22-D). More distantly related aspartic proteases are encoded by retroviruses.

The pepsin family is most active in the low pH range 1–5. All of the enzymes contain two especially reactive aspartate carboxyl groups.[378] One of them (Asp 215 in pepsin) reacts with site-directed diazonium compounds and the other (Asp 32) with site-directed epoxides.[379] It is attractive to think that one of these carboxyl groups might be the nucleophile in a double displacement mechanism. The second carboxyl could then be the proton donor to the cleaving group. The acyl-enzyme would be an acid anhydride. However, X-ray studies on pepsin[363,380] and on related fungal enzymes such as **penicillopepsin**[381] and others[373,375] suggested a different possibility: A water molecule, hydrogen bonded to one of the active site Asp carboxylates or bridging between them, becomes the nucleophile. A proton, held by the carboxylate pair, protonates the substrate carbonyl to facilitate nucleophilic attack.[381] This is illustrated in Eq. 12-23 but without detail. Several intermediate sequences of reaction steps are possible.[382] At the beginning of the sequence one of the two symmetrically placed carboxylates is protonated while the other is not. Also notice that although the active site is symmetric, the substrate is bound asymmetrically as determined by its hydrogen bonding into an extended binding site.



(12-23)

A characteristic feature of catalysis by the aspartic proteases is a tendency, with certain substrates, to catalyze transpeptidation reactions of the following type.

Acetyl-Phe-Tyr + Acetyl-Phe* → Acetyl-Phe*-Tyr

(12-24)

Here Phe* is an isotopically labeled residue. Although such reactions suggested the possibility of some kind of activated amino group on the tyrosine that is cut off in the initial cleavage of the unlabeled substrate, it is more likely that the released tyrosine stays in the active site,[383] while the acetyl-Phe fragment exchanges with acetyl*-Phe.

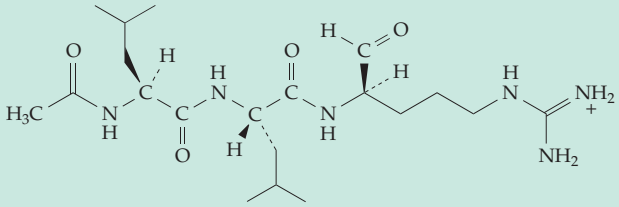
BOX 12-C SYNTHETIC PROTEASE INHIBITORS

One of the goals of synthetic medicinal chemistry is to design potent inhibitors of clinically important proteases. Elastase inhibitors may be useful for treatment of emphysema, pancreatitis, and arthritis,[a,b] while inhibitors of the angiotensinogen-converting enzyme or of renin (Box 22-D) can help control blood pressure. Inhibition of thrombin, factor Xa, or other blood clotting factors (Fig. 12-17) may prevent blood clots and inhibition of the cytosolic tryptase may provide a new treatment for asthma. Inhibition of the cysteine protease cathepsin K may help combat osteoporosis and inhibition of cysteine proteases of corona viruses may fight the common cold. Cysteine proteases of schistosomes are also targets for protease inhibitors.[c]

Many chemical approaches are used in designing inhibitors. Often, a naturally occurring inhibitor provides a starting point. The availability of high-resolution structures of the target enzymes and of various enzyme-inhibitor complexes assists in the rational design of tight-binding inhibitors. Use of combinatorial chemistry (Chapter 3)[d] is another source of potential inhibitors. To be of practical use in medicine many criteria of stability, solubility, and low toxicity must be met. While most inhibitors are disappointing as drugs, their use in laboratory experimentation has clarified a great deal of biochemistry.

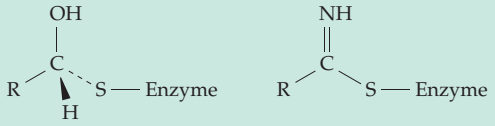
A straightforward approach is to hunt for short polypeptides that meet the specificity requirement of an enzyme but which, because of peculiarities of the sequence, are acted upon very slowly. Such a peptide may contain unusual or chemically modified amino acids. For example, the peptide **Thr-Pro-nVal-NMeLeu-Tyr-Thr** (nVal = norvaline; NMeLeu = *N*-methylleucine) is a very slow elastase substrate whose binding can be studied by X-ray diffraction and NMR spectroscopy.[e] Thiol proteases are inhibited by **succinyl-Gln-Val-Val-Ala-Ala-*p*-nitroanilide**, which includes a sequence common to a number of naturally occurring peptide inhibitors called **cystatins**.[f] They are found in various animal tissues where they inhibit cysteine proteases.

A group of inhibitors such as **leupeptin** have C-terminal aldehyde groups. Small oligopeptides with this structure and with appropriate specificity-determining side chains form tetrahedral hemiacetals, which may mimic transition state structures, at the active sites of the target enzymes.[g,h]



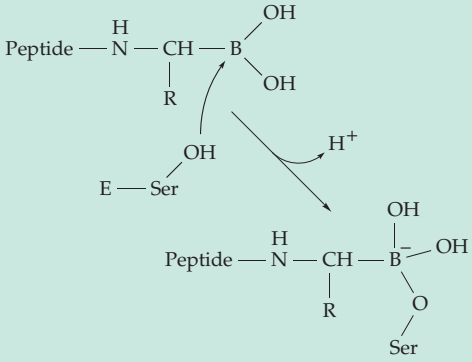
Leupeptin (*Actinomyces*)

Leupeptin is a slow, tight-binding inhibitor of trypsin. Some peptide aldehydes are potent, reversible inhibitors of cysteine proteases forming hemithioacetals with the active site cysteine.[i] Similarly, peptide nitriles form thioimidate adducts.[h]



Hemithioacetal Thioimidate

The peptide **boronic acids** form adducts with the active site serine of serine proteases.[j–l]



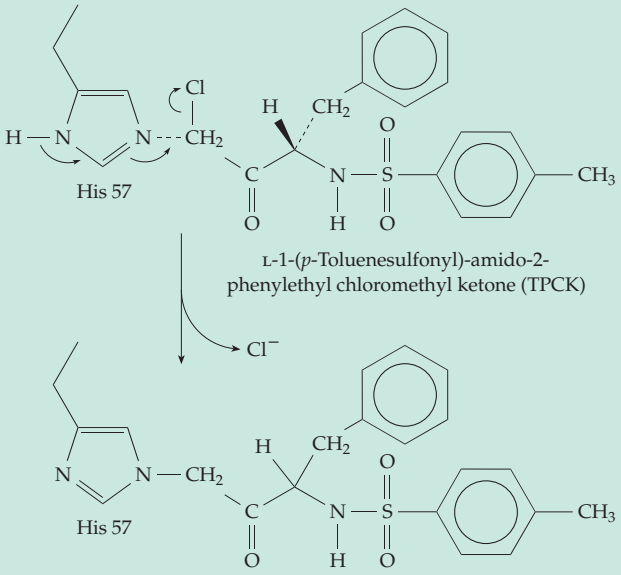
Adduct

However, both X-ray crystallography[m] and ^{11}B NMR[n] have shown that imidazole of the catalytic triad may also add to the boronic acid and that a tetrahedral adduct with both the serine oxygen and histidine nitrogen covalently bonded to boron can also be formed.[m] Thus, in reversibly inhibited enzymes a mixture of different chemical species may exist. Inhibitors can be designed to bind more tightly by providing additional bonding opportunities. For example, a suitably placed cyano group on a phenylalanine ring in the P_1 position of thrombin

BOX 12-C (continued)

or other serine proteases can form a hydrogen bond to the peptide NH of Gly 219 (see Fig. 12-9).[o]

Numerous synthetic active-site directed or enzyme-activated irreversible inhibitors have been designed.[p] For example, the following chloroketone inhibits chymotrypsin but does not act on trypsin. The corresponding structure with a lysine side chain (TLCK) inhibits only trypsin. These **affinity labeling compounds** initially bind noncovalently at the active site.

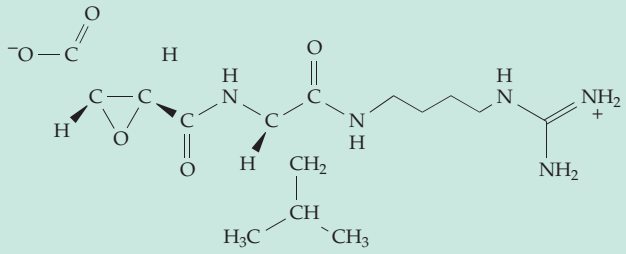


However, α-chloroketones are powerful alkylating agents and the bound inhibitor attacks His 57 of the catalytic triad system. The reaction is probably more complex than is indicated in the foregoing equation and may involve an epoxy ether intermediate.[q] Many other peptide chloromethyl ketone inhibitors have been devised.[q,r]

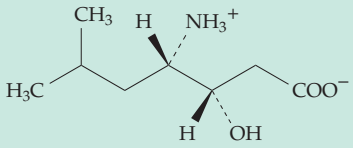
Isocoumarins inactivate many serine proteases. For example, 7-amino-4-chloro-3-methoxyisocoumarin acylates serine 195 of elastases as follows.[s]

Many other enzyme-activated inhibitors are being developed.[c,d,t]

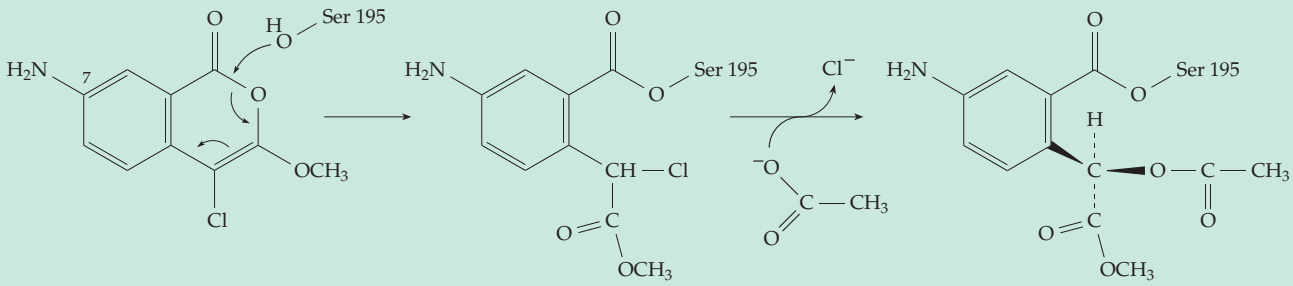
Epoxy groups, such as that of E-64, a compound isolated from the culture medium of a species of *Aspergillus*, react irreversibly with the active site thiolate group of cysteine proteases.[i,u,v] Related epoxides may become useful medications against abnormal cathepsin levels.



All of the aspartic proteases are inhibited by **pepstatin**, a peptide produced by some species of *Actinomyces* and which contains two residues of the unusual amino acid **statine** (**sta**).[w] Pepstatin has the sequence Isovaleryl-L-Val-L-Val-Sta-L-Ala-Sta.



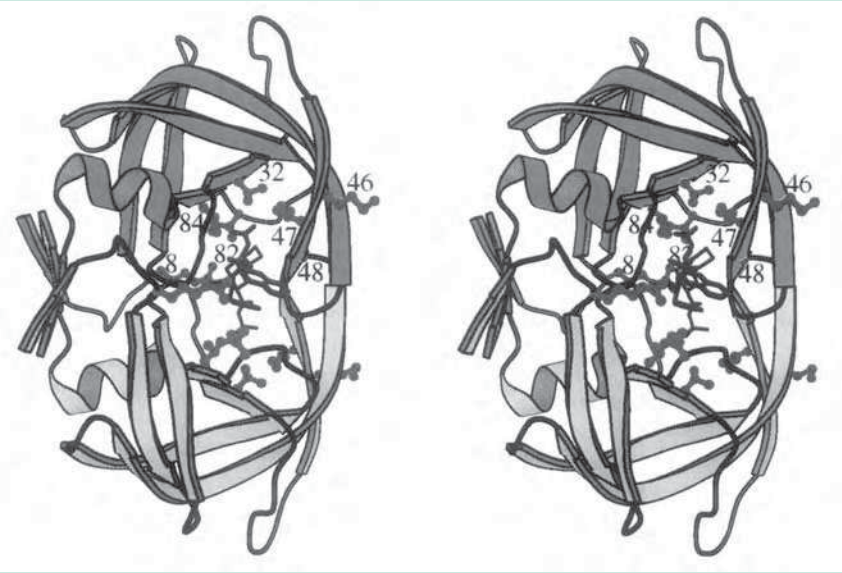
The statine residue mimics the noncovalently bonded tetrahedral intermediate, permitting formation of a very tight complex. Pepstatin is a poor inhibitor of human renin but its existence has inspired the synthesis of numerous related compounds, some of which are effective renin inhibitors.[x,y] Some of these inhibitors use the human angiotensinogen sequence with a secondary alcohol group mimicking the tetrahedral intermediate.[x,z]



BOX 12-C SYNTHETIC PROTEASE INHIBITORS (continued)

These aspartic protease inhibitors are also "lead compounds" in the development of inhibitors of HIV protease.[aa–cc] As in statine-based inhibitors, the site of occupancy by the catalytic H_2O (green in Eq. 12-23) is occupied in the inhibitor by something that mimics a tetrahedral intermediate with –CHOH–, $-PO_2H-$, etc.[bb] Tremendous efforts are being expended in designing these inhibitors and considerable success has been achieved. However, the rapid development of mutant strains of the virus with drug-resistant proteases presents a major challenge.[cc,dd]

Mercaptans of suitable structure bind tightly to Zn^{2+} in the active sites of metalloproteases. For example, **captopril**[ee] is a tight-binding competitive inhibitor of the angiotensinogen-converting enzyme that is effective in lowering blood pressure and the first of many related inhibitors.[ff] As mentioned in the text, a variety of inhibitors that mimic the geometry of a tetrahedral intermediate or transition state are also potent inhibitors of metalloproteases.



Stereoscopic ribbon structure of the HIV-1 protease with the synthetic inhibitor Sequinivir[cc] bound in the active site. One of the two identical subunits (top) is shaded darker than the second (bottom). When mutated, the amino acid side chains shown in ball-and-stick form with residue numbers shown for the top subunit led to drug-resistant viruses. Courtesy of Alex Wlodawer, National Cancer Institute.[cc]

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Pepsin is secreted as the inactive pepsinogen, which is activated by H^+ ions at a pH below 5. Determination of its crystal structure revealed that in the proenzyme the N-terminal 44-residue peptide segment lies across the active site, blocking it.[384] At low pH the salt bridges that stabilize the proenzyme are disrupted and the active site is opened up to substrates.

While the cellular aspartate proteases are over 300 residues in length, the retroviral proteases are less than one-half this size.[385–388] That of the human HIV-I protease contains only 99 residues. These enzymes are cut from a polyprotein (encoded by the viral ***gag*** and ***pol*** genes (Fig. 28-26). The *pol* gene encodes four other essential enzymes as well, and these are cut apart at eight different sites by action of the protease.[388] Despite its small size, it displays sequence homologies with the larger cellular aspartate proteases and has a related three-dimensional structure. Each chain has only one active site aspartate; the functional enzymes are dimers and the catalytic mechanism appears to be similar to that of pepsin.[388–392] A great deal of effort is being devoted to designing synthetic HIV protease inhibitors, which are used in the treatment of AIDS (Box 12-C).

5. Metalloproteases

The pancreatic **carboxypeptidases** are characterized by the presence of one firmly bound **zinc ion** in each molecule. The Zn^{2+} can be removed and can be replaced by other metal ions such as Co^{2+} and Ni^{2+}, in some cases with reconstitution of catalytic activity. The human pancreas synthesizes and secretes proenzymes for two forms of carboxypeptidase A, with a preference for C-terminal hydrophobic residues, as well as carboxypeptidases B, which prefer C-terminal basic residues. Additional A and B forms[393] as well as more specialized nondigestive carboxypeptidases are also known. In eukaryotic cells the latter participate in processing of proteins. Following removal of N-terminal signal sequences, processing often continues with removal of basic residues from the C termini by **carboxypeptidases N**, **H** (also called E or enkephalin convertase),[394] and **M**, all of which are metalloenzymes.[395,396] Carboxypeptidase N removes C-terminal arginines from many biologically important peptides. It also circulates in the plasma and protects the body by inactivating such potent inflammatory peptides as the kinins and anaphylotoxins.[397] Carboxypeptidase H is located in secretory granules, while carboxypeptidase M is membrane associated.[396] Dipeptidyl carboxypeptidase (**angiotensin-converting enzyme**) removes the C-terminal Pro–Phe dipeptide from angiotensinogen to generate the potent pressor agent angiotensin I (Box 22-D) and cleaves dipeptides from many other substrates as well.[398] A **D-alanyl-D-alanyl carboxypeptidase** cleaves D-alanine from the ends of cell wall peptides (Chapter 20).[399]

Another well-known zinc-containing enzyme is **thermolysin**, a nonspecific *endopeptidase* widely used in laboratories. Produced by a themophilic bacterium, it is unusually resistant to heat. It contains four bound calcium ions in addition to the active site zinc.[400–402] The active site structure resembles that of pancreatic carboxypeptidase A and the two enzymes appear to act by similar mechanisms.[401,403,404] The mammalian zinc endopeptidase **neprilysin**, an integral membrane protein involved in inactivation of enkephalins and other signaling peptides, also resembles thermolysin.[405] A related neutral endopeptidase is the product of a gene called *PEX* (phosphate-regulating gene with homologies to endopeptidases on the X chromosome). The absence of the *PEX* gene product causes **X-linked hypophosphatemic rickets** which leads to excessive loss of phosphate from the body with defective mineralization of bone.[406] The **mitochondrial processing peptidase**, which removes signal sequences from the N termini of mitochondrial proteins, also contains Zn^{2+} at its active site.[407,408] However, it is an αβ heterodimer and a member of an additional family of enzymes, one of which includes human insulin-degrading enzyme.

In both carboxypeptidase A and thermolysin the active site Zn^{2+} is chelated by two imidazole groups and a glutamate side chain (Fig. 12-16). In carboxypeptidase A, Arg 145, Tyr 248, and perhaps Arg 127 form hydrogen bonds to the substrate. A water molecule is also bound to the Zn^{2+} ion. The presence of the positively charged side chain of Arg 145 and of a hydrophobic pocket accounts for the preference of the enzyme for C-terminal amino acids with bulky, nonpolar side chains. The Zn^{2+} in thermolysin is also bound to two imidazole groups and that in D-alanyl-D-alanyl carboxypeptidase to three.

The presence of a zinc ion in the metalloproteases immediately suggested a role in catalysis. Unlike protons, which have a weak affinity for the oxygen of an amide carbonyl group, a metal ion can form a strong complex. If held in position by other ligands from the protein, a properly placed zinc ion might be expected to greatly enhance the electrophilic nature of the carbon atom of the C=O group. However, it has been difficult to establish the exact mechanism of action.[410] Carboxypeptidase A cleaves both peptides and ester substrates. For peptides, K_m is the same for various metals while k_{cat} changes, but the converse is true for ester substrates.[411] From its position Glu 270 (Glu 143 in thermolysin) seems to be the logical nucleophile to attack the substrate to form an acyl-enzyme intermediate, an anhydride. Using the following specific *ester* substrate, Makinen *et al.* showed that at very low temperatures of −40° to −60°C, in solvents such as 50:50 ethylene glycol:water, an acyl-enzyme intermediate can be detected spectroscopically. It could even be separated from free enzyme[412] by gel

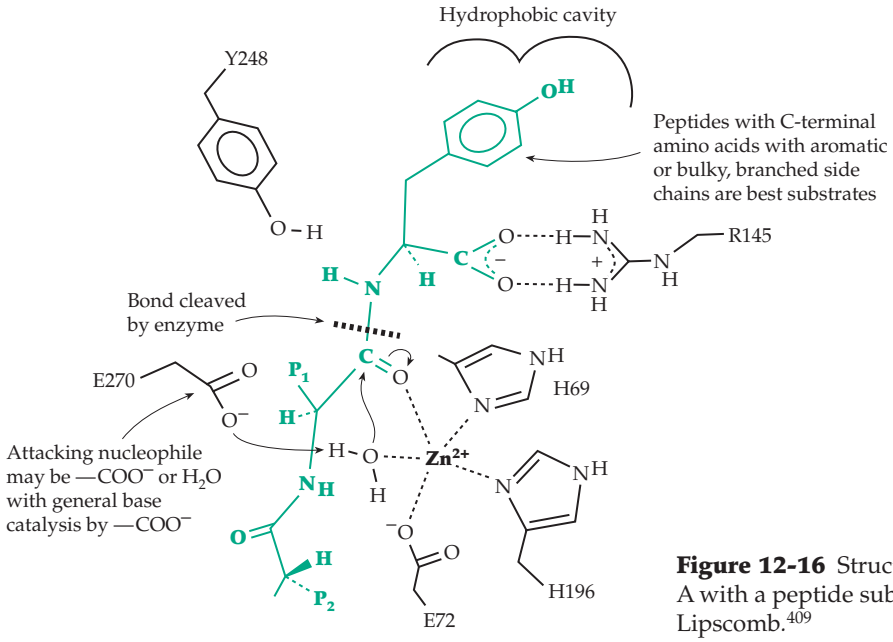
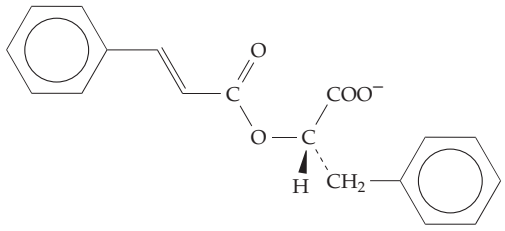


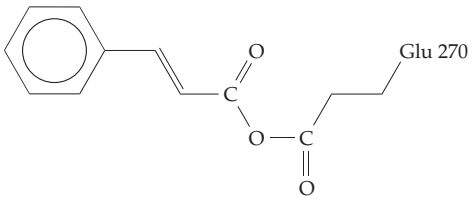
Figure 12-16 Structure of the active site of carboxypeptidase A with a peptide substrate present. See Christianson and Lipscomb.[409]

filtration at −60°C and its cyanoborohydride reduction product was characterized.[413]



O-(*trans*-*p*-cinnamoyl)-L-β-phenyllactate

The intermediate appears to be the acid anhydride formed by Glu-270.[414,415] The conformation of the intermediate was deduced by ENDOR spectroscopy and its formation and reaction interpreted according to stereoelectronic principles.[416]



If the anhydride mechanism is correct, the water molecule bound to the Zn^{2+} probably provides an HO^- ion necessary for the hydrolysis of the intermediate anhydride.[417]

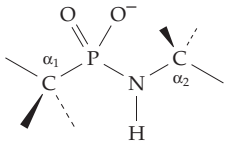
Although these results seem convincing there are objections.[410] The mechanism deduced for hydrolysis of an ester may be different than that for a peptide.[418] Furthermore, many observations favor an alternative mechanism. A hydroxide ion derived from a water molecule bound to the zinc ion may be the initial attacking nucleophile.[404,419–422] Both X-ray crystallographic studies and EPR investigations[403,414] show that the zinc in carboxypeptidases can coordinate at least five surrounding atoms. As shown in Fig. 12-16, the Zn^{2+} could hold the attacking water molecule and, at the same time, provide the positive charge for stabilizing the resulting tetrahedral intermediate. Glu 270 (or in thermolysin His 231) acts as a base to deprotonate the bound H_2O. Mock and Stanford argue that the H_2O is probably displaced when the peptide carbonyl binds and that the H_2O is then deprotonated and adds to the polarized carbonyl.[404]

The pH dependence of the action of carboxypeptidase A is determined by pK_a values of ~6 and ~9.5 for the free enzyme[422] and of ~6.4 and ~9 for k_{cat}.[420] For thermolysin the values for k_{cat} are ~5 and ~8.[404] Assignment of pK_a values has been controversial. They may all be composites of two or more microscopic constants but probably, at least for carboxypeptidase, the low pK_a is largely that of Glu 270 while the high one represents largely the dissociation of a proton from the zinc-bound H_2O.

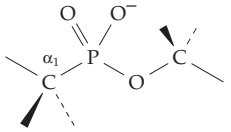
Earlier studies of carboxypeptidase had indicated that Tyr 248 moves its position dramatically upon substrate binding, and it was suggested that its phenolic –OH group protonates the leaving group in the acylation step. However, a mutant in which Tyr 248 was replaced by phenylalanine still functions well.[423]

Phosphonamidates,[424,425] phosphonate esters, and oxo-methylene substrate analogs, which presumably mimic the geometry of the tetrahedral intermediate or transition state of the intermediate or transition state

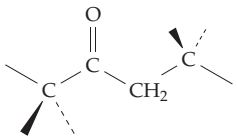
of the catalytic cycle, are effective inhibitors of zinc proteases. X-ray studies of complexes of such inhibitors with both thermolysin and carboxypeptidase A support the suggestion that the Zn^{2+} binds the carbonyl oxygen of the amide bond that is to be cleaved in a



Phosphonamidate



Phosphonate ester



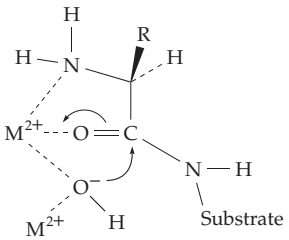
Oxo-methylene analog

substrate and that a glutamate side chain could activate a Zn^{2+}-bound water to form an attacking nucleophile. A similar conclusion was reached from the structure of a thermolysin–product complex.[426]

Another large group of zinc proteases are the **matrix metalloproteases** which act on proteins of the extracellular matrix, such as collagen, proteoglycans, and fibronectin.[427–429c] These enzymes have been classified as **collagenases**, **gelatinases** (which act on denatured collagen), **stromelysins** (which are activated in response to inflammatory stimuli),[430–432] and a membrane type. The group also includes the digestive enzyme **astacin**, from the crayfish,[433] and metalloproteases from snake venoms.[429,434] The matrix metalloproteases are essential to the remodeling of the extracellular matrix that occurs during wound healing, tissue growth, differentiation, and cell death. An example of tissue remodeling is the development of dental enamel (Box 8-G). The proteinaceous matrix formed initially must be digested away, perhaps by the metalloprotease **enamelysin**, and replaced by the dense mineral of enamel.[435] Excessive secretion of collagenase by fibroblasts is observed in **rheumatoid arthritis** and other inflammatory conditions.

At least 64 different matrix metalloproteins are known.[427] Each enzyme consists of three domains. An 80- to 90-residue N-terminal propeptide domain contains a cysteine whose $-S^-$ group binds to the active site zinc, screening it from potential substrates. The central catalytic domain is followed by a hinge region and a C-terminal domain that resembles the serum iron binding and transporting **hemopexin**.[427,436] The mechanism of action is probably similar to that of thermolysin.[430]

Many aminopeptidases are metalloenzymes.[437] Most studied is the cytosolic **leucine aminopeptidase** which acts rapidly on N-terminal leucine and removes other amino acids more slowly. Each of the subunits of the hexameric enzyme contains *two* divalent metal ions, one of which must be Zn^{2+} or Co^{2+}.[438,439] A methionine aminopeptidase from *E. coli* contains two Co^{2+} ions[440,441] and a proline-specific aminopeptidase from the same bacterium two Mn^{2+}.[442] In all of these enzymes the metal ions are present as dimetal pairs similar to those observed in phosphatases and discussed in Section D,4 and to the Fe–Fe pairs of hemerythrin and other diiron proteins (Fig. 16-20). A hydroxide ion that bridges the metal ions may serve as the nucleophile in the aminopeptidases.[438] A bound bicarbonate ion may assist.[438a]



A metalloenzyme **peptide deformylase** removes the formyl groups from the N termini of bacterial proteins. Although the active site is similar to that of thermolysin,[443] the Zn^{2+} form of peptide deformylase is unstable. Both Ni^{2+} and Fe^{2+} form active, stable enzymes.[444,445]

6. ATP-Dependent Proteases

Much of metabolism is driven by the Gibbs energy of hydrolysis of ATP so it shouldn't be surprising that ATP is sometimes rather directly involved in hydrolytic degradation of polypeptide chains. Much of protein processing occurs in the endoplasmic reticulum,[446] which also assists in sorting unneeded and defective proteins for degradation in the ubiquitin–proteasome system. Proteasomal degradation occurs in the cytosol, in the nucleus, and along the ER. However, the predominant location is the *centrosome*.[446a]

Polyubiquitination of proteins requires ATP (Box 10-C) and additional ATP is utilized in the proteasomes (Box 7-A) during the selection of polyubiquitinated proteins for hydrolysis.[447,448] With 28 subunits the 26S proteasome is complex and not fully understood.[448] The ATP-hydrolyzing subunits appear to all be in the cap regions. Is the ATP used to open and close the entry pores? To induce conformational changes in all subunits as part of a catalytic cycle? Or to unfold folded proteins to help them enter the proteasomes?[449]

Some answers may be obtained from smaller bacterial, mitochondrial, and chloroplast ATP-dependent proteases. Cells of *E. coli* contain at least nine proteases, which have been named after the musical scale as Do, Re, Mi, Fa, So, La, Ti, Di, and Ci.[450,451] Most are serine proteases but two, Ci and Pi, are metalloproteins. Protease **La** (**Lon protease**, encoded by gene ***lon***) has attracted particular attention because the hydrolysis of two molecules of ATP occurs synchronously with cleavage of a peptide linkage in the protein chain.[452] This enzyme, as well as protease **Ti** (more often called **Clp**, for caseinolytic protease),[451,453,454] is ATP-dependent.[451]

The Lon protease of *E. coli* is a large 88-kDa serine protease with the catalytic domain, containing active site Ser 679, in the C-terminal half. The N-terminal portion contains two ATP-binding motifs and a linker region.[455,456] A homologous protein known either as Lon or as PIM1 is present in mitochondria.[457,458] The *E. coli* proteases Clp (Ti) include ClpAP and ClpXP, which are heterodimers, each containing the catalytic subunit ClpP and either ATPase ClpA or ClpX. The active enzyme may be designated **ClpAP**.[459–461] A homolog of ClpP has been found in human mitochondria.[461a] The ATPase Clp forms seven-subunit rings resembling the rings of proteasomes (Box 7-A), while the catalytic subunit ClpA forms six-membered rings.[451,454] Despite the mismatch in symmetry and the fact that they are serine proteases, these enzymes appear similar to proteasomes. However, each catalytic subunit has an ATPase neighbor. Why is it needed? The related **ClpB** is both an ATPase and a chaperonin. It is essential for survival of *E. coli* at high temperatures.[461b]

Another ATP-dependent protease identified among heat shock proteins of *E. coli* is known as **Hs1V–Hs1U** or (ClpQ–ClpY). It has a threonine protease active site and is even more closely remeniscient of eukaryotic proteasomes.[462–463a] Also active in *E. coli* is another ring-like protease, a membrane-bound zinc endopeptidase **FtsH** (or HflB).[463b] Similar eukaryotic proteases also exist.[460]

7. The Many Functions of Proteases

Many of the enzymes considered in the preceding sections function within the *digestive* tract. Others function in the *processing* of newly formed peptide chains, while others act in the *intracellular degradation* of proteins. Yet others are secreted from cells and function in the external surroundings. Both processing and intracellular degradation can be viewed as parts of *biosynthetic loops* (Chapter 17) that synthesize mature proteins, and then degrade them when they have served their function or have become damaged. The pathways involved are varied and complex, a natural result of thousands of enzymes and other compounds acting on accessible and chemically appropriate parts of the proteins. Evolutionary selection has evidently led to the particular set of pathways that we observe for any organisms.

Because they must often cleave large **polyproteins**, many viruses encode processing proteases.[464–466b] For example, the entire RNA genome of the poliovirus encodes a large polyprotein which is cut by two virally encoded chymotrypsin-like cysteine proteases within Tyr-Gly and Gln-Gly sequences;[341,465] Asn-Ser sequences are also cut, apparently autocatalytically. As we have already seen, retroviruses encode their own aspartic protease. Most cellular and secreted proteins of bacteria or eukaryotes also undergo processing. This ranges from removal of *N*-formyl groups to cutting off signal peptides, addition or formation of prosthetic groups, internal cleavages, and modifications at the C termini. A variety of peptidases are required.

Degradation of proteins, which converts them back to amino acids as well as other products, takes place in part in the cytosol via the ubiquitin-protease system. Proteolysis also occurs in ER and external cell spaces through the action of membrane-bound and secreted proteases. Loosely folded proteins, which can arise in various ways, are subject to rapid degradation. For example, synthesis of a polypeptide chain on a ribosome may be accidentally disrupted with formation of a protein with a shorted chain. Mature proteins may become damaged. For example, certain histidine residues are readily attacked by oxidizing reagents. Oxidative damage may mark proteins for rapid degeneration.[450] In *E. coli*, proteases So and Re attack oxidized glutamine synthetase much more rapidly than the intact native enzyme.[450,467] In mammalian cells the proteasomes degrade oxidized as well as poorly folded proteins.[468] However, proteasomal digestion is not complete and peptide fragments may be secreted. Peptide fragments of proteins are also formed in the ER and may be secreted. Thus, a variety of small peptide hormones and other biologically active peptides are both generated and inactivated in or around cells.[469–471] Some receptors are activated by proteases.[471a]

Circulating proenzymes of the blood clotting factors, of the complement system (Chapter 31), represent a specialized group of secreted **signaling proteins** that are able to initiate important defensive cascades. Proteases also act more directly in defense systems of the body. For example, serine proteases cause lysis of the target cells of cytotoxic T lymphocytes[472] (Chapter 31) and activated neutrophils[473] (Chapter 18). At the same time, pathogenic bacteria often secrete proteases that assist in attack on their hosts[474] and schistosomes secrete an elastase that helps them penetrate skin and invade their hosts.[475]

8. Protease Inhibitors of Animals and Plants

Premature conversion of proenzymes such as trypsinogen into active proteases in the pancreas would be disastrous. To prevent this, the pancreas also produces inhibitors. The complex cascades that control blood clotting would also be unstable were it not for the presence in blood of numerous inhibitory proteins. Indeed, inhibitors of proteases are found everywhere in animals, plants, and microorganisms.[476–478] They are usually proteins but small antibiotic protease inhibitors are also produced by microorganisms. Protein inhibitors are usually specific for a given type of enzyme: The **serpins** inhibit serine proteases, and the **cystatins** inhibit cysteine proteases.[479–483] Other inhibitors block the action of metalloproteases[484–486] and aspartic proteases. Inhibitors help not only host organisms but also parasites. For example, *Ascaris suum*, a very large nematode that is thought to infect 1/4 of the human population of the earth and nearly all of the pigs, secretes a pepsin inhibitor.[487] However, the large 720-kDa serum **α-macroglobulins** (Box 12-D) inhibit all classes of proteases.

About 20 families of protein inhibitors of proteases have been described.[488] The egg white **ovomucoids** comprise one family. Turkey ovomucoid is a three-domain protein whose 56-residue third domain is a potent inhibitor of most serine proteases.[488,489] The 58-residue **pancreatic trypsin inhibitor**[490] is a member of another family of small proteins. A 36-residue insect (locust) protease inhibitor is even smaller.[491]

Inhibitors that block the action of trypsin and other proteases are found in many plants. The inhibitor activity is usually highest in seeds and tubers, but synthesis of inhibitors can be induced in other parts of plants e.g. tomato and potato by wounding.[492] See also Chapter 31. Legumes contain small 60- to 76-residue inhibitors (Bowman–Birk inhibitors) each containing seven disulfide linkages, and they are relatively stable toward cooking and toward acid denaturation in the stomach.[493,494] Although they interfere with protein digestion they seem to have an anticarcinogen effect.[493] Soybeans also contain a larger 181-residue trypsin inhibitor (Kunitz type).[495]

Several inhibitor–protease complexes have been crystallized and details of their interactions are known. For example, the pancreatic trypsin inhibitor binds at the active site of trypsin with $K_f > 10^{13}$ M^{-1} at neutral pH.[496] The two molecules fit snugly together,[490,497] the inhibitor being bound as if it were a peptide substrate with one edge of the inhibitor molecule forming an antiparallel β structure with a peptide chain in the enzyme. Lysine 15, which forms part of this β structure, enters the specific P_1 binding site for a basic amino acid in a substrate. Thus, the protease inhibitor is a modified substrate which may actually undergo attack at the active site. However, the fit between the two molecules is so tight that it is hard for a water molecule to enter and complete the catalytic act. The complex reacts very slowly, keeping the enzyme inactive. There is not enough inhibitor to interfere with the large amounts of trypsin formed from trypsinogen in the small intestine so that trypsin can function there.

Most protease inhibitors act by mechanisms similar to that of the pancreatic trypsin inhibitor. They are very slow substrates with a **reactive loop** that carries suitable P_1, P_2, and P_1' residues that meet the specificity requirements of the enzyme. Additional noncovalent interactions prevent dissociation and make the energy barrier for hydrolysis so high that the reaction is extremely slow.[488,494,495]

The serpins are larger ~400-residue proteins.[498–500] They also form complexes in which hydrolysis and release of the serpin occurs very slowly. However, structural analysis before reaction and after release showed that a major rearrangement occurs in the serpin structure. The P_1 through ~P_{15} residues of the cleaved reactive loop are inserted into the center of the main β sheet of the serpin, leaving the P_1 and P_1' residues ~7 nm apart.[499,500] It seems likely that the rearrangement begins during formation of the tight inhibited complex, which cannot be dissociated by boiling in a sodium dodecyl sulfate (SDS) solution and which may be an acyl-enzyme.[501,502] A serpin molecule can act only once.

Blood contains several serpins. They are abundant, accounting for about 10% of the total protein of human plasma.[478,500] The most abundant is the **α1-protease inhibitor** or α_1-antitrypsin, a 394-residue glycoprotein component of the α-globulin fraction of blood serum.[500a] There is no trypsin in tissues, but this inhibitor blocks the action of other serine proteases, including cathepsin G and **leukocyte elastase**.[503] Hereditary absence of α_1-protease inhibitor often leads to severe **pulmonary emphysema** at an early age. Elastase released by neutrophils at sites of inflammation degrades many components of connective tissue including elastin, collagen, and proteoglycans. Without the presence of protease inhibitor too much damage is done to surrounding tissue. A lack of this inhibitor is one of the commonest genetic defects among persons of European ancestry, affecting 1 in 750 persons born.[504–506] The most serious known mutation is a replacement of guanine by adenine at a specific point in the DNA and a resultant replacement of Glu 342 with lysine in the protease inhibitor. This in some manner adversely affected the processing and secretion of the protein.

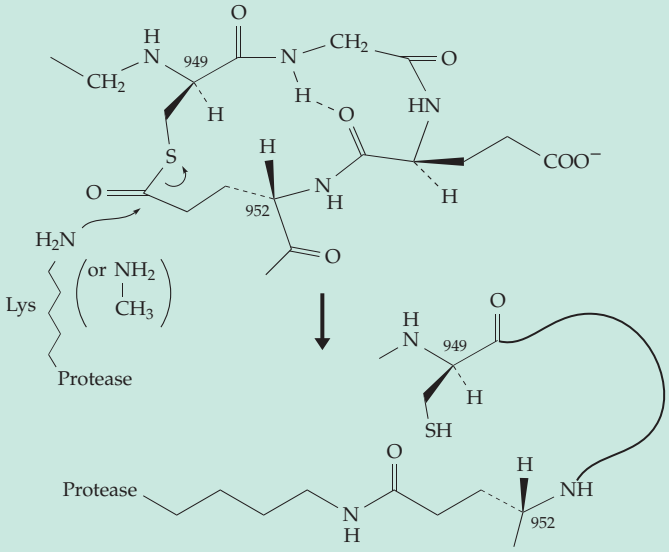
At its reactive site the α_1-protease inhibitor has the sequence

Ala-Ile-Pro-Met*-Ser-Ile-Pro-Pro,

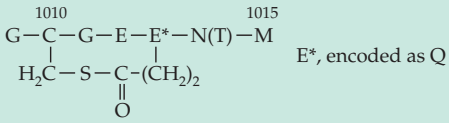
the Met-Ser pair marked by the asterisk fitting into the P_1-P_1' sites (Fig. 12-14). The methionine (Met 358) in

BOX 12-D MOLECULAR MOUSETRAPS

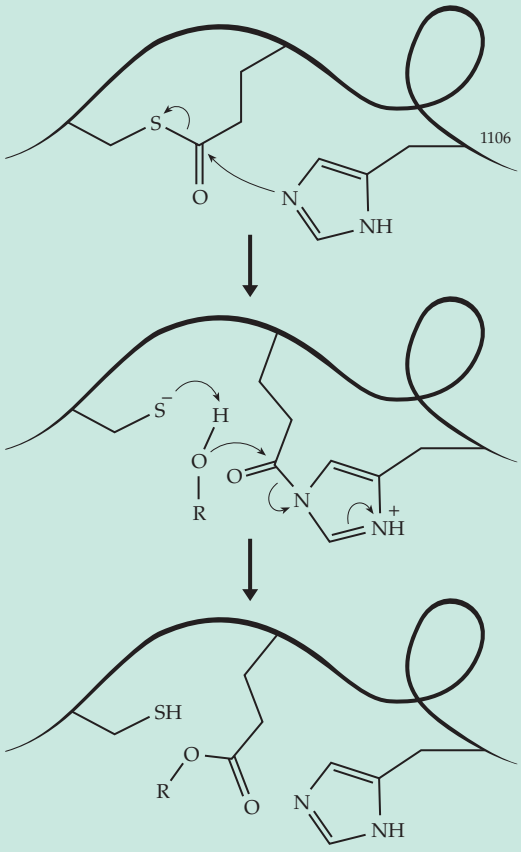
The large 720-kD α_2-macroglobulin of human serum, as well as related proteins of vertebrate and invertebrate circulatory systems and of egg whites of birds and reptiles, is a trap for proteases.[a–f] Human macroglobulin is a homotetramer consisting of two pairs of identical 180-kDa subunits, each pair being held together in an antiparallel configuration by two disulfide bridges. Each subunit contains a "bait region" with cleavage sites appropriate for nearly all known endoproteases[f] and also a thioester linkage as explained later. Electron microscopic reconstructions of the native protein and its complexes with proteases show that a major structural transformation occurs.[e,f] The macroglobulin traps two protease molecules of the size of trypsin, or one larger one such as plasmin, in an internal cavity. The internal thioesters, which are formed between Cys 949 and Gln 952 (with loss of NH_3) in each subunit, become reactive[g] and form covalent bonds with ε-amino groups of various lysine side chains of the trapped proteases.



The serum proteins C3 and C4, members of the complement system (Chapter 31), are converted into their active forms, C3b and C4b, by proteolytic removal of short N-terminal peptide fragments. Both C3b and C4b bind tightly to cell surfaces, a feature that helps to direct the complement system's attack to the surfaces of invading organisms. This tight binding also involves covalent attachment of macromolecules by reaction with a preformed thioester just as with α_2-macroglobulin.[h–j] In fact, the thioester linkage was first discovered in the complement proteins. Both C3 and C4 contain the thioester within the following sequence, which is the same as that in α_2-macroglobulin:



Here the side chains have been added for the thioester-forming cysteine and glutamate and the sequence numbers are for C3. The thioester-forming glutamate is labeled E* because it is not encoded as glutamate but as glutamine, suggesting a mechanism of thioester formation. Protein C4 exists as two subforms, C4A and C4B. Both C3 and C4A react predominately with lysine amino groups, but C4B reacts with –OH groups of cell surface polysaccharides.[h] It has a histidine at position 1106. There is good evidence that it is adjacent to the C=O group of the thioester and reacts to form an acyl-imidazole which is more reactive with hydroxyl groups than is a thioester:



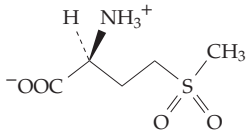
Activation of C3 and C4 apparently allows the preformed thioester, which is buried in the interior

BOX 12-D (continued)

of these proteins, to become exposed on the external surface where it can react. The high group transfer potential of the thioester ensures that the reaction will go to completion.

[a] Sottrup-Jensen, L. (1989) *J. Biol. Chem.* **264**, 11539–11542
[b] Fothergill, J. (1982) *Nature (London)* **298**, 705–706
[c] Jacobsen, L., and Sottrup-Jensen, L. (1993) *Biochemistry* **32**, 120–126
[d] Andersen, G. R., Koch, T. J., Dolmer, K., Sottrup-Jensen, L., and Nyborg, J. (1995) *J. Biol. Chem.* **270**, 25133–25141
[e] Boisset, N., Taveau, J.-C., Pochon, F., and Lamy, J. (1996) *J. Biol. Chem.* **271**, 25762–25769
[f] Qazi, U., Gettins, P. G. W., Strickland, D. K., and Stoops, J. K. (1999) *J. Biol. Chem.* **274**, 8137–8142
[g] Gettins, P. G. W. (1995) *Biochemistry* **34**, 12233–12240
[h] Law, S. K. A., and Dodds, A. W. (1997) *Protein Sci.* **6**, 263–274
[i] Khan, S. A., Sekulski, J. M., and Erickson, B. W. (1986) *Biochemistry* **25**, 5165–5171
[j] Dodds, A. W., Ren, X.-D., Willis, A. C., and Law, S. K. A. (1996) *Nature (London)* **379**, 177–179

this sequence, as well as another nearby methionine residue, is very susceptible to oxidation to sulfoxides:



Methionine sulfoxide

The oxidation, which may be caused by such agents as myeloperoxidase (Chapter 16) released from leukocytes,[507] inactivates the inhibitor. This may be physiologically important in permitting the proteases to be *uninhibited* in the immediate vicinity of the leukocyte. Cigarette smoke also inactivates α_1-protease inhibitor by oxidation of the same methionine residues and the lungs of smokers contain the oxidized inhibitor.[508] However, the major cause of emphysema among smokers appears to be an increase in released neutrophil elastase.[509] One approach to the treatment of emphysema involves weekly intravenous injection of α_1-antitrypsin.[510] This treatment may be improved by use of genetically engineered oxidation-resistant variants of the antitrypsin such as Met 385→Val.[504,511] Efforts are also being made to introduce an α_1-antitrypsin gene into lung epithelial cells.[510,512]

Blood plasma also contains at least nine other protease inhibitors. One of these, the thrombin inhibitor **antithrombin III** (Section 9), contains the sequence Arg-Ser-Leu at the P_1, P_1', and P_2' sites. A tragic case of a person born with a Met 385 → Arg mutation in α_1-antitrypsin has been reported.[513] This converted the antitrypsin to an antithrombin causing a bleeding disorder that was eventually fatal.

9. Coagulation of Blood

The clotting of blood following injury and the subsequent dissolving of the clot are familiar phenomena that involve several cascades of proteolytic enzymes together with a number of accessory **cofactors**.[514–516] The first step in clotting results from "activation" of blood platelets which aggregate to form a platelet plug that slows bleeding.[514,517] The clot, which is formed by the insoluble **fibrin**, grows on the platelet surfaces and strengthens the plug. The initial rapid formation of a clot occurs via the **tissue factor pathway** (or extrinsic pathway; right side of Fig. 12-17) which is triggered by the exposure in injured tissues of **tissue factor** (TF), a transmembrane glycoprotein[518–522] and a member of the cytokine receptor superfamily.[518] Human TF is a 263-residue protein with a single membrane-spanning region and a small 20-residue C-terminal cytoplasmic domain. The 219-residue extracellular domain consists largely of two IgG-like domains (Fig. 12-18).[519,523] This protein stimulates the conversion of fibrinogen[524–526] into the insoluble fibrin through the action of three proteases–factor VIIa, factor Xa, and thrombin. These enzymes are generated from proenzymes VII, X, and prothrombin, respectively in a cascade mechanism.

Factor VII binds tightly to TF,[527,527a,b] which also binds Ca^{2+} and phospholipid of the cell membranes. Within this complex a plasma protease, such as thrombin or factor VIIa or Xa, cleaves a single Arg-Ile bond in VII to form active VIIa.[528–530] The TF•VIIa complex is a very active protease which cleaves a specific peptide bond in factor X to form Xa[531–533] which continues the cascade. Notice that there are autocatalytic features: VII can be converted to the active VIIa by the action of Xa and the accessory factor Va is generated from the precursor, factor V, in part through the action of thrombin.[514,534–535] Factors Xa and Va together with Ca^{2+} and phospholipid form the active **prothrombinase** complex which attacks prothrombin to form the active enzyme thrombin.[536,537] The roles of factor Va and Ca^{2+} appear to be to hold the prothrombin and the activated protease Xa together on the phospholipid surface.[538] This localizes the clotting. Factor Xa is unusually specific, cleaving only after arginine in the sequence. Its activation of prothrombin results from cleavage of two

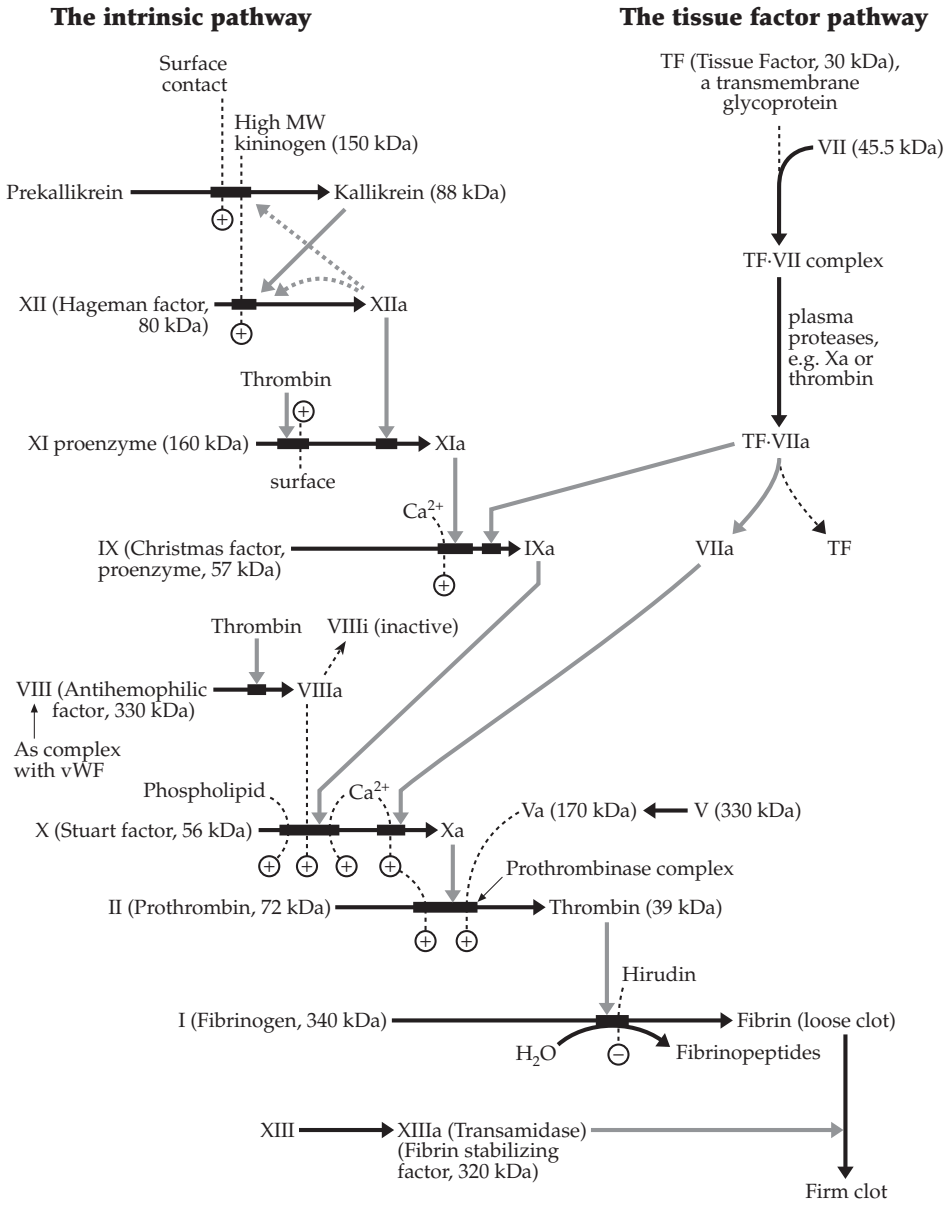


Figure 12-17 Major components of the human blood clotting cascades. The site of action of the leech anticoagulant protein hirudin is also indicated.

peptide bonds, which releases the 39-kDa thrombin from the much larger, immobilized 72-kDa proenzyme.[236,531]

Thrombin, like most other clotting factors, is also a serine protease. However, the clotting factors are multidomain proteins that are more elaborate and more specific[539] than the digestive enzymes. Prothrombin, as well as factors VII, IX, and X and the anticoagulant protein C, contain at their N-terminal ends several residues of γ-carboxyglutamate (Gla), an amino acid generated in a posttranslational modification that depends upon vitamin K (Chapter 15). In human prothrombin there are ten of these in the following N-terminal sequence, where E* represents Gla. Since many of these enzymes are also dependent upon activation by calcium ions (see Fig. 12-17), it is thought that the function of Gla is to assist in the binding of

```
                10          20          30
ANTFLE*E*VRKGNLE*RE*CVE*E*TCSYE*E*AAFE*ALE*SS
```

Ca^{2+} which helps to tie these proteins to the phospholipids of platelet surfaces. In factors VII, IX, X, and protein C this Ca^{2+}-binding domain is followed by two epidermal growth factor (EGF)-like domains, each containing one residue of *erythro*-β-hydroxyaspartate or hydroxyasparagine formed by hydroxylation of an aspartate or asparagine residue in the first EGF-like domain.[540,540a,b] The C-terminal catalytic domain of each enzyme contains the protease active site.

Fibrinogen is an elongated molecule with an $(\alpha\beta\gamma)_2$ structure.[524,541,541a] Thrombin cleaves specific Arg-Gly bonds in the α and β chains releasing short (14- to16-residue) "fibrinopeptides" from the N termini of the peptide chains. This leaves Gly-Pro-Arg "knobs" at

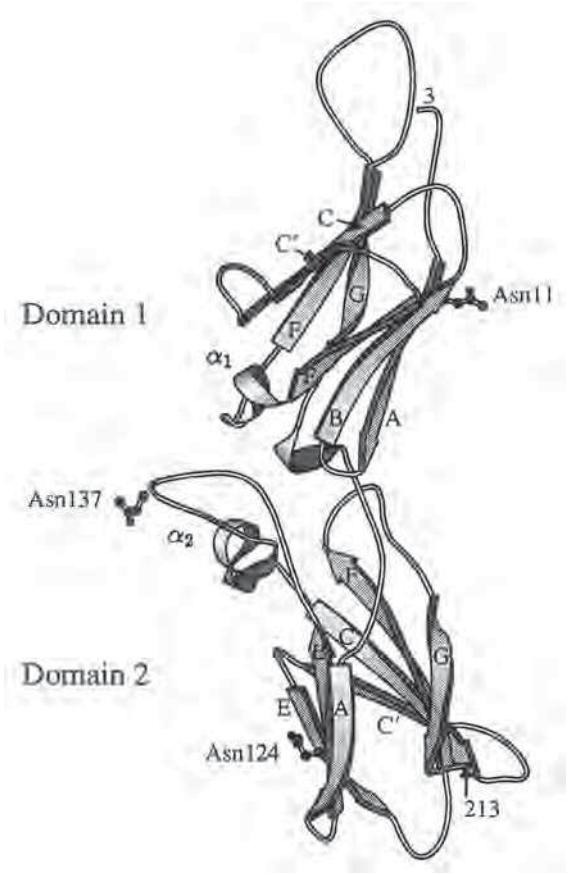


Figure 12-18 A ribbon drawing of the three-dimensional structure of the extracellular region (residues 3 to 213) of human tissue factor. Strands of domain 1 and 2 are labeled A to G. Two helices are labeled α_1, and α_2 and the three asparagines that provide the glycosylation sites are also marked. MolScript drawing from Harlos *et al.*[520]

the N termini of the α-chains and these fit into complementary "holes" in the γ chains to form noncovalently linked aggregates.[542–544] The clot that forms is unstable, but it is soon crosslinked by the action of the **transamidase** (transglutaminase or **factor XIIIa**; Eq. 2-23).[545,546] The fibrin monomers, von Willebrand factor (discussed in following paragraphs), fibronectin, collagen, and other proteins all become crosslinked.

The slower **intrinsic mechanism** first described in 1964[547,548] consists of a cascade involving six proteases (Fig. 12-17, left side). Again, autocatalytic cycles are present in the activation by XIIa of both prekallikrein[549] and XII and in activation by thrombin of factors XI and VIII. This intrinsic pathway is initiated by the serine protease proenzymes prekallikrein and factor XII together with the accessory protein high-molecular-mass-H- kininigen.[549] Activation occurs when blood contacts surfaces such as glass or kaolin (a clay).[547,548,550] Factor XI can also be activated by thrombin. Heredity absence of factor XI leads to bleeding problems, especially after surgery, but absence of XII does not. This fact suggests that direct activation of XI by thrombin is important and that the kallikrein–factor XII pathway is usually less important.

Factor IX (Christmas factor) is next in the intrinsic mechanism cascade. It can be activated either by XIa or by VIIa of the tissue factor pathway. The absence of a functional factor IX leads to the inherited X-linked bleeding disorder **hemophilia B** which affects 1 in 30,000 males. The condition can be mild or very serious[551,552] and may be caused by a variety of mutations or by incorrect splicing of the messenger RNA for the 416-residue factor IX. The level of factor IX in blood increases with age, almost doubling by old age.[552a]

Factor IXa causes a rapid activation of factor X only if Ca^{2+}, phospholipid,[553,554] and the accessory factor VIIIa[555] are present. The IXa•VIIIa complex acts on X about 2×10^5 times faster than does IXa alone. This complex cleaves the same bonds in X as does the VIIa•Va complex formed in the tissue factor pathway.[514] The 2332-residue factor VIII and factor V have similar structures that include three repeats of a domain homologous to the blue copper-containing plasma protein **ceruloplasmin** (Chapter 16).[556–559] Tyrosine 1680 of VIII apparently must be converted to a sulfate ester for full activity.[560]

The absence of factor VIII in about 1/10,000 males born, leads to the severe X-linked bleeding disorder **hemophilia A**. Human factor VIII is encoded by a 186-kb gene containing 26 coding exons. Severe cases of hemophilia are usually a result of point mutations that produce stop codons in this gene and therefore a shortened protein. Milder cases may result from an amino acid substitution.[561] Factor VIII circulates in the plasma as a complex with the **von Willebrand factor**, (vWF), a large multimeric protein derived by proteolytic cleavage, glycosylation, and sulfation of a 2813-residue precursor.[562–566] The mature 2050-residue vWF stabilizes factor VIII in the blood. Another important function of vWF is to bind platelets to damaged endothelial surfaces.[567,568] Like fibrinogen, vWF contains RGD sequences specific for binding to adhesion receptors.[569,570] It also binds to collagen. A serious inherited bleeding disorder caused by deficiency of vWF was first discovered among inhabitants of islands in the Gulf of Bothnia, Finland, in 1926 by von Willebrand.[563,571] If mild forms of the disease are included, vWD deficiency is the commonest bleeding problem. However, abnormalities have been identified in almost every one of the proteins in the coagulation cascades.[514]

Until recently, these bleeding diseases were treated by regular injections of the appropriate factors isolated from human plasma. This was both costly and carried a high risk of infection by HIV, hepatitis, or other contaminating viruses. Now, cloned genes are used for commercial production of the factors.[572,573] Experiments in animals, designed to lead to eventual gene

replacement therapy, have been somewhat successful.[573,574] It is of interest to compare the relative concentrations of several of the clotting factors in plasma and to compare these with their positions in the cascade of the tissue factor (extrinsic) pathway.[515]

	mg / L	Mass kDa	μM
VII	0.5	45.5	0.01
V	10.0	330	0.03
Prothrombin	150	72	2.1
Fibrinogen	3,000	340	8.8

Why do we have the intrinsic pathway when the tissue factor pathway provides rapid clot formation? The answer seems to be that the tissue factor pathway is needed immediately after injury but that it is turned off quickly by the **anticoagulation systems** of the body. As a result the protease **plasmin** begins to dissolve (lyse) the clot within a few hours. The intrinsic pathway is apparently needed to maintain the clot for a longer period.[514]

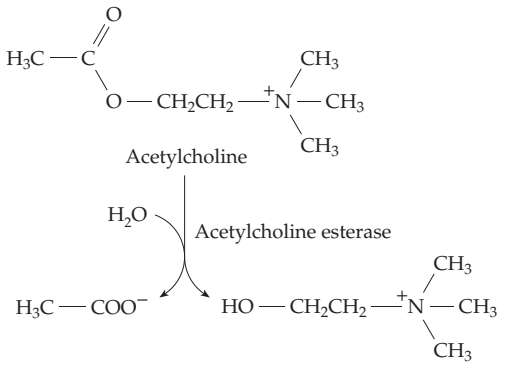
What prevents the clotting mechanism, with its autocatalytic cycles, from running out of control? In part the answer lies in the localization of the enzymatic activation to tissue surfaces near a wound. The flow of blood rapidly dilutes components that escape into the general circulation and liver cells take up and destroy the active proteases. A variety of circulating antiproteases including the **tissue factor pathway inhibitor**[575] act on these escaped enzymes. Two anticoagulation systems that are localized on vessel walls[576] also come into action very quickly. The circulating polysaccharide heparin (Chapter 4) forms a complex with the serpin **antithrombin**. Antithrombin traps thrombin as an inactive complex or compound[577,578a] and heparin greatly accelerates the inactivation.[578] Kallikrein and factors IXa, Xa, XIa, and XIIa are also inhibited.

Thrombin is an allosteric protein which exists as a mixture, in nearly equal amounts, of a fast-acting form that cleaves fibrinogen and is stabilized by Na^+, and a slow-acting form that initiates the second anticoagulant cascade.[579] The slow acting form, bound to **thrombomodulin**,[527a,540b,580,580a,b,581] an endothelial cell surface protein, attacks the proenzyme of another serum protease called **protein C**.[582,582a] Activated protein C (APC) inactivates the accessory clotting factors Va and VIIIa. The accessory factor **protein S**[583] is also needed for rapid inactivation. A blood clot is temporary and its dissolution begins as soon as it is formed, largely through the action of plasmin, a protease derived from the circulating 791-residue **plasminogen**[584–588] through the action of yet other proteases. Plasminogen often becomes crosslinked to cell surface proteins by transglutaminase.[586] Plasminogen activators include **urokinase**,[589] a protease present in kidney tissue and urine, and **tissue plasminogen activator** (tPA),[590,591] a 527-residue protease which is now produced by recombinant DNA technology. It is sometimes used to dissolve blood clots in emergency situations, such as myocardial infarction and pulmonary embolism,[592] but can also cause serious bleeding problems. Plasmin is inhibited by the plasma **α_2 antiplasmin** and is inactivated by action of clotting factor XIIa. Likewise, tPA is inhibited by several protease inhibitors present in tissues and in plasma. One is a fast-acting serpin called plasminogen activator inhibitor.[593] Another anticoagulant compound of medical interest is **hirudin**, a 65-residue peptide from the leech. It binds very tightly to thrombin (K_d = 1 pM) preventing its action.[594–596] Insects also produce antithrombin.[597,598] Ticks[599,600] and some insects[598] inject proteins that inhibit Xa selectively.[599] Anticoagulants are of great medical importance and much effort is being devoted to the design of better inhibitors of thrombin,[601–603] factor Xa,[604] and other components of the blood coagulation cascade.

Inherited deficiencies of the anticoagulant pathways with associated problems of thrombosis are known. These include problems with protein C,[576,605] plasminogen,[606] and antithrombin.[607,608]

10. Esterases and Lipases

A group of esterases hydrolyze simple oxygen esters. Some of these are designed to hydrolyze a particular ester or small group of esters, while others have a more nonspecific action. **Acetylcholinesterase**[609–611a] is specific for acetylcholine (Eq. 12-25), a neurotransmitter that is released at many nerve synapses and neuromuscular junctions (Chapter 30). The acetylcholine, which is very toxic in excess, must be destroyed rapidly to prepare the synapse for transmission of another impulse:



(12-25)

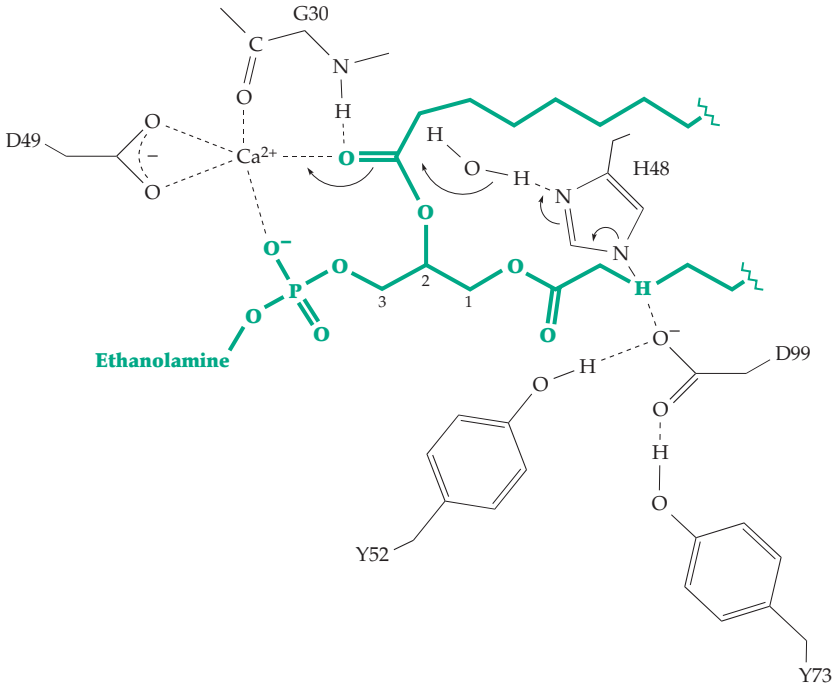
The more widely distributed **butyrylcholinesterase**[612] is less specific but prefers butyrylcholine. Acetylcholinesterase is a very efficient catalyst:[613–615] $k_{cat} = 1.6 \times 10^4\ s^{-1}$

and $k_{cat}/K_m = 2 \times 10^8\ M^{-1}s^{-1}$ at 25°C. It exists as a series of molecular forms containing varying numbers of 68-kDa catalytic subunits, 100-kDa structural subunits and subunits with triple-helical ~120-kDa collagen-like "tails."[609,616] The subunits are joined by disulfide bridges to give aggregates that range from simple dimers of catalytic subunits to tailed forms containing 8–12 catalytic subunits and non-collagenous structural subunits as well. The tailed forms are secreted from cells and may be designed to take up residence in the basal lamina of synapses, whereas the dimers are apparently attached to phosphatidylinositol anchors in the membranes (Fig. 8-13).[617] Human liver **carboxylesterases** are relatively nonspecific enzymes that hydrolyze ester groups of various drugs and toxins including cocaine and heroin. Products are often excreted in the urine.[618] **Thioesterases** function in biosynthesis of fatty acids, polyketides (Chapter 21), and many other substances.[619]

Lipases[620,621] hydrolyze triacylglycerols. The pancreatic digestive lipase[622,623] acts faster on emulsified fats than on glycerol esters in true solution but requires the cooperation of a small 10-kDa **colipase**.[624] **Cholesterol esterase** hydrolyzes not only cholesterol esters but also esters of fat-soluble vitamins, phospholipids, and triacylglycerols.[625,626] Other lipases include gastric[627] and hepatic lipases and a **lysosomal acid lipase**[628] which also attacks neutral lipids. Plasma **lipoprotein lipase**[629,630] digests fats in the chylomicrons and from the very low-density lipoproteins of blood. **Hormone-sensitive lipase** hydrolyzes stored triacylglycerols in the cytosol in response to catecholamines, ACTH, and other hormones.[631] The **phospholipases** attack phospholipids, while **cutinase**,[632] produced by some fungi, cleaves the ester linkages in the cutin (Chapter 21) of plant surfaces. Fungal lipases are important industrial commodities. Numerous structural and mechanistic studies have been made with them.[620,629,633–635] The gene for a lipase from *Candida rugosa* has been synthesized using codons that maximize its expression in *Saccharomyces cerevisiae* and which allow for further genetic engineering of the lipase.[636]

All of these esterases appear to act by mechanisms closely related to those of proteases. Acetylcholinesterase contains an active site serine that reacts with organophosphorus compounds (Box 12-E) and is part of an Asp-His-Ser catalytic triad which lies in a deep "gorge" as well as an oxyanion hole.[637] A surprise is the absence of an essential carboxylate group that might bind the positively charged trimethylammonium group of acetylcholine. Instead, the lining of the gourge is rich in aromatic side chains which may interact with the methyl groups of the substrate and by their polarizability stabilize the charge.[611,638] Most lipases, including cutinase, also have an Asp-His-Ser or Glu-His-Ser triad as well as some form of oxyanion hole.[620,632,639] Like the serine proteases, the lipases have bell-shaped pH optima.

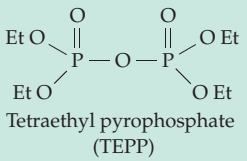
Phospholipase A_2 cleaves the ester linkage at the 2 position in phospholipids.[640–642] One isoenzyme form is secreted by the pancreas as a proenzyme whose N-terminal seven residues are removed by trypsin to give an active 125-residue enzyme. Phospholipase A_2 of a similar type is abundant in venoms of reptiles and bees. The venom and pancreatic enzymes have closely similar three-dimensional structures.[643–645] Although the folding pattern is different from that of chymotrypsin, imidazole (His 48) and carboxylate (Asp 99) groups are present in the active site in an orientation resembling that of the catalytic triad of serine protease. The enzyme requires calcium ions, one of which binds at an appropriate point for complexation with the substrate carbonyl as it is converted to an oxyanion intermediate. The backbone NH of Gly 30 may also serve as an oxyanion ligand. However, in most phospholipases there is no active site serine. Instead, a water molecule is positioned to serve as the attacking nucleophile in formation of the oxyanion as is indicated in the following scheme, which shows a truncated phosphatidylethanolamine as the substrate.[643] Phospholipase A_2 is up to 1000 or more times as active on phospholipids in micelles as on dissolved substrates.[621,646,647] Apparently, the

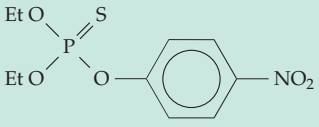


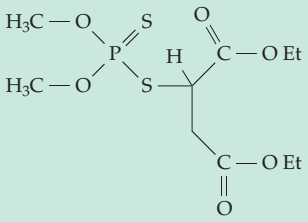
BOX 12-E INSECTICIDES

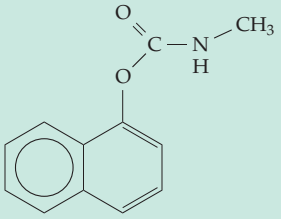
Over 200 organic insecticides, designed to kill insects without excessive danger to humans and animals, are presently in use.[a–e] Many of these compounds act by inhibiting cell respiration; others uncouple ATP synthesis from electron transport. The chlorinated hydrocarbons such as DDT act on nerves in a manner that is still not fully understood. One of the largest classes of insecticides acts on the enzyme acetylcholinesterase of nerve synapses. Like chymotrypsin, it contains an active site serine residue that reacts with organophosphorus compounds. The extreme toxicity of esters of pyrophosphate and of dialkylphosphonofluoridates was recognized in the 1930s and led to their development in Germany and in England as insecticides and as nerve gases. Among the most notorious is diisopropylphosphonofluoridate (diisopropylfluorophosphate; DFP), for which the LD_{50} (dose lethal to 50% of the animals tested) is only 0.5 mg kg^{-1} intravenously. This exceedingly dangerous compound can cause rapid death by absorption through the skin.

The following structures are a few of the organophosphorus compounds and other acetylcholinesterase inhibitors that are selectively toxic to insects.


Tetraethyl pyrophosphate (TEPP)

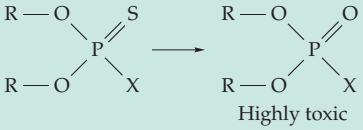

Parathion


Malathion


Carbaryl

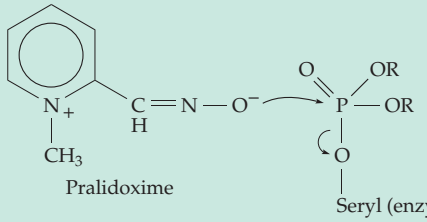
The characteristic high group transfer potential of a phospho group in pyrophosphate linkage, which makes ATP so useful in cells, also permits tetraethyl pyrophosphate to phosphorylate active sites of acetylcholinesterases. While TEPP is very toxic, it is rapidly hydrolyzed; all harmful residues are gone within a few hours after use.

Two insecticides that have been used widely are **parathion** and **malathion**. They are less toxic than DFP or TEPP and do not become effective insecticides until they undergo bioactivation during which conversion from a P = S to a P = O compound occurs:


Highly toxic

The desulfuration reaction involves microsomal oxidases of the liver, the sulfur being oxidized ultimately to sulfate.[f] The reactivity of parathion with cholinesterases depends upon the high group transfer potential imparted by the presence of the excellent leaving group, the *p*-nitrophenolate anion. If the P– O linkage to this group is hydrolyzed before the desulfuration takes place, the phosphorus compound is rendered harmless. Thus, the design of an effective insecticide involves finding a compound which insects activate rapidly but which is quickly degraded by higher animals. Other factors, such as rate of penetration of the insect cuticle and rate of excretion from the organism, are also important.

The phosphorylated esterases formed by the action of organophosphorus inhibitors are very stable, but some antidotes can reverse the inhibition. The oxime of 2-formyl-1-methylpyridinium ion (pralidoxime) is very effective.[g] Its positive charge permits it to bind to the site normally occupied by the quarternary nitrogen of acetylcholine and to displace the dialkylphospho group:


Pralidoxime — Seryl (enzyme)

Carbaryl, a widely used methyl carbamate, is a pseudosubstrate of acetylcholinesterase that reacts 10^5 to 10^6 times more slowly than do normal substrates. The carbamoylated enzyme formed is not as stable as the phosphorylated enzymes and the inhibition is reversible.

A basic problem is that most insecticides are designed to attack the central nervous system of the insect, the system that depends heavily upon acetylcholine. However, in human beings the readily accessible peripheral nervous system also depends upon acetylcholine, e.g., in neuronuscular junctions. The danger of poisoning is great. Another approach is to attack the glutamate-dependent peripheral system in insects, e.g., with inhibitors of glutamate decarboxylase. Glutamate functions as a neurotransmitter in the human body but only in the well-

BOX 12-E (continued)

protected central nervous system.

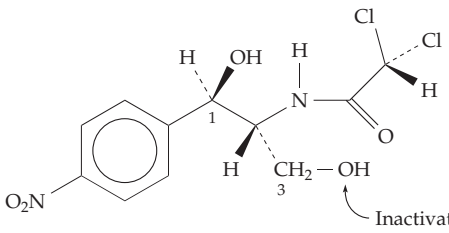
Another important problem is the development of insects resistant to insecticides. This often arises as a result of increased levels of carboxylesterases which hydrolyze both organophosphates and carbaryl.[h,i] A mutation that changed a single active site glycine to aspartate in a carboxylesterase of a blowfly changed the esterase to an organophosphorus hydrolase which protected the fly against insecticides.[j]

[a] Büchel, K. H., ed. (1983) *Chemistry of Pesticides*, Wiley, New York
[b] Hassall, K. A. (1990) *The Biochemistry and Uses of Pesticides*, 2nd ed., VCH Publ., Weinheim
[c] Kamrin, M. A., ed. (1997) *Pesticide Profiles*, CRC Press, Boca Raton, Florida
[d] Casida, J. E. (1973) *Ann. Rev. Biochem.* **42**, 259–278
[e] Wilkinson, C. F., ed. (1976) *Insect Biochemistry and Physiology*, Plenum, New York
[f] Nakatsugawa, T., Tolman, N. M., and Dahm, P. A. (1969) *Biochem. Pharmacol.* **18**, 1103–1114
[g] Wilson, I. B., and Ginsburg, S. (1955) *Biochim. Biophys. Acta.* **18**, 168–170
[h] Raymond, M., Callaghan, A., Fort, P., and Pasteur, N. (1991) *Nature (London)* **350**, 151–153
[i] Karunaratne, S. H. P. P., Hemingway, J., Jayawardena, K. G. I., Dassanayaka, V., and Vaughan, A. (1995) *J. Biol. Chem.* **270**, 31124–31128
[j] Newcomb, R. D., Campbell, P. M., Ollis, D. L., Cheah, E., Russell, R. J., and Oakeshott, J. G. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7464–7468

substrate-binding cavity of the protein is designed to accommodate phospholipid molecules in the preferred conformation found in the micelles.[643] Most of the other lipases have lids which close over the active sites and impede access of substrates. Binding to a phospholipid surface apparently induces a conformational change that opens the lid. This allows substrate to enter from the lipid surface.[620,621,646] Cutinases, which do not display interfacial activation, do not have lids. Phospholipase A_2 activity is stimulated by an applied electrical field, a result that suggests that its activity *in vivo* may be regulated in part by the membrane potential.[648]

11. Other Acyltransferases

Acyl groups are frequently transferred from amides or esters to various acceptors in biosynthetic reactions. Among the many known acyltransferases are the ribosomal **peptidyl transferases** (Chapter 29), a transacylase involved in bacterial peptidoglycan synthesis (Chapter 20), transglutaminase (Eq. 2-23),[649,650] γ-glutamylcyclotransferase (Box 11-B), and transacylation reactions involving acyl-CoA derivatives. Examples of the latter are *N*-acetylation[651] or myristoylation (Chapter 8) of proteins, the formation of acetylcholine from choline[652] and of acetylcarnitine from carnitine (Eq. 17-4), and acetylation of the antibiotic chloramphenicol. The high group transfer potential of thioesters ensures that these reactions proceed to completion. **Chloramphenicol acetyltransferase** (**CAT**),[653] the enzyme that catalyzes acetylation and inactivation of the antibiotic by bacteria, is much used in studies of gene expression (Chapter 28). A catalytic histidine removes the proton from the 3-OH group and a serine hydroxyl provides an oxyanion hole to accommodate the anticipated tetrahedral intermediate.[652,654] The steroidal antibiotic fusidic acid (Chapter 22, Section G) is a competitive inhibitor.[655] A related transferase is aspartate carbamoyltransferase (Fig. 7-20; Chapter 24).



Chloramphenicol, a broad-spectrum *Streptomyces* antibiotic

Penicillin and related antibiotics are inactivated by β-lactamases (Box 20-G), some of which resemble serine proteases in forming acyl enzymes with active site serine side chains.[656,657] Others are zinc metallogenzymes.[658,659] **Amidohydrolases** such as **asparaginase** and **glutaminase,**[660,661] deacetylases,[662] and many other hydrolases can also be described as acyltransferases.

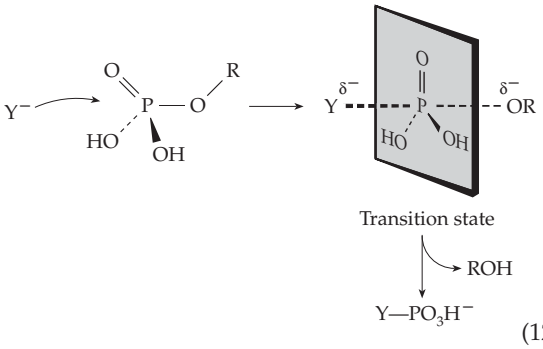
D. Displacement on a Phosphorus Atom

Nucleophilic displacements on phosphorus (Table 10-1, reaction type 1C) are involved in virtually every aspect of cellular energetics and in many aspects of biosynthesis. One large group of such enzymes are **phosphotransferases**, which transfer **phospho** (also called **phosphono** or, traditionally in biochemistry, **phosphoryl**) groups from one nucleophilic center to another. When transfer is to water the enzymes are called **phosphatases**, and when from one group in a molecule to another in the same molecule, **mutases**.

Enzymes that transfer a phospho group from ATP to water are **ATPases** and those that transfer the phospho group from ATP to some other nucleophile are **kinases**. Substituted phospho groups can also be transferred. Thus, **nucleases**, members of a larger class of **phosphodiesterases**, hydrolyze nucleic acids by transfer of nucleotidyl groups to a hydroxyl group of water. **Polynucleotide polymerases** transfer nucleotidyl groups to growing polynucleotide chains. An intramolecular nucleotidyl transferase is adenylate cyclase. **Topoisomerases** carry out a sequence of phosphotransferase reactions.

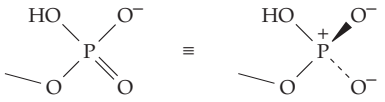
1. Questions about Mechanisms

Consider the following general equation for transfer to nucleophile Y^- of a phospho group attached in an ester or an anhydride linkage to form ROH, which could be an alcohol, carboxylic acid, or a phosphoric acid such as ATP.



(12-26)

This equation could also represent a half-reaction in a double-displacement process. As with displacements on saturated carbon atoms, two basic mechanisms can be imagined. The first is S_N2-like or **associative**.[663–667] The transition state might be represented where the bonds from the phosphorous atom to Y and to O are approximately equally formed. In an **in-line** displacement, where Y, P, and –OR are colinear, this mechanism leads to inversion if a chiral phospho group is used. There is another possibility for the associative mechanism. Whereas a carbon atom can form only four stable covalent bonds, phosphorus is able to form five. While nucleophilic attack on carbon leads to a *transient* five-bonded transition state (Eq. 12-2), attack on phosphorus could produce a relatively long-lived pentacovalent intermediate (Eq. 12-27). Notice that two transition states are involved. Remember that our conventional way of drawing phosphate esters with a double bond from phosphorus to one of the oxygens is misleading. All of the P–O bonds share some of the double-bond character and the phosphate group has many characteristics of a completely single-bonded structure:

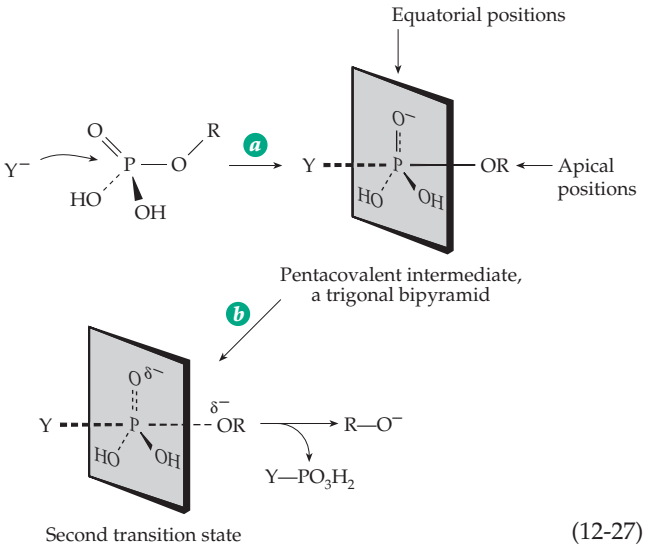


Likewise, in the transition state structures of Eq. 12-27, step *b*, the P–O bonds, except for that to –OR, are equivalent and all have partial double-bond character.

Geometric complexities. The geometry of the pentacovalent intermediate in Eq. 12-27 is that of a **trigonal bipyramid**. In this structure the bond angles in the **equatorial** plane are 120°, whereas all of the angles between any of those bonds and the two that attach to the groups in the **apical** positions are 90°. This disparity arises naturally from the fact that it is impossible to place five points on the surface of a sphere all equidistant one from the other. The attack of Y^- from the side opposite O–R (an in-line attack) leads to a trigonal bipyramid in which –O–R and –Y occupy the two apical positions. However, if Y^- attacks a face opposite one of the other oxygens (**adjacent** attack), –O–R will take an equatorial position.

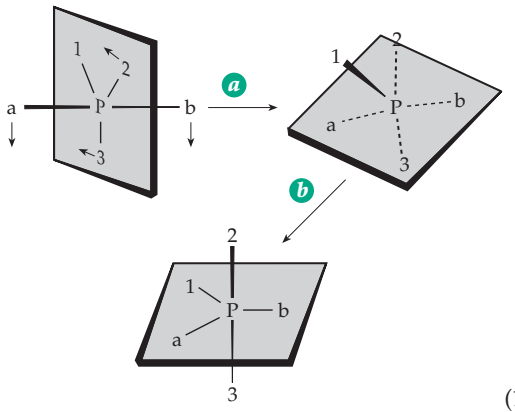
The chemical reactivities of groups in the apical and equatorial positions of pentacovalent intermediates are different.[664] In particular, elimination of a nucleophilic group to form a tetrahedral phosphate is easier from an apical position than from an equatorial position. For the in-line displacement of Eq. 12-27 elimination of RO^- should be easy. However an adjacent attack would leave –OR in an equatorial position. Before it could be eliminated, the intermediate would probably have to undergo a **permutational rearrangement** by which –OR would be transferred from an equatorial to an apical position.

One type of permutational rearrangement, known as **pseudorotation**, can be visualized as in Eq. 12-28.[668,669] The axial groups a and b move back while equatorial groups 2 and 3 move forward, still in the same equato-



(12-27)

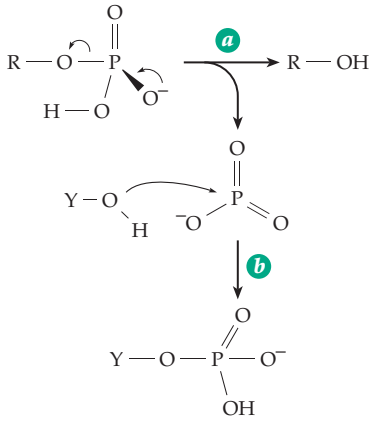
rial plane. Equatorial group 1 does not move. This decreases the 120° bond angles between the equatorial groups and increases the bond angles between group 1 and the axial groups until all four bond angles to group 1 are equivalent. The resulting **square pyramid** is a high-energy transition state structure in the pseudorotation process and can either revert to the original structure or, by continued motion of the groups in the same directions as before, to the structures shown at



(12-28)

the bottom of Eq. 12-28. In the final structure, groups 2 and 3 are axial and the original axial groups a and b are equatorial. Pseudorotation is slow enough that it could be rate limiting in enzymatic reactions. Stereoelectronic effects could also affect these rates.[670,671] However, much evidence indicates that enzymes almost always avoid these complexities by using in-line mechanisms.

Metaphosphate ions. An alternative to an associative mechanism is an S_N1-like or **dissociative** mechanism which can occur by elimination of a **metaphosphate ion** (Eq. 12-29, step *a*). A nucleophilic reagent can then add to the eliminated metaphosphate in step *b*. The formation of metaphosphate is analogous to formation of carbocation during the action of lysozyme (Eq. 12-11). This dissociative mechanism could lead either to racemization or to inversion of a chiral phosphate. Metaphosphate ions have been shown to exist. They are generated in certain nonenzymatic elimination reactions in aprotic solvents[672–674] and they are reactive electrophiles able to react as in Eq. 12-29. However, there is some doubt that they can exist free in aqueous media.[675] Jencks and associates concluded from studies of linear Gibbs energy relationships that in the transition state for nonenzymatic phospho transfer reactions there is a large amount of bond breaking and a small amount of new bond formation in the transition state[676] but no free metaphosphate. There is a large dependence of the rate on the pK_a of the leaving group, but there is still a small dependence on the pK_a of the entering nucleophile. Thus, we have *a*



(12-29)

dissociative mechanism but without free metaphosphate. The concept is supported by studies of kinetic isotope effects.[677,678]

Coping with negative charges. We visualize phospho group transfers as involving attack by a nucleophile bearing at least a partial negative charge. However, phospho groups also carry one or two negative charges and that in ATP even more. Therefore, it does not seem surprising that many phosphotransferases are metalloenzymes, sometimes containing bound Zn^{2+} which neutralizes some of the negative charge. Furthermore, enzymes usually accept ATP as a substrate only when it is accompanied by a divalent metal ion,[679] usually Mg^{2+}. Another way in which enzymes deal with the negative charges on phospho groups is to have arginine side chains in appropriate positions to interact by forming strong ion paired-hydrogen bonds. It is often assumed that it is essential to neutralize the charge on the phospho group to avoid electrostatic repulsion from a partial or complete negative charge on the attacking nucleophilic center. However, with a dissociative mechanism and a metaphosphate-like intermediate the transition state may be reached without charge neutralization.[679,680] Interactions of Mg^{2+} with phosphorus-containing substrates, like those of fixed positive charges in the protein may also be essential for binding the substrate correctly to an enzyme.

2. Magnetic Resonance Studies

There have been many investigations of phosphotransferases by NMR and EPR methods.[681,682] One approach is to use paramagnetic ions such as Mn^{2+}, Cu^{2+}, or Cr^{3+} to induce nuclear relaxation in substrate and coenzyme molecules at active sites of enzymes. Flavin radicals and specifically introduced nitroxide spin labels can serve as well. Paramagnetic ions greatly increase the rate of magnetic relaxation of nearby nuclei (Chapter 3, Section I). Thus, small amounts of

Mn^{2+} in a sample lead to broadening of lines in ordinary ^{1}H, ^{13}C, and ^{31}P NMR spectra.

Useful information about enzymes can sometimes be obtained by observing effects of paramagnetic ions on the NMR signal of protons in the solvent water. The relaxation time of solvent protons is usually greater than 1 s. However, in the ion $Mn(H_2O)_6^{2+}$ the protons of the coordinated water molecules relax much more rapidly, both T_1 and T_2 being $\sim 10^{-1}$ s. Since the coordinated water molecules usually exchange rapidly with the bulk solvent, a small number of manganese ions can cause a significant increase in relaxation rate for all of the water protons. Broadening of the proton band is observed and differences in T_1 and T_2 can be measured by appropriate methods. Paramagnetic relaxation effects usually increase as the inverse sixth power of the internuclear distance. Knowing the Mn^{2+}–H distance to be 0.287 ± 0.005 nm for hydrated Mn^{2+}, it has been possible to relate the effects on T_1 and T_2 to the number of H_2O molecules coordinated at any one time to a protein-bound metal ion and to their rate of exchange with the bulk solvent.

Relaxation effects on ^{1}H, ^{13}C, and ^{31}P, while more difficult to observe, can provide geometric information about active sites. The theory is complex, but under some conditions the paramagnetically induced relaxation can be described adequately by Eq. 12-30.

$$r = C[T_{1M} f(\tau_c)]^{1/6} \qquad (12\text{-}30)$$

Here r is the internuclear distance, C is a combination of physical constants, and T_{1M} is the longitudinal relaxation time. The complex function $f(\tau_c)$ depends upon the correlation time τ_c, the resonance frequency of the nucleus being observed, and the frequency of precession of the electron spins at the paramagnetic centers. The value of τ_c can be estimated (Chapter 3) and, in turn, the distance r according to Eq. 12-30.

Such studies on creatine kinase (Eq. 12-31) utilized both a bound Mn^{2+} ion and a nitroxide spin label to estimate distances of various protons from the nitroxide.[683] Together with EPR measurements (Box 8-C), which gave the Mn^{2+}–nitroxide distance, a model of the ATP•Mn^{2+} complex in the active site was constructed. Additional EPR experiments on Mn^{2+} complexes with ATP and ADP containing ^{17}O in the α, β, or γ phospho groups showed that in the enzyme•ATP• creatine complex the metal ion is bound to all three phospho groups of ATP. It remained coordinated with the two phospho groups of ADP and also that of the phosphocreatine product in the enzyme•ADP•creatine-*P* complex as well as in the transition state, which is pictured occurring via a metaphosphate ion.[684]

$$\text{Creatine-}P + \text{ADP} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{ATP} + \text{creatine} \qquad (12\text{-}31)$$

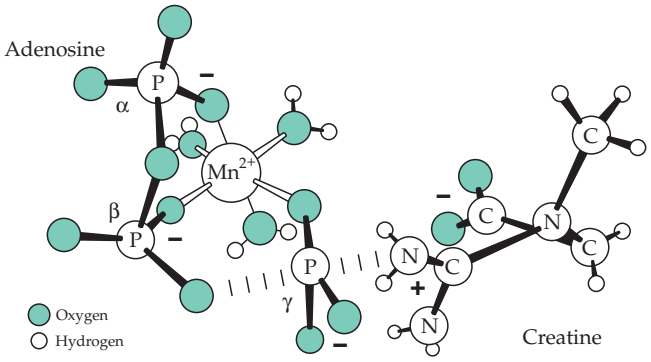


Figure 12-19 Proposed transition state structure formed from Mn^{2+}, ATP, and creatine bound in the active site of muscle creatine kinase. Based on EPR spectroscopy with regiospecifically ^{17}O-labeled substrates. The electrical charges have been added in one possible constellation. However, hydrogen atoms bound to phospho groups are not shown. After Leyh *et al.*[684]

Phosphorus-31 NMR has been used to measure **internal equilibrium constants** within enzyme-substrate (ES) complexes.[663,685–687] By having both substrate and product concentrations high enough to saturate the enzyme, all of the enzyme exists as ES and enzyme–product (EP) complexes in equilibrium with each other. For a phosphotransferase at least one substrate and one product contain phosphorus. Although the NMR resonances are broadened by binding to the large, slowly tumbling protein, their areas can be measured satisfactorily and can be used to calculate an equilibrium constant such as that for Eq. 12-32:

$$\text{ES} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{EP}$$

$$K' = [\text{EP}]/[\text{ES}] \qquad (12\text{-}32)$$

An example is illustrated in Fig. 12-20. In this experiment[685] the relative areas of the ^{31}P signals of ADP (one for free ADP and one, slightly more intense, for MgADP) and of the signal for phosphoenolpyruvate (PEP) were measured in the absence of enzyme and in the presence of a catalytic amount of pyruvate kinase (Fig. 12-20A). The results verified that the equilibrium constant for the overall reaction (Eq. 12-33) is very high (3300).

$$\text{Phosphoenolpyruvate (PEP)} + \text{ADP} \rightarrow \text{ATP} + \text{pyruvate} \qquad (12\text{-}33)$$

However, with an excess of enzyme (Fig. 12-20B) the internal constant was estimated as

$$K' = \frac{[E \bullet MgATP \bullet pyruvate]}{[E \bullet MgADP \bullet PEP]} = 0.5 - 1.0 \qquad (12\text{-}34)$$

This is a difficult measurement and reinvestigation by another group[686] indicated that the amount of the PEP-containing complex had been overestimated and that K′=10.

If the rates of the forward and backward reactions in Eq. 12-34 are of the same order as the spin-lattice relaxation times (T_1) of the ^{31}P in the bound substrate and product, the rate constants k_f and k_r can be evaluated by **saturation-transfer NMR**.[688] This is done by irradiating one resonance, e.g., that of the γ-P of ATP, and observing whether this causes a loss of intensity of the resonance for the product which is receiving its phospho group from ATP. This technique was used to observe the creatine kinase reaction (Eq. 12-31) in living muscle in both relaxed and contracting states. For resting muscle the observed forward flux was 1.7×10^{-3} M s^{-1} and the backward flux 1.2×10^{-3} M s^{-1}. Thus, this reaction, which supplies ATP for contraction of the muscle from stored phosphocreatine, appears to be operating at or near equilibrium. This had been assumed but had previously been difficult to prove. Two-dimensional NMR techniques can now be used for this kind of measurement.[689]

When ^{31}P is bonded to ^{18}O the chemical shift of the ^{31}P is altered by 0.0206 ppm from that when the phosphorus is bonded to ^{16}O. This allows ^{18}O labels introduced into phospho groups to serve as tracers which can be followed continuously during reactions.[683] The technique is useful in studies of stereochemistry (see Section 2) and for examination of **positional isotope exchange**.[690] This latter technique is often used with ATP containing ^{18}O in the β,γ-bridge position. If an enzyme transfers the terminal (γ) phospho group to an acceptor via a phosphoenzyme but without loss of the ADP, we may expect positional isomerization. The ^{18}O will move between the β,γ-bridge position and a nonbonding position as the phospho group is repeatedly transferred back and forth between ATP and the acceptor and as the phospho group rotates.[682,690]

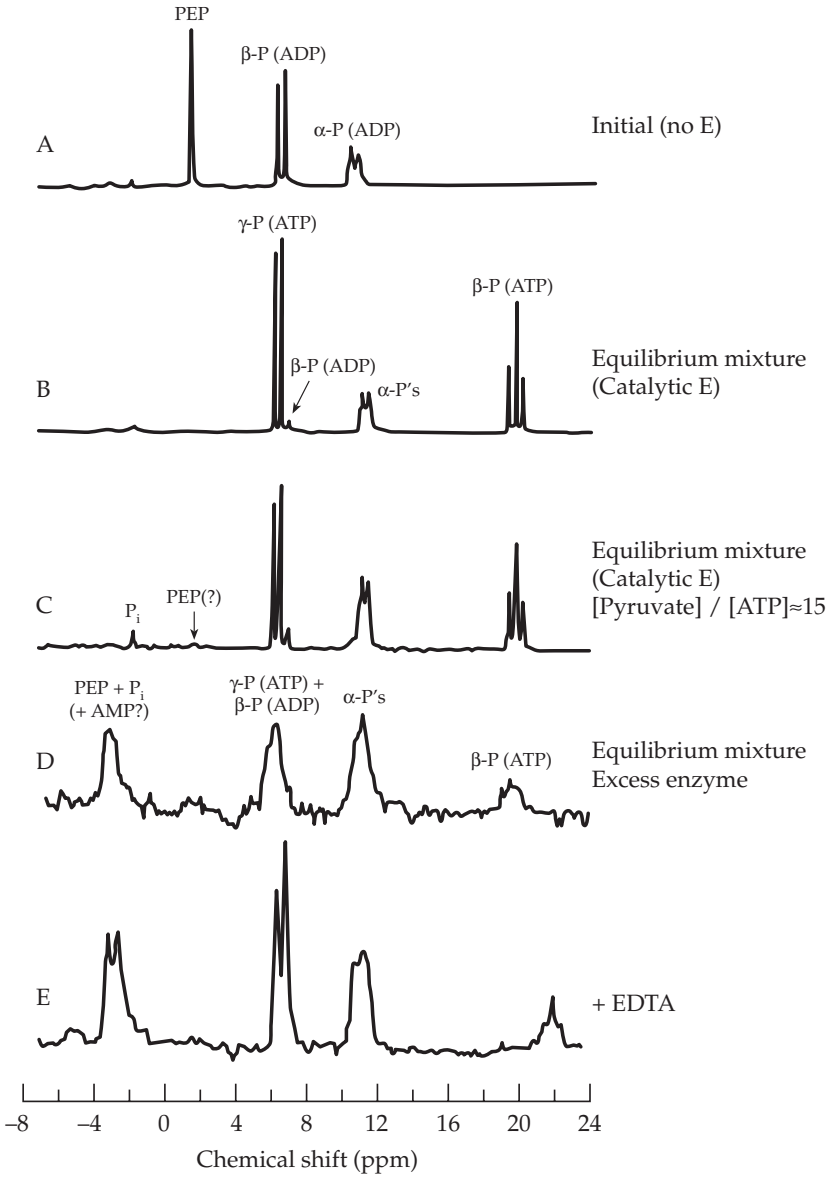
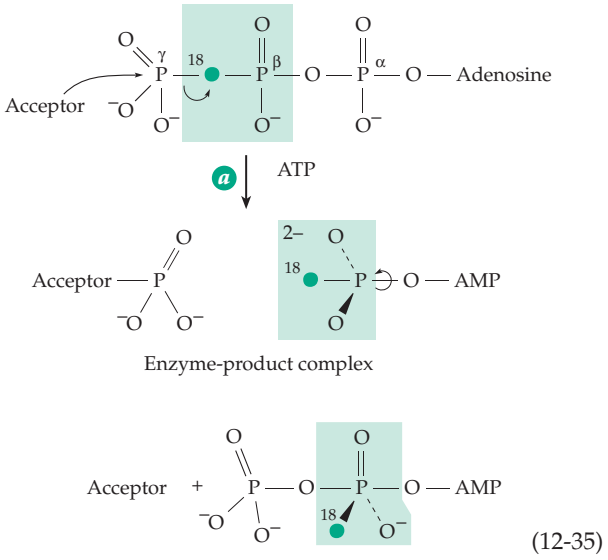


Figure 12-20 Equilibria in pyruvate kinase reaction as studied by ^{31}P NMR at 40.3 MHz, pH 8.0, 15°C. (A–C) Equilibria with low enzyme in levels ~15% ^2H_2O. (A) ^{31}P NMR spectrum of 1.5 ml of reaction mixture; PEP, 13.3 mM; ADP, 14.1 mM; $MgCl_2$, 20 mM; potassium Hepes buffer, 100 mM; KC, 50 mM without enzyme. (B) Equilibrium mixture after the addition of ~1 mg of pyruvate kinase to the reaction mixture. (C) Equilibrium after the addition of potassium pyruvate (final concentration of 200 mM) to the sample of the spectrum in (B). (D,E) Equilibrium with enzyme concentrations in excess of the substrates. Sample volumes ~1.1 ml with 10% ^2H_2O. (D) Equilibrium mixture set up with enzyme (2.8 mM active sites); 2.8 mM PEP; 2.4 mM ADP; 5.7 mM $MgCl_2$; 100 mM potassium Hepes; 100 mM KCl. (E) Spectrum after the addition of 50 µl of 400 mM EDTA (pH readjusted to 8.0) to the sample of spectrum D. The EDTA removes metal ions, stopping the catalytic reactions and sharpening the resonances. From Nageswara Rao *et al.*[685]

Equation 12-35 shows one part of this isomerization. The negative oxygens have been omitted here to avoid implying a known state of protonation or a localization of charge.

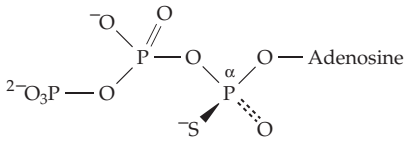


(12-35)

Figure 12-21 illustrates the use of the technique in investigating the possible participation of metaphosphate in a nonenzymatic reaction.

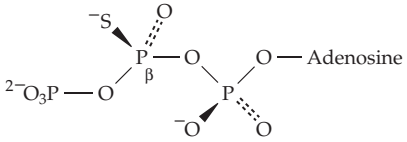
3. Stereochemistry

Evidence for an in-line S_N2-like mechanism of most enzymatic phospho group transfer reactions comes largely from study of chiral phospho groups.[663,692–695] A chiral phosphate can be introduced at either the α or β phosphorus of ATP by substitution of one of the oxygen atoms by sulfur. A chiral phospho group in the β position can be formed by substituting one oxygen by S and a second by ^{18}O.

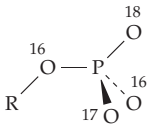


ATP αS (*R* configuration at P)

Notice that the negative charge is largely localized on sulfur in these phosphorothioate compounds.[696] More general is the use of ^{17}O and ^{18}O to form a chiral phospho group:



ATP βS (*S* configuration at P)



An ester chiral at the phosphorus atom; *R* configuration

Considerable ingenuity was required in both the synthesis of these chiral compounds[695,697] and the stereochemical analysis of the products formed from them by enzymes.[698–700] In one experiment the phospho group was transferred from chiral phenyl phosphate to a diol acceptor using *E. coli* alkaline phosphatase as a catalyst (Eq. 12-36). In this reaction transfer of the phospho group occurred without inversion. The chirality of the product was determined as follows. It was cyclized by a nonenzymatic in-line displacement to give equimolar ratios of three isomeric cyclic diesters. These were methylated with diazomethane to a mixture of three pairs of diastereoisomers triesters. These diastereoisomers were separated and the chirality was determined by a sophisticated mass spectrometric analysis.[692] A simpler analysis employs ^{31}P NMR spectroscopy and is illustrated in Fig. 12-22. Since alkaline phosphatase is relatively nonspecific, most phosphate esters produced by the action of phosphotransferases can have their phospho groups transferred without inversion to 1,2-propanediol and the chirality can be determined by this method.

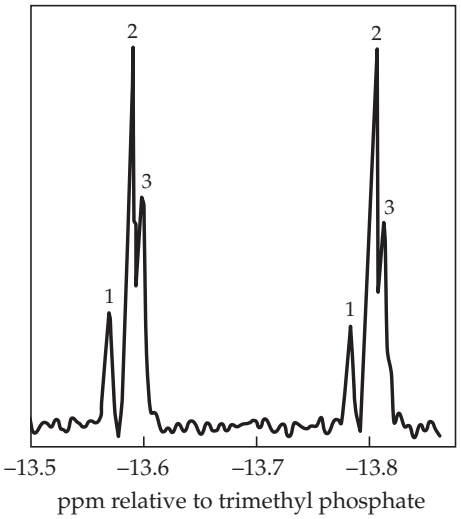
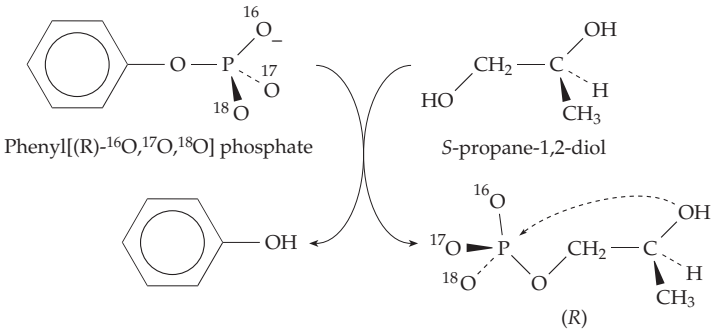


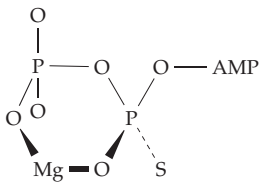
Figure 12-21 The ^{31}P NMR spectrum at 101.2 MHz of P_α of isotopically labeled ADP. This was recovered from an experiment in which ADP containing 87 atom % of ^{18}O in all four oxygens around P_β was allowed to undergo partial (~20%) nonenzymatic hydrolysis to AMP and P_i. Peaks 1 represent the species containing no ^{18}O bonded to P_α. Peaks 2 represent all species with ^{18}O in the P_α–O–P_β bridge, and peaks 3 represent species with ^{18}O in nonbridging positions at P_α. These have undergone positional isotope exchange. From Lowe and Tuck.[691]



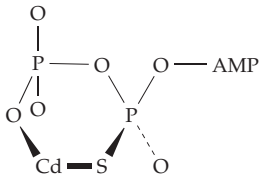
(12-36)

Although inversion was not observed with the *E. coli* alkaline phosphatase, it has been observed for ribonucleases and many other hydrolytic enzymes and for most kinases transferring phospho groups from ATP. The difference lies in the existence of a phosphoenzyme intermediate in the action of alkaline phosphatase (see Eq. 12-38). Each of the two phosphotransferase steps in the phosphatase action apparently occurs with inversion. The simplest interpretation of all the experimental results is that *phosphotransferases usually act by in-line S_N2-like mechanisms which may involve metaphosphate-ion-like transition states that are constrained to react with an incoming nucleophile to give inversion*. An adjacent attack with pseudorotation would probably retain the original configuration and is therefore excluded.

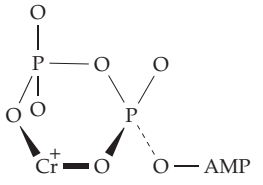
The substrate for many phosphotransferases is MgATP. Which of the possible isomers of this chelate complex is utilized by these enzymes? Since Mg^{2+} associates and dissociates rapidly from the complexes there are several possibilities: a tridentate complex with oxygens from α, β, and γ phospho groups coordinated with the metal ion, an α,β-bidentate, a β,γ-bidentate, or a monodentate complex. Most evidence suggests that β,γ-bidentate complexes of the following types are the true substrates.



Mg–ATP βS complex with Λ screw sense, *R* configuration at P



Cd–AT βS complex with Λ screw sense, *S* configuration at P

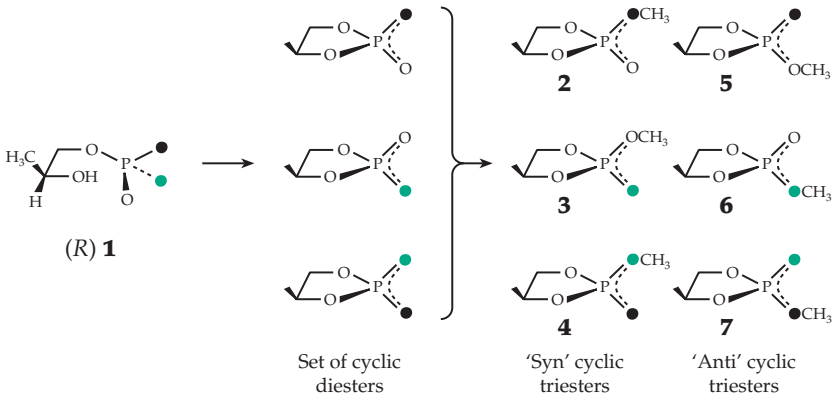


Cr–ATP complex with Δ screw sense

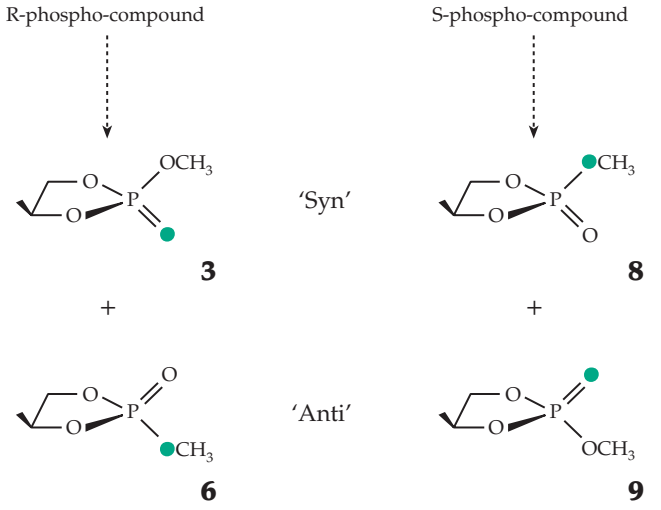
The first structure drawn is for a Mg–ATP *S* complex with a chiral β-phosphothioate group. The Mg^{2+} is expected to bond to oxygen. However, in the second complex, in which Cd^{2+} has been substituted for Mg^{2+}, it is expected that the Cd^{2+} will bond to sulfur. Therefore, the stereochemically equivalent structure will be obtained only when the compound has the *S* configuration at the phosphorus. In the third of the foregoing complexes, Mg^{2+} has been replaced by Cr^{3+} to give an **exchange-inert** complex of ATP in which the Cr^{3+} will remain attached firmly to the same two oxygen atoms under most experimental conditions. Notice also that in this complex, the AMP portion occupies a different position than in the first two complexes. It is called the Δ **screw-sense isomer**,[702] while the upper two complexes are Λ screw-sense isomers. We may reasonably expect that an enzyme, which may provide additional ligands to the metal ions, will prefer one or the other of these screw-sense isomers. For α,β-bidentate ATP complexes both α and β phosphorous atoms become chiral centers and even more isomers are possible.

The use of exchange inert Cr^{3+} and Co^{3+} complexes of ATP has been developed by Cleland and associates.[702–705] The β,γ-bidentate chromium complexes were separated into the Λ and Δ isomers.[704] Each was separated further into a pair of "ring-puckering isomers". These metal complexes are all competitive inhibitors of MgATP and both ring puckering isomers of the Δ screw sense are very slow substrates of various kinases. The Λ isomers serve as very slow substrates for pyruvate kinase, adenylate kinase, and fructose-6-phosphate kinase.[703] The Δ exo isomer is the strongest inhibitor of creatine kinase, suggesting this same conformation for the MgATP substrate. The disastereoisomers of ATPαS and ATPβS have also been tested with kinases in the presence of either Mg^{2+} or Cd^{2+}. With creatine kinase[695,706] the isomers with the R configuration at phosphorus are preferred in the presence of Mg^{2+} but those with the *S* configuration are preferred in the presence of Cd^{2+}. This might be related to the previously mentioned preferences of the metals for O vs. S ligands, and would suggest that this enzyme prefers the Λ isomer (see foregoing structures). However, EPR studies with the corresponding Mn^{2+} complexes suggest that the Δ isomer is preferred by the enzyme.[693] There are 12 isomers of monoammine Cr(III)ATP. Their use has provided information about the location of water molecules in metal complexes of kinases.[707]

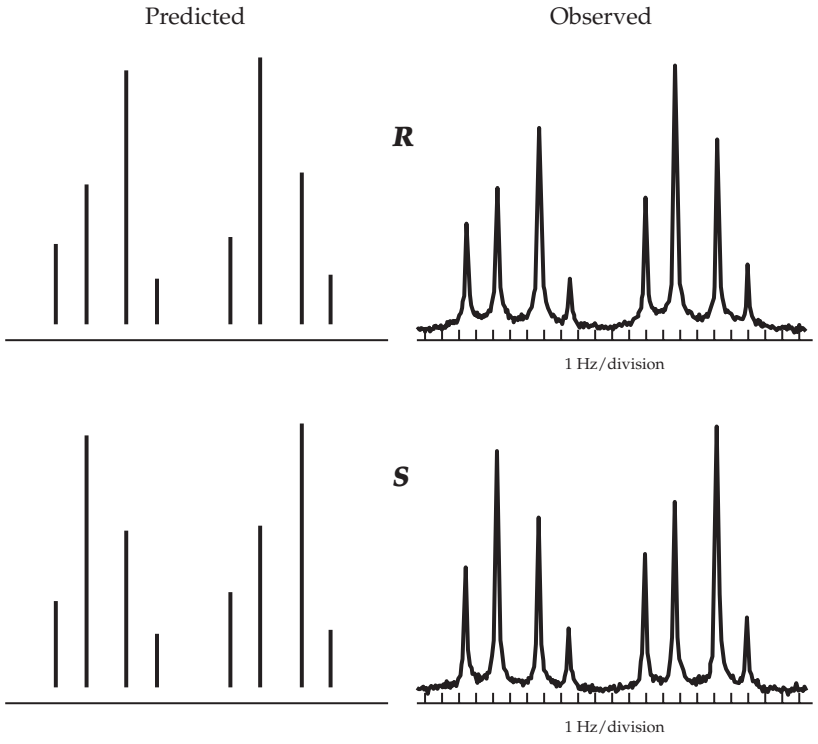
In the following sections we will consider several individual phosphotransferases.



The product 1-phospho-(*S*)-propane-1,2-diol **1** (here shown as *R* at phosphorus) is converted by in-line ring closure to an equimolar mixture of three cyclic diesters. These are methylated to give six cyclic triesters. Of these, only **3** and **6** give sharp ^{31}P resonance because the ^{17}O in the others broadens the lines.



Two cyclic triesters **3** and **6** from an *R*-phospho compound and two others **8** and **9** from an *S*-phospho compound will give sharp ^{31}P NMR resonances.

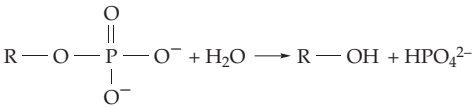


Predicted and observed ^{31}P NMR spectra of the mixtures of syn and anti cyclic triesters derived from labeled samples of 1 phospho-(*S*)-propane-1,2-diols that are *R* and *S* at phosphorus.

Figure 12-22 Method for determining chirality of phospho groups containing ^{16}O, ^{17}O, and ^{18}O. From Buchwald and Knowles.[701]

4. Phosphatases

The phosphatases catalyze hydrolysis of phosphate esters to produce inorganic phosphate:[667]

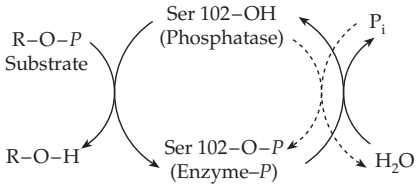


(12-37)

The **acid phosphatases** and **alkaline phosphatases** are nonspecific and cleave many different phosphate esters, whereas **glucose-6-phosphatase**, **fructose-1,6-bisphosphatase**, and many others are specific for single substrates. The nonspecific phosphatases may provide inorganic phosphate ions in places where they are needed, e.g., in mineralizing bone. All phosphatases help to drive metabolic cycles (Chapter 17).

The alkaline phosphatases are found in bacteria, fungi, and higher animals but not in higher plants. In *E. coli* alkaline phosphatase is concentrated in the periplasmic space. In animals it is found in the brush border of kidney cells, in cells of the intestinal mucosa, and in the osteocytes and osteoblasts of bone. It is almost absent from red blood cells, muscle, and other tissues which are not involved extensively in transport of nutrients.

The alkaline phosphatase of *E. coli* is a dimer of 449-residue subunits which requires Zn^{2+}, is allosterically activated by Mg^{2+}, and has a pH optimum above 8.[667,708–711] At a pH of ~4, incubation of the enzyme with inorganic phosphate leads to formation of a phosphoenzyme. Using ^{32}P-labeled phosphate, it was established that the phosphate becomes attached in ester linkages to serine 102. The same active site sequence Asp-Ser-Ala is found in mammalian alkaline phosphatases. These results, as well as the stereochemical arguments given in Section 2, suggest a double-displacement mechanism of Eq. 12-38:



(12-38)

The active site contains two Zn^{2+} ions and one Mg^{2+} ion which are held by imidazole and carboxylate groups. The inorganic phosphate in an enzyme–product complex is bound to both zinc ions (Fig. 12-23). The Ser 102 side chain is above one Zn. In the enzyme-*P* intermediate it would be linked to the phospho group as an ester which would then be hydrolyzed, reversibly, by a water molecule bound to Zn.[712–713a] This water presumably dissociates to Zn^{+}–OH and its bound hydroxyl ion carries out the displacement. This reaction may be preceded by a proton transfer to an oxygen atom of the phospho group.[714]

Acid phosphatases, which have pH optima of ~5 and are inhibited by fluoride ion, occur in bacteria, fungi, plants, and animals. In bone, the acid phosphatase content is high in the osteoclasts which function in the resorption of calcium from bone. The highest content of acid phosphatase in humans is in the prostate and an elevated serum level has long been used as a diagnostic indicator of prostatic cancer.[715] Acid phosphatase is a periplasmic enzyme in *E. coli*.[716] Phosphoenzymes have been trapped from both plant and animal acid phosphatases.[717] For example, a brief incubation with ^{32}P-labeled *p*-nitrophenyl phosphate followed by rapid denaturation in an alkaline medium gave a covalently labeled protein from which ^{32}P-containing N δ-phosphohistidine was isolated. In agreement with kinetic evidence that a phosphoenzyme is a true intermediate, there is no inversion of chiral phospho groups by liver or prostate acid phosphatases.[718]

Some acid phosphatases from animals and plants are violet in color and contain iron (Chapter 16) and an Mn^{3+}-containing acid phosphatase has been isolated from sweet potatoes.[720] These enzymes have dimetal centers, often containing one Zn^{2+} and one Fe^{3+} with bridging carboxylate and hydroxide ions between the metals. Imidazole, tyrosinate, and carboxylate side chains hold the metals as in Fig. 16-20. A water molecule bound to the Fe^{3+} is thought to dissociate with a low pK_a of 4.8 to give an $Fe^{3+} \bullet {}^{-}OH$ complex. The hydroxyl ion can then attack the phospho groups, one

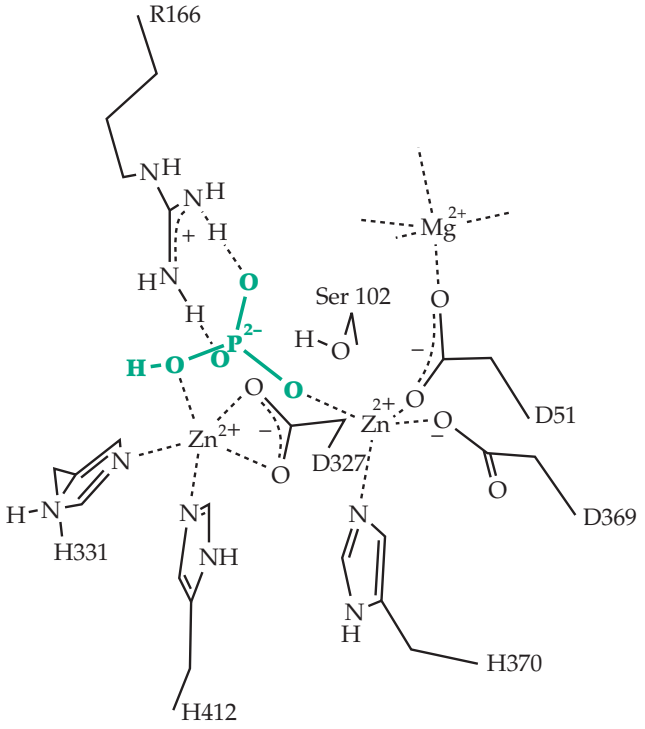


Figure 12-23 Schematic drawing of the product inorganic phosphate bound in the active site of *E. coli* alkaline phosphatase. See Ma and Kantrowitz.[719]

of whose oxygen atoms is coordinated with the Zn^{2+}.

The mechanism resembles that proposed for a **phosphotriesterase** (Fig. 12-24). The triesterase catalyzes detoxification of organophosphorus toxins such as parathion (Box 12-E) and seems to have evolved rapidly from a homologous protein of unknown function.[721] The phosphotriesterase contains two Zn^{2+} ions in a dimetal center. An unusual structural feature is a carbamate group, formed from Lys 169 and CO_2, which provides a bridging ligand for the metal pair.[721–725] A carbamylated lysine also functions in ribulose bisphosphate carboxylase (Fig. 13-11).

Phosphatases specific for such substrates as glucose-6-phosphate, fructose-1,6-bisphosphate, and phosphoglycolate help to drive metabolic cycles (Chapter 17). The 335-residue **fructose-1,6-bisphosphatase** associates to form a tetramer with D_2 symmetry.[726–730] The allosteric enzyme exists in two conformational states (see Chapter 11). Activity is dependent upon Mg^{2+} or other suitable divalent cation, e.g., Mn^{2+} or Zn^{2+}, and is further enhanced by K^+ or NH_3^+. While the dimetal sites depicted in Figs. 12-23 and 12-24 are quite rigid and undergo little change upon formation of complexes with substrates or products, the active site of fructose-1,6-bisphosphatase is more flexible. There are three metal-binding sites but they contain no histidine side chains and have been seen clearly only in a product complex.[727,728] Perhaps because of the need for flexibility involved in allosteric changes, the active site is not fully formed until the substrate binds. **Fructose-2,6-bisphosphatase** forms one domain of a bifunctional kinase-phosphatase (Chapter 11). It has two imidazole rings, as well as side chains from a glutamate and two arginine residues at the catalytic and substrate-binding site.[728a]

The 357-residue mammalian glucose-6-phosphatase plays an important role in metabolism (Chapter 17). Defects in the enzyme cause a glycogen storage disease (Box 20-D) and severe disruption of metabolism.[731] However, the molecular basis of its action is not well-known. Furthermore, the active site of the enzyme is located in the lumen of the endoplasmic reticulum[732] and glucose-6-phosphate must pass in through the plasma membrane. An additional glucose-6-phosphate transporter subunit may be required to allow the substrate to leave the cytoplasm.[73]

Pyrophosphatases, which are present in all cells, and catalyze hydrolysis of inorganic pyrophosphate (PP_i) to orthophosphate (P_i) (see Chapter 6, Section D), also drive metabolic sequences. The very active pyrophosphatase of *E. coli* has a turnover number of over $2 \times 10^4\ s^{-1}$ at 37°C. The 1000 molecules per cell are sufficient to immediately hydrolyze any pyrophosphate produced by bacterial metabolism.[733] The much studied soluble pyrophosphatases of *E. coli*,[734,735] yeast,[736] and other organisms[736a,b] are metalloenzymes that are most active with Mg^{2+}. Two Mg^{2+} ions are held, mostly by carboxylate side chains, while a third apparently enters the active site as magnesium pyrophosphate, perhaps $MgP_2O_7^-$. As with other metallohydrolases, a metal-bound hydroxyl ion may serve as the attacking nucleophile.

At least three distinct families of **protein phosphatases** remove phosphate groups from serine, threonine, and tyrosine side chains in proteins.[737] Their role in control of numerous biochemical processes has been discussed in Chapter 11, Section C,2. The catalytic domains or subunits of the protein phosphatases act together with regulatory domains or separate regulatory subunits to control thousands of reactions. For example, protein phosphatase 1 (PP1) together with a glycogen-targeting subunit dephosphorylates inactive glycogen kinase (see Fig. 11-4). Belonging to the same family is **calcineurin** (PP2B), a phosphatase activated by Ca^{2+} through binding to calmodulin (Box 6-D). There are two families of Ser/Thr phosphatases. Their polypeptide folding patterns differ, but the active sites have similar dimetal centers resembling those in Figs. 12-24 and 16-20 with $Mn^{2+} + Fe^{2+}$, $Zn^{2+} + Fe^{3+}$, and probably other pairs of metals.[737–740] The family containing PP1 has weak sequence homology with the purple acid phosphatases.[740] Another common feature of these enzymes is a conserved His-Asp dyad (His 125 and Asp 95 in PP1) which is thought to be a proton donor, protonating the leaving group ($-O^-$) in a manner

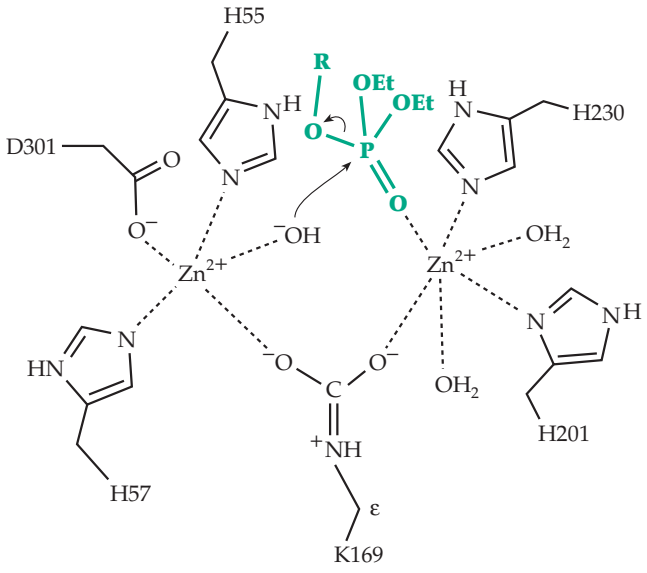


Figure 12-24 Hypothetical event in the action of a phosphotriesterase. A carbamylated lysine (lower center), as well as a water molecule, bridge the two Zn^{2+} ions, which are held by imidazole and aspartate carboxylate groups. The bound H_2O can be deprotonated to give the HO^- complex shown. The substrate may displace the HO^- ion from the right-hand zinc and thereby move close to the bound HO^- which attacks as indicated. Based on Cd^{2+}-containing structure and discussion by Benning *et al.*[722]

reminiscent of the serine proteases. The reaction is evidently initiated by attack of an $^{-}$OH ion held by the dimetal center. This would resemble the mechanism pictured in Fig. 12-24 except that the phospho group would carry two negative charges.

The protein tyrosine phosphatases also exist as several families with numerous functions in control of transcription, growth, differentiation, and metabolism.[741–743] These enzymes function by a double-displacement mechanism, as in Eq. 12-38, but with a cysteine side chain rather than serine. The cysteine is present in the conserved sequence (H/V)**C**X_5**R**(S/T). The arginine binds the phospho group and helps to stabilize the transition state, which probably is metaphosphate-like.[742]

5. Ribonucleases (RNases)

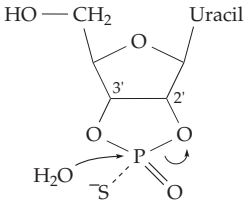
Many hydrolases act on phosphodiester linkages, which abound in nature.[725,744] Some are digestive enzymes but others serve more specific metabolic functions. **Ribonuclease A** (**RNase A**), the pancreatic digestive enzyme responsible for breakdown of RNA, was one of the first enzymes for which a structure was deduced. By 1963 Moore and Stein and their associates, who had earlier developed ion exchange methods for separating amino acids and peptides (Fig. 3-6), had determined the sequence of the 124-residue bovine enzyme.[745] They observed that Lys 41 was unusually reactive with dinitrofluorobenzene and that photooxidation of His 12 and His 119, which are almost at opposite ends of the peptide chain, inactivates the enzyme. They concluded that both histidines are at the active site, a conclusion that was later substantiated by X-ray crystallography.[746–748] A segment 12 nucleotides in length can fit into the cleft in the enzyme that contains the active site. The negatively charged phosphates of the RNA backbone form 8 – 9 electrostatic bonds to lysine and arginine side chains of the enzyme.[749,750] However, the only close interactions of the nucleic acid bases with the enzyme occur at the site of cleavage as shown in Fig. 12-25. The four residues His 12, Lys 41, Thr 45, and His 119 are strictly conserved in the RNAse A superfamily.[749] The carboxylate of Asp 121 apparently helps to orient the proper tautomer of His 119 for catalysis.[751]

Ribonuclease A was the first enzyme to be synthesized in the laboratory. Fully active ribonuclease has been synthesized,[752] as have new modified enzymes. For example a 63-residue peptide made up of five segments of the native RNase sequence retained measurable catalytic activity.[753] Using total synthesis, unnatural amino acids, such as 4-fluorohistidine, have been incorporated at specific positions in RNAse.[752]

Cleavage of a phosphodiester linkage in the substrate chain occurs in two steps. In the first or *transesterification* step, the hydroxyl group on the 2' position of the ribose ring is thought to be deprotonated by attack of either the imidazole of His 12, as shown in Fig. 12-25, or by the adjacent amino group of Lys 41. (In the latter case His 12 would have to remove a proton from the $-NH_3^+$ group of Lys 41 before it could attack.) In either case the positive charge of Lys 41 would help to neutralize the negative charge on the phosphate. The deprotonation of the 2'–OH may occur synchronously with its attack on the adjacent 3' phospho group. An in-line displacement of the oxygen attached to the 5' carbon of the next nucleotide unit is thought to be assisted by His 119. Its protonated imidazolium group may transfer a proton to a phosphate oxygen atom prior to or synchronously with formation of the new P–O bond in step *a* (Fig. 12-25).[754] The intermediate formed in step *a* is a cyclic 2', 3'-diphosphate which then undergoes hydrolysis by attack of a water molecule in step *b* to give the free nucleoside 3'-phosphate. The overall reaction is a two-step double-displacement, analogous to that with chymotrypsin, except that a neighboring group in the substrate rather than an amino acid side chain is the nucleophilic catalyst. The pH dependence of the enzyme is in agreement with this mechanism because there are two pK_a values of ~5.4 and ~6.4 which regulate the catalytic activity. Microscopic pK_a values of His 12 and His 119 have been measured by NMR spectroscopy as ~ 6.1 and ~ 6.3 and are shifted somewhat by binding of nucleotides.

A bacterial peptidase splits a 20-residue fragment containing His 12 from the N-terminal end of RNase A. This "S-peptide" can be recombined with the rest of the molecule, which is inactive, to give a functional enzyme called ribonuclease S. In a similar way, residues 119–124 of RNase A can be removed by digestion with carboxypeptidase to give an inactive protein which lacks His 119. In this case, a synthetic peptide with the sequence of residues 111 – 124 of RNase A forms a complex with the shortened enzyme restoring full activity.[755]

Stereochemical studies support in-line mechanisms for both the transesterification and hydrolysis steps of ribonuclease catalysis. For example, chiral uridine 2',3'-cyclic phosphorothioates are hydrolyzed with inversion of configuration, with the diastereoisomer shown yielding a 2'-monophosphothioate of the *R* configuration at phosphorus.



Uridine 2',3'-cyclic phosphorothioate

Transesterification step is also in-line[757] as it is shown in Fig. 12-25. Study of kinetic isotope effects in H_2O–D_2O mixtures suggested that two protons may move synchronously as the enzyme–substrate complex passes through the transition state.[758] Although RNase A is one of the most studied of all enzymes, there are still uncertainties about the mechanism. Is a proton removed first by His 12 (Fig. 12-25), as has long been assumed, or is a proton transferred first from His 119 to the oxygen of the phospho group?[759,760] Is the reaction concerted, as suggested by kinetic isotope effects,[761] or is there a pentacovalent intermediate?

The specificity of RNase A for a pyrimidine on the 3' side of the phosphodiester bond that is cleaved is evidently ensured by the pair of hydrogen bonds from O-2' of the pyrimidine to the backbone NH of Thr 45 and a second from the N-4' proton to the side chain OH of the same threonine (Fig. 12-25). Other nucleases, such as ribonuclease T_2,[762] with different specificities also make use of hydrogen bonding of the base at the 3' side of the cleavage point with backbone amide groupings.

Various bacterial ribonucleases as well as the fungal ribonucleases T_1, U_1, and U_2 (see also Fig. 5-43) have amino acid sequences related to that of RNase A[763,764,764a] but with distinctly different three-dimensional structures. The active sites contain Glu, His, and Arg side chains. For RNase T_1, Glu 58 and His 92 appear to provide acid–base catalysis with assistance from Tyr 38, Arg 77, and His 40.[763,765] A glutamate carboxylate also appears to be the catalytic base in the related RNase, called **barnase**, from *Bacillus amyloliquefaciens*.[766]

In addition to extracellular digestive enzymes, the RNase family contains many intracellular enzymes that are involved in turnover of RNA.[767,768] RNase H digests away RNA primers during DNA synthesis (Chapter 27). RNase H activity is also present in a domain of viral reverse transcriptases and is absolutely essential for the replication of HIV and other retroviruses.[769] The structures of the reverse transcriptase RNase H domain and of the *E. coli* enzyme are similar.[769–771] Unlike RNase A, the RNases H are metalloenzymes which apparently contain two Mg^{2+} ions held by carboxylate groups and utilize a metal bound HO^- ion as in previously discussed phosphatases. Secreted RNases sometimes have specific functions. For example, the 123-residue **angiogenin** is homologous to pancreatic RNases but acts to induce formation of new blood vessels (angiogenesis).[772–774] This is essential to growth of solid cancers as well as for normal growth. The enzyme is a very poor catalyst but its RNase activity appears essential for its biological function. Mutation of any of the catalytic residues His 13, Lys 40, or His 114 abolishes all angiogenic activity. A neurotoxin secreted by eosinophils[775] is one of a group of selectively toxic RNases.[776,777] Intracellular RNases are often found as complexes of specific inhibitor proteins.[778]

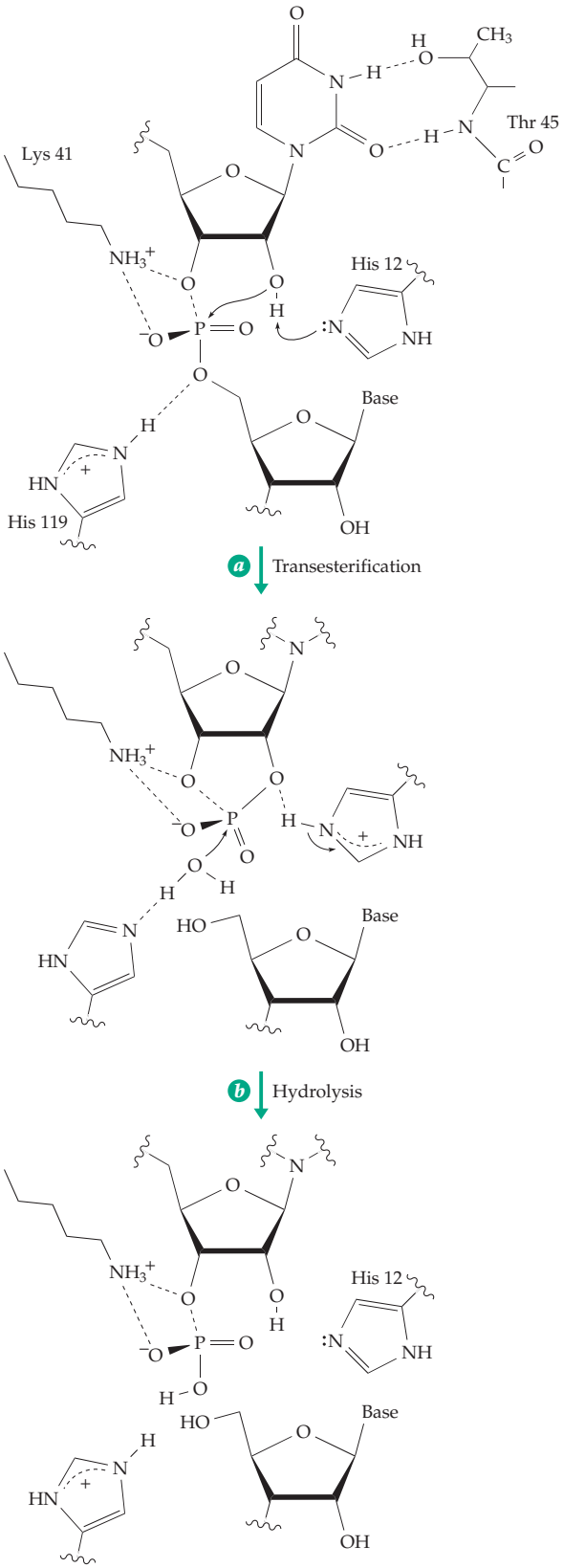


Figure 12-25 Proposed two-step in-line reaction mechanism for ribonuclease A. The hydrogen bonding that provides recognition of the pyrimidine base at the 3' end created by the cleavage is also shown. See Wladkowski *et al.*[756]

6. Ribonuclease P, Ribozymes, and Peptidyl Transferase

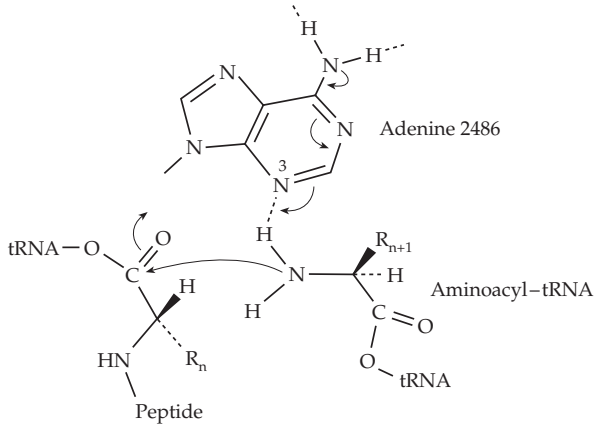
A very different ribonuclease participates in the biosynthesis of all of the transfer RNAs of *E. coli*. **Ribonuclease P** cuts a 5' leader sequence from precursor RNAs to form the final 5' termini of the tRNAs. Sidney Altman and coworkers in 1980 showed that the enzyme consists of a 13.7-kDa protein together with a specific 377-nucleotide RNA component (designated M1 RNA) that is about five times more massive than the protein.[779] Amazingly, the M1 RNA alone is able to catalyze the ribonuclease reaction with the proper substrate specificity.[780–782a] The protein apparently accelerates the reaction only about twofold for some substrates but much more for certain natural substrates. The catalytic center is in the RNA, which functions well only in a high salt concentration. A major role of the small protein subunit may be to provide counterions to screen the negative charges on the RNA and permit rapid binding of substrate and release of products.[783] Eukaryotes, as well as other prokaryotes, have enzymes similar to the *E. coli* RNase P. However, the eukaryotic enzymes require the protein part as well as the RNA for activity.[784]

Thomas Cech and associates independently discovered another class of catalytic RNA molecules. These are **self-splicing RNAs** that cut out intervening sequences from themselves to generate ribosomal RNA precursors (see Chapter 28).[785–787] They act only once and are therefore not enzymes. However, the introns that are cut out during self-splicing are **ribozymes** which, like the RNA from ribonuclease P, can act catalytically and have properties similar to those of protein enzymes. They exhibit the kinetic properties of enzymes and are denatured by heat. The RNAs are folded into compact structures resembling those of globular proteins. Like tRNA, they contain loops and hydrogen-bonded stems. Phylogenetic comparisons (Chapter 29) of the M1 RNA of ribonuclease P isolated from various species have allowed prediction of precise secondary structures.[783,788,789] A simplified M1 RNA consisting of 263 nucleotides from conserved regions of the molecule is catalytically effective.[783] Tetrahymena ribozyme also has a complex structure with a 247-nucleotide catalytic core formed by two structural domains (Fig. 12-26).[790,791] The crystal structure of a third ribozyme, one found in the RNA of the human pathogen hepatitis delta virus (HDV), has also been determined.[792] It is a smaller 72-nucleotide self-cleaved molecule with a very different structure from that in Fig. 12-26. It makes use of a double pseudoknot (see Fig. 5-29) to bind the RNA into a compact, tightly hydrogen-bonded structure with a deep active site cleft. It is the fastest known naturally occurring self-cleaving RNA and is able to react at a rate of more than 1 s^{-1} at its optimum temperature of 65°C.

Smaller self-cleaving RNAs have been found among plant viruses and viroids. Many of them have a common catalytic core which can be converted into 30- to 40-nucleotide ribozymes. Only 17 nucleotides and three hydrogen-bonded helical stems are required to form the self-cleaving "hammerhead" domain, which has a structural similarity to the catalytic core of the *Tetrahymena* ribozyme. The **hammerhead ribozymes** (Fig. 12-27) represent one form of small ribozyme.[793–797] Another is the **hairpin ribozyme** shown in Fig. 12-28,[798,801] which also shows the even smaller lead-dependent "**leadzyme**," a ribozyme that doesn't occur in nature.

In an intact viral self-cleaving RNA the entire catalytic center is formed from a single strand. Stems II and III of the hammerhead ribozyme (Fig. 12-27C) are closed by large loops. In the ribozyme shown, loop III has been cut off and stem II has been closed by a tight loop to form a compact catalytic RNA that will cut a substrate having a suitable nucleotide sequence for binding to the ribozyme. Only 12 bases in this ribozyme are highly conserved. By varying the sequences in the ribozyme half of stems I and III, catalysts that cleave after any sequence GUX, where X=A, C, or V, can be designed. Such catalysts are useful in the laboratory and potentially also in medicine.

What groups of a ribozyme bind to substrates and what groups participate in catalysis? Like peptides, RNAs have amide groups that can hydrogen bond to substrates. Adenine and cytosine can supply protonated amino groups which could participate in acid–base catalysis. This is evidently the case in the **peptidyl transferase** centers of ribosomes. The RNA in these centers catalyzes a transesterification in which an aminoacyl group is transferred from an aminoacyl-tRNA anto the growing polypeptide chain attached to a second tRNA molecule. The reaction is evidently catalyzed by a universally conserved adenine ring located at position 2486 in the *Haloarcula marismortii* 23S RNA (position 2451 in *E. coli*). There are no protein groups within 1.8 nm of the location of peptide bond synthesis.[798a] The active site adenine appears to be much more basic than normal. A high pK_a of 7.6 controls the peptidyl transferase, and also controls the methylation of the active site adenine by dimethylsulfate.[798b] The site of protonation is thought to be largely N3 of adenine 2486, which is probably the basic center involved in catalysis. The peptidyltransferase reaction may be initiated as follows.[798a]



A similar catalytic mechanism is probably used by the small 85-nucleotide hepatitis delta virus ribozyme whose catalytic base is thought to be N3 of cytosine 75, which is associated with a pK_a of ~6.1.[798c] Both this HDV ribozyme and the ribosomal RNA resemble serine proteases with histidine as the catalytic base. However, the self-splicing RNA of *Tetrahymena* initiates a nucleophilic attack with the 3'–OH group of a guanosine molecule that is bound to a site in the P7 region (Fig. 12-16A) and which acts as a cofactor (see Fig. 28-18). Ribonuclease P and all group I and II self-cleaving introns also use an external nucleophile such as a guanosine –OH and form 3'-OH and *O*-phosphate or

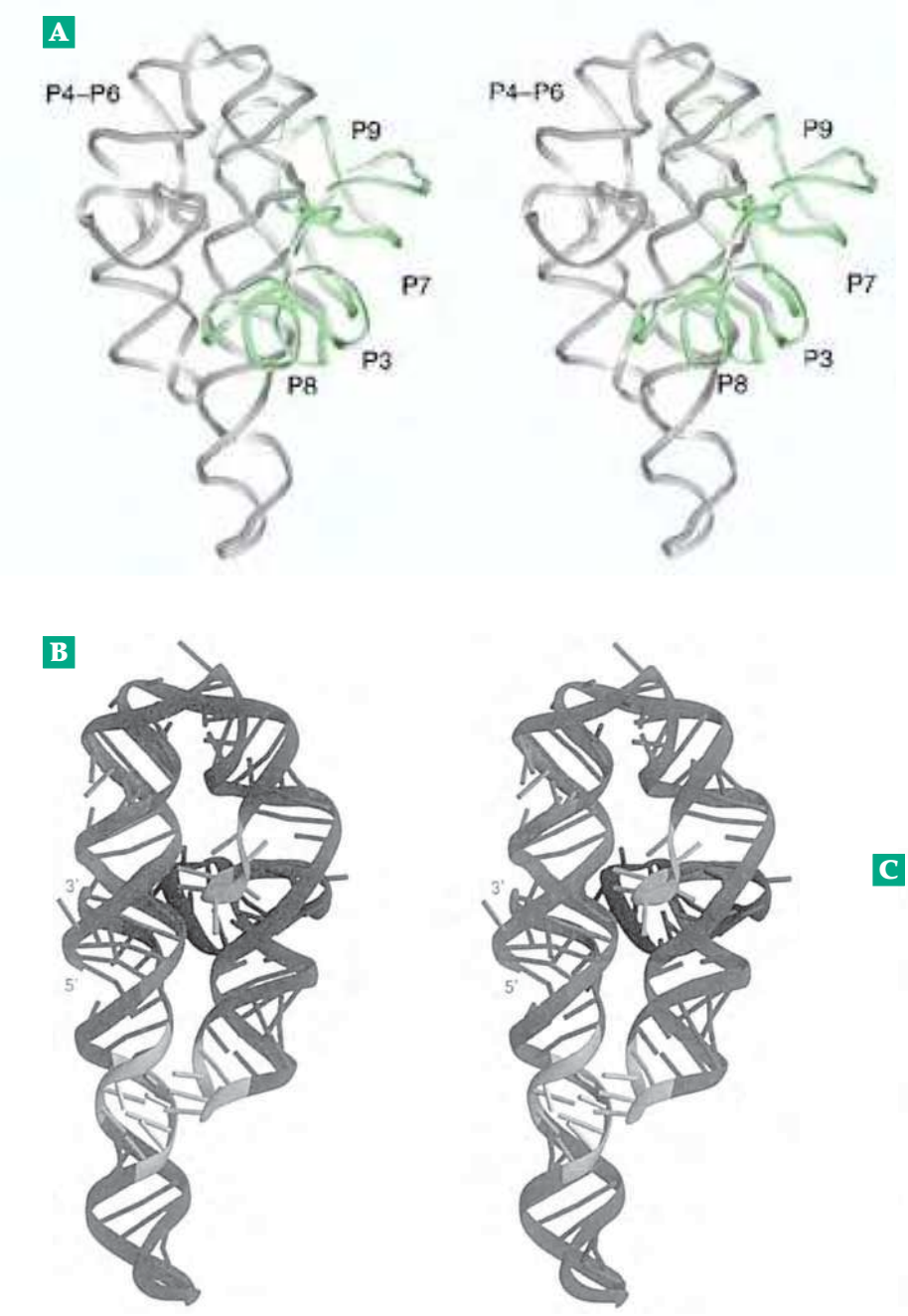


Figure 12-26 (A,B) Stereoscopic views of the *Tetrahymena* ribozyme. (A) Ribbon tracing of the phosphodiester backbone. The successive conserved structural elements, from the 5' and 3' ends are designated P1 to P9. The P4 – P6 region forms one major domain, while the P3 and P5 – P9 regions form a second domain which folds around the first. From Golden *et al.*[790] (B) Structure of the P4 – P6 domain viewed from the back side as pictured in (A). From Cate *et al.*[791] The active site lies between this domain and the larger one which folds over it leaving the active site in a deep crevice.[790] The active site is centered above the "tetraloop" GAAA (residues G150 to A153), which is shown with lighter shading (in B) near the upper center of the P4 – P6 domain. (C). Details of one of three layers of hydrogen-bonded interactions between purine and pyrimidine bases involved in interaction of the tetraloop with the adjacent helix in the P5 region. Cleavage occurs in the P1 domain, which folds into the active site and is not shown in these drawings. Courtesy of Thomas Cech.

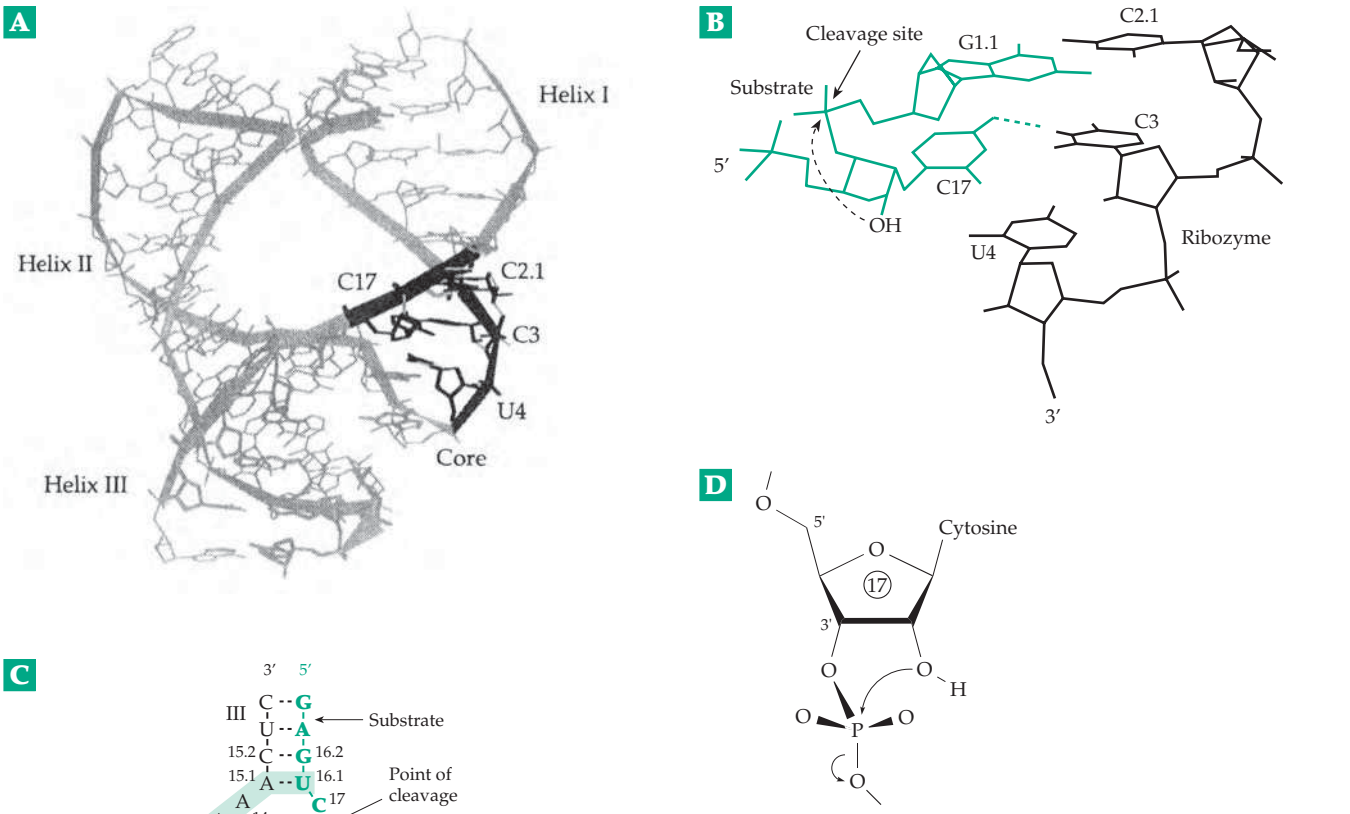


Figure 12-27 (A) Structure of a hammerhead ribozyme. The cleavage site region is drawn with dark lines. (B) The cleavage region showing the cleavage site in the substrate strand. From Baidya and Uhlenback.[793] (C) Diagram of a hammerhead ribozyme with standard numbering of nucleotides. The three helical stems are labeled I, II, and III. From Bevers *et al.*[796] (D) Simplified cleavage mechanism which resembles step *a* of Fig. 12-25 and ignores the known participation of metal ions.

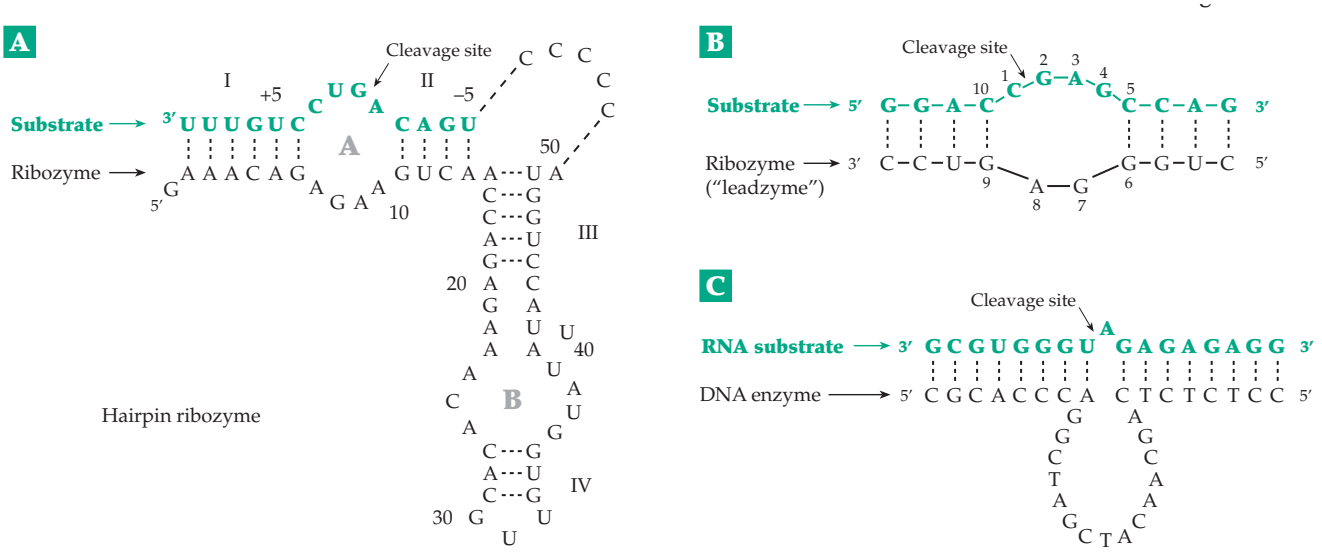
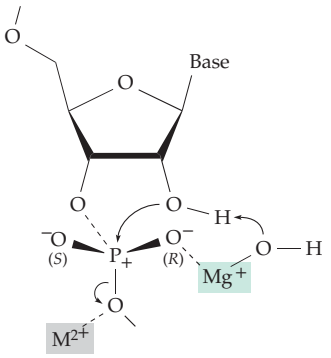


Figure 12-28 (A) A hairpin ribozyme formed from the minus strand of a satellite RNA associated with tobacco ringspot virus. On the basis of hydroxyl radical footprinting (see Fig. 5-50), to identify protected areas a folding pattern that brings domains A and B together to form a compact catalytic core has been proposed.[798] (B) A "leadzyme," a ribozyme dependent upon Pb^{2+} for cleavage of RNA.[802,803] (C) An RNA-cleaving DNA enzyme.[804]

phosphodiester ends at the cleavage points. Hammerhead ribozymes utilize the 2'-OH of the ribose at the cleavage site to form a 2',3'-cyclic phosphate ester as in step *a* of the ribonuclease A reaction (Fig. 12-25) and as indicated in Fig. 12-27D. Ribozymes act by in-line mechanisms, causing inversion of the configuration at the phosphorus.[799,800]

Most ribozymes, as well as RNase P,[805,806] require one or two metal ions for activity.[807] Magnesium ions predominate and many Mg^{2+} ions are bound at distinct sites in crystalline ribozymes. Hammerhead and hairpin ribozymes work reasonably well with monovalent ions. One proposed mechanism is for an HO^- ion bound to the Mg^{2+} to remove H^+ from the 2'– OH of the ribose ring as follows:



When the *pro-R* oxygen of the phospho group (labeled *R* in the accompanying structure) was replaced by sulfur the rate of cleavage decreased 10^3-fold. However, the rate was restored fully when Cd^{2+} was added, presumably because of the high affinity of cadmium ions for sulfur ligands.[808] This supports the possibility of a dual role for Mg^{2+} in activating a water molecule to provide HO^- and in stabilizing negative charges on the phospho group by interaction with the *pro-R* oxygen in the transition state. Several investigators have suggested that *two* metal ions may be needed. One possibility is for a mechanism similar to that proposed for alkaline phosphatase (Fig. 12-25) and other phosphotransferases.[809,810] The *Tetrahymena* ribozyme functions best if both Mg^{2+} and Mn^{2+} are present.[811] A second metal may act as a Lewis acid facilitating loss of the 5'-OH as indicated by M^{2+} (in gray) in the preceding diagram. Metal ions may also participate in conformational changes as well as have structural functions in ribozymes.[812,813] Studies of the kinetics of action of hammerhead ribozymes have suggested that the intrinsic ribozyme-substrate binding energy is utilized for catalysis.[814] This may be possible because the ribozyme is only partially folded in the ground state but it folds into a tighter conformation in the transition state.[815]

Methods have been devised for generating enormous numbers of RNA molecules with random sequences and for selecting those with unusual catalytic activities.[816–819] Among the new catalysts produced in this way are very small ribozymes that cleave RNA specifically in the presence of Pb^{2+} (Fig. 12-28B).[802,803] The leadzyme is more active with neodymium (Nd^{3+}) + Pb^{2+} than with lead alone, suggesting a two-metal mechanism.[820] Other artificial ribozymes include RNA ligases,[817] acyltransferases,[821] and DNA hydrolases.[822,823] Is it possible to find a DNA enzyme? Without the 2'–OH of ribose to form hydrogen bonds it seemed doubtful, but an RNA-cleaving DNA enzyme has been selected from a population of ~10^{14} different small DNA molecules. The DNA enzyme (Fig. 12-28C) will cleave RNA, whose sequence fulfills the base pairing requirements of two 8-deoxynucleotide recognition domains. Cleavage occurs between an unpaired purine and a paired pyrimidine using a metal-dependent mechanism that gives a 2',3'-cyclic phosphate as in ribonuclease A cleavage.[804]

7. Deoxyribonucleases (DNases)

A multitude of nucleases cleave DNA, single- or double-stranded. They range from the pancreatic digestive enzyme DNase I through specialized nucleases that function during DNA repair and the hundreds of restriction endonucleases that have become so valuable in modern laboratory work. Some nucleases leave a 3'-phosphate ester at a cut end in a DNA chain, while others leave a 5'-phosphate end.[824] Many nucleases are dealt with in later chapters. Only a few will be mentioned here.

One of the most studied enzymes of this group is the 149-residue micrococcal (staphylcoccal) nuclease from *Micrococcus* which cleaves either RNA or single- or double-stranded DNA. The relatively nonspecific enzyme cuts nearly randomly at the 5' side of the phosphodiester linkages, leaving 3'-phosphate groups. It enhances the uncatalyzed hydrolysis rate at least 10^{16}-fold.[825–827] The crystal structure showed that the majority of the acidic and basic side chains of the protein interact with each other through clusters of hydrogen bonds. At the active site the side chains of both Arg 35 and Arg 87 form pairs of hydrogen bonds to the 5'-phosphate group of the specific inhibitor deoxythymidine 3',5'-diphosphate (Fig. 12-29). While Arg 87 appears to be in a position to protonate the leaving group $-O^-$, ^{13}C NMR experiments showed that all of the arginine side chains had pK_a values above 11.6. However, Tyr 85 has a pK_a of 9.5, which appears to control k_{cat}/K_m.[828] A Y85F mutant lacks this pK_a. The X-ray structure also suggests that Glu 43 may be the attacking nucleophile and that it may deprotonate a water molecule bound to the Glu 43 carboxylate. The resulting HO^- probably carries out a direct in-line attack as shown in Fig. 12-29. Mutants such as E43D, E43Q, and E43S have greatly decreased activity,[829] in

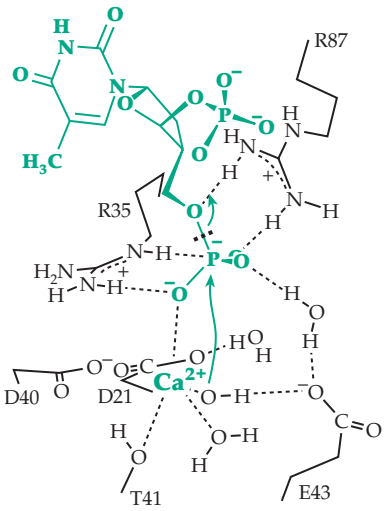


Figure 12-29 Drawing showing the hydrogen-bonding interactions between the guanidinium ions of arginines 35 and 87 of the micrococcal (staphylococcal) nuclease with the 5'-phosphate of the inhibitor thymidine 3',5'-diphosphate in the complex of E + I + Ca^{2+}. A possible mechanism is illustrated. A hydroxyl ion bound to Ca^{2+} carries out an in-line attack on the phosphorus. See Libson *et al.*[826]

agreement with this mechanism. The nearby Ca^{2+} is essential. For this nonspecific nuclease there is no hydrogen bonding of a purine or pyrimidine base of the substrate to the enzyme.

The digestive enzyme, pancreatic DNAse I, makes single-stranded cuts in double-stranded (ds) DNA. An exposed strand of peptide chain from the enzyme binds into the minor groove of B-type DNA.[830] Because this groove becomes too narrow in long (A+T) rich sequences, they are cleaved slowly. Certain hypersensitive sites are cleaved very rapidly, perhaps because the DNA at these regions is bent or is able to bend to give a very good fit to the enzyme active site. A histidine which is hydrogen bonded to a nearby carboxylate of a glutamate side chain appears to be a catalytic base that acts upon a water molecule as in phospholipase A (Section D,10), displacing the 3' oxygen of the phosphodiester linkage. An imidazolium group from a second histidine is hydrogen bonded to an aspartate carboxylate and a tyrosine –OH to form a catalytic triad that can protonate the 3' $-O^-$ as it is displaced.[831] Two Mg^{2+} ions are also required. Both are held by different carboxylate side chains and may also interact with oxygen atoms of the phospho group to neutralize charge and stabilize the transition state.

In contrast to DNAse I, the **restriction endonucleases**, which are discussed in Chapter 5, Section H,2 and in Chapter 26, have precise substrate sequence specificities. Three of the best known restriction endonucleases are called *Eco*RI,[832–834] an enzyme which binds to and cuts both strands of the palindromic sequence 5'-GAATTC; *Eco*RV,[835] which cuts both strands in the center of the sequence 5'-GATATC; and *Bam*H1, which binds to the sequence 5'-GGATCC and cleaves after the 5' G on each strand.[836] A high-resolution structure is also known for *Cfr*10I, which recognizes the less strict sequence 5'-PuCCGGPy and cleaves both strands after the 5' Pu.[837] All of these enzymes require Mg^{2+} and have active sites containing carboxylate groups. Two-metal mechanisms have been suggested.

Restriction endonuclease *Eco*RI is able to cut a chain in dsDNA which has a chiral phophorothioate group at the specific cleavage site.[838] The reaction occurs with inversion of configuration at phosphorus, suggesting direct in-line attack by a hydroxyl ion generated from H_2O.

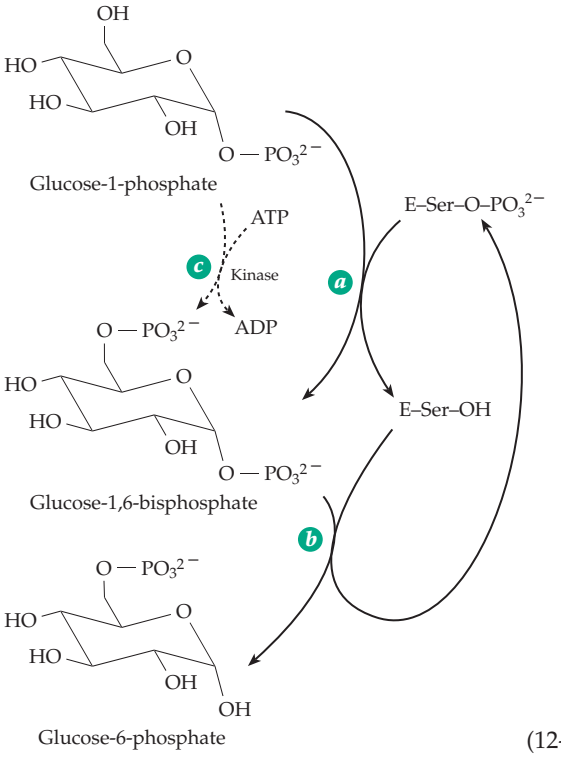
Attempts are being made to design semisynthetic restriction endonucleases specific for single-stranded DNA or RNA. For example, an oligonucleotide with a sequence complementary to a sequence adjacent the linkage that is to be cut can be covalently linked to a relatively nonspecific nuclease. Such an enzyme derived from micrococal nuclease cuts a single-stranded chain of either DNA or RNA adjacent to the double-stranded region of the ES complex.[839]

8. Mutases

Phosphotransferases that shift phospho groups from one position within a substrate to another are often called **mutases**. For example, **phosphoglucomutase** catalyzes the interconversion of glucose 1-phosphate and glucose 6-phosphate, an important reaction that bridges glycogen metabolism and glycolysis (Fig. 11-2). This 561-residue protein operates through formation of an intermediate **phosphoenzyme**.[840–842] The phospho group becomes attached to the OH of Ser 116 and can be transferred either to the 6 or the 1 position of a glucose phosphate (step *a* and reverse of step *b* in Eq. 12-39).

The two-step reaction accomplishes the reversible isomerization of glucose 1-phosphate and glucose 6-phosphate via **glucose 1,6-bisphosphate**. Evidently, the glucose bisphosphate, without leaving the enzyme is reoriented to allow transfer of the phospho group to either the 1- or 6-position.[841] The phospho enzyme is relatively unstable and can undergo hydrolysis to free enzyme and P_i. To prevent loss of active enzyme in this way, a separate reaction (catalyzed by a kinase; Eq. 12-39, step *c*)[843] generates glucose 1,6-bisphosphate, which rephosphorylates any free enzyme formed by hydrolysis of the phospho enzyme. The glucose 1,6-bisphosphate can be regarded as a cofactor or **cosubstrate** for the reaction.

Phosphoglycerate mutase, which interconverts 2-phosphoglycerate and 3-phosphoglycerate in glycolysis (Fig. 10-3, step *c*), functions by a similar mecha-



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nism.[843–845] However, the enzyme-bound phospho group is carried on an imidazole group. The essential cosubstrate required by some phosphoglycerate mutases is 2,3-bisphosphoglycerate.[845] It is formed from the glycolytic intermediate 1,3-bisphosphoglycerate (Fig. 10-3) by action of another mutase, **bisphosphoglycerate mutase**.[843,846]

1,3-bisphosphoglycerate → 2,3-bisphosphoglycerate (12-40)

This is also the pathway for synthesis of 2,3-biphosphoglycerate in red blood cells where it serves as an important allosteric regulator (Chapter 7). Two human phosphoglycerate mutase isoenzymes are known. One is found in muscle and the other in brain and other tissues.[844] A hereditary lack of the muscle type enzyme is one of the known types of glycogen storage diseases (Box 20-D).

Human **phosphomannomutase**, which catalyzes the interconversion of mannose 1- and 6- phosphates, appears to carry the phospho group on an aspartate side chain in the sequence **D**XDX (T/V), which is conserved in a family of phosphomutases and phosphatases.[847] The first aspartate in the sequence is phosphorylated during the enzymatic reaction.

9. Molecular Properties of Kinases

Kinases transfer phospho groups from polyphosphates such as ATP to oxygen, nitrogen, or sulfur atoms of a second substrate.[848] Examples include **hexokinase**, the enzyme responsible for synthesis of glucose 6-phosphate from free glucose and ATP (Fig. 11-2, step *a*); **phosphofructokinase**, which forms fructose 1,6 bisphosphate in the glycolysis pathway (Fig. 11-2, step *b*); and **phosphoglycerate kinase**, and **pyruvate kinase**, both of which form ATP from ADP in the glycolysis pathway (Fig. 10-3, steps *b*, *c*, and *f*). There are many others. Kinases vary greatly in size and in three-dimensional structure. For example, a small **adenylate kinase**, which phosphorylates AMP to ADP (Eq. 6-65), is a 22-kDa monomer of 194 residues. Pyruvate kinase is a tetramer of 60-kDa subunits and muscle phosphofructokinases are tetramers of 75- to 85-kDa subunits. The three-dimensional structures also vary. While all kinases consist of two domains built around central β sheets (Fig. 12-30), there are several different folding patterns.[849] The two-domain structures all have deep clefts which contain the active sites. Both adenylate kinase (Fig. 12-30) and hexokinase crystallize in two or more forms with differing conformations.[850] This and other evidence suggests that as a kinase binds and recognizes its two correct substrates, the active site cleft closes by a hinging action that brings together the reacting molecules in the correct orientation.[851–853] In the crystal structure shown in Fig. 12-30 both ADP and AMP are bound in a nonproductive complex. If the ADP were replaced with ATP (or the AMP with a second ADP) to form a productive complex the two reacting phospho groups would be ~0.8 nm apart. A reaction could not occur without further closing of the active site cleft.[854] Evidence for domain closure has been obtained for many other kinases. For example, substrate complexes of phosphoglycerate kinase have been crystallized in both "open" forms and "closed" forms in which a 30° hinge-bending movement has brought the ligands together for an in-line phospho group transfer.[855]

For many enzymes an ATP binding site has been revealed by study of nonhydrolyzable analogs of ATP such as "AMP-PNP" whose structure is shown in Fig. 12-31.[856] AMP-PNP has been used in thousands of investigations of ATP-dependent processes.[857] For example, the structure of a phosphoglycerate • Mg^{2+} • AMP-PNP complex in the active site of phosphoglycerate kinase has been determined.[858] Modeling of a transition state complex indicates that all three negatively charged oxygens of the ATP portion of structure are stabilized by hydrogen bonding.[852] Related analogs such as AMP-PCH_2P (Fig. 12-3) have been used in similar ways.[859] Another analog Mg^{2+} • Ap_5A (Fig. 12-31) is a bisubstrate inhibitor which binds to adenylate kinases, fixing the enzymes in a closed conformation that is thought to resemble the transition state.[859,860]

The Mg^{2+} complex of ATP is regarded as the true substrate for kinases. The metal usually also binds both to the phospho groups of ATP and to groups on

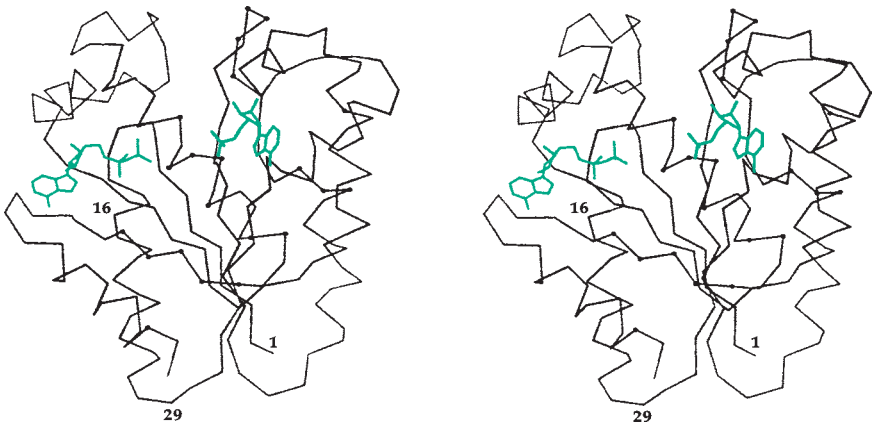


Figure 12-30 Stereoscopic α-carbon plots of a 194-residue subunit of adenylate kinase from the archaebacterium *Sulfolobus acidocaldarius* with ADP (left side) and AMP (right side) bound into the active site. From Vonrheim *et al.*[854] Courtesy of G. E. Schulz.

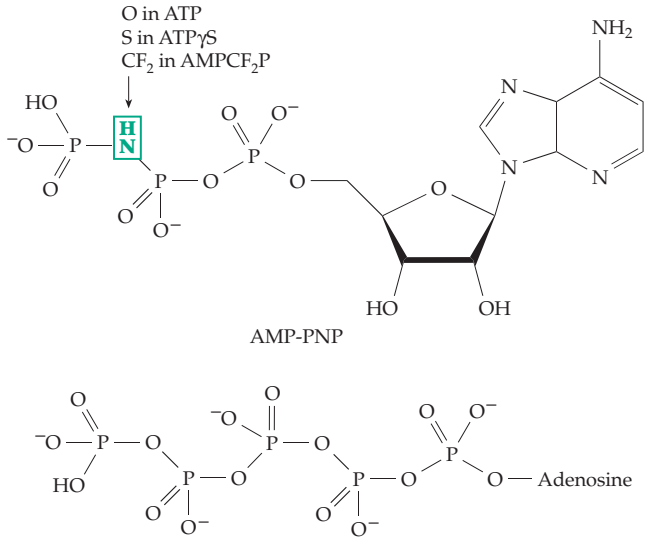
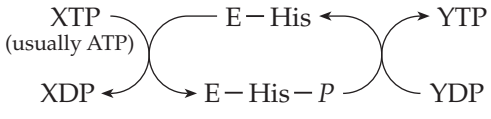


Figure 12-31 Some useful analogs of ATP.

the enzyme. Crystallographic investigations as well as studies with exchange-inert ATP complexes (Section 2) have suggested that the metal ion is bound initially to the terminal (γ) and adjacent (β) phospho groups of ATP. However, this metal bridge could prevent rather than assist the reaction. For some phosphotransferase reactions with exchange-inert complexes the product appears to be the α,β-bidentate complex of ADP (see Section 2) suggesting that movement of the metal ion from the β,γ- to the α,β-bidentate ATP complex may occur either prior to or during transfer of the phospho group. Theoretical calculations also suggest a movement in the metal complexation as the reaction progresses.[861] EPR studies that involved observation of hyperfine coupling between ^{17}O in substrates and Mn^{2+} in the active site led Reed and Leyh to conclude that the activating metal ion is bound to all three phospho groups in the transition state.[849]

Adenylate kinase performs the essential function of recovering AMP formed by many enzymatic processes and converting it to ADP (Eq. 6-65) which can be reconverted to ATP by oxidative or substrate level phosphorylation. The enzyme is present in all organisms. In vertebrates different isoenzymes function in the cytosol, mitochondrial intermembrane space, and mitochondrial matrix.[862,863] A group of other **nucleotide** and **deoxynucleotide kinases** convert nucleoside monophosphates into diphosphates.[864,865] Some of them, e.g., **uridylate kinase** are similar in structure and properties to adenylate kinase.[866,867] Another member of the adenylate kinase family is phosphoribulokinase, an important photosynthetic enzyme (see Fig. 17-14, step *a*).[868]

Most kinases transfer chiral phospho groups with inversion and fail to catalyze partial exchange reactions that would indicate phosphoenzyme intermediates. However, **nucleoside diphosphate kinase** contains an active site histidine which is phosphorylated to form a phosphoenzyme.[869] The enzyme catalyzes phosphorylation of nucleoside diphosphates other than ADP by a nucleotide triphosphate, usually ATP.



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Here, X is usually adenosine and Y is any ribonucleoside or deoxyribonucleoside. This enzyme supplies all of the nucleotide triphosphates except ATP for use in the many cellular processes that require them.[870–872] The enzyme aligns the substrate, holding the phospho group with a pair of arginines and a magnesium ion. The phospho group is aligned for an in-line displacement by N^{δ} of His 122, part of a hydrogen-bonded His–Glu dyad. Formation of the phosphoenzyme occurs in less than 1 ms without significant conformational change other than a 30° rotation of the histidine ring.[870,873]

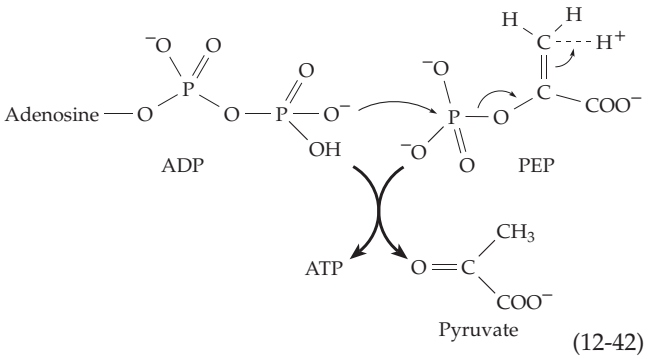
Hexokinase, the enzyme that phosphorylates glucose to glucose 6-phosphate, exists as four isoenzymes in mammals. Hexokinases I, II, and III are large ~ 100-kDa monomers with similar amino acid sequences, a single active site, and complex allosteric regulatory properties. The three-dimensional structure is known for hexokinase I, which is the pacemaker of glycolysis in the brain.[874,875] Hexokinase IV (glucokinase) is a 50-kDa protein that is found in liver and in the β cells of the pancreas. It has a low K_m and is not inhibited by glucose 6-phosphate, properties that allow for rapid uptake of glucose after a meal.[876] Its properties are similar to those of a major isoenzyme of yeast,[877] a dimer of identical 50-kDa subunits whose structure is also known.[878] The glucose-binding residues are conserved in yeast hexokinase, in glucokinase, and in brain hexokinase. The sequence of the latter suggests that it may have arisen by a doubling of a shorter hexokinase gene.[879]

Fructose 6-phosphate kinase (phosphofructokinase) has attracted much attention because of its regulatory properties (Chapter 11).[880–882] Prokaryotic forms are somewhat simpler.[883] The related fructose 6-phosphate 2-kinase is a component of a bifunctional kinase-phosphatase (Fig. 11-2, steps *d* and *e*) and has a structure similar to that of adenylate kinase.[884]

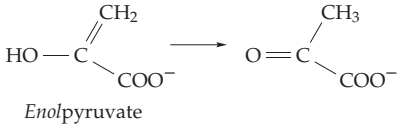
Phosphoglycerate kinase is encoded by the mammalian X chromosome. Several mutant forms are associated with hemolytic anemia and mental disorders.[885,886] Mutation of Glu 190, which is in the hinge region far from the active site, to Gln or Asn markedly reduces enzymatic activity.[887]

Creatine kinase transfers a phospho group to a nitrogen atom of the quanidinium group of creatine (Eq. 12-31 and Fig. 12-19). Several isoenzymes participate in its function of buffering the ATP level in tissues such as muscle fibers, neurons, photoreceptors, and spermatozoa which experience high and fluctuating energy needs.[888] A form from the mitochondrial intermembrane space of chicken heart is an octomer of 380-residue subunits.[889] The structurally and mechanistically similar **arginine kinase** has an analogous function in many invertebrates, e.g., in the horseshoe crab, which provided enzyme for a structure determination.[890]

The ~500-residue subunits of **pyruvate kinase** consist of four domains,[891] the largest of which contains an 8-stranded barrel similar to that present in triose phosphate isomerase (Fig. 2-28). Although these two enzymes catalyze different types of reactions, a common feature is an enolic intermediate. One could imagine that pyruvate kinase protonates its substrate phosphoenolpyruvate (PEP) synchronously with the phospho group transfer (Eq. 12-42). However, the enzyme catalyzes the rapid conversion of the enolic form of pyruvate to the oxo form (Eq. 12-43) adding the proton sterospecifically to the *si* face. This and other evidence favors the enol as a true intermediate



(12-42)



(12-43)

and a product of the phosphotransfer step.[891] Pyruvate kinase requires not only two equivalents of a divalent cation such as Mg^{2+} or Mn^{2+} but also a monovalent cation, usually K^+. However, Li^+, Na^+, NH_4^+, Rb^+, Tl^+, and others can substitute. The monovalent cation induces an essential conformational change. Using ^{205}Tl NMR it was found that the thallium ion binds about 0.6 nm from Mn^{2+} that is also present in the active site.[892] All three metals interact directly with the γ phospho group of ATP.[891] Pyruvate kinase is a regulated allosteric enzyme present in four isoenzymic forms in mammals.[893–894a]

Protein kinases, which were discussed in Chapter 11, phosphorylate selected – OH groups of serine, threonine, and tyrosine side chains in proteins. Examination of the sequences in the complete genome of yeast (*Saccharomyces cerevisiae*) indicates the presence of at least 113 protein kinase genes, which account for ~ 2% of the total DNA.[895] Higher eukaryotes have more. While structures of these enzymes vary widely, they share a common two-domain catalytic core structure.[896,897] The best known, and one of the simplest of them, is the catalytic subunit of cyclic AMP-dependent protein kinase.[897,898] The substrate MgATP binds into the active site cleft with the γ-phospho group protruding to meet the appropriate site of a bound protein substrate (Fig. 12-32). Most protein kinases are regulated by an "activation loop" that must be correctly placed before the ES complex can be formed. As discussed in Chapter 11, regulation of the cAMP-dependent kinase depends upon inhibition by a regulatory subunit. In the cAMP-dependent kinases and many other protein kinases the activation loop (not shown in Fig. 12-32), which helps to form the substrate site, contains a phosphothreonine residue which is essential for activity. It is a stable feature of the cAMP-activated kinase, incorporated at position 197, but for some tyrosine kinases it is generated by autophosphorylation.[899]

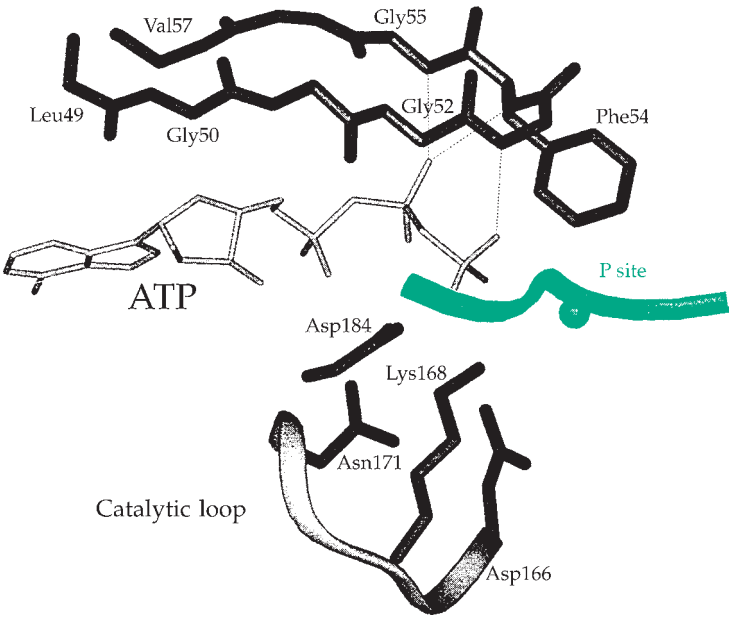


Figure 12-32 The active site of cyclic AMP-dependent protein kinase with bound ATP and a segment of an inhibitory peptide (green) blocking the substrate site. The small ball is an alanine side chain; it would be Ser or Thr in a substrate. The ATP is clamped by the glycine-rich loop at the top of the figure. Hydrogen bonds from peptide NH groups bind the β and γ phospho groups of ATP. A magnesium ion (not shown) also binds to the β and γ phosphate oxygens and to the invariant Asp 184. The Asp 166 carboxylate is probably the catalytic base for deprotonation of the substrate –OH. From Grant *et al.*[896] Courtesy of Susan S. Taylor.

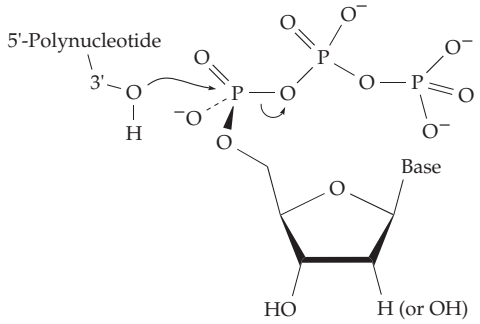
For the enzyme to be activated the phosphothreonine must form a hydrogen-bonded ion pair with Lys 189 and be hydrogen bonded to His 87, tying together critical regions of the catalytic domain. The regulatory subunits, unless occupied by cAMP (Chapter 11) are competitive inhibitors of the substrates.[900] Cyclic GMP-activated kinases also have distinct functions.[901] In **tryosine kinases**[902–904] C-terminal src-homology domains (Fig. 7-30 and Chapter 11) fold over and interact in an inhibitory fashion until an appropriate activating signal is received (see Fig. 11-13).

A relative of the kinases is **adenylate cyclase**, whose role in forming the allosteric effector 3',5'-cyclic AMP (cAMP) was considered in Chapter 11. This enzyme catalyzes a displacement on P_α of ATP by the 3'-hydroxyl group of its ribose ring (see Eq. 11-8 , step *a*). The structure of the active site is known.[905] Studies with ATPαS suggest an in-line mechanism resembling that of ribonuclease (step *a*, Eq. 12-25). However, it is Mg^{2+} dependent, does not utilize the two-histidine mechanism of ribonuclease A, and involves an aspartate carboxylate as catalytic base.[906] All isoforms of adenylate cyclase are activated by the α subunits of some G proteins (Chapter 11). The structures[907] of $G_{s\alpha}$ and of its complex with adenylate kinase[905] have been determined. The $G_{s\alpha}$ activator appears to serve as an allosteric effector.

Guanylate cyclases, which form cyclic GMP, occur in particulate and soluble forms.[908] The latter have been of great interest because they are activated by nitric oxide (NO). The soluble guanylate cyclases are αβ heterodimers. The C-terminal regions of both α and β subunits are homologous to the catalytic domain of adenylate cyclase. The N-terminal domain of the α subunits contains heme whose Fe atom is coordinated by a histidine imidazole.[908,908a] This iron atom is apparently the receptor for NO, a major gaseous hormone, which is discussed in Chapter 18.

10. Nucleotidyl Transferases

An important group of enzymes transfer substituted phospho groups, most often nucleotidyl groups. The nucleases, ATPases, and GTPases, which have already been discussed, belong to this group as do the nucleic acid synthesizing enzymes, the **DNA** and **RNA polymerases**,[909–911] **reverse transcriptase**,[912] and **topoisomerases**. As with other phosphotransferases, the nucleotidyl transfers occur with inversion[913,913a] and crystallographic investigations also support in-line mechanisms as illustrated in the following scheme. Two metal ions assist.



The inorganic pyrophosphate formed is hydrolyzed to inorganic phosphate by pyrophosphatase. Specific information about the polymerases and topoisomerases is given in Chapters 27 and 28.

E. The Adenylate Kinase Fold, the P Loop, and ATPases and GTPases

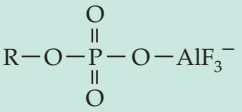
A magnesium–ATP-binding fragment consisting of ~40 residues at the N terminus of adenylate kinase contains sequences homologous to those in the GTP-binding "G proteins" such as the protooncogenes *ras* (Fig. 11-7A) and also to sequences in myosin and in mitochondrial ATP synthase.[914] This includes the glycine-rich "P loop" which extends from Gly 15 to Gly 22 in the porcine cytosolic enzyme and contains a highly conserved lysine [Lys 21 of porcine adenylate

BOX 12-F THE TOXICITY OF ALUMINUM

Aluminum, in the form of oxides and silicate minerals, is the most abundant element in the earth's crust. Yet it appears to be actively excluded from most living organisms. It seems surprising that a naturally occuring Al^{3+}-dependent enzyme hasn't been found, but there is no evidence that Al is an essential element. Until recently, it was usually regarded as harmless. The aluminum salts known as alums are used in baking powders and have been added to pickles and other foods. Aluminum sulfate is often used as a coagulant to clarify turbid drinking water. Because of the insolubility of $Al(OH)_3$ and of aluminum phosphate the concentration of Al^{3+} is very low at neutral pH. However, small amounts of $AlOH^{2+}$, $Al(OH)_2^+$, and $Al(OH)_4^-$ are present in water. Fluoride complexes such as AlF^{2+} may also be present. Soluble complexes, such as that with citrate, may permit some Al^{3+} to be absorbed by the body.[a,b]

The toxicity of aluminum has been recognized most clearly by the development of bone disease caused by deposition of Al in bones of patients on hemodialysis[a–c] and in infants on intravenous therapy.[d,e] Excessive Al in the water used for dialysis may also cause brain damage. Dietary aluminum may be one cause of Alzheimer's disease,[f–h] but this is controversial as is a possible role of aluminum in vaccines in causing inflammation in muscle.[i,j] Solubilization of soil aluminum by acid rain has been blamed for the decline of forests in Europe and North America,[j] for the death of fish in acid waters,[k] and for very large reductions in yield for many crops.[l,m] An aluminum-resistant strain of buckwheat makes and secretes from its roots large amounts of oxalate which binds and detoxifies the Al^{3+} ions.[m]

Al^{3+} has a radius somewhat less than that of Fe^{3+} (Table 6-10) and it may sometimes occupy empty Fe^{3+} binding sites. Thus, the transferrin[n] in blood carries some Al^{3+}, although citrate is probably a more important carrier.[o] Al^{3+} binds preferentially to oxygen ligands and can compete with Mg^{2+}. However, the slower rate of ligand exchange reactions with Al^{3+} may interfere with the proper functioning of the metal. Brain hexokinase is strongly inhibited[p] by Al^{3+} and the binding of Al^{3+} to tubulin decreases the rates of GTP hydrolysis and of Ca^{2+}-induced depolymerization of microtubules.[q] Aluminofluoride ions, such as AlF_4^-, react with phosphates to form ions such as,



which may be potent competitive inhibitors of enzymes acting on ATP, GTP, or other phosphate-containing substrate.[r,s] However, Fe^{3+} can be replaced by Al^{3+} in a purple acid phosphatase (Chapter 16) with retention of good catalytic activity.[t]

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[a] Martin, R. B. (1986) *Clinical Chemistry* **32**, 1797–1806
[b] Macdonald, T. L., and Martin, R. B. (1988) *Trends Biochem. Sci.* **13**, 15–19
[c] Andress, D. L., Kopp, J. B., Maloney, N. A., Coburn, J. W., and Sherrard, D. J. (1987) *N. Engl. J. Med.* **316**, 292–296
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[e] Bishop, N. J., Morley, R., Chir, B., Day, J. P., and Lucas, A. (1997) *N. Engl. J. Med.* **336**, 1557–1561
[f] Good, P. F., and Perl, D. P. (1993) *Nature (London)* **362**, 418
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[h] Walker, P. R., LeBlanc, J., and Sikorska, M. (1989) *Biochemistry* **28**, 3911–3915
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[ij] Malakoff, D. (2000) *Science* **288**, 1323–1324
[j] Godbold, D. L., Fritz, E., and Hüttermann, A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3888–3892
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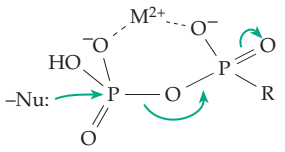
[n] Roskams, A. J., and Connor, J. R. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9024–9027
[o] Martin, R. B., Savory, J., Brown, S., Bertholf, R. L., and Wills, M. R. (1987) *Clinical Chemistry* **33**, 405–407
[p] Viola, R. E., Morrison, J. F., and Cleland, W. W. (1987) *Biochemistry* **19**, 3131–3137
[q] Macdonald, T. L., Humphreys, W. G., and Martin, R. B. (1987) *Science* **236**, 183–186
[r] Troullier, A., Girardet, J.-L., and Dupont, Y. (1992) *J. Biol. Chem.* **267**, 22821–22829
[s] Chabre, M. (1990) *Trends Biochem. Sci.* **15**, 6–10
[t] Merkx, M., and Averill, B. A. (1999) *J. Am. Chem. Soc.* **121**, 6683–6689

kinase and Lys 17 of the archaeal enzyme (Fig. 12-30)] and of the *ras* oncogene product).[915] The lysine side chain appears to interact with the β and / or γ phospho group of ATP or GTP. The peptide chain of the P loop wraps around the β phospho group as the chain turns from the first β strand into the first helix. This can be seen in Fig. 12-30, in which the P loop surrounds the β phospho group of ADP. Two peptide NH groups also bind to the β phospho group on a side opposite to that occupied by Mg^{2+}. A similar loop in the dinucleotide-binding domain of a dehydrogenase can be seen hydrogen bonded to the P_α and P_β phospho groups of NAD^+ in Fig. 2-13. Consensus sequences for three groups of glycine-rich loops are:[896]

Dinucleotide-binding	G X G X X G
P loop	G X X X X G K (S/T)
Protein kinase	G X G X X G X V

The protein kinase loop is seen at the top of Fig. 12-32 and extends from Gly 50 to Val 57. These conserved loops help to hold the ATP in place and to orient it correctly. Do they have any other significance? The answer is not clear. These glycine-rich loops fold across the β–γ diphosphate linkage that is broken when ATP or GTP is hydrolyzed. Cleavage of both of these molecules is associated with movement. Kinases close around ATP, and parts of G proteins (Fig. 11-7) move when these regulatory devices function.[915,916] Cleavage of ATP causes movement in the ATPase heads of muscle myosin, providing the force for muscular contraction (Chapter 19). Somewhat the opposite occurs in mitochondrial ATP synthase (Chapter 18) when movement in the synthase heads snaps ADP and inorganic phosphate together to form ATP. Common to all of these processes is a movement of charge within the ATP (or its cleavage products) from the attack nucleophile into the neighboring phospho group, as is indicated by the arrows in the following diagram.



Not shown in this diagram is an accompanying flow of positive charge which may include movement of a metal ion, addition and loss of protons, and which may induce conformational changes.[914] The latter are essential not only to muscle contraction and ATP synthesis but also to many other processes that depend upon the Gibbs energy of cleavage of ATP and related compounds. This includes the pumping of ions against concentration gradients (Chapter 8), the action of **topoisomerases** (Chapter 27) which function to alter the supercoiling in DNA, and the functioning of the phosphotransferase system by which sugars and other compounds are brought into bacterial cells (Chapter 8).

F. Displacements on Sulfur Atoms

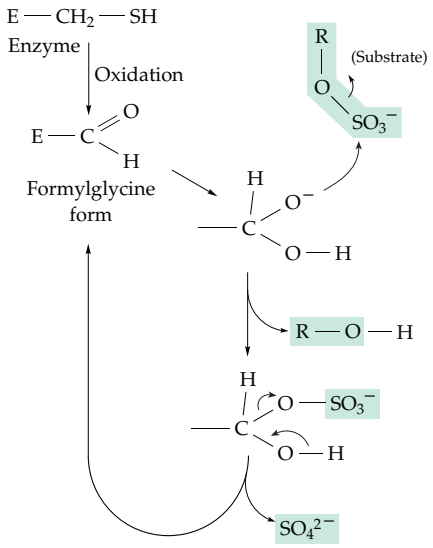
Nucleophilic displacement reactions occur on sulfur atoms in various oxidation states. A common reaction is **thiol–disulfide** exchange (Eqs. 10-9, 11-7), a reaction in which a nucleophilic thiolate anion attacks one of the sulfur atoms of a disulfide. Proteins such as **thioredoxin** of *E. coli* and **thioltransferases** (Box 15-C), which contain internal disulfide bridges, can be reduced by disulfide exchange with thiols such as glutathione (Box 11-B). The reduced proteins may then undergo similar exchange reactions that cleave disulfide linkages in other molecules. An example is glutathione reductase (Fig. 15-12). Thioltransferases may also serve as **protein disulfide isomerases** (Chapter 10, Section D,3). Nucleophilic displacements on sulfur or on selenium atoms are steps in a variety of enzymatic reactions. Among these are glutathione peroxidase (Eq. 15-59) and thiosulfate: cyanide sulfotransferase (Eq. 24-45).

While esters of sulfuric acid do not play as central a role in metabolism as do phosphate esters, they occur widely. Both oxygen esters ($R–O–SO_3^-$, often referred to as **O-sulfates**) and derivatives of sulfamic acid ($R–NH–SO_3^-$, **N-sulfates**) are found, the latter occurring in mucopolysaccharides such as heparin. Sulfate esters of mucopolysaccharides and of steroids are ubiquitous and sulfation is the most abundant known modification of tyrosine side chains. Choline sulfate and ascorbic acid 2-sulfate are also found in cells. Sulfate esters of phenols and many other organic sulfates are present in urine.

Sulfotransferases[917–920a] transfer sulfo groups to O and N atoms of suitable acceptors (reaction type 1D, Table 10-1). Usually, transfer is from the "active sulfate," **3'-phosphoadenosine 5'-phosphosulfate** (**PAPS**),[921] whose formation is depicted in Eq. 17-38. **Sulfatases** catalyze hydrolysis of sulfate esters. The importance of such enzymes is demonstrated by the genetic **mucopolysaccharidoses**. In four of these disease-specific sulfatases that act on iduronate sulfate, heparan *N*-sulfate, galactose-6-sulfate, or *N*-acetylglucosamine-4-sulfate are absent. Some of these, such as heparan *N*-sulfatase deficiency, lead to severe mental retardation, some cause serious skeletal abnormalities, while others are mild in their effects.[922]

Sulfatases are unusual in having a residue of **formylglycine** at the active site. This is generated oxidatively from cysteine in human enzymes[923,923a] and from serine in some bacterial sulfatases.[924,924a] Absence of this modification results in a multiple sulfatase deficiency disease. A probable mechanism of sulfatase

based on a crystal structure determination is given by Eq. 12-44.[923]



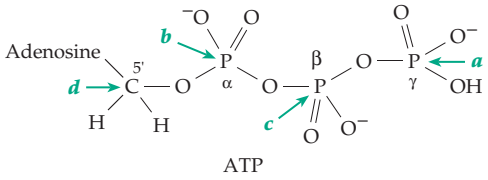
(12-44)

G. Multiple Displacement Reactions and the Coupling of ATP Cleavage to Endergonic Processes

A combination of successive displacement reactions of two types is required in many enzymatic reactions, including most of those by which the cleavage of ATP is coupled to biosynthesis. To harness the group transfer potential of ATP to drive an endergonic metabolic process there must be a mechanism of **coupling**. Otherwise, hydrolysis of ATP within a cell would simply generate heat. *An essential part of the coupling mechanism usually consists of a nucleophilic displacement on phosphorus followed by displacement on carbon.* Likewise, the synthesis of ATP and related compounds often begins with a displacement on carbon followed by one on phosphorus.

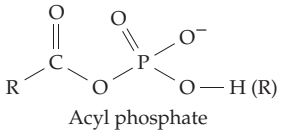
1. Transfer of Phospho, Pyrophospho, and Adenylyl Groups from ATP

The first step in coupling ATP cleavage to any process is transfer of part of the ATP molecule to a nucleophile Y, usually by displacement on one of the three phosphorus atoms. The nucleophilic attack may be (*a*) on the terminal phosphorus (P_γ) with displacement of ADP or (*b*) on the internal phosphorus (P_α) with displacement of inorganic pyrophosphate. In the first case, $Y–PO_3H^-$ is formed; in the latter, **Y-adenylyl** (sometimes shortened to Y-adenyl) is formed. More rarely, displacement occurs (*c*) on the central phosphorus (P_β) with transfer of a pyrophospho group to the nucleophile. Still less frequent (*d*) is a displacement on C-5' as shown in Eq. 17-37. If the nucleophile Y in any of these displacement reactions is H_2O, the resulting hydrolysis tends to go to completion, i.e., the phospho, adenylyl, and pyrophospho groups of ATP all have high group transfer potentials (Table 6-6). If Y is an –OH group in an ordinary alcohol the transfer reaction also tends to go to completion because the group transfer potential of a simple phosphate ester is relatively low. Consequently, phosphorylation by ATP is often used as a means of introducing an essentially irreversible step in a metabolic pathway.

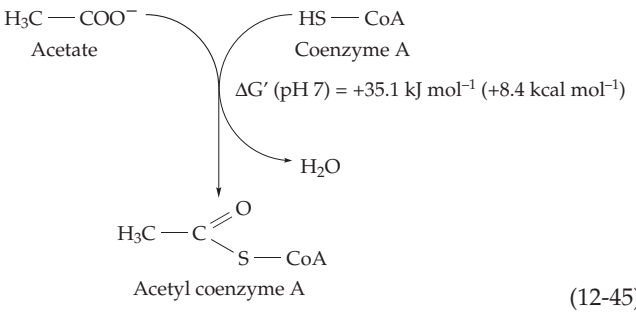


2. Acyl Phosphates

Transfer of a phospho or adenylyl group from ATP to the oxygen atom of a carboxylate group yields an **acyl phosphate**, a type of metabolic intermediate of special significance. Acyl phosphates are mixed anhydrides of carboxylic and phosphoric acids in which *both the acyl group and the phospho group have high group transfer potentials.* As a consequence, acyl phosphates can serve as metabolic intermediates through which the group transfer potential of ATP is transferred into other molecules and is harnessed to do chemical work.



A typical example is the synthesis of acetyl coenzyme A (Eq. 12-45). See Fig. 14-1 for the complete structure of –SH group-containing coenzyme A.

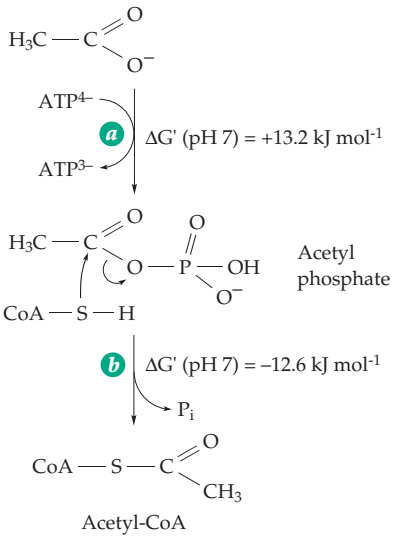


(12-45)

Because the acetyl group in the product also has a high group transfer potential, $\Delta G'$ for this reaction is highly positive and the formation of acetyl-CoA will not occur spontaneously. However, the sum of $\Delta G'$ for the reaction in Eq.12-45 plus that for the hydrolysis of ATP (Eq. 12-46) is nearly zero (+ 0.6kJ mol^{-1}).

$$ATP^{4-} + H_2O \rightarrow ADP^{3-} + HPO_4^{2-} + H^+$$
$$\Delta G' = -34.5 \text{ kJ mol}^{-1} (-8.3 \text{ kcal mol}^{-1}) \quad (12\text{-}46)$$

Coupling of the two reactions is accomplished by first letting an oxygen atom of the nucleophilic carboxylate group attack P_γ of ATP to form acetyl phosphate (Eq. 12-47, step *a*). In the second step (step *b*) the sulfur atom of the –SH group of coenzyme A (often abbreviated CoA–SH) attacks the carbon atom of the acetyl phosphate with displacement of the good leaving group P_i. While $\Delta G'$ for Eq. 12-47, step *a*, is moderately positive (meaning that a relatively low concentration of acetyl phosphate will accumulate unless the [ATP] / [ADP] ratio is high), the equilibrium in Eq. 12-47, step *b*, favors the products.

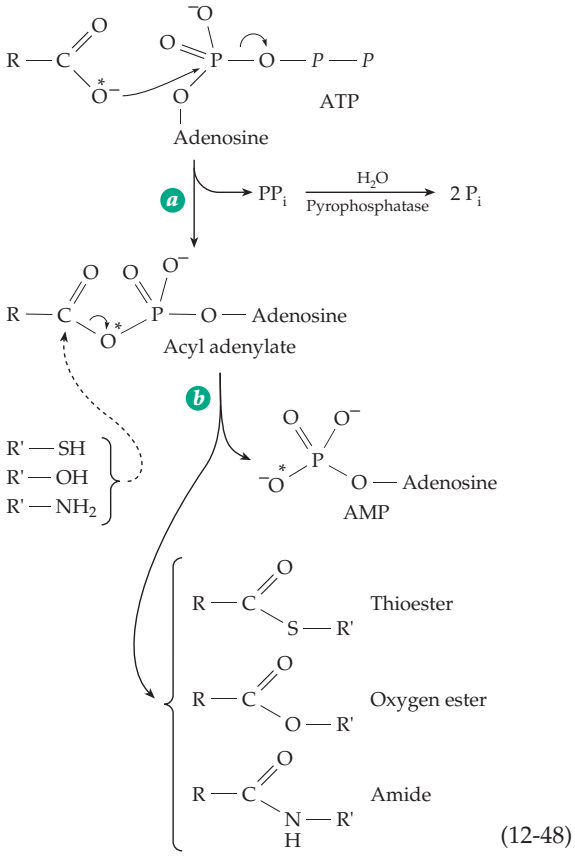


(12-47)

The two reactions of Eq. 12-47 are catalyzed by **acetate kinase**[925] and an ***S*-acetyltransferase**, respectively. The sequence represents an essential first stage in bacterial utilization of acetate for growth. It is also used in a few bacteria in reverse as a way of *generating* ATP in fermentation reactions. On the other hand, most eukaryotic cells make acetyl-CoA from acetate by coupling the synthesis to cleavage of ATP to AMP and P_i. A single enzyme **acetyl-CoA synthetase** (acetate thiokinase) catalyzes both steps in the reaction (Eq. 10-1). The sequence parallels that of Eq. 12-47, but the initial displacement is on P_α of ATP to form **acetyl adenylate**. This intermediate remains tightly bound to the enzyme until the second step in the sequence takes place. When ^{18}O is present in the acetate (designated by the asterisks in Eq. 12-48) it appears in the phospho group of AMP as expected for the indicated mechanism.

3. General Mechanism of Formation of Thioesters, Esters, and Amides

The sequences of Eqs. 12-47 and 12-48 are general ones used by cells for linking carboxylic acids to –OH, –SH, and – NH_2 groups to form oxygen esters, thioesters, or amides, respectively. ATP can be cleaved at either P_α or P_γ. If cleavage is at P_α (Eq. 12-48) the hydrolysis of inorganic pyrophosphate (PP_i) to P_i provides an additional coupling of ATP cleavage to biosynthesis as is discussed in Chapter 17, Section H.

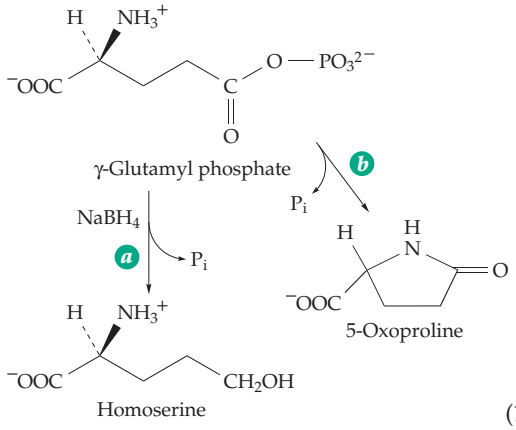


(12-48)

An enzyme catalyzing a reaction similar to that of acetyl-CoA synthetase is **succinyl-CoA synthetase** (succinate thiokinase).[926,927] The enzyme from *E. coli* has been studied most. The first step is formation of a phosphoenzyme by transfer of the γ phospho group from ATP to N^ε of histidine 246 in the α subunit of the 140-kDa $\alpha_2\beta_2$ tetramer.[926] The phospho group is then transferred to succinate to form succinyl phosphate, which reacts with coenzyme A, as in step *b*, Eq. 12-48,

to form succinyl-CoA. Crystallographic studies suggest that for this step the succinyl phosphate and the coenzyme A may be bound to opposite α subunits in the tetramer.[926] However, in some bacteria and in eukaryotes the enzyme appears to operate as an αβ heterodimer.[927]

Glutamine synthetase,[928,929] a large enzyme containing 12 identical 468-residue subunits with 622 symmetry (as in Fig. 7-12), has a major regulatory function in nitrogen metabolism, which is discussed in Chapter 24. Apparently, the intermediate acyl phosphate (γ-glutamyl phosphate) has a transient existence and all three reactants–glutamate, NH_4^+, and ATP–must be bound to the enzyme concurrently before the active site becomes functional. Early evidence for the acyl phosphate[930,931] included reduction by sodium borohydride to an alcohol (Eq. 12-49*a*) and isolation of the internal amide of glutamic acid **5-oxoproline** (Eq. 12-49*b*).



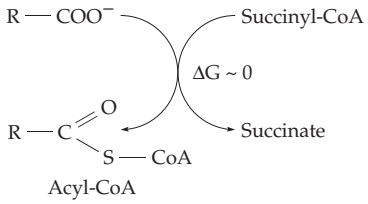
(12-49)

Ammonium ions appear to be bound at a specific site and to be deprotonated by the Asp 50 carboxylate.[929]

The **aminoacyl-tRNA synthetases** join amino acids to their appropriate tRNA molecules for protein synthesis. They have the very important task of selecting both a specific amino acid and a specific tRNA and joining them. The enzymes differ in size and other properties. However, they all appear to function by a common basic chemistry that makes use of cleavage of ATP at P_α (Eq. 12-48) via an intermediate aminoacyl adenylate and that is outlined also in Eq. 17-36. These enzymes are discussed in Chapter 29 .

4. Coenzyme A Transferases

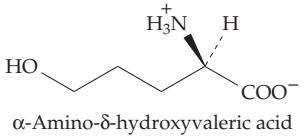
The following problem in energy transfer arises occasionally: A thioester, such as succinyl-CoA, is available to a cell and the energy available in its unstable linkage is needed for synthesis of a different thioester. It would be possible for a cell to first form ATP or GTP, using a synthetase reaction in reverse; then the ATP or GTP formed could be used to make the new linkage by the action of another acyl-CoA synthetase. However, special enzymes, the **CoA transferases**, function more directly (Eq. 12-50). The mechanism is not obvious.



(12-50)

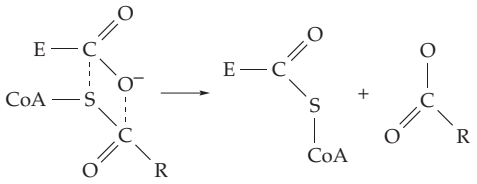
How can the CoA be transferred from one acyl group to another while still retaining the high group transfer potential of the acyl group?

The following experiments shed some light. Kinetic studies of **succinyl-CoA–acetoacetate CoA transferase** indicate a ping-pong mechanism. The enzyme alternates between two distinct forms, one of which has been shown to contain bound CoA.[932–934] The E-CoA intermediate formed from enzyme plus acetoacetyl-CoA was reduced with ^3H-containing sodium borohydride and the protein was completely hydrolyzed with HCl. Tritium-containing α-amino-δ-hydroxyvaleric acid was isolated. Since thioesters (as well as oxygen esters) are cleaved in a two-step process



to alcohols by reduction with borohydride, it was concluded that the intermediate E-CoA is a thioester of the Glu 344 side chain. In exchange reactions ^{18}O from labeled succinate entered both the E-CoA intermediate and the carboxyl group of acetoacetate.

A mechanism involving formation of a transient anhydride is similar to reactions discussed in preceding sections.[717,720] The student should be able to write out the step-by-step detail. Does this mechanism explain the ^{18}O exchange data? A second possibility is a **4-center reaction** (Eq. 12-51). However, mechanisms of this type have not been demonstrated for enzymatic reactions.



(12-51)

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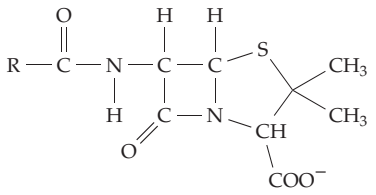
Study Questions

1. Outline the reactions by which glyceraldehyde 3-phosphate is converted to 3-phosphoglycerate with coupled synthesis of ATP in the glycolysis pathway. Show important mechanistic details.

2. Papain is a protein-hydrolyzing (proteolytic) enzyme with an –SH group and an imidazole group at the active site. Write a reasonable structure for a "tetrahedral intermediate" that would be expected to arise during formation of an acyl enzyme intermediate.

3. Adenylate kinase catalyzes the interconversion of ATP, AMP, and ADP.
   a) Draw a reasonable structure for a penta-covalent intermediate derived from ATP and AMP.
   b) Draw a reasonable structure for the transition state leading from ATP + AMP to two molecules of ADP in an S_N2-like reaction.

4. Penicillin inhibits a D-alanyl-D-alanine transpeptidase that catalyzes the reaction



Penicillin

$$\text{R}'\text{-D-alanyl-D-alanine} + \text{H}_2\text{N-R} \rightarrow \text{R}'\text{-D-alanyl-NH-R} + \text{D-alanine}$$

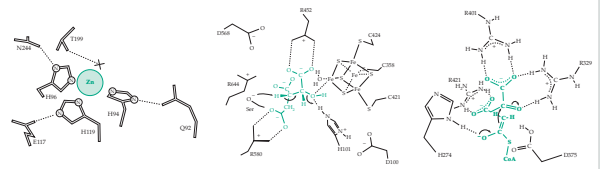
   where R and R′ are different parts of a bacterial peptidoglycan. Write a step-by-step mechanism for this reaction and indicate how penicillin may inhibit the enzyme by combining with it irreversibly. See Strynodka *et al., Nature* **359**, 700–705, 1992, for related reaction of penicillin with a penicillinase.

5. Write out step-by-step chemical mechanisms for the following enzymatic reaction. Use small arrows to indicate directions of electron flow. Remember to have all electrons move in the same direction in any single structure.

$$\text{Amino acid} + \text{tRNA} + \text{ATP} \rightarrow \text{Aminoacyl-tRNA} + \text{AMP} + \text{PP}_\text{i}$$

6. Trypsin in which Asp 102 has been replaced by Asn has 10^4 times less catalytic activity than natural trypsin at neutral pH. From the crystal structure of the mutant enzyme it appears that the imidazole group of His 57 is held by the Asn side chain in the wrong tautomeric form for catalysis. Explain. Compare this incorrect tautomeric form with that in the initial structure shown in Fig. 12-11.

7. A recent discovery in biochemistry is that RNA can act as an enzyme in chemical reactions, usually reactions involving RNA hydrolysis. Discuss the features of RNA structure that might favor evolution of enzymes composed entirely of a single polyribonucleotide chain, and describe a proposed mechanism for RNA-catalyzed hydrolysis of RNA molecules.

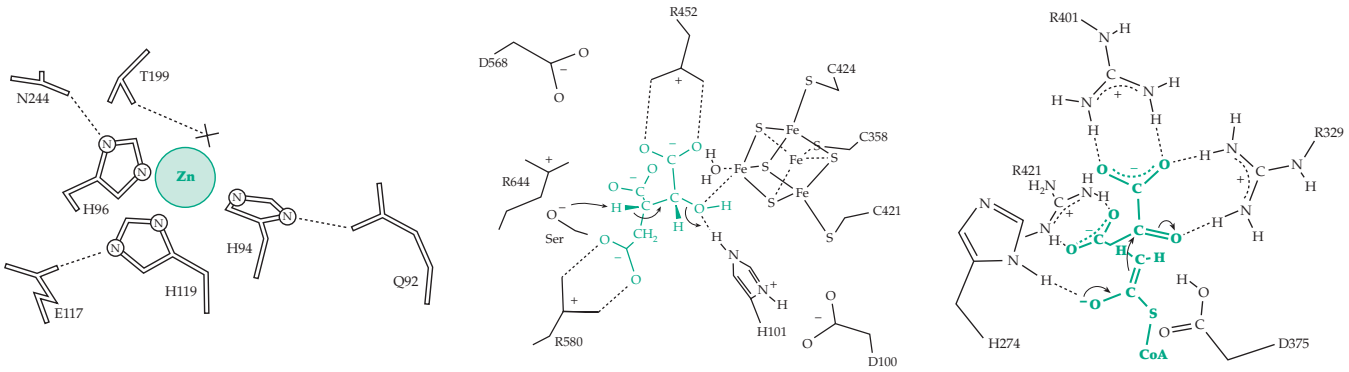


One of the simplest biochemical addition reactions is the hydration of carbon dioxide to form carbonic acid, which is released from the zinc-containing **carbonic anhydrase** (left, Fig. 13-1) as HCO_3^-. **Aconitase** (center, Fig. 13-4) is shown here removing a water molecule from isocitrate, an intermediate compound in the citric acid cycle. The H_2O that is removed will become bonded to an iron atom of the Fe_4S_4 cluster at the active site as indicated by the black H_2O. An enolate anion derived from acetyl-CoA adds to the carbonyl group of oxaloacetate to form citrate in the active site of **citrate synthase** (right, Fig. 13-9) to initiate the citric acid cycle.

Contents

Enzymatic Addition, Elimination, Condensation, and Isomerization: Roles for Enolate and Carbocation Intermediates

13

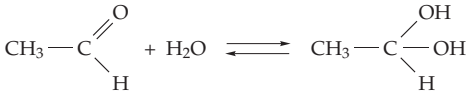


In Chapter 12 we considered reactions by which living cells are able to transfer groups from one molecule to another using nucleophilic displacements. We also showed how transfer reactions can be utilized by ligases to join two molecules together with the Gibbs energy of cleavage of ATP or of a related molecule driving the reaction. In this chapter we will examine addition reactions, which provide a simple way of joining two molecules by means of C–O, C–N, C–S, or C–C bonds. Among these are the aldol and Claisen-type condensations by which C–C bonds are formed. We will also consider elimination reactions and decarboxylations, which are the reverse of addition and condensation reactions, as well as mechanistically related isomerizations. Many reactions of these types occur in the major pathways of metabolism.

A. Addition of R–OH, R–NH_2, and R–SH to Polarized Double Bonds

Next to nucleophilic displacement, the commonest mechanistic processes in enzymatic catalysis are addition to double bonds and elimination to form double bonds. These often involve addition of a nucleophile together with a proton to a highly polarized double bond such as C=O or C=N–. In other reactions, which are discussed in Section C,2, the nucleophile attacks one end of a C=C bond that is polarized by conjugation with C=O or C=N.

Alcohols, amines, and thiols add readily to the electrophilic carbon of the carbonyl group to form **hemiacetals**, **carbinolamines, hemiketals,** and **hemimercaptals**. An example is the formation of ring structures of sugars (Eq. 4-1). Water can also add to carbonyl groups and most aliphatic carbonyl compounds exist in water as an equilibrium mixture with a covalent hydrate (Eq. 13-1). For example, acetaldehyde in aqueous solution consists of a mixture of about 50% free aldehyde and 50% hydrate in rapidly reversible equilibrium[1,2] and formaldehyde is over 99.9% hydrated.[3]

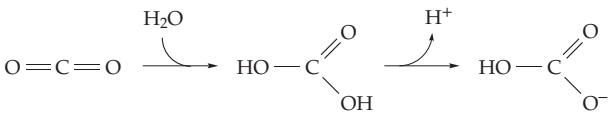


(13-1)

Addition reactions often occur as parts of more complex reactions. For example, a thiol group of glyceraldehyde-3-phosphate dehydrogenase reacts with the aldehyde substrate to form a hemimercaptal, which is subsequently oxidized to a thioester (see Fig. 15-6).

1. Carbonic Anhydrase

Another simple addition reaction is the hydration of CO_2 to form the bicarbonate ion. Without catalysis the reaction may require several seconds,[4,5] the apparent first-order rate constant being ~0.03 s^{-1} at 25°C. Cells must often hasten the process. The specific catalyst carbonic anhydrase is widespread in its distribution



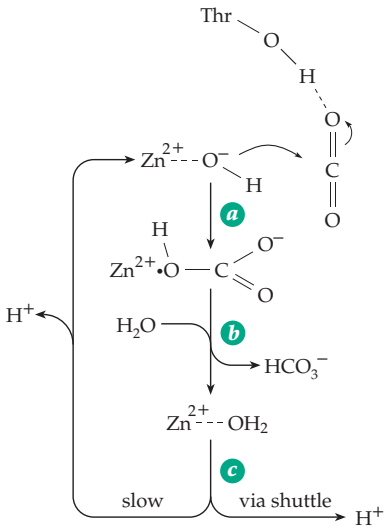
(13-2)

and is especially active in tissues (e.g., red blood cells, and lungs) that are involved in respiration. One liter of mammalian blood contains 1–2 g of this enzyme, a monomeric 30-kDa protein containing ~260 amino acids and one tightly bound ion of Zn^{2+}. Erythrocytes contain two isoenzymes (I and II) of carbonic anhydrase and the human body contains at least eight distinct isoenzymes (I – VIII).[5–8] They are found wherever there is a high demand for CO_2 or bicarbonate. Isoenzyme I, II, III, and VII are cytosolic. Carbonic anhydrase I is specific to erythrocytes, while isoenzyme II is present in most cells. Hereditary lack of carbonic anhydrase II is associated with **osteopetrosis** (marble bone disease), a condition involving failure of bone resorption and the calcification of other tissues.[9] The generation of acidity according to Eq. 13-2 is presumably required for dissolution of bone by the osteoclasts.

Isoenzymes III and VII have a more specialized distribution. Carbonic anhydrase III is abundant in adipocytes which use bicarbonate in fatty acid synthesis.[7] Isoenzyme V is present in the mitochondrial matrix and is also abundant in both adipocytes and liver.[7,8] Isoenzyme IV is a larger membrane-associated form, while VI is secreted into the saliva.[10] Carbonic anhydrase has also been identified in *E. coli*.,[11] in a methanobacterium,[12] and in green plants.[13,13a] A 60-kDa carbonic anhydrase called **nacrein** is found in the organic matrix of the nacreous layer of the pearl oyster, the layer that forms aragonite (orthorhombic calcium carbonate) in the shell and in pearls.[14]

X-ray studies of carbonic anhydrases I and II, from human blood, revealed that both have an ellipsoidal shape of dimensions ~4.1 x 4.1 x 4.7 nm.[15,16] The zinc atom in each molecule lies in a deep pocket ~1.2 nm from the surface and is surrounded by three histidine side chains and one H_2O or OH^- ion, the four ligands forming a distorted tetrahedron (Fig. 13-1). The coordinating imidazole group from His 119 is hydrogen bonded to a carboxylate group of Glu 117, a feature reminiscent of the charge-relay system of serine protease. This carboxylate group is also bound into a more extended hydrogen-bonded network, part of which is indicated in Fig. 13-1. The other imidazole groups also form hydrogen bonds to protein groups and the zinc-bound H_2O is involved in an extensive hydrogen-bonded network with several other bound water molecules and protein side chains.[17a] Most of these structural features are conserved in the other mammalian isoenzymes.[5] However, from X-ray absorption spectroscopy (EXAFS) it appears that spinach carbonic anhydrase contains one or more sulfur ligands to the zinc,[13] while the enzymes from the archaeon *Methanosarcina thermophila* have a left-handed β helix structure (see Figs. 2-17 and 13-3).[12] Nevertheless, this enzyme has the same three-histidine Zn–OH structure found in the mammalian enzymes.

Carbonic anhydrase II is among the most rapid enzymes known, with the turnover number at 25°C for hydration of CO_2 being ~10^6 s^{-1}. The same enzyme catalyzes hydration of acetaldehyde (Eq. 13-1) but at a 1000-fold slower rate. A pK_a of ~7 controls the activity. This appears to represent the loss of H^+ from the Zn^{2+}–OH_2 complex[18] to give Zn^+–OH. The latter is in effect a stabilized hydroxide ion existing at a pH at which OH^- is normally not present in quantity. It is this hydroxide ion that adds to the CO_2 or to the aldehyde substrate (Eq. 13-3, step *a*).[18–20] In step *b* a water molecule replaces the departing bicarbonate.[17] A variety of data



(13-3)

indicate that proton transfers mediated by the enzyme are essential parts of the carbonic anhydrase mechanism.[18–19b,21–23] One proposal is that the nearby imidazole group of His 64 (not shown in Fig. 13-1) deprotonates the bound H_2O via a hydrogen-bonded network of bound water molecules (Eq. 13-3, step *c*). The side chain of Thr 199 may function in a cyclic proton transfer in step *c*.[19] The proton generated in step *c* is released to the solvent in a process that is catalyzed by buffer anions or by amines such as histamine. The latter binds at the edge of the active site and forms an additional hydrogen-bonded pathway to the zinc-bound H_2O.[24] A different proton shuttle pathway has been proposed for the slower carbonic anhydrase III.[25]

Related to the reaction catalyzed by carbonic anhydrase is the addition of an amino group to CO_2 (Eq. 2-21) to form a carbamino group (–NH–COO^-). This reaction is essential to the functioning of hemoglobin, which must carry large amounts of CO_2, as carbamino groups, from tissues to the lungs, (Eq. 7-47) and to some enzymes such as ribulose bisphosphate carboxylase (Figs. 13-10, 13-11).

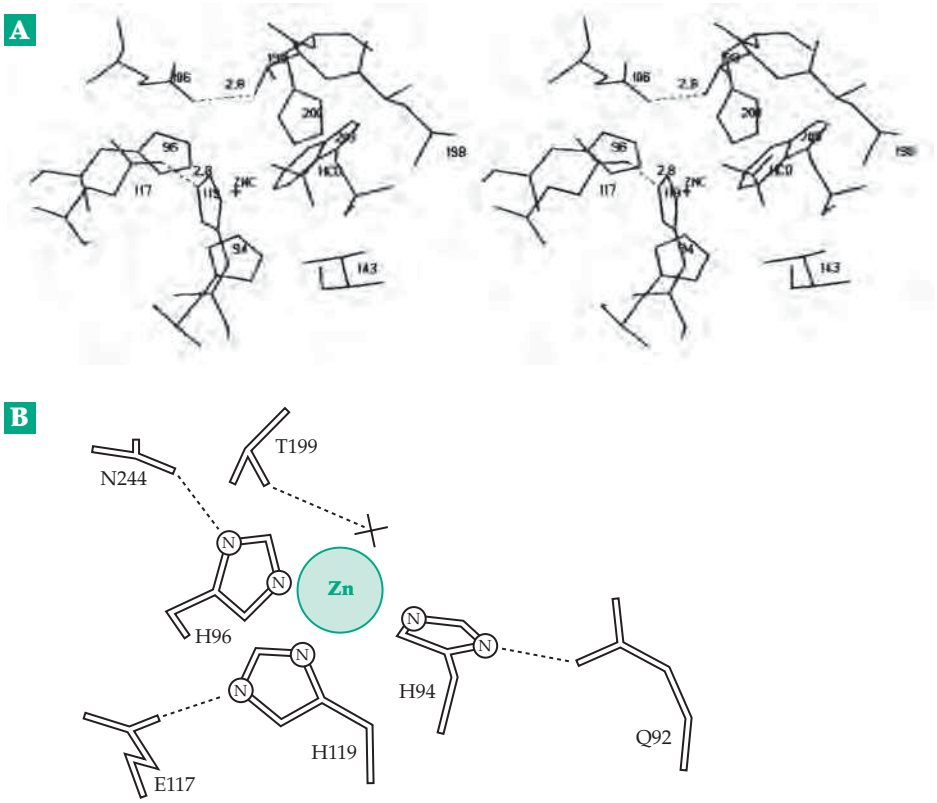
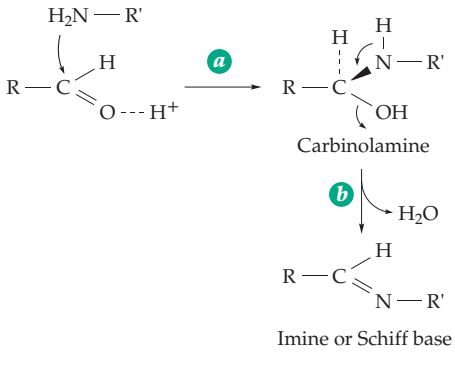


Figure 13-1 (A) Stereoscopic view showing the binding pocket for HCO_3^- in the active site of human carbonic acid anhydrase I. Also shown are the hydrogen-bonded pairs E117–H119 and E106–T199. ZNC, zinc ion; HCO, bicarbonate. From Kumar and Kannan.[16] (B) View of the active site of carbonic anhydrase II. The orientation is a little different than in (A). The location of H_2O or ^-OH bound to the zinc ion is marked X. Hydrogen bonds from the three histidines that form coordinate bonds to the zinc, Q92, E117, the backbone carbonyl of N244, and the hydrogen bond from the zinc-bound hydroxyl to the T199 side chain are shown as dashed lines. From Kiefer *et al.*[17]

2. Imines (Schiff Bases)

As we have seen already, many enzymatic reactions depend upon formation of **imines**, which are commonly called Schiff bases. The two-step formation of Schiff bases consists of addition of an amino group to a carbonyl group to form a carbinolamine followed by elimination of water (Eq. 13-4).[26] One group of **aldolases** (Section D) have, at their active centers,



(13-4)

lysine side chains which form Schiff bases with the ketone substrates prior to the principal reaction of breaking or forming a C – C bond. Similarly, the initial reaction of the aldehyde coenzyme **pyridoxal phosphate** with amino acid substrates is the formation of Schiff bases (Fig. 14-4). Indeed, *the groups C=O and H_2N– are inherently complementary* and their interaction through imine formation is extremely common.

Schiff bases often form within a fraction of a second, but one or both steps of Eq. 13-4 may require catalysis to achieve enzymatic velocities.[27] The reaction is usually completely reversible and formation constants are often low enough that a carbonyl compound present in small amounts will not react extensively with an amine unless the two are brought together on an enzyme surface. If the amino group is hydrogen bonded to the enzyme, the proton may remain on the Schiff base nitrogen, enhancing the electron-accepting properties of the C=N group. The pK_a values of Schiff bases with aliphatic aldehydes are usually 1– 2 units lower than those of the corresponding primary amines. However, the local environment may sometimes cause the pK_a to be increased. Most Schiff bases are reduced readily by sodium borohydride or sodium cyanoborohydride to form secondary amines in which the original aldehydes are bound covalently to the original amino groups (Eq. 3-34). This provides a method for locating sites of Schiff base formation in enzymes. An isotopically substituted aldehyde or amine can be employed, or an isotopic label can be introduced, by using ^2H- or ^3H-containing sodium borohydride in the reduction.

3. Stereochemistry of Addition to Trigonal Carbon Atoms

Adducts formed by enzymatic addition of nucleophiles to carbonyl groups are usually chiral. For example, addition of an amino group to the "front" side of the carbonyl carbon in Eq. 13-5 (the *si* face as defined in Chapter 9) creates a carbinolamine of the *S* configuration. Addition of the amino group from behind the plane of the paper (the *re* face) would lead to a carbinolamine of the *R* configuration.

BOX 13-A ZINC

The average human ingests 10–15 mg of zinc a day.[a] Although it is poorly absorbed, the concentrations of zinc in tissues are relatively high and the metal plays an essential role in a multitude of enzymes. The total zinc content of a 70-kg person is 1.4–2.3 g. A typical tissue concentration of Zn^{2+} is 0.3–0.5 mM; an unusually high content of ~15 mM is found in the prostate gland.

Zinc ion is much more tightly bound to most organic ligands than is Mg^{2+} (Table 6-9). It has a filled 3*d* shell and tends to form four ionic bonds with a tetrahedral geometry, often with nitrogen- or sulfur-containing ligands.[b] Unlike Mg^{2+}, which interacts rapidly and reversibly with enzymes, Zn^{2+} tends to be tightly bound within over 300 **metalloenzymes**.[c–e] A common feature is the surrounding of the Zn^{2+} at the active center by three imidazole groups, the fourth coordination position being free for interaction with substrate. The second nitrogen of the imidazole ring in many instances is hydrogen bonded to a main chain carbonyl group of the peptide, a feature that is also shared by histidines in other metalloproteins.

The most important chemical function of Zn^{2+} in enzymes is probably that of a Lewis acid providing a concentrated center of positive charge at a nucleophilic site on the substrate.[f] This role for Zn^{2+} is discussed for carboxypeptidases (Fig.12-16) and thermolysin,[g] alkaline phosphatase (Fig. 12-23),[h] RNA polymerases, DNA polymerases, carbonic anhydrase (Fig. 13-1),[i] class II aldolases (Fig. 13-7), some alcohol dehydrogenases (Fig. 15-5), and superoxide dismutases (Fig.16-22). Zinc ions in enzymes can often be replaced by Mn^{2+}, Co^{2+}, and other ions with substantial retention of catalytic activity.[f,j]

In addition to its function in catalysis, zinc often plays an important structural role, e.g., in the **zinc finger** transcriptional regulators (Fig. 5-38).[k] Zinc ions bind to insulin and stabilize its hexameric structure (Fig. 7-18).[l] Six Zn^{2+} ions are present in the hexagonal tail plate of the T-even bacteriophage (Box 7-C) and appear to be essential for invasion of bacteria.[m] In carnivores, the **tapetum**, the reflecting layer behind the retina of the eye of many animals, contains crystals of the Zn^{2+}–cysteine complex. Since zinc ions have no color their presence has often been overlooked. Zinc ions will doubtless be found in many more places within cells. Zinc is usually the major component of the bound metals in the **metallothioneins** (Box 6-E). These small 6.6-kDa proteins which contain ~33% cysteine and bind as many as six ions of Cd^{2+}, Hg^{2+}, Cu^{2+}, or Zn^{2+} per molecule are present in all animal tissues as well as in plants and some bacteria.[j]

From a nutritional viewpoint, Cu^{2+} competes with zinc ion, as does the very toxic Cd^{2+}. The latter accumulates in the cortex of the kidney. Dietary cadmium in concentrations less than those found in human kidneys shortens the lives of rats and mice. However, some marine diatoms contain a cadmium-dependent carbonic anhydrase.[n] Although zinc deficiency was once regarded as unlikely in humans, it is now recognized as occurring under a variety of circumstances[o,p] and is well-known in domestic animals.[q] Consumption of excessive amounts of protein as well as alcoholism, malabsorption, sickle cell anemia, and chronic kidney disease can all be accompanied by zinc deficiency.

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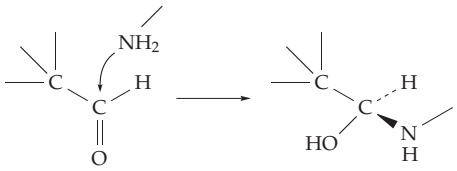
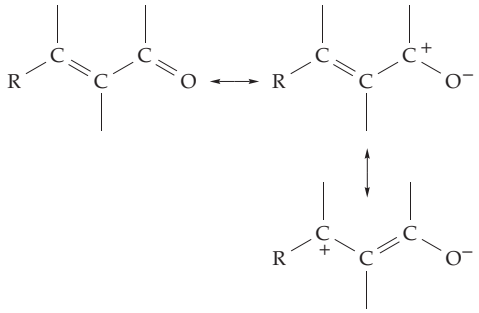


Addition from "front" (*re* face of the carbonyl carbon) — *S* configuration (13-5)

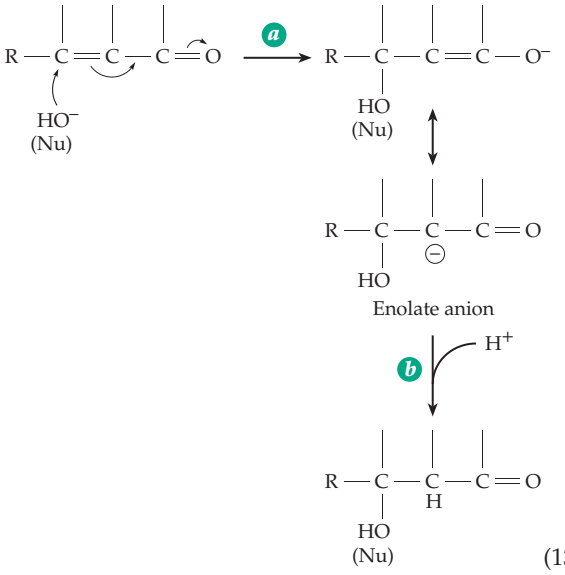
4. Addition to Carbon–Carbon Double Bonds, Often Reversible Reactions

Most of the reactions that we will consider in this chapter involve addition of a proton to a carbon atom or removal of a proton attached to a carbon atom. A frequent metabolic reaction is addition of water to a carbon–carbon double bond that is conjugated with a carbonyl group. This transmits the polarization of the carbonyl group to a position located two carbon atoms further along the chain.



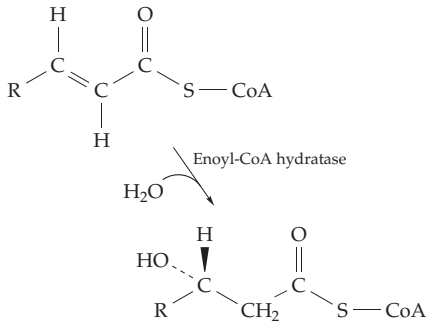
Because of this effect bases can add to a carbon–carbon double bond in a position β to the carbonyl group as in Eq. 13-6, step *a*. The product of addition of HO^- is the anion of an **enol** in which the negative charge is distributed by resonance between the oxygen of the carbonyl group and the carbon adjacent to the carbonyl. A stable product results if a proton adds to the latter position (Eq. 13-6, step *b*). Many nucleophilic groups (Nu in Eq. 13-6) other than HO^-, may add, the reaction being known as a **Michael addition**. If a neutral nucleophile Nu adds, the product may lose a proton, or transfer it to the adjacent carbon in Eq. 13-6, step *b*, to give a neutral end product. The reverse of an addition reaction of this type is also known as an **elimination reaction**.

Enzymatic reactions involving addition to a C=C bond adjacent to a carbonyl group (or in which elimination occurs α,β to a carbonyl) are numerous. Except for some enzymes acting by free radical mechanisms, the nucleophilic group always adds at the β position suggesting that the mechanism portrayed by Eq. 13-7 is probable. It is noteworthy that *frequently in a metabolic sequence a carbonyl group is deliberately introduced to facilitate elimination or addition at adjacent carbon atoms*. The carbonyl may be formed by oxidation of a hydroxyl group or it may be provided by a thioester formed with coenzyme A or with an **acyl carrier protein** (Chapter 14).



(13-6)

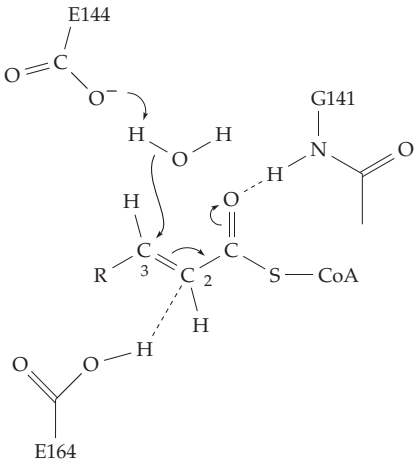
Enoyl-CoA hydratase. A specific example of the reaction in Eq. 13-6 is the addition of water to *trans*-α,β-unsaturated CoA derivatives (Eq. 13-7). It is catalyzed by enoyl–CoA hydratase (crotonase) from mitochondria and is a step in the β oxidation of fatty acids (Fig. 10-4).



L-β-Hydroxyacyl-CoA (13-7)

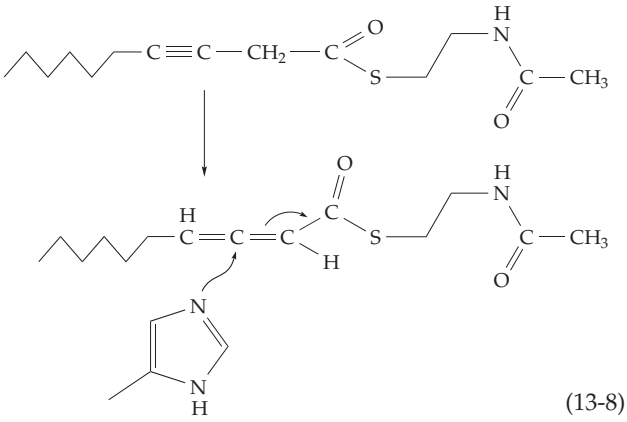
The enzyme is a hexamer, actually a dimer of trimers made up of 291-residue polypeptide chains.[28] Acetoacetyl-CoA is a competitive inhibitor which binds into the active site and locates it. From the X-ray structure of the enzyme–inhibitor complex it can be deduced that the carboxylate group of E144 abstracts a proton from a water molecule to provide the hydroxyl ion that binds to the β position (Eq. 13-6, step *a*) and that the E164 carboxyl group donates a proton to the intermediate enolate anion in step *b*.[28] The hydroxyl group

and the proton enter from the same side of the double bond in a *syn* addition. The X-ray structure shows that the E144 and E164 side chains are on the same side of the double bond, accounting for this stereochemistry.[28,28a]



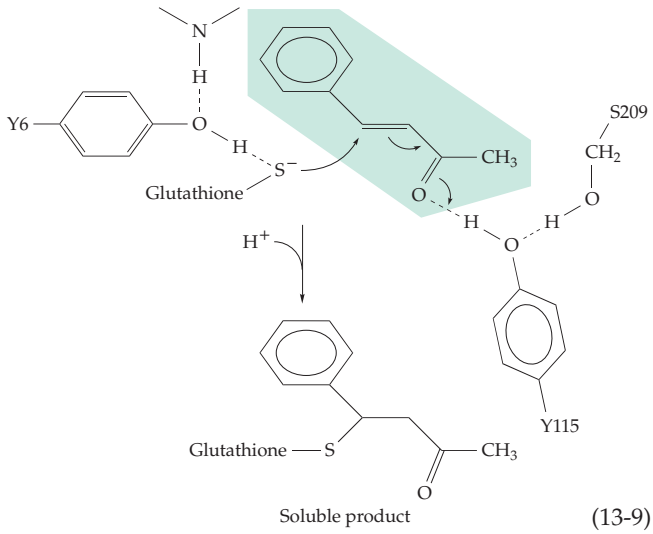
Cinnamoyl-CoA thiol esters (R = phenyl in the foregoing structure) contain a good light-absorbing group (chromophore). Binding to the protein induces distinct shifts in the ultraviolet absorption spectrum and also in ^{13}C NMR and Raman spectra. These shifts suggest that binding induces an enhanced positive charge at C-3 and formation of a strong hydrogen bond to the carbonyl group.[29] The X-ray studies indicate that this bond is to the G141 peptide nitrogen as shown. These results favor an enolate anion intermediate as in Eq. 13-6. However, kinetic isotope effects[30] as well as studies of proton exchange have suggested a concerted mechanism with water adding at the same time that a proton binds at C-2.[30–31a]

A closely related *E. coli* protein is a 79-kDa multifunctional enzyme that catalyzes four different reactions of fatty acid oxidation (Chapter 17). The amino-terminal region contains the enoyl hydratase activity.[32] A quite different enzyme catalyzes dehydration of thioesters of β-hydroxyacids such as 3-hydroxydecanoyl-acyl carrier protein (see Eq. 21-2) to both form and isomerize enoyl-ACP derivatives during synthesis of unsaturated fatty acids by *E. coli*. Again, a glutamate side chain is the catalytic base but an imidazole group of histidine has also been implicated.[33] This enzyme is inhibited irreversibly by the *N*-acetylcysteamine thioester of 3-decynoic acids (Eq. 13-8). This was one of the first enzyme-activated inhibitors to be studied.[34]



(13-8)

Glutathione S-transferases. Addition of glutathione (Box 11-B) to a large variety of different substrates containing electrophilic centers, such as that at the β position in an α,β-unsaturated ketone (Eq. 13-9), is catalyzed by the ubiquitous group of enzymes called glutathione *S*-transferases. There are six classes of

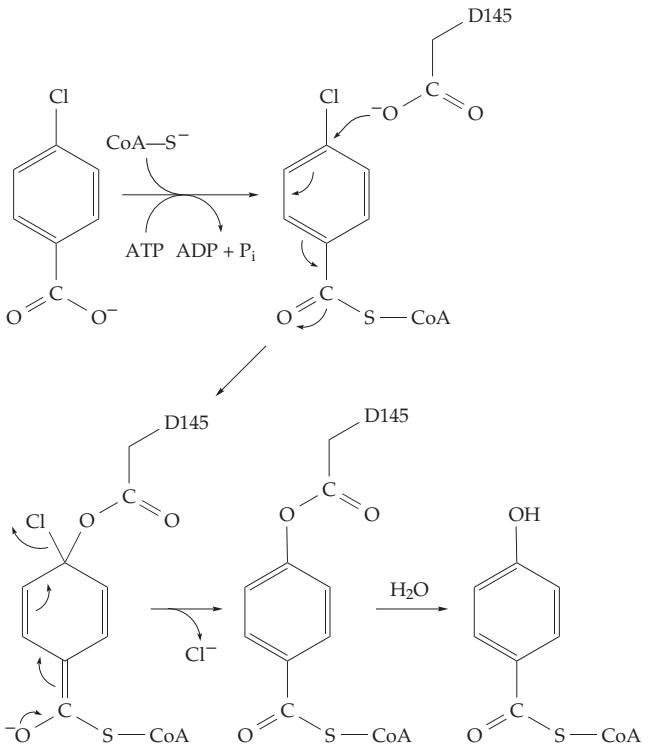


(13-9)

eukayotic glutathione *S*-transferases—one membrane-associated microsomal[35] and five cytosolic.[36–40] They play an important role in detoxifying many xenobiotics and other relatively hydrophobic compounds by converting them to more soluble compounds that can be degraded and excreted easily (see Box 11-B). A pair of tyrosines appear to participate in catalysis, as is indicated in Eq. 13-9.[38,41] Glutathione *S*-transferases have attracted attention because of their role in detoxifying anticancer drugs. Cancer cells can become resistant to drugs as a result of excessive synthesis of these detoxifying enzymes. At the same time glutathione transferases protect human patients from drugs. In plants these enzymes may provide protection from insecticides and herbicides.[41a] Glutathione transferases of pathogenic organisms such as schistosomes are appropriate targets for new drugs as well as for vaccines.[42] Nonenzymatic Michael addition of glutathione to such compounds as **4-hydroxynonenal**, a product of peroxidation of the polyunsaturated arachidonate (see Eq. 21-15) are also biochemically important.

Chlorobenzoyl-CoA dehalogenase. The enzymatic release of chloride ion from 4-chloroxybenzoyl-CoA

would be hard to explain by a simple nucleophilic displacement by HO^-. However, *addition* of HO^-, followed by elimination of Cl^-, would be chemically reasonable. Nevertheless, single-turnover studies revealed a more complex mechanism involving formation of a covalent adduct with the enzyme.[43,44] A carboxylate group adds and Cl^- is eliminated to form of an oxygen ester, which is then hydrolyzed to the final product (Eq. 13-10). Studies of mutant enzymes[45] together with X-ray crystallography[46] support this mechanism. As with enoyl-CoA hydratase, binding of substrate to chlorobenzoyl-CoA dehalogenase causes alterations in ultraviolet, NMR, and Raman spectra that can be interpreted as indicating enhanced polarization of the benzoyl group.[47,48] For example, changes in the C = O stretching frequency suggest that the bond is elongated, presumably as a result of hydrogen bonding to the N – H of G114, which lies at the N terminus of an α helix and experiences the additional polarizing effect of the helix dipole (Fig. 2-20).[48,49] This effect can be compared with that mentioned previously for enoyl-CoA hydratase.

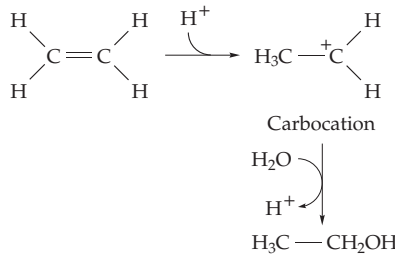


(13-10)

In fact, close sequence and structural homologies show that chlorobenzoyl-CoA dehalogenase, enoyl-CoA hydratase, and a variety of other hydratases, isomerases, synthases, lyases, and hydrolases belong to a large family of related proteins.[49a,49b,49c]

5. Addition to Double Bonds Adjacent to Carboxylate Groups

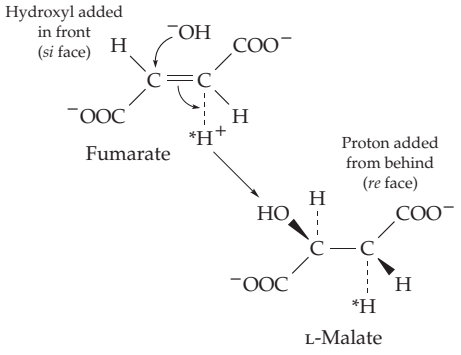
Biochemical reactions often involve addition to C = C bonds that are not conjugated with a true carbonyl group but with the poorer electron acceptor $-COO^-$. While held on an enzyme a carboxylate group may be protonated, making it a better electron acceptor. Nevertheless, there has been some doubt as to whether the carbanion mechanism of Eq. 13-6 holds for these enzymes. Some experimental data suggested a quite different mechanism, one that has been established for the nonenzymatic hydration of alkenes. An example is the hydration of ethylene by hot water with dilute sulfuric acid as a catalyst (Eq. 13-11), an industrial method of preparation of ethanol. The electrons of the double bond form the point of attack by a proton, and the resulting carbocation readily abstracts a hydroxyl



(13-11)

ion from water. Direct addition of OH^- to ethylene to form a carbanion is not favored, because there is no adjacent carbonyl group to stabilize the negative charge.

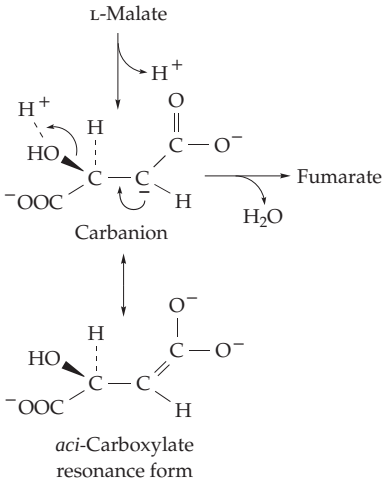
Fumarate hydratase. The most studied enzyme of this group is probably the porcine mitochondrial fumarate hydratase (fumarase; see also Chapter 9), a tetramer of 48.5-kDa subunits[50] with a turnover number of ~2 x 10^3 s^{-1}. It accelerates the hydration reaction more than 10^{15}-fold.[51] A similar enzyme, the 467-residue **fumarase C** whose three-dimensional structure is known,[52,53] is found in cells of *E. coli* when grown aerobically. The product of the fumarate hydratase reaction is L-malate (*S*-malate). The stereospecificity is extremely high. If the reaction is carried out in ^2H_2O an atom of ^2H is incorporated into the *pro-R* position, i.e., the proton is added strictly from the *re* face of the trigonal carbon (Eq. 13-12). To obtain L-malate the hydroxyl must have been added from the opposite side of the double bond. Such *anti* (*trans*) addition is much more common in both nonenzymatic and enzymatic reactions than is addition of both H and OH (or –Y) from the same side (*syn*, *cis*, or adjacent addition).[54] For concerted addition it is a natural result of stereoelectronic control. Almost *all* enzymatic addition and elimination reactions involving free carboxylic acids are anti with the proton entering from the *re* face,



(13-12)

suggesting that there has been conservation throughout evolution of a single basically similar mechanism.[54,55]

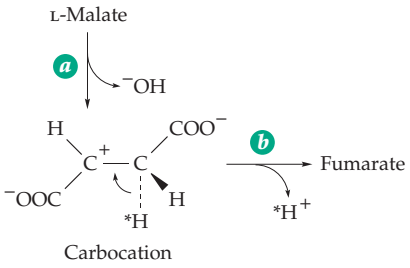
The pH dependence of the action of fumarate hydratase indicates participation of both an acidic and a basic group with pK_a values of 5.8 and 7.1.[56] See Chapter 9 for additional information. However, either anion or carbocation mechanisms might be possible. That the cleavage of the C–H bond is not rate limiting is suggested by the observation that malate containing ^{2}H in the *pro-R* position is dehydrated at the same rate as ordinary malate. If the anion mechanism (Eq. 13-13) is correct, the ^{2}H from the *pro-R* position of specifically labeled malate might be removed rapidly, while the loss of OH^- could be slower. If so, the ^{2}H would be "washed out" of L-malate faster than could happen by conversion to fumarate followed by rehydration to malate. In fact, the opposite was observed.



(13-13)

The hydroxyl group was lost rapidly and the ^{2}H more slowly. This result suggested the carbocation ion mechanism of Eq. 13-14.

While no *primary* isotope rate effect was observed, when the hydrogen at C-2 or the *pro-S* hydrogen at C-3 of malate was replaced by ^{2}H or ^{3}H distinct secondary isotope effects were seen. Thus, k(^{1}H) / k(^{2}H) = 1.09 for both the *pro-S* and the C-3 hydrogen atoms.[57] These findings appeared to support the carbocation

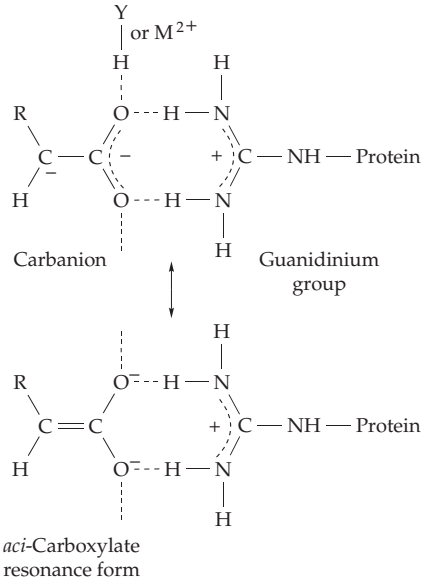


(13-14)

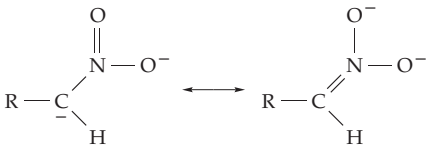
mechanism for the reasons considered in Chapter 12 and suggested that step *b* of Eq. 13-14 is rate limiting. The relative values of V_{max} for hydration of fumarate, fluorofumarate, and difluorofumarate (104, 410, and 86 mol ml^{-1} min^{-1} mg^{-1}) also seemed to support the carbocation mechanism.[58]

However, it has become clear that protons removed from a substrate to a basic group in a protein need not exchange rapidly with solvent (see Eq. 9-102). In fact, the proton removed by fumarate hydratase from malate is held by the enzyme for relatively long periods of time. Its rate of exchange between malate and solvent is slower than the exchange of a bound fumarate ion on the enzyme surface with another substrate molecule from the medium.[59] Thus, the overall rate is determined by the speed of dissociation of products from the enzyme and we cannot yet decide whether removal of a proton precedes or follows loss of OH^-.

Two new lines of evidence suggest that proton abstraction comes first. A careful study of both ^{18}O and ^{2}H isotope effects[60] supports the carbanion intermediate, as does the strong inhibition by anions of 3-nitropropionate and 3-nitro-2-hydroxypropionate.[61] To provide a good electron sink the carboxylate group adjacent to the proton that is removed by fumarate hydratase must either be actually protonated in the enzyme–substrate (ES) complex or paired with and hydrogen bonded to a positively charged group.



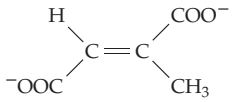
The anion formed by removal of the 3-H is analogous to the enolate anion of Eq. 13-6 and has a strong structural similarity to the readily formed anions of organic nitro compounds. The nitronate anions may, perhaps, be regarded as transition state inhibitors.



Nitronate anion

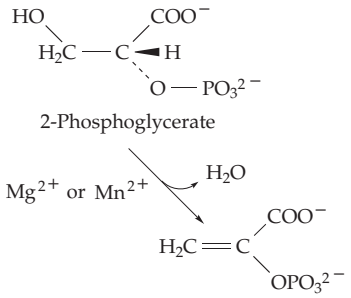
There is still a third possible mechanism for the fumarate hydratase reaction. The proton and hydroxyl groups may be added *simultaneously* in a concerted reaction.[62] However, observed kinetic isotope effects are not consistent with this mechanism.[60] In 1997 the structure of fumarase C of *E. coli* was reported.[52,53] Each active site of the tetrameric enzyme is formed using side chains from three different subunits. The H188 imidazole is hydrogen bonded to an active site water molecule and is backed up by the E331 carboxylate which forms a familiar catalytic pair. However, these results have not clarified the exact mode of substrate binding nor the details of the catalytic mechanism. Structural studies of fumarate hydratase from yeast[53a] and the pig[53b] are also in progress.

Some other fumarate-forming reactions. Other enzymes that catalyze elimination reactions that produce fumarate are **aspartate ammonia-lyase** (aspartase),[63] **argininosuccinate lyase** (Fig. 24-10, reaction *g*),[64,65] and **adenylosuccinate lyase** (Fig. 25-15). In every case it is NH_3 or an amine, rather than an OH group, that is eliminated. However, the mechanisms probably resemble that of fumarate hydratase. Sequence analysis indicated that all of these enzymes belong to a single **fumarase-aspartase family**.[64,65] The three-dimensional structure of aspartate ammonia-lyase resembles that of fumarate hydratase, but the catalytic site lacks the essential H188 of fumarate hydratase. However, the pK_a values deduced from the pH dependence of V_{max} are similar to those for fumarase.[64] **3-Methylaspartate lyase** catalyzes the same kind of reaction to produce ammonia plus *cis*-mesaconate.[63] Its sequence is not related to that of fumarase and it may contain a dehydroalanine residue (Chapter 14).[66]



cis-Mesaconate

Enolase. A key reaction in the metabolism of sugars is the dehydration of 2-phosphoglycerate to form **phosphoenolpyruvate** (PEP), the phospho derivative of the enolic form of pyruvic acid:



(13-15)

A carbanionic intermediate has often been suggested for this enzyme.[67,68] However, despite measurements of kinetic isotope effects and many other experiments it has been difficult to establish a detailed mechanism.[68]

Enolase has a complex metal ion requirement,[68a,69] usually met by Mg^{2+} and Mn^{2+}. From NMR studies of the relaxation of water protons, it was concluded that a Mn^{2+} ion coordinates two rapidly exchangeable water molecules in the free enzyme. When substrate binds, one of these water molecules may be immobilized and may participate in an addition reaction that forms phosphoenolpyruvate (reverse of reaction 13-15). A tightly bound "conformational" metal ion is located in the known three-dimensional structure in such a

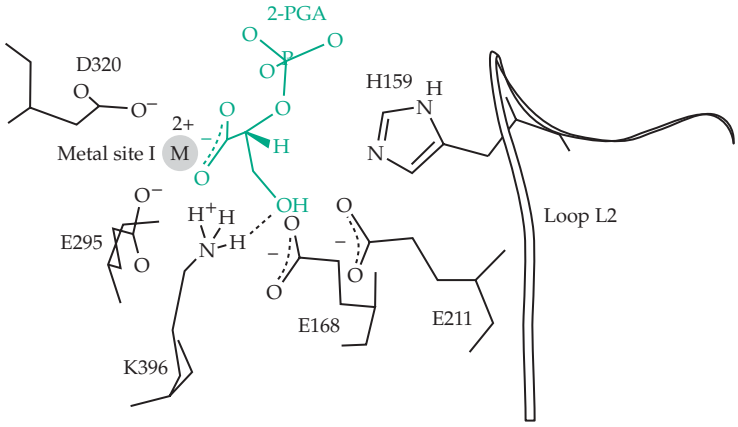


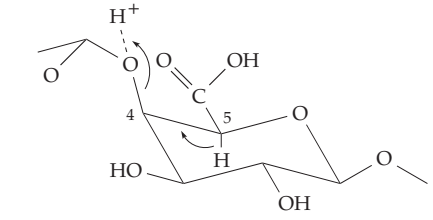
Figure 13-2 View of the active site of yeast enolase containing a bound molecule of 2-phospho-D-glycerate. The catalytic magnesium ion is at the left but the "conformational" metal is not visible here. The imidazole group of His 159 serves as the catalytic base and the $-NH_3^+$ of Lys 396 or Lys 345[73b] as the catalytic acid. From Vinarov and Nowak.[69]

way that it might function in this manner. A more loosely bound "catalytic" metal ion is also essential.[69–72]

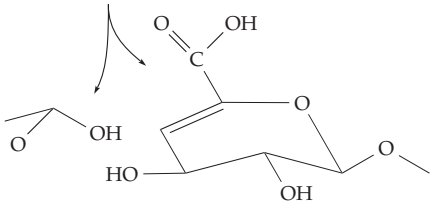
Figure 13-2 shows a view of the active site of yeast enolase occupied by a molecule of bound 2-phosphoglycerate. Histidine 159 is probably the catalytic base that removes the α-proton to form an *aci*-anion which is stabilized by interaction with the catalytic Mg^{2+} ion. A protonated lysine 396 amino group may be the catalytic acid.[68a,69] The active site is surrounded by a complex hydrogen-bonded network.[73] As is discussed in Section B, a large number of other enzymes belong to an **enolase superfamily** of enzymes. Among them is **glucarate dehydratase**, which initiates a pathway for catabolism of D-glucarate and galactarate in *E. coli*.[73a]

Pectate lyase and related enzymes. A group of polysaccharide lyases cleave the chains of polymers of uronic acids with 1,4 linkages such as pectins, hyaluronan, heparin,[74,75] and dermatan sulfate (Fig. 4-11). These bacterial enzymes also employ an elimination mechanism.[76] The geometry of the β-linked galacturonic acid units of pectin is favorable for *anti* elimination of the 5-H and the *O*-glycosyl group in the 4 position (Eq. 13-16). However, the corresponding **hyaluronate lyase** (hyaluronidase) acting on glucuronic acid residues causes a *syn* elimination. These results suggest the formation of anionic intermediates which can eliminate a substituent from either the equatorial or axial position of the sugar ring. Hyaluronate lyase from the pathogenic *Streptococcus pneumoniae* apparently utilizes an imidazole group as the catalytic base and a tyrosine side chain as the proton donor in the reaction.[76a,76b]

Pectate lyase C from the plant pathogen *Erwinia*, which causes soft-rot in many different plants, has a parallel β barrel structure (Fig. 13-3)[77] which is similar to that of the tailspike protein shown in Fig. 2-17 and represents what may be a very large structural family of proteins.[78] The location of the active site is not



Galacturonic acid unit of pectin



(13-16)

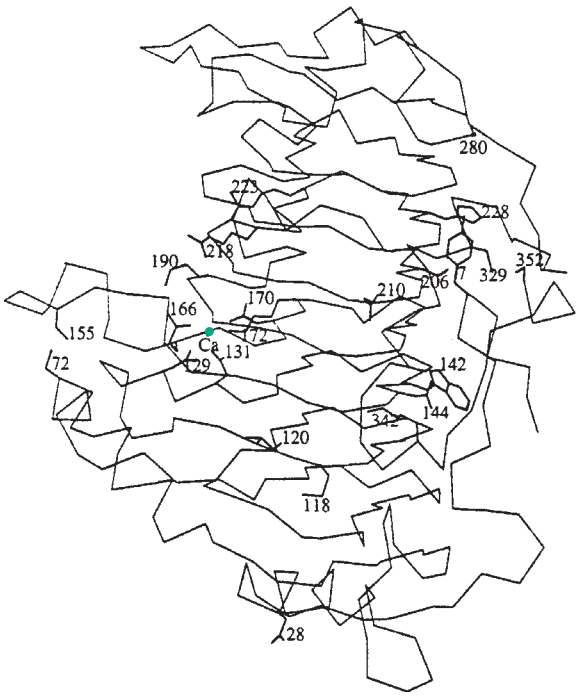


Figure 13-3 The three-dimensional structure of pectate lyase C showing locations of amino acids substituted by oligonucleotide-directed mutation of the cloned gene. The green dot labeled Ca is the Ca^{2+}-binding site.[79] Courtesy of Frances Jurnak.

obvious from the structure but on the basis of the effects of a large number of mutations, it is thought to be near the Ca^{2+}- binding site labeled in Fig. 13-3. Replacement of any of three Asp or Glu and three Lys residues in this region leads to loss of or reduction in catalytic activity.[79]

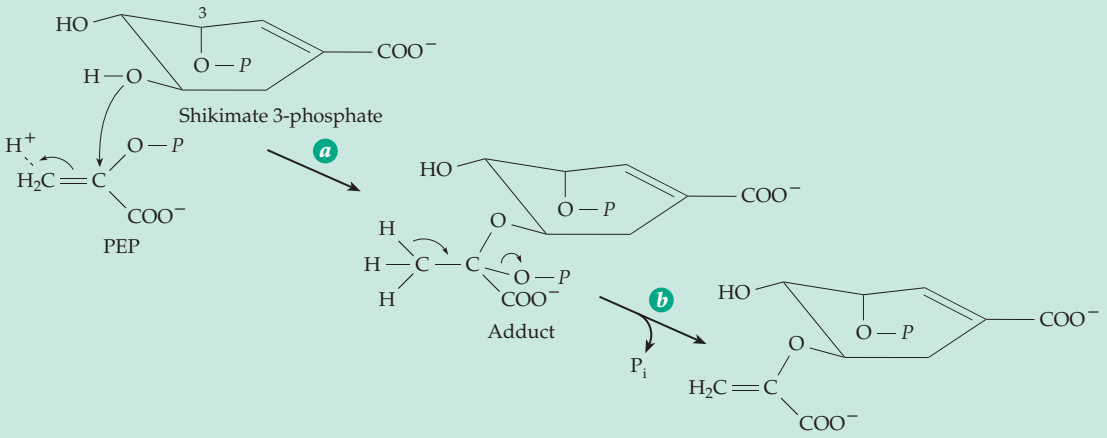
6. Aconitase and Related Iron – Sulfur Proteins

Two consecutive reactions of the citric acid cycle (Fig. 10-6), the dehydration of citrate to form *cis*-aconitate and the rehydration in a different way to form isocitrate (Eq. 13-17), are catalyzed by aconitase (aconitate hydratase). Both reactions are completely stereospecific. In the first (Eq. 13-17, step *a*), the *pro-R* proton from C-4 (stereochemical numbering) of citrate is removed and in step *c* isocitrate is formed. Proton addition is to the *re* face in both cases.

As with fumarate hydratase, the enzyme holds the abstracted proton (for up to 7×10^{-5} s) long enough so that a *cis*-aconitate molecule sometimes diffuses from the enzyme and (if excess *cis*-aconitate is present) is replaced by another. The result is that the new *cis*-aconitate molecule sometimes receives the proton (intermolecular proton transfer). The proton removed

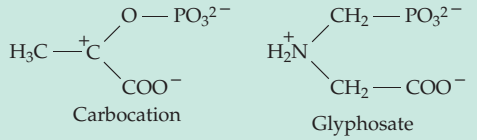
BOX 13-B EPSP SYNTHASE AND THE HERBICIDE GLYPHOSATE

The reversible reaction of phosphoenolpyruvate (PEP) with shikimate 3-phosphate is a step in the synthesis of the aromatic amino acids (see Fig. 25-1). The chemical mechanism indicated



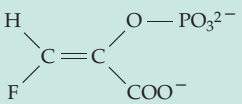
5-Enolpyruvoylshikimate-3-phosphate (EPSP)

was proposed by Leaven and Sprinson in 1964.[a] Step *a* is unusual[b] because it involves protonation on the methylene carbon of PEP and addition of a nucleophile at C-2, the opposite of the addition in the enolase reaction (Eq. 13-15, reverse). It is likely that formation of a cationic intermediate precedes that of the adduct shown. However, the structure of the adduct has been confirmed by isolation from the active site and by synthesis[c] and it has been observed in the active site by NMR spectroscopy.[d] The three-dimensional structure of EPSP synthase is known.[e,m] It is inhibited by the commercial herbicide **glyphosate** [*N*-(phosphonomethyl) glycine] whose structure is somewhat similar to that of the proposed carbocation that arises from PEP. The inhibitor (*Z*)-3-fluorophosphoenolpyruvate is a pseudosubstrate that reacts in step *a* to give a stable adduct unable to go through step *b* to form a



Carbocation

Glyphosate



(*Z*)-3-Fluorophosphonoenolypyruvate

A related mechanism is utilized in the biosynthesis of UDP-muramic acid (Eq. 20-6).[j] There is an enolpyruvoyl adduct analogous to that of EPSP synthase; a proposed enolpyruvoyl-enzyme adduct with Cys 115 is not on the major path.[k,l] However, this enzyme is not inhibited by glyphosate.[i]

[a] Levin, J. G., and Sprinson, D. B. (1964) *J. Biol. Chem.* **239**, 1142–1150

[b] Barlow, P. N., Appleyard, R. J., Wilson, B. J. O., and Evans, J. N. S. (1989) *Biochemistry* **28**, 7985–7991

[c] Anderson, K. S., Sikorski, J. A., Benesi, A. J., and Johnson, K. A. (1988) *J. Am. Chem. Soc.* **110**, 6577–6579

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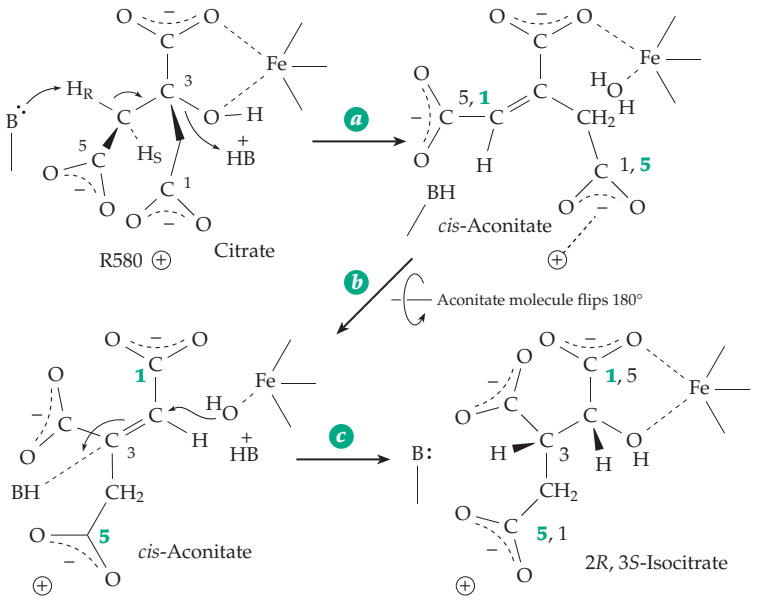
[m] Lewis, J., Johnson, K. A., and Anderson, K. S. (1999) *Biochemistry* **38**, 7372–7379

from citrate is often returned to the molecule in Eq. 13-17, step *b*, but the position of reentry is different from that of removal. Apparently, after the initial proton removal, the *cis*-aconitate that is formed "flips over" so that it can be rehydrated (Eq. 13-17, step *c*) with participation of the same groups involved in dehydration but with formation of the new product.[80,80a]

Aconitase contains iron in the form of an Fe_4S_4 iron-sulfur cluster (Fig. 13-4).[81–83] However, the enzyme is usually isolated in a form that does not show its maximum activity until it has been incubated with ferrous iron (Fe^{2+}). The inactive form of the enzyme is thought to contain an Fe_3S_4 cluster (Chapter 16) which is converted back to the Fe_4S_4 cluster by the incubation



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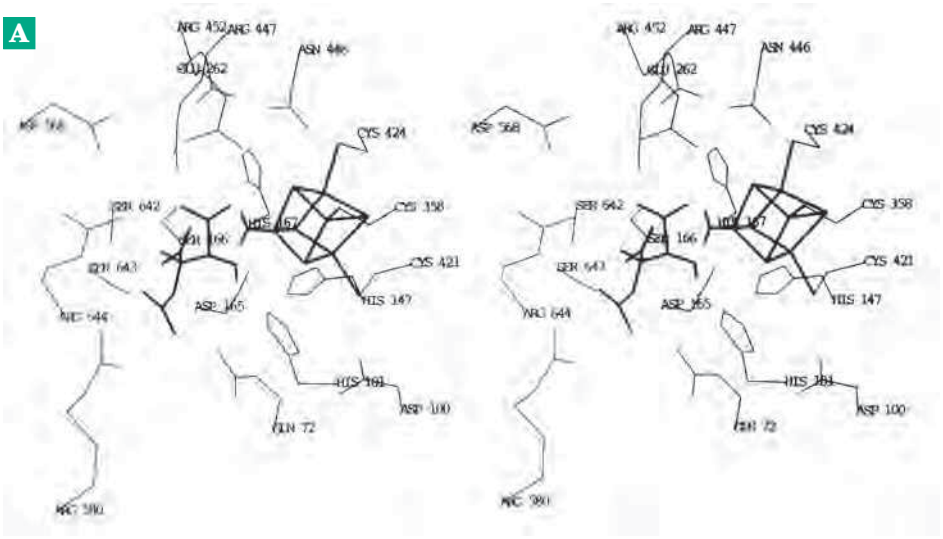


Figure 13-4 (A) Stereoscopic view of the active site of mitochondrial aconitase with a molecule of L-isocitrate placed by modeling next to the Fe_4S_4 cluster. This cluster still has a water molecule bound to one iron atom as in the free enzyme.[83] Courtesy of C. D. Stout. (B) Interpretive diagram.

with Fe^{2+}.[84–87] From Mössbauer spectroscopy it was deduced that the iron atom that is lost upon inactivation is the binding site for the –OH group of citrate or isocitrate and of an adjacent carboxylate.[87] Since iron can engage in oxidation–reduction processes, aconitase could act by a mechanism different from others discussed here. However, an Fe–OH group could act much as does the Zn–OH of carbonic anhydrase (Eq. 13-4). Any redox chemistry may be involved in control of the enzyme.

X-ray studies have confirmed the proximity of the Fe_4S_4 cluster to bound substrate analogs.[83,88] The isocitrate shown in Fig. 13-4 was fitted into the active site by modeling. It shows the water molecule bound to the Fe_4S_4 cluster as observed for the free enzyme.[83] Notice that this cluster is held by three cysteine side chains (C358, C421, and C424) rather than the usual four (Chapter 16). The fourth iron atom is free to bind to water. When the enzyme acts on isocitrate this water must be displaced by the one generated from the substrate. The Fe probably acts as a polarizing electrophile that assists in the elimination but it is apparently the histidine–aspartate pair (H101–D100) that serves as the catalytic acid in generating the H_2O. What is the catalytic base? The only candidate seen in the structure is the Ser 642 –OH, dissociated to $-O^-$. Can this be correct? Mutational analysis supports the essential role of this side chain.[80a] The peptide NH and guanidinium groups of R644 appear to provide an "oxyanion hole" (Chapter 12) that stabilizes the negative charge.[86] Mutations also support the role of the His-Asp pair.[88,89] Another His–Asp pair (H167–D165) is located directly behind the bound substrate and Fe–OH_2 in Fig. 13-4A and is apparently also essential.[88]

Aconitase exists as both mitochondrial and cytosolic isoenzyme forms of similar structure. However, the cytosolic isoenzyme has a second function. In its apoenzyme form, which lacks the iron–sulfur cluster, it acts as the much-studied **iron regulatory factor**, or iron-responsive element binding protein (IRE-BP). This protein binds to a specific stem-loop structure in the messenger RNA for proteins involved in iron transport and storage (Chapter 28).[86,90]

Other enzymes in the aconitase family include **isopropylmalate isomerase** and **homoaconitase** enzymes functioning in the chain elongation pathways to leucine and lysine, both of which are pictured in Fig. 17-18.[90] There are also iron–sulfur dehydratases, some of which may function by a mechanism similar to that of aconitase. Among these are the two fumarate hydratases, **fumarases A and B,** which are formed in place of fumarase C by cells of *E. coli* growing anaerobically.[91,92] Also related may be bacterial L-serine and L-threonine dehydratases. These function without the coenzyme pyridoxal phosphate (Chapter 14) but contain iron–sulfur centers.[93–95] A **lactyl-CoA dehydratase** and some related iron-sulfur enzymes (pp. 861-862) may act via a mechanism related to that of vitamin B_{12}.

7. Addition to or Formation of Isolated Double Bonds

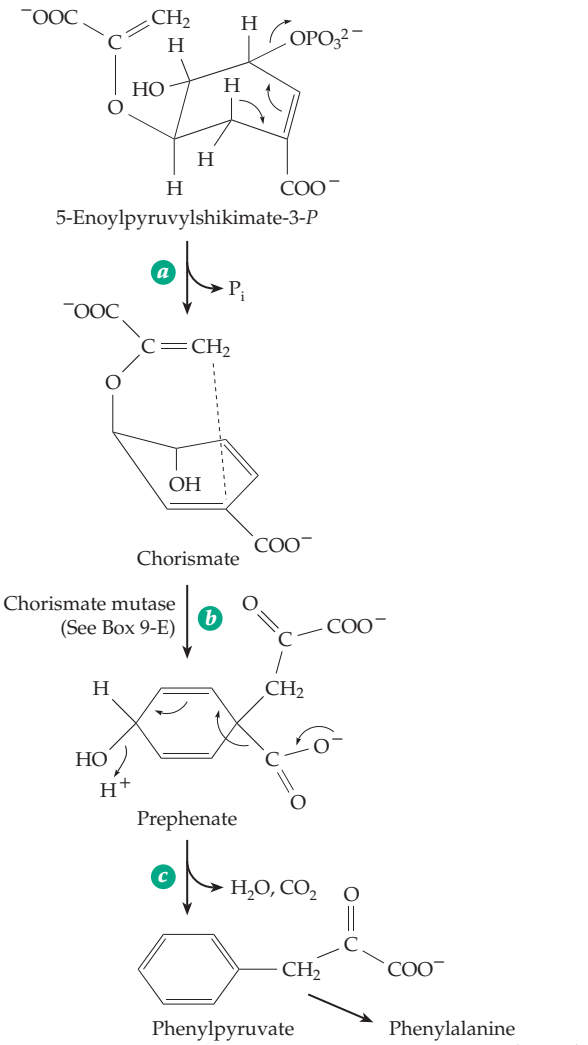
Only a few examples are known in which an enzyme induces addition to a double bond that is *not conjugated* with a carbonyl or carboxyl group. Pseudomonads have been observed to catalyze stereospecific hydration of oleic acid to D-10-hydroxystearate.[96] The addition is *anti* and the proton enters from the *re* face.

8. Conjugative and Decarboxylative Elimination Reactions

Elimination can occur if the electrophilic and nucleophilic groups to be removed are located not on adjacent carbon atoms but rather are separated from each other by a pair of atoms joined by a double bond. Such a conjugative elimination of phosphate is the last step in the biosynthesis of **chorismate** (Eq. 13-18, step *a*). Chorismate is converted to **prephenate** (see Box 9-E) which undergoes a conjugative and decarboxylative elimination (Eq. 13-18, step *c*) with loss of both water and CO_2 to form **phenylpyruvate**, the immediate biosynthetic precursor of phenylalanine. These reactions provide a good example of how *elimination reactions can be used to generate aromatic groups*. In fact, this is the usual method of synthesis of aromatic rings in nature.

Notice the stereochemistry of Eq. 13-18, step *a*. Orbital interaction rules predict that if the elimination is a concerted process it should be syn. The observed anti elimination suggests a more complex mechanism involving participation of a nucleophilic group of the enzyme.[97]

Elimination usually involves loss of a proton together with a nucleophilic group such as –OH, $-NH_3^+$, phosphate, or pyrophosphate. However, as in Eq. 13-18, step *c*, electrophilic groups such as $-COO^-$ can replace the proton. Another example is the conversion of **mevalonic acid-5-pyrophosphate** to **isopentenyl pyrophosphate** (Eq. 13-19): This is a key reaction in the biosynthesis of isoprenoid compounds such as cholesterol and vitamin A (Chapter 22). The phosphate ester formed in step *a* is a probable intermediate and the reaction probably involves a carbocationic intermediate generated by the loss of phosphate prior to the decarboxylation.



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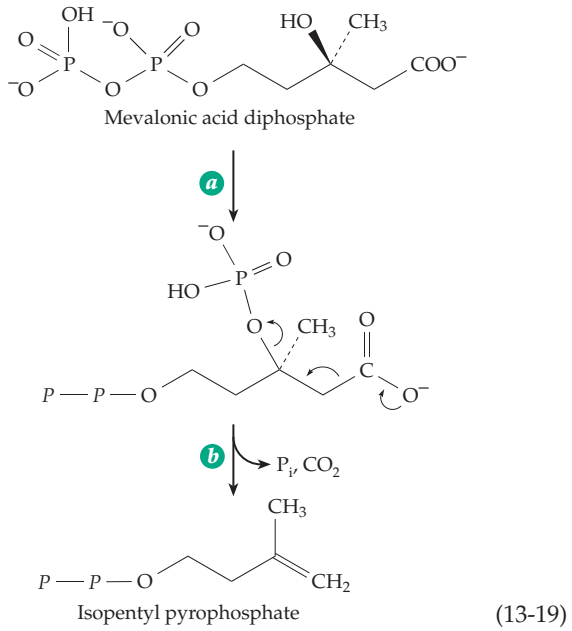
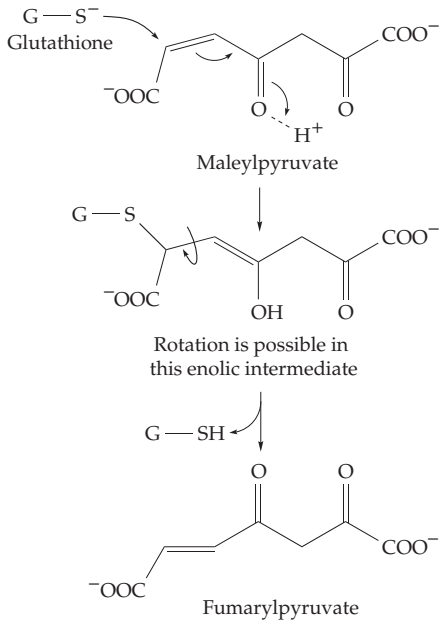


(13-19)

9. Isomerization Assisted by Addition

An interesting use is made of addition to a double bond by glutathione-dependent ***cis–trans* isomerases**.[76] One of them converts **maleate** to fumarate with a turnover number of 300 s^{-1}. Similar enzymes, which participate in bacterial breakdown of aromatic compounds (Fig. 25-7), isomerize **maleylacetoacetate** and **maleylpyruvate** to the corresponding fumaryl derivatives (Eq. 13-20). The – SH group of bound glutathione is thought to add to the double bond. Rotation can then occur in the enolic intermediate. Thiocyanate ion catalyzes the isomerization of maleic acid nonenzymatically, presumably by a similar mechanism.



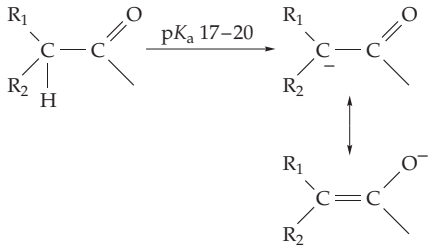
(13-20)

10. Reversibility of Addition and Elimination Reactions

Many addition and elimination reactions, e.g., the hydration of aldehydes and ketones, and reactions catalyzed by lyases such as fumarate hydratase are strictly reversible. However, biosynthetic sequences are often nearly irreversible because of the elimination of inorganic phosphate or pyrophosphate ions. Both of these ions occur in low concentrations within cells so that the reverse reaction does not tend to take place. In decarboxylative eliminations, carbon dioxide is produced and reversal becomes unlikely because of the high stability of CO_2. Further irreversibility is introduced when the major product is an aromatic ring, as in the formation of phenylpyruvate.

B. Enolic Intermediates in Enzymatic Reactions

Enzymologists have freely proposed enolate anions, enols, and enamines as intermediates for many years. Such intermediates have been demonstrated for some nonenzymatic acid- or base-catalyzed reactions, but how can enzymes form enolates at pH 7 without the use of strong acids or bases? The microscopic pK_a value of an α-hydrogen in a ketone or aldehyde is about 17–20.[72,98,99]



(13-21)

It will be similar for an acyl-CoA. However, for a carboxylic acid, protonated on oxygen, the pK_a will be much higher (~22–25) and for carboxylate anions even higher (~29–32).[100]

1. Mandelate Racemase and Related Enzymes

The degradation of mandelic acid by the bacterium *Pseudomonas putida* (Chapter 25) is initiated by mandalate racemase, another $(\alpha/\beta)_8$-barrel protein.[101] X-ray structures of bound inhibitors together with modeling suggest that the side chain of Lys 264 is the catalytic base that abstracts the α-H from *S*-mandelate (Fig. 13-5) and that the catalytic pair of His 297 and Asp 270 acts as proton donor, or, in the reverse direction, as catalytic base for deprotonation of *R*-mandelate.[102–104] The enzyme is structurally a member of the enolase superfamily[105] and requires Mg^{2+} for activity. The pH dependence of the reaction velocity k_{cat} reveals two pK_a values in the ES complex; ~6.4 and ~10.0. These are the same for *S*- and *R*- isomers. How can the pK_a values be assigned? Do they each belong in part to His 297 and in part to Lys 166? Do other adjacent groups also share? In the K166R mutant the lower pK_a is raised to 8.0. The D270N mutant has lost all but 0.01% of its catalytic activity.[104]

The carboxylate group of the mandelate interacts with side chains of E317, K264, and a Mg^{2+} ion (Fig. 13-5). These may serve both to protonate the carboxylate and also to help stabilize an *aci* anion formed upon dissociation of the α-hydrogen (Eq. 13-22). Both pK_a values for mandelic acid and its enolic form and the equilibrium constants for enolization in water are known[107] and are given beside the arrows in Eq. 13-22. It is difficult to imagine how a base with a pK_a near neutrality could remove an α-proton with a pK_a of 22.0, which is 14 pK units away from the pK_a of the catalytic base. This corresponds to a thermodynamic barrier (Eq. 9-97) of 14 x 5.7 = 80 kJ/mol, making the reaction impossibly slow. In addition to this thermodynamic barrier the *rates* of dissociation of carbon acids are known to be slow, presumably because of the lack of hydrogen bond formation between the C–H proton and the catalytic base (Eq. 9-97). This *intrinsic* barrier (Chapter 9) for simple ketones has been estimated as 45 kJ/mol for a total barrier of ~125 kJ. However, the observed $\Delta G^{\ddagger}$ as estimated from Eq. 9-81 is only ~57 kJ/mol. The enzyme must catalyze the reaction by lowering the very high thermodynamic barrier and perhaps also by lowering the intrinsic barrier.[72,108]

Protonation of the carboxylate greatly decreases the microscopic pK_a for loss of the α-hydrogen as a proton to form the enolic *aci* acid (Eq. 13-22). Double protonation, although depending upon a pK_a of –8,

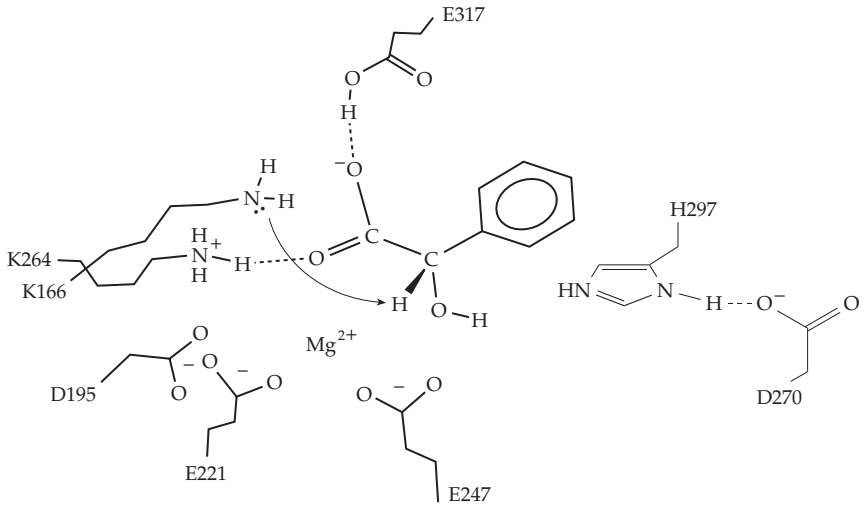
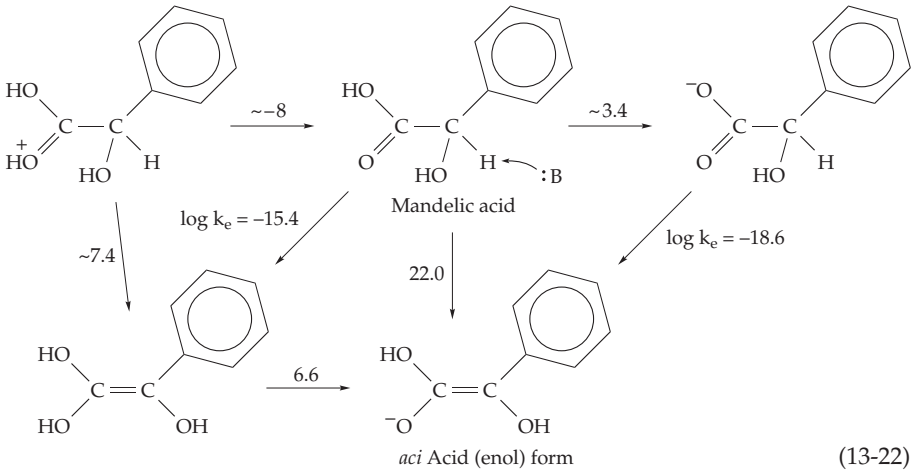


Figure 13-5 An *S*-mandelate ion in the active site of mandelate racemase. Only some of the polar groups surrounding the active site are shown. The enzyme has two catalytic acid–base groups. Lysine 166 is thought to deprotonate *S*-mandelate to form the *aci* anion, while His 297 deprotonates *R*-mandelate to form the same anion.[106]

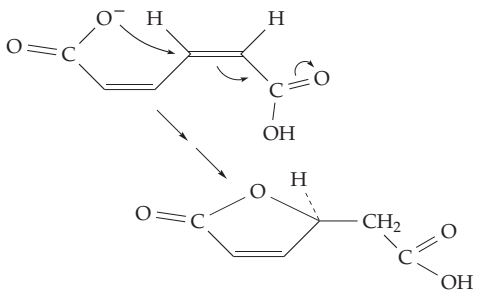


(13-22)

would reduce the pK_a of the α-hydrogen to ~ 7.4, making it very easy to enolize[109] and solving the thermodynamic problem. However, what forces could keep the substrate molecule in this unlikely state of protonation? Gerlt and Gassman proposed formation of a strong, short hydrogen bond to a carboxylate oxygen.[110] Formation of such a bond would lead to an increased positive charge on the alpha carbon, lowering the pK_a and therefore the thermodynamic barrier. It could also lower the intrinsic barrier,[108] for example, by permitting, to some extent, the formation of a C—H---N hydrogen bond in the transition state.[111] Polarization by the Mg^{2+} ion may also be involved. The very strong electrostatic forces within the active site may be sufficient to explain the formation of enolate anions or enols as intermediates in many different enzymes.[108,112]

A number of other racemases and epimerases may function by similar mechanisms. While some amino acid racemases depend upon pyridoxal phosphate (Chapter 14), several others function without this coenzyme. These include racemases for aspartate,[113] glutamate,[114–115a] proline, phenylalanine,[116] and diaminopimelate epimerase.[117] Some spiders are able to interconvert D and L forms of amino acid residues in intact polypeptide chains.[118,119]

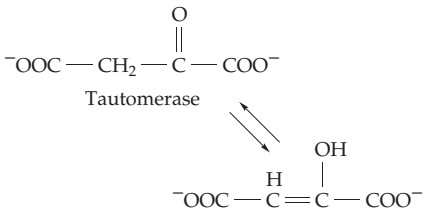
Another enzyme of the mandelate pathway of degradation of aromatic rings (Fig. 25-8) is the ***cis,cis*-muconate lactonizing enzyme** which catalyzes the reaction of Eq. 13-23. It has a three-dimensional structure almost identical to that of mandelate racemase but has incorporated an additional feature that allows formation, by addition, of the intermediate *aci*-acid.[101,105]



(13-23)

2. Isomerases

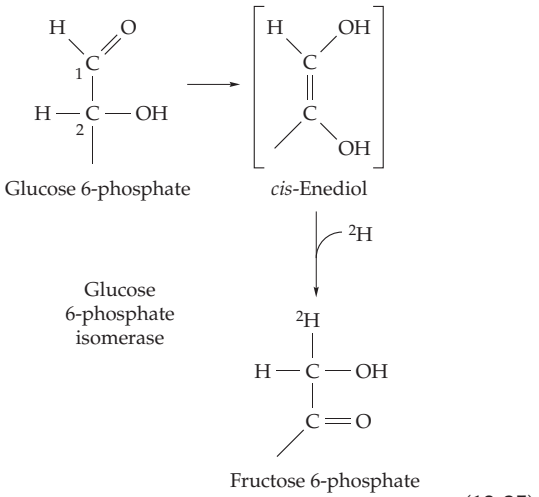
The isomerases that catalyze the simplest reactions are **tautomerases** that promote the oxo-enol (keto-enol) transformation. The widely distributed oxaloacetate tautomerase (Eq. 13-24) is especially active in animal tissues.[97,120] Oxaloacetate exists to a substantial extent in the enolic form: at 38°, ~ 6% enol, 13% oxo, and ~ 81% covalent hydrate.[120,121] A mammalian **phenylpyruvate tautomerase** has also been investigated.[122]



(13-24)

The oxidation of one functional group of a molecule by an adjacent group in the same molecule is a feature of many metabolic sequences. In most cases an enolic intermediate, formed either from a ketone as in Eq. 13-25 or by a dehydration reaction (see Eq. 13-32), is postulated.

Aldose–ketose interconversions. A metabolically important group of enzymes catalyze the interconversion of aldose sugars with the corresponding 2-ketoses (Table 10-1, reaction 4C). Several sugar phosphates undergo rapid isomerization. **Glucose 6-phosphate isomerase** (Eq. 13-25) appears to function in all cells with a high efficiency.[123–125a] The 132 kDa dimeric protein from muscle converts glucose 6-phosphate to



(13-25)

fructose 6-phosphate with a turnover number of ~ 10^3 s^{-1}. Hereditary defects in this enzyme cause a variety of problems that range from mild to very severe.[126] **Mannose 6-phosphate isomerase** also forms fructose 6-phosphate, while **ribose 5-phosphate isomerase**[127] interconverts the 5-phosphates of D-ribose and D-xylulose in the pentose phosphate pathways (Chapter 17). Other enzymes, most often metalloenzymes, catalyze the isomerization of free sugars.

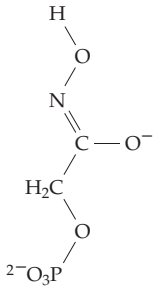
These enzymes vary widely in secondary and tertiary structure.[127a] Mannose-6-phosphate isomerase is a 45 kDa Zn^{2+}-containing monomer. The larger 65 kDa **L-fucose isomerase**, which also acts on D-arabinose, is a hexameric Mn^{2+}-dependent enzyme.[127a] **L-Arabinose isomerase** of *E. coli*, which interconverts arabinose and L-ribulose, is a hexamer of 60-kDa subunits[128] while the **D-xylose isomerase** of *Streptomyces* is a tetramer of 43-kDa subunits.[129] The nonenzymatic counterpart of the isomerization catalyzed by the enzyme is the base-catalyzed **Lobry deBruyn–Alberda van Ekenstein transformation** (Eq. 13-25).[130]

In 1895, Emil Ficher proposed an enediol intermediate for this isomerization. As would be expected, the enzyme-catalyzed isomerization of glucose-6-phosphate in ^2H_2O is accompanied by incorporation of deuterium into the product fructose 6-phosphate at C-1. In the reverse reaction ^2H-containing fructose 6-phosphate was found to react at only 45% of the rate of the ^1H-containing compound. Thus, the primary deuterium isotope effect expected for a rate-limiting cleavage of the C–H bond was observed (see Chapter 12, Section B,3).

When fructose 6-phosphate containing both ^2H and ^{14}C in the 1 position was isomerized in the presence of a large amount of nonlabeled fructose 6-phosphate, the product glucose 6-phosphate contained not only ^{14}C but also ^2H, and the distribution indicated that the ^2H had been transferred from the C-1 position into the C-2 position.[123] It was concluded that in over half the turnovers of the enzyme, ^2H removed from C-1 is put back on the same molecule at C-2. This intramolecular transfer of a proton suggests a syn transfer, the proton being removed and put back on the same side of the molecule. The carrier of the proton may be a histidine side chain.[131] This result, together with the known configuration of glucose at C-2, indicates that the intermediate is the *cis*-enediol and that addition of a proton at either C-1 or C-2 of the enediol is to the *re* face. However, mannose-6-phosphate isomerase catalyzes addition to the *si* face.

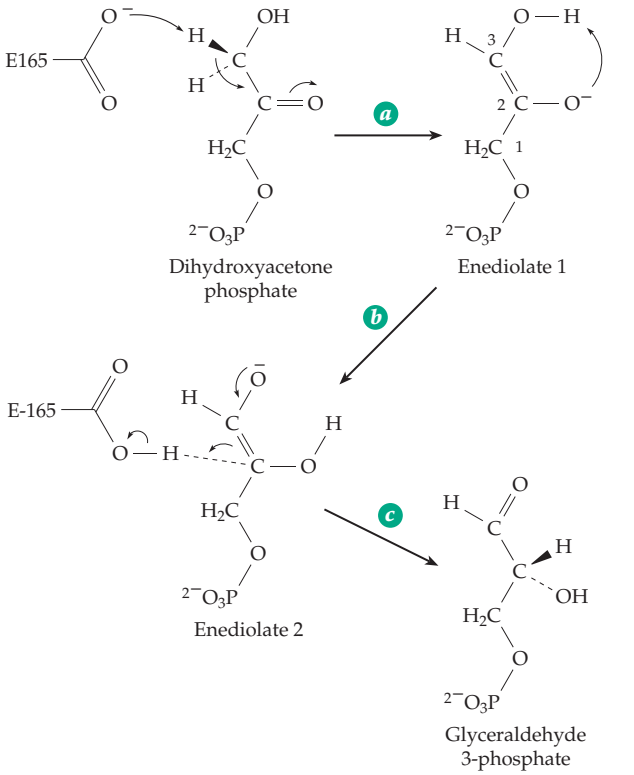
Catalysis of ring opening by isomerases. Glucose-6-phosphate isomerase catalyzes a second reaction, namely, the opening of the ring of the α-anomer of glucose 6-phosphate (one-half of the mutarotation reaction; Eq. 10-88). Noltmann suggested a concerted acid–base catalysis by two side chain groups as is indicated in Eq. 9-90. An NMR study showed that the isomerization of the α anomer occurs at least ten times faster than that of the β. Thus, β-glucose 6-phosphate is first converted to α-glucose 6-phosphate before it can be isomerized to fructose 6-phosphate.[132]

Triose phosphate isomerase. This dimeric 53-kDa enzyme interconverts the 3-phosphate esters of glyceraldehyde and dihydroxyacetone and is the fastest enzyme participating in glycolysis. Its molecular activity at 25° is ~2800 s^{-1} in the direction shown in Eq. 13-26 and ~250 s^{-1} in the reverse direction (the predominant direction in metabolism)[133] and is thought to operate at the diffusion-controlled limit (Chapter 9).[133] Each of the identical subunits consists of a striking $(\alpha/\beta)_8$ barrel (Fig. 13-6) with an active site at the carboxyl ends of the β strands.[134–137] Structures of the enzyme containing bound inhibitors such as phosphoglycolohydroxamate have also been determined (Fig. 13-6).[138] Triose phosphate isomerase is also one of the most investigated of all enzymes. Not only are its catalytic properties unusual but also there are known defects in the human enzyme[137] and it is also a potential target for antitrypanosomal drugs.[139]



Phosphoglycolohydroxamate (PGH), a tight-binding inhibitor (K_d = 15μM) that resembles enediolate 1 of Eq. 13-26

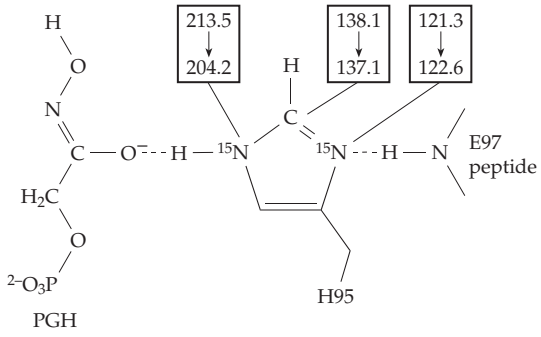
Although its high catalytic activity might favor intramolecular transfer of the proton removed by triose phosphate isomerase, little such transfer has



(13-26)

been observed.[140] This suggested that a relatively weak base such as a carboxylate group might serve as the proton acceptor at the C-3 position of dihydroxyacetone phosphate. Covalent labeling experiments and later X-ray studies have implicated Glu 165, whose carboxylate group is thought to remove the *pro-R* hydrogen atom from the hydroxymethyl group of dihydroxyacetone phosphate as indicated in Eq. 13-26. When this residue is replaced by Asp, most activity is lost.[141] Kinetic studies suggest that this carboxyl group has a pK_a of ~3.9 in the free enzyme. However, k_{cat} is independent of pH up to pH 10.[140] The pK_a of the phosphate also affects the binding (K_m), with only the phosphate dianion being a substrate. Study of the infrared spectrum of dihydroxyacetone bound to the enzyme revealed a shift of the carbonyl bands at 1733 cm^{-1} by about 20 cm^{-1} to ~1713 cm^{-1}. This might indicate a polarization and stretching of the carbonyl group by a positively charged histidine or lysine side chain of the protein.[142] The His 95 side chain is appropriately placed (Fig. 13-6) to function in this way[143] and its replacement by glutamine decreases catalytic activity by a factor of 400.[144] The $-NH_3^+$ group of lysine 12, another essential residue, is apparently needed for substrate binding.[136,145] After product is released, an isomerization within the enzyme is usually required to prepare it for acceptance of a new substrate. This may involve movement of protons between side chain groups or a conformational change or both. In the case of triose phosphate isomerase, a very rapid isomerization requiring about ~ 1 ms has been detected.[146]

To investigate further the function of His 95, Lodi and Knowles recorded the ^{13}C and ^{15}N NMR spectra of the three histidine rings[148] both in unligated enzyme and in the phosphoglycolohydroxamate (PGH) complex. The results were a surprise. The His 95 resonances



did not change at all when the pH was changed from below 5 to 9.9, and the chemical shift values (shown above) indicate clearly that the ring is **unprotonated** in both free enzyme and in the complex. The key chemical shift values are shown on the diagram to the

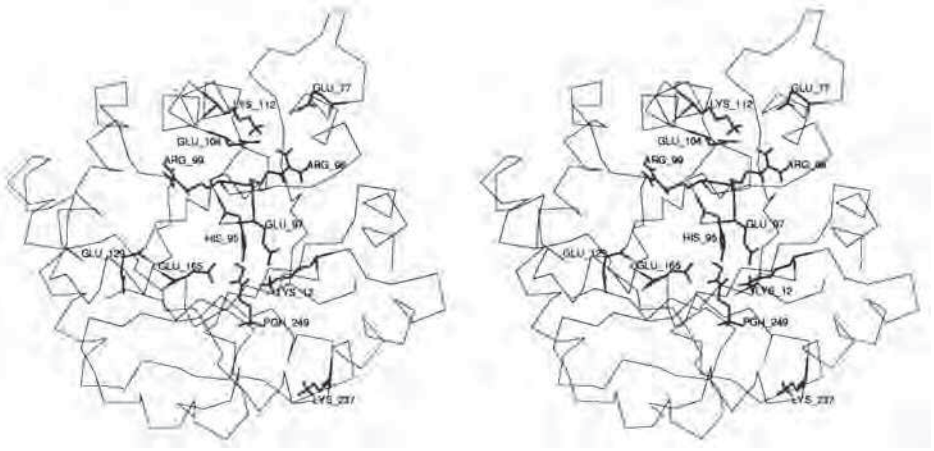
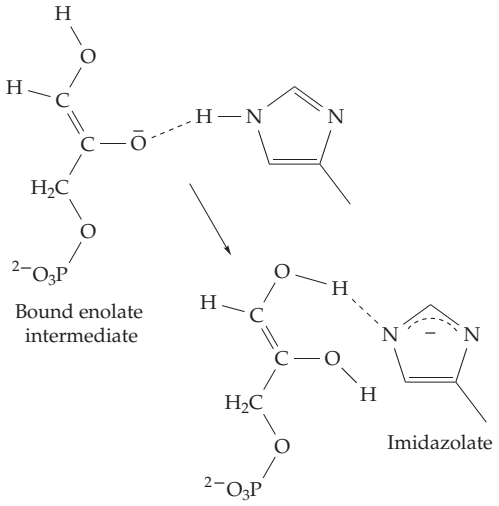


Figure 13-6 Stereoscopic view into the active site of triose phosphate isomerase showing side chains of some charged residues; PGH, a molecule of bound phosphoglycolohydroxamate, an analog of the substrate enolate.[138] The peptide backbone, as an alpha-carbon plot, is shown in light lines.[147] The $(\alpha / \beta)_8$-barrel structure is often called a TIM barrel because of its discovery in this enzyme. Courtesy of M. Karplus.

left; the arrows indicate the changes upon formation of the PGH complex. These results suggest that H95 is never protonated and that if it acts as the catalytic acid it does so by dissociating to an imidazolate ion (Eq. 13-27).[148]

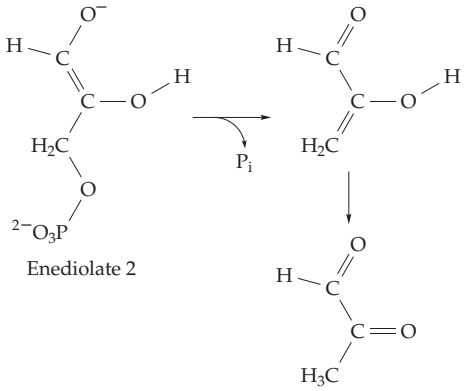


(13-27)

The imidazole would donate a proton to the enolate ion and then remove a proton from the resulting enediol. Although the proposed chemistry is unusual, it is argued that the high pK_a of the neutral imidazole acting as a proton donor would be matched with the high pK_a of the enol, permitting rapid reaction.[148] Nevertheless, two other possibilities exist. Theoretical calculations support the idea that the proton transfer between the two oxygens in the enediolate may occur *without catalysis by a proton donor*[149] as indicated in step *b* of Eq. 13-26. Another possibility is that a transient protonation of H95 by the adjacent E97 carboxyl group occurs and allows the histidine to participate in the proton transfer. Study of kinetic isotope effects has indicated coupled motion of protons and proton tunneling.[143]

Another detail should be mentioned. The active site of triose phosphate isomerase is formed by a series of loops connecting the α helices and β strands of the barrel. One of those loops, consisting of residues 167–176, folds over the active site after the substrate is bound to form a hinged lid that helps to hold the substrate in the correct orientation for reaction.[150–152] When the lid, which can be seen in Fig. 13-6, closes, the peptide NH of G171 forms a hydrogen bond to a phosphate oxygen atom of the substrate. This is only one of many known enzymes with deeply buried active sites that close in some similar fashion before a rapid reaction occurs.

Although enzymes tend to be extremely specific they are not always completely able to avoid side reactions. Triose phosphate isomerase releases small amounts of **methylglyoxal** (Eq. 13-28), presumably as

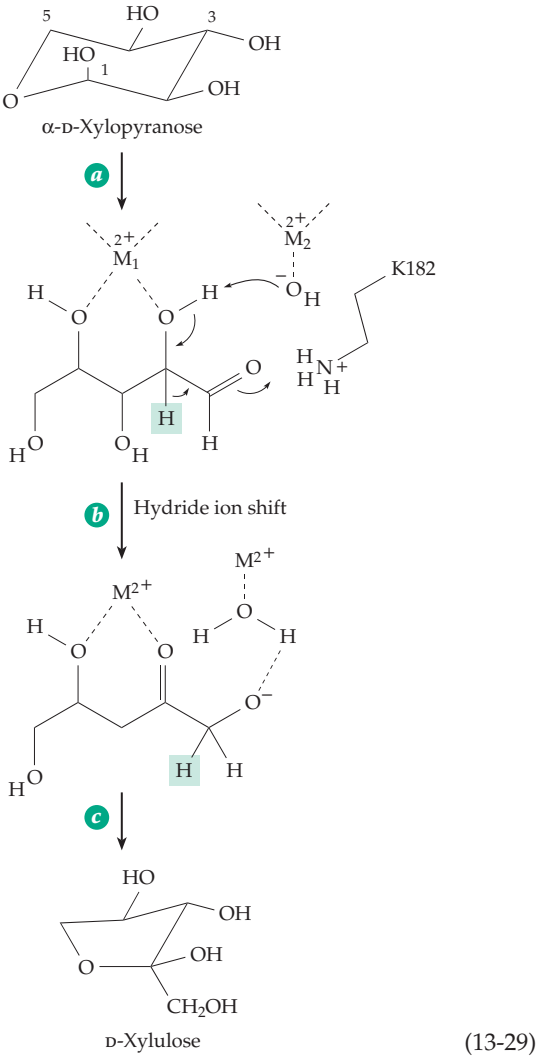


(13-28)

a result of elimination of phosphate from enediolate 2 of Eq. 13-26. Deletion of four residues from the hinged lid produced an enzyme in which this side reaction was increased 5.5-fold.[146]

Xylose isomerase and the hydride shift mechanism. This bacterial enzyme, which isomerizes D-xylose to D-xylulose, has an $(\alpha/\beta)_8$ barrel similar to that of triose phosphate isomerase. It is of industrial importance because it also catalyzes isomerization of D-glucose to the sweeter sugar D-fructose, a reaction used in preparation of high-fructose syrup. For this purpose the isomerase is often immobilized on an insoluble matrix such as diethylaminoethyl cellulose.[153] This enzyme, as well as other isomerases that act on free sugars, requires a metal ion such as Mg^{2+}, Co^{2+}, or Mn^{2+}. The three-dimensional structures, solved in several different laboratories,[129,154–158] show that there are two metal ions (ordinarily Mg^{2+}) about 0.5 nm apart and held by an array of glutamate and aspartate side chains. One glutamate carboxylate forms short ionic bonds to both metal ions and an essential histidine is also coordinated with metal ion 2. The three-dimensional structure is superficially similar to that of triose phosphate isomerase but there are major differences in properties. Xylose isomerase requires metal ions, acts on unphosphorylated non-ionic substrates, and does not catalyze detectable exchange of protons with the solvent. Furthermore, *X-ray structures do not show the presence of any catalytic base that could initiate formation of an intermediate enolate ion.* These facts suggested an alternative isomerization mechanism, one involving a hydride ion shift and well-known from studies of the Cannizarro reaction.[159] Because the non-ionic substrates and inhibitors bind weakly it has been difficult to obtain a clear picture of events in the active site. The substrate is the α-anomer of D-xylopyranose[155] and the enzyme catalyzes the opening of the sugar ring (step *a*, Eq. 13-29).[157] The details of this process are not clear but acid-base catalysis as in Eq. 9-87 is probable.

There is a consensus[156–158] that the open form of

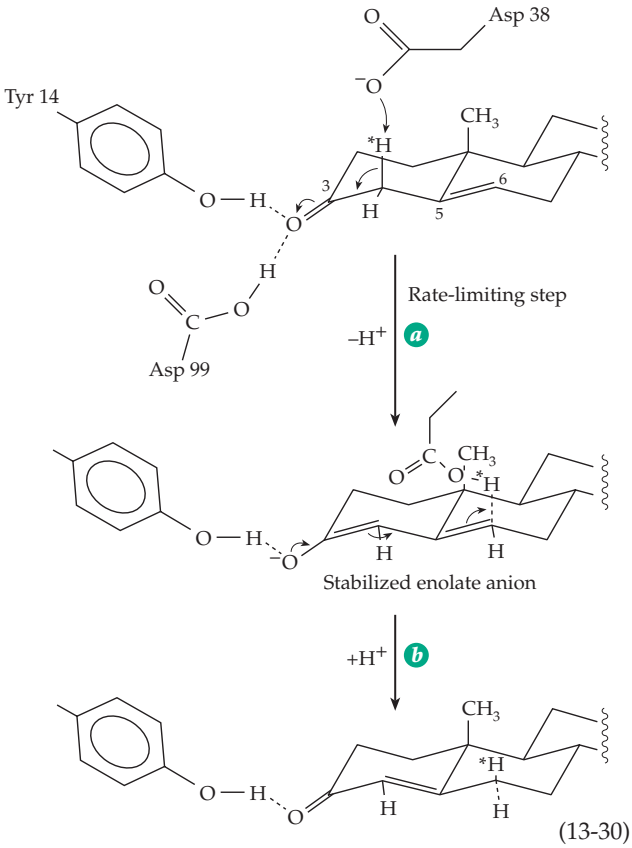


(13-29)

the sugar is bound to one of the two metal ions as shown in Eq. 13-29 and that a bound hydroxide ion on the second metal provides the catalytic base to remove a proton from the –OH at position 2. The isomerization occurs with the shaded hydrogen atom being shifted as H^- to the carbon atom of the carbonyl group. During the reaction metal ion 2 moves apart from metal ion 1 by ~0.09 nm,[129,157,158] another fact that has made analysis of X-ray data difficult. Protonation of the alkoxide ion at C-2 is followed by ring closure (step *c*, Eq. 13-29).

3-Oxosteroid isomerases. Cholesterol serves in the animal body as a precursor of all of the steroid hormones, including the 3-oxosteroids progesterone and testosterone (Chapter 22). The 3-hydroxyl group of cholesterol is first oxidized to an oxo group. This is followed by an essentially irreversible migration of the double bond in the 5,6-position into conjugation with the carbonyl group (Eq. 13-30) catalyzed by a 3-oxo-Δ^5-steroid isomerase. The small 125-residue enzyme from the bacterium *Pseudomonas testosteroni* has been studied by a great variety of methods.[160–164] The subunits associate as dimers or higher oligomers. The enzyme has a high content of nonpolar amino acid residues and is soluble in high concentrations of ethanol. This property is compatible with the location of the eukaryotic enzyme in the endoplasmic reticulum. The substrate binding site is a hydrophobic cavity.

Oxosteroid isomerase has a remarkably high molecular activity (~0.75 x 10^5 s^{-1}). Substrates containing ^2H in the 4 position react only one-fourth as rapidly as normal substrates. The large isotope effect suggested that cleavage of the C–H bond to form an enzyme-stabilized enolate anion is rate limiting (Eq. 13-30, step *a*).[160] The proton in the axial position at C-4 is removed preferentially but without complete stereospecificity.[165] The abstracted proton must be carried by a group on the enzyme and returned to the 6 position of the substrate, again in an axial orientation (Eq. 13-30, step *b*), a suprafacial transfer. Studies of the binding of spin-labeled substrate analogs, molecular modeling, and directed mutation indicate that Asp 38 is this proton carrier.[164,166] Little exchange of the proton with solvent is observed, presumably because of the extreme rapidity of the isomerase action. However, competitive inhibitors such as nortestosterone, whose double-bond arrangement is that of the product in Eq. 13-30, undergo exchange of one of the hydrogens at C-4 with the medium. The ultraviolet absorption band of the inhibitor at 248 nm is shifted to 258 nm upon combination with the enzyme, presumably as a result of formation of the

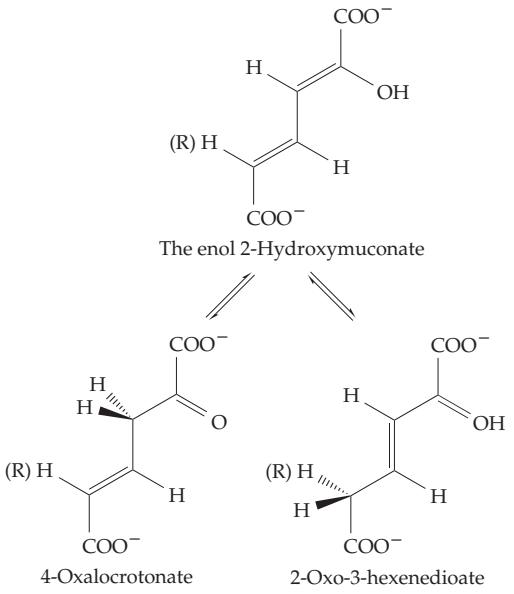


(13-30)

enolate anion. Tyrosine 14 has been identified as the catalytic acid, able to stabilize the enolate anion as depicted in Eq. 13-30.[161,162] A high-resolution NMR structure[167] and X-ray crystal structures[167a,b] have revealed an adjacent Asp 99 side chain that may assist.[167c]

Enzymatic isomerization of *cis*-aconitate to *trans*-aconitate apparently also involves proton abstraction,[165] with resonance in the anion extending into the carboxylic acid group. Its mechanism may be directly related to that of the oxosteroid isomerase. However, there are other 1,3-proton shifts in which neither a carbonyl nor a carboxyl group is present in the substrate (Eqs. 13-55, 13-56).

4-Oxalocrotonate tautomerase. This bacterial enzyme, which functions in the degradation of toluene (Chapter 25), is actually an isomerase. It catalyzes rapid interconversion of an unconjugated unsaturated α-oxoacid such as 4-oxalocrotonate with an intermediate enol (which may leave the enzyme) and the isomeric conjugated oxoacid (Eq. 13-31).[168–170] A related 5-carboxymethyl-2-hydroxymuconate isomerase



(13-31)

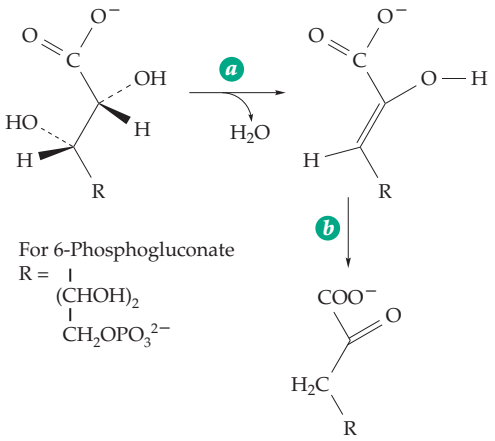
catalyzes the same reaction when R = $-CH_2COO^-$ in Eq. 13-31. It is an unusually small enzyme consisting of only 62 amino acid residues. The pH dependence reveals pK_a values of 6.2 and 9.0 in the free enzyme and 7.7 and 8.5 in the ES complex.[171]

The pK_a of 6.2 has been associated with the amino-terminal proline 1.[172] 4-Oxalocrotonate tautomerase is one of a small group of enzymes that have been synthesized nonenzymatically with both L amino acids and as a mirror image constructed with D amino acids.[173]

3. Internal Oxidation–Reduction by Dehydration of Dihydroxyacids

When a carboxylic acid contains hydroxyl groups in both the α and β positions, dehydration leads to formation of an enol that can tautomerize to 2-oxo-3-deoxy derivatives of the original acid (Eq. 13-32). Thus, phosphogluconate dehydratase yields 2-oxo-3-deoxyphosphogluconate as the product. When the reaction is carried out in ^2H_2O the ^2H is incorporated with a random configuration at C-3, indicating that the enzyme catalyzes only the dehydration and that the tautomerization of the enol to the ketone is nonenzymatic.

The reaction of Eq. 13-32 initiates a unique pathway of sugar breakdown, the Entner–Douderoff pathway (Eq. 17-18), in certain organisms. The 6-phosphogluconate is formed by oxidation of the aldehyde group of glucose 6-phosphate. This pattern of oxidation of a sugar to an aldonic acid followed by dehydration according to Eq. 13-32 occurs frequently in metabolism. A related reaction is the dehydration of 2-phosphoglycerate by enolase (Eq. 13-15). In this case the product is phosphoenolpyruvate, a stabilized form of the enolic intermediate of Eq. 13-32 (when R = H).



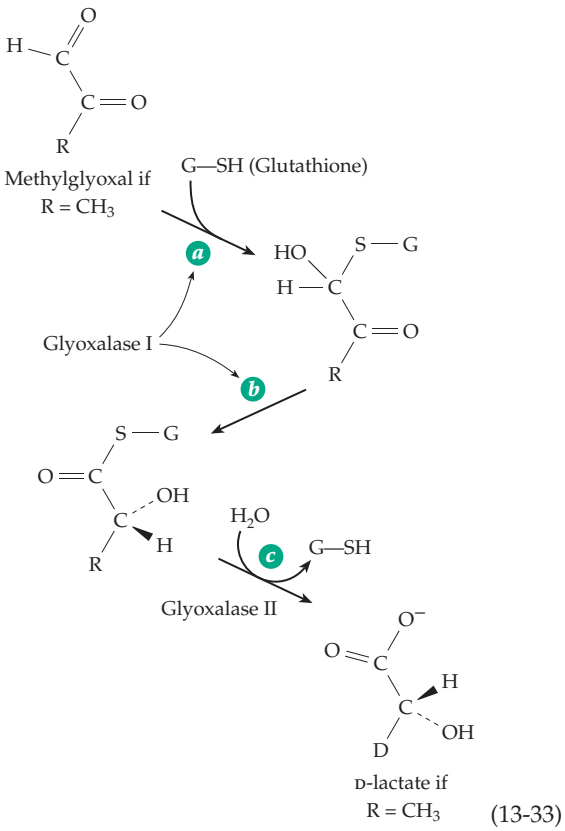
(13-32)

4. Formation and Metabolism of Methylglyoxal (Pyruvaldehyde)

The rather toxic methylglyoxal is formed in many organisms and within human tissues.[174] It arises in part as a side reaction of triose phosphate isomerase (Eq. 13-28) and also from oxidation of acetone (Eq. 17-7) or aminoacetone, a metabolite of threonine (Chapter 24).[175] In addition, yeast and some bacteria, including *E. coli*, have a **methylglyoxal synthase** that converts dihydroxyacetone to methylglyoxal, apparently using a mechanism similar to that of triose phosphate isomerase. It presumably forms enediolate 2 of Eq. 13-26, which eliminates inorganic phosphate to yield methyl-

glyoxal as in Eq. 13-28.[176,176a] Methylglyoxal is converted to D-lactate by the two-enzyme **glyoxalase** system (Eq. 13-33).[177] The combined action of methylglyoxal synthase and the glyoxalases provides a bypass to the usual glycolysis pathway (Fig. 10-3). Although it does not provide energy to the cell, it releases inorganic phosphate from sugar phosphates that may accumulate under conditions of low phosphate because the free P_i concentration is too low to support the glyceraldehyde 3-phosphate dehydrogenase reaction (step *a* of Fig. 10-3).

Glyoxalase acts not only on methylglyoxal but also on other α-oxo-aldehydes. It is thought to be an important enzyme system that protects cells against these potentially dangerous metabolites. Glyoxalase consists of a pair of enzymes, **glyoxalase I** and **glyoxalase II**, which catalyze the reactions of Eq. 13-33. Each subunit of the 183-residue human glyoxalase I contains one tightly bound Zn^{2+} ion.[178] However, the *E.coli* enzyme[178a] is inactive with Zn^{2+} and maximally active with Ni^{2+}. The enzyme requires glutathione as a cofactor, and in step *a* of the reaction (Eq. 13-33) the glutathione adds to form a thiohemiacetal[179] which is then isomerized in step *b*. During this step retention of the abstracted proton is so complete that it was earlier thought to function by an intramolecular hydride ion shift as in xylose isomerase. More recent evidence favors an enolate anion intermediate. However, the three-dimensional structure is not related to that of other isomerases and the exact mechanism remains uncertain. Glyoxylase II, which catalyzes step *c* of Eq. 13-33, is an esterase.[175,180]

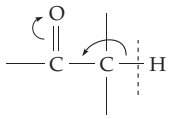


(13-33)

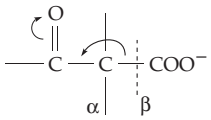
C. Beta Cleavage and Condensation

In Chapter 12 and in the preceding sections of this chapter we examined displacement and addition reactions involving nucleophilic centers on O, N, or S. Bonds from carbon to these atoms can usually be broken easily by acidic or basic catalysis. The breaking and making of C–C bonds does not occur as readily and the "carbon skeletons" of organic molecules often stick together tenaciously. Yet living cells must both form and destroy the many complex, branched carbon compounds found within them.

A major mechanistic problem in cleavage or formation of carbon–carbon bonds is the creation of a nucleophilic center on a carbon atom. The problem is most often solved by using the *activating influence of a carbonyl group to generate a resonance-stabilized enolate anion*. Just as the presence of a carbonyl group facilitates cleavage of an adjacent C–H bond, so it can also assist the cleavage of a C–C bond. The best known reactions of this type are the **aldol cleavage** and the



Cleavage of C—H bond facilitated by a carbonyl group



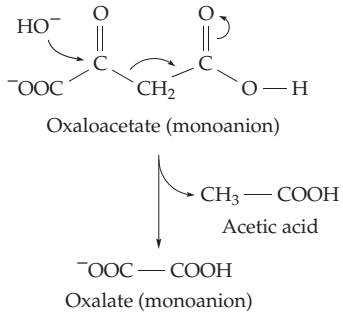
β Cleavage with release of CO_2

decarboxylation of **β-oxo acids**. The latter has been referred to as β decarboxylation and its reverse as β carboxylation. In this book these terms have been extended to include other reactions by which bonds between the α and β carbon atoms of a carbonyl compound are broken or formed, and these will be referred to as **β cleavage and β condensation**.

The β *condensation* reactions consist of displacement or addition reactions in which an enzyme-bound enolate anion acts as the nucleophile. We can group these condensation reactions into three categories as indicated by reaction types 5A, 5B, and 5C of Table 10-1.

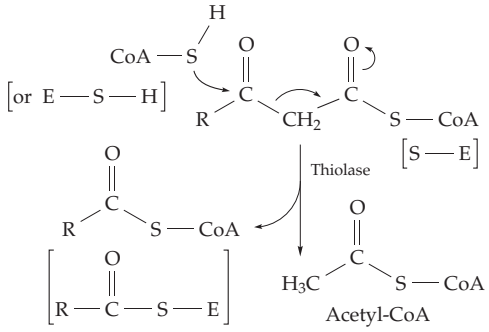
1. Displacement on a Carbonyl Group

A β-oxo acid, with proper catalysis, is susceptible to hydrolysis by attack of water on the carbonyl group. An example is the reaction catalyzed by **oxaloacetate acetylhydrolase** which has been isolated from *Aspergillus niger* (Eq. 13-34). A related cleavage is catalyzed by ribulose bisphosphate carboxylase (see Eq. 13-48).



(13-34)

The **thiolases**[181] are lyases that cleave β-oxoacyl derivatives of CoA by displacement with a thiol group of another CoA molecule (Eq. 13-35). This is the chain cleavage step in the β oxidation sequence by which fatty acid chains are degraded (Fig. 10-4). Biosynthetic thiolases catalyze the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, (see Eq. 17-5), a precursor to cholesterol and related compounds and to poly-β-hydroxybutyrate (Box 21-D). Because acetyl-CoA is a thioester, the reaction is usually described as a **Claisen condensation**. A related reaction, involving decarboxylation of malonyl-CoA, is a step in fatty acid synthesis. Since the thiolases are inhibited by –SH reagents it has been suggested that a thiol group in the enzyme reacts initially with the β-carbonyl group as in Eq. 13-35 to give an enzyme-bound *S*-acyl intermediate. The acyl group is then transferred to CoA in a second step.



(13-35)

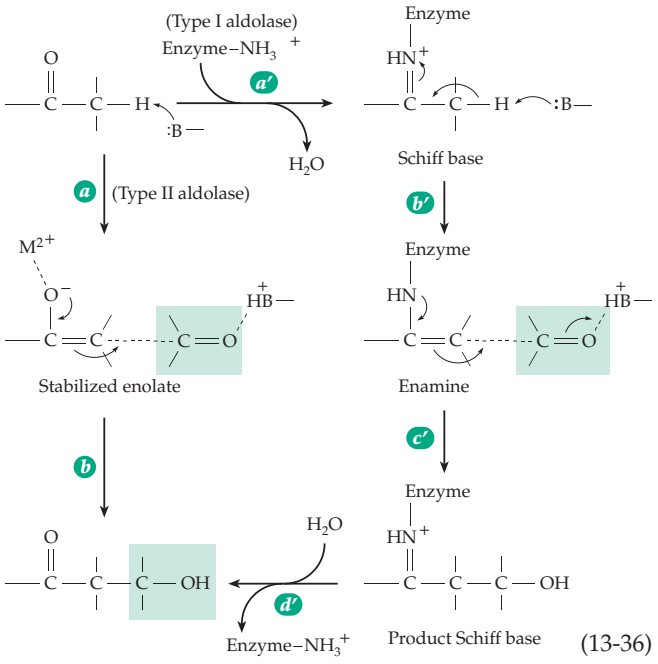
A very similar reaction is catalyzed by 3-hydroxy-3-methylglutaryl-CoA lyase (HMG-CoA lyase), which functions in the formation of acetoacetate in the human body (Eq. 17-5, step *c*) and also in the catabolism of leucine (Fig. 24-18)[182,183] and in the synthesis of **3-hydroxy-3-methylglutaryl-CoA**, the presursor of cholesterol (Eq. 17-5, step *b*)[183a]

2. Addition of an Enolate Anion to a Carbonyl Group or an Imine; Aldolases

The **aldol condensation** (Eq. 13-36), which is also illustrated in Box 10-D, is one of the more common reactions by which C–C bonds are formed[183b] and, in the reverse reaction, cleaved in metabolism. Aldolases are classified into two major types. The type II aldolases are metal-ion dependent, the metal ion stabilizing the intermediate enolate ion (Eq. 13-36, steps *a* and *b*). Type I aldolases, which include the most studied mammalian enzymes, have a more complex mechanism involving intermediate Schiff base forms (Eq. 13-36, steps *a′*, *b′*, *c′*, *d′*).[184] The best known members of this group are the **fructose bisphosphate aldolases** (often referred to simply as aldolases), which cleave fructose-1,6-P_2 during glycolysis (Fig. 10-2, step *e*).

These enzymes have been found in all plant and animal tissues examined and are absent only from a few specialized bacteria. Three closely related isoenzymes are found in vertebrates.[185,186] The much studied rabbit muscle aldolase A is a 158-kDa protein tetramer of identical peptide chains.[186,187] Aldolase B, which is lacking in hereditary fructose intolerance, predominates in liver and isoenzyme C in brain.[185]

Treatment with sodium borohydride of the enzyme-substrate complex of aldolase A and dihydroxyacetone phosphate leads to formation of a covalent linkage between the protein and substrate. This and other evidence suggested a Schiff base intermediate (Eq. 13-36). When ^{14}C-containing substrate was used, the borohydride reduction (Eq. 3-34) labeled a lysine side chain in the active site. The radioactive label was followed through the sequence determination and was found on Lys 229 in the chain of 363 amino acids.[186,188–188b] The enzyme is another $(\alpha / \beta)_8$-barrel protein and the side chain of Lys 229 projects into the interior of the barrel which opens at the C-terminal ends of the strands. The conjugate base form of another lysine, Lys 146, may represent the basic group B in Eq. 13-36,

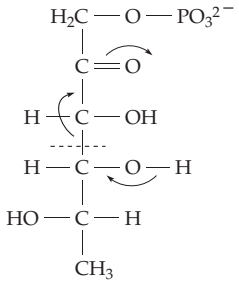


(13-36)

step *b*. Another possibility is that the adjacent phosphate group of the substrate acts as the acid–base catalyst for this step.[189] Aldolase A has been altered by mutations into a monomeric form that retains high catalytic activity,[190] something that has not often been accomplished for oligomeric enzymes.

The **type II aldolases** are not inactivated by sodium borohydride in the presence of substrate. A probable function of the essential metal ion is to polarize the carbonyl group as indicated in Eq. 13-36. In both yeast and *E. coli* aldolase the Zn^{2+} is held by 3 imidazole groups.[191,191a] An arginine side chain is a conserved residue involved in substrate binding in several class II aldolases.[192] Some blue-green algae contain both types of aldolase, as do the flagellates *Euglena* and *Chlamydomonas*.

The catabolism of L-fucose by *E. coli* requires cleavage of L-fuculose-1-phosphate to form dihydroxyacetone phosphate and D-lactaldehyde by a class II aldolase.[193]



L-Fuculose-1-phosphate

The mechanism of chain cleavage proposed on the basis of the structure and modeling[193] is illustrated in Fig. 13-7.

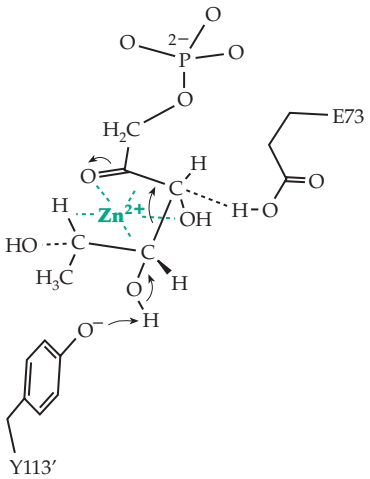
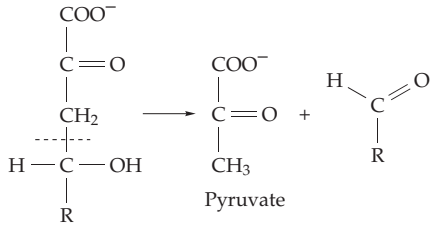


Figure 13-7 Interaction of the bound zinc ion of L-fuculose-1-phosphate aldolase and catalytic side chains with the substrate in the active site of the enzyme as revealed by X-ray crystallography and modeling. See Dreyer and Schulz.[193]

Special aldolases of both classes cleave and form C–C bonds throughout metabolism. Several of them act on 2-oxo-3-deoxy substrates forming pyruvate as one product (Eq. 13-37). The aldehyde product varies. In the Entner–Doudoroff pathway of carbohydrate metabolism (Chapter 21) 3-deoxy-2-oxo-6-phosphogluconate (KDPG) is cleaved to pyruvate and 3-phosphoglyceraldehyde.[194] The same products arise from the corresponding phosphogalactonate derivative.[195] The subunits of the trimeric KDPG aldolase have an $(\alpha/\beta)_8$-barrel structure similar to that of eukaryotic fructose 1,6-bisphosphate aldolase.[194] The 8-carbon sugar acid "KDO" of bacterial cell walls (Fig. 4-26) is cleaved by another aldolase. The catabolism of hydroxyproline leads to 4-hydroxy-2-oxoglutarate, which is cleaved to pyruvate and glyoxylate.[196] In the catabolism of deoxynucleotides, another aldolase converts 2-deoxyribose 5-phosphate to acetaldehyde and glyceraldehyde 3-phosphate.[197]

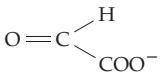
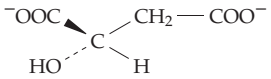
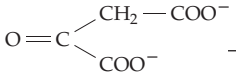
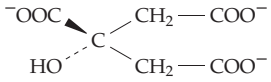
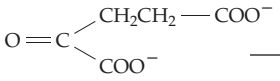
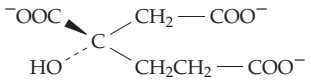
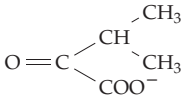
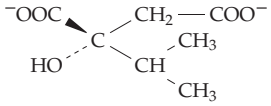
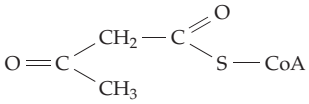
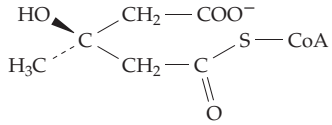


(13-37)

Closely related to aldolases is **transaldolase**, an important enzyme in the pentose phosphate pathways of sugar metabolism and in photosynthesis. The mechanism of the transaldolase reaction (Eq. 17-15) is similar to that used by fructose-1,6-bisphosphate aldolase with a lysine side chain forming a Schiff base and catalytic aspartate and glutamate side chains.[198]

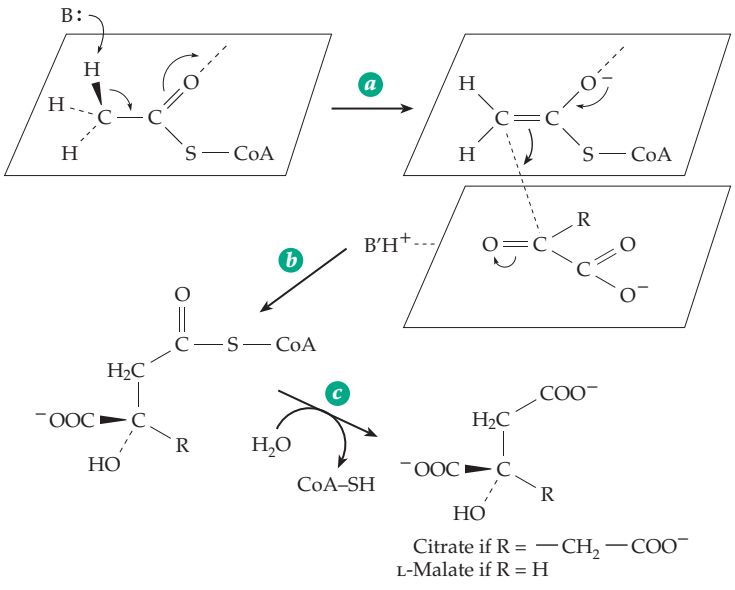
Polycarboxylic acid synthases. Several enzymes, including **citrate synthase,** the key enzyme which catalyzes the first step of the citric acid cycle, promote condensations of acetyl-CoA with ketones (Eq. 13-38). An α-oxo acid is most often the second substrate, and a thioester intermediate (Eq. 13-38) undergoes hydrolysis to release coenzyme A.[199] Because the substrate acetyl-CoA is a thioester, the reaction is often described as a Claisen condensation. The same enzyme that catalyzes the condensation of acetyl-CoA with a ketone also catalyzes the second step, the hydrolysis of the CoA thioester. These polycarboxylic acid synthases are important in biosynthesis. They carry out the initial steps in a *general chain elongation process* (Fig. 17-18). While one function of the thioester group in acetyl-CoA is to activate the methyl hydrogens toward the aldol condensation, the subsequent hydrolysis of the thioester linkage provides for overall irreversibility and "drives" the synthetic reaction.

TABLE 13-1
Products Arising from Reactions of Acetyl-CoA with a Second Substrate with Catalysis by a Polycarboxylate Synthase

Ketone substrate	Product	Further metabolites
 Glyoxylate	 L-Malate	Carbohydrates, etc., via glyoxylate pathway
 Oxaloacetate	*Si*-Citrate synthase  Citrate	2-Carbon unit from acetyl-CoA occupies *pro-S* position
 2-Oxoglutarate	 Homocitrate	Lysine via α-aminoadipic acid
 2-Oxoisovalerate	 α-Isopropylmalate	Leucine
 Acetoacetyl-CoA	 *S*-3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA)	Free acetoacetate; Isoprenoid compounds

The stereochemistry of the reaction is also illustrated in Eq. 13-38. These enzymes may be classified by designating the face of the carbonyl group to which the enolate anion adds. The *si* face is up in Eq. 13-38. The common citrate synthase of animal tissues[200] and that of *E. coli*[201] condense with the *si* face and are designated (*si*)-citrate synthases. A few anaerobic bacteria use citrate (*re*)-synthase having the opposite stereochemistry.[202] Many citrate synthases are ~100-kDa dimers[203,204] but some are hexamers[203,205] and are allosterically inhibited by NADH. The second substrates and products of several related reactions of acetyl-CoA are summarized in Table 13-1.

The *si*-citrate synthase of pigs is a dimer of 437-residue chains, each of which is organized into a large rigid domain and a smaller



(13-38)

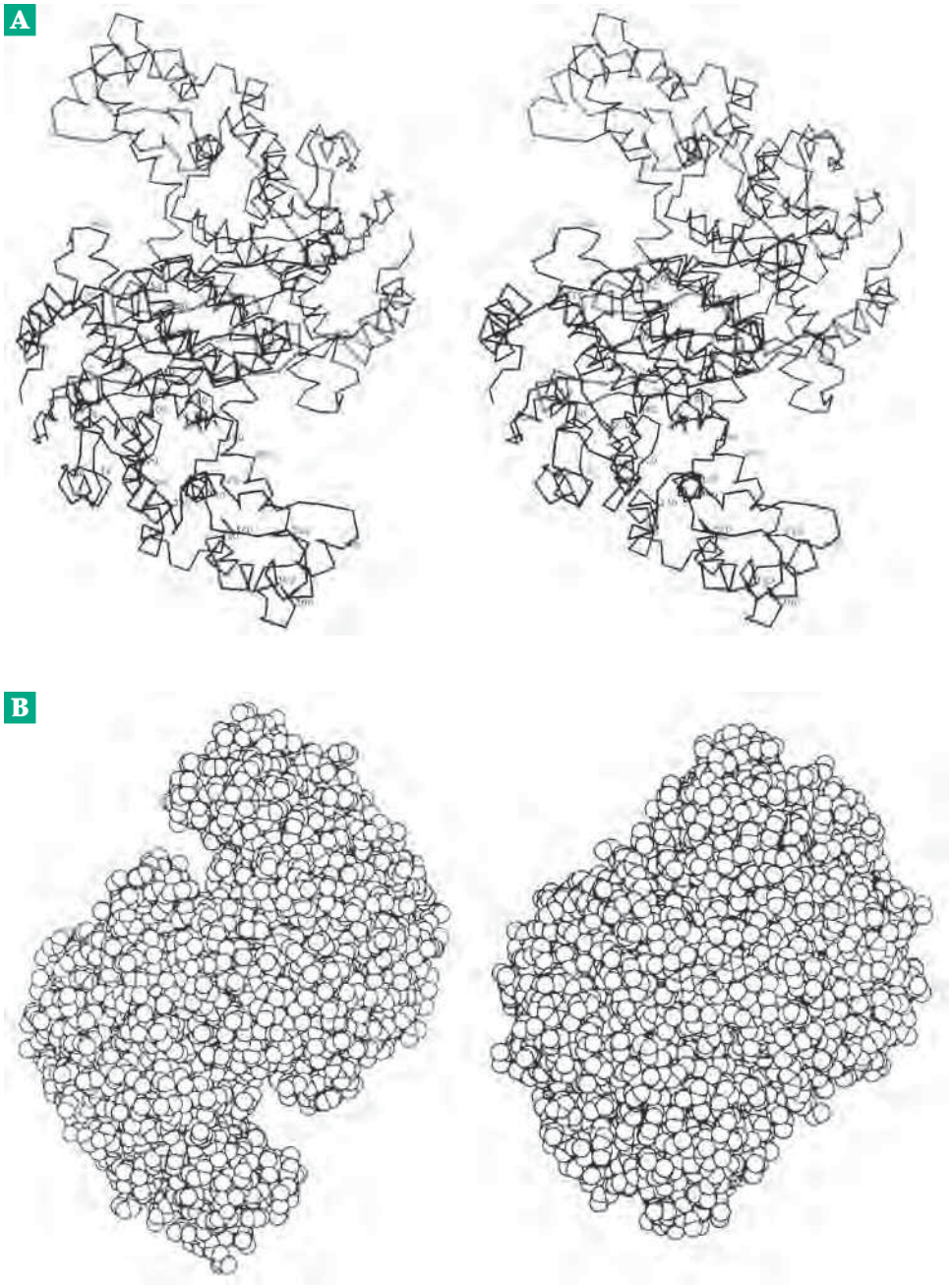
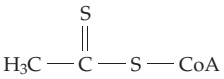


Figure 13-8 Three-dimensional structure of citrate synthase. (A) Stereoscopic alpha carbon trace of the dimeric pig enzyme in its open conformation.[209] A molecule of citrate is shown in the lower subunit. The view is down the two-fold axis. Courtesy of Robert Huber. (B) Space-filling representation of the open (left) and closed (right) forms of the same enzyme.[200] Courtesy of Stephen J. Remington.

flexible domain. The active site lies between the domains.[206] When oxaloacetate binds into the cleft the smaller domain undergoes a complex motion that closes the enzyme tightly around the substrate (Fig. 13-8).[200,207,208] The bound oxaloacetate is shown in Fig. 13-9. It is surrounded by a large number of polar side chains, including several from histidine and arginine residues. Of these, Arg 401 and Arg 421 of the second subunit bind the substrates' two carboxylate groups. In this tight complex the imidazole of His 320 is in the correct position to protonate the carbonyl oxygen of the oxaloacetate. The domain movement has also brought the groups that bind the acetyl-CoA into their proper position creating, by "induced fit," the acetyl-CoA binding site. This conformational change also accounts for the observation of an ordered kinetic mechanism with oxaloacetate binding before acetyl-CoA. The imidazole of His 274 is correctly oriented to abstract a proton from the methyl group of acetyl-CoA to generate the intermediate enolate anion. When oxaloacetate alone is bound into the active site the carbonyl stretching frequency, observed by infrared spectroscopy, is shifted from 1718 cm^{-1} for free oxaloacetate to 1697 cm^{-1}. This decrease of 21 cm^{-1} suggests a strong polarization of the C = O bond by its interaction with His 320 in the ground state.[211] This interaction is seen also in the ^{13}C NMR spectrum, the carbonyl resonance being shifted downfield by 6.8 ppm upon

binding to the protein.[212] The enolate anion mechanism is also supported by kinetic isotope effects.[213]

The methyl protons of dithia-acetyl-CoA are much more reactive than those of acetyl-CoA (pK_a ~12.5 vs ~20). When citrate acts on this acetyl-CoA analog together with oxaloacetate, the expected enolate anion

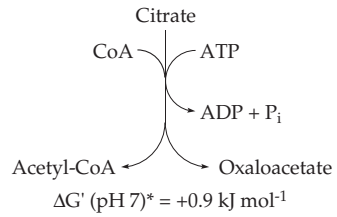


Dithia-acetyl-CoA

forms rapidly as is indicated by the appearance of a 306 nm absorption band. However, it condenses very slowly with the oxaloacetate.[214] The binding equilibria, kinetics, and X-ray structures of complexes for a variety of other analogs of acetyl-CoA have also been studied.[210,215-216a] It appears that Asp 375 and His 274 work together to generate the enolate anion (Fig. 13-9). NMR measurements indicate formation of an unusually short hydrogen bond, but its significance is uncertain.[216a,216b] Malate synthase (Table 13-1) operates with a very similar mechanism but has an entirely different amino acid sequence and protein fold.[216c]

Other Claisen condensations are involved in synthesis of fatty acids and polyketides[217] (Chapter 21) and in formation of 3-hydroxy-3-methylglutaryl-CoA, the precursor to the polyprenyl family of compounds (Chapter 22). In these cases the acetyl group of acetyl-CoA is transferred by a simple displacement mechanism onto an –SH group at the active site of the synthase to form an acetyl-enzyme.[218,219] The acetyl-enzyme is the actual reactant in step *b* of Eq. 17-5 where this reaction, as well as that of HMG-CoA lyase, is illustrated.

Citrate cleaving enzymes. In eukaryotic organisms the synthesis of citrate takes place within the mitochondria, but under some circumstances citrate is exported into the cytoplasm. There it is cleaved by **ATP-citrate lyase**. To ensure that the reaction goes to completion, cleavage is coupled to the hydrolysis of ATP to ADP and inorganic phosphate (Eq. 13-39). The value of *G'* given here is extremely dependent upon the concentration of Mg^{2+} as a result of strong chelation of Mg^{2+} by citrate.[220] The reaction sequence is complex but can be understood in terms of an initial ATP-dependent synthesis of



$\Delta G'$ (pH 7)* = +0.9 kJ mol^{-1} (13-39)

citryl-CoA using a mechanism similar to that in Eq. 12-47. There is evidence for both phosphoenzyme and citryl enzyme intermediates (Eq. 13-40).[221] Native ATP-citrate lyase is a tetramer of 110-kDa subunits. It usually contains some phosphoserine and phosphothreonine residues but they apparently have little effect on activity.[222] Phosphorylation is catalyzed by cAMP-dependent and by insulin-dependent protein kinases.[223,224] A related reaction is the ATP-dependent cleavage of malate to acetyl-CoA and glyoxylate. It requires two enzymes, malyl-CoA being an intermediate.[225]

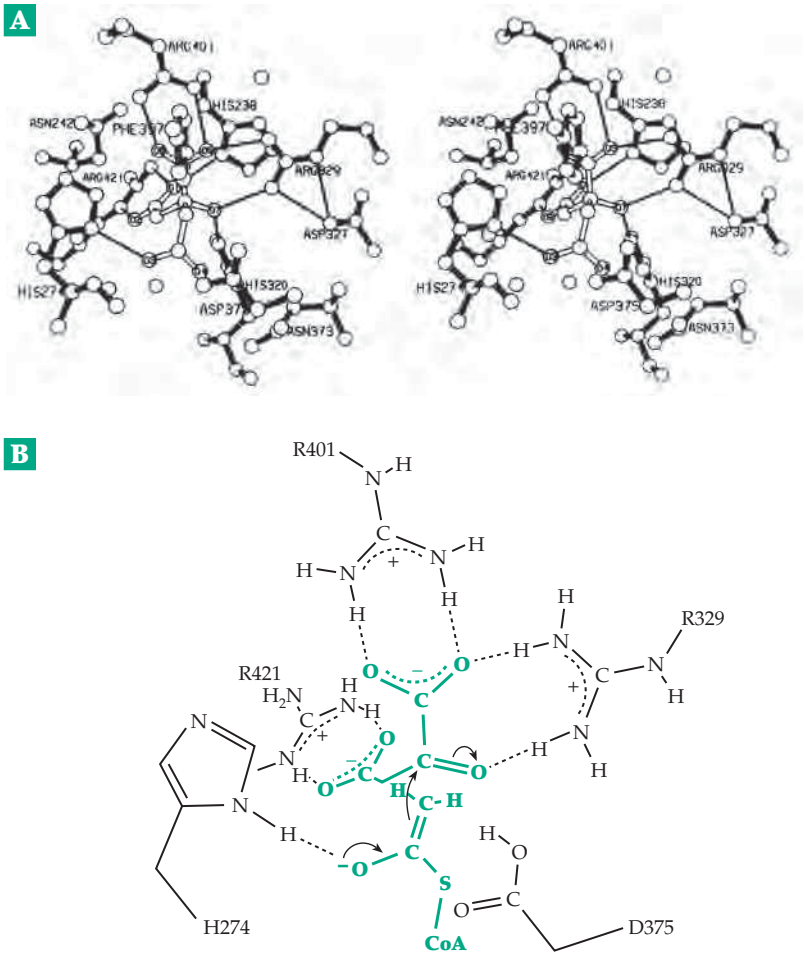
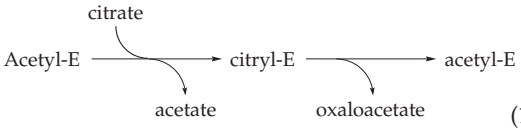


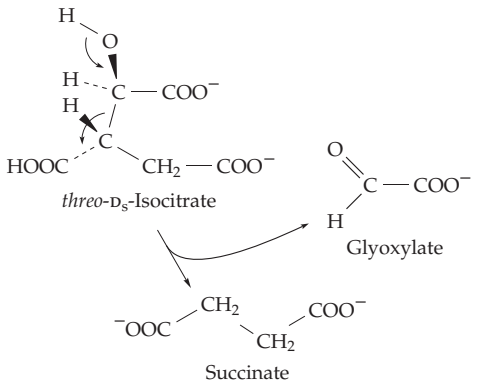
Figure 13-9 Active site of pig citrate synthase. (A) Stereoscopic view with a molecule of citrate in the active site.[200] Courtesy of Stephen J. Remington. (B) Interpretive view of the enolate anion of acetyl-CoA and oxaloacetate bound in the active site. Based on work by Kurz *et al.*[210]

A substrate-induced citrate lyase found in bacteria such as *E. coli* and *Klebsiella* promotes the anaerobic dissimilation of citrate splitting it to oxaloacetate and acetate.[226] The large ~585-kDa protein from *Klebsiella*[225] has the composition $(\alpha\beta\gamma)_6$, where the α, β, and γ subunits have masses of ~ 55-, 30-, and 10-kDa, respectively. The subunit carries an unusual covalently bound derivative of coenzyme A[227] (see also Chapter 14). The 10-kDa γ subunit serves as an **acyl-carrier protein**, the –SH of its prosthetic group being acetylated by a separate ATP-dependent ligase. The resulting acetyl-enzyme undergoes an acyl exchange (analogous to a CoA-transferase reaction; Eq. 12-50) to form a citryl enzyme before the aldol cleavage takes place (Eq. 13-40).



(13-40)

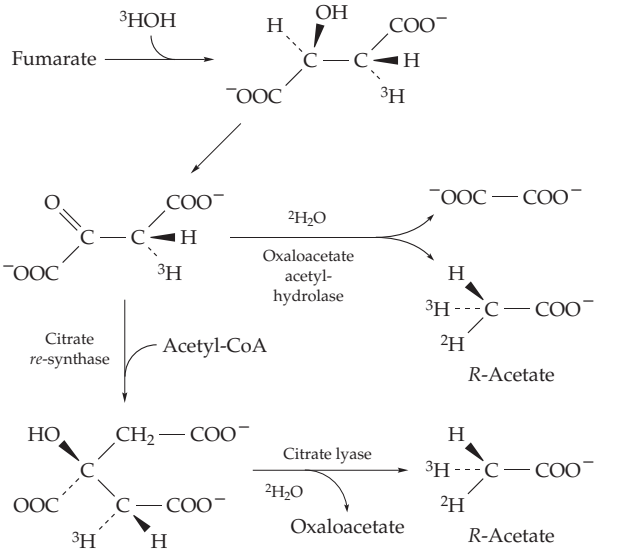
The first unique enzyme of the important glyoxylate pathway (Chapter 17), **isocitrate lyase**, cleaves isocitrate to succinate and glyoxylate (Eq. 13-41).[228] The carboxylate group that acts as electron acceptor would presumably be protonated by the enzyme.



(13-41)

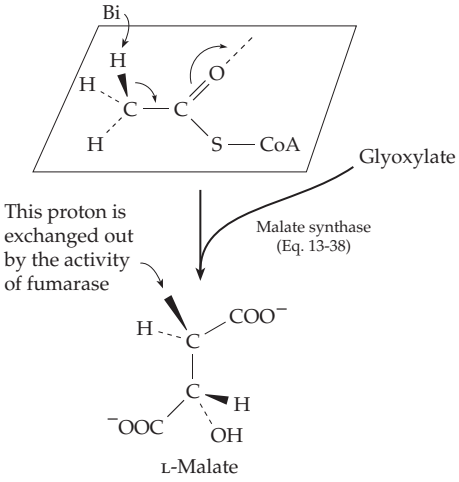
3. Chiral Acetates and Their Use in Stereochemical Studies

Consider the series of enzyme-catalyzed reactions shown in Eq. 13-42. Fumarate is hydrated in ^{3}H-containing water to malate which is oxidized to oxaloacetate. Hydrolysis of the latter with oxaloacetate acetylhydrolase (Eq. 13-34) in ^2H_2O gives oxalate and chiral (*R*) acetate. The identical product can be obtained by condensing oxaloacetate with acetyl-CoA using citrate (*re*)-synthetase. The resulting citrate is cleaved in ^2H_2O using a citrate lyase having the *si* specificity.[229,230] Acetate of the opposite chirality can be formed enzymatically beginning with [2,3-^{3}H]fumarate hydrated by fumarate hydratase in ordinary water. Chiral acetates have also been prepared nonenzymatically,[229] and their configuration has been established unequivocally.



(13-42)

During the action of both oxaloacetate acetylhydrolase and citrate lyase (Eq. 13-42) inversion of configuration occurs about the carbon atom that carries the negative charge in the departing enolate anion. That inversion also occurs during catalysis by citrate (*re*)-synthase and other related enzymes has been demonstrated through the use of chiral acetates.[229,230] The findings with malate synthase[199] are illustrated in Eq. 13-38. Presumably, a basic group B of the enzyme removes a proton to form the planar enolate anion. The second substrate glyoxylate approaches from the other side of the molecule and condenses as is shown. Since any one of the three protons in either *R* or *S* chiral acetyl-CoA might have been abstracted by base B, several possible combinations of isotopes are possible in the L-malate formed. One of the results of the experiment using chiral (*R*) acetyl-CoA is illustrated in Eq. 13-43. The reader can easily tabulate the results of removal of the ^{2}H or ^{3}H. However, notice that if the base –B: removes ^{2}H (D) or ^{3}H (T) the reaction will be much slower because of the kinetic isotope effects which are expected to be $^{H}k/^{D}k \approx 7$ and $^{H}k/^{T}k \approx 16$. A second important fact is that the *pro-R* hydrogen at C-3 in malate is specifically exchanged out into water by the action of fumarate hydratase. From the distribution of tritium in the malate and fumarate formed using the two chiral acetates, the inversion by malate synthase was established. See Kyte[231] for a detailed discussion.

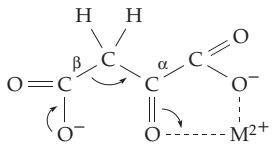


(13-43)

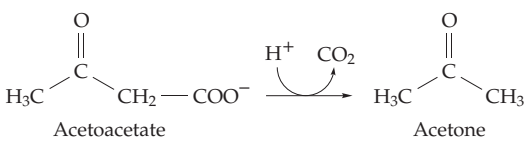
4. Addition of an Enolate Ion to Carbon Dioxide and Decarboxylation

The addition of an enolate anion to CO_2 to form a β-oxoacid represents one of the commonest means of incorporation of CO_2 into organic compounds. The reverse reaction of **decarboxylation** is a major mechanism of biochemical formation of CO_2. The equilibrium constants usually favor decarboxylation but the cleavage of ATP can be coupled to drive carboxylation when it is needed, e.g., in photosynthesis.

Decarboxylation of β-oxoacids. Beta-oxoacids such as oxaloacetic acid and acetoacetic acid are unstable, their decarboxylation being catalyzed by amines, metal ions, and other substances. Catalysis by amines depends upon Schiff base formation,[232] while metal ions form chelates in which the metal assists in electron withdrawal to form an enolate anion.[233–235]



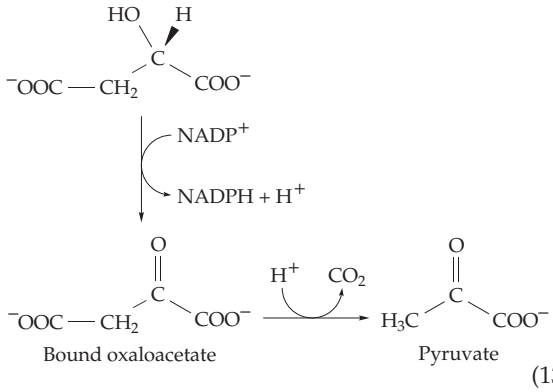
Can we apply any of this information from nonenzymatic catalysis to decarboxylating enzymes? Some decarboxylases do form Schiff bases with their substrates, and some are dependent on metal ions.[235] The acetone-forming fermentation of *Clostridium acetobutylicum* requires large amounts of acetoacetate decarboxylase (Eq. 13-44).



(13-44)

The enzyme is inactivated by borohydride in the presence of substrate, and acid hydrolysis of the inactivated enzyme yielded ε-*N*-isopropyllysine. Decarboxylation occurs from a Schiff base by a mechanism analogous to that of the aldol cleavage shown in Eq. 13-36.[236] Mechanistically related is 4-oxalocrotonate decarboxylase.[236a]

Linked oxidation and decarboxylation. Metabolic pathways often make use of oxidation of a β-hydroxy acid to a β-oxoacid followed by decarboxylation in the active site of the same enzyme. An example is conversion of L-malate to pyruvate (Eq. 13-45). The Mg^{2+} or Mn^{2+}-dependent decarboxylating malic dehydrogenase that catalyzes the reaction is usually called **the malic enzyme**. It is found in most organisms.[237–240] While a concerted decarboxylation and dehydrogenation may sometimes occur,[241–242] the enzymes of this group appear usually to operate with bound oxoacid intermediates as in Eq. 13-45.

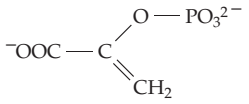


(13-45)

Other reactions of this type are the oxidation of isocitrate to 2-oxoglutarate in the citric acid cycle (Fig. 17-4, steps *d* and *e*),[243] oxidation of 6-phosphogluconate to ribulose 5-phosphate (Eq. 17-12),[244] and corresponding reactions of isopropylmalate dehydrogenase[245,246] and tartrate dehydrogenase.[247,248] Crystallographic studies of isocitrate dehydrogenase using both photolabile "caged" isocitrate[247] and slow mutant forms[243] with polychromatic Laue crystallography (Chapter 3) have demonstrated the rapid formation of the anticipated intermediate **oxalosuccinate**.

Phosphoenolpyruvate, a key metabolic intermediate. A compound of central importance in metabolism is the phosphate ester of the enol form of pyruvate, commonly known simply as phosphoenolpyruvate (PEP).[249] It is formed in the glycolysis pathway by dehydration of 2-phosphoglycerate (Eq. 13-15) or by decarboxylation of oxaloacetate. Serving as a preformed enol from which a reactive enolate anion can be released for condensation reactions,[250,251] PEP

is utilized in metabolism in many ways.

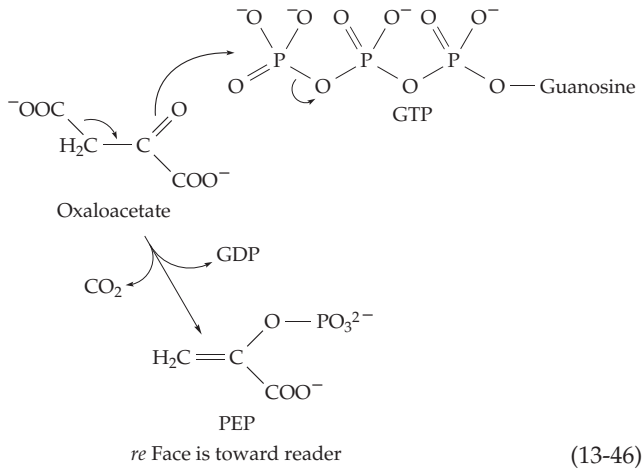


Phosphoenolpyruvate (PEP)

In animals and in many bacteria, PEP is formed by decarboxylation of oxaloacetate. In this reaction, which is catalyzed by **PEP carboxykinase** (PEPCK), a molecule of GTP, ATP, or inosine triphosphate captures and phosphorylates the enolate anion generated by the decarboxylation (Eq. 13-46).[252] The stereochemistry is such that CO_2 departs from the *si* face of the forming enol.[253] The phospho group is transferred from GTP with inversion at the phosphorus atom.[254] The enzyme requires a divalent metal ion, preferably Mn^{2+}. In fact, kinetic studies of the GTP-dependent avian mitochondrial enzyme indicate two metal-binding sites, one on the polyphosphate group of the bound GTP and one on carboxylate side chains of the protein.[252,255] The three-dimensional structure of the ATP-dependent *E. coli* enzyme reveals a nucleotide binding site similar to the ATP site of adenylate kinase (Fig. 12-30).[256] A definite binding site for CO_2 is also present in the enzyme.[257]

PEPCK is also activated by low concentrations of Fe^{2+} and this activation depends upon a protein that has been identified as glutathione peroxidase (Eq. 15-58). By destroying H_2O_2 the latter may allow the Fe^{2+} to prevent oxidation of an SH group on PEP carboxykinase.[258] Synthesis of PEPCK is stimulated by glucagon, evidently through a direct action of cAMP on transcription of the structural gene.[259] Transcription is also stimulated by glucocorticoids and thyroid hormone and is inhibited by insulin.

In some organisms, such as the parasitic *Ascaris suum*, PEPCK functions principally as a means of synthesis of oxaloacetate by reaction of PEP with CO_2 and GDP. These organisms lack pyruvate kinase, which allows for buildup of the high PEP concentration needed to drive this reaction.[260] In a similar way PEP can be converted to oxaloacetate by **PEP carboxytransphosphorylase**, an enzyme found only in propionic acid bacteria and in *Entamoeba*. The reaction (Eq. 13-47) is accompanied by synthesis of inorganic pyrophosphate which may be cleaved to "pull" the reaction in the indicated direction.



Oxaloacetate

GTP

PEP

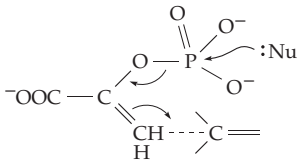
re Face is toward reader

(13-46)

$$PEP + CO_2 + P_i \rightarrow PP_i + \text{oxaloacetate} \qquad (13\text{-}47)$$

Oxaloacetate is also decarboxylated without phosphorylation of the enolate anion formed but with release of free pyruvate. Both pyruvate kinase and PEPCK can act as oxaloacetate decarboxylases.[261]

In the important reactions discussed in the following sections enolate ions are intermediates in carbon-carbon bond formation. Other examples are given in Eqs. 20-7 and 20-8, and Fig. 25-1, in which C–C bonds are formed by action of PEP as a carbon nucleophile,



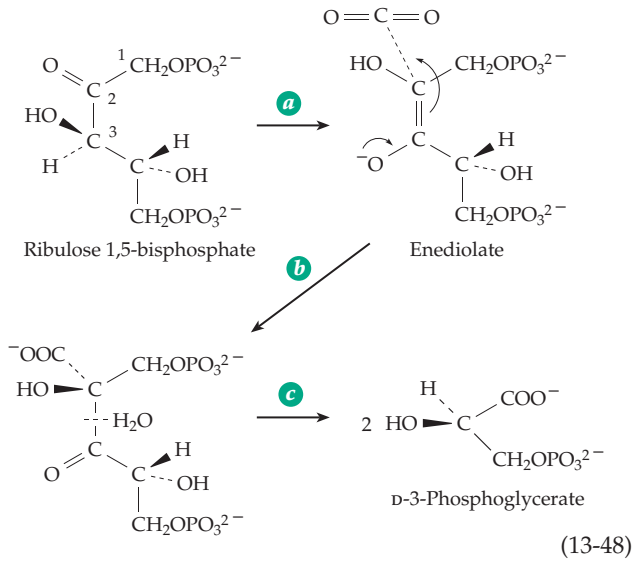
e.g., in aldol-like condensations.[242a] Yet another unusual type of reaction, involving formation of an enol ether linkage is illustrated in Box 13-B and in Eq. 25-3.

Ribulose bisphosphate carboxylase. The major route of incorporation of CO_2 into organic compounds is via photosynthesis. When ^{14}C-labeled CO_2 enters chloroplasts of green plants, the first organic ^{14}C-containing compound detected is 3-phosphoglycerate. Two molecules of this compound are formed through the action of **ribulose-1,5-bisphosphate carboxylase** (abbreviated **rubisco**), an enzyme present in chloroplasts and making up 16% of the protein of spinach leaves. This enzyme, whose structure is illustrated in Fig. 13-10, is thought to be the most abundant protein on earth. Because O_2 competes with CO_2 as a substrate, the enzyme also catalyzes an "oxygenase" reaction. It is therefore often called **ribulose-1,5-bisphosphate carboxylase/oxygenase**.[262] The rubisco from most plants is a 500- to 560-kDa L_8S_8 oligomer as shown in Fig. 13-10. The large subunit is encoded in the chloroplast DNA. However, a family of nuclear genes encode the small subunits, which are synthesized as larger precursors, with N-terminal extensions being removed to give the mature subunits.[263,264] Two types of small subunits occupy different positions in the

quasi-symmetric spinach rubisco.[264] In *Euglena* the small subunit is synthesized as a polyprotein precursor containing eight copies of the subunit.[265] Rubisco from the hydrogen-oxidizing bacterium *Alcaligenes eutrophus* has a similar quaternary structure,[266] but the enzyme from *Rhodospirillum rubrum* is a simple dimer.[267] In dinoflagellates the rubisco gene is present in nuclear DNA rather than in the chloroplasts.[268]

The carboxylation reaction catalyzed by rubisco differs from others that we have considered in that the carboxylated product is split by the same enzyme (Eq. 13-48). The mechanism shown in Eq. 13-48 was suggested by Bassham *et al*.[271] Ribulose bisphosphate, for which the enzyme is absolutely specific, is first converted to its enolic form, a 2,3-enediol. Loss of a proton from the 3-OH group forms the endiolate anion needed for the carboxylation in step *b*. The product of this step is a β-oxoacid which undergoes enzyme-catalyzed hydrolytic cleavage (Eq. 13-48, step *c*; see also Eq. 13-34). Support for this mechanism came from the observation that 2-carboxyarabinitol bisphosphate (Fig. 13-11B) is a potent inhibitor, possibly a transition state analog.[272] This inhibitor, bound into the active site of the enzyme from spinach, is seen in both Figs. 13-10B and 13-11A. An expanded version of Eq. 13-48 is given in Fig. 13-12, which is based upon modeling together with a variety of X-ray structures including that shown in Fig. 13-11.

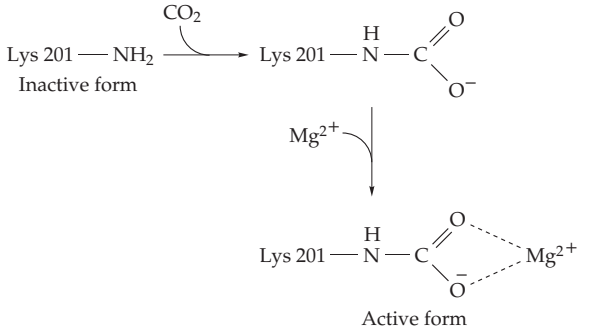
An essential Mg^{2+} ion is held by carboxylates of D203 and E204 and by modified K201. It also coordinates three molecules of H_2O in the free enzyme.[272a] Catalytic roles for the H294 and H327 imidazole groups are still being elucidated.[272b] Lysine 175 protonates C2 of the aci anion generated by C–C bond cleavage (step *e*, Fig. 13-12).[272c,276] Like many enzymes, rubisco exists in two major conformational states: open and closed.[272d]



(13-48)

Chemical studies also support the indicated mechanism. For example, the β-oxoacid intermediate formed in step *b* of Eq. 13-48 or Fig. 13-12 has been identified as a product released from the enzyme by acid denaturation during steady-state turnover.[273,274] Isotopic exchange with ^3H in the solvent[275] and measurement of ^{13}C isotope effects[277] have provided additional verification of the mechanism. The catalytic activity of the enzyme is determined by ionizable groups with pK_a values of 7.1 and 8.3 in the ES complex.[278]

The apparent value of K_m for total CO_2 (CO_2 + HCO_3^-) is high, 11–30 mM, but for the true substrate CO_2 it is only 0.45 mM. In intact chloroplasts the affinity for substrate is distinctly higher, with the K_m for total CO_2 dropping to ~0.6 mM. The difference appears to result largely from a regulatory reaction of CO_2 in which the side chain amino group of lysine 201 of the large subunit forms a carbamate (Eq. 13-49). Although carbamylation is spontanous, it is enhanced by an ATP-dependent process catalyzed by **rubisco activase.**[279] The carbamylation converts the side chain of Lys 201 into a negatively charged group that binds to an essential divalent metal, usually Mg^{2+}, in the active center[282] as is shown in Fig. 13-11, A and B. The nature of the reaction is uncertain. One possibility is that the activase is a chaperonin.[280] It appears to assist the enzyme in removing inhibitory sugars that arise by side reactions in the active site.[281] Rubisco is also regulated by the level of a natural inhibitor which has been identified as 2-carboxyarabinitol 1-phosphate. This is the same as the inhibitor shown in Fig. 13-11 but with one less phosphate group and consequent weaker binding.[273a]



(13-49)

In most plants photosynthesis is also strongly inhibited by O_2. This observation led to the discovery that O_2 competes directly for CO_2 at the active site of rubisco in a process called **photorespiration**. Chloroplasts inhibited by oxygen produce **glycolate** in large amounts[282a] as a result of the reaction of the intermediate enediolate ion formed in step *b* of Eq. 13-48 with O_2

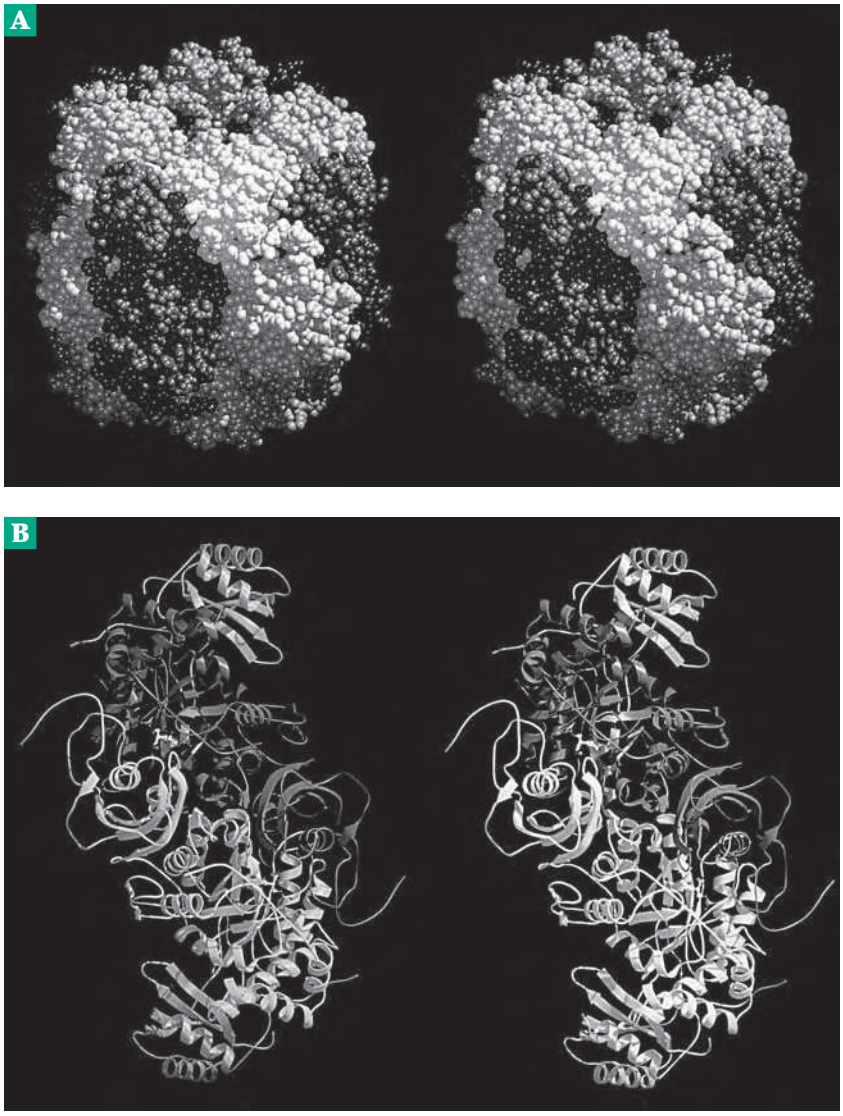


Figure 13-10 Stereoscopic view of ribulose bisphosphate carboxylase (rubisco) from spinach. (A) The symmetric L_8S_8 molecule contains eight 475-residue large subunits (in two shades of gray) and eight 123-residue small subunits (lighter gray in upper half of image). (B) One L_2S_2 substructure containing two active sites shared between adjacent large subunits with the bound inhibitor 2-carboxy-D-arabinitol 1,5-bisphosphate.[269] In the upper LS unit the S subunit (top) is light and the L subunit is dark. Courtesy of Inger Andersson. Similar structures have been determined for enzymes from tobacco[269a] and from the cyanobacterium *Synechoccus*.[270]

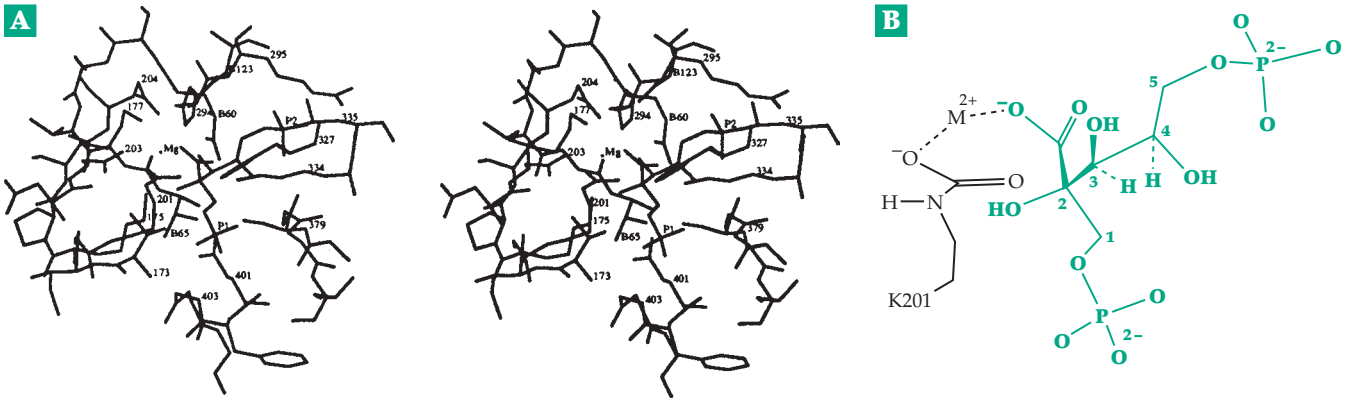


Figure 13-11 (A) Overview of the active site of spinach rubisco showing bound 2-carboxy-D-arabinitol 1,5-bisphosphate and Mg^{2+} and residues within hydrogen-bonding distance of these ligands. The hydroxyl groups at C2 and C3 of the inhibitor are in *cis* conformation. [269] Courtesy of Inger Andersson. (B) Structure of the inhibitor 2-carboxy-D-arabinitol 1,5-bisphosphate. A part of the carbamylated lysine 201 and the essential metal ion are also shown.

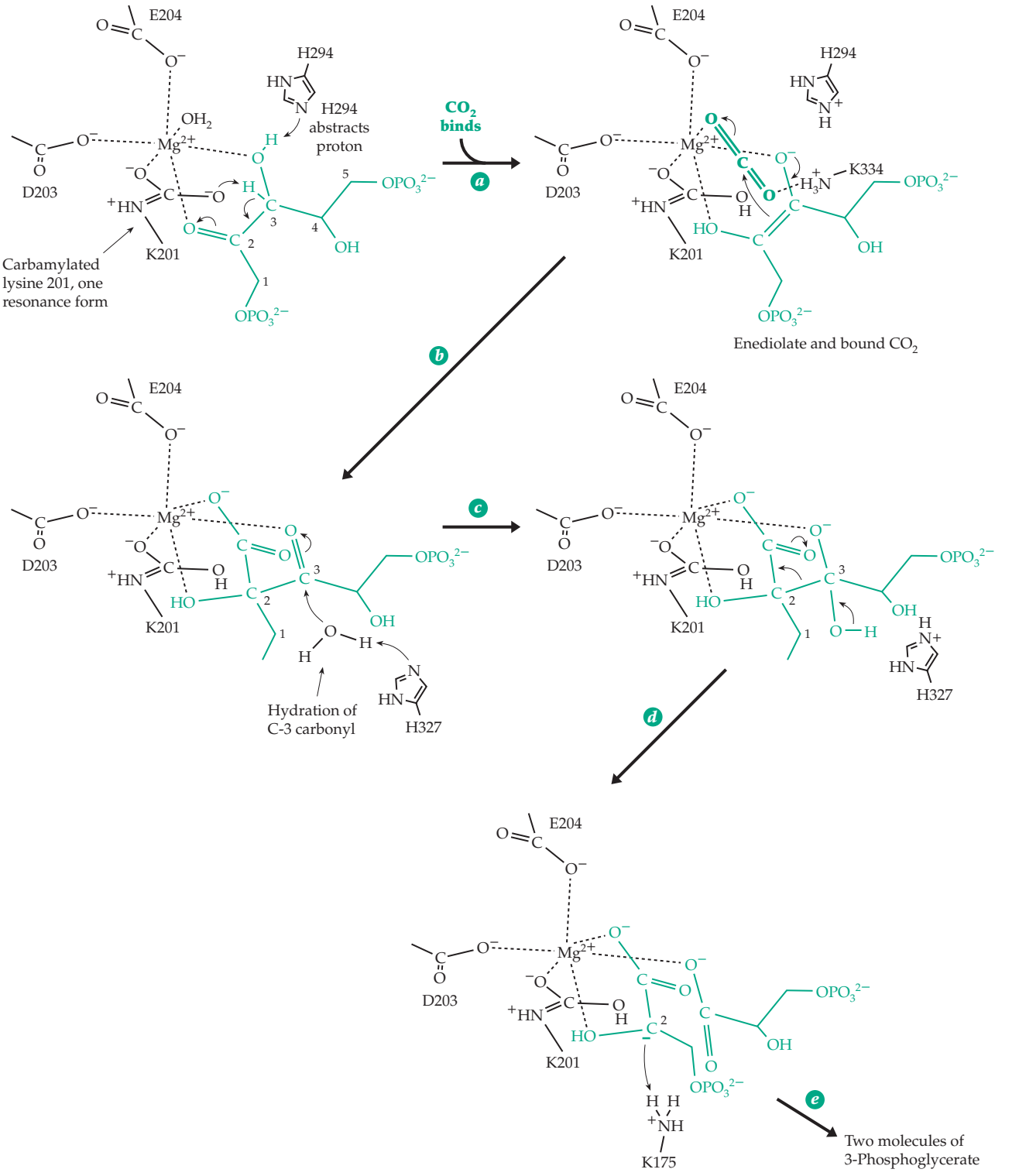
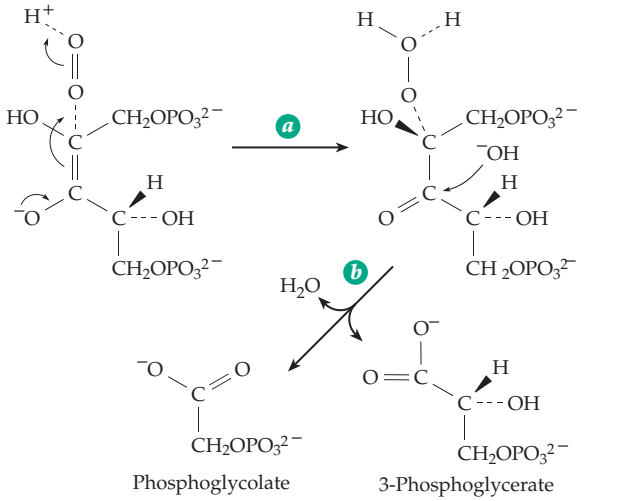


Figure 13-12 Proposed mechanism of action of ribulose bisphosphate carboxylase (rubisco). This is an abbreviated version of the mechanism as presented by Taylor and Andersson.[276] The binding of ribulose 1,5-bisphosphate occurs after carbamylation of lysine 201 and binding of a magnesium ion. Formation of an enediolate intermediate in step *a* is probably catalyzed by the carbamate group as indicated. Removal of a proton from the 3-OH, perhaps by H294, and addition of a proton to form an –OH group at C2 are also necessary. The CO_2 may bind to the Mg^{2+} and be polarized by interaction with other side chains prior to reaction. Carboxylation occurs by addition of the enediolate to CO_2 in step *b*. The hydration of the resulting 3-oxo group (step *c*) is necessary for cleavage of the C–C bond in step *d*. The participation of the newly formed carboxylate as an *aci* anion coordinated to Mg^{2+} is presumably involved. Protonation, with stereochemical inversion, is thought to involve K175, as shown. The two product molecules dissociate in step *e*.

(Eq. 13-50, step *a*). The peroxide formed in this way breaks up under the hydrolytic action of the enzyme to form phosphoglycolate and 3-phosphoglycerate (Eq. 13-50, step *b*).

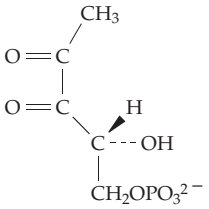


(13-50)

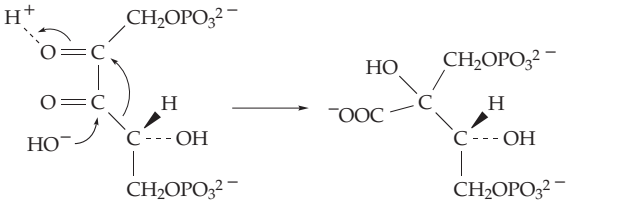
Molecular oxygen usually reacts rapidly with only those organic substrates, such as dihydroflavins, that are able to form stable free radicals. However, the endiolate anion of Eq. 13-50 may be able to donate a single electron to O_2 to form a superoxide–organic radical pair prior to formation of the peroxide (see also Eq. 15-30). Similar oxygenase side reactions have been observed for a variety of other enzymes that utilize carbanion mechanisms.[283] The reaction of rubisco with O_2 is of both theoretical and practical interest, the latter because of its significance in lowering the yield in photosynthesis (Chapter 23).

The simpler dimeric rubisco from *Rhodospirillum* is very inefficient in carboxylation and catalyzes much more oxygenation than do rubiscos of higher plants.[283a] Mutant enzymes that have impaired carboxylase and enhanced oxygenase are also known.[284,284a]

The small subunits of rubisco may help suppress undesirable side reactions.[285] For example, the following deoxypentodiulose phosphate can be formed by β elimination from the second intermediate of Eq. 13-48.

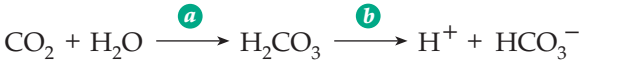


Some side products of the normal oxgenase reaction arise by elimination of a peroxide ion from the first intermediate of Eq. 13-50. The resulting dicarbonyl bisphosphate can rearrange to give a carboxytetritol bisphosphate (Eq. 13-51).[284]



(13-51)

Carbon dioxide or bicarbonate ion? An important question in the consideration of carboxylation and decarboxylation reactions is whether the reactant or the product is CO_2 or HCO_3^-. An approach to answering the question was suggested by Krebs and Roughton,[286]



(13-52)

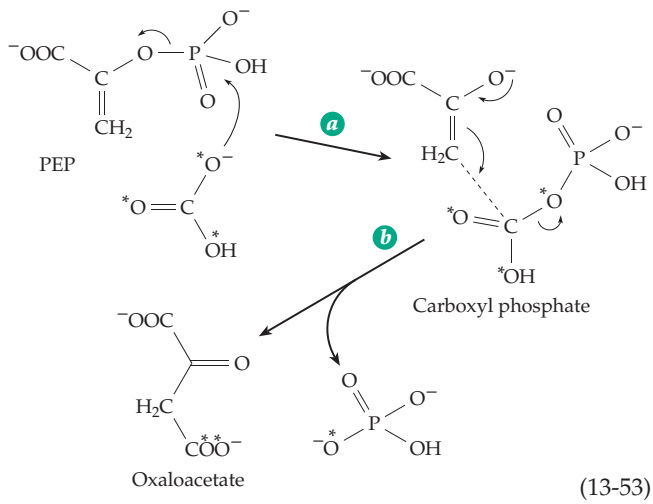
who pointed out that the attainment of equilibrium between free CO_2 and HCO_3^- (Eq. 13-52) may require several seconds. If an enzyme produces CO_2 as a product and progress of the reaction is followed manometrically, the pressure will rise higher than the equilibrium value as the CO_2 is evolved. Later when substrate is exhausted and the CO_2 equilibrates with bicarbonate, the pressure will fall again. The addition of carbonic anhydrase, which catalyzes Eq. 13-52, step *a*, abolishes the "overshoot." If bicarbonate is the primary product of a decarboxylation, there is a lag in the appearance of free CO_2.

A second approach is to use [^{18}O]bicarbonate and to follow the incorporation of ^{18}O into a carboxylated substrate. If CO_2 is the primary substrate only two labeled oxygen atoms enter the compound, whereas if HCO_3^- is the reactant three are incorporated.[287] A third technique is measurement of the rate of incorporation of CO_2 or bicarbonate in the carboxylated product. Over a short interval of time, e.g., 1 min, different kinetics will be observed for the incorporation of CO_2 and of bicarbonate.[288] Using these methods, it was established that the product formed in Eq. 13-46 and the reactant in Eq. 13-47 is CO_2. However, the carboxylation enzymes considered in the next section use bicarbonate as the substrate.

5. Incorporation of Bicarbonate into Carboxyl Groups

An important enzyme with a biosynthetic function in many bacteria and in all higher plants is **PEP carboxylase**,[289] which catalyzes the reaction of Eq. 13-53. This enzyme, in effect, accomplishes the reverse of Eq. 13-46 by converting the three-carbon PEP, by reaction with bicarbonate, into the four-carbon oxaloacetate. The latter is needed for "priming" of the citric acid cycle and for biosynthesis of such amino acids as aspartate and glutamate. That the enzyme functions in this way is indicated by the fact that mutants of *Salmonella* defective in the enzyme do not grow unless oxaloacetate or some other intermediate in the citric acid cycle is added to the medium. The enzyme from *S. typhimurium* is a 400-kDa tetramer with complex regulatory properties. The corresponding enzyme from spinach has 12 subunits and 12 bound Mn^{2+} ions. The enzyme also has a special function in the C_4 plants,[290] in which it is a component of a carbon dioxide concentrating system (Chapter 23).

When [^{18}O]bicarbonate is a substrate, two labeled oxygen atoms enter the oxaloacetate, while the third appears in P_i. A concerted, cyclic mechanism could explain these results. However, study of kinetic isotope effects,[291] use of a substrate with a chiral thiophospho group,[292] and additional ^{18}O exchange studies[293] have ruled out this possibility. A transient carboxyl phosphate (Eq. 13-53) is evidently an intermediate.[294,295] The incorporation of the ^{18}O from bicarbonate into phosphate is indicated by the asterisks. The carboxyl group enters on the *si* face of PEP. However, there is another possibility.[295,296] The carboxyl phosphate, while in the active site adjacent to the enolate anion, may eliminate phosphate, the enolate ion adding to the resulting CO_2 to form the final product. According to this mechanism the group transfer potential of the phosopho group in PEP is

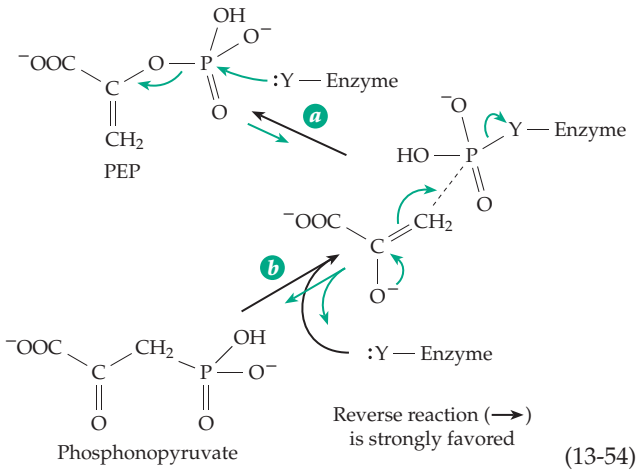


(13-53)

utilized to concentrate the CO_2 and to localize it next to the enolate anion generated by the same process.

PEP carboxylase is lacking from animal tissues and fungi. In these creatures PEP is converted to pyruvate, which is then carboxylated to oxaloacetate with coupled cleavage of ATP by the action of **pyruvate carboxylase** (Eq. 14-3), an enzyme that not only utilizes bicarbonate ion but also contains **biotin**. However, there are mechanistic similarities between its action and that of PEP carboxylase.

PEP mutase and the synthesis of phosphonates. The lipids of some organisms, such as *Tetrahymena*, contain aminoethylphosphonate, a compound with a C–P bond (Chapter 8). There are also many other naturally occurring phosphono compounds and huge quantities of synthetic phosphonates, present in detergents, herbicides, and insecticides, are metabolized by bacteria.[297] Here we will consider only one step in the biosynthesis of phosphonates, the conversion of PEP into phosphonopyruvate (Eq. 13-54), a reaction catalyzed by **PEP mutase**. The phospho group is moved



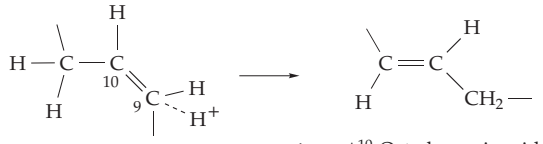
(13-54)

from the oxygen atom of PEP to the methylene carbon atom. When attached to carbon the phospho group is designated **phosphono**. The reaction occurs with retention of the configuration around phosphorus and a phosphoenzyme intermediate seems to be involved[298,299] as shown in Eq. 13-54. The equilibrium in this reaction strongly favors PEP. One further product formed from phosphonopyruvate is phosphonoethylamine, a component of phosphonolipids.[300] It is easy to imagine a synthetic route involving transamination followed by decarboxylation. Another mutase, similar to PEP mutase, shifts the carboxyphospho group $^{-}OOC–PO_2^{-}–$ exactly as in Eq. 13-54 as part of the biosynthetic pathway of a natural herbicide formed by *Streptomyces hygroscopicus*.[301,302] Soil bacteria contain specialized enzymes that catalyze the hydrolytic cleavage of P–C bonds.[302a]

D. Some Isomerization and Rearrangement Reactions

A few metabolic reactions do not fit into any of the categories discussed so far and apparently do not depend upon a coenzyme. Some of these involve transfer of alkyl groups or of hydrogen atoms from one carbon to another. The hydrogen atoms move by direct transfer without exchange with the medium. All of the reactions could involve carbocations but there is often more than one mechanistic possibility.

A simple 1,3-proton shift is shown in Table 10-1 as reaction type 6A. An example is the isomerization of oleic acid to *trans*-Δ^{10}-octadecenoic acid (Eq. 13-55) catalyzed by a soluble enzyme from a pseudomonad.[303]

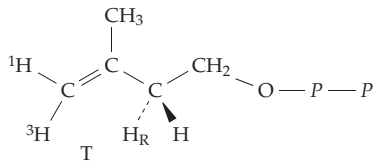


Oleic acid
(*cis*-Δ^9-octadecenoic acid)

trans-Δ^{10}-Octadecenoic acid

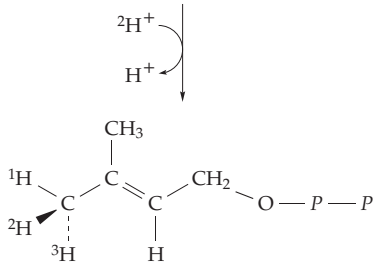
(13-55)

A second example is isomerization of **isopentenyl diphosphate** to **dimethylallyl diphosphate** (Eq. 13-56).[304–307] The stereochemistry has been investigated using the ^{3}H-labeled compound shown in Eq. 13-56. The *pro-R* proton is lost from C-2 and a proton is added to the *re* face at C-4. When the reaction was carried out in ^2H_2O a chiral methyl group was produced as shown.[304] A concerted proton addition and abstraction is also possible, the observed *trans* stereochemistry being expected for such a mechanism. However, the fact that the enzyme is strongly inhibited by the cation of 2-(diethylamino) ethyl pyrophosphate, an analog of a probable carbocation intermediate. Cysteine and glutamate side chains are essential.[307]



T

Isopentenyl diphosphate

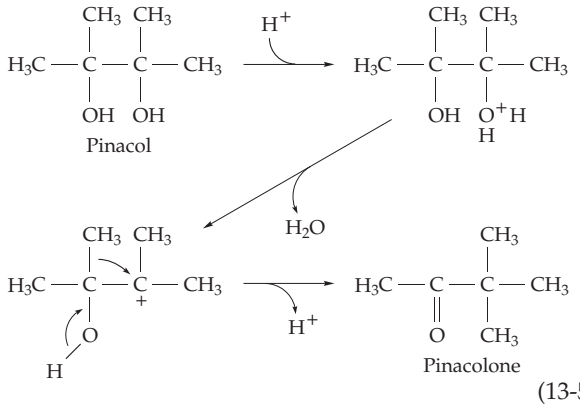


Dimethylallyl diphosphate

(13-56)

Reaction type 6B of Table 10-1 is allylic rearrangement with simultaneous condensation with another molecule. The reaction, which is catalyzed by **prenyltransferases**,[307a] occurs during the polymerization of polyprenyl compounds (Fig. 22-1,Eqs. 22-2, 22-3). Experimental evidence favors a carbocation mechanism for all of these reactions.[308,309] See Chapter 22.

Reaction type 6C (Table 10-1) occurs during the biosynthesis of leucine and valine (Fig. 24-17). The rearrangement is often compared with the nonenzymatic acid-catalyzed pinacol–pinacolone rearrangement in which a similar shift of an alkyl group takes place (Eq. 13-57). The enzyme-catalyzed rearrangement



Pinacol

Pinacolone

(13-57)

presumably gives the structure drawn in brackets in Table 10-1. The same enzyme always catalyzes reduction with NADH to the diol, the Mg^{2+}-dependent enzyme being called **acetohydroxy acid isomeroreductase**. Rearrangement has never been observed without the accompanying reduction.[310–313] More complex rearrangements that occur during biosynthesis of sterols are described in Chapter 22.

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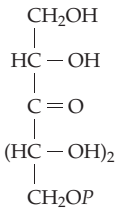
Study Questions

1. Discuss the role of carbonyl groups in facilitating reactions of metabolism.

2. Write a step-by-step sequence showing the chemical mechanisms involved in the action of a type I aldolase that catalyzes cleavage of fructose 1,6-bisphosphate. The enzyme is inactivated by sodium borohydride in the presence of the substrate. Explain this inactivation.

3. Some methylotrophic bacteria dehydrogenate methanol to formaldehyde. The latter undergoes an aldol condensation to form a hexulose-6-phosphate:



   Write a reasonable cycle of biochemical reactions (utilizing this compound) by which three molecules of methanol can yield one molecule of glyceraldehyde 3-phosphate, a compound that can be either catabolized for energy or utilized for biosynthesis.

4. Malate is formed in the glyoxylate pathway by reaction of glyoxylate with Acetyl-CoA. Indicate the chemical mechanism of the reactions involved and structure of an intermediate species.

5. Acetyl-CoA is condensed with acetoacetyl-CoA to give an intermediate which is eventually converted to mevalonate.
   a) Show the sequence of reactions leading from acetyl-CoA to mevalonate with the structures of important intermediates.
   b) Mevalonate is an important intermediate on the pathway leading to what *class* of compounds?

6. a) Show the structures of the reactants for the 3-hydroxy-3-methylglutaryl-CoA synthase reaction.
   b) Free coenzyme A is liberated in the above reaction. From which of the two reactants did it come? Explain the metabolic significance of the liberation of free CoA.

7. Phosphoenolpyruvate carboxykinase catalyzes the following reaction:

   Oxaloacetate + GTP→ CO_2 + phosphoenolpyruvate + GDP

   Illustrate the probable mechanism of the reaction.

8. Write a complete step-by-step mechanism for the action of ATP-citrate lyase which catalyzes the following reaction (Eq. 13-39):

   ATP + citrate + CoA – SH → Acetyl – CoA + oxaloacetate + ADP + P_i

9. Malonyl-CoA synthetase forms its product from free malonate and MgATP. Both phospho-enzyme and malonyl-enzyme intermediates have been detected. Suggest a sequential mechanism of action. See Kim, Y. S., and Lee, J. K. (1986) *J. Biol. Chem.* **261**, 16295–16297.

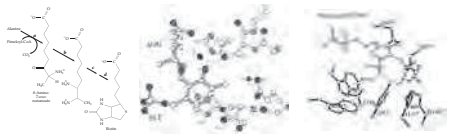
10. The carboxylation of acetone with HCO_3^- to form acetoacetate (the reverse of Eq. 13-44) is not a thermodynamically spontaneous process ($\Delta G°'$ ~17 kJ mol^{-1}). An anaerobic strain of *Xanthobacter* couples this reaction to the cleavage of ATP to AMP + 2 P_i. Suggest possible mechanisms. See Sluis, M. K., and Ensign, S. A. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8456–8461.

11. The reaction of Eq. 10-11 begins with a hydroxylation, which can be viewed as insertion of an oxygen atom into a C–H bond. Draw the probable intermediate formed in this reaction and indicate how it can be converted to the final products.

12. Cyanase from *E. coli* decomposes cyanate (NCO^-) to ammonia and CO_2. Isotopic labeling studies show that the second oxygen atom in the product CO_2 comes not from H_2O but from a second substrate, bicarbonate ion. The correct equation for the enzymatic process is:

   $NCO^- + H^+ + HCO_3^- \rightarrow NH_2 - COO^- + CO_2$

   Propose a sequence of steps for this enzymatic reaction, which has been investigated by Anderson and coworkers. Johnson, W. V., and Anderson, P. M. (1987) *J. Biol. Chem.* **262**, 9021–9025 and Anderson, P. M., Korte, J. J., Holcomb, T. A., Cho, Y.-g, Son, C.-m, and Sung, Y.-c. (1994) *J. Biol. Chem.* **269**, 15036–15045.



The vitamin **biotin** is formed in nature (left) by condensation of L-alanine with pimeloyl-CoA to form 8-amino-7-oxononanoate (AON). This compound is seen at the upper left of the center structure joined as a Schiff base with the coenzyme **pyridoxal phosphate** (PLP). This is a product complex of the enzyme AON synthase (see Webster *et al., Biochemistry* **39**, 516-528, 2000) Courtesy of D. Alexeev, R. L. Baxter, and L. Sawyer. Biotin synthesis requires three other enzymes (steps *b*, *c*, *d*). Step *b* is catalyzed by a PLP-dependent transaminase. At the left is **thiamin diphosphate**, in the form of its 2-(1-hydroxyethyl) derivative, an intermediate in the enzyme pyruvate decarboxylase (Dobritzsch *et al., J. Biol. Chem.* **273**, 20196-20204, 1998). Courtesy of Guoguang Lu. Thiamin diphosphate functions in all living organisms to cleave C–C bonds adjacent to C=O groups.

Contents

Coenzymes: Nature's Special Reagents

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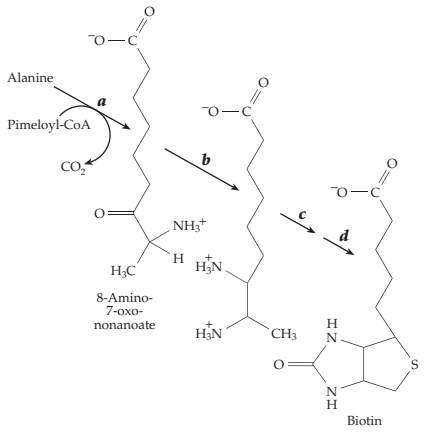
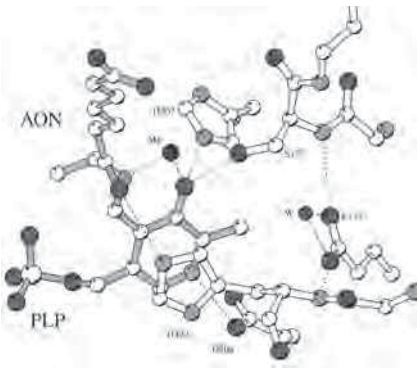
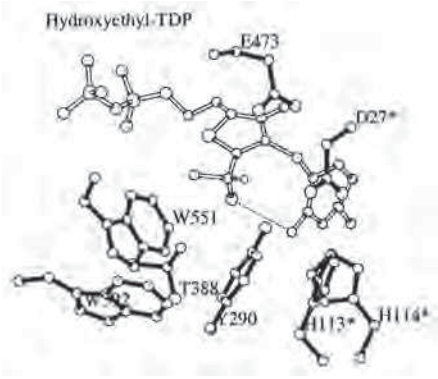






Most of the reactions discussed in Chapters 12 and 13 are catalyzed by enzymes that contain only those functional groups found in the side chains of the constituent amino acids. **Coenzymes** are nonprotein molecules that function as essential parts of enzymes. Coenzymes often serve as "special reagents" needed for reactions that would be difficult or impossible using only simple acid–base catalysis. In many instances, they also serve as **carriers**, alternating catalysts that accept and donate chemical groups, hydrogen atoms, or electrons. Coenzymes will be considered here in three groups:

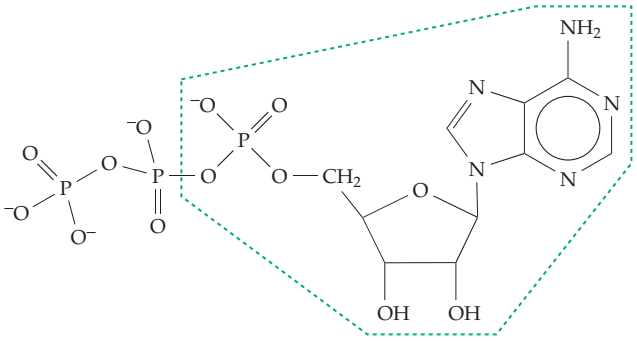
1. Compounds of high group transfer potential such as ATP and GTP that function in energy coupling within cells. Because it is cleaved and then dissociates from the enzyme to which it is bound, ATP may be regarded as a substrate rather than a coenzyme. However, as a phosphorylated form of ADP it may also be viewed as a carrier of high-energy phospho groups.

2. Compounds, often derivatives of **vitamins** that, while in the active site of the enzyme, alter the structure of a substrate in a way that permits it to react more readily. Coenzyme A, pyridoxal phosphate, thiamin diphosphate, and vitamin B_{12} coenzymes fall into this group.

3. Oxidative coenzymes with structures of precisely determined oxidation–reduction potential. Examples are NAD^+, $NADP^+$, FAD, and lipoic acid. They serve as carriers of hydrogen atoms or of electrons. Some of these coenzymes, such as NAD^+ and $NADP^+$, can usually dissociate rapidly and reversibly from the enzymes with which they function. Others, including FAD, are much more tightly bound and rarely if ever dissociate from the protein catalyst. Heme groups are covalently linked to proteins such as cytochrome *c* and cannot be dissociated without destroying the enzyme. Very tightly bound coenzyme groups are often called **prosthetic groups**, but there is no sharp line that divides prosthetic groups from the loosely bound coenzymes. For example, NAD^+ is bound weakly to some proteins but tightly to others. Oxidative coenzymes are discussed in Chapter 15.

A. ATP and the Nucleotide "Handles"

The role of ATP in "driving" biosynthetic reactions has been considered in Chapter 12, where attention was focused on the polyphosphate group which undergoes cleavage. What about the adenosine end? Here is a shapely structure borrowed from the nucleic acids. What is it doing as a carrier of phospho groups? At least part of the answer seems to be that the adenosine monophosphate (AMP) portion of the molecule is a "handle" which can be "grasped" by catalytic proteins. For some enzymes, such as acetyl-CoA synthetase (Eq. 12-45), the handle is important because the intermediate acyl adenylate must remain bound to the protein. Without the large adenosine group, there would be little for the protein to hold onto.



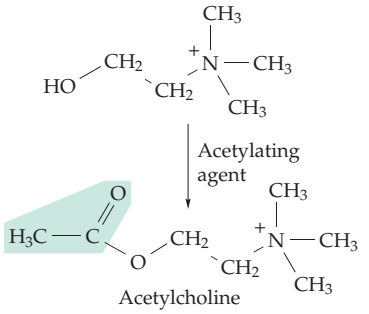
The AMP "handle" in ATP

AMP is only one of several handles to which nature attaches phospho groups to form di- and triphosphate derivatives. Like AMP, the other handles are nucleotides, the monomer units of nucleic acids. Thus, one enzyme requiring a polyphosphate as an energy source selects ATP, another CTP, or GTP. The nucleotide handles not only carry polyphosphate groups but also are present in other coenzymes, such as CoA, NAD^+, $NADP^+$, and FAD. In addition, they serve as carriers for small organic molecules. For example, **uridine diphosphate glucose** (UDP-Glc) Chapter 10, is a carrier of active glucosyl groups important in sugar metabolism and **cytidine diphosphate choline** is an intermediate in synthesis of phospholipids.

Recalling that acetyl adenylate (acetyl-AMP) is an intermediate in synthesis of acetyl-CoA, and comparing the biosynthesis of sugars, phospholipids, and acetyl-CoA, we see that in each case the enzyme involved requires a different nucleotide handle. The handle may provide a means of recognition which can help an enzyme to pick the right bit of raw material out of the sea of molecules surrounding it. Figure 5-6 shows the shapes of the four purine and pyrimidine bases forming the most common nucleotide handles. The distinctive differences both in shape and in hydrogen bond patterns are obvious. In binding to proteins, the hydrogen bond-forming groups in the purine and pyrimidine bases sometimes interact with precisely positioned groups in the protein. However, in some enzymes the "handle" is not precisely bound. This seems to be the case for adenine, which makes surprisingly few hydrogen bonds to proteins.[1] Hydroxyl groups of the ribose or deoxyribose ring also often form hydrogen bonds and the negatively charged oxygen atoms of the 5'-phosphate may interact with positively charged protein side chains.

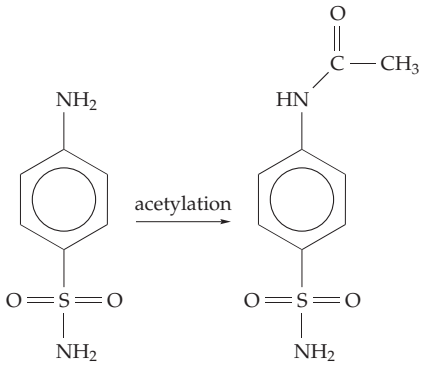
B. Coenzyme A and Phosphopantetheine

The existence of a special coenzyme required in biological acetylation was recognized by Fritz Lipmann in 1945.[2–3a] The joining of acetyl groups to other molecules is a commonplace reaction within living cells, one example being the formation of the neurotransmitter **acetylcholine**. In the laboratory acetylation is carried out with reactive compounds such as acetic anhydride or acetyl chloride.



Acetylcholine (14-1)

Lipmann wondered what nature used in their place. His approach in seeking the biological "active acetate" is one that has been used successfully in solving many biochemical problems. He first set up a test system to examine the ability of extracts prepared from fresh liver tissue to catalyze the acetylation of sulfanilamide (Eq. 14-2). A specific color test was available for quantitative determination of very small amounts of the



Sulfanilamide (14-2)

product. The rate of acetylation of sulfanilamide under standard conditions was taken as a measure of the activity of the biochemical acetylation system. Lipmann soon discovered that the reaction required ATP and that the ATP was cleaved to ADP concurrently with the formation of acetyl-sulfanilamide. He also found that dialysis or ultrafiltration rendered the liver extract almost inactive in acetylation. Apparently some essential material passed out through the semipermeable dialysis membrane. When the dialysate or ultrafiltrate was concentrated and added back, acetylation activity was restored. The unknown material was not destroyed by boiling, and Lipmann postulated that it was a new coenzyme which he called **coenzyme A** (CoA). Now the test system was used to estimate the amount of the

BOX 14-A DISCOVERY OF THE VITAMINS

Several mysterious and often fatal diseases which resulted from vitamin deficiencies were prevalent until the past century. Sailors on long sea voyages were often the victims. In the Orient, the disease **beriberi** was rampant and millions died of its strange paralysis called "polyneuritis." In 1840, George Budd predicted that the disease was caused by the lack of some organic compound that would be discovered in "a not too distant future."[a] In 1893, C. Eijkman, a Dutch physician working in Indonesia, observed paralysis in chicks fed white rice consumed by the local populace. He found that the paralysis could be relieved promptly by feeding an extract of rice polishings. This was one of the pieces of evidence that led the Polish biochemist Casimir Funk to formulate the "vitamine theory" in about 1912. Funk suggested that the diseases **beriberi**, **pellagra**, **rickets**, and **scurvy** resulted from lack in the diet of four different vital nutrients. He imagined them all to be amines, hence the name **vitamine**.

In the same year in England, F. G. Hopkins announced that he had fed rats on purified diets and discovered that amazingly small amounts of **accessory growth factors**, which could be obtained from milk, were necessary for normal growth.[b] By 1915, E. V. McCollum and M. Davis at the University of Wisconsin had recognized that rat growth depended on not one but at least two accessory factors. The first, soluble in fatty solvents, they called **A**, and the other, soluble in water, they designated **B**. Factor B cured beriberi in chicks. Later, when it was shown that vitamine A was not an amine, the "e" was dropped and **vitamin** became a general term.

Progress in isolation of the vitamins was slow, principally because of a lack of interest. According to R. R. Williams, when he started his work on isolation of the antiberiberi factor in 1910 most people were convinced that his efforts were doomed to failure, so ingrained was Pasteur's idea that diseases were caused only by bacteria. In 1926, Jansen isolated a small amount of thiamin, but it was not until 1933 that Williams, working almost without financial support, succeeded in preparing a large amount of the crystalline compound from rice polishings. Characterization and synthesis followed rapidly.[c,d]

It was soon apparent that the new vitamin alone would not satisfy the dietary need of rats for the B factor. A second thermostable factor (B_2) was required in addition to thiamin (B_1), which was labile and easily destroyed by heating. When it became clear that factor B_2 contained more than one component, it was called **vitamin B complex**. There was some confusion until relatively specific animal tests for each one of the members had been devised. **Riboflavin** was found to be most responsible for the stimulation of rat growth, while **vitamin B_6** was needed to prevent a facial dermatitis or "rat pellagra." **Pantothenic acid** was especially effective in curing a chick dermatitis, while **nicotinamide** was required to cure human pellagra. **Biotin** was required for growth of yeast.

The antiscurvy (antiscorbutic) activity was called **vitamin C**, and when its structure became known it was called **ascorbic acid**. The fat-soluble factor preventing rickets was designated **vitamin D**. By 1922, it was recognized that another fat-soluble factor, **vitamin E**, is essential for full-term pregnancy in the rat. In the early 1930s **vitamin K** and the **essential fatty acids** were added to the list of fat-soluble vitamins. Study of the human blood disorders "tropical macrocytic anemia" and "pernicious anemia" led to recognition of two more water-soluble vitamins, **folic acid** and **vitamin B_{12}**. The latter is required in minute amounts and was not isolated until 1948. Have all the vitamins been discovered? Rats can be reared on an almost completely synthetic diet. However, there is the possibility that for good health humans require some as yet undiscovered compounds in our diet. Furthermore, it is quite likely that we receive some essential nutrients that we cannot synthesize from bacteria in our intestinal tracts. An example may be the pyrroloquinoline quinone (PQQ).[e]

Why do we need vitamins? Early clues came in 1935 when nicotinamide was found in NAD^+ by H. von Euler and associates and in $NADP^+$ by Warburg and Christian. Two years later, K. Lohman and P. Schuster isolated pure **cocarboxylase**, a dialyzable material required for decarboxylation of pyruvate by an enzyme from yeast. It was shown to be thiamin diphosphate (Fig. 15-3). Most of the water-soluble vitamins are converted into coenzymes or are covalently bound into active sites of enzymes. Some lipid-soluble vitamins have similar functions but others, such as vitamin D and some metabolites of vitamin A, act more like hormones, binding to receptors that control gene expression or other aspects of metabolism.

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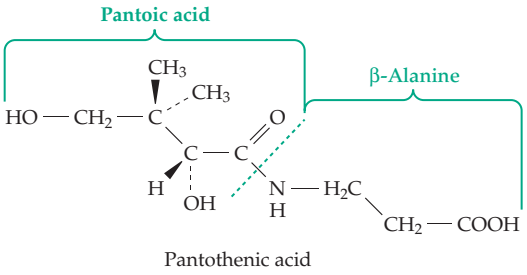
[a] Hughes, R. E. (1973) *Medical History* **17**, 127–134
[b] Harris, L. D. (1937) *Vitamins in Theory and Practice*, Cambridge Univ. Press, London and New York
[c] Karlson, P. (1984) *Trends Biochem. Sci.* **9**, 536–537
[d] Williams, R. R., and Spies, T. D. (1938) *Vitamin B_1 and Its Use in Medicine*, Macmillan, New York
[e] Killgore, J., Smidt, C., Duich, L., Romero-Chapman, N., Tinker, D., Reiser, K., Melko, M., Hyde, D., and Rucker, R. B. (1989) *Science* **245**, 850–852

coenzyme in a given volume of dialysate or in any other sample. When small amounts of CoA were supplied to the test system, only partial restoration of the acetylation activity was observed and the amount of restoration was proportional to the amount of CoA. With test system in hand to monitor various fractionation methods, Lipmann soon isolated the new coenzyme in pure form from yeast and liver.

Coenzyme A (Fig. 14-1) is a surprisingly complex molecule. The handle is AMP with an extra phospho group on its 3'-hydroxyl. The phosphate of the 5'-carbon is linked in anhydride (pyrophosphate) linkage to another phosphoric acid, which is in turn esterified with **pantoic acid**. Pantoic acid is linked to **β-alanine** and the latter to **β-mercaptoethylamine** through amide linkages, the reactive SH group being attached to a long (1.9-nm) semi-flexible chain. Coenzyme A can be cleaved by hydrolysis to **pantetheine** (Fig. 14-1), **pantetheine 4'-phosphate**, and **pantothenic acid**. These three compounds are all **growth factors**.

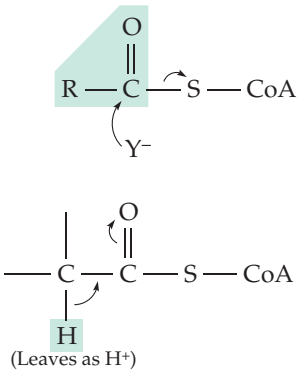
Pantothenic acid is a vitamin, which is essential to human life. Its name is derived from a Greek root that reflects its universal occurrence in living things. The bacterium *Lactobacillus bulgaricus*, which converts milk



to yogurt, needs the more complex pantetheine for growth. It finds a ready supply of pantetheine in milk and has lost its ability to synthesize this compound. However, it can convert pantetheine to CoA. Pantetheine 4'-phosphate is required for growth of *Acetobacter suboxydans*. While it is not a dietary essential for most organisms, it is found in a covalently bound form in several enzymes.

While CoA was discovered as the "acetylation coenzyme," it has a far more general function. It is required, in the form of acetyl-CoA, to catalyze the synthesis of citrate in the citric acid cycle. It is essential to the β oxidation of fatty acids and carries propionyl and other acyl groups in a great variety of other metabolic reactions. About 4% of all known enzymes require CoA or one of its esters as a substrate.[4]

Coenzyme A has two distinctly different biochemical functions, which have already been considered briefly in Chapters 12 and 13 and can be summarized as follows:



1. Activation of acyl group, R—C(=O)—, toward transfer by nucleophilic displacment
2. Activation of a hydrogen atom adjacent to the carbonyl of a thioester

These functions depend, to a considerable extent, on the fact that the properties of the carbonyl group of a thioester are closely similar to those of an isolated carbonyl group in a ketone.[5]

Synthesis of fatty acids in bacteria requires a small **acyl carrier protein (ACP)** whose functions are similar to those of CoA. However, it contains pante-

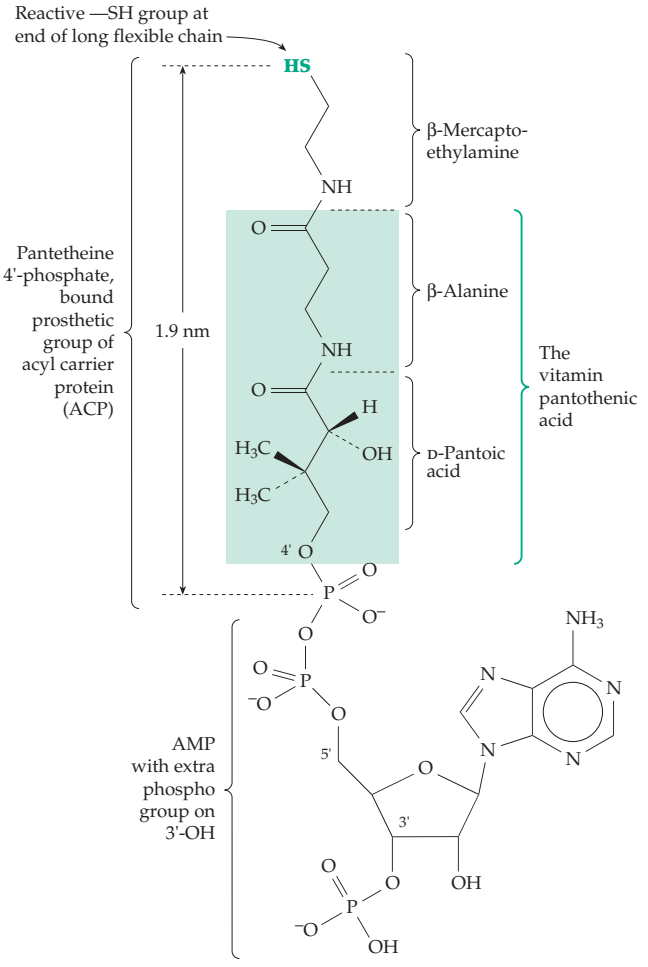
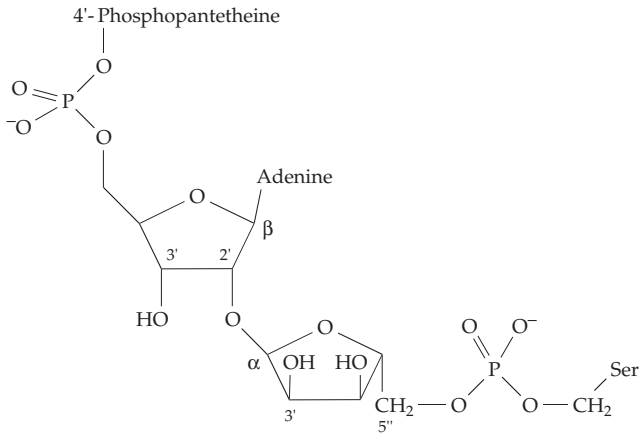


Figure 14-1 Coenzyme A, an acyl-activating coenzyme containing the vitamin pantothenic acid.

theine 4'-phosphate covalently bonded through phosphodiester linkage to a serine side chain. In *E. coli* this is at position 36 in the 77-residue protein.[6,7] Here the nucleotide handle of CoA has been replaced with a larger and more complex protein which can interact in specific ways with the multiprotein fatty acid synthase complex described in Chapter 21. In higher organisms ACP is usually not a separate protein but a domain in a large synthase molecule. Bound phosphopantetheine is also found in enzymes involved in synthesis of peptide antibiotics (Chapter 29)[8,9] and polyketides (Chapter 21).[10,11] It is also present in subunits of cytochrome oxidase and of ATP synthase of *Neurospora* but appears to play only a structural role, being needed for proper assembly of these multimeric proteins.[12] In a citrate-cleaving enzyme, and in a bacterial malonate decarboxylase, phosphopantethine is attached to a serine side chain as 2′-(5″-phosphoribosyl)-3′-dephospho-CoA.[13–14a]

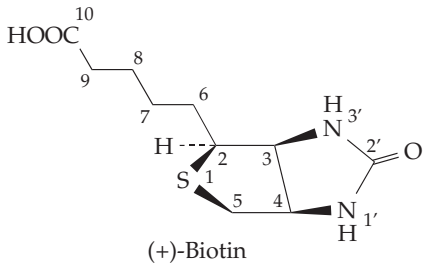


Attachment of phosphopantetheine to proteins is catalyzed by a phosphotransferase that utilizes CoA as the donor. A phosphodiesterase removes the phosphopantetheine, providing a turnover cycle.[15–15b] A variety of synthetic analogs have been made.[4,16] The reactive center of CoA and phosphopantetheine is the SH group, which is carried on a flexible arm that consists in part of the β-alanine portion of pantothenic acid. A mystery is why pantoic acid, a small odd-shaped molecule that the human body cannot make, is so essential for life. The hydroxyl group is a potential reactive site and the two methyl groups may enter into formation of a "trialkyl lock" (p. 485), part of a sophisticated "elbow" or shoulder for the SH-bearing arm.

When it binds to citrate synthase acetyl-CoA appears to bind only after the enzyme has undergone a conformational change that closes the enzyme around its other substrate oxaloacetate (Fig. 13-8). The adenine ring of the long CoA handle is tightly bonded to the protein through hydrophobic interactions and hydrogen-bond recognition interactions. Additional specific hydrophobic interactions allow the enzyme to hold the acetyl group in a precise position where it can be acted upon by the catalytic groups. In the case of CoA transferase (Eq. 12-50) smaller thiols can replace CoA but with much lower catalytic rates. The acyl-CoA derivatives are weakly bound, the expected intrinsic binding energy of the pantetheine portion of the molecule apparently being used to increase k_{cat}.[17] From a study of kinetics and equilibria it was concluded that the binding energy of the interaction of the nucleotide portion of coenzyme A with the enzyme is utilized to increase the rate of formation and to stabilize the covalently linked E-CoA (see Eq. 12-50). On the other hand, binding of the pantoic acid part of the molecule decreases the stability of the transition state for breakup of the complex by ~40 kJ/mol, which corresponds to a 10^7-fold increase in the reaction rate.[16]

C. Biotin and the Formation of Carboxyl Groups from Bicarbonate

By 1901 it was recognized that yeast required for its growth an unknown material which was called **bios**. This was eventually found to be a mixture of pantothenic acid, inositol, and a third component which was named **biotin**. Biotin was also recognized as a factor promoting growth and respiration of the clover root nodule organism *Rhizobium trifolii* and as vitamin H, a material that prevented dermatitis and paralysis in rats that were fed large amounts of uncooked egg white. Isolation of the pure vitamin was a heroic task accomplished by Kögl in 1935. In one preparation 250 kg of dried egg yolk yielded only 1.1 mg of crystalline biotin.[18]

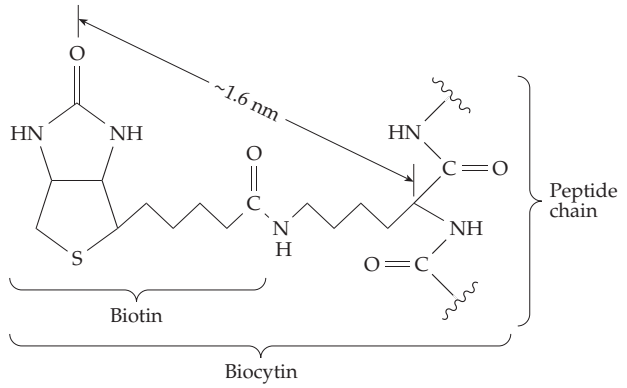


Biotin contains three chiral centers and therefore has eight stereoisomers.[18,19] Of these, only one, the dextrorotatory (+)-biotin, is biologically active.[19,20] The vitamin is readily oxidized to the sulfoxide and sulfone. The sulfoxide can be reduced back to biotin by a molybdenum-containing reductase in some bacteria (see also Chapter 16, Section H).[20a] Biotin is synthesized from pimeloyl-CoA (see chapter banner, p. 719 and Eq. 24-39). Four enzymes are required. Two of them, a

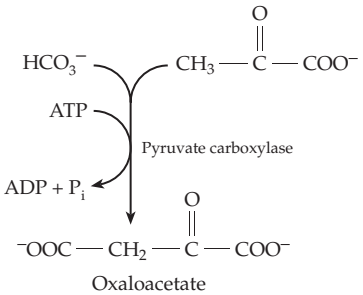
synthase that catalyzes step *a* (banner) and a transaminase that catalyzes step *b*, contain the coenzyme pyridoxal phosphate (PLP).[20b] The final step is insertion of a sulfur atom from an iron-sulfur center.

1. Biotin-Containing Enzymes

Within cells the biotin is covalently bonded to proteins, its double ring being attached to a 1.6-nm flexible arm. The first clue to this fact was obtained from isolation of a biotin-containing material **biocytin**, ε-N-biotinyl-L-lysine, from autolysates (self-digests) of rapidly growing yeast.[21] It was subsequently shown that the lysine residue of the biocytin was originally present in proteins at the active sites of biotin-containing enzymes usually within the sequence AMKM[22] or VMKM.[23] Other conserved features also mark the attachment site.[24]



Biotin acts as a **carboxyl group carrier** in a series of carboxylation reactions, a function originally suggested by the fact that aspartate partially replaces biotin in promoting the growth of the yeast *Torula cremonis*. Aspartate was known to arise by transamination from oxaloacetate, which in turn could be formed by carboxylation of pyruvate. Subsequent studies showed that biotin was needed for an enzymatic ATP-dependent reaction of pyruvate with bicarbonate ion to form oxaloacetate (Eq. 14-3). This is a β carboxylation coupled to the hydrolysis of ATP.



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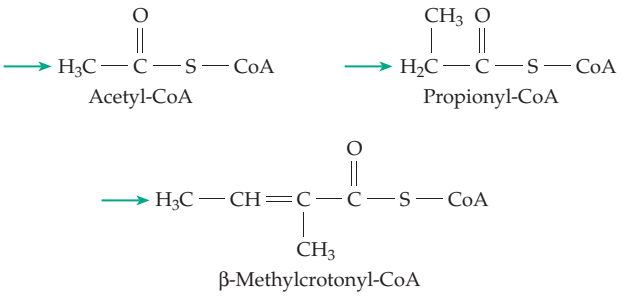
In addition to pyruvate carboxylase, other biotin-requiring enzymes act on **acetyl-CoA**, **propionyl-CoA**, and **β-methylcrotonyl-CoA**, using HCO_3^- to add carboxyl groups at the sites indicated by the arrows in the accompanying structures. Because of the presence of the C=C double bond conjugated with the carbonyl group, the carboxylation of β-methylcrotonyl-CoA is electronically analogous to β carboxylation.



Human cells, as well as those of other higher eukaryotes, carry genes for all four of these enzymes.[25–29] Acetyl-CoA carboxylase is a cytosolic enzyme needed for synthesis of fatty acids but the other three enzymes enter mitochondria when they function. The biotin-dependent carboxylases, which are listed in Table 14-1, have a variety of molecular sizes and subunits but show much evolutionary conservation in their sequences and chemical mechanisms.[22,25] In higher eukaryotes all of the catalytic apparatus of the enzymes is present in single large 190- to 200-kDa subunits. The 251-kDa subunit of yeast acetyl-CoA carboxylase consists of 2337 amino acid residues.[30] That of rats contains 2345[31] and that of the alga *Cyclotella* 2089.[32] Human cytosolic acetyl-Co carboxylase has 2347 residues while a mitochondria-associated form has an extra 136 residues, most of them in a hydrophobic N-terminal extension.[32a,b] Plants have two forms of the enzyme, one cytosolic and one located in plastids. In wheat, they have 2260 and 2311 residues, respectively.[32c,32d] Animal and fungal pyruvate carboxylases are also large ~500-kDa tetramers.[32e] The yeast enzyme consists of 1178-residue monomers.[33] In contrast, the 560-kDa human propionyl-CoA carboxylase is an $\alpha_4\beta_4$ tetramer.[34,35]

In bacteria and in at least some plant chloroplasts,[36,37] acetyl-CoA carboxylase consists of three different kinds of subunit and four different peptide chains. The much studied *E. coli* enzyme is composed of a 156-residue **biotin carboxyl carrier protein**,[38] a 449-residue **biotin carboxylase**, whose three-dimensional structure in known,[39,39a] and a **carboxyltransferase** subunit consisting of 304 (α)- and 319 (β)- residue chains. These all associate as a dimer of the three subunits (eight peptide chains).[40–42]

Biotin becomes attached to the proper ε-amino groups at the active centers of biotin enzymes by the action of **biotin holoenzyme synthetase** (biotinyl

protein ligase), which utilizes ATP to form an intermediate biotinyl-AMP.[43–47a] Hereditary deficiency of this enzyme has been observed in a few children and has been treated by administration of extra biotin.[48] The *E. coli* biotin holoenzyme synthetase, whose three-dimensional structure is known, has a dual function. It is also a repressor of transcription of the biotin biosynthetic operon.[45] Intracellular degradation of biotin-containing proteins yields biotin-containing oligopeptides as well as biocytin. These are acted on by **biotinidase** to release free biotin.[49,50] The action of this enzyme in recycling biotin may be a controlling factor in the rate of formation of new biotin-dependent enzymes.

2. The Mechanism of Biotin Action

It may seem surprising that a coenzyme is needed for these carboxylation reactions. However, unless the cleavage of ATP were coupled to the reactions, the equilibria would lie far in the direction of decarboxylation. For example, the measured apparent equilibrium constant K' for conversion of propionyl-CoA to *S* methylmalonyl-CoA at pH 8.1 and 28°C[51] is given by Eq. 14-4.

$$K' = \frac{[\text{ADP}][\text{P}_i][\text{methylmalonyl–CoA}]}{[\text{ATP}][\text{HCO}_3^-][\text{propionyl–CoA}]} = 5.7$$

$$\Delta G' = -4.36 \text{ kJ mol}^{-1} \qquad (14\text{-}4)$$

TABLE 14-1
Enzymes Containing Bound Biotin

1. Catalyzing beta carboxylation using HCO_3^- with coupled cleavage of ATP to ADP + P_i

   Acetyl-CoA carboxylase
   Propionyl-CoA carboxylase
   Pyruvate carboxylase
   β-Methylcrotonyl-CoA carboxylase
   (δ carboxylation)

2. Carboxyl group transfer without cleavage of ATP

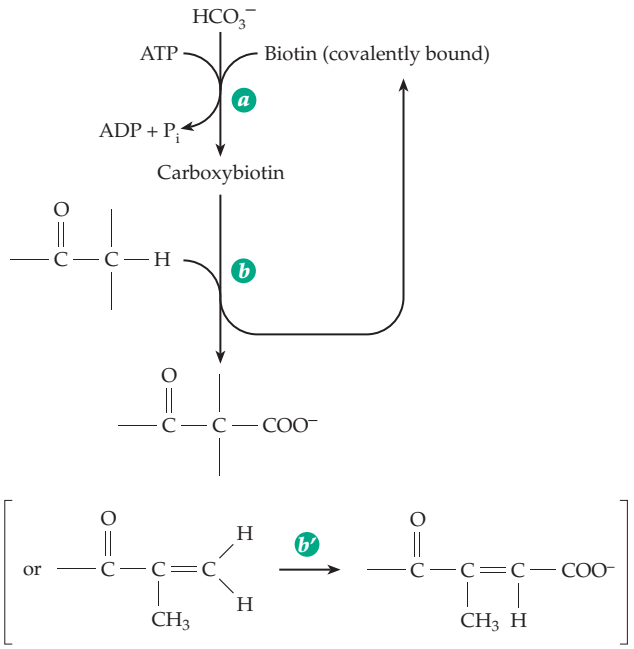
   Carboxyltransferase of *Propionobacterium*

3. Biotin-dependent Na^+ pumps

   Oxaloacetate decarboxylase
   Methylmalonyl-CoA decarboxylase
   Glutaconyl-CoA decarboxylase

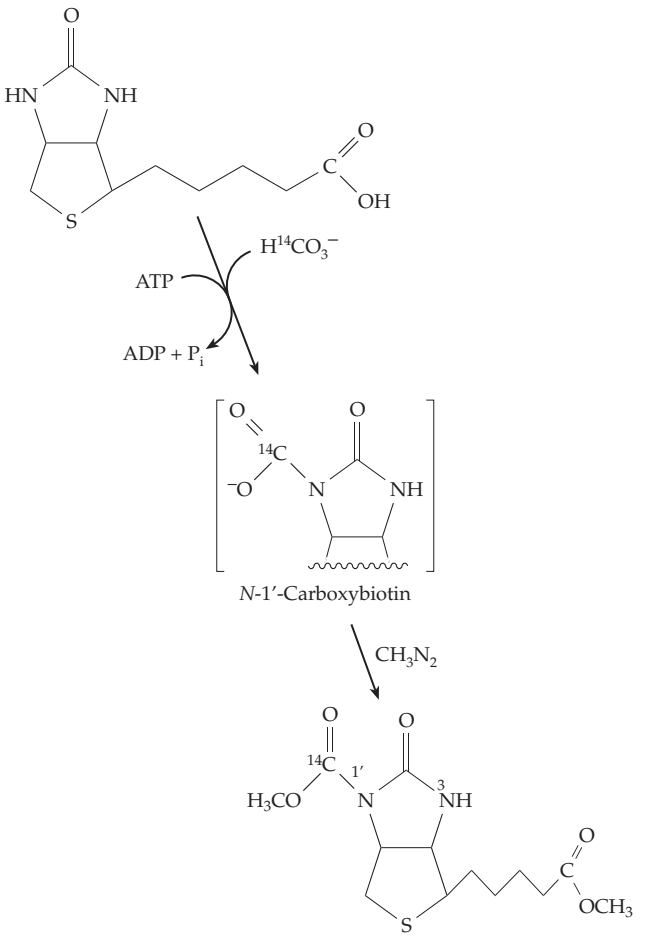
4. Other
   Malonate decarboxylase
   Urea carboxylase

The function of biotin is to mediate the coupling of ATP cleavage to the carboxylation, making the overall reaction exergonic. This is accomplished by a two-stage process in which a **carboxybiotin** intermediate is formed (Eq. 14-5). There is *one known biotin-containing enzyme that does not utilize ATP*. Propionic acid bacteria contain a **carboxyltransferase** which transfers a carboxyl group reversibly from methylmalonyl-CoA to pyruvate to form oxaloacetate and propionyl-CoA (see Fig. 17-10). This huge enzyme consists of a central hexameric core of large 12S subunits to which six 5S dimeric subunits and twelve 123-residue biotinylated peptides are attached.[25,52] No ATP is needed because free HCO_3^- is not a substrate. However, biotin serves as the carboxyl group carrier in this enzyme too.



(14-5)

Carboxybiotin. The structure of biotin suggested that bicarbonate might be incorporated reversibly into its position 2'. However, this proved not to be true and it remained for F. Lynen and associates to obtain a clue from a "model reaction." They showed that purified β-methylcrotonyl-CoA carboxylase promoted the carboxylation of *free* biotin with bicarbonate ($H^{14}CO_3^-$) and ATP. While the carboxylated biotin was labile, treatment with diazomethane (Eq. 14-6) gave a stable dimethyl ester of ***N*-1'-carboxybiotin**.[53,54] The covalently bound biotin at active sites of enzymes was also successfully labeled with $^{14}CO_2$. Treatment of the labeled enzymes with diazomethane followed by hydrolysis with trypsin and pepsin gave authentic *N*-1'-carboxybiocytin. It was now clear that the cleavage of ATP is required to couple the CO_2 from HCO_3^- to the biotin to form carboxybiotin. The enzyme must

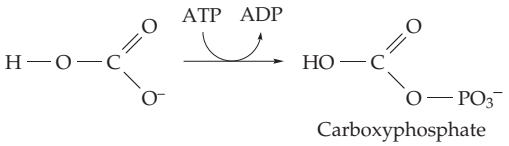


(14-6)

then transfer the carboxyl group from carboxybiotin to the substrate that is to be carboxylated. Enzymatic transfer of a carboxyl group from chemically synthesized carboxybiotin onto specific substrates confirmed the proposed mechanism.[55]

The biotin carboxyl carrier subunit of *E. coli* acetyl-CoA carboxylase contains the covalently bound biotin.[55a,b] The larger biotin carboxylase subunit catalyzes the ATP-dependent attachment of CO_2 to the biotin and the carboxyltransferase subunit catalyzes the final transcarboxylation step (Eq. 14-5, step *b*) by which acetyl-CoA is converted into malonyl-CoA. The biotin, which is attached to the carrier protein, is presumably able to move by means of its flexible arm from a site on the carboxylase to a site on the transcarboxylase.

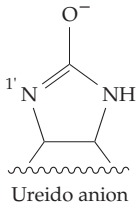
Carboxyphosphate. During the initial carboxylation step ^{18}O from labeled bicarbonate enters the P_i that is split from ATP. This suggested transient formation of **carboxyphosphate** by nucleophilic attack of HCO_3^- on ATP (Eq. 14-7). The carboxyl group of this reactive mixed anhydride[56] could then be transferred to biotin. This mechanism is supported by the fact that biotin carboxylase catalyzes the transfer of a



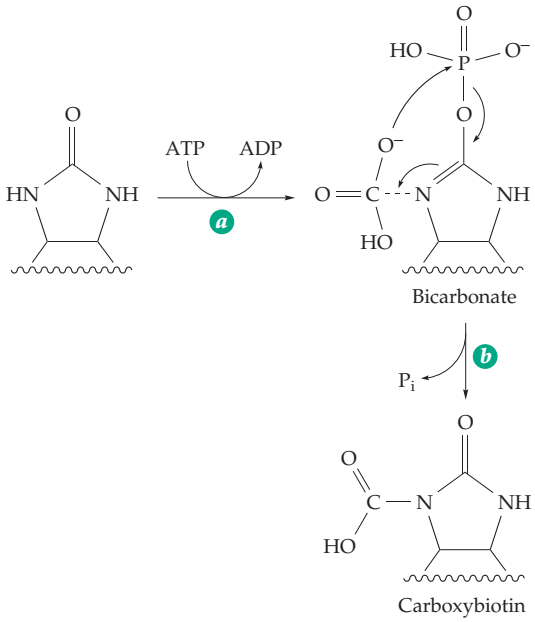
(14-7)

phospho group to ADP from carbamoyl phosphate, an analog of carboxyphosphate in a reaction that is analogous to the reverse of that in Eq. 14-7 and also by a slow bicarbonate-dependent ATPase activity that does not depend upon biotin.[57,58,58a]

The simplest mechanism for transfer of the carboxyl group of carboxyphosphate to biotin would appear to be nucleophilic displacement of the phosphate leaving group by N1' of biotin. The enzyme could presumably first catalyze removal of the N1' hydrogen to form a ureido anion.[59] Another reasonable possibility would be for the terminal phospho group of ATP to be transferred to biotin to form an *O*-phosphate[60,61]

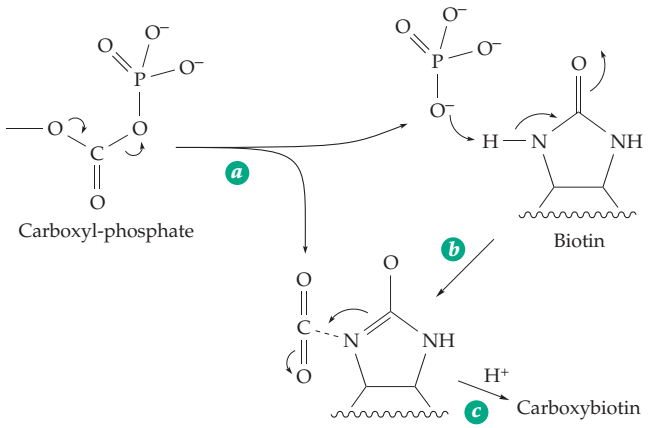


(Eq. 14-8, step *a*) which could react with bicarbonate as in Eq. 14-8, step *b*. Cleavage of the enol phosphate by attack of HCO_3^- would simultaneously create a nucleophilic center at N1' and carboxyphosphate ready to react with N1'. However, this could not easily explain the ATPase activity in the absence of biotin.



(14-8)

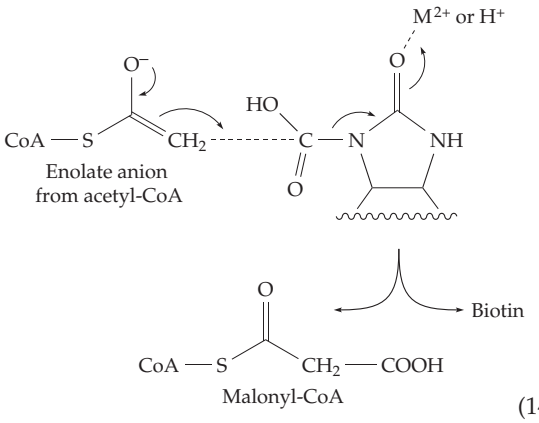
Either of the foregoing mechanisms requires that the ureido anion of biotin attack the rather unreactive carbon atom of carboxyphosphate. Another alternative, which is analogous to that suggested for PEP carboxylase (Eq. 13-53) is for carboxyphosphate to eliminate inorganic phosphate to give the more electrophilic CO_2 (Eq. 14-9, step *a*). The very basic inorganic phosphate trianion PO_4^{3-} that is eliminated could remove the proton from N1' of biotin to create the biotin ureido anion (step *b*), which could then add to CO_2 (step *c*).[22]



(14-9)

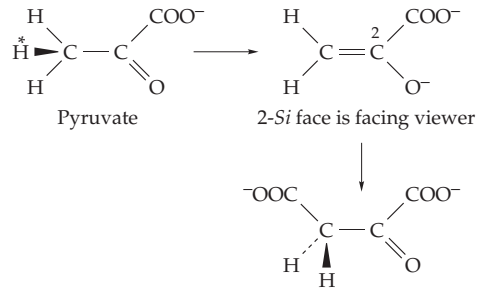
Have we checked all of the possibilities for the mechanism of biotin carboxylation? Kruger and associates[62,63] suggested that biotin, as a ureido anion, might add to bicarbonate to form a highly unstable intermediate which, however, could be phosphorylated by ATP (Eq. 14-10, steps *a* and *b*). This intermediate could undergo elimination of inorganic phosphate (step *c*), driving the reaction to completion. The observed transfer of isotope from ^{18}O-containing bicarbonate into ADP would be observed.

The β carboxylation step. Once formed, the carboxybiotin "head group" could swing to the carboxyltransferase site where transfer of the carboxyl group into the final product takes place. This might occur either by nucleophilic attack of an enolate anion on the carbonyl carbon (Eq. 14-11) or on CO_2 generated by reversal of the reactions of Eq. 14-9, steps *b* and *c*.

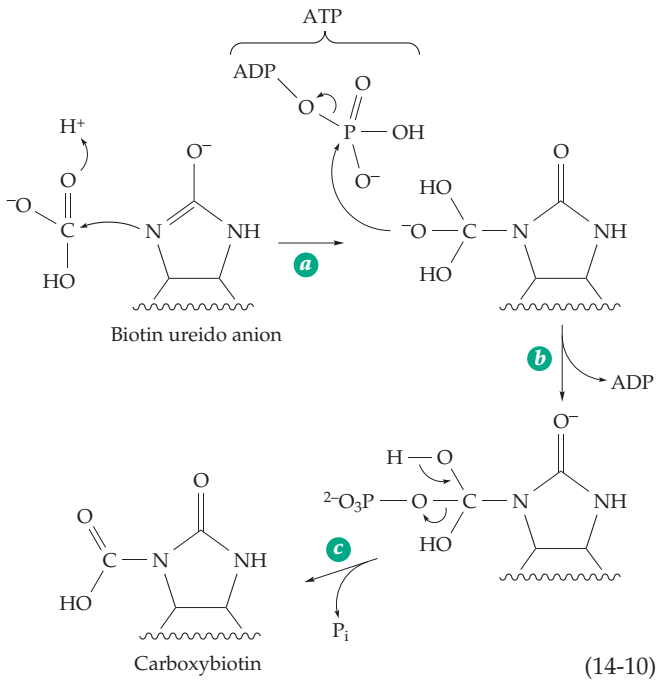


(14-11)

When pyruvate with a chiral methyl group is carboxylated by pyruvate carboxylase the configuration at C-3 is retained. The carboxyl enters from the 2-*si* side, the same side from which the proton (marked H*) was removed to form the enolate anion (Eq. 14-12). Comparable stereochemistry has been established for other biotin-dependent enzymes.[64,65]



(14-12)



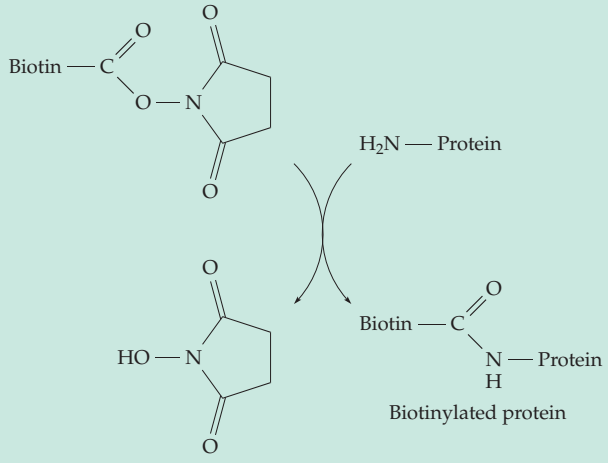
(14-10)

These enzymes do not catalyze any proton exchange at C-3 of pyruvate or at C-2 of an acyl-CoA unless the biotin is first carboxylated. This suggested that removal of the proton to the biotin oxygen and carboxylation might be synchronous. However, ^{13}C and ^{2}H kinetic isotope effects and studies of ^{3}H exchange[66] support the existence of a discrete enolate anion intermediate as shown in Eq. 14-11.[65,67] This mechanism is also consistent with the observation that propionyl-CoA

BOX 14-B THE BIOTIN-BINDING PROTEINS AVIDIN AND STREPTAVIDIN

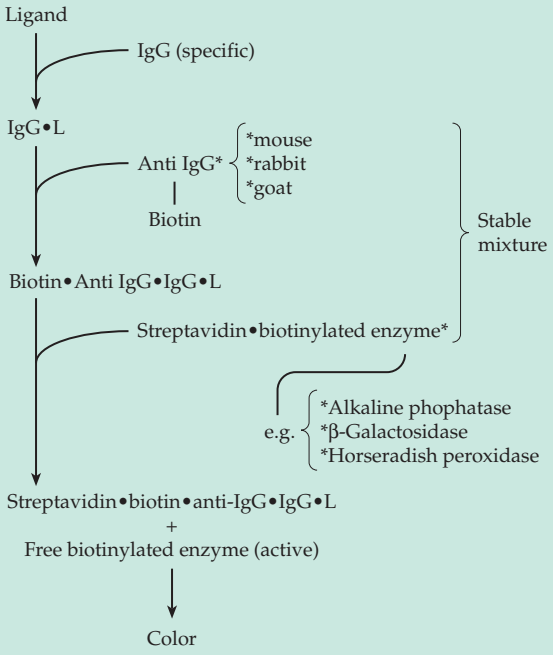
A biochemical curiosity is the presence in egg white of the glycoprotein **avidin**.[a,b] Each 68-kDa subunit of this tetrameric protein binds one molecule of biotin tenaciously with $K_f \sim 10^{15}\ M^{-1}$. Nature's purpose in placing this unusual protein in egg white is uncertain. Perhaps it is a storage form of biotin, but it is more likely an antibiotic that depletes the environment of biotin. A closely similar protein **streptavidin** is secreted into the culture medium by *Streptomyces avidinii*.[c] Its sequence is homologous to that of avidin. It has a similar binding constant for biotin and the two proteins have similar three-dimensional structures.[a,d–j] Biotin binds at one end of a β barrel formed from antiparallel strands and is held by multiple hydrogen bonds and a conformational alteration that allows a peptide loop to close over the bound vitamin.

Historically, avidin was important to the discovery of biotin. The bonding between avidin and biotin is so tight that inclusion of raw egg white in the diet of animals is sufficient to cause a severe biotin deficiency. Avidin has also been an important tool to enzymologists interested in biotin-containing enzymes. Avidin invariably inhibits these enzymes and inhibition by avidin is diagnostic of a biotin-containing protein. Recently, avidin and streptavidin have found widespread application in affinity chromatography, in immunoassays, and in the staining of cells and tissues.[d,k–p] These uses are all based on the ability to attach biotin covalently to side chain groups of proteins, polysaccharides, and other substances. The carboxyl group of the biotin "arm," which lies at the surface of the complex with avidin or streptovidin, can be converted to any of a series of reactive derivatives. For example, *p*-nitrophenyl or *N*-hydroxysuccinimide esters of biotin can be used to attach biotin to amino groups of proteins to form biotinylated proteins.



Other reactive derivatives can be used to attach biotin to phenolic, thiol, or carbonyl groups.

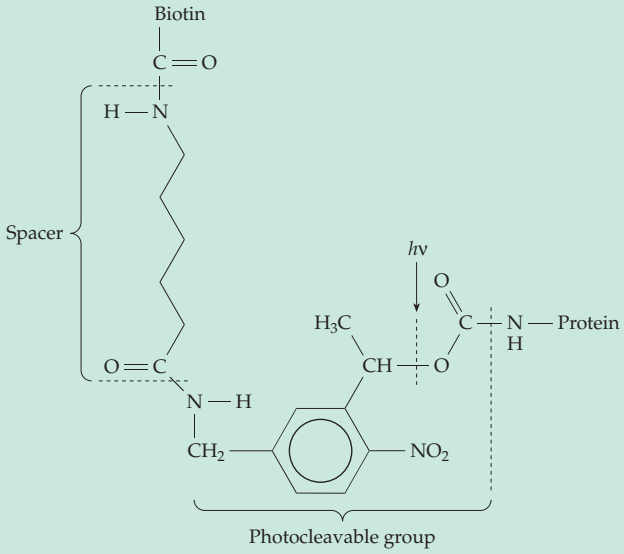
The affinity of avidin or streptavidin for the resulting biotinylated materials is still very high. This fact has permitted the application of this "biotin–avidin technology" to numerous aspects of research and diagnostic medicine. For example, a specific antibody (IgG) can be utilized for immunoassay of a hormone or other ligand. A second antibody, specific for the IgG–ligand complex, can be produced in a biotinylated form. A steptavidin complex of a biotinylated enzyme such as alkaline phosphatase, β-galactosidase, or horseradish peroxidase, for which a sensitive colorimetric assay is available, is allowed to react with the biotin•anti-IgG•IgG•ligand complex. The streptavidin now releases enzyme in proportion to the amount of ligand originally present. Since the biotinylated anti IgG and streptavidin•biotinylated enzyme can be stored as a stable mixture, the assay is simple and fast.



Avidin technology can also be applied to the isolation of proteins and other materials from cells. Because the irreversibility of the binding of biotin may be a problem, photocleavable biotin derivatives have been developed.[q] In the following structure, the biotin derivative has been joined to a protein (as in the first equation in this box) and is ready for separation, perhaps on a column containing immobilized avidin or streptavidin. After separation the biotin together with the linker and photocleavable group are cut off by a short irradiation with ultra-

BOX 14-B (continued)

violet light, leaving the protein in a free form.



[a] Livnah, O., Bayer, E. A., Wilshek, M., and Sussman, J. L. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5076–5080

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[p] Laundon, C. H., and Griffith, J. D. (1987) *Biochemistry* **26**, 3759–3762

[q] Olejnik, J., Sonar, S., Krzymańska-Olejnik, E., and Rothschild, K. J. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7590–7594

carboxylase and transcarboxylase both catalyze elimination of HF from β-fluoropropionyl-CoA to form the unsaturated acrylyl-CoA.[67] The elimination presumably occurs via an enolate anion intermediate as in Eq. 13-28.

A bound divalent metal ion, usually Mn^{2+}, is required in the transcarboxylation step. A possible function is to assist in enolization of the carboxyl acceptor. However, measurement of the effect of the bound Mn^{2+} on ^{13}C relaxation times in the substrate for pyruvate carboxylase indicated a distance of ~0.7 nm between the carbonyl carbon and the Mn^{2+}, too great for direct coordination of the metal to the carbonyl oxygen.[68] Another possibility is that the metal binds to the carbonyl of biotin as indicated in Eq. 14-11. Pyruvate carboxylase utilizes two divalent metal ions and at least one monovalent cation.[68a]

What is the role of the sulfur atom in biotin? Perhaps it interacts with CO_2, helping to hold it in a correct orientation for reaction. Perhaps it helps to keep the ureido ring of biotin planar, or perhaps it has no special function.[69]

3. Control Mechanisms

Most pyruvate carboxylases of animal and of yeast are allosterically activated by acetyl-CoA, but those of bacteria are usually not. The enzyme from chicken liver has almost no activity in the absence of acetyl-CoA, which appears to increase greatly the rate of formation of carboxyphosphate and to slow the side reaction by which carboxyphosphate is hydrolyzed to bicarbonate and phosphate.[70] The acetyl-CoA carboxylases of rat or chicken liver aggregate in the presence of citrate to form ~8000-kDa rods. Citrate is an allosteric activator for this enzyme but it acts only on a phosphorylated form and the primary control mechanism.[71,72] This enzyme in plants is a target for a group of herbicides that are selectively toxic to grasses.[73]

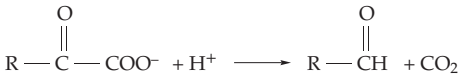
4. Pumping Ions with the Help of Biotin

Biotin-dependent **decarboxylases** act as sodium ion pumps in *Klebsiella*[74] and in various anaerobes.[22,75] For example, oxaloacetate is converted to pyruvate and bound carboxybiotin.[74,74a] The latter is decarboxylated

to CO_2 at the same time that two Na^+ ions are transported from the inside to the outside of the cell. The function of this pump, like that of the Na^+, K^+-ATPase (Fig. 8-25) is to provide an electrochemical gradient that drives the transport of other ions and molecules through the membrane. Similar ion pumps are operated by decarboxylation of methylmalonyl-CoA[76] and glutaconyl-CoA.[77] Yeast (*Saccharomyces cerevisiae*) cannot make biotin and requires an unusually large amount of the vitamin when urea, allantoin, allantoic acid, and certain other compounds are supplied as the sole source of nitrogen for growth. The reason is that in this organism urea must first be carboxylated by the biotin-containing **urea carboxylase**[78] (see Eq. 24-25) before it can be hydrolyzed to NH_3 and CO_2.

D. Thiamin Diphosphate

In Chapter 13 we considered the breaking of a bond between two carbon atoms, one of which is also bonded to a carbonyl group. These β cleavages are catalyzed by simple acidic and basic groups of the protein side chains. On the other hand, the decarboxylation of 2-oxo acids (Eq. 14-13) and the cleavage and formation of α-hydroxyketones (Eq. 14-14) depend upon thiamin diphosphate (**TDP**).[79–85] These reactions represent a second important method of making and breaking carbon–carbon bonds which we will designate **α condensation and α cleavage**. The common feature of all thiamin-catalyzed reactions is that the bond broken (or formed) is *immediately adjacent to the carbonyl group*, not one carbon removed, as in β cleavage reactions. No simple acid–base catalyzed mechanisms can be written; hence the need for a coenzyme.



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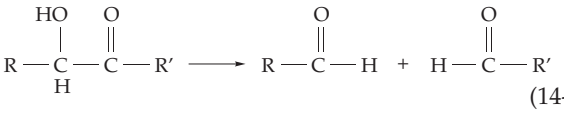
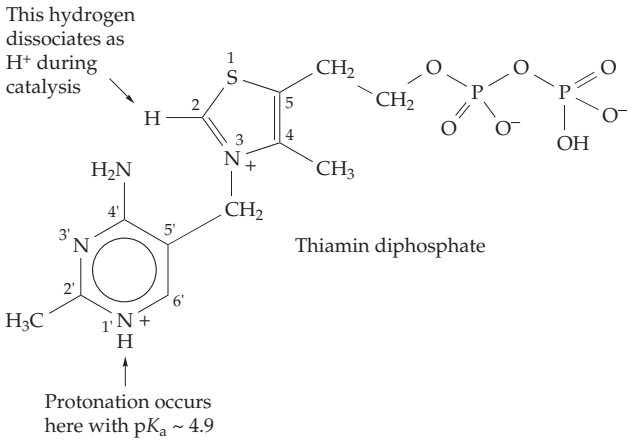


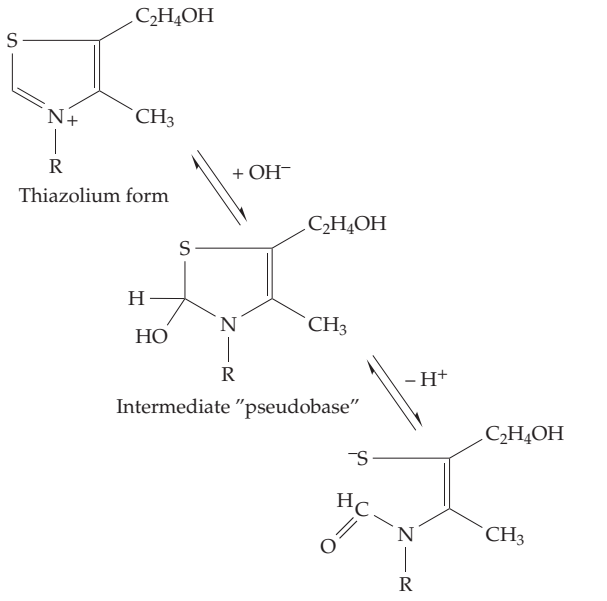
(14-14)

1. Chemical Properties of Thiamin

The weakly basic portion of thiamin or of its coenzyme forms is protonated at low pH, largely on N-1 of the pyrimidine ring.[86–88] The pK_a value is ~ 4.9. In basic solution, thiamin reacts in two steps with an opening of the thiazole ring (Eq. 14-15) to give the anion of a thiol form which may be crystallized as the sodium salt.[79,84] This reaction, like the competing reaction described in Eq. 7-19, and which leads to a yellow unstable form of the thiamin anion, is an example of a cooperative two-proton dissociation with linked structural changes. A very low concentration of the



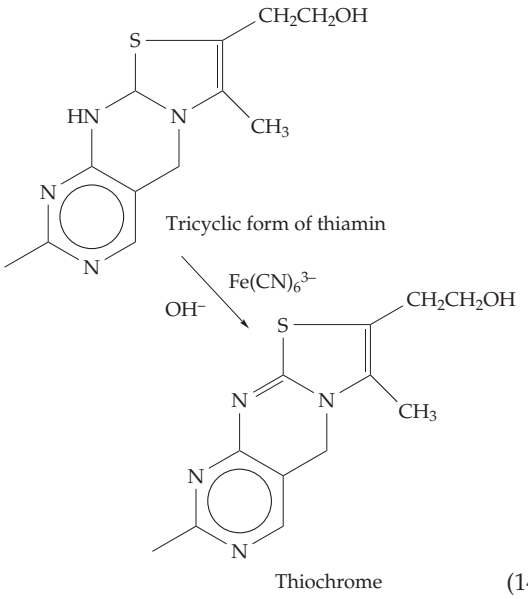
intermediate "pseudobase" is present during the titration. This property, which is unusual among small molecules, was instrumental in leading Williams *et al.* to the correct structure for the vitamin.[89] A still unanswered question is, What biological significance is associated with these reactions? Perhaps the thiol form depicted in Eq. 14-15 or the "yellow form" (Eq. 7-19) becomes attached to active sites of some proteins through disulfide linkages.



(14-15)

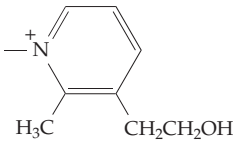
Thiamin is unstable at high pH[90,91] and is destroyed by the cooking of foods under mildly basic conditions. The thiol form undergoes hydrolysis and oxidation by air to a disulfide. The tricyclic form (Eq. 7-19) is oxidized to **thiochrome**, a fluorescent compound

whose formation from thiamin by treatment with alkaline hexacyanoferrate (III) (ferricyanide; Eq. 14-16) is the basis of a much used fluorimetric assay.



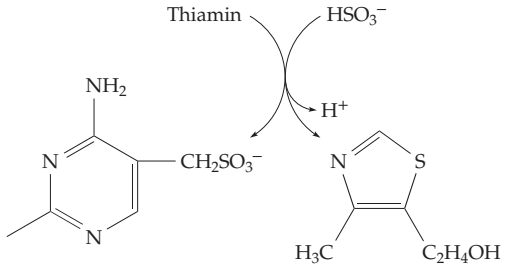
(14-16)

Treatment of thiamin with boiling 5N HCl deaminates it to the hydroxy analogue **oxythiamin**, a potent antagonist. **Pyrithiamin**, another competitor containing



in place of the thiazolium ring, is very toxic especially to the nervous system.

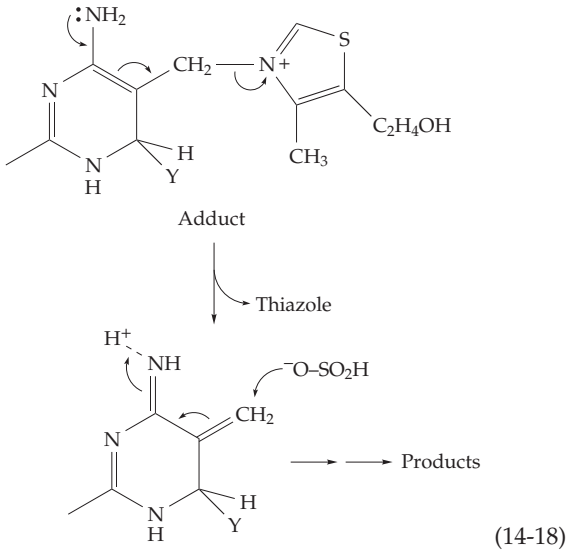
In a solution of sodium sulfite at pH 5, thiamin is cleaved by what appears to be a nucleophilic displacement reaction on the methylene group to give the free thiazole and a sulfonic acid.



(14-17)

In fact the mechanism of the reaction is more complex and is evidently initiated by addition of a nucleophile Y, such as $^-$OH or bisulfite, followed by elimination of the thiazole (Eq. 14-18).

A similar cleavage is catalyzed by thiamin-degrading enzymes known as thiaminases which are found in a number of bacteria, marine organisms, and plants. In a bacterial thiaminase, group Y of Eq. 14-18 is a cysteine –SH.[92,92a]

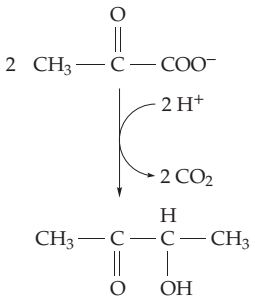


(14-18)

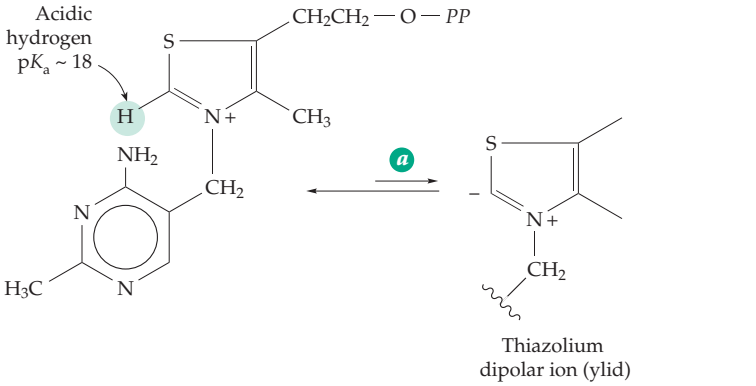
Thiamin is synthesized in bacteria, fungi, and plants from 1-deoxyxylulose 5-phosphate (Eq. 25-21), which is also an intermediate in the nonmevalonate pathway of polyprenyl synthesis. However, thiamin diphosphate is a coenzyme for synthesis of this intermediate (p. 736), suggesting that an alternative pathway must also exist. Each of the two rings of thiamin is formed separately as the esters 4-amino-5-hydroxymethylpyrimidine diphosphate and 4-methyl-5-(β-hydroxyethyl) thiazole monophosphate. These precursors are joined with displacement of pyrophosphate to form thiamin monophosphate.[92b] In eukaryotes this is hydrolyzed to thiamin, then converted to thiamin diphosphate by transfer of a diphospho group from ATP.[92b,c] In bacteria thiamin monophosphate is converted to the diphosphate by ATP and thiamin monophosphate kinase.[92b]

2. Catalytic Mechanisms

The first real clue to the mechanism of thiamin-dependent cleavage came in about 1950 when Mizuhara showed that at pH 8.4 thiamin catalyzes the nonenzymatic conversion of pyruvate into acetoin (Eq. 14-19).[93] Following Mizuhara's lead, Breslow investigated the same reaction using the then new NMR method.[94] He made the surprising discovery that the hydrogen atom in the 2 position of the thiazolium ring, between the sulfur and the nitrogen atoms, exchanged easily with deuterium of ^2H_2O. The pK_a of this proton has been estimated as ~18, low enough to permit rapid

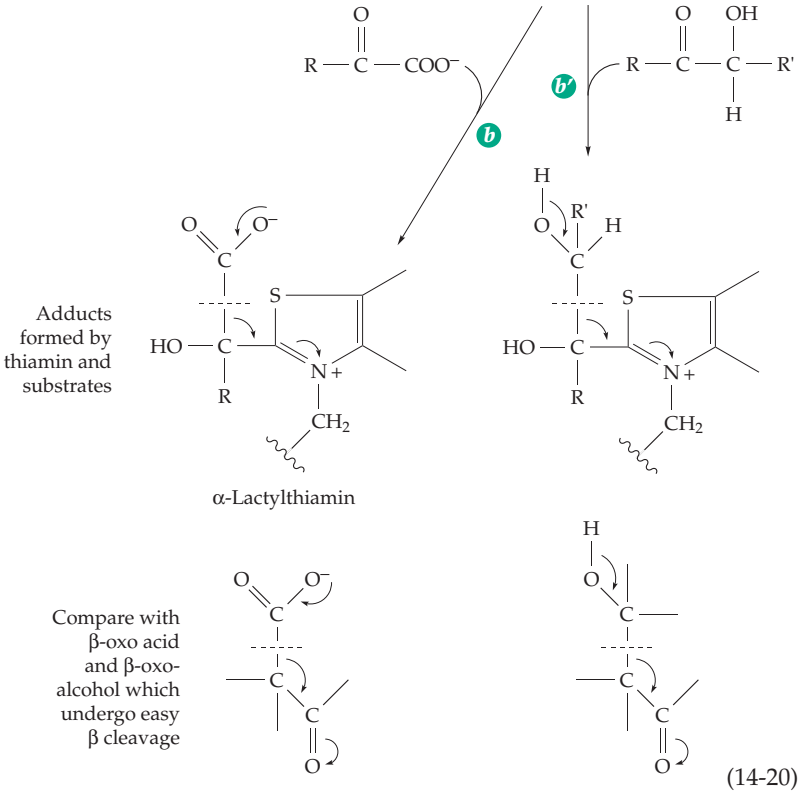


(14-19)



dissociation and replacement with ^2H.[84,95,96] The resulting **thiazolium dipolar ion** (or **ylid**) formed by this dissociation (Eq. 14-20, step *a*) is stabilized by the electrostatic interaction of the adjacent positive and negative charges. Breslow suggested that this dipolar ion is the key intermediate in reactions of thiamin-dependent enzymes.[94,97] The anionic center of the dipolar ion can react with a substrate such as an 2-oxo acid or 2-oxo alcohol by addition to the carbonyl group (Eq. 14-20, step *b* or 14-20, step *b'*). The resulting adducts are able to undergo cleavage readily, as indicated by the arrows showing the electron flow toward the =N$^+$– group.

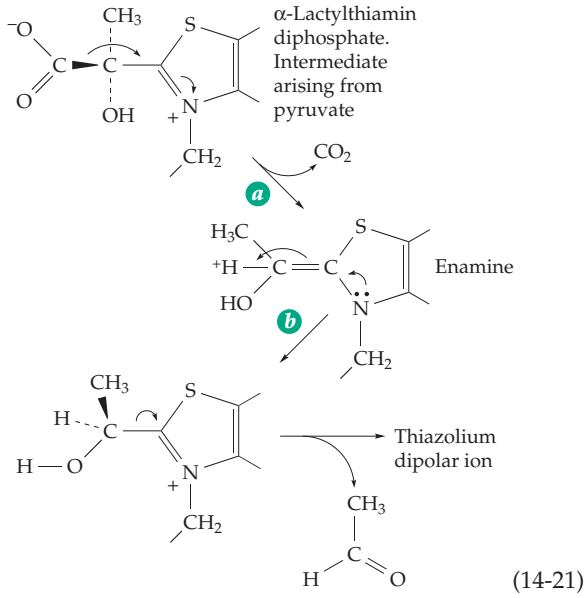
Below the structures of the adducts in Eq. 14-20 are those of a 2-oxo acid and a β-ketol with arrows indicating the electron flow in decarboxylation and in the aldol cleavage. The similarities to the thiamin-dependent cleavage reaction are especially striking if one remembers that in some aldolases and decarboxylases the substrate carbonyl group is first converted to an N-protonated Schiff base before the bond cleavage.



(14-20)

We see that *the essence of the action of thiamin diphosphate as a coenzyme is to convert the substrate into a form in which electron flow can occur from the bond to be broken into the structure of the coenzyme*. Because of this alteration in structure, a bond breaking reaction that would not otherwise have been possible occurs readily. To complete the catalytic cycle, the electron flow has to be reversed again. The thiamin-bound cleavage product (an enamine) from either of the adducts in Eq. 14-20 can be reconverted to the thiazolium dipolar ion and an aldehyde as shown in step *b* of Eq. 14-21 for decarboxylation of pyruvate to acetaldehyde.

The adducts α-lactylthiamin and α-lactylthiamin diphosphate have both been synthesized.[84,98–100] As long as α-lactylthiamin is kept as a dry solid or at low pH, it is stable. However, it decarboxylates readily in neutral solution (Eq. 14-21). Decarboxylation is much

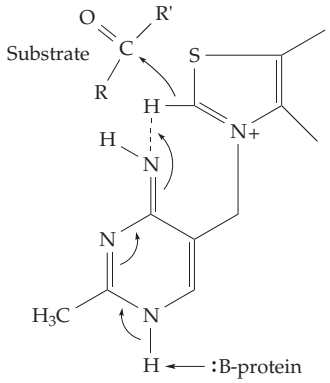


(14-21)

more rapid in methanol, a fact that was predicted by Lienhard and associates.[101] They suggested that decarboxylation is easier in a solvent of low polarity because the transition state has a lower polarity than does lactylthiamin. An enzyme could assist the reaction by providing a relatively nonpolar environment.

The crystal structures of thiamin-dependent enzymes (see next section) as well as modeling[102,103] suggest that lactylthiamin pyrophosphate has the conformation shown in Eq. 14-21. If so, it would be formed by the addition of the ylid to the carbonyl of pyruvate in accord with stereoelectronic principles, and the carboxylate group would also be in the correct orientation for elimination to form the enamine in Eq. 14-21, step *b*.[82–83a] A transient 380- to 440-nm absorption band arising during the action of pyruvate decarboxylase has been attributed to the enamine.

What is the role of the pyrimidine portion of the coenzyme in these reactions? The pyrimidine ring has a large inductive effect on the basicity of the thiazolium nitrogen and may increase the rate of dissociation of the C-2 proton somewhat.[104] More significant is the fact that the $-NH_2$ group is properly placed to function as a basic catalyst in the generation of the thiazolium dipolar ion. However, the amino group of thiamin is not very basic ($pK_a \sim 4.9$) and the site of protonation at low pH is largely N-1 of the pyrimidine ring. In the protonated form the $-NH_2$ group is even less basic because of electron withdrawal into the ring. Studies of thiamin analogs suggested another possibility. Schellenberger[105] found pyruvate decarboxylase inactive when TDP was substituted by analogs with modified aminopyrimidine rings, e.g., with methylated or dimethylated amino groups or with N1 of the ring replaced by carbon (an aminopyridyl analog). More recently the experiment has been repeated with additional enzymes[106] and X-ray studies have shown that the analogs bind into the active site of transketolase in a normal way.[107] Of the compounds studied *only an aminopyridyl analog of TDP having a nitrogen atom at 1'* (but CH at 3') *had substantial catalytic activity.* Jordan and Mariam showed that *N*-1'-methylthiamin is a superior catalyst in nonenzymatic catalysis.[108] These results are consistent with the speculative scheme illustrated in the following drawing from the first edition of this book.[109] The $-NH_2$ group of the N1-protonated aminopyrimidine has lost a proton to form a normally *minor tautomer* in which the resulting imino group would be quite basic. Assisted by a basic group from the protein, it could abstract the proton from the thiazolium ring to form the ylid.



Crystallographic studies show that the catalytic base (:B-protein) is the carboxylate group of a conserved glutamate side chain (E59' in Fig. 14-2). Kern *et al*. used NMR spectroscopy of thiamin diphosphate present in native and mutant pyruvate decarboxylase and transketolase to monitor the exchange rates of the C2-H proton of the thiazolium ring.[109a] The results confirmed the importance of the conserved glutamate side chain for dissociation of the C2-H proton. Participation of other catalytic groups from the enzyme may also be important. However, these groups are not conserved in the whole family of enzymes. For example, glutamine 122, which is within hydrogen-bonding distance of both the substrate and thiamin amino group, is replaced by histidine in transketolase.[110] A variety of kinetic studies involving mutants,[111,111a] alternative substrates,[112,113] and isotope effects in substrates[114–116] and solvent[117,118] have not yet resolved the details of the proton transfers that occur within the active site.

3. Structures of Thiamin-Dependent Enzymes

By 1998, X-ray structures had been determined for four thiamin diphosphate-dependent enzymes: (1) a bacterial pyruvate oxidase,[119,120] (2) yeast and bacterial pyruvate decarboxylases,[121–122c] (3) transketolase,[110,123,124] and (4) benzoylformate decarboxylase.[124a] The reactions catalyzed by these enzymes are all quite different, as are the sequences of the proteins. However, the thiamin diphosphate is bound in a similar way in all of them. A conserved pattern of hydrogen bonds holds the diphosphate group to the protein and also provides ligands to a metal ion. This is normally Mg^{2+}, which is held in nearly perfect octahedral coordination by two phosphate oxygen atoms, a conserved aspartate carboxylate, a conserved asparagine amide, and a water molecule. The thiamin rings are in a less polar region. The amino group of the pyrimidine is adjacent to the 2–CH of the thiazole and N1' of the pyrimidine is apparently protonated and hydrogen bonded to the carboxylate group of a conserved glutamate side

chain. This is shown in Fig. 14-2. Substitution of the corresponding glutamate 51 of yeast pyruvate decarboxylase by glutamine or alanine greatly reduced or eliminated catalytic activity.[125]

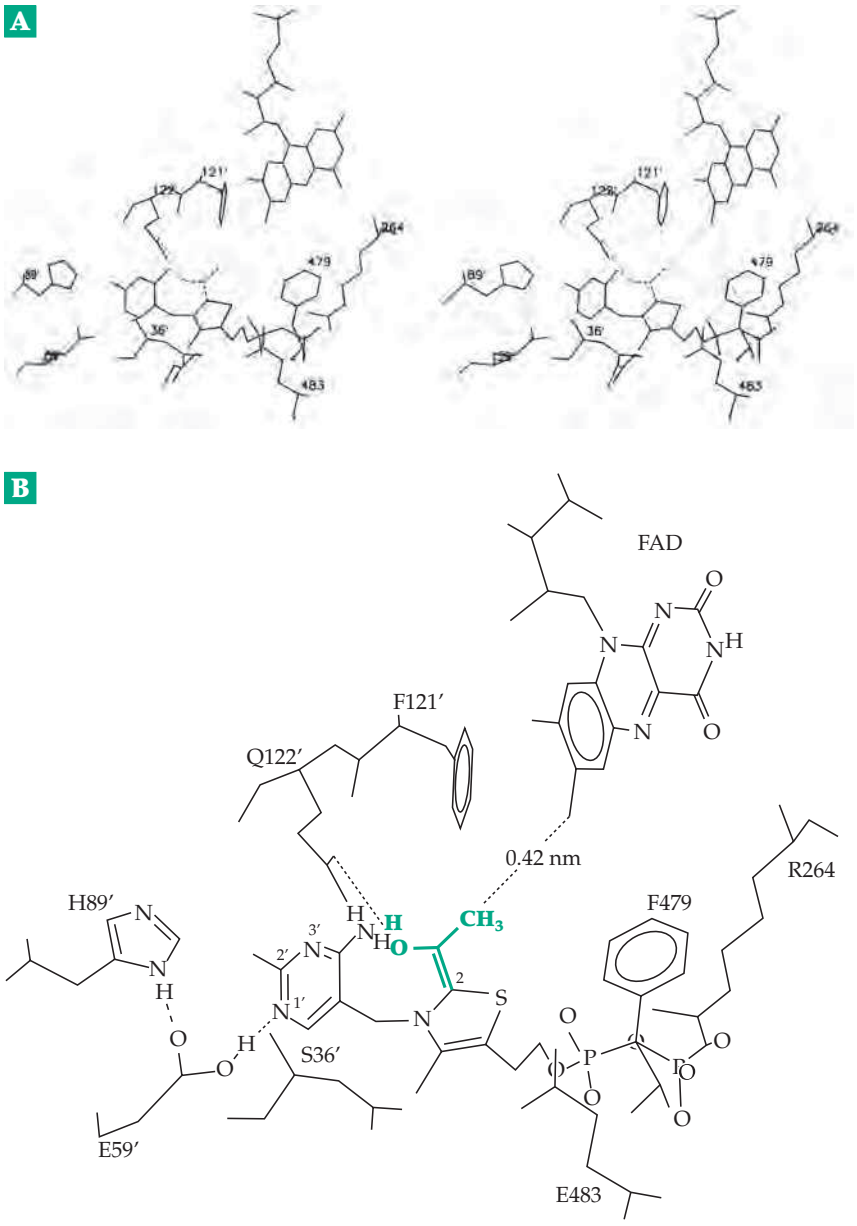
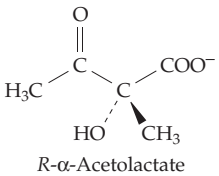


Figure 14-2 (A) Stereoscopic view of the active site of pyruvate oxidase from the bacterium *Lactobacillus plantarium* showing the thiamin diphosphate as well as the flavin part of the bound FAD. The planar structure of the part of the intermediate enamine that arises from pyruvate is shown by dotted lines. Only some residues that may be important for catalysis are displayed: G35', S36', E59', H89', F121', Q122', R264, F479, and E483. Courtesy of Georg E. Schulz.[119] (B) Simplified view with some atoms labeled and some side chains omitted. The atoms of the hypothetical enamine that are formed from pyruvate, by decarboxylation, are shown in green.

4. The Variety of Enzymatic Reactions Involving Thiamin

Most known thiamin diphosphate-dependent reactions (Table 14-2) can be derived from the five half-reactions, *a* through *e*, shown in Fig. 14-3. Each half-reaction is an α cleavage which leads to a thiamin- bound enamine (center, Fig. 14-3) The decarboxylation of an α-oxo acid to an aldehyde is represented by step *b* followed by *a* in reverse. The most studied enzyme catalyzing a reaction of this type is yeast **pyruvate decarboxylase,** an enzyme essential to alcoholic fermentation (Fig. 10-3). There are two ~250-kDa isoenzyme forms, one an α_4 tetramer and one with an $(\alpha\beta)_2$ quaternary structure. The isolation of α-hydroxyethylthiamin diphosphate from reaction mixtures of this enzyme with pyruvate[52] provided important verification of the mechanisms of Eqs. 14-14, 14-15. Other decarboxylases produce aldehydes in specialized metabolic pathways: indolepyruvate decarboxylase[126] in the biosynthesis of the plant hormone **indole-3-acetate** and benzoylformate decarboxylase in the mandelate pathway of bacterial metabolism (Chapter 25).[124a,127]

Formation of α-ketols from α-oxo acids also starts with step *b* of Fig. 14-3 but is followed by condensation with another carbonyl compound in step *c*, in reverse. An example is decarboxylation of pyruvate and condensation of the resulting active acetaldehyde with a second pyruvate molecule to give *R*-α-acetolactate, a reaction catalyzed by **acetohydroxy acid synthase** (acetolactate synthase).[128] Acetolactate is the precursor to valine and leucine. A similar ketol condensation, which is catalyzed by the same synthase, is



R-α-Acetolactate

required in the biosynthesis of isoleucine (Fig. 24-17). Since this

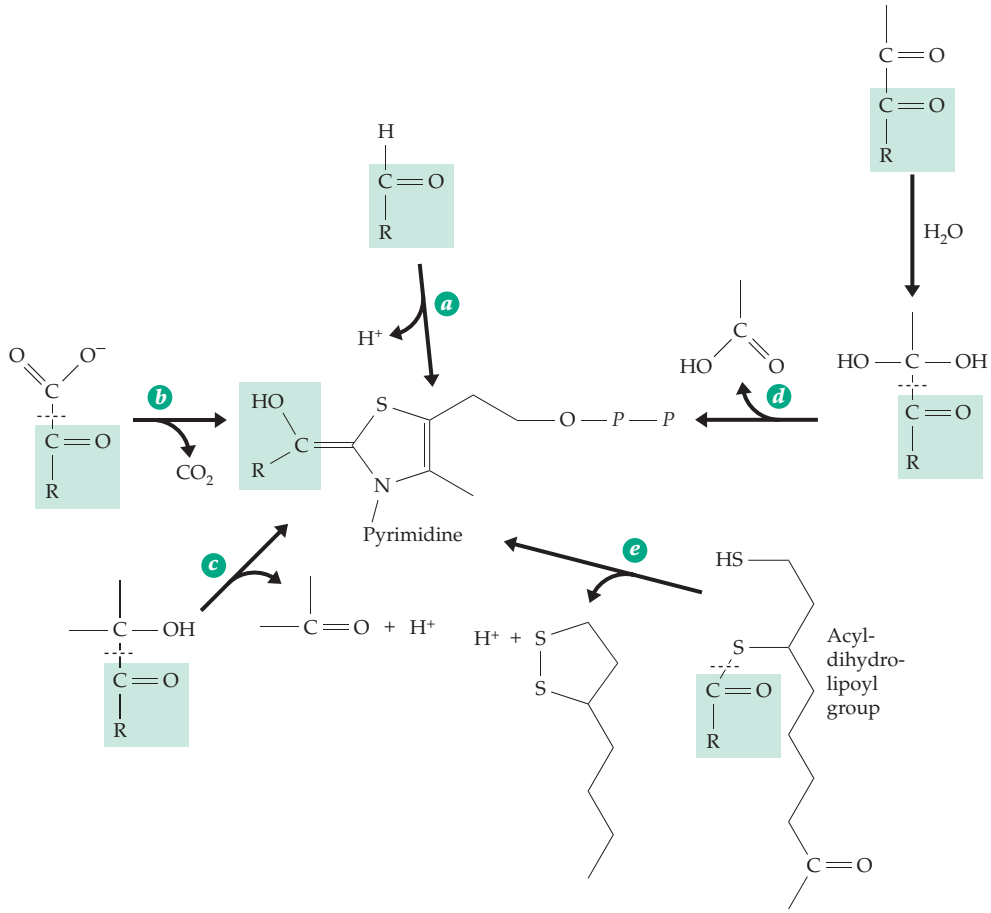


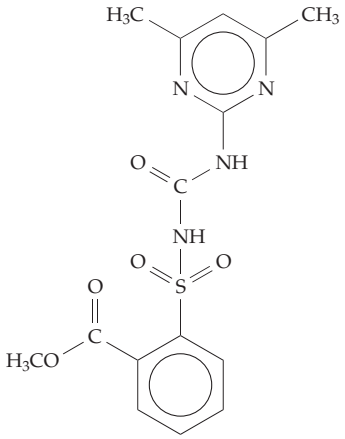
Figure 14-3 Half-reactions making up the thiamin-dependent α cleavage and α condensation reactions.

TABLE 14-2
Enzymes Dependent upon Thiamin Diphosphate as a Coenzyme

1. Nonoxidative
   - Pyruvate decarboxylase*
   - Indolepyruvate decarboxylase
   - Benzoylformate decarboxylase*
   - Glyoxylate carboligase
   - Acetohydroxy acid synthase (acetolactate synthase)
   - 1-Deoxy-D-xylulose 5-phosphate synthase
   - Transketolase*
   - Phosphoketolase
2. Oxidative decarboxylase
   - Pyruvate oxidase (FAD)*
   - Pyruvate dehydrogenase (Lipoyl, FAD, NAD^+) multienzyme complex
   - Pyruvate:ferredoxin oxidoreductase
   - Indolepyruvate:ferredoxin oxidoreductase

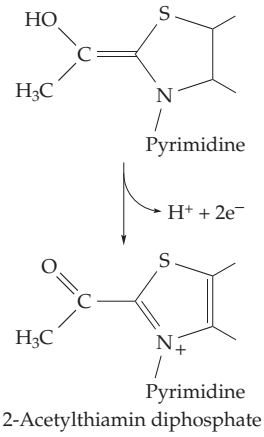
* Three-dimensional structures for these enzymes had been determined by 1998.

synthase is not present in mammals it is a popular target for herbicides.[129,130] It is inhibited by many of the most widely used herbicides including sulfometuron methyl, whose structure is shown here.



Sulfometuron methyl

Acetolactate is a β-oxo acid and is readily decarboxylated to acetoin, a reaction of importance in bacterial fermentations (Eq. 17-26). Acetoin, of both *R* and *S*



(14-22)

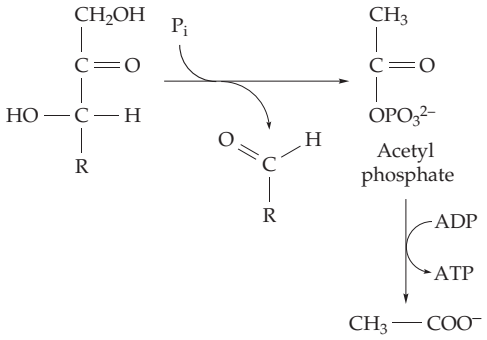
configurations, is also formed by pyruvate decarboxylases acting on acetaldehyde.[103,131] The ketol condensation of two molecules of glyoxylate with decarboxylation to form tartronic semialdehyde (see Fig. 17-6) is an important reaction in bacterial metabolism. It is catalyzed by **glyoxylate carboligase,**[132] another thiamin diphosphate-dependent enzyme. Formation of 1-deoxy-D-xylulose 5-phosphate, an intermediate in the nonmevalonate pathway of isoprenoid synthesis, is formed in a thiamin diphosphate-catalyzed condensation of pyruvate with glyceraldehyde 3-phosophate (Fig. 22-2)[132a] However, there is an unresolved problem. As previously mentioned, the same intermediate is thought to be a precursor to thiamin diphosphate (Eq. 25-21). This suggests the presence of an alternative pathway.

Ketols can also be formed enzymatically by cleavage of an aldehyde (step *a*, Fig. 14-3) followed by condensation with a second aldehyde (step *c*, in reverse). An enzyme utilizing these steps is **transketolase** (Eq. 17-15),[132b] which is essential in the pentose phosphate pathways of metabolism and in photosynthesis. α-Diketones can be cleaved (step *d*) to a carboxylic acid plus active aldehyde, which can react either via *a* or *c* in reverse. These and other combinations of steps are often observed as side reactions of such enzymes as pyruvate decarboxylase. A related thiamin-dependent reaction is that of pyruvate and acetyl-CoA to give the α-diketone, **diacetyl**, $CH_3COCOCH_3$.[133] The reaction can be viewed as a displacement of the CoA anion from acetyl-CoA by attack of thiamin-bound active acetaldehyde derived from pyruvate (reverse of step *d*, Fig. 14-3 with release of CoA).

5. Oxidative Decarboxylation and 2-Acetylthiamin Diphosphate

The oxidative decarboxylation of pyruvate to form acetyl-CoA or acetyl phosphate plays a central role in the metabolism of our bodies and of most other organisms. This reaction is usually formulated as the reverse of step *e* of Fig. 14-3, which shows the cleavage of an **acyl-dihydrolipoyl** derivative. However, there is a possibility that the lipoyl group functions not as shown in Fig. 14-3 but as an oxidant that converts the TDP enamine to 2-acetylthiamin diphosphate (Eq. 14-22)[134,135] and only after that as an acyl group carrier. A related reaction that is known to proceed through acetyl-TDP is the previously mentioned bacterial pyruvate oxidase. As seen in Fig. 14-2, this enzyme has its own oxidant, FAD, which is ready to accept the two electrons of Eq. 14-22 to produce bound acetyl-TDP. The electrons may be able to jump directly to the FAD, with thiamin and flavin radicals being formed at an intermediate stage.[135a] The electron transfers as well as other aspects of oxidative decarboxylation are discussed in Chapter 15, Section C.

A reaction that is related to that of transketolase but is likely to function via acetyl-TDP is **phosphoketolase**, whose action is required in the energy metabolism of some bacteria (Eq. 14-23). A product of phosphoketolase is acetyl phosphate, whose cleavage can be coupled to synthesis of ATP. Phosphoketolase presumably catalyzes an α cleavage to the thiamin-containing enamine shown in Fig. 14-3. A possible mechanism of formation of acetyl phosphate is elimination of H_2O from this enamine, tautomerization to 2-acetylthiamin, and reaction of the latter with inorganic phosphate.



(14-23)

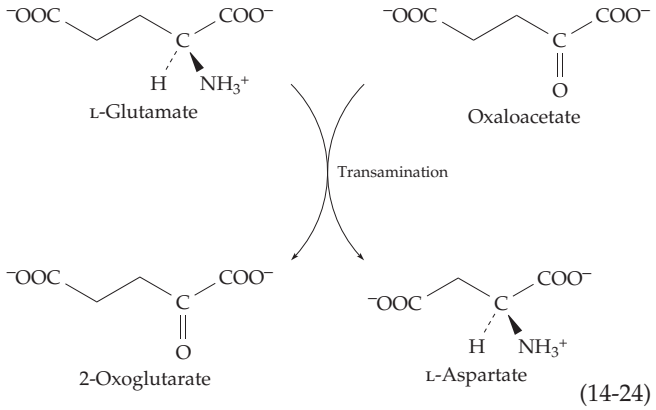
6. Thiamin Coenzymes in Nerve Action

The striking paralysis caused by thiamin deficiency together with studies of thiamin analogs as metabolites suggested a special action for this vitamin in nerves.[136] The thiamin analog **pyrithiamin** (p. 731) both induces paralytic symptoms and displaces thiamin from nerve preparations. The nerve poison **tetrodotoxin** (Chapter 30) blocks nerve conduction by inhibiting inward diffusion of sodium, but it also promotes release of thiamin from nerve membranes. Evidence for a metabolic significance of thiamin triphosphate comes from identification of soluble and membrane-associated thiamin triphosphatases[137] as well as a kinase that

forms protein-bound thiamin triphosphate in the brain.[138] Mono-, tri-, and tetraphosphates also occur naturally in smaller amounts. One might speculate about a possible role for the rapid interconversion of cationic and yellow anionic forms of thiamin via the tricyclic form (Eq. 7-19) in some aspect of nerve conduction.

E. Pyridoxal Phosphate

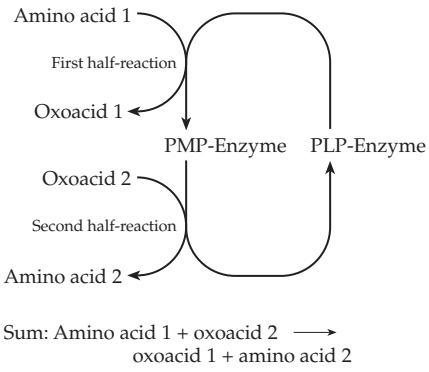
The phosphate ester of the aldehyde form of vitamin B_6, **pyridoxal phosphate** (pyridoxal-*P* or PLP), is required by many enzymes catalyzing reactions of amino acids and amines. The reactions are numerous, and pyridoxal phosphate is surely one of nature's most versatile catalysts. The story begins with biochemical **transamination**, a process of central importance in nitrogen metabolism. In 1937, Alexander Braunstein and Maria Kritzmann, in Moscow, described the transamination reaction by which amino groups can be transferred from one carbon skeleton to another.[139,140] For example, the amino group of glutamate can be transferred to the carbon skeleton of oxaloacetate to form aspartate and 2-oxoglutarate (Eq. 14-24).



(14-24)

This transamination reaction is a widespread process of importance in many aspects of the nitrogen metabolism of organisms. A large series of **transaminases** (**aminotransferases**), for which glutamate is most often one of the reactants, have been shown to catalyze the reactions of other oxoacids and amino acids.[141–143]

In 1944, Esmond Snell reported the nonenzymatic conversion of pyridoxal into pyridoxamine (Box 14-C) by heating with glutamate. He recognized that this was also transamination and proposed that pyridoxal might be a part of a coenzyme needed for aminotransferases and that these enzymes might act via two half-reactions that interconverted pyridoxal and pyridoxamine (Eq. 14-25). The hypothesis was soon verified and the coenzyme was identified as pyridoxal 5'-phosphate or pyridoxamine 5'-phosphate (Fig. 14-5).[144,145]



(14-25)

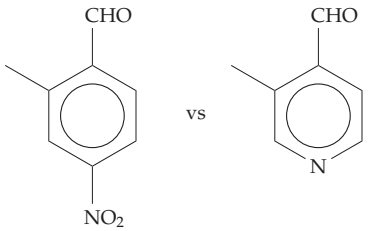
At about the same time, Gunsalus and coworkers noticed that the activity of **tyrosine decarboxylase** produced by lactic acid bacteria was unusually low when the medium was deficient in pyridoxine. Addition of pyridoxal plus ATP increased the decarboxylase activity of cell extracts.[146] PLP was synthesized and was found to be the essential coenzyme for this and a variety of other enzymes.[147]

1. Nonenzymatic Models

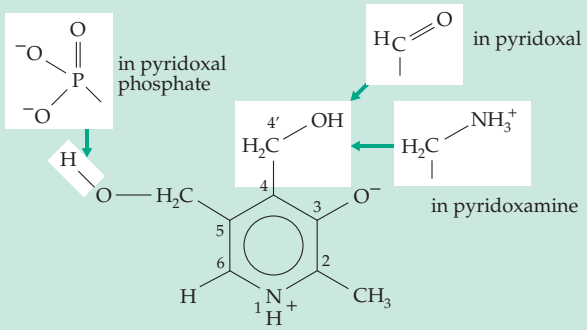
Pyridoxal or PLP, in the complete absence of enzymes, not only undergoes slow transamination with amino acids but also catalyzes many other reactions of amino acids that are identical to those catalyzed by PLP-dependent enzymes. Thus, *the coenzyme itself can be regarded as the active site of the enzymes* and can be studied in nonenzymatic reactions. The latter can be thought of as *models* for corresponding enzymatic reactions. From such studies Snell and associates drew the following conclusions.[148]

a. The aldehyde group of PLP reacts readily and reversibly with amino acids to form Schiff bases (Fig. 14-4) which react further to give products.

b. For an aldehyde to be a catalyst, a strong electron-attracting group, e.g., the ring nitrogen of pyridine (as in PLP), must be *ortho* or *para* to the –CHO group. A nitro group, also strongly electron attracting, can replace the pyridine nitrogen in model reactions.



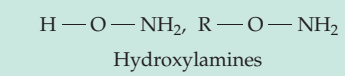
BOX 14-C THE VITAMIN B_6 FAMILY: PYRIDOXINE, PYRIDOXAL, AND PYRIDOXAMINE



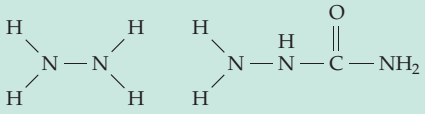
Pyridoxine (pyridoxol)
Vitamin B_6 alcohol

Pyridoxine, the usual commercial form of vitamin B_6, was isolated and synthesized in 1938. However, studies of bacterial nutrition soon indicated the existence of other naturally occurring forms of the new vitamin which were more active than pyridoxine in promoting growth of certain lactic acid bacteria. The amine **pyridoxamine** and the aldehyde **pyridoxal** were identified by Snell, who found that pyridoxal could be formed from pyridoxine by mild oxidation and that pyridoxamine could be formed from pyridoxal by heating in a solution with glutamic acid via a transamination reaction. These simple experiments also suggested the correct structures of the new forms of vitamin B_6. Animal tissues contain largely pyridoxal, pyridoxamine, and their phosphate esters. The lability of the aldehyde explains the ease of destruction of the vitamin by excessive heat or by light. On the other hand, plant tissues contain mostly pyridoxine, which is more stable. Kinases use ATP to form the phosphate esters, which are interconvertible within cells.[a–e] Pyridoxine 5'-phosphate can be oxidized to PLP[d,e,f] and the latter may undergo transamination to PMP. The acid–base chemistry and tautomerism of pyridoxine were discussed in Chapter 6, Section E,2.

Many poisonous substances as well as useful drugs react with PLP-requiring enzymes. Thus, much of the toxic effect of the "carbonyl reagents" hydroxylamine, hydrazine, and semicarbazide stems from their formation of stable derivatives analogous to Schiff bases with PLP.

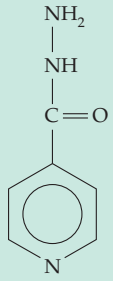


Hydroxylamines



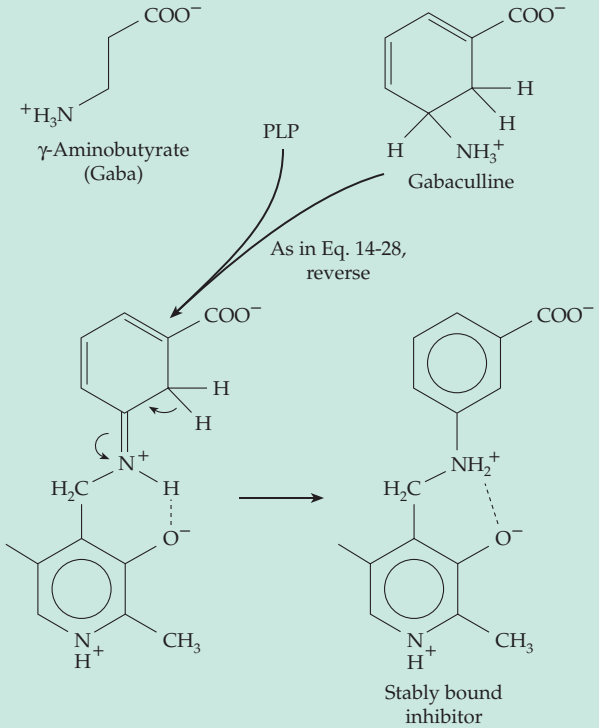
Hydrazine Semicarbazide

Isonicotinyl hydrazide (INH), one of the most effective drugs against tuberculosis, is inhibitory to pyridoxal kinase, the enzyme that converts pyridoxal to PLP.[c] Apparently, the drug reacts with pyridoxal to form a hydrazone which blocks the enzyme. Pyridoxal kinase is not the primary target of INH in mycobacteria. However, patients on long-term isonicotinyl hydrazide therapy sometimes suffer symptoms of vitamin B_6 deficiency.[g]



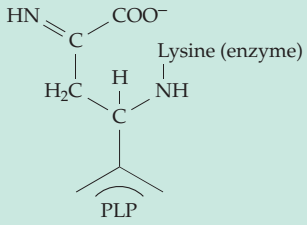
Isonicotinyl hydrazide
(isoniazid)

PLP-dependent enzymes are inhibited by a great variety of enzyme-activated inhibitors that react by several distinctly different chemical mechanisms.[h] Here are a few. The naturally occurring **gabaculline** mimics γ-aminobutyrate (Gaba) and inhibits γ-aminobutyrate aminotransferase as well as other PLP-dependent enzymes. The inhibitor follows the normal catalytic pathway as far as the ketimine. There, a proton is lost from the inhibitor permitting formation of a stable benzene ring and leaving the inhibitor stuck in the active site:



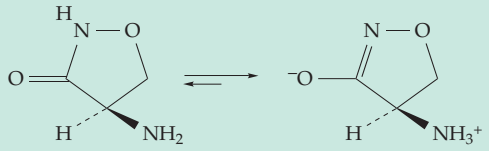
BOX 14-C (continued)

Beta-chloroalanine and serine *O*-sulfate can undergo β elimination (as in Eq. 14-29) in active sites of glutamate decarboxylase or aspartate aminotransferase. The enzymes then form free aminoacrylate, a reactive molecule that can undergo an aldol-type condensation with the external aldimine to give the following product.[i]



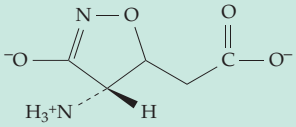
Nucleophilic groups from enzymes can add to double bonds, e.g., in an aminoacrylate Schiff base, or to multiple bonds present in the inhibitor. An example is γ-vinyl γ-aminobutyrate (4-amino-5-hexenoic acid), another inhibitor of brain γ-aminobutyrate aminotransferase which is a useful anticonvulsant drug.

Another enzyme-activated inhibitor is the streptomyces antibiotic **D-cycloserine** (oxamycin), an antitubercular drug that resembles D-alanine in structure. A potent inhibitor of alanine racemase, it also inhibits the non-PLP, ATP-dependent, **D-alanyl-D-alanine synthetase** which is needed in the biosynthesis of the peptidoglycan of bacterial cell walls.

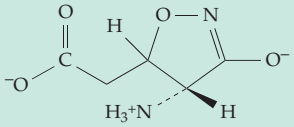


D-Cycloserine

L-Cycloserine inhibits many PLP enzymes and is toxic to humans. This observation led Khomutov *et al.* to synthesize the following more specific "cycloglutamates," structural analogs of glutamic acid with fixed conformations.[j,k] Nature apparently anticipated the synthetic chemist, because it has been reported that the mushroom *Tricholoma muscarium* contains one of the same compounds. It is said to impart two interesting properties to the mushroom: a delicious flavor and a lethal action on flies that alight on the mushroom's surface![l]

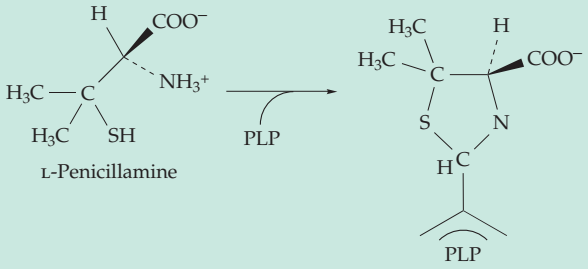


A cycloglutamate that inhibits aspartate aminotransferase



An isomeric cycloglutamate tricholomic acid found in certain mushrooms

The substituted cysteine derivative L-penicillamine causes convulsions and low glutamate decarboxylase levels in the brain, presumably because the Schiff base formed with PLP can then undergo cyclization, the SH group adding to the C=N to form a stable thiazolidine ring.



L-Penicillamine

Toxopyrimidine, the alcohol derived from the pyrimidine portion of the thiamin molecule, is a structural analog of pyridoxal. When fed to rats or mice it induces running fits which can be stopped by administration of vitamin B_6. Phophorylation of toxopyrimidine by pyridoxal kinase may produce an antagonistic analog of PLP. In a similar fashion, 4-deoxypyridoxine, which was tested as a possible anticancer drug, caused convulsions and other symptons of vitamin B_6 deficiency in humans. A host of synthetic PLP derivatives have been made, some of which are effective in blocking PLP enzymes.[h]

[a] Lepkovsky, S. (1979) *Fed. Proc.* **38**, 2699–2700
[b] McCormick, D. B., Gregory, M. E., and Snell, E. E. (1961) *J. Biol. Chem.* **236**, 2076–2084
[c] Snell, E. E., and Haskell, B. E. (1970) *Comprehensive Biochemistry* **21**, 47–71
[d] McCormick, D. B. and Chen, H. (1999) *J. Nutr.* **129**, 325–327
[e] Hanna, M. L., Turner, A. J., and Kirkness, E. F. (1997) *J. Biol. Chem.* **272**, 10756–10760
[f] Ngo, E. O., LePage, G. R., Thanassi, J. W., Meisler, N., and Nutter, L. M. (1998) *Biochemistry* **37**, 7741–7748
[g] Lui, A., and Lumeng, L. (1986) in *Vitamin B_6, Pyridoxal Phosphate: Chemical, Biochemical and Medical Aspects*, Vol. 1B (Dolphin, D., Poulson, R., and Avramovíc, O., eds), pp. 601–674, Wiley, New York
[h] Walsh, C. T. (1986) in *Vitamin B_6, Pyridoxal Phosphate: Chemical, Biochemical and Medical Aspects*, Vol. 1B (Dolphin, D., Poulson, R., and Avramovíc, O., eds), pp. 43–70, Wiley, New York
[i] Likos, J. J., Ueno, H., Fedhaus, R. W., and Metzler, D. E. (1982) *Biochemistry* **21**, 4377–4386
[j] Khomutov, R. M., Koveleva, G. K., Severin, E. S., and Vdovina, L. V. (1967) *Biokhim.* **32**, 900–907
[k] Sastchenko, L. P., Severin, E. S., Metzler, D. E., and Khomutov, R. M. (1971) *Biochemistry* **10**, 4888–4894
[l] Iwasaki, H., Kamiya, T., Oka, O., and Veyanagi, J. (1969) *Chem. Pharm. Bull.* **17**, 866–872

c. The presence of an – OH group adjacent to the – CHO group greatly enhances the catalytic activity. Since certain metal ions, such as Cu^{2+} and Al^{3+}, increase the rates in model systems and are known to chelate with Schiff bases of the type formed with PLP, it was concluded that either a metal ion or a proton formed a chelate ring and helped to hold the Schiff base in a planar conformation (Fig. 14-6). *However, such a function for metal ions has not been found in PLP-dependent enzymes.*

d. In model systems the 5-hydroxymethyl and 2-methyl groups are not needed for catalysis. However, in enzymes the 5-CH_2OH group is essential for attachment of the phosphate handle. The 2-CH_3 group is usually not necessary for coenzymatic activity.

Many investigations of nonenzymatic reactions of PLP and related compounds have been and are still being conducted[149,150]

2. A General Mechanism of Action of PLP

Based upon consideration of the various known PLP-dependent enzymes of amino acid metabolism, Braunstein and Shemyakin in 1952 proposed a general mechanism of PLP action[151,152] which, in most details, was the same as the one proposed independently by Snell and associates on the basis of the nonenzymatic reactions.[148] The general mechanism, which has been verified by studies of many enzymes, can be stated as follows: *Pyridoxal phosphate reacts to convert the amino group of a substrate into a Schiff base that is electronically the equivalent of an adjacent carbonyl* (Fig. 14-4). However, a Schiff base of an amino acid with a simple aldehyde (for example, acetaldehyde) has the opposite polarity from that of C=O (see the following structures). Such an imine could not substitute for a carbonyl group in activating an α-hydrogen nor in facilitating C–C bond cleavage in the amino acid. It is necessary to have the strongly electron-attracting pyridine group conjugated with the C=N group in such a way that electrons can flow from the substrate into the coenzyme.

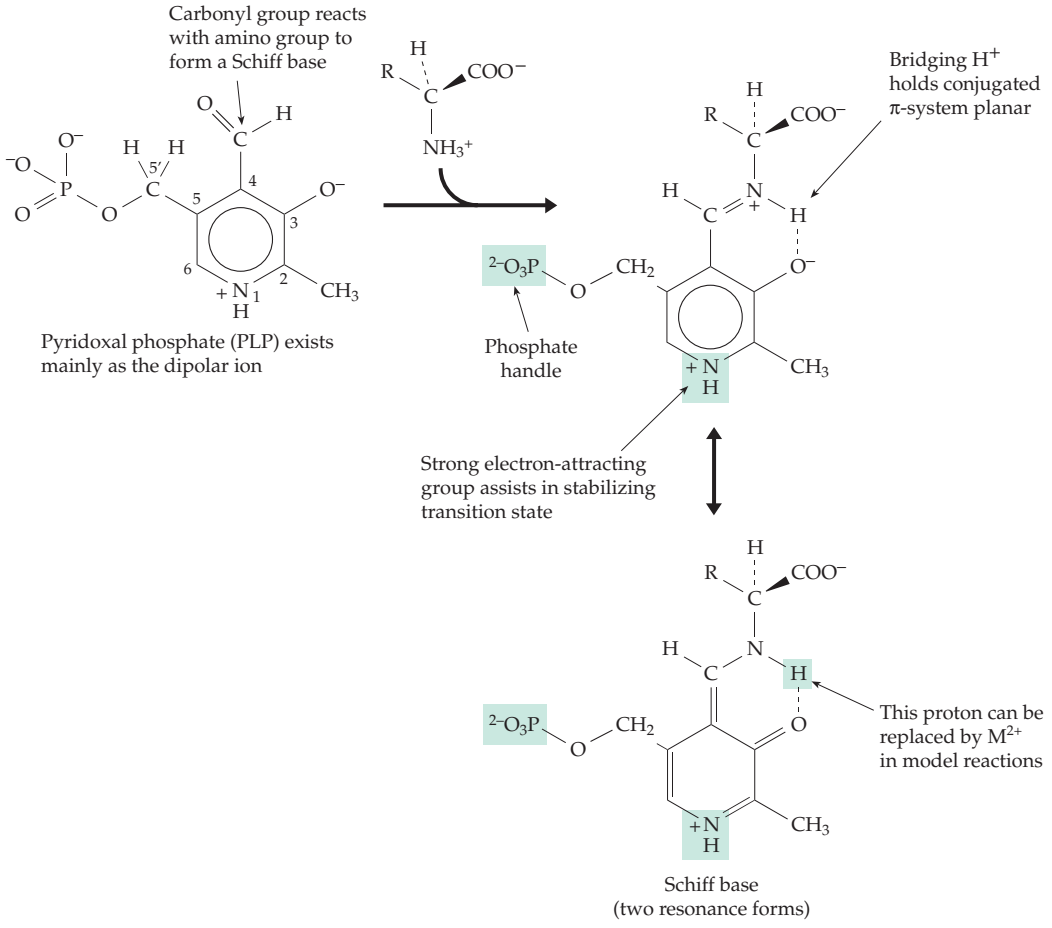
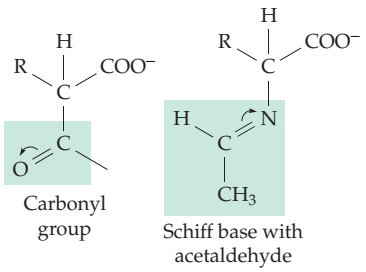
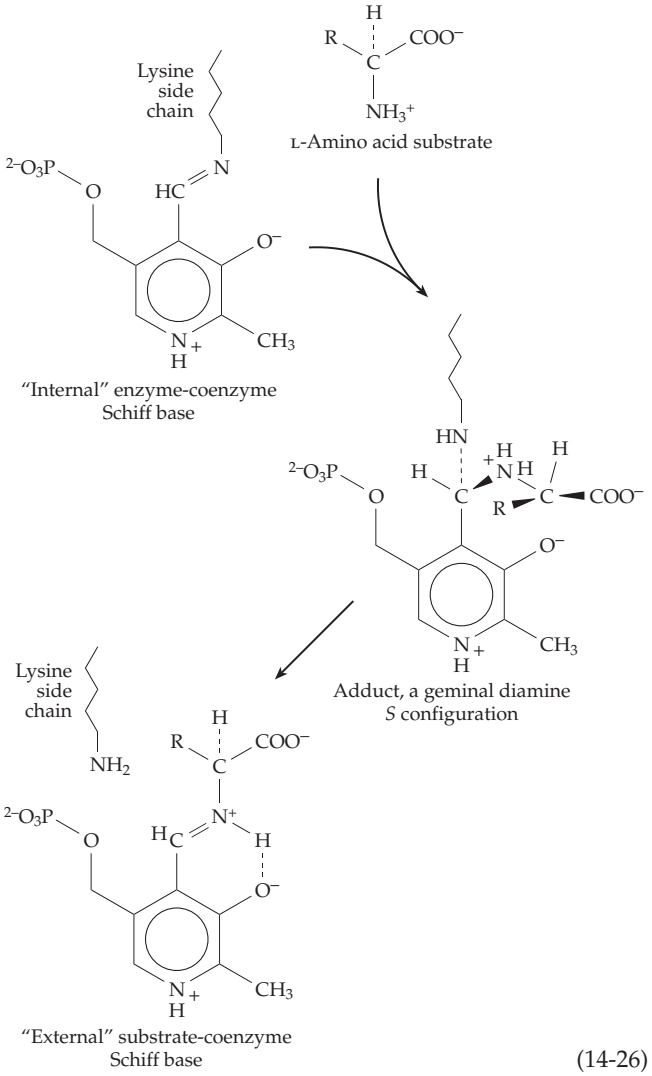


Figure 14-4 Pyridoxal 5'-phosphate (PLP), a special coenzyme for reactions of amino acids.



Before discussing the reactions of Schiff bases of PLP we should consider one fact that was not known in 1952. PLP is bound into an enzyme's active site as a Schiff base with a specific lysine side chain before a substate binds . This is often called the **internal aldimine**. When the substrate binds it reacts with the internal Schiff base by a two-step process called **transimination** (Eq. 14-26) to form the substrate Schiff base, which is also called the **external aldimine**.



(14-26)

3. The Variety of PLP-Dependent Reactions

In Fig. 14-5 the reactions of PLP-amino acid Schiff bases are compared with those of β-oxo-acids. Beta-hydroxy-α-oxo acids and Schiff bases of PLP with β-hydroxy-α-amino acids can react in similar ways. The reactions fall naturally into three groups (*a*,*b*,*c*) depending upon whether the bond cleaved is from the α-carbon of the substrate to the hydrogen atom, to the carboxyl group, or to the side chain. A fourth group of reactions of PLP-dependent enzymes (*d*) also involve removal of the α-hydrogen but are mechanistically more complex. Some of the many reactions catalyzed by these enzymes are listed in Table 14-3.

***Loss of the α-hydrogen* (Group *a*).** Dissociation of the α-hydrogen from the Schiff base leads to a **quinonoid–carbanionic intermediate** whose structure in depicted in Fig. 14-5. The name reflects the characteristics of the two resonance forms drawn. Like an enolate anion, this intermediate can react in several ways (1-4).

(1) **Racemization**. A proton can be added back to the original alpha position but without stereospecificity. A racemase which does this is important to bacteria. They must synthesize D-alanine and D-glutamic acid from the corresponding L-isomers for use in formation of their peptidoglycan envelopes.[153–154a] The combined actions of alanine racemase plus D-alanine aminotransferase, which produces D-glutamate as a product, provide bacteria with both D amino acids. A fungal alanine racemase is necessary for synthesis of the immunosuppresant cyclosporin (Box 9-F).[155,155a] High concentrations of free D-alanine are found in certain regions of the brain and also in various glands.[156]

The carboxyl group of an amino acid can also activate the α-hydrogen. This may be the basis for an aspartate racemase and other racemases that are *not* dependent upon PLP.[156–158] See also Chapter 13, Section B,4.

(2) **Cyclization**. A second kind of reaction is represented by the conversion of *S*-adenosylmethionine to **aminocyclopropanecarboxylic acid**, a precursor to the plant hormone **ethylene** (see Chapter 24).[159] The quinonoid intermediate cyclizes with elimination of methylthioadenosine to give a Schiff base of the product (Eq. 14-27).[160–161a] The cyclization step appears to be a simple S_N2-like reaction.[162]

(3) **Transamination**. A proton can add to the carbon attached to the 4 position of the PLP ring (Fig. 14-5) to form a second Schiff base, often referred to as a **ketimine** (Eq. 14-28). The latter can readily undergo hydrolysis to **pyridoxamine phosphate** (PMP) and an α-oxo acid. This sequence represents one of the

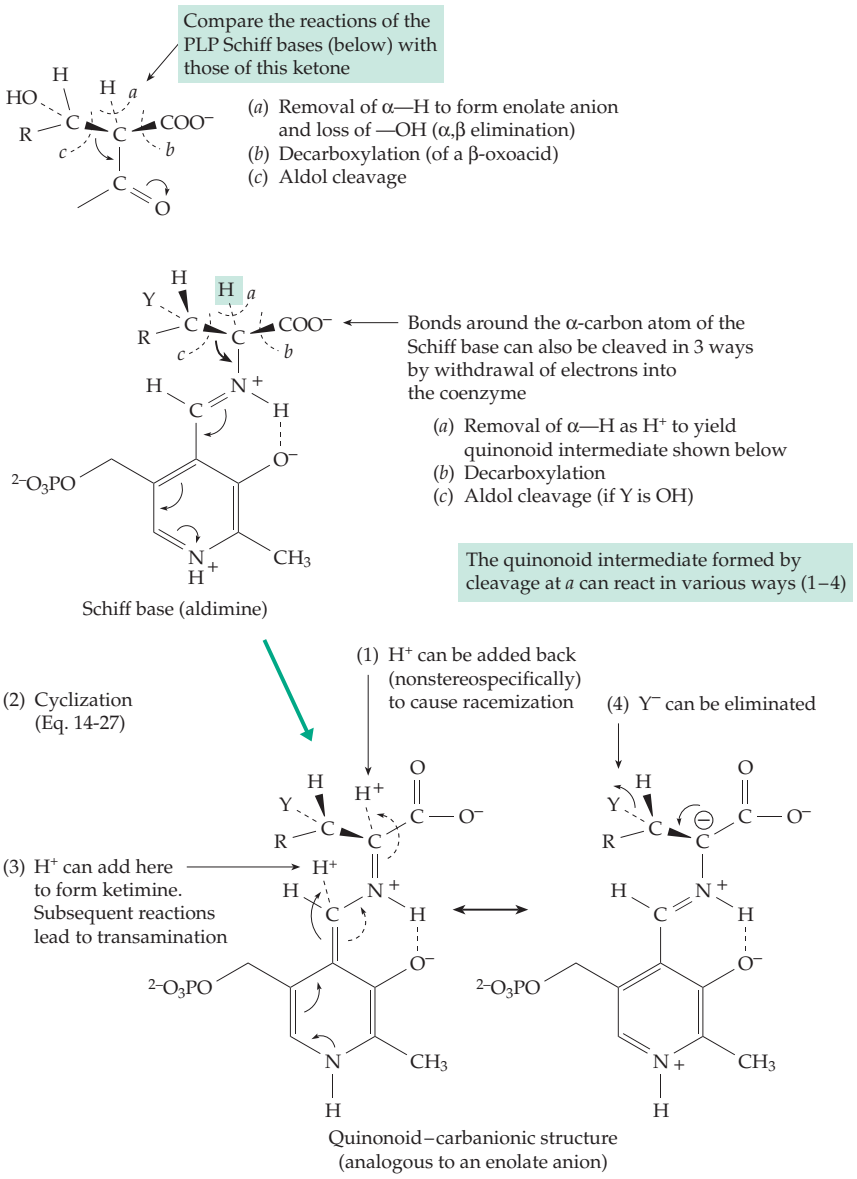


Figure 14-5 Some reactions of Schiff bases of pyridoxal phosphate. (*a*) Formation of the quinonoid intermediate, (*b*) elimination of a β substituent, and (*c*) transamination. The quinonoid–carbanionic intermediate can react in four ways (1–4) if enzyme specificity and substrate structure allow.

two half-reactions (Eqs. 14-24 and 14-25) required for enzymatic transamination.

Transaminases participate in metabolism of most of the amino acids, over 60 different enzymes have been identified.[142,163] Best studied are the **aspartate aminotransferases**, a pair of cytosolic and mitochondrial isoenzymes which can be isolated readily from animal hearts. Their presence in heart muscle and brain in high concentration is thought to be a result of their functioning in the malate–aspartate shuttle (Chapter 18). The sequences of the two proteins differ greatly, with only 50% of the residues being the same in both isoenzymes. However, these differences are largely on the outside surface, the folding pattern (Fig. 2-6) and internal structure (Fig. 14-6) are almost identical.[142,163–167a] Three-dimensional structures of aspartate aminotransferases of *E. coli*, yeast, chickens, and mammals are extremely similar, even though sequence identity may be as low as 20%.[167a] Most other transaminases also use the L-gutamate–oxoglutarate pair as one of the product–reactant pairs but a few prefer smaller substrates with uncharged side chains. An example is serine: pyruvate (or alanine:glyoxylate) aminotransferase, an important mitochondrial and peroxisomal enzyme in both animals and plants.[168] Other specialized aminotransferases act on aromatic amino acids,[168a] the branched chain amino acids valine, leucine, and isoleucine,[168b,c] and D-amino acids.[168d] Many of them are highly specific for individual amino acids such as phosphoserine,[168e] ornithine,[168f,g] *N*-acetylornithine,[168h,i] and 8-amino-7-oxononanoate (see banner, p. 719).[168j] An apparently internal transamination, which requires PMP and PLP, converts glutamate-1-semialdehyde into δ-aminolevulinate (see Eq. 24-44) in the pathway of porphyrin biosynthesis used by bacteria and plants.[168k]

(4) **Elimination and β replacement**. When a good leaving group is present in the β position of the amino acid it can be eliminated (Fig. 14-5, Eq. 14-29).[170] A large number of enzymes catalyze such reactions. Among them are **serine** and **threonine dehydratases,** which eliminate OH^- as H_2O;[171–173a] **tryptophan indole-lyase** (tryptophanase) of bacteria, which eliminates indole;[174–176c] **tyrosine phenol-lyase** (elimination of phenol);[177–178a] and **alliinase** of garlic (elimination of 1-propenylsulfenic acid).[179,180] Cystathionine, a precursor to methionine, eliminates L-homocysteine through the action of **cystathionine β lyase** (cystathionase).[181,182] Ammonia is eliminated from the β position of 2,3-diaminopropionate by a bacterial lyase.[183]

TABLE 14-3
Some Enzymes That Require Pyridoxal Phosphate as a Coenzyme

(*a*) Removing alpha hydrogen as H^+
- (1) Racemization
  - Alanine racemase*
- (2) Cyclization
  - Aminocyclopropane carboxylate synthase
- (3) Amino group transfer
  - Aspartate aminotransferase*
  - Alanine aminotransferase
  - D-Amino acid aminotransferase*
  - Branched chain aminotransferase
  - Gamma-aminobutyrate aminotransferase
  - ω-Amino acid:pyruvate aminotransferase*
  - Tyrosine aminotransferase
  - Serine:pyruvate aminotransferase
- (4) Beta elimination or replacement
  - D- and L- Serine dehydratases (deaminases)
  - Tryptophan indole-lyase (tryptophanase)*
  - Tyrosine phenol-lyase*
  - Alliinase
  - Cystathionine β-lyase (cystathionase)*
  - *O*-Acetylserine sulfhydrylase (cysteine synthase)
  - Cystathionine β-synthase
  - Tryptophan synthase*

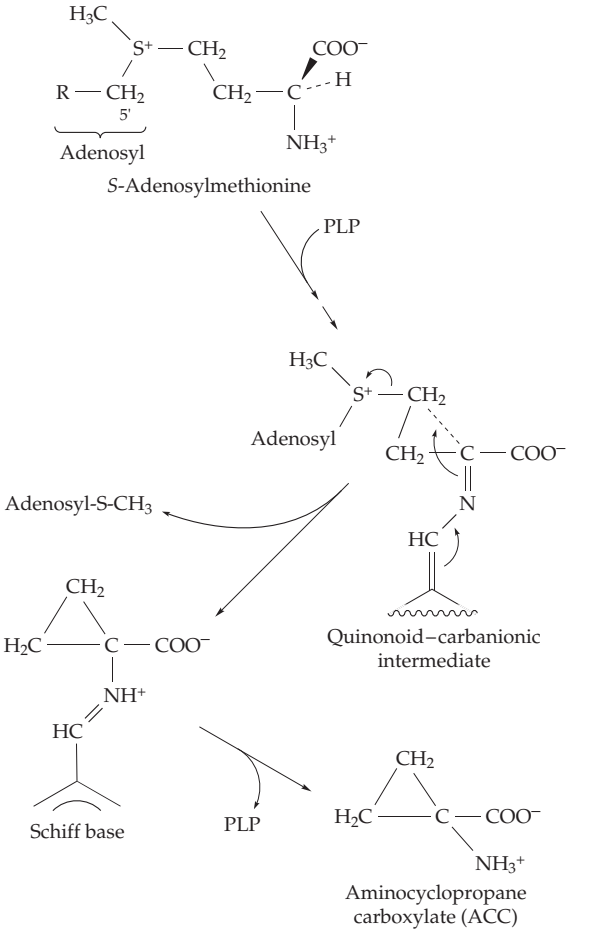
(*b*) Removal of alpha carboxylate as CO_2
- Diaminopimelate decarboxylase
- Glycine decarboxylase (requires lipoyl group)
- Glutamate decarboxylase
- Histidine decarboxylase
- Dopa decarboxylase
- Ornithine decarboxylase*
- Tyrosine decarboxylase
- Dialkylglycine decarboxylase (a decarboxylating transaminase)*

(*c*) Removal or replacement of side chain (or –H) by aldol cleavage
- Serine hydroxymethyltransferase
- Threonine aldolase
- δ-Aminolevulinate synthase
- Serine palmitoyltransferase
- 2-Amino-3-oxobutyrate-CoA ligase

(*d*) Reactions of ketimine intermediates
- Aspartate γ-decarboxylase
- Selenocysteine lyase
- *Nif S* protein of nitrogenase
- Gamma elimination and replacement
  - Cystathionine γ-synthase
  - Cystathionine γ-lyase
  - Threonine synthase

(*e*) Other enzymes
- Lysine 2,3-aminomutase
- Glycogen phosphorylase*
- Pyridoxamine phosphate (PMP) in synthesis of 3,6-dideoxy hexoses

* The three-dimensional structures of these and other PLP-dependent enzymes were determined by 2000.



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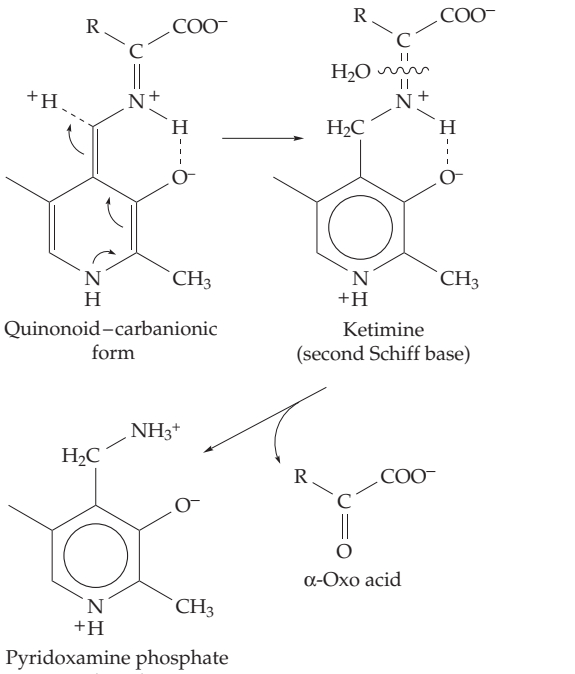

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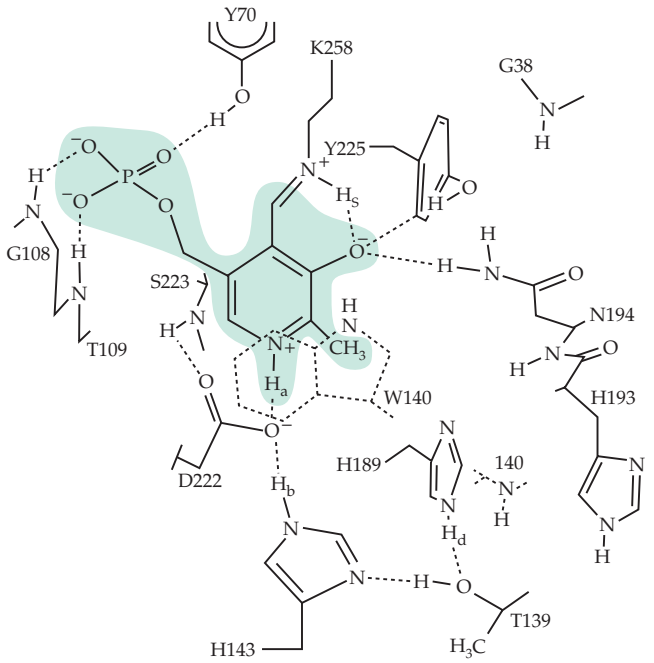
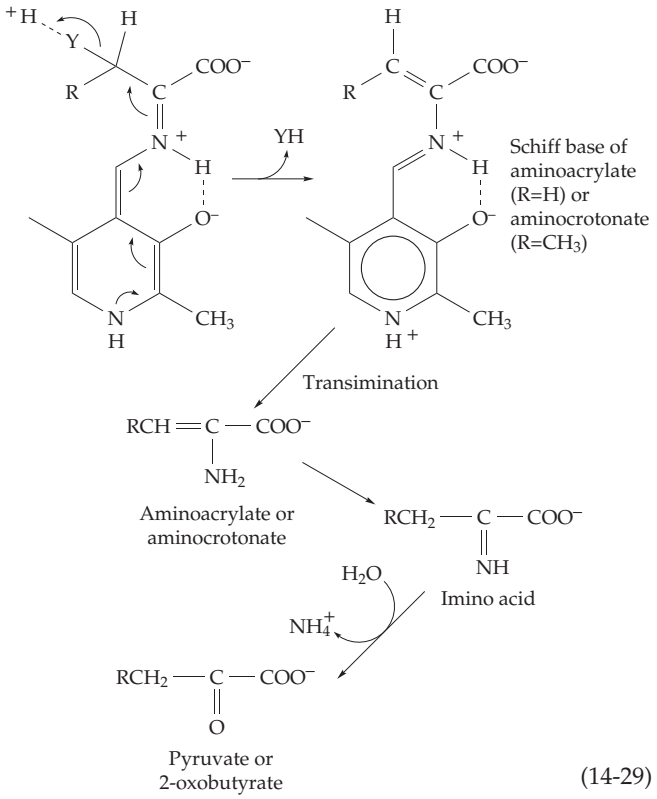
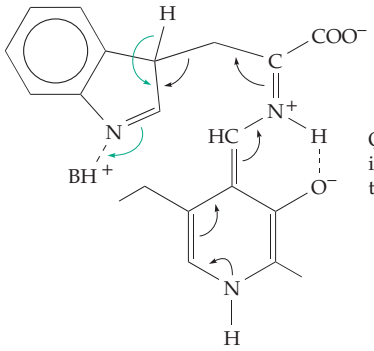


Figure 14-6 Drawing showing pyridoxal phosphate (shaded) and some surrounding protein structure in the active site of cytosolic aspartate aminotransferase. This is the low pH form of the enzyme with an *N*-protonated Schiff base linkage of lysine 258 to the PLP. The tryptophan 140 ring lies in front of the coenzyme. Several protons, labeled H_a, H_b, and H_d, are represented in ^1H NMR spectra by distinct resonances whose chemical shifts are sensitive to changes in the active site.[169]



(14-29)

Beta replacement is catalyzed by such enzymes of amino acid biosynthesis as **tryptophan synthase** (Chapter 25),[184] ***O*-acetylserine sulfhydrylase** (cysteine synthase),[185–186a] and **cystathionine β-synthase** (Chapter 24).[187–188c] In both elimination and β replacement an unsaturated Schiff base, usually of aminoacrylate or aminocrotonate, is a probable intermediate (Eq. 14-29). Conversion to the final products is usually assumed to be via hydrolysis to free aminoacrylate, tautomerization to an imino acid, and hydrolysis of the latter, e.g., to pyruvate and ammonium ion (Eq. 14-29). However, the observed stereospecific addition of a



Quinonoid–carbanionic intermediate with tautomerized indole ring

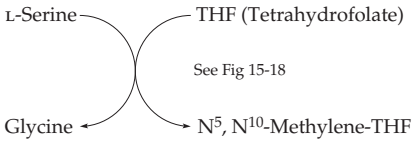
proton at the β-C atom of 2-oxobutyrate[189] suggests that these steps may occur with the participation of groups from the enzyme. Before indole can be eliminated by tryptophan indolelyase the indole ring must presumably be tautomerized to the following form of the quinonoid intermediate. The same species may be created by tryptophan synthase upon addition of indole to the enzyme-bound aminoacrylate. The green arrows on the structure indicate the tautomerization that would occur to convert the indole ring to the structure found in tryptophan. The three-dimensional structure of tryptophan synthase is shown in Fig. 25-3. It is a complex of two enzymes with a remarkable tunnel through which the intermediate indole can pass.[184,190] An unusual PLP-dependent β replacement is used to synthesize a transfer RNA ester of **selenocysteine** prior to its insertion into special locations in a few proteins (Chapter 15, Section G, and Chapter 29).

***Decarboxylation* (Group *b*).** The bond to the carboxyl group of an amino acid substrate is broken in reactions catalyzed by **amino acid decarboxylases**.[191,192] These also presumably lead to a transient quinonoid-carbanionic intermediate. Addition of a proton at the original site of decarboxylation followed by breakup of the Schiff base completes the sequence. Decarboxylation of amino acids is nearly irreversible and frequently appears as a final step in synthesis of amino compounds. For example, in the brain glutamic acid is decarboxylated to **γ-aminobutyric acid** (Gaba),[193–196b] while 3,4-dihydroxyphenylalanine (dopa) and 5-hydroxy-

tryptophan are acted upon by an **aromatic amino acid decarboxylase** to form, respectively, the neurotransmitters **dopamine** and **serotonin**.[197–199b] Histidine is decarboxylated to **histamine**.[200–202] However, not all histidine decarboxylases use PLP as a coenzyme (Section F).

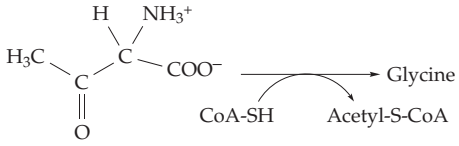
Arginine is converted by a PLP-dependent decarboxylase to agmatine (Fig. 24-12) which is hydrolyzed to **1,4-diaminopropane**.[191] This important cell constituent is also formed by hydrolysis of arginine to **ornithine** (Fig 24-10) and decarboxylation of the latter.[203–206c] **Lysine** is formed in bacteria by decarboxylation of *meso*-diamino-pimelic acid (Fig. 24-14). **Glycine** is decarboxylated oxidatively in mitochondria in a sequence requiring lipoic acid and tetrahydrofolate as well as PLP (Fig. 15-20).[207–209b] A **methionine** decarboxylase has been isolated in pure form from a fern.[210] The bacterial **dialkylglycine** decarboxylase is both a decarboxylase and an aminotransferase which uses pyruvate as its second substrate forming a ketone and L-alanine as products (See Eq. 14-37)[210a, 210b]

***Side chain cleavage* (Group *c*).** In a third type of reaction the side chain of the Schiff base of Fig. 14-5 undergoes aldol cleavage. Conversely, a side chain can be added by β condensation. The best known enzyme of this group is **serine hydroxymethyltransferase**, which converts serine to glycine and formaldehyde.[211–213b] The latter is not released in a free form but is transferred by the same enzyme specifically to **tetrahydrofolic acid** (Eq. 14-30), with which it forms a cyclic adduct.



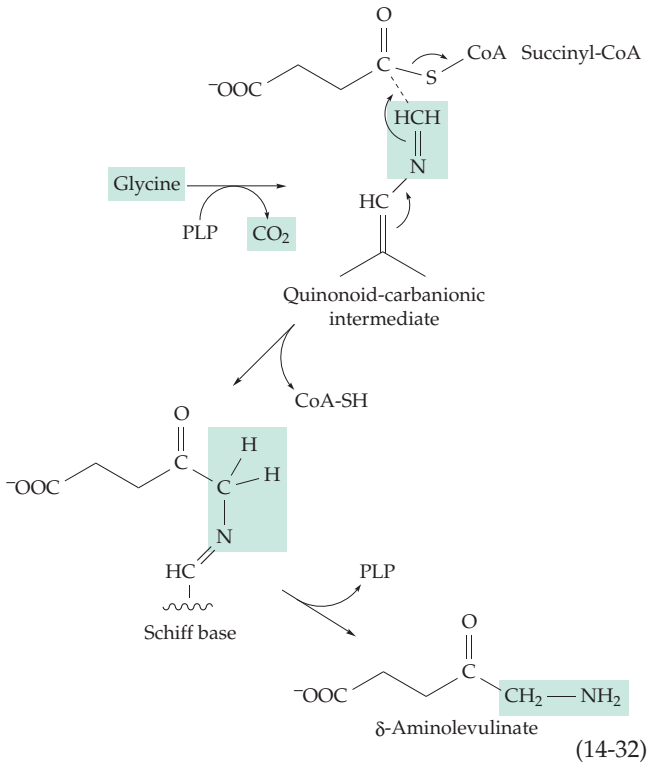
(14-30)

Threonine is cleaved to acetaldehyde by the same enzyme. A related reaction is indicated in Fig. 24-27 (top). In a more important pathway of degradation of threonine the hydroxyl group of its side chain is dehydrogenated to form 2-amino-3-oxobutyrate which is cleaved by a PLP-dependent enzyme to glycine and acetyl-CoA (Eq. 14-31).[214,215]



(14-31)

Conversely, ester condensation reactions join acyl groups from CoA derivatives to Schiff bases derived from glycine or serine. Succinyl-CoA is the acyl donor



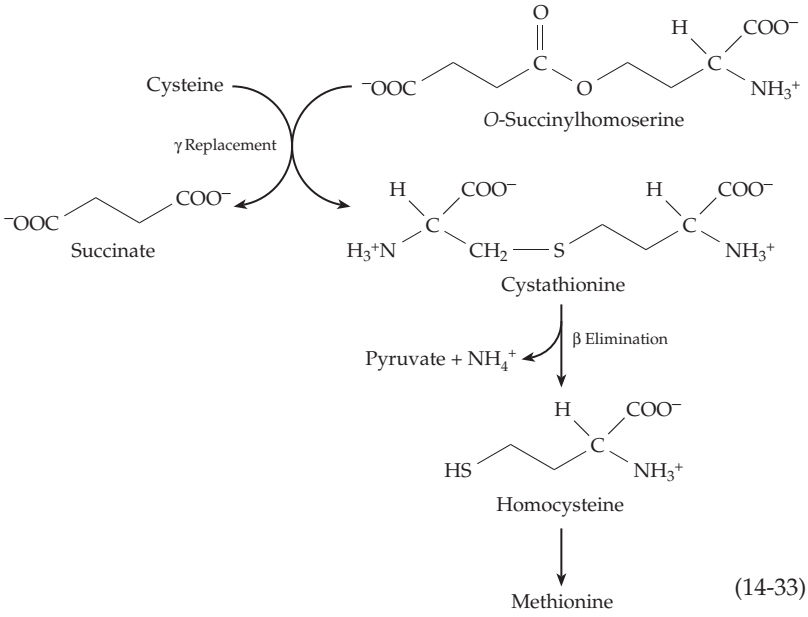
(14-32)

in Eq. 14-32 for the second known pathway for biosynthesis of **δ-aminolevulinic acid**, an intermediate in porphyrin synthesis (Chapter 24).[216–218b] The enzyme does not catalyze decarboxylation of glycine in the absence of succinyl-CoA, and the decarboxylation probably follows the condensation as indicated in Eq. 14-32.[219] In a similar reaction in the biosynthesis of **sphingosine** serine is condensed with palmitoyl-CoA and decarboxylated to form an aminoketone intermediate (Fig. 21-6).[219a] 8-Amino-7-oxonanonoate synthase forms a precursor of biotin (see banner, p. 719).[220]

***Ketimine intermediate as electron acceptor* (Group *d*).** The fourth group of PLP-dependent reactions are thought to depend upon formation of the ketimine intermediate of Eq. 14-28. In this form the original α-hydrogen of the amino acid has been removed and the C=NH$^+$ bond of the ketimine is polarized in a direction that favors electron withdrawal from the amino acid into the imine group. This permits another series of enzymatic reactions analogous to those of the β-oxo acid shown at the top of Fig. 14-5. Both elimination and C–C bond cleavage α,β to the C=N group of the ketimine can occur.

Enzymes of this group catalyze elimination of γ substituents from amino acids as illustrated in Fig. 14-7. Eliminated groups may be replaced by other substituents, either in the α or the β positions. The ketimine formed initially by such an enzyme (step *a*) undergoes elimination of the γ substituent (β with respect to the C=N group) along with a proton from the β position of the original amino acid to form an

unsaturated intermediate which can react in one of three ways, depending upon the enzyme. Addition of HY′ leads to γ replacement (step *c*), while addition of a proton at the α position leads, via reaction step *d*, to an α,β-unsaturated Schiff base. The latter can react by addition of HY′ (β replacement, step *e*) or it can break down to an α-oxo acid and ammonium ion (step *f*), just as in the β elimination reactions of Eq. 14-29. An important γ replacement reaction is conversion of *O*-acetyl-, *O*-succinyl-, or *O*-phosphohomoserine to cystathionine (Eq. 14-33). This **cystathionine γ-synthase** reaction[220a] lies on the pathway of biosynthesis of methionine by bacteria, fungi, and



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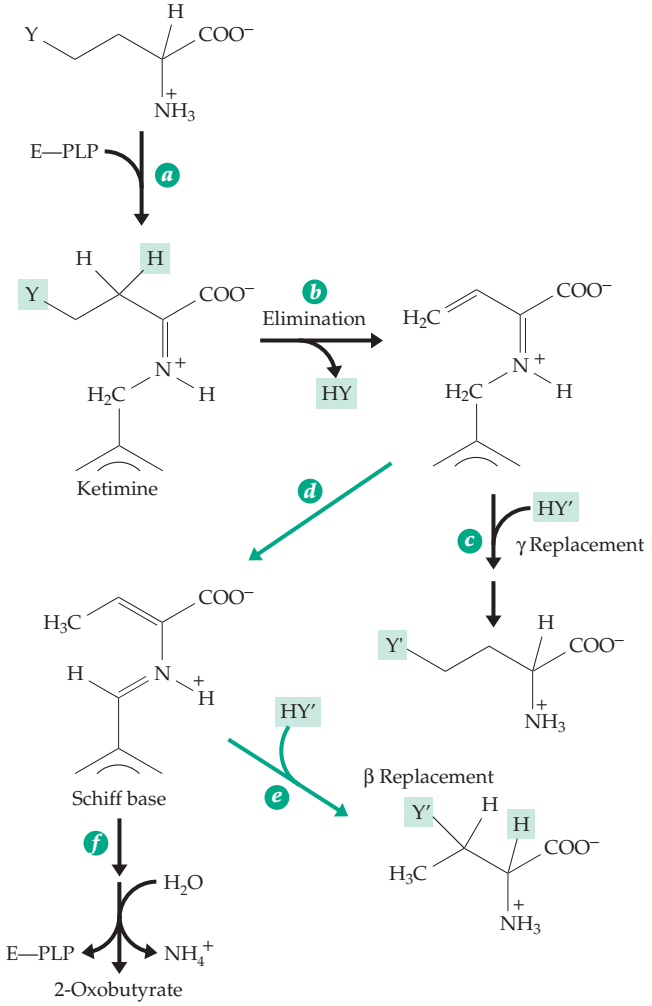


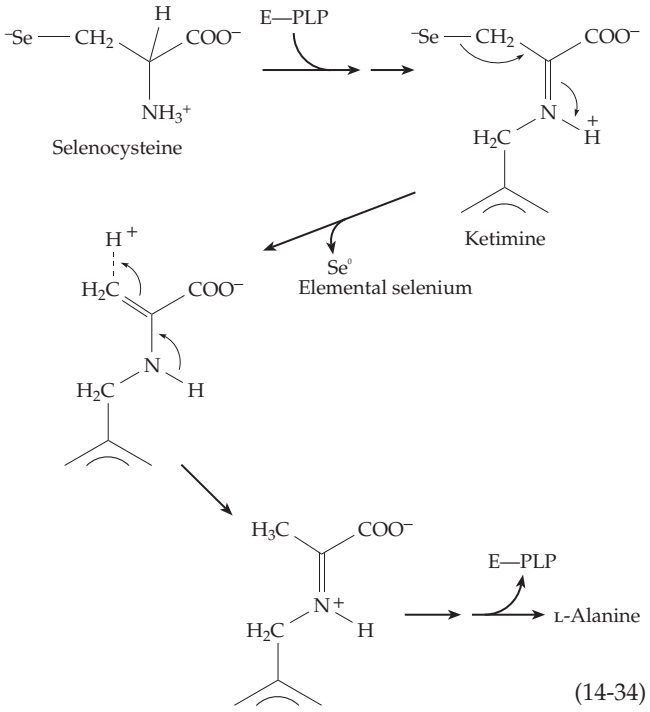
Figure 14-7 Some PLP-dependent reactions involving elimination of a γ substituent. Replacement by another γ substituent or by a substituent in the β position is possible, as is deamination to an α-oxo acid.

higher plants. Subsequent reactions include β elimination from cystathionine of **homocysteine**[220b] which is then converted to methionine (Eq. 14-33). Threonine is formed from ***O*-phosphohomoserine** via γ elimination followed by β replacement with HO^-, a reaction catalyzed by **threonine synthase** (Fig. 24-13).[220c]

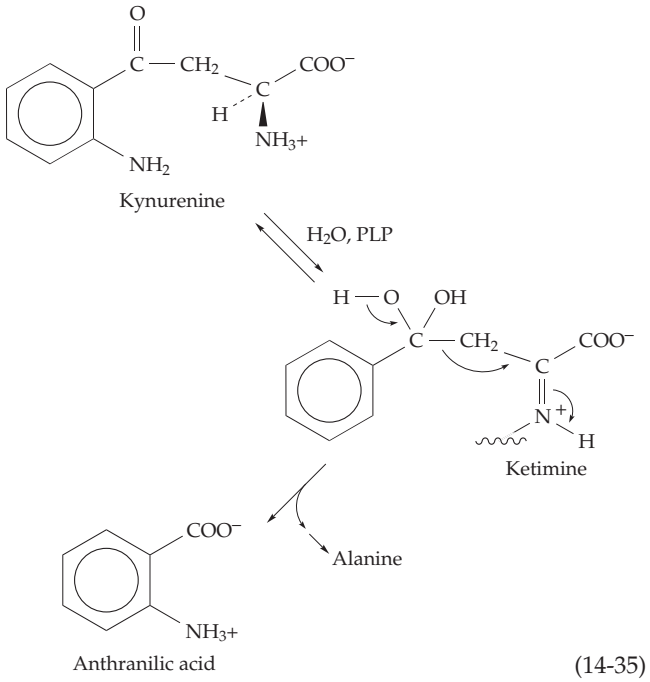
The loss of a β-carboxyl group as CO_2 can also occur through a ketimine or quinonoid intermediate. For example, the bacterial **aspartate β-decarboxylase**[221] converts aspartate to alanine and CO_2. **Selenocysteine** is utilized to create the active sites of several enzymes (Chapter 15). Excess selenocysteine is degraded by the PLP-dependent **selenocysteine lyase**,[222–223b] which evidently eliminates elemental selenium from a ketimine or quinonoid state of an intermediate Schiff base (Eq. 14-34). A similar reaction may occur in the biosynthesis of iron–sulfur clusters. The **Nif S** protein is essential for formation of Fe_4S_4 clusters in the nitrogen-fixing enzyme nitrogenase. This enzyme is in some way involved in transferring the sulfur atom of cysteine into an iron–sulfur cluster.[224] Alanine is the other product suggesting transfer of S^0 into the cluster using the sequence of Eq. 14-34.

Another related reaction that goes through a ketimine is the conversion of the amino acid **kynurenine** to alanine and anthranilic acid.[225] It presumably depends upon hydration of the carbonyl group prior to β cleavage (Eq. 14-35). An analogous thiolytic cleavage utilizes CoA to convert 2-amino-4-ketopentanoate to acetyl-CoA and alanine.[226]

Glycogen phosphorylase. While PLP is ideally designed to catalyze reactions of amino compounds it was surprising to find it as an essential cofactor for glycogen phosphorylase (Fig. 11-5). The PLP is linked as a Schiff base in the same way as in other PLP-depen-



(14-34)

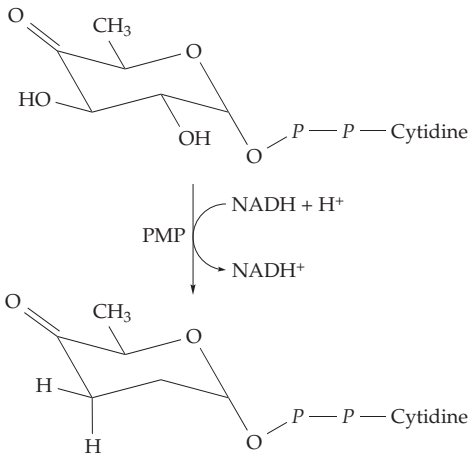


(14-35)

dent enzymes, but there is no obvious function for the coenzyme ring. As suggested in Chapter 12, the phosphate group probably acts as an acid–base catalyst. It has been estimated that 50% of the vitamin B_6 in our body is present as PLP in muscle phosphorylase.[227] Studies of vitamin B_6-deficient rats suggest that PLP in phosphorylase serves as a reserve supply, much of which can be taken for other purposes during times of deficiency.

4. Pyridoxamine Phosphate as a Coenzyme

If PLP is a cofactor designed to react with amino groups of substrates, might not pyridoxamine phosphate (PMP) act as a coenzyme for reactions of carbonyl compounds? An example of this kind of function has been found[228–230] in the formation of 3,6-dideoxyhexoses needed for bacterial cell surface antigens (Fig. 4-15). Glucose (as cytidine diphosphate glucose; CDP-glucose) is first converted to 4-oxo-6-deoxy-CDP-glucose. The conversion of the latter to 3,6-dideoxy-CDP-glucose (Eq. 14-36) requires PMP as well as NADH or NADPH.



(14-36)

The student may find it of interest to propose a mechanism for this reaction, taking into account the expected direct transfer of a hydrogen from NADH as described in Chapter 15, before consulting published papers. Part of the reaction cycle appears to involve a free radical derived from the PMP. This is discussed further in Chapter 20 together with free radical-forming PLP enzymes

5. Stereochemistry of PLP-Requiring Enzymes

According to stereoelectronic principles, the bond in the substrate amino acid that is to be broken by a PLP-dependent enzyme should lie in a plane perpendicular to the plane of the cofactor–imine π system (Fig. 14-8). This would minimize the energy of the transition state by allowing maximum σ–π overlap between the breaking bond and the ring–imine π system. It also would provide the geometry closest to that of the planar quinonoid intermediate to be formed, thus minimizing molecular motion in the approach to the transition state. Figure 14-8 shows three orientations of an amino acid in which the α-hydrogen, the carboxyl group, and the side chain, respectively, are positioned for cleavage. For each orientation shown, another geometry suitable for cleavage of the same bond is