

# Enzymes—Kinetics and Specificity

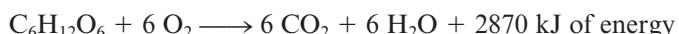


## ESSENTIAL QUESTIONS

At any moment, thousands of chemical reactions are taking place in any living cell. Enzymes are essential for these reactions to proceed at rates fast enough to sustain life.

### What are enzymes, and what do they do?

Living organisms seethe with metabolic activity. Thousands of chemical reactions are proceeding very rapidly at any given instant within all living cells. Virtually all of these transformations are mediated by **enzymes**—proteins (and occasionally RNA) specialized to catalyze metabolic reactions. The substances transformed in these reactions are often organic compounds that show little tendency for reaction outside the cell. An excellent example is glucose, a sugar that can be stored indefinitely on the shelf with no deterioration. Most cells quickly oxidize glucose, producing carbon dioxide and water and releasing lots of energy:



(−2870 kJ/mol is the standard-state free energy change [ $\Delta G^\circ'$ ] for the oxidation of glucose.) In chemical terms, 2870 kJ is a large amount of energy, and glucose can be viewed as an energy-rich compound even though at ambient temperature it is not readily reactive with oxygen outside of cells. Stated another way, glucose represents **thermodynamic potentiality**: Its reaction with oxygen is strongly exergonic, but it doesn't occur under normal conditions. On the other hand, enzymes can catalyze such thermodynamically favorable reactions, causing them to proceed at extraordinarily rapid rates (Figure 13.1). In glucose oxidation and countless other instances, enzymes provide cells with the ability to exert *kinetic control over thermodynamic potentiality*. That is, living systems use enzymes to accelerate and control the rates of vitally important biochemical reactions.

► The space shuttle must accelerate from zero velocity to a velocity of more than 18,000 miles per hour in order to enter earth orbit. Its three main engines are powered by energy released in the reaction:  $2\text{H}_2 + \text{O}_2 \rightarrow 2 \text{ H}_2\text{O}$ . The rate of  $\text{O}_2$  consumption per engine is 13,000 moles per second.

*There is more to life than increasing its speed.*

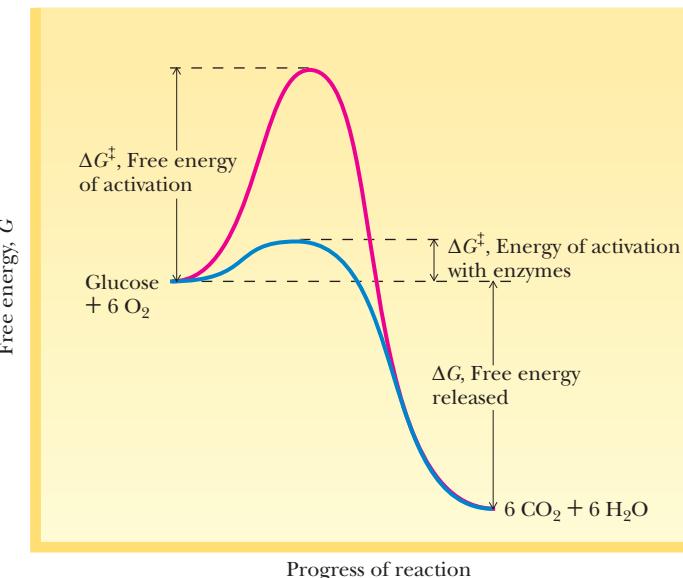
Mahatma Gandhi (1869–1948)

## KEY QUESTIONS

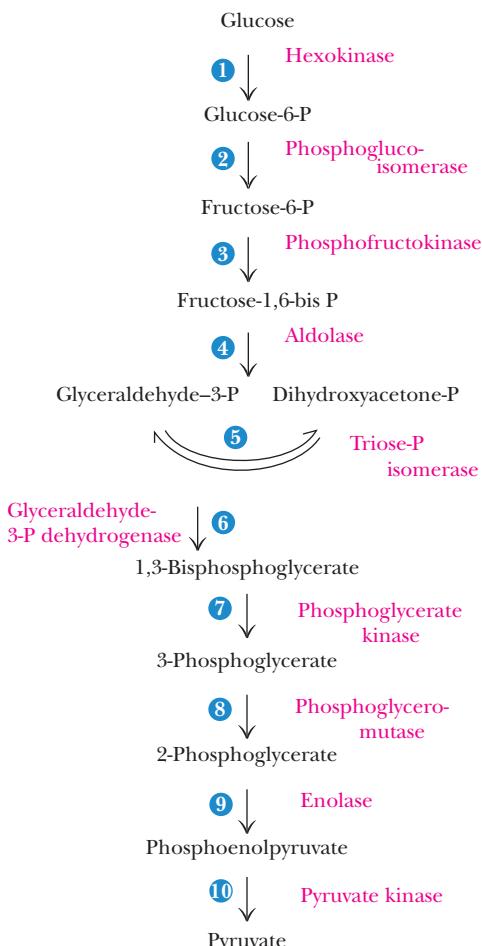
- 13.1 What Characteristic Features Define Enzymes?
- 13.2 Can the Rate of an Enzyme-Catalyzed Reaction Be Defined in a Mathematical Way?
- 13.3 What Equations Define the Kinetics of Enzyme-Catalyzed Reactions?
- 13.4 What Can Be Learned from the Inhibition of Enzyme Activity?
- 13.5 What Is the Kinetic Behavior of Enzymes Catalyzing Bimolecular Reactions?
- 13.6 How Can Enzymes Be So Specific?
- 13.7 Are All Enzymes Proteins?
- 13.8 Is It Possible to Design an Enzyme to Catalyze Any Desired Reaction?



Online homework and a Student Self Assessment for this chapter may be assigned in OWL.



**FIGURE 13.1** Reaction profile showing the large  $\Delta G^\ddagger$  for glucose oxidation. Enzymes lower  $\Delta G^\ddagger$ , thereby accelerating rate.



**FIGURE 13.2** The breakdown of glucose by *glycolysis* provides a prime example of a metabolic pathway.

## Enzymes Are the Agents of Metabolic Function

Acting in sequence, enzymes form metabolic pathways by which nutrient molecules are degraded, energy is released and converted into metabolically useful forms, and precursors are generated and transformed to create the literally thousands of distinctive biomolecules found in any living cell (Figure 13.2). Situated at key junctions of metabolic pathways are specialized **regulatory enzymes** capable of sensing the momentary metabolic needs of the cell and adjusting their catalytic rates accordingly. The responses of these enzymes ensure the harmonious integration of the diverse and often divergent metabolic activities of cells so that the living state is promoted and preserved.

### 13.1 What Characteristic Features Define Enzymes?

Enzymes are remarkably versatile biochemical catalysts that have in common three distinctive features: **catalytic power**, **specificity**, and **regulation**.

#### Catalytic Power Is Defined as the Ratio of the Enzyme-Catalyzed Rate of a Reaction to the Uncatalyzed Rate

Enzymes display enormous catalytic power, accelerating reaction rates as much as  $10^{21}$  over uncatalyzed levels, which is far greater than any synthetic catalysts can achieve, and enzymes accomplish these astounding feats in dilute aqueous solutions under mild conditions of temperature and pH. For example, the enzyme jack bean *urease* catalyzes the hydrolysis of urea:



At 20°C, the rate constant for the enzyme-catalyzed reaction is  $3 \times 10^4/\text{sec}$ ; the rate constant for the uncatalyzed hydrolysis of urea is  $3 \times 10^{-10}/\text{sec}$ . Thus,  $10^{14}$  is the ratio of the catalyzed rate to the uncatalyzed rate of reaction. Such a ratio is defined as the relative **catalytic power** of an enzyme, so the catalytic power of urease is  $10^{14}$ .

## Specificity Is the Term Used to Define the Selectivity of Enzymes for Their Substrates

A given enzyme is very selective, both in the substances with which it interacts and in the reaction that it catalyzes. The substances upon which an enzyme acts are traditionally called **substrates**. In an enzyme-catalyzed reaction, none of the substrate is diverted into nonproductive side reactions, so no wasteful by-products are produced. It follows then that the products formed by a given enzyme are also very specific. This situation can be contrasted with your own experiences in the organic chemistry laboratory, where yields of 50% or even 30% are viewed as substantial accomplishments (Figure 13.3). The selective qualities of an enzyme are collectively recognized as its **specificity**. Intimate interaction between an enzyme and its substrates occurs through molecular recognition based on structural complementarity; such mutual recognition is the basis of specificity. The specific site on the enzyme where substrate binds and catalysis occurs is called the **active site**.

## Regulation of Enzyme Activity Ensures That the Rate of Metabolic Reactions Is Appropriate to Cellular Requirements

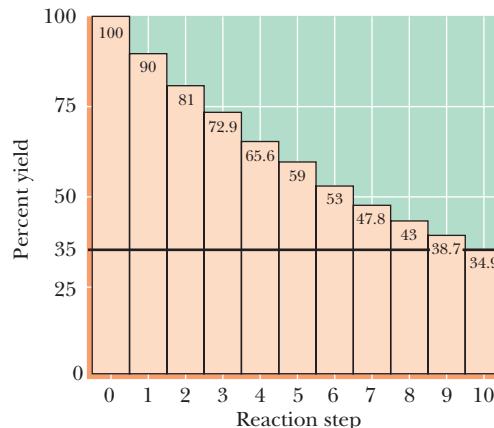
Regulation of enzyme activity is essential to the integration and regulation of metabolism. Enzyme regulation is achieved in a variety of ways, ranging from controls over the amount of enzyme protein produced by the cell to more rapid, reversible interactions of the enzyme with metabolic inhibitors and activators. Chapter 15 is devoted to discussions of this topic. Because most enzymes are proteins, we can anticipate that the functional attributes of enzymes are due to the remarkable versatility found in protein structures.

## Enzyme Nomenclature Provides a Systematic Way of Naming Metabolic Reactions

Traditionally, enzymes were named by adding the suffix *-ase* to the name of the substrate upon which they acted, as in *urease* for the urea-hydrolyzing enzyme or *phosphatase* for enzymes hydrolyzing phosphoryl groups from organic phosphate compounds. Other enzymes acquired names bearing little resemblance to their activity, such as the peroxide-decomposing enzyme *catalase* or the proteolytic enzymes (*proteases*) of the digestive tract, *trypsin* and *pepsin*. Because of the confusion that arose from these trivial designations, an International Commission on Enzymes was established to create a systematic basis for enzyme nomenclature. Although common names for many enzymes remain in use, all enzymes now are classified and formally named according to the reaction they catalyze. Six classes of reactions are recognized (Table 13.1). Within each class are subclasses, and under each subclass are sub-subclasses within which individual enzymes are listed. Classes, subclasses, sub-subclasses, and individual entries are each numbered so that a series of four numbers serves to specify a particular enzyme. A systematic name, descriptive of the reaction, is also assigned to each entry. To illustrate, consider the enzyme that catalyzes this reaction:



A phosphate group is transferred from ATP to the C-6-OH group of glucose, so the enzyme is a *transferase* (class 2, Table 13.1). Subclass 7 of transferases is *enzymes transferring phosphorus-containing groups*, and sub-subclass 1 covers those *phosphotransferases with an alcohol group as an acceptor*. Entry 2 in this sub-subclass is **ATP:D-glucose-6-phosphotransferase**, and its classification number is **2.7.1.2**. In use, this number is written preceded by the letters **E.C.**, denoting the Enzyme Commission. For example, entry 1 in the same sub-subclass is **E.C.2.7.1.1, ATP:D-hexose-6-phosphotransferase**, an ATP-dependent enzyme that transfers a phosphate to the 6-OH of hexoses (that is, it is non-specific regarding its hexose acceptor). These designations can be cumbersome, so in



**FIGURE 13.3** A 90% yield over 10 steps, for example, in a metabolic pathway, gives an overall yield of 35%. Therefore, yields in biological reactions *must be substantially greater*; otherwise, unwanted by-products would accumulate to unacceptable levels.

| Systematic Classification of Enzymes According to the Enzyme Commission |  |             |   |
|---|--|-------------|---|
| E.C. Number   | Systematic Name and Subclasses                         | E.C. Number | Systematic Name and Subclasses  |
| 1   | <i>Oxidoreductases</i> (oxidation–reduction reactions) | 4           | <i>Lyases</i> (bond cleavage by means other than hydrolysis or oxidation) |
| 1.1   | Acting on CH—OH group of donors                        | 4.1         | C—C lyases  |
| 1.1.1   | With NAD or NADP as acceptor                           | 4.1.1       | Carboxy lyases  |
| 1.1.3   | With O <sub>2</sub> as acceptor                        | 4.1.2       | Aldehyde lyases   |
| 1.2   | Acting on the >C=O group of donors                     | 4.2         | C—O lyases  |
| 1.2.3   | With O <sub>2</sub> as acceptor                        | 4.2.1       | Hydrolases  |
| 1.3   | Acting on the CH—CH group of donors                    | 4.3         | C—N lyases  |
| 1.3.1   | With NAD or NADP as acceptor                           | 4.3.1       | Ammonia lyases  |
| 2   | <i>Transferases</i> (transfer of functional groups)    | 5           | <i>Isomerase</i> s (isomerization reactions)                              |
| 2.1   | Transferring C-1 groups                                | 5.1         | Racemases and epimerases  |
| 2.1.1   | Methyltransferases                                     | 5.1.3       | Acting on carbohydrates   |
| 2.1.2   | Hydroxymethyltransferases and formyltransferases       | 5.2         | <i>Cis-trans</i> isomerasers  |
| 2.1.3   | Carboxyltransferases and carbamoyltransferases         | 6           | <i>Ligases</i> (formation of bonds with ATP cleavage)                     |
| 2.2   | Transferring aldehydic or ketonic residues             | 6.1         | Forming C—O bonds   |
| 2.3   | Acyltransferases                                       | 6.1.1       | Amino acid–RNA ligases  |
| 2.4   | Glycosyltransferases                                   | 6.2         | Forming C—S bonds   |
| 2.6   | Transferring N-containing groups                       | 6.3         | Forming C—N bonds   |
| 2.6.1   | Aminotransferases                                      | 6.4         | Forming C—C bonds   |
| 2.7   | Transferring P-containing groups                       | 6.4.1       | Carboxylases  |
| 2.7.1   | With an alcohol group as acceptor                      |             |   |
| 3   | <i>Hydrolases</i> (hydrolysis reactions)               |             |   |
| 3.1   | Cleaving ester linkage                                 |             |   |
| 3.1.1   | Carboxylic ester hydrolases                            |             |   |
| 3.1.3   | Phosphoric monoester hydrolases                        |             |   |
| 3.1.4   | Phosphoric diester hydrolases                          |             |   |

everyday usage, trivial names are commonly used. The glucose-specific enzyme E.C.2.7.1.2 is called *glucokinase*, and the nonspecific E.C.2.7.1.1 is known as *hexokinase*. *Kinase* is a trivial term for enzymes that are ATP-dependent phosphotransferases.

## Coenzymes and Cofactors Are Nonprotein Components Essential to Enzyme Activity

Many enzymes carry out their catalytic function relying solely on their protein structure. Many others require nonprotein components, called **cofactors** (Table 13.2). Cofactors may be metal ions or organic molecules referred to as **coenzymes**. Coenzymes and cofactors provide proteins with chemically versatile functions not found in amino acid side chains. Many coenzymes are vitamins or contain vitamins as part of their structure. Usually coenzymes are actively involved in the catalytic reaction of the enzyme, often serving as intermediate carriers of functional groups in the conversion of substrates to products. In most cases, a coenzyme is firmly associated with its enzyme, perhaps even by covalent bonds, and it is difficult to separate the two. Such tightly bound coenzymes are referred to as **prosthetic groups** of the enzyme. The catalytically active complex of protein and prosthetic group is called the **holoenzyme**. The protein without the prosthetic group is called the **apoenzyme**; it is catalytically inactive.

**TABLE 13.2** Enzyme Cofactors: Some Metal Ions and Coenzymes and the Enzymes with Which They Are Associated

| Metal Ions and Some Enzymes That Require Them |   | Coenzymes Serving as Transient Carriers of Specific Atoms or Functional Groups |   |  |
|---|---|--|---|--|
| Metal Ion                                     | Enzyme  | Coenzyme   | Entity Transferred  | Representative Enzymes Using Coenzymes |
| Fe <sup>2+</sup> or Fe <sup>3+</sup>          | Cytochrome oxidase                                | Thiamine pyrophosphate (TPP)   | Aldehydes   | Pyruvate dehydrogenase                 |
|   | Catalase  | Flavin adenine dinucleotide (FAD)  | Hydrogen atoms  | Succinate dehydrogenase                |
|   | Peroxidase  | Nicotinamide adenine dinucleotide (NAD)  | Hydride ion (:H <sup>-</sup> )                            | Alcohol dehydrogenase                  |
| Cu <sup>2+</sup>                              | Cytochrome oxidase                                |  |   |  |
| Zn <sup>2+</sup>                              | DNA polymerase                                    | Coenzyme A (CoA)   | Acyl groups   | Acetyl-CoA carboxylase                 |
|   | Carbonic anhydrase                                | Pyridoxal phosphate (PLP)  | Amino groups  | Aspartate aminotransferase             |
|   | Alcohol dehydrogenase                             |  |   |  |
| Mg <sup>2+</sup>                              | Hexokinase  | 5'-Deoxyadenosylcobalamin (vitamin B <sub>12</sub> )                           | H atoms and alkyl groups                                  | Methylmalonyl-CoA mutase               |
|   | Glucose-6-phosphatase                             |  |   |  |
| Mn <sup>2+</sup>                              | Arginase  | Biotin (biocytin)  | CO <sub>2</sub>   | Propionyl-CoA carboxylase              |
| K <sup>+</sup>                                | Pyruvate kinase (also requires Mg <sup>2+</sup> ) | Tetrahydrofolate (THF)   | Other one-carbon groups, such as formyl and methyl groups | Thymidylate synthase                   |
| Ni <sup>2+</sup>                              | Urease  |  |   |  |
| Mo  | Nitrate reductase                                 |  |   |  |
| Se  | Glutathione peroxidase                            |  |   |  |

## 13.2 | Can the Rate of an Enzyme-Catalyzed Reaction Be Defined in a Mathematical Way?

**Kinetics** is the branch of science concerned with the rates of reactions. The study of **enzyme kinetics** addresses the biological roles of enzymatic catalysts and how they accomplish their remarkable feats. In enzyme kinetics, we seek to determine the maximum reaction velocity that the enzyme can attain and its binding affinities for substrates and inhibitors. Coupled with studies on the structure and chemistry of the enzyme, analysis of the enzymatic rate under different reaction conditions yields insights regarding the enzyme's mechanism of catalytic action. Such information is essential to an overall understanding of metabolism.

Significantly, this information can be exploited to control and manipulate the course of metabolic events. The science of pharmacology relies on such a strategy. **Pharmaceuticals**, or **drugs**, are often special inhibitors specifically targeted at a particular enzyme in order to overcome infection or to alleviate illness. A detailed knowledge of the enzyme's kinetics is indispensable to rational drug design and successful pharmacological intervention.

### Chemical Kinetics Provides a Foundation for Exploring Enzyme Kinetics

Before beginning a quantitative treatment of enzyme kinetics, it will be fruitful to review briefly some basic principles of chemical kinetics. **Chemical kinetics** is the study of the rates of chemical reactions. Consider a reaction of overall stoichiometry:



Although we treat this reaction as a simple, one-step conversion of A to P, it more likely occurs through a sequence of elementary reactions, each of which is a simple molecular process, as in



where I and J represent intermediates in the reaction. Precise description of all of the elementary reactions in a process is necessary to define the overall reaction mechanism for  $A \rightarrow P$ .

Let us assume that  $A \rightarrow P$  is an elementary reaction and that it is spontaneous and essentially irreversible. Irreversibility is easily assumed if the rate of P conversion to A is very slow or the concentration of P (expressed as  $[P]$ ) is negligible under the conditions chosen. The **velocity**,  $v$ , or **rate**, of the reaction  $A \rightarrow P$  is the amount of P formed or the amount of A consumed per unit time,  $t$ . That is,

$$v = \frac{d[P]}{dt} \quad \text{or} \quad v = -\frac{d[A]}{dt} \quad (13.1)$$

The mathematical relationship between reaction rate and concentration of reactant(s) is the **rate law**. For this simple case, the rate law is

$$v = \frac{-d[A]}{dt} = k[A] \quad (13.2)$$

From this expression, it is obvious that the rate is proportional to the concentration of A, and  $k$  is the proportionality constant, or **rate constant**.  $k$  has the units of  $(\text{time})^{-1}$ , usually  $\text{sec}^{-1}$ .  $v$  is a function of  $[A]$  to the first power, or in the terminology of kinetics,  $v$  is first-order with respect to A. For an elementary reaction, the **order** for any reactant is given by its exponent in the rate equation. The number of molecules that must simultaneously interact is defined as the **molecularity** of the reaction. Thus, the simple elementary reaction of  $A \rightarrow P$  is a **first-order reaction**. Figure 13.4 portrays the course of a first-order reaction as a function of time. The rate of decay of a radioactive isotope, like  $^{14}\text{C}$  or  $^{32}\text{P}$ , is a first-order reaction, as is an intramolecular rearrangement, such as  $A \rightarrow P$ . Both are **unimolecular reactions** (the molecularity equals 1).

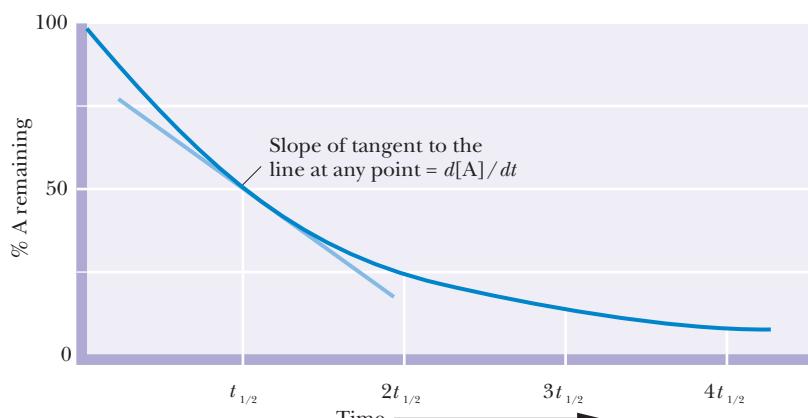
## Bimolecular Reactions Are Reactions Involving Two Reactant Molecules

Consider the more complex reaction, where two molecules must react to yield products:



Assuming this reaction is an elementary reaction, its molecularity is 2; that is, it is a **bimolecular reaction**. The velocity of this reaction can be determined from the rate of disappearance of either A or B, or the rate of appearance of P or Q:

$$v = \frac{-d[A]}{dt} = \frac{-d[B]}{dt} = \frac{d[P]}{dt} = \frac{d[Q]}{dt} \quad (13.3)$$



**FIGURE 13.4** Plot of the course of a first-order reaction.

The half-time,  $t_{1/2}$ , is the time for one-half of the starting amount of A to disappear.

The rate law is

$$v = k[A][B] \quad (13.4)$$

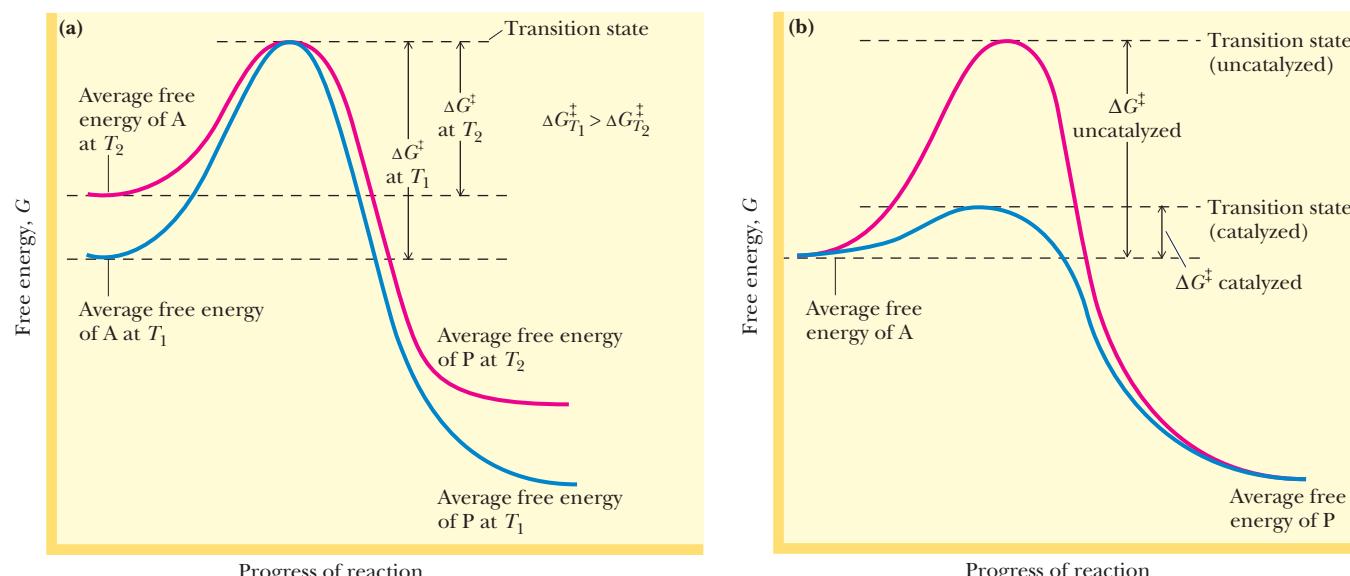
Since A and B must collide in order to react, the rate of their reaction will be proportional to the concentrations of both A and B. Because it is proportional to the product of two concentration terms, the reaction is **second-order** overall, first-order with respect to A and first-order with respect to B. (Were the elementary reaction  $2A \rightarrow P + Q$ , the rate law would be  $v = k[A]^2$ , second-order overall and second-order with respect to A.) Second-order rate constants have the units of  $(\text{concentration})^{-1}(\text{time})^{-1}$ , as in  $M^{-1} \text{ sec}^{-1}$ .

Molecularities greater than 2 are rarely found (and greater than 3, never). (The likelihood of simultaneous collision of three molecules is very, very small.) When the overall stoichiometry of a reaction is greater than two (for example, as in  $A + B + C \rightarrow$  or  $2A + B \rightarrow$ ), the reaction almost always proceeds via unimolecular or bimolecular elementary steps, and the overall rate obeys a simple first- or second-order rate law.

At this point, it may be useful to remind ourselves of an important caveat that is the first principle of kinetics: *Kinetics cannot prove a hypothetical mechanism*. Kinetic experiments can only rule out various alternative hypotheses because they do not fit the data. However, through thoughtful kinetic studies, a process of elimination of alternative hypotheses leads ever closer to the reality.

## Catalysts Lower the Free Energy of Activation for a Reaction

In a first-order chemical reaction, the conversion of A to P occurs because, at any given instant, a fraction of the A molecules has the energy necessary to achieve a reactive condition known as the **transition state**. In this state, the probability is very high that the particular rearrangement accompanying the  $A \rightarrow P$  transition will occur. This transition state sits at the apex of the energy profile in the energy diagram describing the energetic relationship between A and P (Figure 13.5). The average free energy of A molecules defines the initial state, and the average free energy of P molecules is the final state along the reaction coordinate. The rate of any chemical reaction is proportional to the concentration of reactant molecules (A in this case) having this transition-state energy. Obviously, the higher this energy is above the average energy, the smaller the fraction of molecules that will have this energy and the slower the reaction will proceed. The height of this energy barrier is called the **free energy of activation**,  $\Delta G^\ddagger$ . Specifically,  $\Delta G^\ddagger$  is the energy required to raise the average energy of 1 mol of reactant (at a given temperature)



**FIGURE 13.5** Energy diagram for a chemical reaction ( $A \rightarrow P$ ) and the effects of (a) raising the temperature from  $T_1$  to  $T_2$  or (b) adding a catalyst.

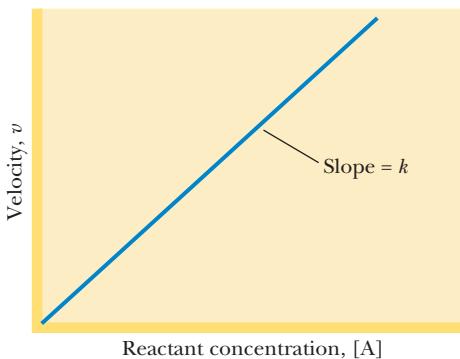
to the transition-state energy. The relationship between activation energy and the rate constant of the reaction,  $k$ , is given by the **Arrhenius equation**:

$$k = Ae^{-\Delta G^\ddagger/RT} \quad (13.5)$$

where  $A$  is a constant for a particular reaction (not to be confused with the reactant species, A, that we're discussing). Another way of writing this is  $1/k = (1/A)e^{\Delta G^\ddagger/RT}$ . That is,  $k$  is inversely proportional to  $e^{\Delta G^\ddagger/RT}$ . Therefore, if the energy of activation decreases, the reaction rate increases.

### Decreasing $\Delta G^\ddagger$ Increases Reaction Rate

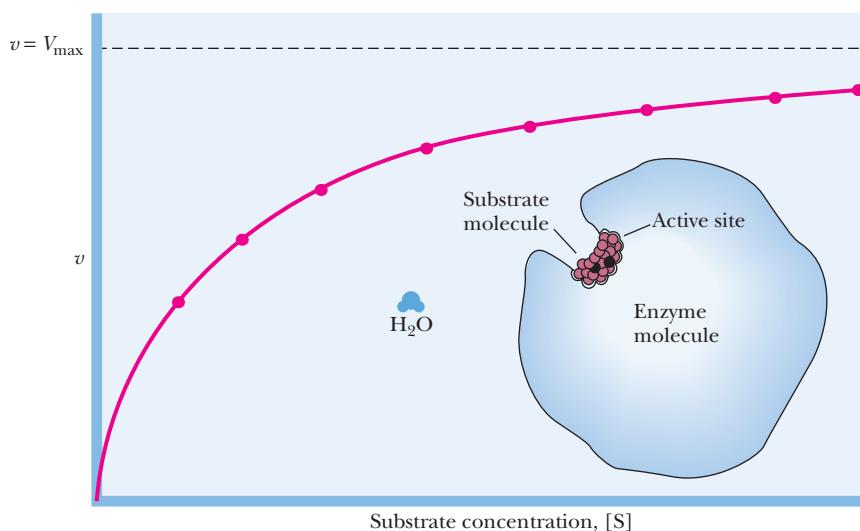
We are familiar with two general ways that rates of chemical reactions may be accelerated. First, the temperature can be raised. This will increase the kinetic energy of reactant molecules, and more reactant molecules will possess the energy to reach the transition state (Figure 13.5a). In effect, increasing the average energy of reactant molecules makes the energy difference between the average energy and the transition-state energy smaller. (Also note that the equation  $k = Ae^{-\Delta G^\ddagger/RT}$  demonstrates that  $k$  increases as  $T$  increases.) The rates of many chemical reactions are doubled by a 10°C rise in temperature. Second, the rates of chemical reactions can also be accelerated by catalysts. Catalysts work by lowering the energy of activation rather than by raising the average energy of the reactants (Figure 13.5b). Catalysts accomplish this remarkable feat by combining transiently with the reactants in a way that promotes their entry into the reactive, transition-state condition. Two aspects of catalysts are worth noting: (1) They are regenerated after each reaction cycle ( $A \rightarrow P$ ), and therefore can be used over and over again; and (2) catalysts have *no* effect on the overall free energy change in the reaction, the free energy difference between A and P (Figure 13.5b).



**FIGURE 13.6** A plot of  $v$  versus  $[A]$  for the unimolecular chemical reaction,  $A \rightarrow P$ , yields a straight line having a slope equal to  $k$ .

### 13.3 What Equations Define the Kinetics of Enzyme-Catalyzed Reactions?

Examination of the change in reaction velocity as the reactant concentration is varied is one of the primary measurements in kinetic analysis. Returning to  $A \rightarrow P$ , a plot of the reaction rate as a function of the concentration of A yields a straight line whose slope is  $k$  (Figure 13.6). The more A that is available, the greater the rate of the reaction,  $v$ . Similar analyses of enzyme-catalyzed reactions involving only a single substrate yield remarkably different results (Figure 13.7). At low concentrations of the substrate S,  $v$  is proportional to  $[S]$ , as expected for a first-order reaction. However,  $v$  does not increase



**FIGURE 13.7** Substrate saturation curve for an enzyme-catalyzed reaction. The amount of enzyme is constant, and the velocity of the reaction is determined at various substrate concentrations. The reaction rate,  $v$ , as a function of  $[S]$  is described mathematically by a rectangular hyperbola. The  $H_2O$  molecule provides a rough guide to scale.

proportionally as  $[S]$  increases, but instead begins to level off. At high  $[S]$ ,  $v$  becomes virtually independent of  $[S]$  and approaches a maximal limit. The value of  $v$  at this limit is written  $V_{\max}$ . Because rate is no longer dependent on  $[S]$  at these high concentrations, the enzyme-catalyzed reaction is now obeying **zero-order kinetics**; that is, the rate is independent of the reactant (substrate) concentration. This behavior is a **saturation effect**: When  $v$  shows no increase even though  $[S]$  is increased, the system is saturated with substrate. Such plots are called **substrate saturation curves**. The physical interpretation is that every enzyme molecule in the reaction mixture has its substrate-binding site occupied by S. Indeed, such curves were the initial clue that an enzyme interacts directly with its substrate by binding it.

## The Substrate Binds at the Active Site of an Enzyme

An enzyme molecule is often (but not always) orders of magnitude larger than its substrate. In any case, its **active site**, that place on the enzyme where S binds, comprises only a portion of the overall enzyme structure. The conformation of the active site is structured to form a special pocket or cleft whose three-dimensional architecture is complementary to the structure of the substrate. The enzyme and the substrate molecules “recognize” each other through this structural complementarity. The substrate binds to the enzyme through relatively weak forces—H bonds, ionic bonds (salt bridges), and van der Waals interactions between sterically complementary clusters of atoms.

## The Michaelis–Menten Equation Is the Fundamental Equation of Enzyme Kinetics

Leonor Michaelis and Maud L. Menten proposed a general theory of enzyme action in 1913 consistent with observed enzyme kinetics. Their theory was based on the assumption that the enzyme, E, and its substrate, S, associate reversibly to form an enzyme–substrate complex, ES:



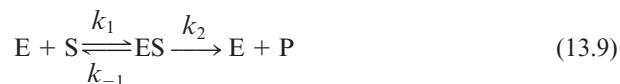
This association/dissociation is assumed to be a rapid equilibrium, and  $K_s$  is the *enzyme:substrate dissociation constant*. At equilibrium,

$$k_{-1}[ES] = k_1[E][S] \quad (13.7)$$

and

$$K_s = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1} \quad (13.8)$$

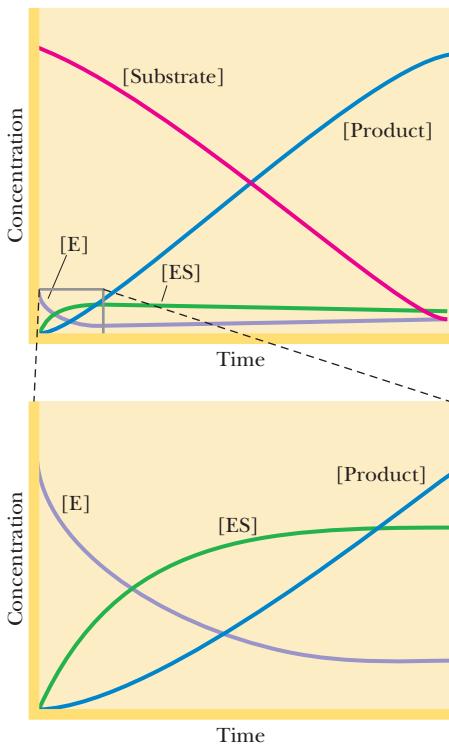
Product, P, is formed in a second step when ES breaks down to yield E + P.



E is then free to interact with another molecule of S.

## Assume That [ES] Remains Constant During an Enzymatic Reaction

The interpretations of Michaelis and Menten were refined and extended in 1925 by Briggs and Haldane, who assumed the concentration of the enzyme–substrate complex ES quickly reaches a constant value in such a dynamic system. That is, ES is formed as rapidly from E + S as it disappears by its two possible fates: dissociation to regenerate



**FIGURE 13.8** Time course for a typical enzyme-catalyzed reaction obeying the Michaelis-Menten, Briggs-Haldane models for enzyme kinetics. The early stage of the time course is shown in greater magnification in the bottom graph.

E + S and reaction to form E + P. This assumption is termed the **steady-state assumption** and is expressed as

$$\frac{d[ES]}{dt} = 0 \quad (13.10)$$

That is, the change in concentration of ES with time,  $t$ , is 0. Figure 13.8 illustrates the time course for formation of the ES complex and establishment of the steady-state condition.

### Assume That Velocity Measurements Are Made Immediately After Adding S

One other simplification will be advantageous. Because enzymes accelerate the rate of the reverse reaction as well as the forward reaction, it would be helpful to ignore any back reaction by which E + P might form ES. The velocity of this back reaction would be given by  $v = k_{-2}[E][P]$ . However, if we observe only the *initial velocity* for the reaction immediately after E and S are mixed in the absence of P, the rate of any back reaction is negligible because its rate will be proportional to [P], and [P] is essentially 0. Given such simplification, we now analyze the system described by Equation 13.9 in order to describe the initial velocity  $v$  as a function of [S] and amount of enzyme.

The total amount of enzyme is fixed and is given by the formula

$$\text{Total enzyme, } [E_T] = [E] + [ES] \quad (13.11)$$

where [E] is free enzyme and [ES] is the amount of enzyme in the enzyme–substrate complex. From Equation 13.9, the rate of [ES] formation is

$$v_f = k_1([E_T] - [ES])[S]$$

where

$$[E_T] - [ES] = [E] \quad (13.12)$$

From Equation 13.9, the rate of [ES] disappearance is

$$v_d = k_{-1}[ES] + k_2[ES] = (k_{-1} + k_2)[ES] \quad (13.13)$$

At steady state,  $d[ES]/dt = 0$ , and therefore,  $v_f = v_d$ . So,

$$k_1([E_T] - [ES])[S] = (k_{-1} + k_2)[ES] \quad (13.14)$$

Rearranging gives

$$\frac{([E_T] - [ES])[S]}{[ES]} = \frac{(k_{-1} + k_2)}{k_1} \quad (13.15)$$

### The Michaelis Constant, $K_m$ , Is Defined as $(k_{-1} + k_2)/k_1$

The ratio of constants  $(k_{-1} + k_2)/k_1$  is itself a constant and is defined as the **Michaelis constant**,  $K_m$

$$K_m = \frac{(k_{-1} + k_2)}{k_1} \quad (13.16)$$

Note from Equation 13.15 that  $K_m$  is given by the ratio of two concentrations ( $([E_T] - [ES])$  and  $[S]$ ) to one ( $[ES]$ ), so  $K_m$  has the units of *molarity*. (Also, because the units of  $k_{-1}$  and  $k_2$  are in  $\text{time}^{-1}$  and the units of  $k_1$  are  $M^{-1}\text{time}^{-1}$ , it becomes obvious that the units of  $K_m$  are  $M$ .) From Equation 13.15, we can write

$$\frac{([E_T] - [ES])[S]}{[ES]} = K_m \quad (13.17)$$

which rearranges to

$$[ES] = \frac{[E_T][S]}{K_m + [S]} \quad (13.18)$$

Now, the most important parameter in the kinetics of any reaction is the **rate of product formation**. This rate is given by

$$v = \frac{d[P]}{dt} \quad (13.19)$$

and for this reaction

$$v = k_2[ES] \quad (13.20)$$

Substituting the expression for [ES] from Equation 13.18 into Equation 13.20 gives

$$v = \frac{k_2[E_T][S]}{K_m + [S]} \quad (13.21)$$

The product  $k_2[E_T]$  has special meaning. When [S] is high enough to saturate all of the enzyme, the velocity of the reaction,  $v$ , is maximal. At saturation, the amount of [ES] complex is equal to the total enzyme concentration,  $E_T$ , its maximum possible value. From Equation 13.20, the initial velocity  $v$  then equals  $k_2[E_T] = V_{\max}$ . Written symbolically, when  $[S] \gg [E_T]$  (and  $K_m$ ),  $[E_T] = [ES]$  and  $v = V_{\max}$ . Therefore,

$$V_{\max} = k_2[E_T] \quad (13.22)$$

Substituting this relationship into the expression for  $v$  gives the **Michaelis–Menten equation**:

$$v = \frac{V_{\max}[S]}{K_m + [S]} \quad (13.23)$$

This equation says that the initial rate of an enzyme-catalyzed reaction,  $v$ , is determined by two constants,  $K_m$  and  $V_{\max}$ , and the initial concentration of substrate.

### When $[S] = K_m$ , $v = V_{\max}/2$

We can provide an operational definition for the constant  $K_m$  by rearranging Equation 13.23 to give

$$K_m = [S] \left( \frac{V_{\max}}{v} - 1 \right) \quad (13.24)$$

Then, at  $v = V_{\max}/2$ ,  $K_m = [S]$ . That is,  $K_m$  is defined by the substrate concentration that gives a velocity equal to one-half the maximal velocity. Table 13.3 gives the  $K_m$  values of some enzymes for their substrates.

### Plots of $v$ Versus $[S]$ Illustrate the Relationships Between $V_{\max}$ , $K_m$ , and Reaction Order

The Michaelis–Menten equation (Equation 13.23) describes a curve known from analytical geometry as a *rectangular hyperbola*. In such curves, as [S] is increased,  $v$  approaches the limiting value,  $V_{\max}$ , in an asymptotic fashion.  $V_{\max}$  can be approximated experimentally from a substrate saturation curve (Figure 13.7), and  $K_m$  can be derived from  $V_{\max}/2$ , so the two constants of the Michaelis–Menten equation can be obtained from plots of  $v$  versus [S]. Note, however, that actual estimation of  $V_{\max}$ , and consequently  $K_m$ , is only approximate from such graphs. That is, according to Equation 13.23, to get  $v = 0.99 V_{\max}$ , [S] must equal  $99 K_m$ , a concentration that may be difficult to achieve in practice.

From Equation 13.23, when  $[S] \gg K_m$ , then  $v = V_{\max}$ . That is,  $v$  is no longer dependent on [S], so the reaction is obeying zero-order kinetics. Also, when  $[S] < K_m$ , then  $v \approx (V_{\max}/K_m)[S]$ . That is, the rate,  $v$ , approximately follows a first-order rate equation,  $v = k'[A]$ , where  $k' = V_{\max}/K_m$ .

**TABLE 13.3**  $K_m$  Values for Some Enzymes

| Enzyme                     | Substrate                     | $K_m$ (mM) |
|----------------------------|-------------------------------|------------|
| Carbonic anhydrase         | CO <sub>2</sub>               | 12         |
| Chymotrypsin               | N-Benzoyltyrosinamide         | 2.5        |
|                            | Acetyl-L-tryptophanamide      | 5          |
|                            | N-Formyltyrosinamide          | 12         |
|                            | N-Acetyltyrosinamide          | 32         |
|                            | Glycyltyrosinamide            | 122        |
|                            |                               |            |
| Hexokinase                 | Glucose                       | 0.15       |
|                            | Fructose                      | 1.5        |
| $\beta$ -Galactosidase     | Lactose                       | 4          |
|                            |                               |            |
| Glutamate dehydrogenase    | NH <sub>4</sub> <sup>+</sup>  | 57         |
|                            | Glutamate                     | 0.12       |
|                            | $\alpha$ -Ketoglutarate       | 2          |
|                            | NAD <sup>+</sup>              | 0.025      |
|                            | NADH                          | 0.018      |
| Aspartate aminotransferase | Aspartate                     | 0.9        |
|                            | $\alpha$ -Ketoglutarate       | 0.1        |
|                            | Oxaloacetate                  | 0.04       |
|                            | Glutamate                     | 4          |
| Threonine deaminase        | Threonine                     | 5          |
|                            |                               |            |
| Arginyl-tRNA synthetase    | Arginine                      | 0.003      |
|                            | tRNA <sup>Arg</sup>           | 0.0004     |
|                            | ATP                           | 0.3        |
| Pyruvate carboxylase       | HCO <sub>3</sub> <sup>-</sup> | 1.0        |
|                            | Pyruvate                      | 0.4        |
|                            | ATP                           | 0.06       |
| Penicillinase              | Benzylpenicillin              | 0.05       |
|                            |                               |            |
| Lysozyme                   | Hexa-N-acetylglucosamine      | 0.006      |

$K_m$  and  $V_{\max}$ , once known explicitly, define the rate of the enzyme-catalyzed reaction, provided:

1. The reaction involves only one substrate, or if the reaction is multisubstrate, the concentration of only one substrate is varied while the concentrations of all other substrates are held constant.
2. The reaction ES→E + P is irreversible, or the experiment is limited to observing only initial velocities where [P] = 0.
3. [S]<sub>0</sub> > [E]<sub>T</sub> and [E]<sub>T</sub> is held constant.
4. All other variables that might influence the rate of the reaction (temperature, pH, ionic strength, and so on) are held constant.

### Turnover Number Defines the Activity of One Enzyme Molecule

The **turnover number** of an enzyme,  $k_{\text{cat}}$ , is a measure of its maximal catalytic activity.  $k_{\text{cat}}$  is defined as the number of substrate molecules converted into product per enzyme molecule per unit time when the enzyme is saturated with substrate. The turnover number is also referred to as the **molecular activity** of the enzyme. For the simple Michaelis–Menten reaction (Equation 13.9) under conditions of initial velocity measurements,

$k_2 = k_{\text{cat}}$ . Provided the concentration of enzyme,  $[E_T]$ , in the reaction mixture is known,  $k_{\text{cat}}$  can be determined from  $V_{\text{max}}$ . At saturating  $[S]$ ,  $v = V_{\text{max}} = k_2 [E_T]$ . Thus,

$$k_2 = \frac{V_{\text{max}}}{[E_T]} = k_{\text{cat}} \quad (13.25)$$

The term  $k_{\text{cat}}$  represents the kinetic efficiency of the enzyme. Table 13.4 lists turnover numbers for some representative enzymes. Catalase has the highest turnover number known; each molecule of this enzyme can degrade 40 million molecules of  $\text{H}_2\text{O}_2$  in 1 second! At the other end of the scale, lysozyme requires 2 seconds to cleave a glycosidic bond in its glycan substrate.

In many situations, the actual molar amount of the enzyme is not known. However, its amount can be expressed in terms of the activity observed. The International Commission on Enzymes defines **one international unit** as the amount that catalyzes the formation of 1 micromole of product in 1 minute. (Because enzymes are very sensitive to factors such as pH, temperature, and ionic strength, the conditions of assay must be specified.) In the process of purifying enzymes from cellular sources, many extraneous proteins may be present. Then, the units of enzyme activity are expressed as enzyme units per mg protein, a term known as **specific activity** (see Table 5.1).

| Enzyme                | Values of $k_{\text{cat}}$ (Turnover Number) for Some Enzymes |
|-----------------------|---|
| Catalase              | 40,000,000  |
| Carbonic anhydrase    | 1,000,000   |
| Acetylcholinesterase  | 14,000  |
| Penicillinase         | 2,000   |
| Lactate dehydrogenase | 1,000   |
| Chymotrypsin          | 100   |
| DNA polymerase I      | 15  |
| Lysozyme              | 0.5   |

## The Ratio, $k_{\text{cat}}/K_m$ , Defines the Catalytic Efficiency of an Enzyme

Under physiological conditions,  $[S]$  is seldom saturating and  $k_{\text{cat}}$  itself is not particularly informative. That is, the in vivo ratio of  $[S]/K_m$  usually falls in the range of 0.01 to 1.0, so active sites often are not filled with substrate. Nevertheless, we can derive a meaningful index of the efficiency of Michaelis–Menten-type enzymes under these conditions by using the following equations. As presented in Equation 13.23, if

$$v = \frac{V_{\text{max}}[S]}{K_m + [S]}$$

and  $V_{\text{max}} = k_{\text{cat}} [E_T]$ , then

$$v = \frac{k_{\text{cat}}[E_T][S]}{K_m + [S]} \quad (13.26)$$

When  $[S] \ll K_m$ , the concentration of free enzyme,  $[E]$ , is approximately equal to  $[E_T]$ , so

$$v = \left( \frac{k_{\text{cat}}}{K_m} \right) [E][S] \quad (13.27)$$

That is,  $k_{\text{cat}}/K_m$  is an apparent second-order rate constant for the reaction of E and S to form product. Because  $K_m$  is inversely proportional to the affinity of the enzyme for its substrate and  $k_{\text{cat}}$  is directly proportional to the kinetic efficiency of the enzyme,  $k_{\text{cat}}/K_m$  provides an index of the catalytic efficiency of an enzyme operating at substrate concentrations substantially below saturation amounts.

An interesting point emerges if we restrict ourselves to the simple case where  $k_{\text{cat}} = k_2$ . Then

$$\frac{k_{\text{cat}}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2} \quad (13.28)$$

But  $k_1$  must always be greater than or equal to  $k_1 k_2 / (k_{-1} + k_2)$ . That is, the reaction can go no faster than the rate at which E and S come together. Thus,  $k_1$  sets the upper limit for  $k_{\text{cat}}/K_m$ . In other words, the catalytic efficiency of an enzyme cannot exceed the diffusion-controlled rate of combination of E and S to form ES. In  $\text{H}_2\text{O}$ , the rate constant for such diffusion is approximately  $10^9/M \cdot \text{sec}$  for small substrates (for example, glyceraldehyde 3-P) and an order of magnitude smaller ( $\approx 10^8/M \cdot \text{sec}$ ) for substrates the size of nucleotides. Those enzymes that are most efficient in their catalysis have  $k_{\text{cat}}/K_m$  ratios

| Enzyme                    | Substrate                     | $k_{\text{cat}}$<br>(sec $^{-1}$ ) | $K_m$<br>(M)         | $k_{\text{cat}}/K_m$<br>(M $^{-1}$ sec $^{-1}$ ) |
|---------------------------|-------------------------------|------------------------------------|----------------------|--|
| Acetylcholinesterase      | Acetylcholine                 | $1.4 \times 10^4$                  | $9 \times 10^{-5}$   | $1.6 \times 10^8$                                |
| Carbonic anhydrase        | CO <sub>2</sub>               | $1 \times 10^6$                    | 0.012                | $8.3 \times 10^7$                                |
|                           | HCO <sub>3</sub> <sup>-</sup> | $4 \times 10^5$                    | 0.026                | $1.5 \times 10^7$                                |
| Catalase                  | H <sub>2</sub> O <sub>2</sub> | $4 \times 10^7$                    | 1.1                  | $4 \times 10^7$                                  |
| Crotonase                 | Crotonyl-CoA                  | $5.7 \times 10^3$                  | $2 \times 10^{-5}$   | $2.8 \times 10^8$                                |
| Fumarase                  | Fumarate                      | 800                                | $5 \times 10^{-6}$   | $1.6 \times 10^8$                                |
|                           | Malate                        | 900                                | $2.5 \times 10^{-5}$ | $3.6 \times 10^7$                                |
| Triosephosphate isomerase | Glyceraldehyde-3-phosphate*   | $4.3 \times 10^3$                  | $1.8 \times 10^{-5}$ | $2.4 \times 10^8$                                |
| $\beta$ -Lactamase        | Benzylpenicillin              | $2 \times 10^3$                    | $2 \times 10^{-5}$   | $1 \times 10^8$                                  |

\* $K_m$  for glyceraldehyde-3-phosphate is calculated on the basis that only 3.8% of the substrate in solution is unhydrated and therefore reactive with the enzyme.

Adapted from Fersht, A., 1985. *Enzyme Structure and Mechanism*, 2nd ed. New York: W. H. Freeman.

approaching this value. Their catalytic velocity is limited only by the rate at which they encounter S; enzymes this efficient have achieved so-called catalytic perfection. All E and S encounters lead to reaction because such “catalytically perfect” enzymes can channel S to the active site, regardless of where S hits E. Table 13.5 lists the kinetic parameters of several enzymes in this category. Note that  $k_{\text{cat}}$  and  $K_m$  both show a substantial range of variation in this table, even though their ratio falls around  $10^8/M \cdot \text{sec}$ .

### Linear Plots Can Be Derived from the Michaelis–Menten Equation

Because of the hyperbolic shape of  $v$  versus [S] plots,  $V_{\text{max}}$  can be determined only from an extrapolation of the asymptotic approach of  $v$  to some limiting value as [S] increases indefinitely (Figure 13.7); and  $K_m$  is derived from that value of [S] giving  $v = V_{\text{max}}/2$ . However, several rearrangements of the Michaelis–Menten equation transform it into a straight-line equation. The best known of these is the **Lineweaver–Burk double-reciprocal plot**:

Taking the reciprocal of both sides of the Michaelis–Menten equation, Equation 13.23, yields the equality

$$\frac{1}{v} = \left( \frac{K_m}{V_{\text{max}}} \right) \left( \frac{1}{[\text{S}]} \right) + \frac{1}{V_{\text{max}}} \quad (13.29)$$

This conforms to  $y = mx + b$  (the equation for a straight line), where  $y = 1/v$ ;  $m$ , the slope, is  $K_m/V_{\text{max}}$ ;  $x = 1/[\text{S}]$ ; and  $b = 1/V_{\text{max}}$ . Plotting  $1/v$  versus  $1/[\text{S}]$  gives a straight line whose  $x$ -intercept is  $-1/K_m$ , whose  $y$ -intercept is  $1/V_{\text{max}}$ , and whose slope is  $K_m/V_{\text{max}}$  (Figure 13.9).

The **Hanes–Woolf plot** is another rearrangement of the Michaelis–Menten equation that yields a straight line:

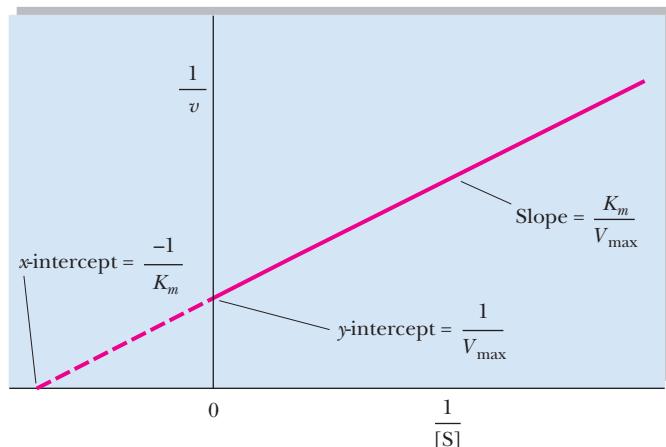
Multiplying both sides of Equation 13.29 by [S] gives

$$\frac{[\text{S}]}{v} = [\text{S}] \left( \frac{K_m}{V_{\text{max}}} \right) \left( \frac{1}{[\text{S}]} \right) + \frac{[\text{S}]}{V_{\text{max}}} = \frac{K_m}{V_{\text{max}}} + \frac{[\text{S}]}{V_{\text{max}}} \quad (13.30)$$

and

$$\frac{[\text{S}]}{v} = \left( \frac{1}{V_{\text{max}}} \right) [\text{S}] + \frac{K_m}{V_{\text{max}}} \quad (13.31)$$

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \left( \frac{1}{[S]} \right) + \frac{1}{V_{\max}}$$



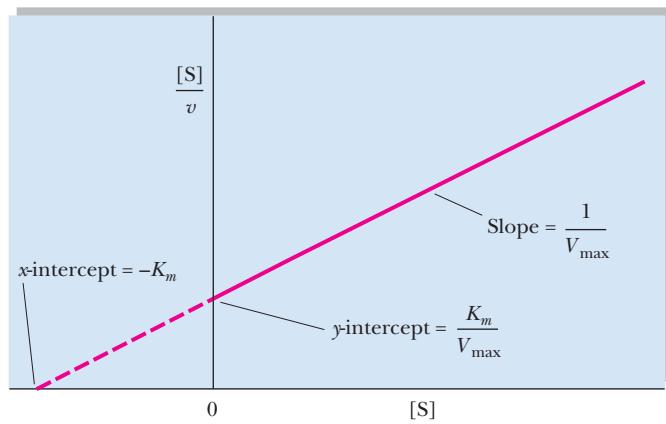
**FIGURE 13.9** The Lineweaver–Burk double-reciprocal plot.

Graphing  $[S]/v$  versus  $[S]$  yields a straight line where the slope is  $1/V_{\max}$ , the  $y$ -intercept is  $K_m/V_{\max}$ , and the  $x$ -intercept is  $-K_m$ , as shown in Figure 13.10. The Hanes–Woolf plot has the advantage of not overemphasizing the data obtained at low  $[S]$ , a fault inherent in the Lineweaver–Burk plot. The common advantage of these plots is that they allow both  $K_m$  and  $V_{\max}$  to be accurately estimated by extrapolation of straight lines rather than asymptotes. Computer fitting of  $v$  versus  $[S]$  data to the Michaelis–Menten equation is more commonly done than graphical plotting.

### Nonlinear Lineweaver–Burk or Hanes–Woolf Plots Are a Property of Regulatory Enzymes

If the kinetics of the reaction disobey the Michaelis–Menten equation, the violation is revealed by a departure from linearity in these straight-line graphs. We shall see in the next chapter that such deviations from linearity are characteristic of the kinetics of regulatory enzymes known as **allosteric enzymes**. Such regulatory enzymes are very important in the overall control of metabolic pathways.

$$\frac{[S]}{v} = \left( \frac{1}{V_{\max}} \right) [S] + \frac{K_m}{V_{\max}}$$

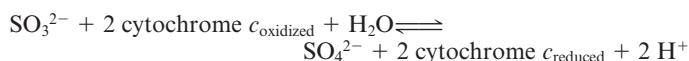


**FIGURE 13.10** A Hanes–Woolf plot of  $[S]/v$  versus  $[S]$ .

## A DEEPER LOOK

### An Example of the Effect of Amino Acid Substitutions on $K_m$ and $k_{cat}$ : Wild-Type and Mutant Forms of Human Sulfite Oxidase

Mammalian sulfite oxidase is the last enzyme in the pathway for degradation of sulfur-containing amino acids. Sulfite oxidase (SO) catalyzes the oxidation of sulfite ( $\text{SO}_3^{2-}$ ) to sulfate ( $\text{SO}_4^{2-}$ ), using the heme-containing protein, cytochrome *c*, as electron acceptor:



*Isolated sulfite oxidase deficiency* is a rare and often fatal genetic disorder in humans. The disease is characterized by severe neurological abnormalities, revealed as convulsions shortly after birth. R. M. Garrett and K. V. Rajagopalan at Duke University Medical Center have isolated the human cDNA for sulfite oxidase from the cells of normal (*wild-type*) and SO-deficient individuals. Expression of these SO cDNAs in transformed *Escherichia coli* cells allowed the isolation and kinetic analysis of wild-type and mutant forms of SO, including one (designated R160Q) in which the Arg at position 160 in the polypeptide chain is replaced by Gln. A genetically engineered version of SO (designated R160K) in which Lys replaces Arg<sup>160</sup> was also studied.

| Kinetic Constants for Wild-Type and Mutant Sulfite Oxidase |                                 |                                 |  |
|--|---------------------------------|---------------------------------|--|
| Enzyme   | $K_m$ sulfite ( $\mu\text{M}$ ) | $k_{cat}$ ( $\text{sec}^{-1}$ ) | $k_{cat}/K_m$ ( $10^6 \text{ M}^{-1} \text{ sec}^{-1}$ ) |
| Wild-type  | 17                              | 18                              | 1.1  |
| R160Q  | 1900                            | 3                               | 0.0016   |
| R160K  | 360                             | 5.5                             | 0.015  |

Replacing R<sup>160</sup> in sulfite oxidase by Q increases  $K_m$ , decreases  $k_{cat}$ , and markedly diminishes the catalytic efficiency ( $k_{cat}/K_m$ ) of the enzyme. The R160K mutant enzyme has properties intermediate between wild-type and the R160Q mutant form. The substrate,  $\text{SO}_3^{2-}$ , is strongly anionic, and R<sup>160</sup> is one of several Arg residues situated within the SO substrate-binding site. Positively charged side chains in the substrate-binding site facilitate  $\text{SO}_3^{2-}$  binding and catalysis, with Arg being optimal in this role.

### Enzymatic Activity Is Strongly Influenced by pH

Enzyme–substrate recognition and the catalytic events that ensue are greatly dependent on pH. An enzyme possesses an array of ionizable side chains and prosthetic groups that not only determine its secondary and tertiary structure but may also be intimately involved in its active site. Furthermore, the substrate itself often has ionizing groups, and one or another of the ionic forms may preferentially interact with the enzyme. Enzymes in general are active only over a limited pH range, and most have a particular pH at which their catalytic activity is optimal. These effects of pH may be due to effects on  $K_m$  or  $V_{max}$  or both. Figure 13.11 illustrates the relative activity of four enzymes as a function of pH. Trypsin, an intestinal protease, has a slightly alkaline pH optimum, whereas pepsin, a gastric protease, acts in the acidic confines of the stomach and has a pH optimum near 2. Papain, a protease found in papaya, is relatively insensitive to pHs between 4 and 8. Cholinesterase activity is pH-sensitive below pH 7 but not between pH 7 and 10. The cholinesterase activity-pH profile suggests that an ionizable group with a  $pK_a$  near 6 is essential to its activity. Might this group be a histidine side chain within its

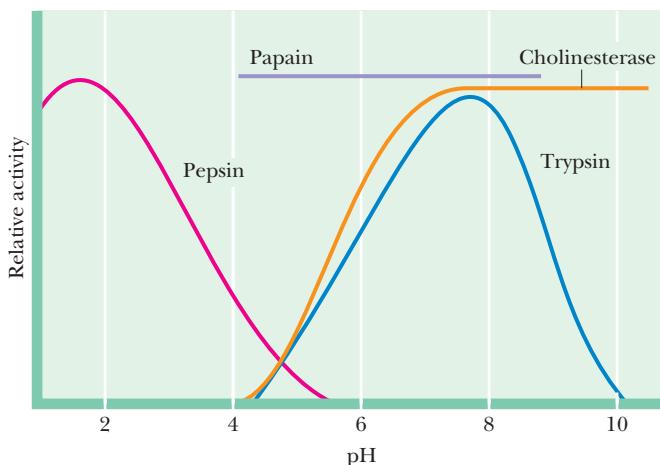


FIGURE 13.11 The pH activity profiles of four different enzymes.

| Optimum pH of Some Enzymes |            |
|----------------------------|------------|
| Enzyme                     | Optimum pH |
| Pepsin                     | 1.5        |
| Catalase                   | 7.6        |
| Trypsin                    | 7.7        |
| Fumarase                   | 7.8        |
| Ribonuclease               | 7.8        |
| Arginase                   | 9.7        |

active site? Although the pH optimum of an enzyme often reflects the pH of its normal environment, the optimum may not be precisely the same. This difference suggests that the pH-activity response of an enzyme may be a factor in the intracellular regulation of its activity.

## The Response of Enzymatic Activity to Temperature Is Complex

Like most chemical reactions, the rates of enzyme-catalyzed reactions generally increase with increasing temperature. However, at temperatures above 50° to 60°C, enzymes typically show a decline in activity (Figure 13.12). Several effects are operating here: (1) the characteristic increase in reaction rate with temperature; (2) a temperature-dependent equilibrium between active enzyme ( $E_{\text{active}}$ ) and the catalytically inactive but not denatured state of the enzyme ( $E_{\text{inact}}$ ); and (3) thermal denaturation of protein structure at higher temperatures. An equilibrium model of these effects proposed by Roy M. Daniel and Michael J. Danson fits well with experimentally observed responses of enzyme activity versus temperature,



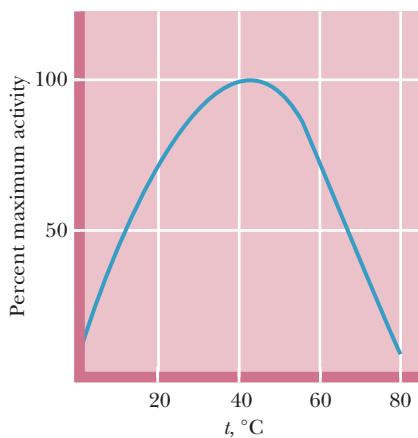
Where X designates the irreversibly inactivated form of the enzyme and  $k_{\text{inact}}$  is the first-order rate constant for thermal denaturation. Most enzymatic reactions double in rate for every 10°C rise in temperature (that is,  $Q_{10} = 2$ , where  $Q_{10}$  is defined as *the ratio of activities at two temperatures 10° apart*) as long as the enzyme is stable and fully active. Some enzymes, those catalyzing reactions having very high activation energies, show proportionally greater  $Q_{10}$  values. The increasing rate with increasing temperature is ultimately offset by the temperature-dependent equilibrium between active and inactive states of the native enzyme, and, ultimately, the denaturation of protein structure at elevated temperatures. The fit of this model with the experimental data supports the existence of  $E_{\text{inact}}$ . Indeed,  $E_{\text{inact}}$  reflects the intrinsic flexibility, and, hence, greater thermal sensitivity of the active site, compared to the rest of the enzyme's structure. Not all enzymes are quite so thermally labile. For example, the enzymes of thermophilic prokaryotes (*thermophilic* = “heat-loving”) found in geothermal springs retain full activity at temperatures in excess of 85°C.

## 13.4 | What Can Be Learned from the Inhibition of Enzyme Activity?

If the velocity of an enzymatic reaction is decreased or **inhibited** by some agent, the kinetics of the reaction obviously have been perturbed. Systematic perturbations are a basic tool of experimental scientists; much can be learned about the normal workings of any system by inducing changes in it and then observing the effects of the change. The study of enzyme inhibition has contributed significantly to our understanding of enzymes.

### Enzymes May Be Inhibited Reversibly or Irreversibly

Enzyme inhibitors are classified in several ways. The inhibitor may interact either reversibly or irreversibly with the enzyme. **Reversible inhibitors** interact with the enzyme through noncovalent association/dissociation reactions. In contrast, **irreversible inhibitors** usually form covalent bonds with side chains or prosthetic groups in the enzyme. That is, the consequence of irreversible inhibition is a decrease in the concentration of active enzyme. The kinetics observed are consistent with this interpretation, as we shall see later.



**FIGURE 13.12** The effect of temperature on enzyme activity.

## Reversible Inhibitors May Bind at the Active Site or at Some Other Site

Reversible inhibitors fall into three major categories: competitive, noncompetitive, and uncompetitive. **Competitive inhibitors** are characterized by the fact that the substrate and inhibitor compete for the same binding site on the enzyme, the so-called **active site** or **substrate-binding site**. Thus, increasing the concentration of S favors the likelihood of S binding to the enzyme instead of the inhibitor, I. That is, high [S] can overcome the effects of I. The effects of the other major types, noncompetitive and uncompetitive inhibition, cannot be overcome by increasing [S]. The three types can be distinguished by the particular patterns obtained when the kinetic data are analyzed in linear plots, such as double-reciprocal (Lineweaver–Burk) plots. A general formulation for common inhibitor interactions in our simple enzyme kinetic model would include



### Competitive Inhibition

Consider the following system:



where an inhibitor, I, binds *reversibly* to the enzyme at the same site as S. S-binding and I-binding are mutually exclusive, *competitive* processes. Formation of the ternary complex, IES, where both S and I are bound, is physically impossible. This condition leads us to anticipate that S and I must share a high degree of structural similarity because they bind at the same site on the enzyme. Also notice that, in our model, EI does not react to give rise to E + P. That is, I is not changed by interaction with E. The rate of the product-forming reaction is  $v = k_2[ES]$ .

It is revealing to compare the equation for the uninhibited case, Equation 13.23 (the Michaelis–Menten equation) with Equation 13.43 for the rate of the enzymatic reaction in the presence of a fixed concentration of the competitive inhibitor, [I]

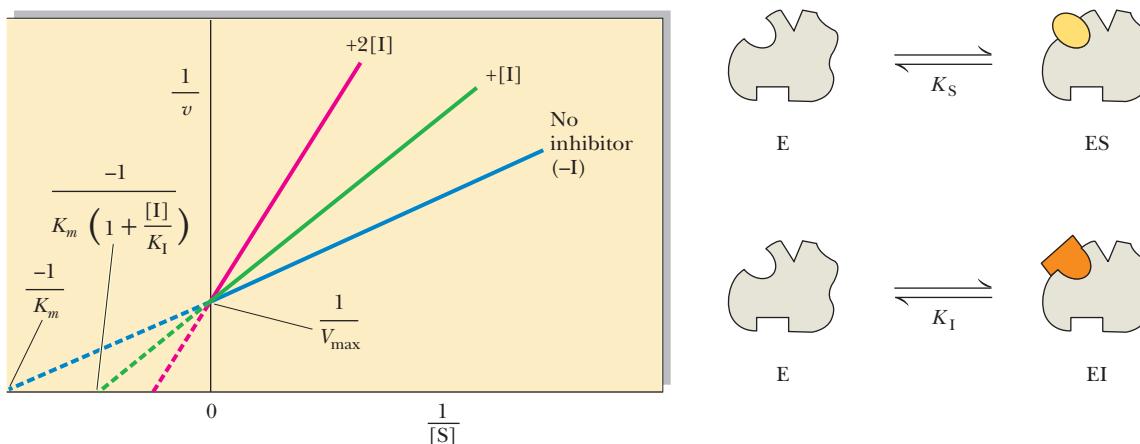
$$v = \frac{V_{\max}[S]}{K_m + [S]}$$

$$v = \frac{V_{\max}[S]}{[S] + K_m \left(1 + \frac{[I]}{K_I}\right)}$$

(see also Table 13.6). The  $K_m$  term in the denominator in the inhibited case is increased by the factor  $(1 + [I]/K_I)$ ; thus,  $v$  is less in the presence of the inhibitor, as expected. Clearly, in the absence of I, the two equations are identical. Figure 13.13 shows a Lineweaver–Burk plot of competitive inhibition. Several features of competitive inhibition are evident. First, at a given [I],  $v$  decreases ( $1/v$  increases). When [S] becomes infinite,  $v = V_{\max}$  and is unaffected by I because all of the enzyme is in the ES form. Note that the value of the  $-x$ -intercept decreases as [I] increases. This  $-x$ -intercept is often termed the *apparent  $K_m$*  (or  $K_{mapp}$ ) because it is the  $K_m$  apparent under these conditions.

| <b>TABLE 13.6</b> The Effect of Various Types of Inhibitors on the Michaelis–Menten Rate Equation and on Apparent $K_m$ and Apparent $V_{\max}$ |  |                                   |                           |
|---|--|-----------------------------------|---------------------------|
| Inhibition Type   | Rate Equation  | Apparent $K_m$                    | Apparent $V_{\max}$       |
| None  | $v = V_{\max}[S]/(K_m + [S])$                            | $K_m$                             | $V_{\max}$                |
| Competitive   | $v = V_{\max}[S]/([S] + K_m(1 + [I]/K_I))$               | $K_m(1 + [I]/K_I)$                | $V_{\max}$                |
| Noncompetitive  | $v = (V_{\max}[S]/(1 + [I]/K_I))/(K_m + [S])$            | $K_m$                             | $V_{\max}/(1 + [I]/K_I)$  |
| Mixed   | $v = V_{\max}[S]/((1 + [I]/K_I)K_m + (1 + [I]/K_I'[S]))$ | $K_m(1 + [I]/K_I)/(1 + [I]/K_I')$ | $V_{\max}/(1 + [I]/K_I')$ |
| Uncompetitive   | $v = V_{\max}[S]/(K_m + [S](1 + [I]/K_I'))$              | $K_m/(1 + [I]/K_I')$              | $V_{\max}/(1 + [I]/K_I')$ |

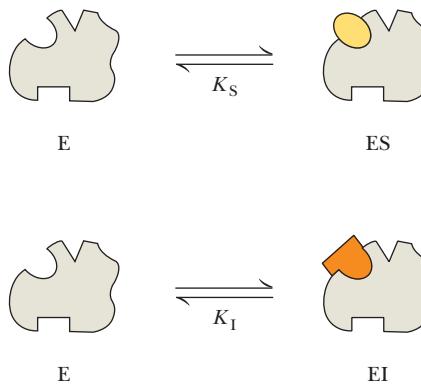
$K_I$  is defined as the enzyme:inhibitor dissociation constant  $K_I = [E][I]/[EI]$ ;  $K_I'$  is defined as the enzyme–substrate complex:inhibitor dissociation constant  $K_I' = [ES][I]/[IES]$ .



**FIGURE 13.13** Lineweaver–Burk plot of competitive inhibition, showing lines for no I, [I], and 2[I]. Note that when [S] is infinitely large ( $1/[S] \approx 0$ ),  $V_{\max}$  is the same, whether I is present or not.

The diagnostic criterion for competitive inhibition is that  $V_{\max}$  is unaffected by I; that is, all lines share a common  $y$ -intercept. This criterion is also the best experimental indication of binding at the same site by two substances. Competitive inhibitors resemble S structurally.

**Succinate Dehydrogenase—A Classic Example of Competitive Inhibition** The enzyme *succinate dehydrogenase* (*SDH*) is competitively inhibited by malonate. Figure 13.14 shows the structures of succinate and malonate. The structural similarity between them is obvious and is the basis of malonate’s ability to mimic succinate and bind at the



### A DEEPER LOOK

## The Equations of Competitive Inhibition

Given the relationships between E, S, and I described previously and recalling the steady-state assumption that  $d[ES]/dt = 0$ , from Equations (13.14) and (13.16) we can write

$$ES = \frac{k_1[E][S]}{(k_2 + k_{-1})} = \frac{[E][S]}{K_m} \quad (13.34)$$

Assuming that  $E + I \rightleftharpoons EI$  reaches rapid equilibrium, the rate of EI formation,  $v_f' = k_3[E][I]$ , and the rate of disappearance of EI,  $v_d' = k_{-3}[EI]$ , are equal. So,

$$k_3[E][I] = k_{-3}[EI] \quad (13.35)$$

Therefore,

$$[EI] = \frac{k_3}{k_{-3}} [E][I] \quad (13.36)$$

If we define  $K_I$  as  $k_{-3}/k_3$ , an enzyme-inhibitor dissociation constant, then

$$[EI] = \frac{[E][I]}{K_I} \quad (13.37)$$

knowing  $[E_T] = [E] + [ES] + [EI]$ . Then

$$[E_T] = [E] + \frac{[E][S]}{K_m} + \frac{[E][I]}{K_I} \quad (13.38)$$

Solving for [E] gives

$$[E] = \frac{K_I K_m [E_T]}{(K_I K_m + K_I [S] + K_m [I])} \quad (13.39)$$

Because the rate of product formation is given by  $v = k_2[ES]$ , from Equation 13.34 we have

$$v = \frac{k_2 [E][S]}{K_m} \quad (13.40)$$

So,

$$v = \frac{(k_2 K_I [E_T][S])}{(K_I K_m + K_I [S] + K_m [I])} \quad (13.41)$$

Because  $V_{\max} = k_2[E_T]$ ,

$$v = \frac{V_{\max}[S]}{K_m + [S] + \frac{K_m[I]}{K_I}} \quad (13.42)$$

or

$$v = \frac{V_{\max}[S]}{[S] + K_m \left(1 + \frac{[I]}{K_I}\right)} \quad (13.43)$$

| Substrate  | Product   | Competitive inhibitor  |
|--|---|--|
| $\begin{array}{c} \text{COO}^- \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{COO}^- \end{array}$ | $\xrightarrow[\text{2H}]{\text{SDH}} \begin{array}{c} \text{COO}^- \\   \\ \text{CH} \\    \\ \text{HC} \\   \\ \text{COO}^- \end{array}$ | $\begin{array}{c} \text{COO}^- \\   \\ \text{CH}_2 \\   \\ \text{COO}^- \end{array}$ |

Succinate                      Fumarate                      Malonate

**FIGURE 13.14** Structures of succinate, the substrate of succinate dehydrogenase (SDH), and malonate, the competitive inhibitor. Fumarate (the product of SDH action on succinate) is also shown.

active site of SDH. However, unlike succinate, which is oxidized by SDH to form fumarate, malonate cannot lose two hydrogens; consequently, it is unreactive.

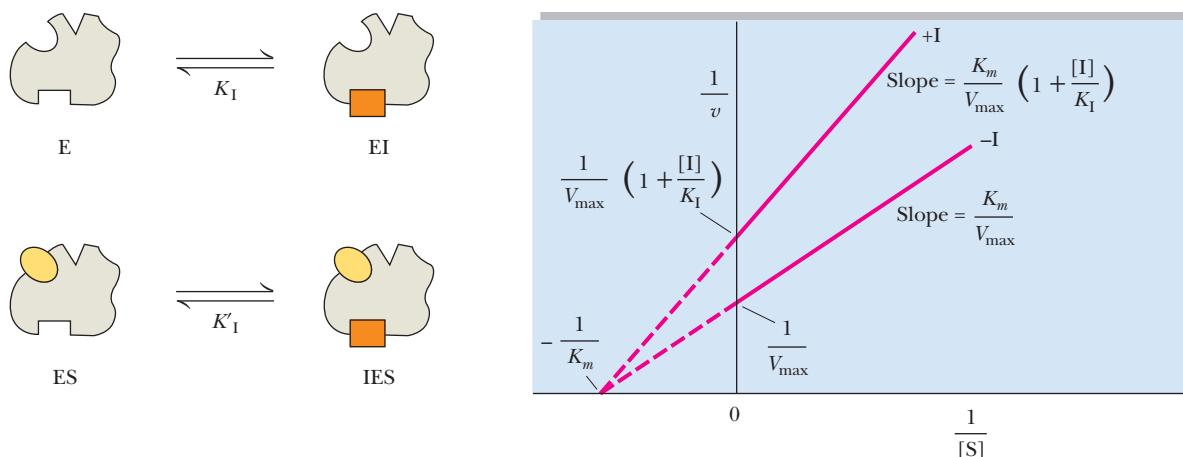
**Noncompetitive Inhibition** Noncompetitive inhibitors interact with both E and ES (or with S and ES, but this is a rare and specialized case). Obviously, then, the inhibitor is not binding to the same site as S, and the inhibition cannot be overcome by raising [S]. There are two types of noncompetitive inhibition: pure and mixed.

**Pure Noncompetitive Inhibition** In this situation, the binding of I by E has no effect on the binding of S by E. That is, S and I bind at different sites on E, and binding of I does not affect binding of S. Consider the system

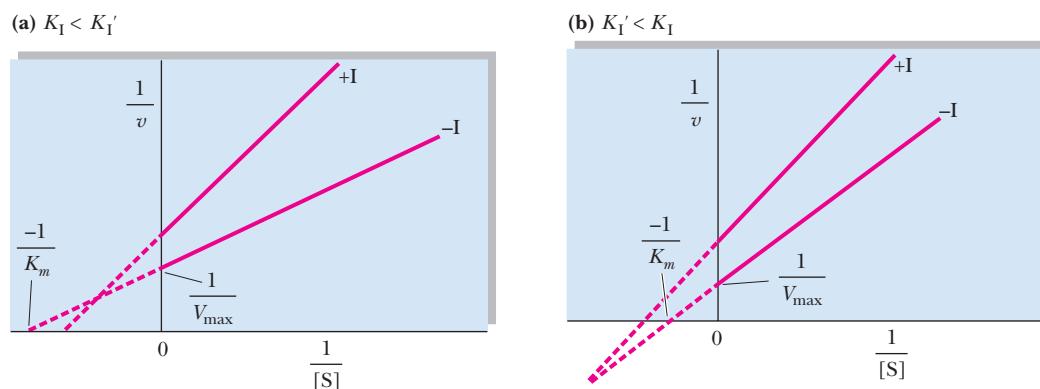


Pure noncompetitive inhibition occurs if  $K_I = K'_I$ . This situation is relatively uncommon; the Lineweaver–Burk plot for such an instance is given in Figure 13.15. Note that  $K_m$  is unchanged by I (the  $x$ -intercept remains the same, with or without I). Note also that the apparent  $V_{max}$  decreases. A similar pattern is seen if the amount of enzyme in the experiment is decreased. Thus, it is as if I lowered [E].

**Mixed Noncompetitive Inhibition** In this situation, the binding of I by E influences the binding of S by E. Either the binding sites for I and S are near one another or conformational changes in E caused by I affect S binding. In this case,  $K_I$  and  $K'_I$ , as defined previously, are not equal. Both the apparent  $K_m$  and the apparent  $V_{max}$  are altered by the presence of I, and  $K_m/V_{max}$  is not constant (Figure 13.16). This inhibitory pattern is commonly encountered. A reasonable explanation is that the inhibitor is binding at a site distinct from the active site yet is influencing the binding of S at the active site.



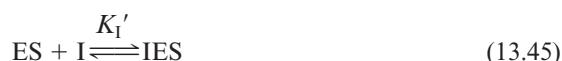
**FIGURE 13.15** Lineweaver–Burk plot of pure noncompetitive inhibition. Note that I does not alter  $K_m$  but that it decreases  $V_{max}$ .



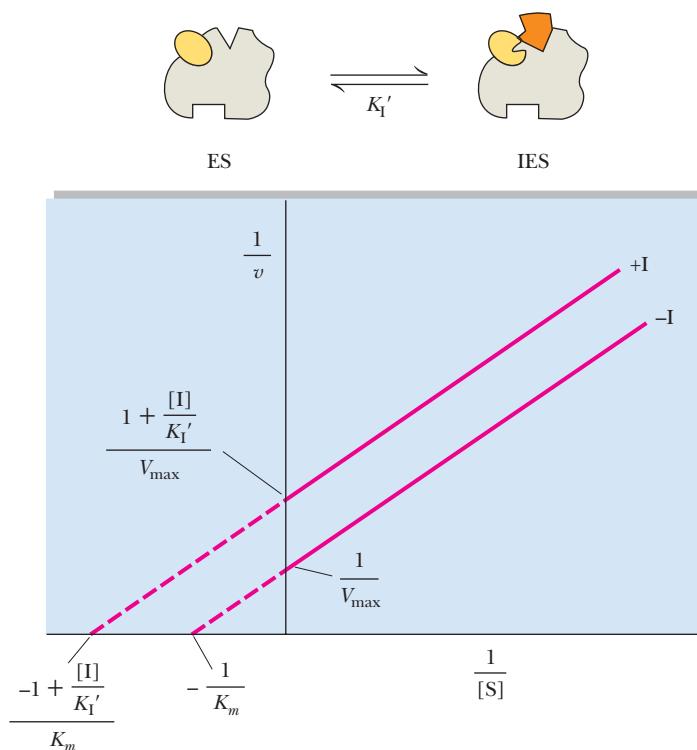
**FIGURE 13.16** Lineweaver–Burk plot of mixed noncompetitive inhibition. Note that both intercepts and the slope change in the presence of I. (a) When  $K_I$  is less than  $K_I'$ ; (b) when  $K_I$  is greater than  $K_I'$ .

Presumably, these effects are transmitted via alterations in the protein’s conformation. Table 13.6 includes the rate equations and apparent  $K_m$  and  $V_{\max}$  values for both types of noncompetitive inhibition.

**Uncompetitive Inhibition** Completing the set of inhibitory possibilities is uncompetitive inhibition. Unlike competitive inhibition (where I combines only with E) or noncompetitive inhibition (where I combines with E and ES), in uncompetitive inhibition, I combines only with ES.



Because IES does not lead to product formation, the observed rate constant for product formation,  $k_2$ , is uniquely affected. In simple Michaelis–Menten kinetics,  $k_2$  is the only rate constant that is part of both  $V_{\max}$  and  $K_m$ . The pattern obtained in Lineweaver–Burk plots is a set of parallel lines (Figure 13.17). A clinically important example is the action of lithium in alleviating manic depression;  $\text{Li}^+$  ions are uncompetitive inhibitors



**FIGURE 13.17** Lineweaver–Burk plot of uncompetitive inhibition. Note that both intercepts change but the slope ( $K_m/V_{\max}$ ) remains constant in the presence of I.

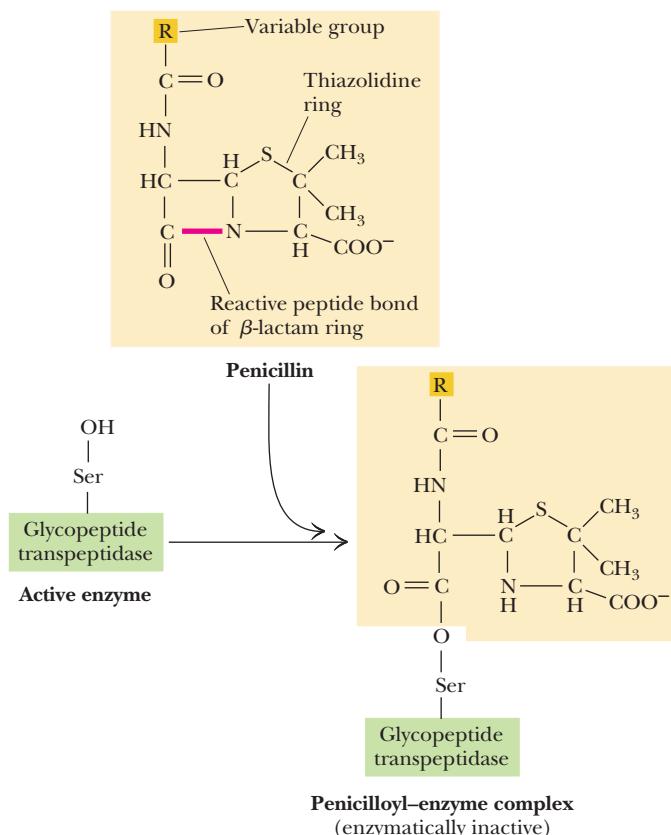
of *myo*-inositol monophosphatase. Some pesticides are also uncompetitive inhibitors, such as Roundup, an uncompetitive inhibitor of 3-enolpyruvylshikimate-5-P synthase, an enzyme essential to aromatic amino acid biosynthesis (see Chapter 25).

## Enzymes Also Can Be Inhibited in an Irreversible Manner

If the inhibitor combines irreversibly with the enzyme—for example, by covalent attachment—the kinetic pattern seen is like that of noncompetitive inhibition, because the net effect is a loss of active enzyme. Usually, this type of inhibition can be distinguished from the noncompetitive, reversible inhibition case because the reaction of I with E (and/or ES) is not instantaneous. Instead, there is a *time-dependent decrease in enzymatic activity* as  $E + I \rightarrow EI$  proceeds, and the rate of this inactivation can be followed. Also, unlike reversible inhibitions, dilution or dialysis of the enzyme:inhibitor solution does not dissociate the EI complex and restore enzyme activity.

**Suicide Substrates—Mechanism-Based Enzyme Inactivators** Suicide substrates are inhibitory substrate analogs designed so that, via normal catalytic action of the enzyme, a very reactive group is generated. This reactive group then forms a covalent bond with a nearby functional group within the active site of the enzyme, thereby causing irreversible inhibition. Suicide substrates, also called *Trojan horse substrates*, are a type of **affinity label**. As substrate analogs, they bind with specificity and high affinity to the enzyme active site; in their reactive form, they become covalently bound to the enzyme. This covalent link effectively labels a particular functional group within the active site, identifying the group as a key player in the enzyme's catalytic cycle.

**Penicillin—A Suicide Substrate** Several drugs in current medical use are mechanism-based enzyme inactivators. For example, the antibiotic **penicillin** exerts its effects by covalently reacting with an essential serine residue in the active site of *glycopeptide transpeptidase*, an enzyme that acts to crosslink the peptidoglycan chains during synthesis of bacterial cell walls (Figure 13.18). Penicillin consists of a thiazolidine ring fused to



**FIGURE 13.18** Penicillin is an irreversible inhibitor of the enzyme *glycopeptide transpeptidase*, also known as *glycoprotein peptidase*, which catalyzes an essential step in bacterial cell wall synthesis.

a  $\beta$ -lactam ring to which a variable R group is attached. A reactive peptide bond in the  $\beta$ -lactam ring covalently attaches to a serine residue in the active site of the glycopeptide transpeptidase. (The conformation of penicillin around its reactive peptide bond resembles the transition state of the normal glycopeptide transpeptidase substrate.) The penicillinoyl–enzyme complex is catalytically inactive. Once cell wall synthesis is blocked, the bacterial cells are very susceptible to rupture by osmotic lysis and bacterial growth is halted.

## 13.5 | What Is the Kinetic Behavior of Enzymes Catalyzing Bimolecular Reactions?

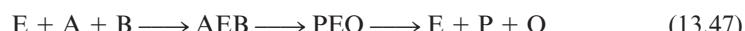
Thus far, we have considered only the simple case of enzymes that act upon a single substrate, S. This situation is not common. Usually, enzymes catalyze reactions in which two (or even more) substrates take part.

Consider the case of an enzyme catalyzing a reaction involving two substrates, A and B, and yielding the products P and Q:



Enzymatic reactions involving two substrates are called **bisubstrate reactions**. In general, bisubstrate reactions proceed by one of two possible routes:

- Both A and B are bound to the enzyme and then reaction occurs to give P + Q:



### HUMAN BIOCHEMISTRY

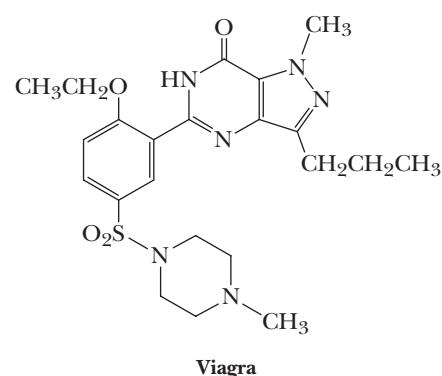
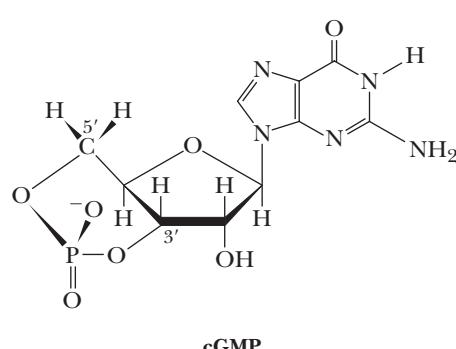
## Viagra—An Unexpected Outcome in a Program of Drug Design

Prior to the accumulation of detailed biochemical information on metabolism, enzymes, and receptors, drugs were fortuitous discoveries made by observant scientists; the discovery of penicillin as a bacteria-killing substance by Fleming is an example. Today, **drug design** is the rational application of scientific knowledge and principles to the development of pharmacologically active agents. A particular target for therapeutic intervention is identified (such as an enzyme or receptor involved in illness), and chemical analogs of its substrate or ligand are synthesized in hopes of finding an inhibitor (or activator) that will serve as a drug to treat the illness. Sometimes the outcome is unanticipated, as the story of **Viagra** (sildenafil citrate) reveals.

When the smooth muscle cells of blood vessels relax, blood flow increases and blood pressure drops. Such relaxation is the result of decreases in intracellular  $[Ca^{2+}]$  triggered by increases in intracellular [cGMP] (which in turn is triggered by nitric oxide, NO; see Chapter 32). Cyclic GMP (cGMP) is hydrolyzed by *phosphodiesterases* to form 5'-GMP, and the muscles contract again. Scientists at Pfizer reasoned that, if phosphodiesterase inhibitors could be found, they

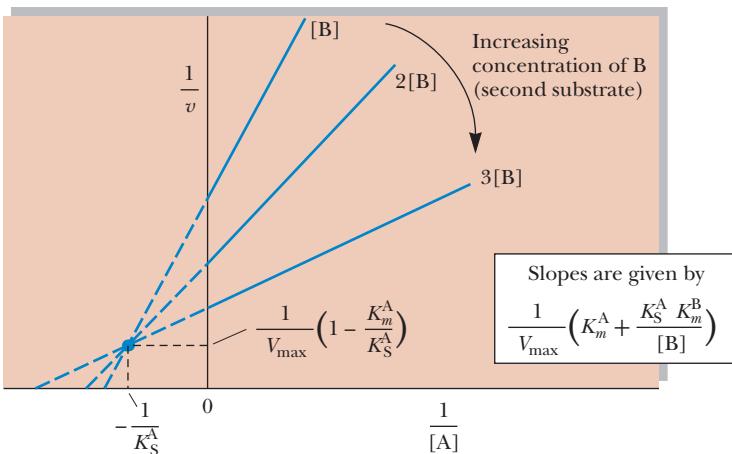
might be useful drugs to treat *angina* (chest pain due to inadequate blood flow to heart muscle) or *hypertension* (high blood pressure). The phosphodiesterase (PDE) prevalent in vascular muscle is PDE 5, one of at least nine different subtypes of PDE in human cells. The search was on for substances that inhibit PDE 5, but not the other prominent PDE types, and Viagra was found. Disappointingly, Viagra showed no significant benefits for angina or hypertension, but some men in clinical trials reported penile erection. Apparently, Viagra led to an increase in [cGMP] in penile vascular tissue, allowing vascular muscle relaxation, improved blood flow, and erection. A drug was born.

In a more focused way, detailed structural data on enzymes, receptors, and the ligands that bind to them has led to **rational drug design**, in which *computer modeling of enzyme-ligand interactions* replaces much of the initial chemical synthesis and clinical prescreening of potential therapeutic agents, saving much time and effort in drug development.



◀ Note the structural similarity between cGMP (left) and Viagra (right).

Double-reciprocal form  
of the rate equation:  $\frac{1}{v} = \frac{1}{V_{\max}} \left( K_m^A + \frac{K_S^A K_m^B}{[B]} \right) \left( \frac{1}{[A]} + \frac{1}{V_{\max}} \left( 1 + \frac{K_m^B}{[B]} \right) \right)$



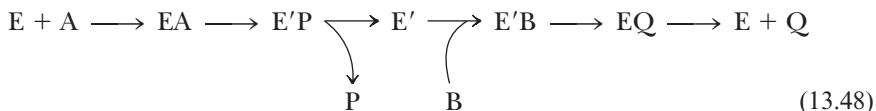
**FIGURE 13.19** Single-displacement bisubstrate mechanism.

Reactions of this type are defined as **sequential** or **single-displacement reactions**. They can be either of two distinct classes:

- random**, where either A or B may bind to the enzyme first, followed by the other substrate, or
- ordered**, where A, designated the *leading substrate*, must bind to E first before B can be bound.

Both classes of single-displacement reactions are characterized by lines that intersect to the left of the  $1/v$  axis in Lineweaver–Burk plots where the rates observed with different fixed concentrations of one substrate (B) are graphed versus a series of concentrations of A (Figure 13.19).

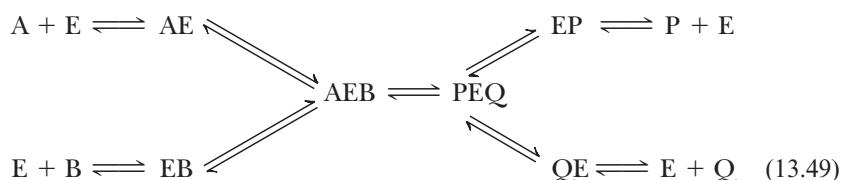
- The other general possibility is that one substrate, A, binds to the enzyme and reacts with it to yield a chemically modified form of the enzyme ( $E'$ ) plus the product, P. The second substrate, B, then reacts with  $E'$ , regenerating E and forming the other product, Q.

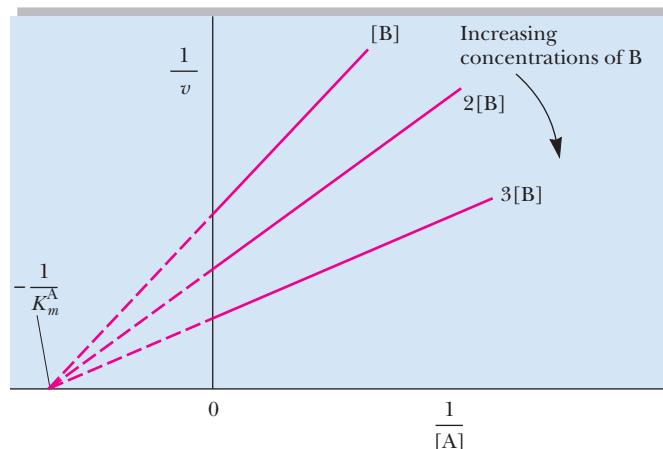


Reactions that fit this model are called **ping-pong** or **double-displacement reactions**. Two distinctive features of this mechanism are the obligatory formation of a modified enzyme intermediate,  $E'$ , and the pattern of parallel lines obtained in double-reciprocal plots of the rates observed with different fixed concentrations of one substrate (B) versus a series of concentrations of A (see Figure 13.22).

### The Conversion of AEB to PEQ Is the Rate-Limiting Step in Random, Single-Displacement Reactions

In this type of sequential reaction, all possible binary enzyme–substrate complexes (AE, EB, PE, EQ) are formed rapidly and reversibly when the enzyme is added to a reaction mixture containing A, B, P, and Q:

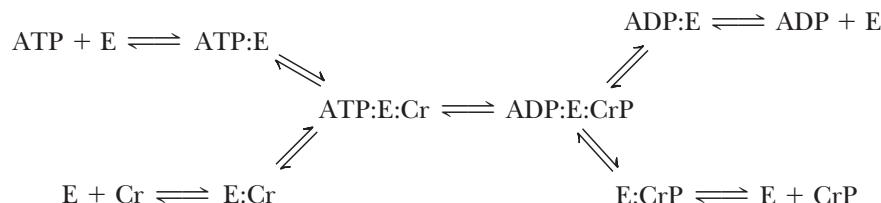




**FIGURE 13.20** Random, single-displacement bisubstrate mechanism where A does not affect B binding, and vice versa.

The rate-limiting step is the reaction  $AEB \rightarrow PEQ$ . It does not matter whether A or B binds first to E, or whether Q or P is released first from QEP. Sometimes, reactions that follow this random order of addition of substrates to E can be distinguished from reactions obeying an ordered, single-displacement mechanism. If A has *no* influence on the binding constant for B (and vice versa) and the mechanism is purely random, the lines in a Lineweaver–Burk plot intersect at the  $1/[A]$  axis (Figure 13.20).

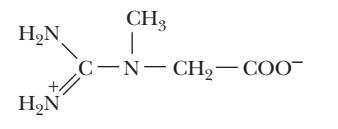
**Creatine Kinase Acts by a Random, Single-Displacement Mechanism** An example of a random, single-displacement mechanism is seen in the enzyme creatine kinase, a phosphoryl transfer enzyme that uses ATP as a phosphoryl donor to form creatine phosphate (CrP) from creatine (Cr). Creatine-P is an important reservoir of phosphate-bond energy in muscle cells (Figure 13.21).



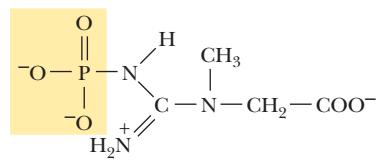
The overall direction of the reaction will be determined by the relative concentrations of ATP, ADP, Cr, and CrP and the equilibrium constant for the reaction. The enzyme can be considered to have two sites for substrate (or product) binding: an adenine nucleotide site, where ATP or ADP binds, and a creatine site, where Cr or CrP is bound. In such a mechanism, ATP and ADP compete for binding at their unique site while Cr and CrP compete at the specific Cr/CrP-binding site. Note that no modified enzyme form ( $E'$ ), such as an E-P intermediate, appears here. The reaction is characterized by rapid and reversible binary ES complex formation, followed by addition of the remaining substrate, and the rate-determining reaction taking place within the ternary complex.

### In an Ordered, Single-Displacement Reaction, the Leading Substrate Must Bind First

In an ordered reaction, the **leading substrate**, A (also called the **obligatory** or **compulsory substrate**), must bind first. Then the second substrate, B, binds. Strictly speaking, B cannot bind to free enzyme in the absence of A. Reaction between A and B occurs in the ternary complex and is usually followed by an ordered release of the products of the



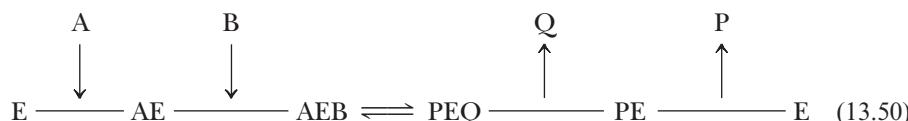
Creatine



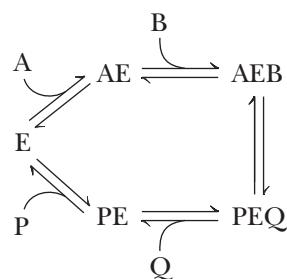
Creatine-P

**FIGURE 13.21** The structures of creatine and creatine phosphate, guanidinium compounds that are important in muscle energy metabolism.

reaction, P and Q. In the following schemes, P is the product of A and is released last. One representation, suggested by W. W. Cleland, follows:

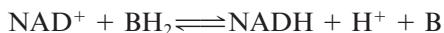


Another way of portraying this mechanism is as follows:

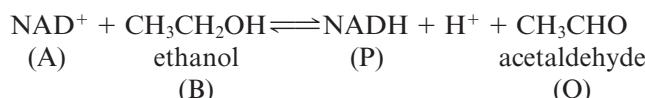


Note that A and P are competitive for binding to the free enzyme, E, but not A and B (or P and B).

**NAD<sup>+</sup>-Dependent Dehydrogenases Show Ordered Single-Displacement Mechanisms** Nicotinamide adenine dinucleotide ( $\text{NAD}^+$ )-dependent dehydrogenases are enzymes that typically behave according to the kinetic pattern just described. A general reaction of these dehydrogenases is



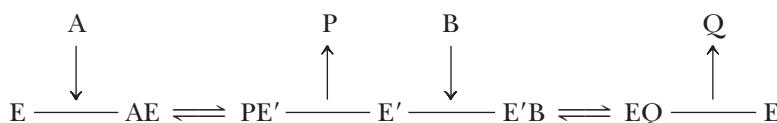
The leading substrate (A) is nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ), and  $\text{NAD}^+$  and NADH (product P) compete for a common site on E. A specific example is offered by alcohol dehydrogenase (ADH):



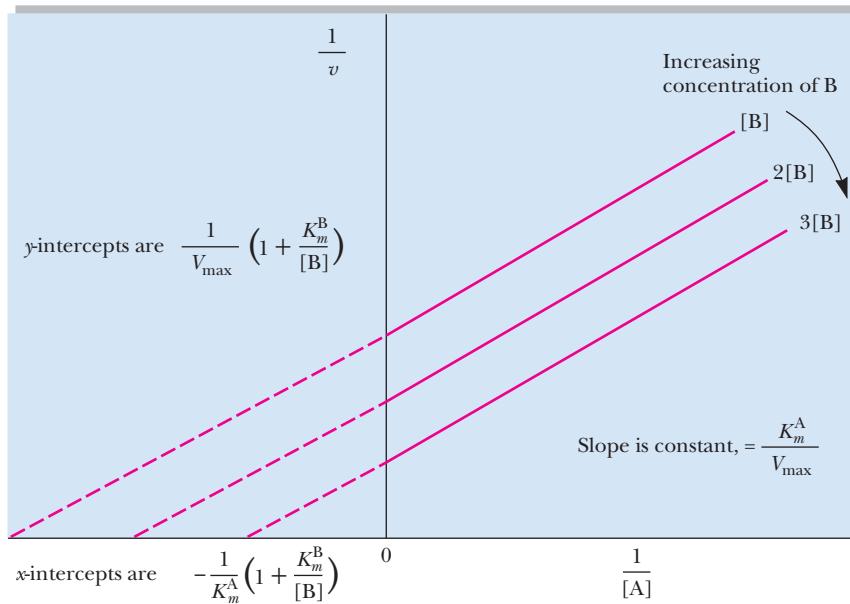
We can verify that this ordered mechanism is not random by demonstrating that no B (ethanol) is bound to E in the absence of A ( $\text{NAD}^+$ ).

## Double-Displacement (Ping-Pong) Reactions Proceed Via Formation of a Covalently Modified Enzyme Intermediate

Double-displacement reactions are characterized by a pattern of parallel lines when  $1/v$  is plotted as a function of  $1/[A]$  at different concentrations of B, the second substrate (Figure 13.22). Reactions conforming to this kinetic pattern are characterized by the fact that the product of the enzyme's reaction with A (called P in the following schemes) is released *prior* to reaction of the enzyme with the second substrate, B. As a result of this process, the enzyme, E, is converted to a modified form, E', which then reacts with B to give the second product, Q, and regenerate the unmodified enzyme form, E:

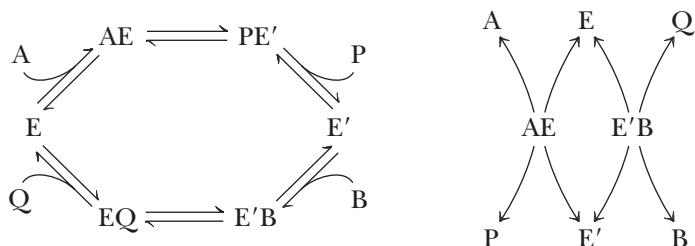


Double-reciprocal form of the rate equation:  $\frac{1}{v} = \frac{K_m^A}{V_{\max}} \left( \frac{1}{[A]} \right) + \left( 1 + \frac{K_m^B}{[B]} \right) \left( \frac{1}{V_{\max}} \right)$



**FIGURE 13.22** Double-displacement (ping-pong) bisubstrate mechanisms are characterized by parallel lines.

or

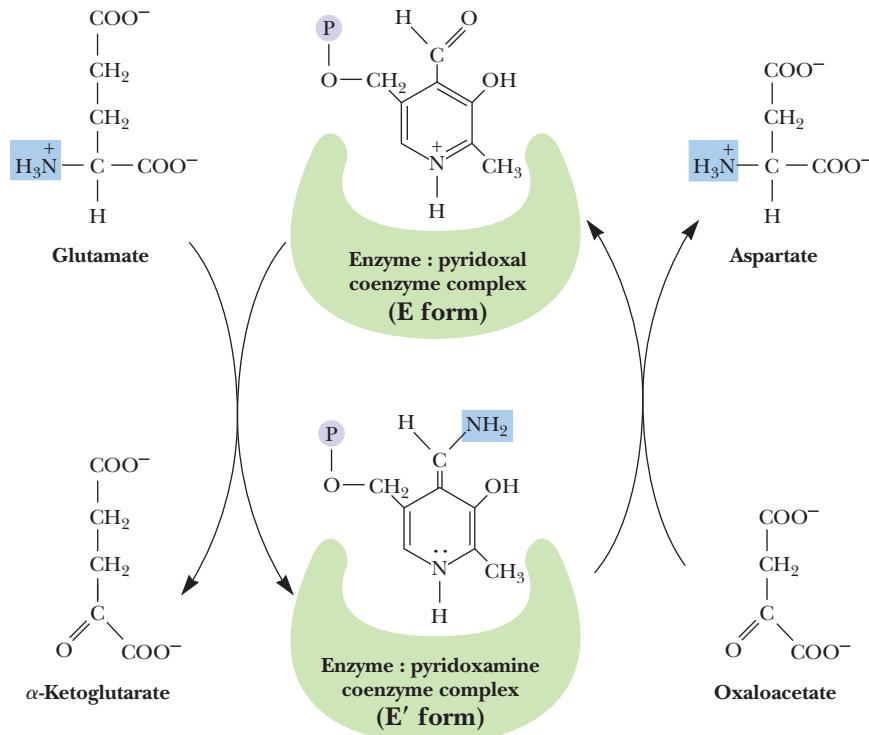


Note that these schemes predict that A and Q compete for the free enzyme form, E, while B and P compete for the modified enzyme form, E'. A and Q do not bind to E', nor do B and P combine with E.

**Aminotransferases Show Double-Displacement Catalytic Mechanisms** One class of enzymes that follow a ping-pong-type mechanism are *aminotransferases* (previously known as transaminases). These enzymes catalyze the transfer of an amino group from an amino acid to an  $\alpha$ -keto acid. The products are a new amino acid and the keto acid corresponding to the carbon skeleton of the amino donor:



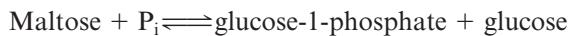
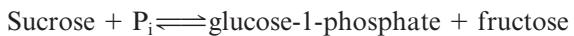
A specific example would be *glutamate:aspartate aminotransferase*. Figure 13.23 depicts the scheme for this mechanism. Note that glutamate and aspartate are competitive for E and that oxaloacetate and  $\alpha$ -ketoglutarate compete for E'. In glutamate:aspartate aminotransferase, an enzyme-bound coenzyme, *pyridoxal phosphate* (a vitamin B<sub>6</sub> derivative), serves as the amino group acceptor/donor in the enzymatic reaction. The unmodified enzyme, E, has the coenzyme in the aldehydic pyridoxal form, whereas in the modified enzyme, E', the coenzyme is actually pyridoxamine phosphate (Figure 13.23). Not all enzymes displaying ping-pong-type mechanisms require coenzymes as carriers for the chemical substituent transferred in the reaction.



**FIGURE 13.23** Glutamate:aspartate aminotransferase, an enzyme conforming to a double-displacement bisubstrate mechanism.

### Exchange Reactions Are One Way to Diagnose Bisubstrate Mechanisms

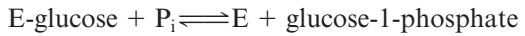
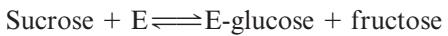
Kineticists rely on a number of diagnostic tests for the assignment of a reaction mechanism to a specific enzyme. One is the graphic analysis of the kinetic patterns observed. It is usually easy to distinguish between single- and double-displacement reactions in this manner, and examining competitive effects between substrates aids in assigning reactions to random versus ordered patterns of S binding. A second diagnostic test is to determine whether the enzyme catalyzes an **exchange reaction**. Consider as an example the two enzymes *sucrose phosphorylase* and *maltose phosphorylase*. Both catalyze the phosphorolysis of a disaccharide and both yield glucose-1-phosphate and a free hexose:



Interestingly, in the absence of sucrose and fructose, sucrose phosphorylase will catalyze the exchange of inorganic phosphate, P<sub>i</sub>, into glucose-1-phosphate. This reaction can be followed by using <sup>32</sup>P<sub>i</sub> as a radioactive tracer and observing the incorporation of <sup>32</sup>P into glucose-1-phosphate:



Maltose phosphorylase cannot carry out a similar reaction. The <sup>32</sup>P exchange reaction of sucrose phosphorylase is accounted for by a double-displacement mechanism where E' is E-glucose:



Thus, in the presence of just <sup>32</sup>P<sub>i</sub> and glucose-1-phosphate, sucrose phosphorylase still catalyzes the second reaction and radioactive P<sub>i</sub> is incorporated into glucose-1-phosphate over time.

Maltose phosphorylase proceeds via a single-displacement reaction that necessarily requires the formation of a ternary maltose:E:P<sub>i</sub> (or glucose:E:glucose-1-phosphate)

complex for any reaction to occur. Exchange reactions are a characteristic of enzymes that obey double-displacement mechanisms at some point in their catalysis.

## Multisubstrate Reactions Can Also Occur in Cells

Thus far, we have considered enzyme-catalyzed reactions involving one or two substrates. How are the kinetics described in those cases in which more than two substrates participate in the reaction? An example might be the glycolytic enzyme *glyceraldehyde-3-phosphate dehydrogenase* (see Chapter 18):



Many other multisubstrate examples abound in metabolism. In effect, these situations are managed by realizing that the interaction of the enzyme with its many substrates can be treated as a series of unisubstrate or bisubstrate steps in a multistep reaction pathway. Thus, the complex mechanism of a multisubstrate reaction is resolved into a sequence of steps, each of which obeys the single- and double-displacement patterns just discussed.

## 13.6 | How Can Enzymes Be So Specific?

The extraordinary ability of an enzyme to catalyze only one particular reaction is a quality known as **specificity**. Specificity means an enzyme acts only on a specific substance, its substrate, invariably transforming it into a specific product. That is, an enzyme binds only certain compounds, and then, only a specific reaction ensues. Some enzymes show absolute specificity, catalyzing the transformation of only one specific substrate to yield a unique product. Other enzymes carry out a particular reaction but act on a class of compounds. For example, *hexokinase* (ATP:hexose-6-phosphotransferase) will carry out the ATP-dependent phosphorylation of a number of hexoses at the 6-position, including glucose. Specificity studies on enzymes entail an examination of the rates of the enzymatic reaction obtained with various **structural analogs** of the substrate. By determining which functional and structural groups within the substrate affect binding or catalysis, enzymologists can map the properties of the active site, analyzing questions such as: Can the active site accommodate sterically bulky groups? Are ionic interactions between E and S important? Are H bonds formed?

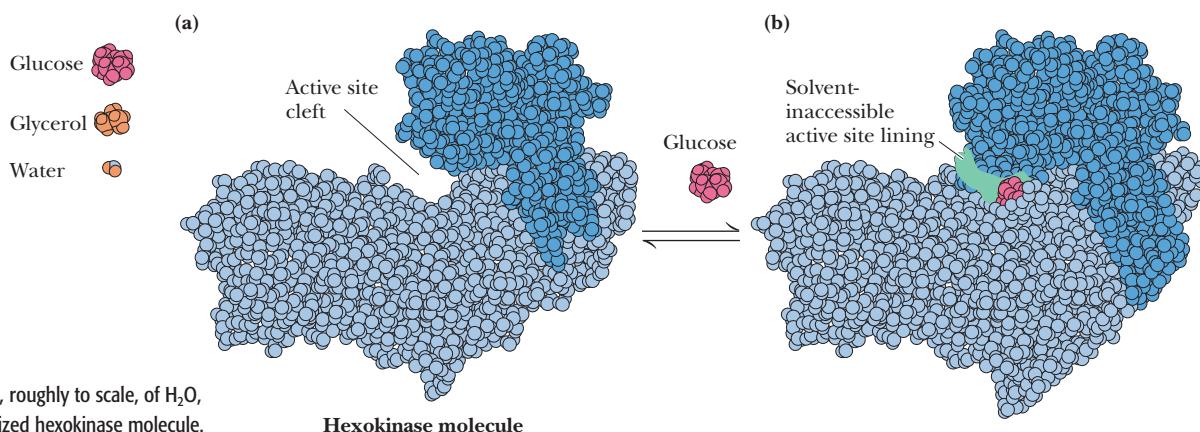
### The “Lock and Key” Hypothesis Was the First Explanation for Specificity

Pioneering enzyme specificity studies at the turn of the 20th century by the great organic chemist Emil Fischer led to the notion of an enzyme resembling a “lock” and its particular substrate the “key.” This analogy captures the essence of the specificity that exists between an enzyme and its substrate, but enzymes are not rigid templates like locks.

### The “Induced Fit” Hypothesis Provides a More Accurate Description of Specificity

Enzymes are highly flexible, conformationally dynamic molecules, and many of their remarkable properties, including substrate binding and catalysis, are due to their structural pliancy. Realization of the conformational flexibility of proteins led Daniel Koshland to hypothesize that the binding of a substrate by an enzyme is an interactive process. That is, the shape of the enzyme’s active site is actually modified upon binding S, in a process of dynamic recognition between enzyme and substrate aptly called **induced fit**. In essence, substrate binding alters the conformation of the protein, so that the protein and the substrate “fit” each other more precisely. The process is truly interactive in

New ideas do not always gain immediate acceptance: “Although we did many experiments that in my opinion could only be explained by the induced-fit theory, gaining acceptance for the theory was still an uphill fight. One (journal) referee wrote, ‘The Fischer Key-Lock theory has lasted 100 years and will not be overturned by speculation from an embryonic scientist.’” *Daniel Koshland, 1996. How to get paid for having fun. Annual Review of Biochemistry 65:1–13.*



**FIGURE 13.24** A drawing, roughly to scale, of  $\text{H}_2\text{O}$ , glycerol, glucose, and an idealized hexokinase molecule.

that the conformation of the substrate also changes as it adapts to the conformation of the enzyme.

This idea also helps explain some of the mystery surrounding the enormous catalytic power of enzymes: In enzyme catalysis, precise orientation of catalytic residues comprising the active site is necessary for the reaction to occur; substrate binding induces this precise orientation by the changes it causes in the protein's conformation.

### "Induced Fit" Favors Formation of the Transition State

The catalytically active enzyme substrate complex is an interactive structure in which the enzyme causes the substrate to adopt a form that mimics the transition state of the reaction. Thus, a poor substrate would be one that was less effective in directing the formation of an optimally active enzyme:transition state conformation. This active conformation of the enzyme molecule is thought to be relatively unstable in the absence of substrate, and free enzyme thus reverts to a different conformational state.

### Specificity and Reactivity

Consider, for example, why hexokinase catalyzes the ATP-dependent phosphorylation of hexoses but not smaller phosphoryl-group acceptors such as glycerol, ethanol, or even water. Surely these smaller compounds are not sterically forbidden from approaching the active site of hexokinase (Figure 13.24). Indeed, water should penetrate the active site easily and serve as a highly effective phosphoryl-group acceptor. Accordingly, hexokinase should display high ATPase activity. It does not. Only the binding of hexoses induces hexokinase to assume its fully active conformation. The hexose-binding site of hexokinase is located between two protein domains. Binding of glucose in the active site induces a conformational change in hexokinase that causes the two domains to close upon one another, creating the catalytic site.

In Chapter 14, we explore in greater detail the factors that contribute to the remarkable catalytic power of enzymes and examine specific examples of enzyme reaction mechanisms.

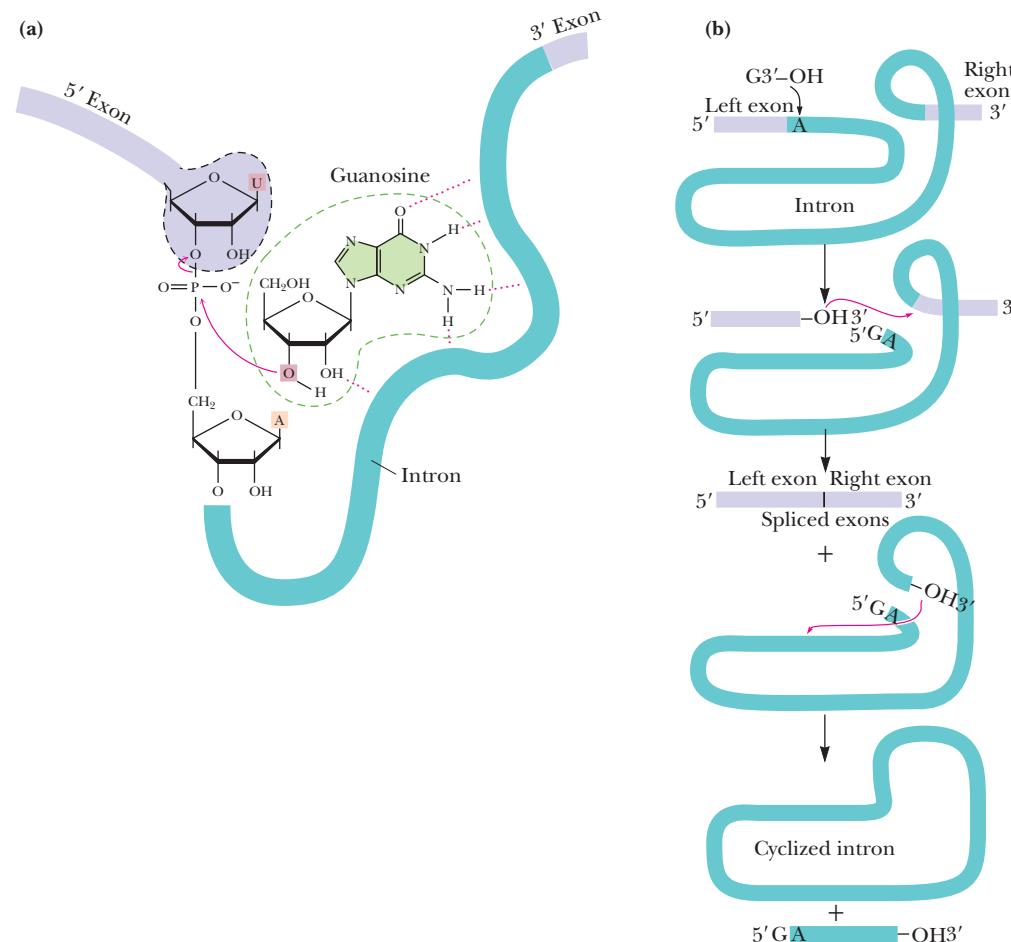
## 13.7 Are All Enzymes Proteins?

### RNA Molecules That Are Catalytic Have Been Term "Ribozymes"

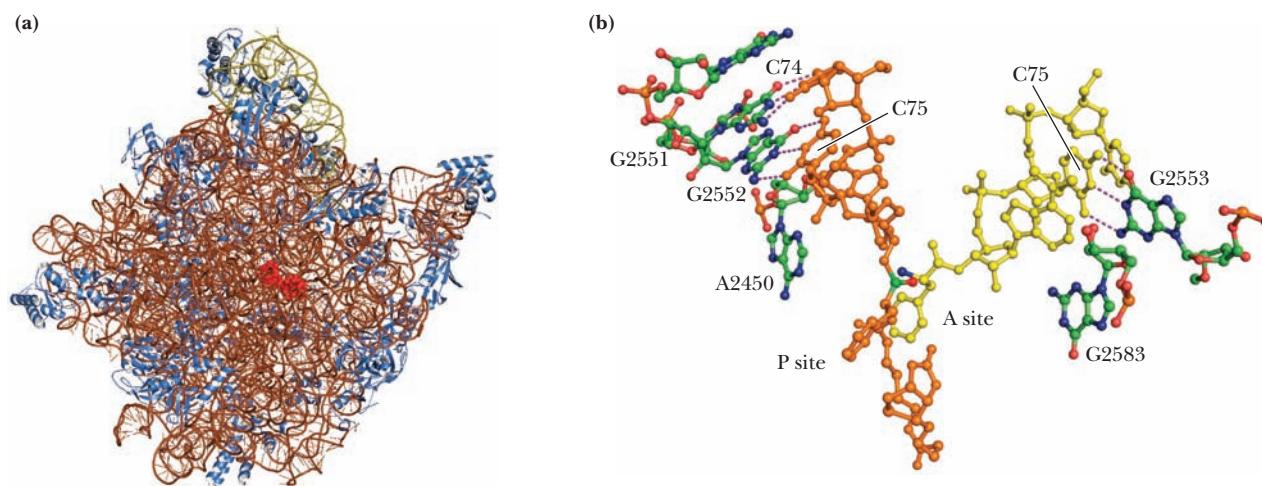
It was long assumed that all enzymes are proteins. However, several decades ago, instances of biological catalysis by RNA molecules were discovered. Catalytic RNAs, or **ribozymes**, satisfy several enzymatic criteria: They are substrate specific, they enhance

the reaction rate, and they emerge from the reaction unchanged. Most ribozymes act in RNA processing, cutting the phosphodiester backbone at specific sites and religating needed segments to form functional RNA strands while discarding extraneous pieces. For example, bacterial RNase P is a ribozyme involved in the formation of mature tRNA molecules from longer RNA transcripts. RNase P requires an RNA component as well as a protein subunit for its activity in the cell. In vitro, the protein alone is incapable of catalyzing the maturation reaction, but the RNA component by itself can carry out the reaction under appropriate conditions. As another example, the introns within some rRNAs and mRNAs are ribozymes that can catalyze their own excision from large RNA transcripts by a process known as **self-splicing**. For instance, in the ciliated protozoan *Tetrahymena*, formation of mature ribosomal RNA from a pre-rRNA precursor involves the removal of an internal RNA segment and the joining of the two ends. The excision of this intron and ligation of the exons is catalyzed by the intron itself, in the presence of  $Mg^{2+}$  and a free molecule of guanosine nucleoside or nucleotide (Figure 13.25). In vivo, the intervening sequence RNA probably acts only in splicing itself out; in vitro, however, it can act many times, turning over like a true enzyme.

**The Ribosome Is a Ribozyme** A particularly significant case of catalysis by RNA occurs in protein synthesis. The **peptidyl transferase reaction**, which is the reaction of peptide bond formation during protein synthesis, is catalyzed by the 23S rRNA of the 50S subunit of ribosomes (see Chapters 10 and 30). The substrates for the peptidyl transferase reaction are two tRNA molecules, one bearing the growing peptide chain (the



**FIGURE 13.25** RNA splicing in *Tetrahymena* rRNA maturation: (a) the guanosine-mediated reaction involved in the autocatalytic excision of the *Tetrahymena* rRNA intron and (b) the overall splicing process. The cyclized intron is formed via nucleophilic attack of the freed 3'-OH end of the intron on the phosphodiester bond that is 15 nucleotides from the 5'-GA end of the spliced-out intron. Cyclization frees a linear 15-mer with a 5'-GA end.

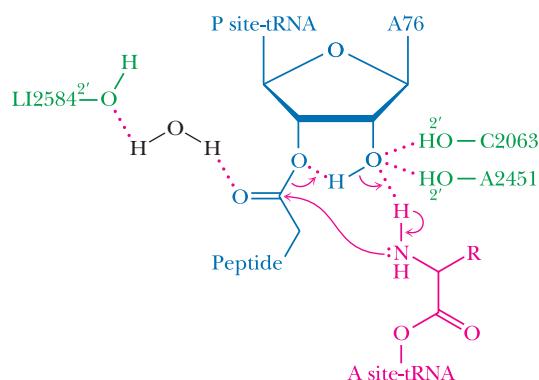


**FIGURE 13.26** (a) The 50S subunit from *H. marismortui* (pdb id = 1FFK). Ribosomal proteins are shown in blue, the 23S rRNA backbone in brown, the 5S rRNA backbone in olive, and a tRNA substrate analog in red. The tRNA analog identifies the peptidyl transferase catalytic center of the 50S subunit. (b) The aminoacyl-tRNA<sub>A</sub> (yellow) and the peptidyl-tRNA<sub>P</sub> (orange) in the peptidyl transferase active site. Bases of the 23S rRNA shown in green and labeled according to their position in the 23S rRNA sequence. Interactions between the tRNAs and the 23S rRNA are indicated by dotted lines. The  $\alpha$ -amino group of the aminoacyl-tRNA<sub>A</sub> (blue) is positioned for the attack on the carbonyl-C (green) of the peptidyl-tRNA<sub>P</sub>. (Adapted from Figure 2 in Beringer, M., and Rodnina, M. V., 2007. The ribosomal peptidyl transferase. *Molecular Cell* 26:311–321.)

peptidyl-tRNA<sub>P</sub>) and the other bearing the next amino acid to be added (the aminoacyl-tRNA<sub>A</sub>). Both the peptidyl chain and the amino acid are attached to their respective tRNAs via ester bonds to the O atom at the CCA-3' ends of these tRNAs (see Figure 11.36). Base-pairing between these C residues in the two tRNAs and G residues in the 23S rRNA position the substrates for the reaction to occur (Figure 13.26). The two Cs at the peptidyl-tRNA<sub>P</sub> CCA end pair with G2251 and G2252 of the 23S rRNA, and the last C (C75) at the 3'-end of the aminoacyl-tRNA<sub>A</sub> pairs with G2553. The 3'-terminal A of the aminoacyl-tRNA<sub>A</sub> interacts with G2583, and the terminal A of the peptidyl-tRNA<sub>P</sub> binds to A2450. Addition of the incoming amino acid to the peptidyl chain occurs when the  $\alpha$ -amino group of the aminoacyl-tRNA<sub>A</sub> makes a nucleophilic attack on the carbonyl C linking the peptidyl chain to its tRNA<sub>P</sub>. Specific 23S rRNA bases and ribose-OH groups facilitate this nucleophilic attack by favoring proton abstraction from the aminoacyl  $\alpha$ -amino group (Figure 13.27). The products of this reaction are a one-residue-longer peptidyl chain attached to the tRNA<sub>A</sub> and the “empty” tRNA<sub>P</sub>.

The fact that RNA can catalyze such important reactions is experimental support for the idea that a primordial world dominated by RNA molecules existed before the

**FIGURE 13.27** The peptidyl transferase reaction. Abstraction of an amide proton from the  $\alpha$ -amino group of the aminoacyl-tRNA<sub>A</sub> (shown in red) by the 2'-O of the terminal A of the peptidyl-tRNA<sub>P</sub> (blue) is aided by hydrogen-bonding interactions with neighboring 23S rRNA nucleotides (green). These interactions facilitate nucleophilic attack by the  $\alpha$ -amino group of the aminoacyl-tRNA<sub>A</sub> on the carbonyl C of the peptidyl-tRNA<sub>P</sub> and peptide bond formation between the incoming amino acid and the growing peptide chain to give a one-residue-longer peptide chain attached to the tRNA<sub>A</sub>. (Adapted from Figure 3 in Beringer, M., and Rodnina, M. V., 2007. The ribosomal peptidyl transferase. *Molecular Cell* 26:311–321.)



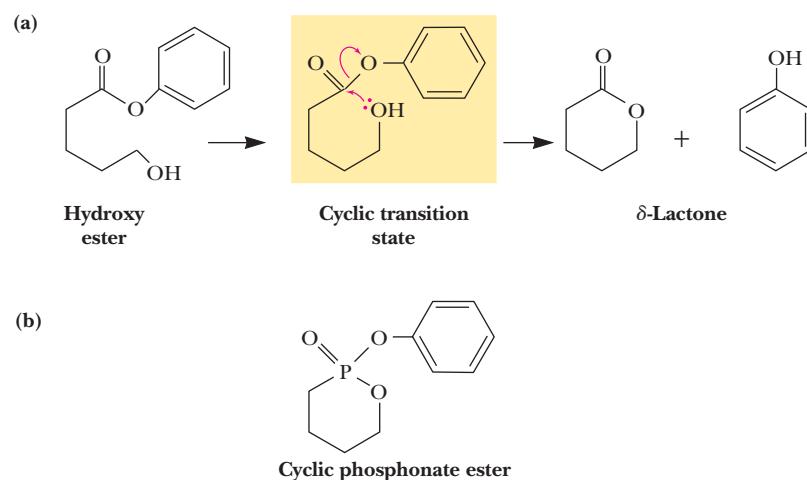
evolution of DNA and proteins. Sidney Altman and Thomas R. Cech shared the 1989 Nobel Prize in Chemistry for their discovery of the catalytic properties of RNA.

## Antibody Molecules Can Have Catalytic Activity

Antibodies are *immunoglobulins*, which, of course, are proteins. Catalytic antibodies are antibodies with catalytic activity (catalytic antibodies are also called **abzymes**, a word created by combining “Ab,” the abbreviation for antibody, with “enzyme.”) Like other antibodies, catalytic antibodies are elicited in an organism in response to immunological challenge by a foreign molecule called an **antigen** (see Chapter 28 for discussions on the molecular basis of immunology). In this case, however, the antigen is purposefully engineered to be *an analog of the transition state in a reaction*. The strategy is based on the idea that a protein specific for binding the transition state of a reaction will promote entry of the normal reactant into the reactive, transition-state conformation. Thus, a catalytic antibody facilitates, or catalyzes, a reaction by forcing the conformation of its substrate in the direction of its transition state. (A prominent explanation for the remarkable catalytic power of conventional enzymes is their great affinity for the transition state in the reactions they catalyze; see Chapter 14.)

One proof of this principle has been to prepare ester analogs by substituting a phosphorus atom for the carbon in the ester group (Figure 13.28). The phosphonate compound mimics the natural transition state of ester hydrolysis, and antibodies elicited against these analogs act like enzymes in accelerating the rate of ester hydrolysis as much as 1000-fold. Abzymes have been developed for a number of other classes of reactions, including C—C bond formation via aldol condensation (the reverse of the aldolase reaction [see Figure 13.2, reaction 4, and Chapter 18]) and the pyridoxal 5'-P-dependent aminotransferase reaction shown in Figure 13.23. This biotechnology offers the real possibility of creating specially tailored enzymes designed to carry out specific catalytic processes.

Catalytic antibodies apparently occur naturally. Autoimmune diseases are diseases that arise because an individual begins to produce antibodies against one of their own cellular constituents. Multiple sclerosis (MS), one such autoimmune disease, is characterized by gradual destruction of the myelin sheath surrounding neurons throughout the brain and spinal cord. Blood serum obtained from some MS patients contains antibodies capable of carrying out the proteolytic destruction of myelin basic protein (MBP). That is, these antibodies were MBP-destructive proteases. Similarly, hemophilia A is a blood-clotting disorder due to lack of the factor VIII, an essential protein for formation of a



**FIGURE 13.28** (a) The intramolecular hydrolysis of a hydroxy ester to yield as products a  $\delta$ -lactone and the alcohol phenol. Note the cyclic transition state. (b) The cyclic phosphonate ester analog of the cyclic transition state.

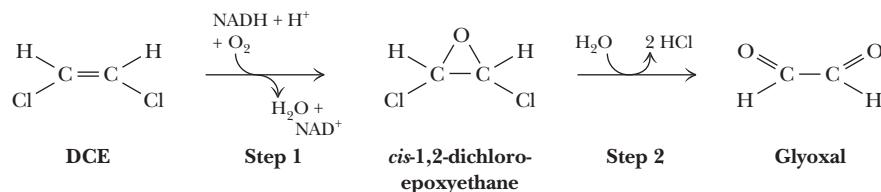
blood clot. Serum from some sufferers of hemophilia A contained antibodies with proteolytic activity against factor VIII. Thus, some antibodies may be proteases.

### 13.8 Is It Possible to Design an Enzyme to Catalyze Any Desired Reaction?

Enzymes have evolved to catalyze metabolic reactions with high selectivity, specificity, and rate enhancements. Given these remarkable attributes, it would be very desirable to have the ability to create **designer enzymes** individually tailored to catalyze any imaginable reaction, particularly those that might have practical uses in industrial chemistry, the pharmaceutical industry, or environmental remediation. To this end, several approaches have been taken to create a desired enzyme *de novo* (*de novo*: literally “anew”; colloquially “from scratch.”) In biochemistry, the synthesis of some end product from simpler precursors.) Most approaches begin with a known enzyme and then engineer it by using *in vitro* mutagenesis (see Chapter 12) to replace active-site residues with a new set that might catalyze the desired reaction. This strategy has the advantage that the known protein structure provides a stable scaffold into which a new catalytic site can be introduced. As pointed out in Chapter 6, despite the extremely large number of possible amino acid sequences for a polypeptide chain, the number of stable tertiary structures that proteins can adopt is rather limited. Yet proteins have an extraordinary range of functional possibilities. So, this approach is rational. A second, more difficult, approach attempts the completely new design of a protein with the desired structure and activity. Often, this approach relies on *in silico* methods, where the folded protein structure and the spatial and reactive properties of its putative active site are modeled, refined, and optimized via computer. Although this approach has fewer limitations in terms of size and shape of substrates, it brings other complications, such as protein folding and stability, to the problem, to say nothing of the difficulties of going from the computer model (*in silico*) to a real enzyme in a cellular environment (*in vivo*).

Enzymes have shown adaptability over the course of evolution. New enzyme functions have appeared time and time again, as mutation and selection according to Darwinian principles operate on existing enzymes. Some enzyme designers have coupled natural evolutionary processes with rational design using *in vitro* mutagenesis. Expression of mutated versions of the gene encoding the enzyme in bacteria, followed by rounds of selection for bacteria producing an enzyme with even better catalytic properties, takes advantage of naturally occurring processes to drive further mutation and selection for an optimal enzyme. This dual approach is whimsically referred to as *semirational design* because it relies on the rational substitution of certain amino acids with new ones in the active site, followed by directed evolution (selection for bacteria expressing more efficient versions of the enzyme).

An example of active-site engineering is the site-directed mutation of an epoxide hydrolase to change its range of substrate selection so that it now acts on chlorinated epoxides (Figure 13.29). Degradation of chlorinated epoxides is a major problem in the



**FIGURE 13.29** *cis*-1,2-Dichloroethylene (DCE) is an industrial solvent that poses hazards to human health; DCE occurs as a pollutant in water sources. Bacterial metabolism of DCE to form *cis*-1,2-dichloroepoxyethane (step 1) occurs readily, but enzymatic degradation of the epoxide to glyoxal and chloride ions (step 2) is limited. Microbial detoxification of DCE in ground water requires enzymes for both steps 1 and 2. Genetic engineering of an epoxide hydrolase to create an enzyme capable of using *cis*-1,2-dichloroepoxyethane as a substrate and catalyzing step 2 is a practical example of *de novo* enzyme design.

removal of toxic pollutants from water resources. Mutation of a bacterial epoxide hydrolase at three active-site residues (F<sup>108</sup>, I<sup>219</sup>, and C<sup>248</sup>) and selection in bacteria for enhanced chlorinated epoxide hydrolase activity yielded an F108L, I219L, C248I mutant enzyme that catalyzed the conversion of *cis*-dichloroepoxyethane to Cl<sup>-</sup> ions and glyoxal with a dramatically increased  $V_{max}/K_m$  ratio.

## SUMMARY



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Living systems use enzymes to accelerate and control the rates of vitally important biochemical reactions. Enzymes provide kinetic control over thermodynamic potentiality: Reactions occur in a timeframe suitable to the metabolic requirements of cells. Enzymes are the agents of metabolic function.

**13.1 What Characteristic Features Define Enzymes?** Enzymes can be characterized in terms of three prominent features: catalytic power, specificity, and regulation. The site on the enzyme where substrate binds and catalysis occurs is called the active site. Regulation of enzyme activity is essential to the integration and regulation of metabolism.

**13.2 Can the Rate of an Enzyme-Catalyzed Reaction Be Defined in a Mathematical Way?** Enzyme kinetics can determine the maximum reaction velocity that the enzyme can attain, its binding affinities for substrates and inhibitors, and the mechanism by which it accomplishes its catalysis. The kinetics of simple chemical reactions provides a foundation for exploring enzyme kinetics. Enzymes, like other catalysts, act by lowering the free energy of activation for a reaction.

**13.3 What Equations Define the Kinetics of Enzyme-Catalyzed Reactions?** A plot of the velocity of an enzyme-catalyzed reaction  $v$  versus the concentration of the substrate S is called a substrate saturation curve. The Michaelis–Menten equation is derived by assuming that E combines with S to form ES and then ES reacts to give E + P. Rapid, reversible combination of E and S and ES breakdown to yield P reach a steady-state condition where [ES] is essentially constant. The Michaelis–Menten equation says that the initial rate of an enzyme reaction,  $v$ , is determined by two constants,  $K_m$  and  $V_{max}$ , and the initial concentration of substrate. The turnover number of an enzyme,  $k_{cat}$ , is a measure of its maximal catalytic activity (the number of substrate molecules converted into product per enzyme molecule per unit time when the enzyme is saturated with substrate). The ratio  $k_{cat}/K_m$  defines the catalytic efficiency of an enzyme. This ratio,  $k_{cat}/K_m$ , cannot exceed the diffusion-controlled rate of combination of E and S to form ES.

Several rearrangements of the Michaelis–Menten equation transform it into a straight-line equation, a better form for experimental determination of the constants  $K_m$  and  $V_{max}$  and for detection of regulatory properties of enzymes.

**13.4 What Can Be Learned from the Inhibition of Enzyme Activity?** Inhibition studies on enzymes have contributed significantly to our understanding of enzymes. Inhibitors may interact either reversibly or irreversibly with an enzyme. Reversible inhibitors bind to the enzyme through noncovalent association/dissociation reactions. Irreversible inhibitors typically form stable, covalent bonds with the enzyme. Reversible inhibitors may bind at the active site of the enzyme (competitive inhibition) or at some other site on the enzyme (noncompetitive inhibition). Uncompetitive inhibitors bind only to the ES complex.

**13.5 What Is the Kinetic Behavior of Enzymes Catalyzing Bimolecular Reactions?** Usually, enzymes catalyze reactions in which two (or even more) substrates take part, so the reaction is bimolecular. Several possibilities arise. In single-displacement reactions, both substrates, A and B, are bound before reaction occurs. In double-displacement (or ping-pong) reactions, one substrate (A) is bound and reaction occurs to yield product P and a modified enzyme form, E'. The second substrate (B) then binds to E' and reaction occurs to yield product Q and E, the unmodified form of enzyme. Graphical methods can be used to distinguish these possibilities. Exchange reactions are another way to diagnose bisubstrate mechanisms.

**13.6 How Can Enzymes Be So Specific?** Early enzyme specificity studies by Emil Fischer led to the hypothesis that an enzyme resembles a “lock” and its particular substrate the “key.” However, enzymes are not rigid templates like locks. Koshland noted that the conformation of an enzyme is dynamic and hypothesized that the interaction of E with S is also dynamic. The enzyme’s active site is actually modified upon binding S, in a process of dynamic recognition between enzyme and substrate called induced fit. Hexokinase provides a good illustration of the relationship between substrate binding, induced fit, and catalysis.

**13.7 Are All Enzymes Proteins?** Not all enzymes are proteins. Catalytic RNA molecules (“ribozymes”) play important cellular roles in RNA processing and protein synthesis, among other things. Catalytic RNAs give support to the idea that a primordial world dominated by RNA molecules existed before the evolution of DNA and proteins.

Antibodies that have catalytic activity (“abzymes”) can be elicited in an organism in response to immunological challenge with an analog of the transition state for a reaction. Such antibodies are catalytic because they bind the transition state of a reaction and promote entry of the normal substrate into the reactive, transition-state conformation.

**13.8 Is It Possible to Design an Enzyme to Catalyze Any Desired Reaction?** Several approaches have been taken to create **designer enzymes** individually tailored to catalyze any imaginable reaction. One rational approach is to begin with a known enzyme and then engineer it using *in vitro* mutagenesis to replace active-site residues with a new set that might catalyze the desired reaction. A second, more difficult approach uses computer modeling to design a protein with the desired structure and activity. A third approach is to couple natural evolutionary processes with rational design using *in vitro* mutagenesis. Expression of mutated versions of the gene encoding the enzyme in bacteria is followed by selection for bacteria producing an enzyme with even better catalytic properties. This dual approach is sometimes called semirational design, because it relies on the rational substitution of certain amino acids with new ones in the active site, followed by directed evolution. Active-site engineering and site-directed mutation have been used to modify an epoxide hydrolase so that it now acts on chlorinated epoxides, substances that are serious pollutants in water resources.

## FOUNDATIONAL BIOCHEMISTRY Things You Should Know After Reading Chapter 13.

- Enzymes are the agents of metabolic function.
- Enzymes exert kinetic control over thermodynamic potentiality.
- Catalytic power is defined as the ratio of the enzyme-catalyzed rate of a reaction to the uncatalyzed rate.
- The substance upon which an enzyme acts is termed the substrate of the enzyme.
- Specificity is the term used to define the selectivity of enzymes for their substrates.
- The specific site on the enzyme to which the substrate binds and where catalysis occurs is called the active site.
- Regulation of enzyme activity ensures that the rate of a metabolic reaction is appropriate to cellular requirements.
- Enzyme nomenclature provides a systematic way of naming metabolic reactions.
- Coenzymes and cofactors are nonprotein components of enzymes that are essential for enzyme activity.
- Tightly bound coenzymes are termed prosthetic groups; the protein without the prosthetic group is called an apoenzyme.
- Enzyme kinetics is the branch of science that studies the properties of enzyme-catalyzed reactions.
- Enzymes, like other catalysts, lower the free energy of activation for a reaction.
- Decreasing the free energy of activation,  $\Delta G^\ddagger$ , increases reaction rate.
- The rate of a simple enzyme-catalyzed reaction can be described by the Michaelis-Menten equation.
- The Briggs-Haldane steady-state assumption ( $d[ES]/dt = 0$ ) provides an improved interpretation of the Michaelis-Menten equation.
- $K_m$ , the Michaelis constant, is defined as  $(k_{-1} + k_2)/k_1$ .
- The velocity of the reaction,  $v$ , is given by  $v = d[P]/dt = k_2[ES]$ .
- The maximal velocity of the reaction,  $V_{\max}$ , is achieved when all of the enzyme, E, is in the ES form.  $V_{\max} = k_2[E_T]$ .
- When  $[S] = K_m$ ,  $v = V_{\max}/2$ .
- The turnover number, or molecular activity, of an enzyme is defined as  $k_{\text{cat}} \cdot k_{\text{cat}} = V_{\max}[E_T]$ .
- The catalytic efficiency of an enzyme is defined as  $k_{\text{cat}}/K_m$ .
- The catalytic efficiency of an enzyme cannot exceed the diffusion-controlled rate of encounters between E and S. Thus, the upper limit on  $k_{\text{cat}}/K_m$  is about  $10^9 \text{ M}^{-1}\text{sec}^{-1}$ .
- Lineweaver-Burk double-reciprocal ( $1/v$  vs.  $1/[S]$ ) plots and Hanes-Woolf ( $[S]/v$  vs.  $[S]$ ) plots convert the hyperbolic Michaelis-Menten  $v$  vs.  $[S]$  response into a straight line.
- Enzyme activity is affected by pH, temperature, and ionic strength, all of which can affect protein conformation.
- Enzyme inhibitors are useful probes of the properties of enzymatic reactions.
- Many drugs and other pharmacological agents are enzyme inhibitors.
- Enzyme inhibitors are classified as competitive (compete with S for binding to the active site), noncompetitive (bind elsewhere on the enzyme) or uncompetitive (bind only to the ES complex).
- Irreversible inhibitors bind irreversibly to or form covalent bonds with the enzyme.
- Bisubstrate reaction mechanisms may be single-displacement (both substrates bind and then the reaction occurs) or double-displacement (the first substrate binds, reaction occurs and product is released and then the second substrate binds, further reaction occurs and the second product is released).
- Single-displacement reactions may be random (it does not matter which substrate binds first) or ordered (one substrate, called the leading substrate, binds first; only when it is bound can the second substrate bind).
- Fischer's "lock and key hypothesis" was the original explanation for enzyme specificity.
- Koshland's "induced fit hypothesis" is a better explanation of enzyme specificity because it considers the conformational change induced in the enzyme (and the substrate) when S is bound by E.
- Not only protein molecules but RNA molecules may be catalytic; catalytic RNA molecules are termed ribozymes.
- The ribosome is a ribozyme.
- Antibodies that have catalytic activity are called abzymes.
- Designer enzymes are enzymes designed to catalyze a reaction for which no naturally occurring enzyme exists.
- Several approaches have been taken to create designer enzymes: rational enzyme design (altering the active site of a known enzyme so that it now catalyzes a new reaction) or *in silico* design (using computers to model an active site that will catalyze a desired reaction and then attempting to create a protein that will have such an active site).
- Designer enzymes have potential applications in organic synthesis (such as the synthesis of new drugs) and environmental remediation (to remove pollutants).

## PROBLEMS

Answers to all problems are at the end of this book. Detailed solutions are available in the *Student Solutions Manual, Study Guide, and Problems Book*.

1. **Exploring the Michaelis-Menten Equation - I** According to the Michaelis-Menten equation, what is the  $v/V_{\max}$  ratio when  $[S] = 4 K_m$ ?
2. **Exploring the Michaelis-Menten Equation - II** If  $V_{\max} = 100 \mu\text{mol}/\text{mL} \cdot \text{sec}$  and  $K_m = 2 \text{ mM}$ , what is the velocity of the reaction when  $[S] = 20 \text{ mM}$ ?

3. **Exploring the Michaelis-Menten Equation - III** For a Michaelis-Menten reaction,  $k_1 = 7 \times 10^7/\text{M} \cdot \text{sec}$ ,  $k_{-1} = 1 \times 10^3/\text{sec}$ , and  $k_2 = 2 \times 10^4/\text{sec}$ . What are the values of  $K_S$  and  $K_m$ ? Does substrate binding approach equilibrium, or does it behave more like a steady-state system?
4. **Graphing the Results from Kinetics Experiments with Enzyme Inhibitors** The following kinetic data were obtained for an enzyme in the absence of any inhibitor (1), and in the presence of two different

inhibitors (2) and (3) at 5 mM concentration. Assume  $[E_T]$  is the same in each experiment.

| $[S]$<br>(mM) | v ( $\mu\text{mol}/\text{mL} \cdot \text{sec}$ )<br>(1) | v ( $\mu\text{mol}/\text{mL} \cdot \text{sec}$ )<br>(2) | v ( $\mu\text{mol}/\text{mL} \cdot \text{sec}$ )<br>(3) |
|---------------|---|---|---|
| 1             | 12  | 4.3   | 5.5   |
| 2             | 20  | 8   | 9   |
| 4             | 29  | 14  | 13  |
| 8             | 35  | 21  | 16  |
| 12            | 40  | 26  | 18  |

Graph these data as Lineweaver–Burk plots and use your graph to find answers to a. and b.

- Determine  $V_{\max}$  and  $K_m$  for the enzyme.
- Determine the type of inhibition and the  $K_I$  for each inhibitor.

**5. How Varying the Amount of Enzyme or the Addition of Inhibitors Affects v Versus [S] Plots** Using Figure 13.7 as a model, draw curves that would be obtained in v versus [S] plots when

- twice as much enzyme is used.
- half as much enzyme is used.
- a competitive inhibitor is added.
- a pure noncompetitive inhibitor is added.
- an uncompetitive inhibitor is added.

For each example, indicate how  $V_{\max}$  and  $K_m$  change.

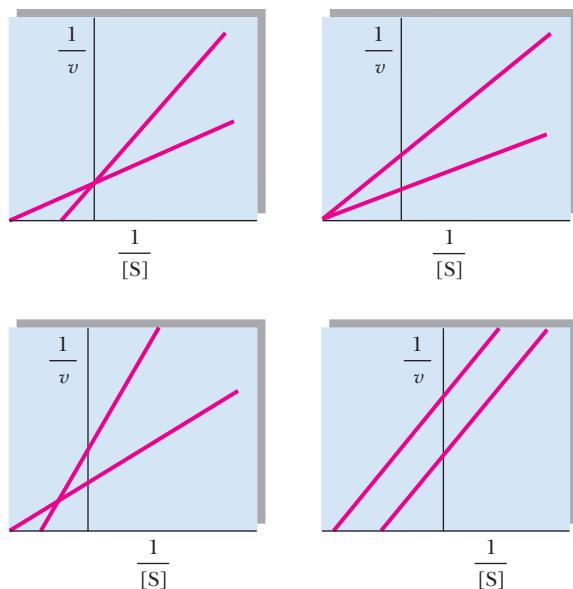
**6. Using Graphical Methods to Derive the Kinetic Constants for an Ordered, Single-Displacement Reaction** The general rate equation for an ordered, single-displacement reaction where A is the leading substrate is

$$v = \frac{V_{\max}[A][B]}{(K_S^A K_m^B + K_m^A[B] + K_m^B[A] + [A][B])}$$

Write the Lineweaver–Burk (double-reciprocal) equivalent of this equation and from it calculate algebraic expressions for the following:

- The slope
- The y-intercepts
- The horizontal and vertical coordinates of the point of intersection when  $1/v$  is plotted versus  $1/[B]$  at various fixed concentrations of A

**7. Interpreting Kinetics Experiments from Graphical Patterns** The following graphical patterns obtained from kinetic experiments have several possible interpretations depending on the nature of the experiment and the variables being plotted. Give at least two possibilities for each.



**8. Using the Equations of Enzyme Kinetics to Treat Methanol Intoxication**

Liver alcohol dehydrogenase (ADH) is relatively nonspecific and will oxidize ethanol or other alcohols, including methanol. Methanol oxidation yields formaldehyde, which is quite toxic, causing, among other things, blindness. Mistaking it for the cheap wine he usually prefers, my dog Clancy ingested about 50 mL of windshield washer fluid (a solution 50% in methanol). Knowing that methanol would be excreted eventually by Clancy's kidneys if its oxidation could be blocked, and realizing that, in terms of methanol oxidation by ADH, ethanol would act as a competitive inhibitor, I decided to offer Clancy some wine. How much of Clancy's favorite vintage (12% ethanol) must he consume in order to lower the activity of his ADH on methanol to 5% of its normal value if the  $K_m$  values of canine ADH for ethanol and methanol are 1 millimolar and 10 millimolar, respectively? (The  $K_I$  for ethanol in its role as competitive inhibitor of methanol oxidation by ADH is the same as its  $K_m$ .) Both the methanol and ethanol will quickly distribute throughout Clancy's body fluids, which amount to about 15 L. Assume the densities of 50% methanol and the wine are both 0.9 g/mL.

**9. Quantitative Relationships Between Rate Constants to Calculate  $K_m$ , Kinetic Efficiency ( $k_{\text{cat}}/K_m$ ) and  $V_{\max}$  – I**

Measurement of the rate constants for a simple enzymatic reaction obeying Michaelis–Menten kinetics gave the following results:

$$k_1 = 2 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$$

$$k_{-1} = 1 \times 10^3 \text{ sec}^{-1}$$

$$k_2 = 5 \times 10^3 \text{ sec}^{-1}$$

- What is  $K_S$ , the dissociation constant for the enzyme–substrate complex?
- What is  $K_m$ , the Michaelis constant for this enzyme?
- What is  $k_{\text{cat}}$  (the turnover number) for this enzyme?
- What is the catalytic efficiency ( $k_{\text{cat}}/K_m$ ) for this enzyme?
- Does this enzyme approach "kinetic perfection"? (That is, does  $k_{\text{cat}}/K_m$  approach the diffusion-controlled rate of enzyme association with substrate?)
- If a kinetic measurement was made using 2 nanomoles of enzyme per mL and saturating amounts of substrate, what would  $V_{\max}$  equal?
- Again, using 2 nanomoles of enzyme per mL of reaction mixture, what concentration of substrate would give  $v = 0.75 V_{\max}$ ?
- If a kinetic measurement was made using 4 nanomoles of enzyme per mL and saturating amounts of substrate, what would  $V_{\max}$  equal? What would  $K_m$  equal under these conditions?

**10. Quantitative Relationships Between Rate Constants to Calculate  $K_m$ , Kinetic Efficiency ( $k_{\text{cat}}/K_m$ ) and  $V_{\max}$  – II**

Triose phosphate isomerase catalyzes the conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate.



The  $K_m$  of this enzyme for its substrate glyceraldehyde-3-phosphate is  $1.8 \times 10^{-5} \text{ M}$ . When  $[\text{glyceraldehydes-3-phosphate}] = 30 \mu\text{M}$ , the rate of the reaction,  $v$ , was  $82.5 \mu\text{mol mL}^{-1} \text{ sec}^{-1}$ .

- What is  $V_{\max}$  for this enzyme?
- Assuming 3 nanomoles per mL of enzyme was used in this experiment ( $[E_{\text{total}}] = 3 \text{ nanomol/mL}$ ), what is  $k_{\text{cat}}$  for this enzyme?
- What is the catalytic efficiency ( $k_{\text{cat}}/K_m$ ) for triose phosphate isomerase?
- Does the value of  $k_{\text{cat}}/K_m$  reveal whether triose phosphate isomerase approaches "catalytic perfection"?
- What determines the ultimate speed limit of an enzyme-catalyzed reaction? That is, what is it that imposes the physical limit on kinetic perfection?

**11. Quantitative Relationships Between Rate Constants to Calculate  $K_m$ , Kinetic Efficiency ( $k_{\text{cat}}/K_m$ ) and  $V_{\max}$  – III**

The citric acid cycle enzyme fumarase catalyzes the conversion of fumarate to form malate.



The turnover number,  $k_{\text{cat}}$ , for fumarase is 800/sec. The  $K_m$  of fumarase for its substrate fumarate is 5  $\mu\text{M}$ .

- In an experiment using 2 nanomole/mL of fumarase, what is  $V_{\text{max}}$ ?
- The cellular concentration of fumarate is 47.5  $\mu\text{M}$ . What is  $v$  when [fumarate] = 47.5  $\mu\text{M}$ ?
- What is the catalytic efficiency of fumarase?
- Does fumarase approach “catalytic perfection”?

**12. Quantitative Relationships Between Rate Constants to Calculate  $K_m$ , Kinetic Efficiency ( $k_{\text{cat}}/K_m$ ) and  $V_{\text{max}}$  - IV** Carbonic anhydrase catalyzes the hydration of  $\text{CO}_2$ :



The  $K_m$  of carbonic anhydrase for  $\text{CO}_2$  is 12 mM. Carbonic anhydrase gave an initial velocity  $v_0 = 4.5 \mu\text{mol H}_2\text{CO}_3$  formed/mL · sec when  $[\text{CO}_2] = 36 \text{ mM}$ .

- What is  $V_{\text{max}}$  for this enzyme?
- Assuming 5 pmol/mL ( $5 \times 10^{-12}$  moles/mL) of enzyme were used in this experiment, what is  $k_{\text{cat}}$  for this enzyme?
- What is the catalytic efficiency of this enzyme?
- Does carbonic anhydrase approach “catalytic perfection”?

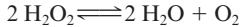
**13. Quantitative Relationships Between Rate Constants to Calculate  $K_m$ , Kinetic Efficiency ( $k_{\text{cat}}/K_m$ ) and  $V_{\text{max}}$  - V** Acetylcholinesterase catalyzes the hydrolysis of the neurotransmitter acetylcholine:



The  $K_m$  of acetylcholinesterase for its substrate acetylcholine is  $9 \times 10^{-5} \text{ M}$ . In a reaction mixture containing 5 nanomoles/mL of acetylcholinesterase and 150  $\mu\text{M}$  acetylcholine, a velocity  $v_0 = 40 \mu\text{mol/mL} \cdot \text{sec}$  was observed for the acetylcholinesterase reaction.

- Calculate  $V_{\text{max}}$  for this amount of enzyme.
- Calculate  $k_{\text{cat}}$  for acetylcholinesterase.
- Calculate the catalytic efficiency ( $k_{\text{cat}}/K_m$ ) for acetylcholinesterase.
- Does acetylcholinesterase approach “catalytic perfection”?

**14. Quantitative Relationships Between Rate Constants to Calculate  $K_m$ , Kinetic Efficiency ( $k_{\text{cat}}/K_m$ ) and  $V_{\text{max}}$  - VI** The enzyme catalase catalyzes the decomposition of hydrogen peroxide:



The turnover number ( $k_{\text{cat}}$ ) for catalase is 40,000,000 sec<sup>-1</sup>. The  $K_m$  of catalase for its substrate  $\text{H}_2\text{O}_2$  is 0.11 M.

## FURTHER READING

### Enzymes in General

Bennett, B. D., Kimball, E. H., Osterhout, R., Van Dien, S. J., and Rabinowitz, J. D., 2009. Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nature Chemical Biology* **5**:593–599.

Creighton, T. E., 1997. *Protein Structure: A Practical Approach and Protein Function: A Practical Approach*. Oxford: Oxford University Press.

Fersht, A., 1999. *Structure and Mechanism in Protein Science*. New York: Freeman & Co. A guide to protein structure, chemical catalysis, enzyme kinetics, enzyme regulation, protein engineering, and protein folding.

Petsko, G. A., and Ringe, D., 2004. *Protein Structure and Function*. Sunderland, MA: New Science Press, LTD; Sinauer Associates, Inc.

### Catalytic Power

Miller, B. G., and Wolfenden, R., 2002. Catalytic proficiency: The unusual case of OMP decarboxylase. *Annual Review of Biochemistry* **71**: 847–885.

- In an experiment using 3 nanomole/L of catalase, what is  $V_{\text{max}}$ ?

- What is  $v$  when  $[\text{H}_2\text{O}_2] = 0.75 \text{ M}$ ?

- What is the catalytic efficiency of fumarase?

- Does catalase approach “catalytic perfection”?

**15. The Effect of the Accumulation of P, the Reaction Product, on the Michaelis–Menten Equation** Equation 13.9 presents the simple Michaelis–Menten situation where the reaction is considered to be irreversible ([P] is negligible). Many enzymatic reactions are reversible, and P does accumulate.

- Derive an equation for  $v$ , the rate of the enzyme-catalyzed reaction S → P in terms of a modified Michaelis–Menten model that incorporates the reverse reaction that will occur in the presence of product, P.
- Solve this modified Michaelis–Menten equation for the special situation when  $v = 0$  (that is, S ⇌ P is at equilibrium, or in other words,  $K_{\text{eq}} = [\text{P}]/[\text{S}]$ ).

(J. B. S. Haldane first described this reversible Michaelis–Menten modification, and his expression for  $K_{\text{eq}}$  in terms of the modified M–M equation is known as the Haldane relationship.)

## Preparing for the MCAT® Exam

**16. Enzyme A follows simple Michaelis–Menten kinetics.**

- The  $K_m$  of enzyme A for its substrate S is  $K_m^S = 1 \text{ mM}$ . Enzyme A also acts on substrate T and its  $K_m^T = 10 \text{ mM}$ . Is S or T the preferred substrate for enzyme A?
- The rate constant  $k_2$  with substrate S is  $2 \times 10^4 \text{ sec}^{-1}$ ; with substrate T,  $k_2 = 4 \times 10^5 \text{ sec}^{-1}$ . Does enzyme A use substrate S or substrate T with greater catalytic efficiency?

**17. Use Figure 13.12 to answer the following questions.**

- Is the enzyme whose temperature versus activity profile is shown in Figure 13.12 likely to be from an animal or a plant? Why?
- What do you think the temperature versus activity profile for an enzyme from a thermophilic prokaryote growing in an 80°C pool of water would resemble?

## ActiveModels Problems

**18.** Examine the ActiveModel for glutamate-oxaloacetate aminotransferase (pdb id = GOT1) and identify the amino acid side chains involved in binding pyridoxal-5'-phosphate

**19.** Using the ActiveModel for hexokinase, explain the conformational change that occurs upon substrate binding.

Wolfenden, R., 2011. Benchmark reaction rates, the stability of biological molecules in water, and the evolution of catalytic power in enzymes. *Annual Review of Biochemistry* **80**:645–667.

Wolfenden, R., and Snider, M. J., 2001. The depth of chemical time and the power of enzymes as catalysts. *Accounts of Chemical Research* **34**:938–945.

### General Reviews of Enzyme Kinetics

Cleland, W. W., 1990. Steady-state kinetics. In *The Enzymes*, 3rd ed. Sigman, D. S., and Boyer, P. D., eds. Volume XIX, pp. 99–158. See also, *The Enzymes*, 3rd ed. Boyer, P. D., ed., Volume II, pp. 1–65, 1970.

Cornish-Bowden, A., 1994. *Fundamentals of Enzyme Kinetics*. Cambridge: Cambridge University Press.

Smith, W. G., 1992. In vivo kinetics and the reversible Michaelis–Menten model. *Journal of Chemical Education* **12**:981–984.

### Graphical and Statistical Analysis of Kinetic Data

Cleland, W. W., 1979. Statistical analysis of enzyme kinetic data. *Methods in Enzymology* **82**:103–138.

Naqui, A., 1986. Where are the asymptotes of Michaelis–Menten? *Trends in Biochemical Sciences* **11**:64–65. A proof that the Michaelis–Menten equation describes a rectangular hyperbola.

Rudolph, F. B., and Fromm, H. J., 1979. Plotting methods for analyzing enzyme rate data. *Methods in Enzymology* **63**:138–159. A review of the various rearrangements of the Michaelis–Menten equation that yield straight-line plots.

Segel, I. H., 1976. *Biochemical Calculations*, 2nd ed. New York: John Wiley & Sons. An excellent guide to solving problems in enzyme kinetics.

### Effect of Active Site Amino Acid Substitutions on $k_{cat}/K_m$

Garrett, R. M., et al., 1998. Human sulfite oxidase R160Q: Identification of the mutation in a sulfite oxidase-deficient patient and expression and characterization of the mutant enzyme. *Proceedings of the National Academy of Sciences U.S.A.* **95**:6394–6398.

Garrett, R. M., and Rajagopalan, K. V., 1996. Site-directed mutagenesis of recombinant sulfite oxidase. *Journal of Biological Chemistry* **271**: 7387–7391.

### Enzymes and Rational Drug Design

Cornish-Bowden, A., and Eisenthal, R., 1998. Prospects for antiparasitic drugs: The case of *Trypanosoma brucei*, the causative agent of African sleeping sickness. *Journal of Biological Chemistry* **273**:5500–5505. An analysis of why drug design strategies have had only limited success.

Kling, J., 1998. From hypertension to angina to Viagra. *Modern Drug Discovery* **1**:31–38. The story of the serendipitous discovery of Viagra in a search for agents to treat angina and high blood pressure.

### Enzyme Inhibition

Cleland, W. W., 1979. Substrate inhibition. *Methods in Enzymology* **63**: 500–513.

Pollack, S. J., et al., 1994. Mechanism of inositol monophosphatase, the putative target of lithium therapy. *Proceedings of the National Academy of Sciences U.S.A.* **91**:5766–5770.

Silverman, R. B., 1988. *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*, Vols. I and II. Boca Raton, FL: CRC Press.

### Catalytic RNA

Altman, S., 2000. The road to RNase P. *Nature Structural Biology* **7**: 827–828.

Cech, T. R., and Bass, B. L., 1986. Biological catalysis by RNA. *Annual Review of Biochemistry* **55**:599–629. A review of the early evidence that RNA can act like an enzyme.

Doherty, E. A., and Doudna, J. A., 2000. Ribozyme structures and mechanisms. *Annual Review of Biochemistry* **69**:597–615.

Frank, D. N., and Pace, N. R., 1998. Ribonuclease P: Unity and diversity in a tRNA processing ribozyme. *Annual Review of Biochemistry* **67**: 153–180.

Narlikar, G. J., and Herschlag, D., 1997. Mechanistic aspects of enzymatic catalysis: Comparison of RNA and protein enzymes. *Annual Review of Biochemistry* **66**:19–59. A comparison of RNA and protein enzymes that addresses fundamental principles in catalysis and macromolecular structure.

Nissen, P., et al., 2000. The structural basis of ribosome activity in peptide bond synthesis. *Science* **289**:920–930. Peptide bond formation by the ribosome: the ribosome is a ribozyme.

Schimmel, P., and Kelley, S. O., 2000. Exiting an RNA world. *Nature Structural Biology* **7**:5–7. Review of the in vitro creation of an RNA capable of catalyzing the formation of an aminoacyl-tRNA. Such a ribozyme would be necessary to bridge the evolutionary gap between a primordial RNA world and the contemporary world of proteins.

Watson, J. D., ed., 1987. Evolution of catalytic function. *Cold Spring Harbor Symposium on Quantitative Biology* **52**:1–955. Publications

from a symposium on the nature and evolution of catalytic biomolecules (proteins and RNA) prompted by the discovery that RNA could act catalytically.

Wilson, D. S., and Szostak, J. W., 1999. In vitro selection of functional nucleic acids. *Annual Review of Biochemistry* **68**:611–647. Screening libraries of random nucleotide sequences for catalytic RNAs.

### Catalytic Antibodies

Hilvert, D., 2000. Critical analysis of antibody catalysis. *Annual Review of Biochemistry* **69**:751–793. A review of catalytic antibodies that were elicited with rationally designed transition-state analogs.

Janda, K. D., 1997. Chemical selection for catalysis in combinatorial antibody libraries. *Science* **275**:945.

Lacroix-Desmazes, S., et al., 2002. The prevalence of proteolytic antibodies against factor VIII in Hemophilia A. *New England Journal of Medicine* **346**:662–667.

Ponomarenko, N. A., 2006. Autoantibodies to myelin basic protein catalyze site-specific degradation of their antigen. *Proceedings of the National Academy of Sciences U.S.A.* **103**:281–286.

Wagner, J., Lerner, R. A., and Barbas, C. F., III, 1995. Efficient adolase catalytic antibodies that use the enamine mechanism of natural enzymes. *Science* **270**:1797–1800.

### Designer Enzymes

Chica, R. A., Doucet, N., and Pelletier, J. N., 2005. Semi-rational approaches to engineering enzyme activity: Combining the benefits of directed evolution and rational design. *Current Opinion in Biotechnology* **16**:378–384.

Jiang, L., Althoff, E. A., Clemente, F. R., et al., 2008. De novo computational design of retro-aldol enzymes. *Science* **319**:1387–1391.

Kaplan, J., and DeGrado, W. F., 2004. De novo design of catalytic proteins. *Proceedings of the National Academy of Sciences U.S.A.* **101**:11566–11570.

Lippow, S. M., and Tidor, B., 2007. Progress in computational protein design. *Current Opinion in Biotechnology* **18**:305–311.

Rui, L., Cao, L., Chen, W., et al., 2004. Active site engineering of the epoxide hydrolase from *Agrobacterium radiobacter* AD1 to enhance aerobic mineralization of *cis*-1,2-dichloroethylene in cells expressing an evolved toluene *ortho*-monooxygenase. *The Journal of Biological Chemistry* **279**:46810–46817.

Siegel, J. B., Zanghellini, A., Lovick, H. M., et al., 2010. Computational design of an enzyme catalyst for a stereoselective bimolecular Diels-Alder reaction. *Science* **329**:309–313. See also the overview by Lutz, S., 2010. Reengineering enzymes. *Science* **309**:285–287.

Walter, K. U., Vamvaca, K., and Hilvert, D., 2005. An active enzyme constructed from a 9-amino acid alphabet. *The Journal of Biological Chemistry* **280**:37742–37746.

Woycechowsky, K. L., et al., 2007. Novel enzymes through design and evolution. *Advances in Enzymology and Related Areas of Molecular Biology* **75**:241–294.

### Specificity

Jencks, W. P., 1975. Binding energy, specificity, and enzymic catalysis: The Circe effect. *Advances in Enzymology* **43**:219–410. Enzyme specificity stems from the favorable binding energy between the active site and the substrate and unfavorable binding or exclusion of nonsubstrate molecules.

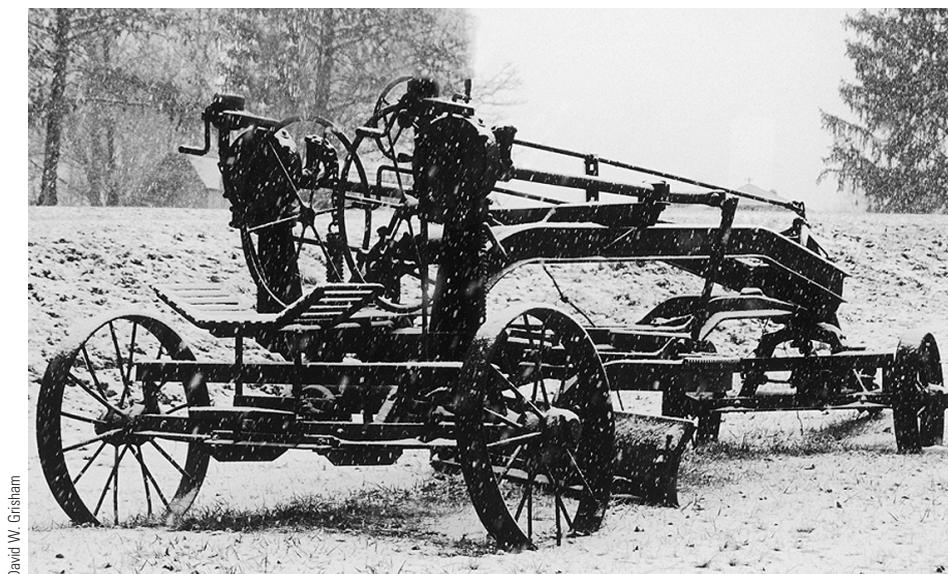
Johnson, K. A., 2008. Role of induced fit in enzyme specificity: A molecular forward/reverse switch. *The Journal of Biological Chemistry* **283**: 26297–26301.

### The Effect of Temperature on Enzyme Activity

Daniel, R. M., and Danson, M. J., 2010. A new understanding of how temperature affects the catalytic activity of enzymes. *Trends in Biochemical Sciences* **35**:584–591.



# Mechanisms of Enzyme Action



## ESSENTIAL QUESTION

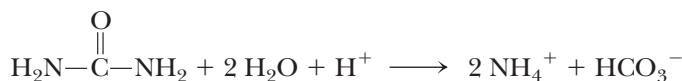
Although the catalytic properties of enzymes may seem almost magical, it is simply chemistry—the breaking and making of bonds—that gives enzymes their prowess. This chapter will explore the unique features of this chemistry. The mechanisms of thousands of enzymes have been studied in at least some detail. In this chapter, it will be possible to examine only a few of these.

**What are the universal chemical principles that influence the mechanisms of enzymes and allow us to understand their enormous catalytic power?**

## 14.1 What Are the Magnitudes of Enzyme-Induced Rate Accelerations?

Enzymes are powerful catalysts. Enzyme-catalyzed reactions are typically  $10^7$  to  $10^{15}$  times faster than their uncatalyzed counterparts (Table 14.1). The most impressive reaction acceleration known is that of fructose-1,6-bisphosphatase, an enzyme found in liver and kidneys that is involved in the synthesis of glucose (see Chapter 22).

These large rate accelerations correspond to substantial decreases in the free energy of activation for the reaction in question. The urease reaction, for example,



shows an energy of activation 84 kJ/mol smaller than that for the corresponding uncatalyzed reaction. To fully understand any enzyme reaction, it is important to account for the rate acceleration in terms of the structure of the enzyme and its mechanism of action.

In all chemical reactions, the reacting atoms or molecules pass through a state that is intermediate in structure between the reactant(s) and the product(s). Consider the transfer of a proton from a water molecule to a chloride anion:



◀ Like the workings of machines, the details of enzyme mechanisms are at once complex and simple.

*No single thing abides but all things flow.  
Fragment to fragment clings and thus  
they grow  
Until we know them by name.  
Then by degrees they change and are no more  
the things we know.*

*Lucretius (ca. 94 B.C.–50 B.C.)*

## KEY QUESTIONS

- 14.1** What Are the Magnitudes of Enzyme-Induced Rate Accelerations?
- 14.2** What Role Does Transition-State Stabilization Play in Enzyme Catalysis?
- 14.3** How Does Destabilization of ES Affect Enzyme Catalysis?
- 14.4** How Tightly Do Transition-State Analogs Bind to the Active Site?
- 14.5** What Are the Mechanisms of Catalysis?
- 14.6** What Can Be Learned from Typical Enzyme Mechanisms?

**TABLE 14.1** A Comparison of Enzyme-Catalyzed Reactions and Their Uncatalyzed Counterparts

| Reaction   | Enzyme                      | Uncatalyzed Rate, $v_u$ (sec $^{-1}$ ) | Catalyzed Rate, $v_e$ (sec $^{-1}$ ) | $v_e/v_u$             |
|--|-----------------------------|--|--------------------------------------|-----------------------|
| Fructose-1,6-bisP $\longrightarrow$ fructose-6-P + P <sub>i</sub>  | Fructose-1,6-bisphosphatase | $2 \times 10^{-20}$                    | 21                                   | $1.05 \times 10^{21}$ |
| (Glucose) <sub>n</sub> + H <sub>2</sub> O $\longrightarrow$ (glucose) <sub>n-2</sub> + maltose   | $\beta$ -amylase            | $1.9 \times 10^{-15}$                  | $1.4 \times 10^3$                    | $7.2 \times 10^{17}$  |
| DNA, RNA cleavage  | Staphylococcal nuclease     | $7 \times 10^{-16}$                    | 95                                   | $1.4 \times 10^{17}$  |
| CH <sub>3</sub> —O—PO <sub>3</sub> <sup>2-</sup> + H <sub>2</sub> O $\longrightarrow$ CH <sub>3</sub> OH + HPO <sub>4</sub> <sup>2-</sup>                                  | Alkaline phosphatase        | $1 \times 10^{-15}$                    | 14                                   | $1.4 \times 10^{16}$  |
| $\text{H}_2\text{N}-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-\text{NH}_2 + 2 \text{H}_2\text{O} + \text{H}^+ \longrightarrow 2 \text{NH}_4^+ + \text{HCO}_3^-$   | Urease                      |  | $3 \times 10^{-10}$                  | $3 \times 10^4$       |
| $\text{R}-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-\text{O}-\text{CH}_2\text{CH}_3 + \text{H}_2\text{O} \longrightarrow \text{RCOOH} + \text{HOCH}_2\text{CH}_3$ | Chymotrypsin                |  | $1 \times 10^{-10}$                  | $1 \times 10^2$       |
| Glucose + ATP $\longrightarrow$ Glucose-6-P + ADP  | Hexokinase                  | $<1 \times 10^{-13}$                   | $1.3 \times 10^{-3}$                 | $>1.3 \times 10^{10}$ |
| $\text{CH}_3\text{CH}_2\text{OH} + \text{NAD}^+ \longrightarrow \text{CH}_3\overset{\text{O}}{\underset{\parallel}{\text{CH}}} + \text{NADH} + \text{H}^+$                 | Alcohol dehydrogenase       | $<6 \times 10^{-12}$                   | $2.7 \times 10^{-5}$                 | $>4.5 \times 10^6$    |
| CO <sub>2</sub> + H <sub>2</sub> O $\longrightarrow$ HCO <sub>3</sub> <sup>-</sup> + H <sup>+</sup>  | Carbonic anhydrase          | $10^{-2}$                              | $10^5$                               | $1 \times 10^7$       |
| Creatine + ATP $\longrightarrow$ Cr-P + ADP  | Creatine kinase             | $<3 \times 10^{-9}$                    | $4 \times 10^{-5}$                   | $>1.33 \times 10^4$   |

Adapted from Koshland, D., 1956. Molecular geometry in enzyme action. *Journal of Cellular Comparative Physiology*, Supp. 1, 47:217; and Wolfenden, R., 2006. Degrees of difficulty of water-consuming reactions in the absence of enzymes. *Chemical Reviews* 106:3379–3396.

In the middle structure, the proton undergoing transfer is shared equally by the hydroxyl and chloride anions. This structure represents, as nearly as possible, the transition between the reactants and products, and it is known as the **transition state**.<sup>1</sup> All transition states contain at least one partially formed bond.

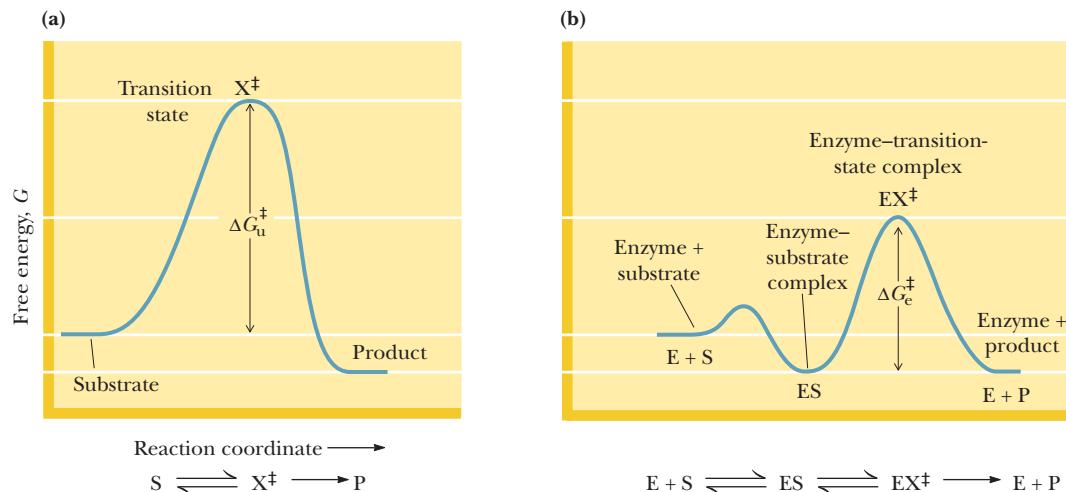
Linus Pauling was the first to suggest (in 1946) that the active sites of enzymes bind the transition state more readily than the substrate and that, by doing so, they stabilize the transition state and lower the activation energy of the reaction. Many subsequent studies have shown that this idea is essentially correct, but it is just the beginning in understanding what enzymes do. Reaction rates can also be accelerated by destabilizing (raising the energy of) the enzyme–substrate complex. Chemical groups arrayed across the active site actually guide the entering substrate toward the formation of the transition state. Thus, the enzyme active site is said to be “preorganized.” Enzymes are dynamic, and fluctuations in the active-site structure are presumed to organize the initial enzyme–substrate complex into a reactive conformation and on to the transition state. Along the way, electrostatic and hydrophobic interactions between the enzyme and the substrate mediate and direct these changes that make the reaction possible. Often, catalytic groups provided by the enzyme participate directly in proton transfers and other bond-making and bond-breaking events.

This chapter describes and elaborates on each of these contributions to the catalytic prowess of enzymes and then illustrates the lessons learned by looking closely at the mechanisms of three well-understood enzymes.

## 14.2 What Role Does Transition-State Stabilization Play in Enzyme Catalysis?

Chemical reactions in which a substrate (S) is converted to a product (P) can be pictured as involving a transition state (which we henceforth denote as X<sup>‡</sup>), a species intermediate in structure between S and P (Figure 14.1). As seen in Chapter 13, the catalytic role

<sup>1</sup>It is important to distinguish **transition states** from **intermediates**. A transition state is envisioned as an extreme distortion of a bond, and thus the lifetime of a typical transition state is viewed as being on the order of the lifetime of a bond vibration, typically 10<sup>-13</sup> sec. Intermediates, on the other hand, are longer lived, with lifetimes in the range of 10<sup>-13</sup> to 10<sup>-3</sup> sec.



**FIGURE 14.1** Enzymes catalyze reactions by lowering the activation energy. Here the free energy of activation for (a) the uncatalyzed reaction,  $\Delta G_u^{\ddagger}$ , is larger than that for (b) the enzyme-catalyzed reaction,  $\Delta G_e^{\ddagger}$ .

of an enzyme is to reduce the energy barrier between substrate and transition state. This is accomplished through the formation of an **enzyme–substrate complex** (ES). This complex is converted to product by passing through a transition state,  $EX^{\ddagger}$  (Figure 14.1). As shown, the energy of  $EX^{\ddagger}$  is clearly lower than that of  $X^{\ddagger}$ . One might be tempted to conclude that this decrease in energy explains the rate enhancement achieved by the enzyme, but there is more to the story.

The energy barrier for the uncatalyzed reaction (Figure 14.1) is of course the difference in energies of the S and  $X^{\ddagger}$  states. Similarly, the energy barrier to be surmounted in the enzyme-catalyzed reaction, assuming that E is saturated with S, is the energy difference between ES and  $EX^{\ddagger}$ . *Reaction rate acceleration by an enzyme means simply that the energy barrier between ES and  $EX^{\ddagger}$  is less than the energy barrier between S and  $X^{\ddagger}$ .* In terms of the free energies of activation,  $\Delta G_e^{\ddagger} < \Delta G_u^{\ddagger}$ .

There are important consequences for this statement. The enzyme must stabilize the transition-state complex,  $EX^{\ddagger}$ , more than it stabilizes the substrate complex, ES. Put another way, enzymes bind the transition-state structure more tightly than the substrate (or the product). The dissociation constant for the enzyme–substrate complex is

$$K_S = \frac{[E][S]}{[ES]} \quad (14.1)$$

and the corresponding dissociation constant for the transition-state complex is

$$K_T = \frac{[E][X^{\ddagger}]}{[EX^{\ddagger}]} \quad (14.2)$$

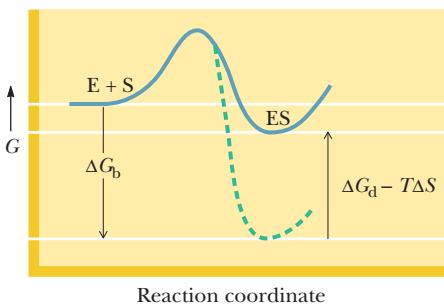
*Enzyme catalysis requires that  $K_T < K_S$ .* According to **transition-state theory** (see references at the end of this chapter), the rate constants for the enzyme-catalyzed ( $k_e$ ) and uncatalyzed ( $k_u$ ) reactions can be related to  $K_S$  and  $K_T$  by

$$k_e/k_u \approx K_S/K_T \quad (14.3)$$

Thus, the enzymatic rate enhancement is approximately equal to the ratio of the dissociation constants of the enzyme–substrate and enzyme–transition-state complexes, at least when E is saturated with S.

## 14.3 How Does Destabilization of ES Affect Enzyme Catalysis?

How is it that  $X^{\ddagger}$  is stabilized more than S at the enzyme active site? To understand this, we must dissect and analyze the formation of the enzyme–substrate complex, ES. There are a number of important contributions to the free energy difference between the



**FIGURE 14.2** The intrinsic binding energy of the enzyme–substrate (ES) complex ( $\Delta G_b$ ) is compensated to some extent by entropy loss due to the binding of E and S ( $T\Delta S$ ) and by destabilization of ES ( $\Delta G_d$ ) by strain, distortion, desolvation, and similar effects. If  $\Delta G_b$  were not compensated by  $T\Delta S$  and  $\Delta G_d$ , the formation of ES would follow the dashed line.

uncomplexed enzyme and substrate ( $E + S$ ) and the ES complex (Figure 14.2). The favorable interactions between the substrate and amino acid residues on the enzyme account for the **intrinsic binding energy**,  $\Delta G_b$ . The intrinsic binding energy ensures the favorable formation of the ES complex, but if uncompensated, it makes the activation energy for the enzyme-catalyzed reaction unnecessarily large and wastes some of the catalytic power of the enzyme.

Compare the two cases in Figure 14.3. Because the enzymatic reaction rate is determined by the difference in energies between ES and  $EX^\ddagger$ , the smaller this difference, the faster the enzyme-catalyzed reaction. Tight binding of the substrate deepens the energy well of the ES complex and actually lowers the rate of the reaction.

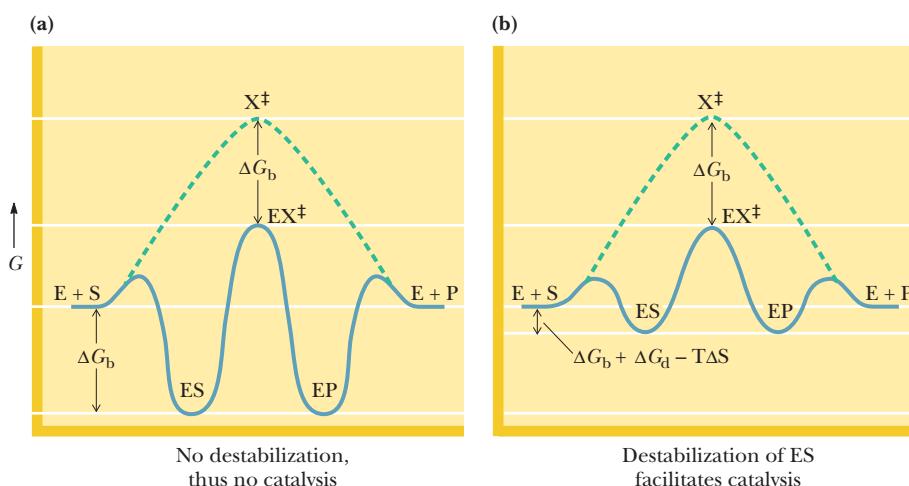
The message of Figure 14.3 is that raising the energy of ES will increase the enzyme-catalyzed reaction rate. This is accomplished in two ways: (1) **loss of entropy** due to the binding of S to E and (2) **destabilization of ES** by strain, distortion, desolvation, or other similar effects. The entropy loss arises from the formation of the ES complex (Figure 14.4), a highly organized (low-entropy) entity compared to  $E + S$  in solution (a disordered, high-entropy situation). Because  $\Delta S$  is negative for this process, the term  $-T\Delta S$  is a positive quantity, and *the intrinsic binding energy of ES is compensated to some extent by the entropy loss that attends the formation of the complex*.

Destabilization of the ES complex can involve **structural strain, desolvation, or electrostatic effects**. Destabilization by strain or distortion is usually just a consequence of the fact (noted previously) that *the enzyme is designed to bind the transition state more strongly than the substrate*. When the substrate binds, the imperfect nature of the “fit” results in distortion or strain in the substrate, the enzyme, or both.

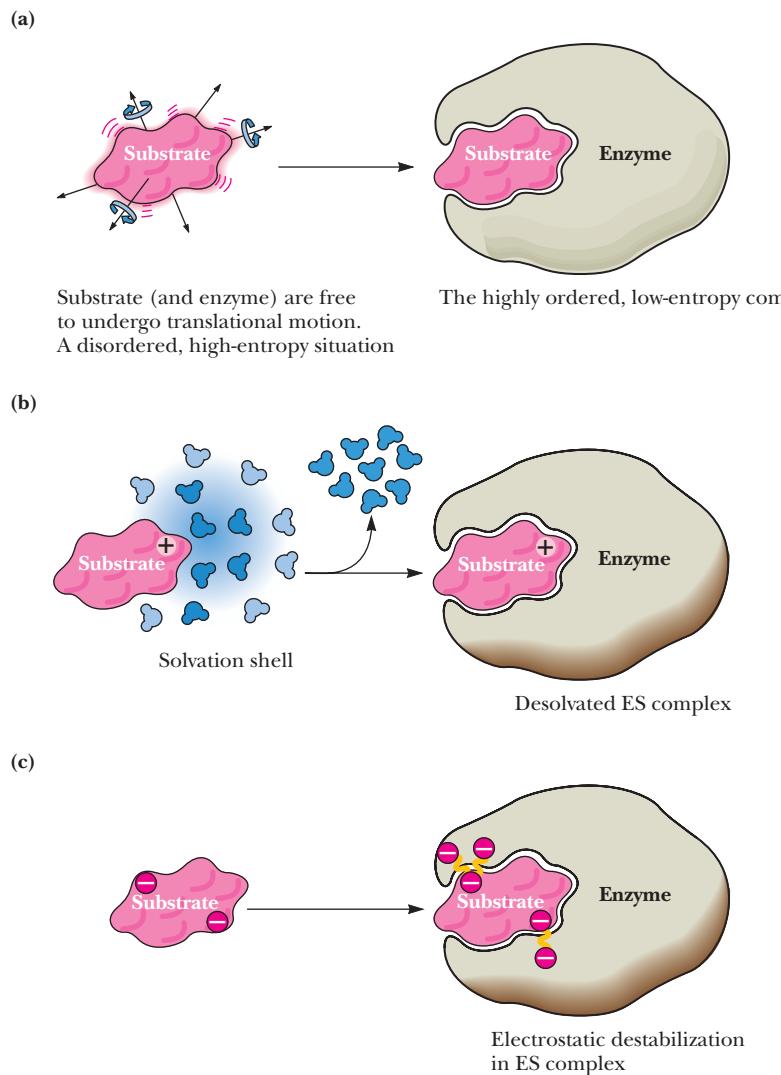
Solvation of charged groups on a substrate in solution releases energy, making the charged substrate more stable. When a substrate with charged groups moves from water into an enzyme active site (Figure 14.4), the charged groups are often desolvated to some extent, becoming less stable and therefore more reactive.

Similarly, when a substrate enters the active site, charged groups may be forced to interact (unfavorably) with charges of like sign, resulting in **electrostatic destabilization** (Figure 14.4). The reaction pathway acts in part to remove this stress. If the charge on the substrate is diminished or lost in the course of reaction, electrostatic destabilization can result in rate acceleration.

Whether by strain, desolvation, or electrostatic effects, destabilization raises the energy of the ES complex, and this increase is summed in the term  $\Delta G_d$ , the free energy of destabilization (Figures 14.2 and 14.3).



**FIGURE 14.3** (a) Catalysis does not occur if the ES complex and the transition state for the reaction are stabilized to equal extents. (b) Catalysis *will* occur if the transition state is stabilized to a greater extent than the ES complex (right). Entropy loss and destabilization of the ES complex  $\Delta G_d$  ensure that this will be the case.



**FIGURE 14.4** (a) Formation of the ES complex results in entropy loss. Before binding, E and S are free to undergo translational and rotational motion. The ES complex is a more highly ordered, low-entropy complex. (b) Substrates typically lose waters of hydration in the formation of the ES complex. Desolvation raises the energy of the ES complex, making it more reactive. (c) Electrostatic destabilization of a substrate may arise from juxtaposition of like charges in the active site. If such charge repulsion is relieved in the course of the reaction, electrostatic destabilization can result in a rate increase.

## 14.4 | How Tightly Do Transition-State Analogs Bind to the Active Site?

Although not apparent at first, there are other important implications of Equation 14.3. It is important to consider the magnitudes of  $K_S$  and  $K_T$ . The ratio  $k_e/k_u$  may even exceed  $10^{16}$ , as noted previously. Given a ratio of  $10^{16}$  and a typical  $K_S$  of  $10^{-4} M$ , the value of  $K_T$  should be  $10^{-20} M$ . The value of  $K_T$  for fructose-1,6-bisphosphatase (see Table 14.1) is an astounding  $7 \times 10^{-26} M$ ! This is the dissociation constant for the transition state from the enzyme, and this very low value corresponds to very tight binding of the transition state by the enzyme.

It is unlikely that such tight binding in an enzyme transition state will ever be determined in a direct equilibrium measurement, however, because the transition state itself is a “moving target.” It exists only for about  $10^{-14}$  to  $10^{-13}$  sec, less than the time required for a bond vibration. On the other hand, the nature of the elusive transition state can be explored using **transition-state analogs**, stable molecules that are chemically and structurally similar to the transition state. Such molecules should bind more strongly than a substrate and more strongly than competitive inhibitors that bear no significant similarity to the transition state. Hundreds of examples of such behavior have been reported. For example, Robert Abeles studied a series of inhibitors of

## A DEEPER LOOK

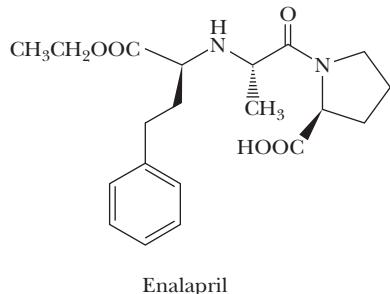
### Transition-State Analogs Make Our World Better

Enzymes (human, plant, and bacterial) are often targets for drugs and other beneficial agents. Transition-state analogs (TSAs), with very high affinities for their enzyme-binding sites, often make ideal enzyme inhibitors, and TSAs have become ubiquitous therapeutic

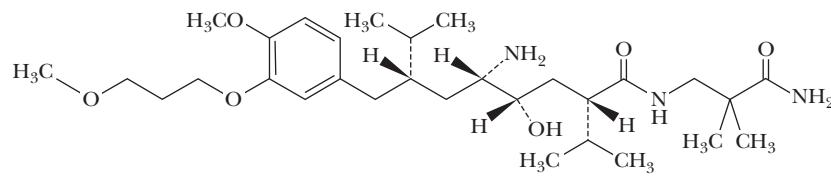
agents that improve the lives of millions and millions of people. A few applications of transition-state analogs for human health and for agriculture are shown here.

#### Enalapril and Aliskiren Lower Blood Pressure

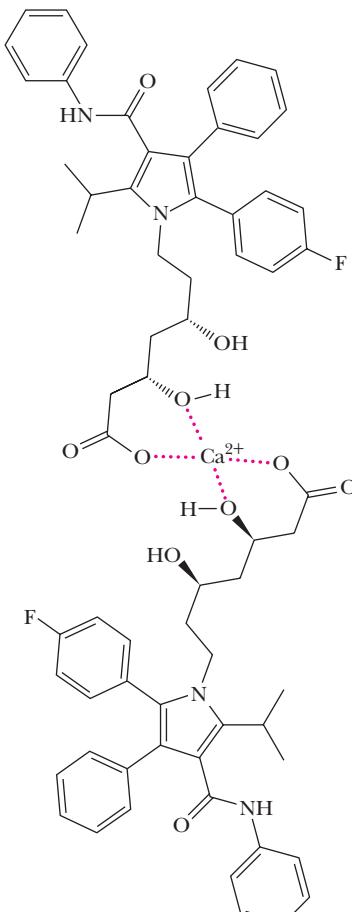
High blood pressure is a significant risk factor for cardiovascular disease, and drugs that lower blood pressure reduce the risk of heart attacks, heart failure, strokes, and kidney disease. Blood pressure is partly regulated by aldosterone, a steroid synthesized in the adrenal cortex and released in response to angiotensin II, a peptide produced from angiotensinogen in two proteolytic steps by renin (an aspartic protease) and angiotensin-converting enzyme (ACE). Vasotec (enalapril) manufactured by Merck and Bovail is an ACE inhibitor. Novartis and Speedel have developed Tekturna (aliskiren) as a renin inhibitor. Both are TSAs.



Enalapril



Aliskiren



Atorvastatin (Lipitor)

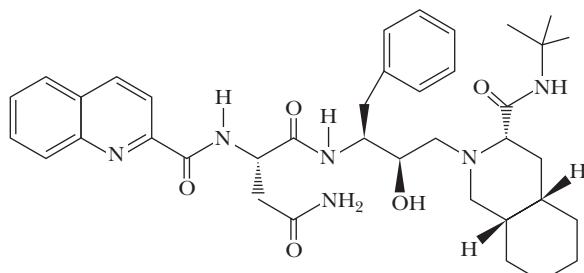


#### Statins Lower Serum Cholesterol

Statins such as Lipitor are powerful cholesterol-lowering drugs, because they are transition-state analog inhibitors of HMG-CoA reductase, a key enzyme in the biosynthetic pathway for cholesterol (discussed in Chapter 24).

### Protease Inhibitors Are AIDS Drugs

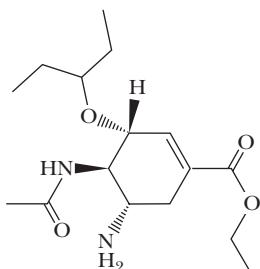
Crixivan (indinavir) by Merck, Invirase (saquinavir) by Roche, and similar “protease inhibitor” drugs are transition-state analogs for the HIV-1 protease, discussed on pages 470–471.



Saquinavir

### Tamiflu Is a Viral Neuraminidase Inhibitor

Influenza is a serious respiratory illness that affects 5% to 15% of the earth’s population each year and results in 250,000 to 500,000 deaths annually, mostly among children and the elderly. Protection from influenza by vaccines is limited by the antigenic variation of the influenza virus. Neuraminidase is a major glycoprotein on the influenza virus membrane envelope that is essential for viral replication and infectivity. Tamiflu is a neuraminidase inhibitor and antiviral agent based on the transition state of the neuraminidase reaction.



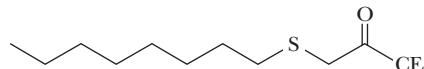
Tamiflu



▲ The 1918 flu pandemic killed more than 20 million people worldwide.

### Juvenile Hormone Esterase Is a Pesticide Target

Insects have significant effects on human health, being the transmitting agents (vectors) for diseases such as malaria, West Nile virus, and viral encephalitis, all carried by mosquitoes, and Lyme disease and Rocky Mountain spotted fever, carried by ticks. One strategy for controlling insect populations is to alter the actions of **juvenile hormone**, a terpene-based substance that regulates insect life cycle processes. Levels of juvenile hormone are controlled by **juvenile hormone esterase (JHE)**, and inhibition of JHE is toxic to insects. OTFP (figure) is a potent transition state analog inhibitor of JHE.



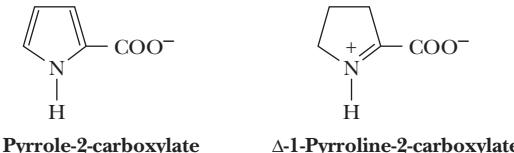
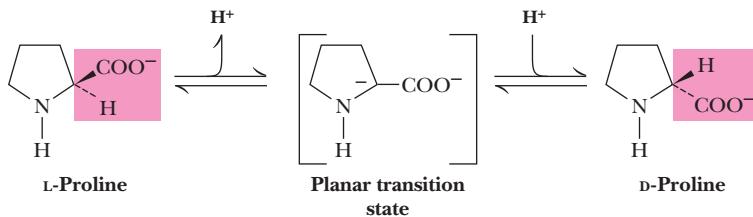
3-Octylthio-1,1,1-trifluoropropan-2-one (OTFP)



### How Many Other Drug Targets Might There Be?

If the human genome contains approximately 20,000 genes, how many of these might be targets for drug therapy? Andrew Hopkins has proposed the term “druggable genome” to conceptualize the subset of human genes that might express proteins able to bind drug-like molecules. The DrugBank database (<http://drugbank.ca>) contains more than 1400 FDA-approved small molecule drugs. Nearly half of these are directed specifically to enzymes. More than 6800 experimental drugs are presently under study and testing. It is easy to imagine that thousands more drugs will eventually be developed, with many of these designed as transition-state analogs for enzyme reactions.

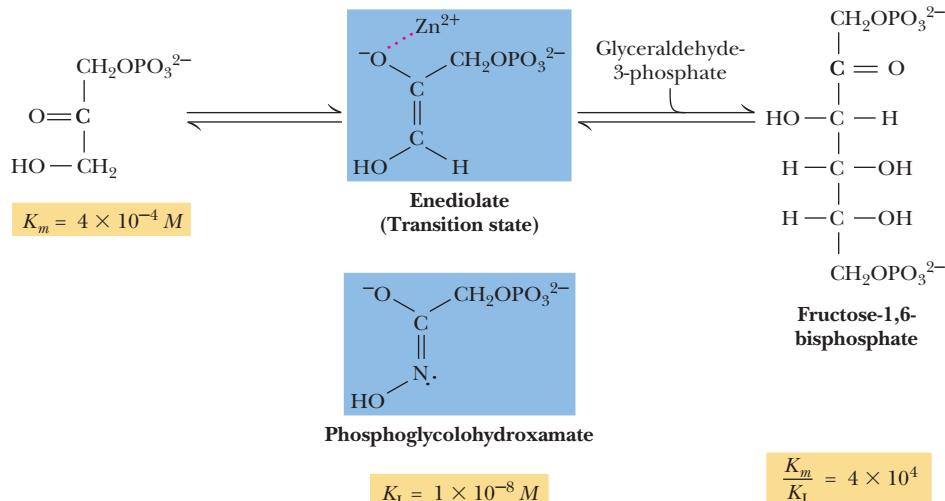
**Proline racemase reaction**



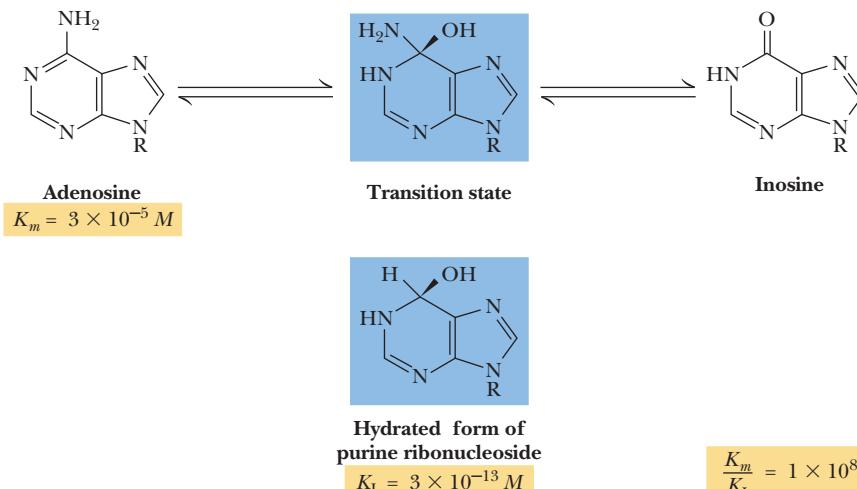
**FIGURE 14.5** The proline racemase reaction. Pyrrole-2-carboxylate and  $\Delta$ -1-pyrroline-2-carboxylate mimic the planar transition state of the reaction.

proline racemase (Figure 14.5) and found that *pyrrole-2-carboxylate* bound to the enzyme 160 times more tightly than L-proline, the normal substrate. This analog binds so tightly because it is planar and is similar in structure to the planar transition state for the racemization of proline. Two other examples of transition-state analogs are shown in Figure 14.6. *Phosphoglycolohydroxamate* binds 40,000 times more tightly to yeast

(a) Yeast aldolase reaction



(b) Calf intestinal adenosine deaminase reaction



**FIGURE 14.6** (a) Phosphoglycolohydroxamate is an analog of the enediolate transition state of the yeast aldolase reaction. (b) Purine riboside, a potent inhibitor of the calf intestinal adenosine deaminase reaction, binds to adenosine deaminase as the 1,6-hydrate. The hydrated form of purine riboside is an analog of the proposed transition state for the reaction.

aldolase than the substrate dihydroxyacetone phosphate. Even more remarkable, the 1,6-hydrate of *purine ribonucleoside* has been estimated to bind to adenosine deaminase with a  $K_I$  of  $3 \times 10^{-13} M$ !

It should be noted that transition-state analogs are only approximations of the transition state itself and will never bind as tightly as would be expected for the true transition state. These analogs are, after all, stable molecules and cannot be expected to resemble a true transition state too closely.

## 14.5 | What Are the Mechanisms of Catalysis?

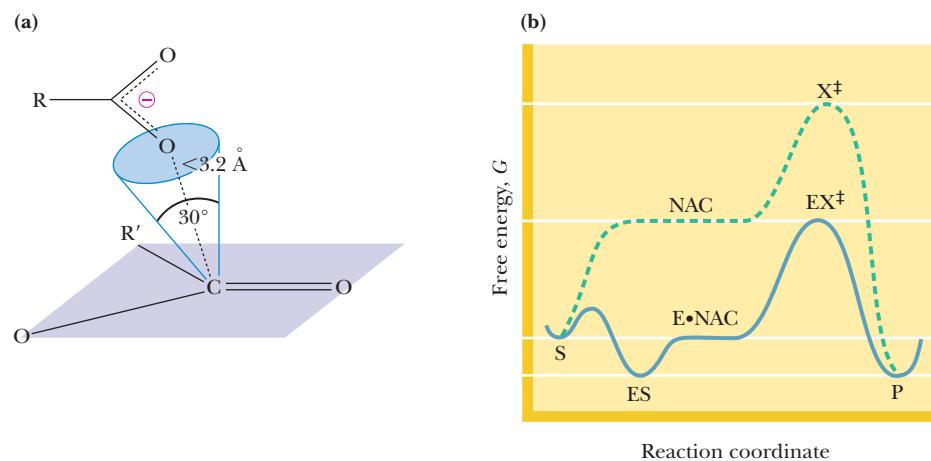
### Enzymes Facilitate Formation of Near-Attack Conformations

Exquisite and beautiful details of enzyme active-site structure and dynamics have emerged from X-ray crystal structures of enzymes and computer simulations of molecular conformation and motion at the active site. Importantly, these studies have shown that the reacting atoms and catalytic groups are precisely positioned for their roles. This “preorganization” of the active site allows it to select and stabilize conformations of the substrate(s) in which *the reacting atoms are in van der Waals contact and at an angle resembling the bond to be formed in the transition state*. Thomas Bruice has termed such arrangements **near-attack conformations (NACs)**, and he has proposed that NACs are the precursors to transition states of reactions (Figure 14.7). In the absence of an enzyme, potential reactant molecules adopt a NAC only about 0.0001% of the time. On the other hand, NACs have been shown to form in enzyme active sites from 1% to 70% of the time.

The **alcohol dehydrogenase (ADH)** reaction provides a good example of a NAC on the pathway to the reaction transition state (Figure 14.8). The ADH reaction converts a primary alcohol to an aldehyde (through an ordered, single-displacement mechanism, see page 432). The reaction proceeds by a proton transfer to water followed by a hydride transfer to  $\text{NAD}^+$ . In the enzyme active site,  $\text{Ser}^{48}$  accepts the proton from the alcohol substrate, the resulting negative charge is stabilized by a zinc ion, and the substrate *pro-R* hydrogen is poised above the  $\text{NAD}^+$  ring prior to hydride transfer (Figure 14.8). Computer simulations of the enzyme–substrate complex involving the deprotonated alcohol show that this intermediate exists as a NAC 60% of the time. The kinetic advantage of such an enzymatic reaction, compared to its nonenzymatic counterpart, is the *ease of formation of the NAC and the favorable free energy difference between the NAC and the transition state* (Figure 14.7).

### Protein Motions Are Essential to Enzyme Catalysis

Proteins are constantly moving. As noted in Chapter 6 (Table 6.2), bonds vibrate, side chains bend and rotate, backbone loops wiggle and sway, and whole domains move with respect to each other. Enzymes depend on such motions to provoke and direct catalytic



**FIGURE 14.7** (a) For reactions involving bonding between O, N, C, and S atoms, NACs are characterized as having reacting atoms within 3.2 Å and an approach angle of  $\pm 15^\circ$  of the bonding angle in the transition state. (b) In an enzyme active site, the enzyme–substrate complex and the NAC are separated by a small energy barrier, and NACs form readily. In the absence of the enzyme, the energy gap between the substrate and the NAC is much greater and NACs are rarely formed. The energy separation between the NAC and the transition state is approximately the same in the presence and absence of the enzyme. (Adapted from Bruice, T., 2002. A view at the millennium: The efficiency of enzymatic catalysis. *Accounts of Chemical Research* 35:139–148.)

## A DEEPER LOOK

### How to Read and Write Mechanisms

The custom among chemists and biochemists of writing chemical reaction mechanisms with electron dots and curved arrows began with two of the greatest chemists of the 20th century. Gilbert Newton Lewis was the first to suggest that a covalent bond consists of a shared pair of electrons, and Sir Robert Robinson was the first to use curved arrows to illustrate a mechanism in a paper in the *Journal of the Chemical Society* in 1922.

Learning to read and write reaction mechanisms should begin with a review of Lewis dot structures in any good introductory chemistry text. It is also important to understand valence electrons and “formal charge.” The formal charge of an atom is calculated as the number of valence electrons minus the “electrons owned” by an atom. More properly

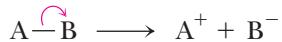
**Formal charge = group number –  
nonbonding electrons – (1/2 shared electrons)**

Students of mechanisms should also appreciate electronegativity—the tendency of an atom to attract electrons. Electronegativities of the atoms important in biochemistry go in the order



Thus, in a C–N bond, the N should be viewed as more electron-rich and C as electron-deficient. An electron-rich atom is termed *nucleophilic* and will have a tendency to react with an electron-deficient (electrophilic) atom.

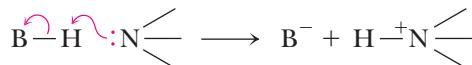
In written mechanisms, a curved arrow shows the movement of an electron pair from its original position to a new one. The tail of the arrow shows where the electron pair comes from, and the head of the arrow shows where the electron pair is going. Thus, the arrow represents the actual movement of a pair of electrons from a filled orbital into an empty one. By convention, an arrow with a full arrowhead  $\curvearrowright$  represents movement of an electron pair, whereas a half arrowhead  $\curvearrowleft$  represents a single electron (for example, in a free radical reaction). For a bond-breaking event, the arrow begins in the middle of the bond, and the arrowhead points at the atom that will accept the electrons:



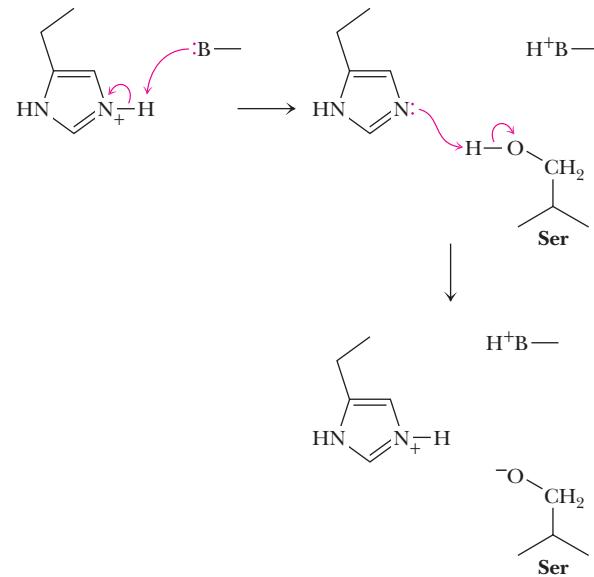
For a bond-making event, the arrow begins at the source of the electrons (for example, a nonbonded pair), and the arrowhead points to the atom where the new bond will be formed:



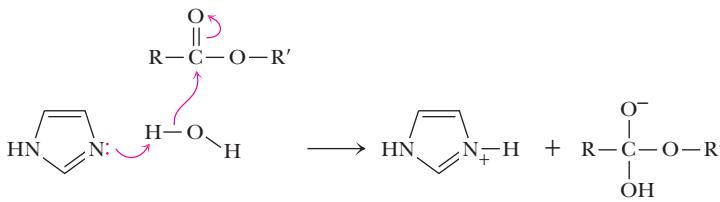
It has been estimated that 75% of the steps in enzyme reaction mechanisms are proton ( $\text{H}^+$ ) transfers. If the proton is donated or accepted by a group on the enzyme, it is often convenient (and traditional) to represent that group as B, for “base,” even if B is protonated and behaving as an acid:



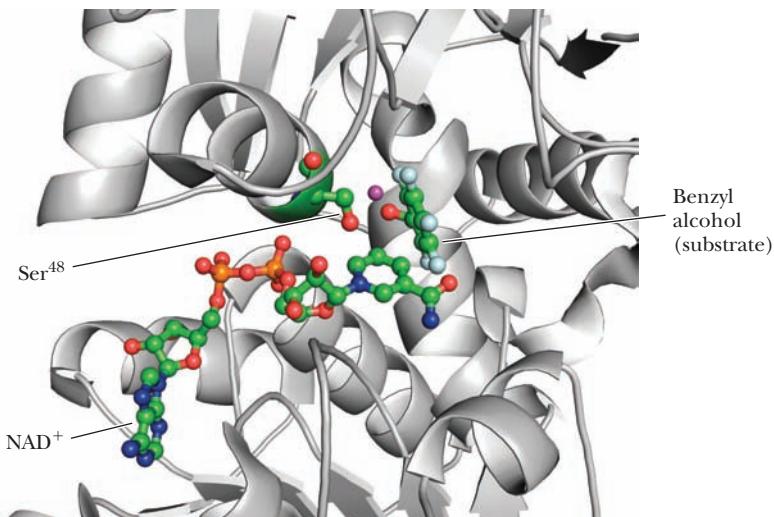
It is important to appreciate that a proton transfer can change a nucleophile into an electrophile, and vice versa. Thus, it is necessary to consider (1) the protonation states of substrate and active-site residues and (2) how  $\text{pK}_a$  values can change in the environment of the active site. For example, an active-site histidine, which might normally be protonated, can be deprotonated by another group and then act as a base, accepting a proton from the substrate:



Water can often act as an acid or base at the active site through proton transfer with an assisting active-site residue:



These concepts provide a sense of what is reasonable and what makes good chemical logic in a reaction. Practice and experience are essential to building skills for reading and writing enzyme mechanisms. Excellent Web sites are available where such skills can be built (<http://www.abdn.ac.uk/curlly-arrows>).

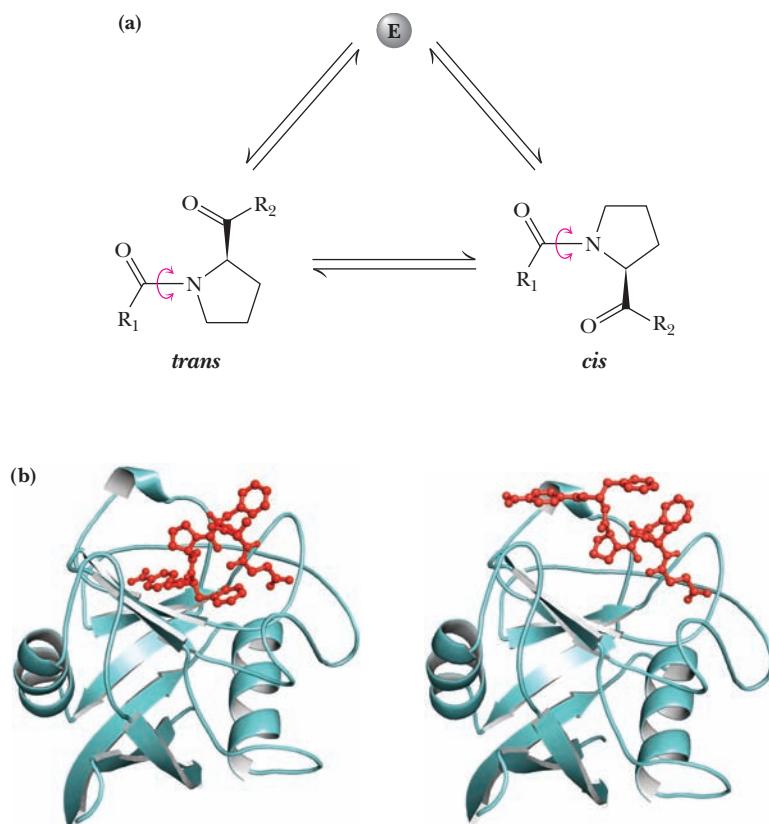


**FIGURE 14.8** The complex of horse liver ADH with benzyl alcohol illustrates the approach to a near-attack conformation. Computer simulations by Bruice and co-workers show that the side-chain oxygen of Ser<sup>48</sup> approaches within 1.8 Å of the hydroxyl hydrogen of the substrate, benzyl alcohol, and that the *pro-R* hydrogen of benzyl alcohol lies 2.75 Å from the C-4 carbon of the nicotinamide ring. The reaction mechanism involves hydroxyl proton abstraction by Ser<sup>48</sup> and hydride transfer from the substrate to C-4 of the NAD<sup>+</sup> nicotinamide ring (pdb id = 1HLD).

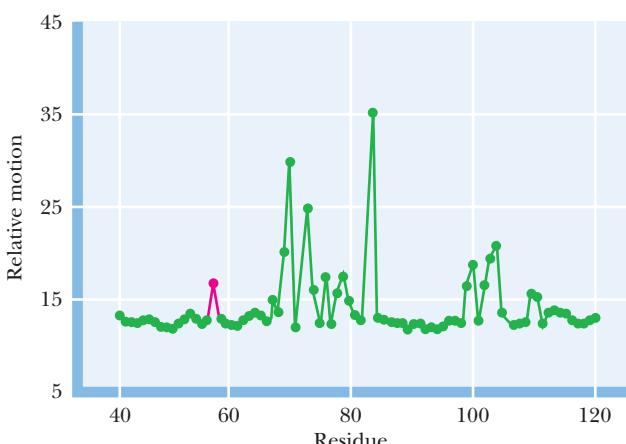
events. Protein motions may support catalysis in several ways: Active site conformation changes can

- assist substrate binding
- bring catalytic groups into position around a substrate
- induce formation of a NAC
- assist in bond making and bond breaking
- facilitate conversion of substrate to product

A good example of protein motions facilitating catalysis is human **cyclophilin A**, which catalyzes the interconversion between *cis* and *trans* conformations of proline in peptides (Figure 14.9). NMR studies of cyclophilin A have provided direct



**FIGURE 14.9** (a) Human cyclophilin A is a **prolyl isomerase**, which catalyzes the interconversion between *trans* and *cis* conformations of proline in peptides. (b) The active site of cyclophilin with a bound peptide containing proline in *cis* and *trans* conformations (pdb id = 1RMH).



**FIGURE 14.10** Catalysis in enzyme active sites depends on motion of active-site residues. NMR studies by Dorothee Kern and her co-workers show that several cyclophilin active-site residues, including Arg<sup>55</sup> (red dot) and Lys<sup>82</sup>, Leu<sup>98</sup>, Ser<sup>99</sup>, Ala<sup>101</sup>, Gln<sup>102</sup>, Ala<sup>103</sup>, and Gly<sup>109</sup> (green dots), undergo greater motion during catalysis than residues elsewhere in the protein. (Adapted from Eisenmesser, E., et al., 2002. Enzyme dynamics during catalysis. *Science* 295:1520–1523.)

measurements of the active-site motions occurring in this enzyme. Certain active-site residues (Lys<sup>82</sup>, Leu<sup>98</sup>, Ser<sup>99</sup>, Ala<sup>101</sup>, Gln<sup>102</sup>, Ala<sup>103</sup>, and Gly<sup>109</sup>) of the enzyme undergo conformation changes during substrate binding, whereas Arg<sup>55</sup> is involved directly in the *cis*-to-*trans* interconversion itself (Figure 14.10).

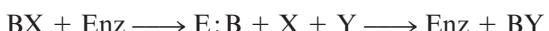
The protein motions that assist catalysis may be quite complex. Stephen Benkovic and Sharon Hammes-Schiffer have characterized an extensive network of coupled protein motions in dihydrofolate reductase. This network extends from the active site to the surface of the protein, and the motions in this network span time scales of femtoseconds ( $10^{-15}$  sec) to milliseconds. Such extensive networks of motion make it likely that the entire folded structure of the protein may be involved in catalysis at the active site.

## Covalent Catalysis

Some enzyme reactions derive much of their rate acceleration from the formation of **covalent bonds** between enzyme and substrate. Consider the reaction:



and an enzymatic version of this reaction involving formation of a **covalent intermediate**:

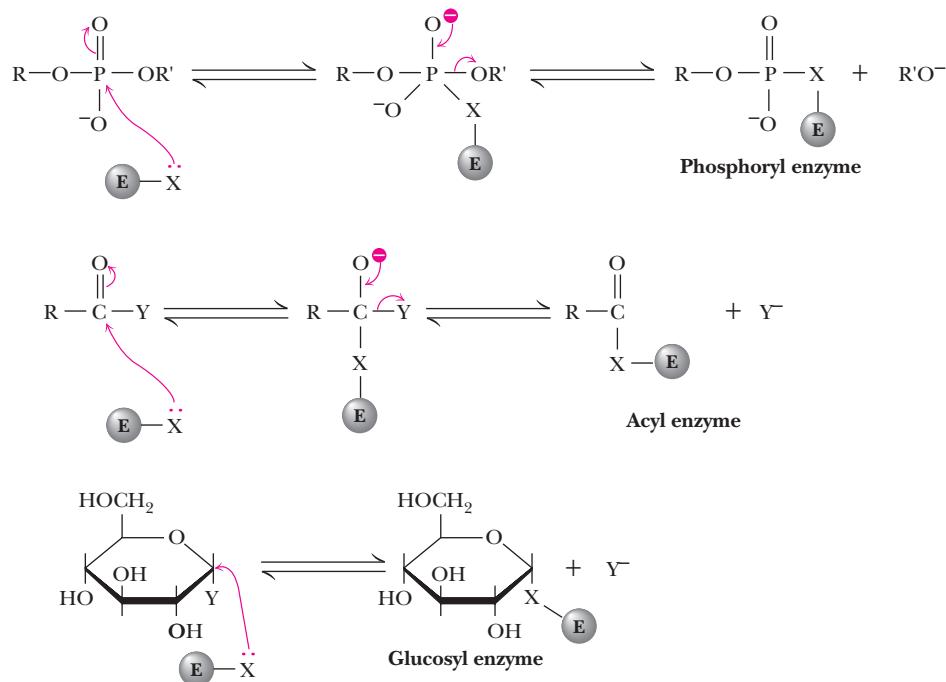


If the enzyme-catalyzed reaction is to be faster than the uncatalyzed case, the acceptor group on the enzyme must be a better attacking group than Y and a better leaving group than X. Note that most enzymes that carry out covalent catalysis have ping-pong kinetic mechanisms.

The side chains of amino acids in proteins offer a variety of **nucleophilic** centers for catalysis, including amines, carboxylates, aryl and alkyl hydroxyls, imidazoles, and thiol groups. These groups readily attack electrophilic centers of substrates, forming covalently bonded enzyme–substrate intermediates. Typical electrophilic centers in substrates include phosphoryl groups, acyl groups, and glycosyl groups (Figure 14.11). The covalent intermediates thus formed can be attacked in a subsequent step by a water molecule or a second substrate, giving the desired product. **Covalent electrophilic catalysis** is also observed, but it usually involves coenzyme adducts that generate electrophilic centers. Hundreds of enzymes are now known to form covalent intermediates during catalysis. Several examples of covalent catalysis will be discussed in detail in later chapters, as noted in Table 14.2.

## General Acid–Base Catalysis

Nearly all enzyme reactions involve some degree of acid or base catalysis. There are two types of acid–base catalysis: (1) **specific acid–base catalysis**, in which the reaction is accelerated by H<sup>+</sup> or OH<sup>−</sup> diffusing in from the solution, and (2) **general acid–base**

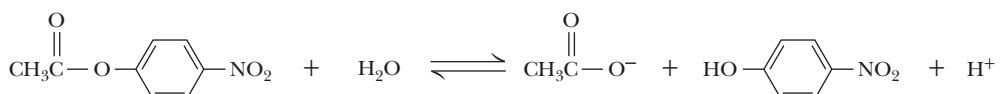
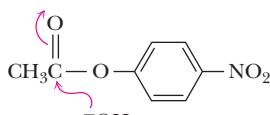
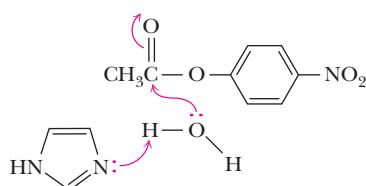


**FIGURE 14.11** Examples of covalent bond formation between enzyme and substrate. In each case, a nucleophilic center ( $X^-$ ) on an enzyme attacks an electrophilic center on a substrate.

**TABLE 14.2** Enzymes That Form Covalent Intermediates

| Enzyme  | Reacting Group                | Covalent Intermediate |
|---|-------------------------------|-----------------------|
| Trypsin   | Serine                        | Acy-Ser               |
| Chymotrypsin (pages 465–466)                          |                               |                       |
| Glyceraldehyde-3-P dehydrogenase (page 589)           | Cysteine                      | Acy-Cys               |
| Phosphoglucomutase (page 493)                         | Serine                        | Phospho-Ser           |
| Phosphoglycerate mutase (page 591)                    | Histidine                     | Phospho-His           |
| Succinyl-CoA synthetase (page 623)                    |                               |                       |
| Aldolase (page 587)                                   | Lysine and other amino groups | Schiff base           |
| Pyridoxal phosphate enzymes (pages 434, 856, and 881) |                               |                       |

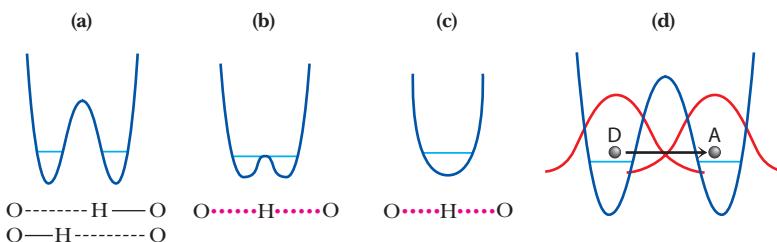
**catalysis**, in which  $\text{H}^+$  or  $\text{OH}^-$  is created *in the transition state* by another molecule or group, which is termed the general acid or general base, respectively. *By definition, general acid-base catalysis is catalysis in which a proton is transferred in the transition state.* Consider the hydrolysis of *p*-nitrophenylacetate by specific base catalysis or with imidazole acting as a general base (Figure 14.12). In the specific base mechanism, hydroxide diffuses into the reaction from solution. In the general base mechanism, the hydroxide that catalyzes the reaction is generated from water in the transition state. The water has been made more nucleophilic without generation of a high concentration of  $\text{OH}^-$  or without the formation of unstable, high-energy species. General acid or general base catalysis may increase reaction rates 10- to 100-fold. In an enzyme, ionizable groups on the protein provide the  $\text{H}^+$  transferred in the transition state. Clearly, an ionizable group will be most effective as an  $\text{H}^+$  transferring agent at or near its  $\text{p}K_a$ . Because the  $\text{p}K_a$  of the histidine side chain is near 7, histidine is often the most effective general acid or base. Descriptions of several cases of general acid-base catalysis in typical enzymes follow.

**Reaction****Specific base mechanism****General base mechanism**

**FIGURE 14.12** Catalysis of *p*-nitrophenylacetate hydrolysis can occur either by specific base hydrolysis (where hydroxide from the solution is the attacking nucleophile) or by general base catalysis (in which a base like imidazole can promote hydroxide attack on the substrate carbonyl carbon by removing a proton from a nearby water molecule).

## Low-Barrier Hydrogen Bonds

As previously noted, the typical strength of a hydrogen bond is 10 to 30 kJ/mol. For an O—H—O hydrogen bond, the O—O separation is typically 0.28 nm and the H bond is a relatively weak electrostatic interaction. The hydrogen is firmly linked to one of the oxygens at a distance of approximately 0.1 nm, and the distance to the other oxygen is thus about 0.18 nm, which corresponds to a bond order of about 0.07. Not all hydrogen bonds are weak, however. As the distance between heteroatoms becomes smaller, the overall bond becomes stronger, the hydrogen becomes centered, and the bond order approaches 0.5 for both O—H interactions (Figure 14.13). These interactions are more nearly covalent in nature, and the stabilization energy is much higher. Notably, the barrier that the hydrogen atom must surmount to exchange oxygens becomes lower as the O—O separation decreases (Figure 14.13). When the barrier to hydrogen exchange has dropped to the point that it is at or below the zero-point energy level of hydrogen, the interaction is referred to as a **low-barrier hydrogen bond (LBHB)**. The hydrogen is now



**FIGURE 14.13** Comparison of conventional (weak) hydrogen bonds (a) and low-barrier hydrogen bonds (b and c). The horizontal line in each case is the zero-point energy of hydrogen. (a) shows an O—H—O hydrogen bond of length 0.28 nm, with the hydrogen attached to one or the other of the oxygens. The bond order for the stronger O—H interaction is approximately 1.0, and the weaker O—H interaction is 0.07. As the O—O distance decreases, the hydrogen bond becomes stronger, and the bond order of the weakest interaction increases. In (b), the O—O distance is 0.25 nm, and the barrier is equal to the zero-point energy. In (c), the O—O distance is 0.23 to 0.24 nm, and the bond order of each O—H interaction is 0.5. (d) If the distance for particle (proton or electron) transfer is sufficiently small, overlap of probability functions (red curves) permit efficient quantum mechanical tunneling between donor (D) and acceptor (A) groups.

free to move anywhere between the two oxygens (or, more generally, two heteroatoms). The stabilization energy of LBHBs may approach 100 kJ/mol in the gas phase and 60 kJ/mol or more in solution. LBHBs require matched  $pK_a$ s for the two electronegative atoms that share the hydrogen. As the two  $pK_a$  values diverge, the stabilization energy of the LBHB is decreased. Widely divergent  $pK_a$  values thus correspond to ordinary, weak hydrogen bonds.

How may low-barrier hydrogen bonds affect enzyme catalysis? A weak hydrogen bond in an enzyme ground state may become an LBHB in a transient intermediate, or even in the transition state for the reaction. In such a case, the energy released in forming the LBHB is used to help the reaction that forms it, lowering the activation barrier for the reaction. Alternatively, the purpose of the LBHB may be to redistribute electron density in the reactive intermediate, achieving rate acceleration by facilitation of “hydrogen tunneling.” Enzyme mechanisms that will be examined later in this chapter (the serine proteases and aspartic proteases) appear to depend upon one or the other of these effects.

## Quantum Mechanical Tunneling in Electron and Proton Transfers

The fundamental premise of transition-state theory, as described in this chapter, is that reactants cannot become products until they acquire enough energy to distort their structures to that of the transition state. However, **quantum mechanical tunneling** offers a path around this energy barrier and many enzymes exploit it. According to quantum theory, if an atom or electron is transferred in a chemical reaction from one site to another across an activation barrier, there is a finite probability that the particle will appear (as part of the product) on the other side of the energy barrier, even though it cannot achieve sufficient energy to reach the transition state (Figure 14.1). The likelihood of tunneling depends on the distance over which the particle must move and the shape (width and height) of the potential energy barrier that separates reactant from product. Wave/particle duality in quantum theory implies that a wavelength  $\lambda$  can be calculated for a particle of mass  $m$  with a given kinetic energy  $E$ , according to the de Broglie equation:

$$\lambda = \frac{h}{\sqrt{2mE}} \quad (14.4)$$

where  $h$  is Planck’s constant. Tunneling can only play a significant role in a reaction when the wavelength of the transferring particle is similar to the distance over which it is transferred. In such cases, the overlap of probability functions for the particle in the reactant and product states is sufficient to permit effective tunneling (Figure 14.13).

In enzyme reactions, only electrons and hydrogen ( $H^+$ ,  $H\cdot$ , or  $H:\cdot$ ) satisfy this condition. Consider the low likelihood of tunneling for atoms larger than hydrogen. For example, the de Broglie wavelength of a carbon atom, with a mass of 12 daltons, is approximately 0.25 Å, a distance too short for tunneling in any reaction involving carbon transfer. Note also that tunneling permits transfer of electrons over substantial molecular distances. Assuming a kinetic energy of 10 kJ/mole, Equation 14.4 yields de Broglie wavelengths for the electron and hydrogen of 38 Å and 0.9 Å, respectively. The latter wavelength is on the order of distances involved in proton, hydrogen, and hydride transfers in enzyme reactions, and it is likely that tunneling is a contributing factor in most, if not all, hydrogen transfer reactions.

## Metal Ion Catalysis

Many enzymes require metal ions for maximal activity. If the enzyme binds the metal very tightly or requires the metal ion to maintain its stable, native state, it is referred to as a **metalloenzyme**. Enzymes that bind metal ions more weakly, perhaps only during the catalytic cycle, are referred to as **metal activated**. One role for metals in metal-activated enzymes and metalloenzymes is to act as electrophilic catalysts, stabilizing the increased

## A DEEPER LOOK

### How Do Active-Site Residues Interact to Support Catalysis?

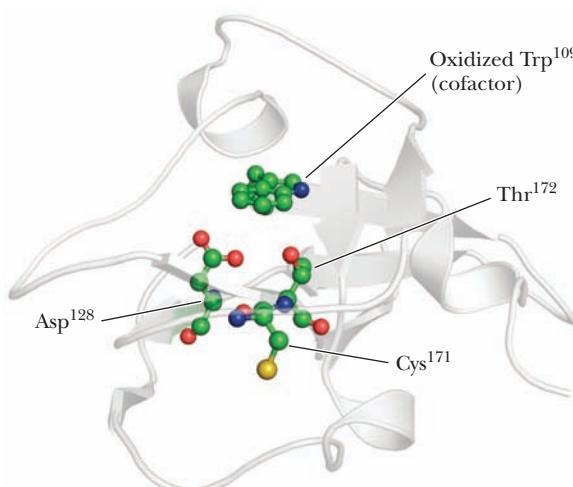
Only about half of the common amino acid residues (that is, His, Cys, Asp, Glu, Arg, Lys, Tyr, Ser, Thr, Asn, and Gln) engage directly in catalytic effects in enzyme active sites. These polar and charged residues provide a relatively limited range of catalytic capabilities. They can act as nucleophiles, facilitate substrate binding, and stabilize transition states. It has been estimated that up to 75% of the steps in enzyme mechanisms involve a simple proton transfer. Is this enough to explain the dramatic catalytic power of enzymes? Or might there be other phenomena at work?

Janet Thornton and Alex Gutteridge have analyzed residue interactions at the active sites of 191 different enzymes. In this group of enzymes, each polar catalytic residue interacts with (on average) 2.3 other polar residues in the active site, whereas noncatalytic, buried polar residues have, on average, interactions with only 1.2 other polar residues. This suggests that some of the interactions between catalytic and noncatalytic residues are functional in some way. At the same time, in only 88 of the enzymes does the key catalytic residue have a direct interaction with a second catalytic residue, indicating that most catalytic residues do not require direct interactions with other catalytic residues to be active.

The catalytic capacities of polar and charged residues can be influenced by other polar and charged residues at the active site and even by hydrophobic residues. The so-called secondary, or noncatalytic, residues at the active site play interesting roles:

- *Raising or lowering catalytic residue pK<sub>a</sub> values through electrostatic or hydrophobic interactions.* In aldkoketoreductase, an Asp–Lys pair facilitates general acid–base catalysis, with Lys<sup>84</sup> lowering the pK<sub>a</sub> of Tyr<sup>58</sup> so that it can donate a proton to the substrate. On the other hand, nearby hydrophobic residues can provide a nonpolar environment that tends to raise the pK<sub>a</sub> values of acidic residues (such as Asp or Glu) and to lower the pK<sub>a</sub> values of basic residues (such as lysine and arginine). Hydrophobic environments can change pK<sub>a</sub> values by as much as 5 or 6 pH units.
- *Orientation of catalytic residues,* as will be seen in the serine proteases, where Asp<sup>102</sup> orients His<sup>57</sup> (see Figure 14.21).

- *Charge stabilization,* as will be seen in chorismate mutase, where active-site arginines stabilize negatively charged carboxyl groups on the substrate (see Figures 14.31 and 14.33).
- *Proton transfers via hydrogen tunneling.* In such quantum mechanical tunneling, the proton transfer is accomplished by molecular motions that lead to degeneracy of a pair of localized proton vibrational states (Figure 14.13). Proton tunneling can be facilitated by nearby molecular motions of secondary residues coupled to the motion and vibration of the bonds in question. David Leys has shown that aromatic amine dehydrogenase probably accomplishes catalysis by coupling local motions (of two secondary residues, C<sup>171</sup> and T<sup>172</sup>) to the vibrational states involved in a proton transfer reaction with D<sup>128</sup>, as shown here.

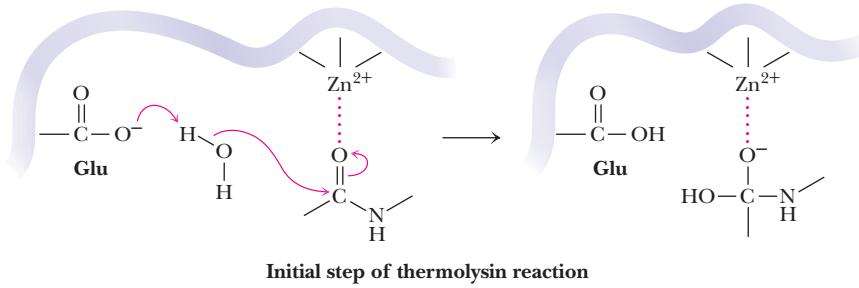


▲ Closeup of the crystal structure of aromatic amine dehydrogenase, showing the relationship of Asp<sup>128</sup>, Thr<sup>172</sup>, and Cys<sup>171</sup>. N atoms are blue; O atoms are red; C atoms are green; S atom is gold (pdb id = 2AH1).

electron density or negative charge that can develop during reactions. Among the enzymes that function in this manner (Figure 14.14) is thermolysin. Another potential function of metal ions is to provide a powerful nucleophile at neutral pH. Coordination to a metal ion can increase the acidity of a nucleophile with an ionizable proton:



The reactivity of the coordinated, deprotonated nucleophile is typically intermediate between that of the un-ionized and ionized forms of free nucleophile. Carboxypeptidase (see Chapter 5) contains an active site Zn<sup>2+</sup>, which facilitates deprotonation of a water molecule in this manner.



**FIGURE 14.14** Thermolysin is an endoprotease (that is, it cleaves polypeptides in the middle of the chain) with a catalytic Zn<sup>2+</sup> ion in the active site. The Zn<sup>2+</sup> ion stabilizes the buildup of negative charge on the peptide carbonyl oxygen, as a glutamate residue deprotonates water, promoting hydroxide attack on the carbonyl carbon. Thermolysin is found in certain laundry detergents, where it is used to remove protein stains from fabrics.

## 14.6 | What Can Be Learned from Typical Enzyme Mechanisms?

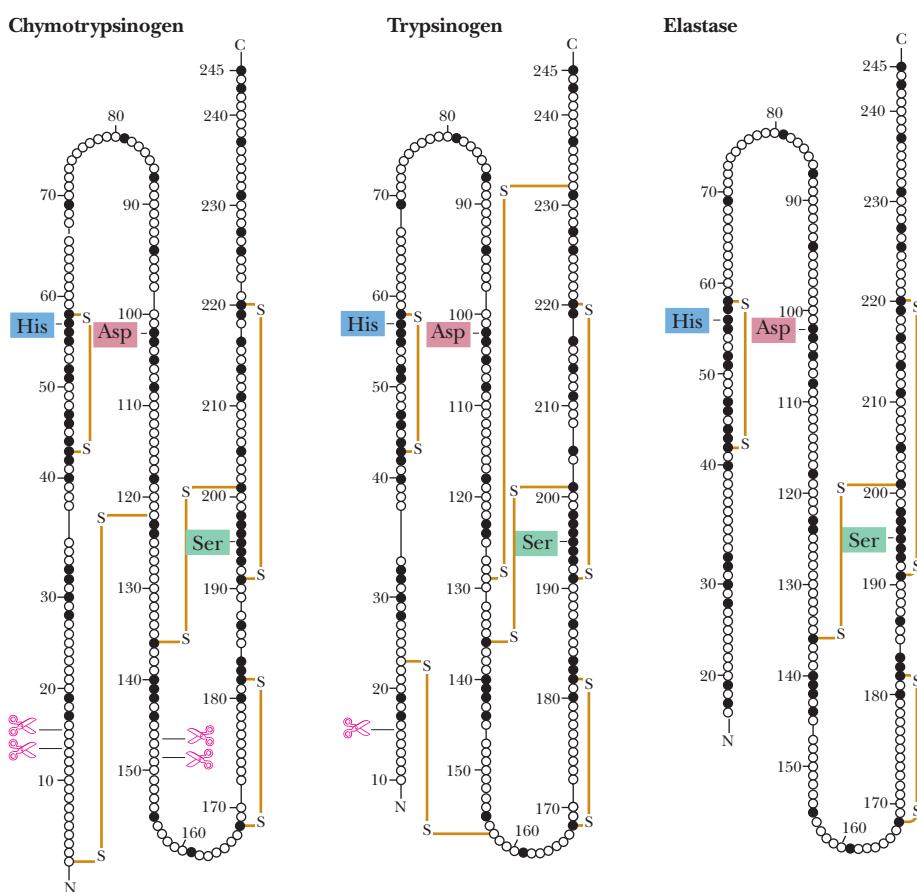
The balance of this chapter will be devoted to several classic and representative enzyme mechanisms, including the serine proteases, the aspartic proteases, and chorismate mutase. Both the serine proteases and the aspartic proteases use general acid–base catalysis chemistry; the serine proteases also employ a covalent catalysis strategy. Chorismate mutase, on the other hand, uses neither of these and depends instead on the formation of a NAC to carry out its reaction. These particular cases are well understood, because the three-dimensional structures of the enzymes and the bound substrates are known at atomic resolution and because great efforts have been devoted to kinetic and mechanistic studies. They are important because they represent reaction types that appear again and again in living systems and because they demonstrate many of the catalytic principles cited previously. Enzymes are the catalytic machines that sustain life, and what follows is an intimate look at the inner workings of the machinery.

### Serine Proteases

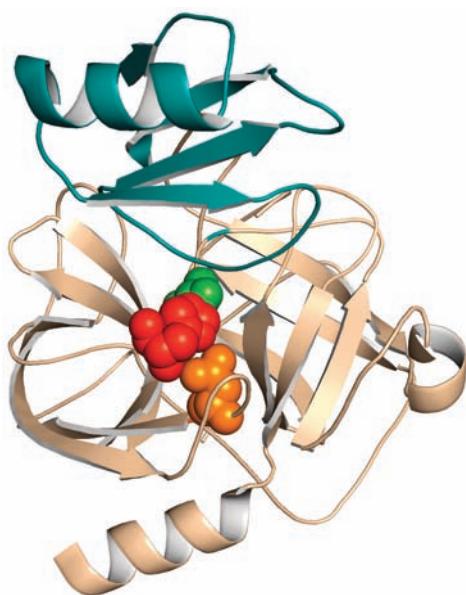
**Serine proteases** are a class of proteolytic enzymes whose catalytic mechanism is based on an active-site serine residue. Serine proteases are one of the best-characterized families of enzymes. This family includes *trypsin*, *chymotrypsin*, *elastase*, *thrombin*, *subtilisin*, *plasmin*, *tissue plasminogen activator*, and other related enzymes. The first three of these are digestive enzymes and are synthesized in the pancreas and secreted into the digestive tract as inactive **proenzymes**, or **zymogens**. Within the digestive tract, the zymogen is converted into the active enzyme form by cleaving off a portion of the peptide chain. Thrombin is a crucial enzyme in the blood-clotting cascade, subtilisin is a bacterial protease, and plasmin breaks down the fibrin polymers of blood clots. Tissue plasminogen activator (TPA) specifically cleaves the proenzyme *plasminogen*, yielding plasmin. Owing to its ability to stimulate breakdown of blood clots, TPA can minimize the harmful consequences of a heart attack, if administered to a patient within 30 minutes of onset. Finally, although not itself a protease, *acetylcholinesterase* is a serine esterase and is related mechanistically to the serine proteases. It degrades the neurotransmitter acetylcholine in the synaptic cleft between neurons.

### The Digestive Serine Proteases

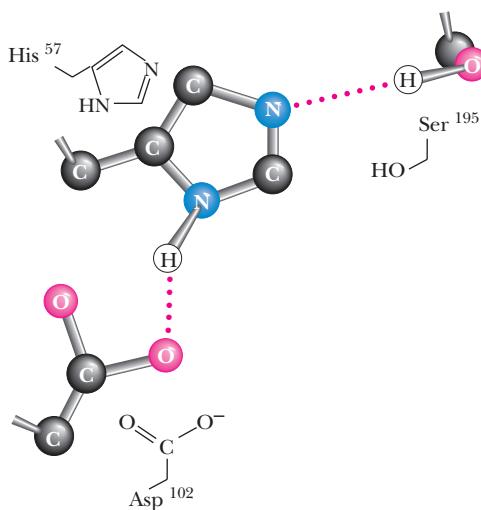
Trypsin, chymotrypsin, and elastase all carry out the same reaction—the cleavage of a peptide chain—and although their structures and mechanisms are quite similar, they display very different specificities. Trypsin cleaves peptides on the carbonyl side of the basic amino acids, arginine or lysine (see Table 5.2). Chymotrypsin prefers to cleave on the carbonyl side of aromatic residues, such as phenylalanine and tyrosine. Elastase is not as specific as the other two; it mainly cleaves peptides on the carbonyl side of small, neutral residues. These three enzymes all possess molecular weights in the range of 25,000, and all have similar sequences (Figure 14.15) and three-dimensional structures. The structure of chymotrypsin is typical (Figure 14.16). The molecule is ellipsoidal in shape and contains an  $\alpha$ -helix at the C-terminal end (residues 230 to 245) and several  $\beta$ -sheet domains. Most of the aromatic and hydrophobic residues are buried in the interior of the protein, and most of the charged or hydrophilic residues are on the surface. Three polar residues—His<sup>57</sup>, Asp<sup>102</sup>, and Ser<sup>195</sup>—form what is known as a **catalytic triad** at the active site (Figure 14.17). These three residues are conserved in trypsin and elastase as well. The active site is actually a depression on the surface of the enzyme, with a pocket that the enzyme uses to identify the residue for which it is specific (Figure 14.18). Chymotrypsin, for example, has a pocket surrounded by hydrophobic residues and large enough to accommodate an aromatic side chain. The pocket in trypsin has a negative charge (Asp<sup>189</sup>) at its bottom, facilitating the binding of positively charged arginine and lysine residues. Elastase, on the other hand, has a shallow pocket with bulky threonine



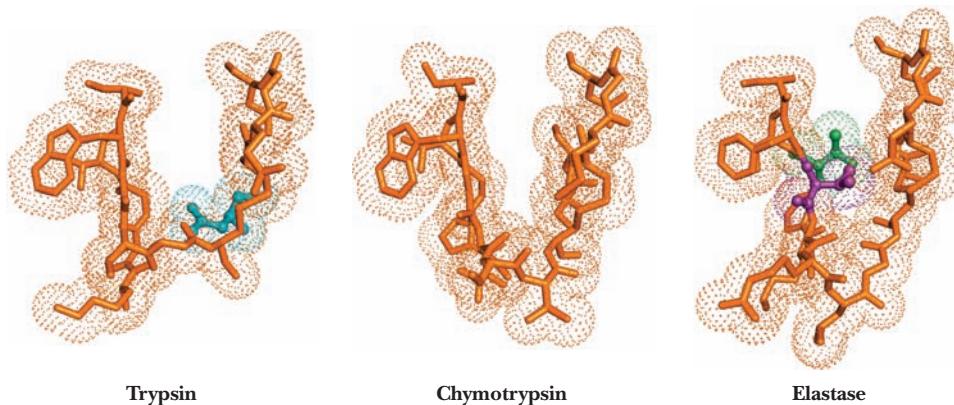
**FIGURE 14.15** Comparison of the amino acid sequences of chymotrypsinogen, trypsinogen, and elastase. Each circle represents one amino acid. Numbering is based on the sequence of chymotrypsinogen. Filled circles indicate residues that are identical in all three proteins. Disulfide bonds are indicated in orange. The positions of the three catalytically important active-site residues (His<sup>57</sup>, Asp<sup>102</sup>, and Ser<sup>195</sup>) are indicated.



**FIGURE 14.16** Structure of chymotrypsin (white) in a complex with eglin C (blue ribbon structure), a target protein. The residues of the catalytic triad (His<sup>57</sup>, Asp<sup>102</sup>, and Ser<sup>195</sup>) are highlighted. His<sup>57</sup> (red) is flanked by Asp<sup>102</sup> (gold) and by Ser<sup>195</sup> (green). The catalytic site is filled by a peptide segment of eglin. Note how close Ser<sup>195</sup> is to the peptide that would be cleaved in the chymotrypsin reaction (pdb id = 1ACB).



**FIGURE 14.17** The catalytic triad of chymotrypsin.

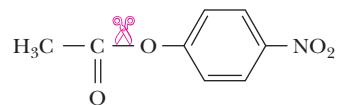


**FIGURE 14.18** The substrate-binding pockets of trypsin (pdb id = 2CMY), chymotrypsin (pdb id = 1ACB), and elastase (pdb id = 3EST). Asp<sup>189</sup> (aqua) coordinates Arg and Lys residues of peptide substrates in the trypsin binding pocket. Val<sup>216</sup> (purple) and Thr<sup>226</sup> (green) make the elastase binding pocket shallow and able to accommodate only small, nonbulky residues.

and valine residues at the opening. Only small, nonbulky residues can be accommodated in its pocket. The backbone of the peptide substrate is hydrogen bonded in anti-parallel fashion to residues 215 to 219 and bent so that the peptide bond to be cleaved is bound close to His<sup>57</sup> and Ser<sup>195</sup>.

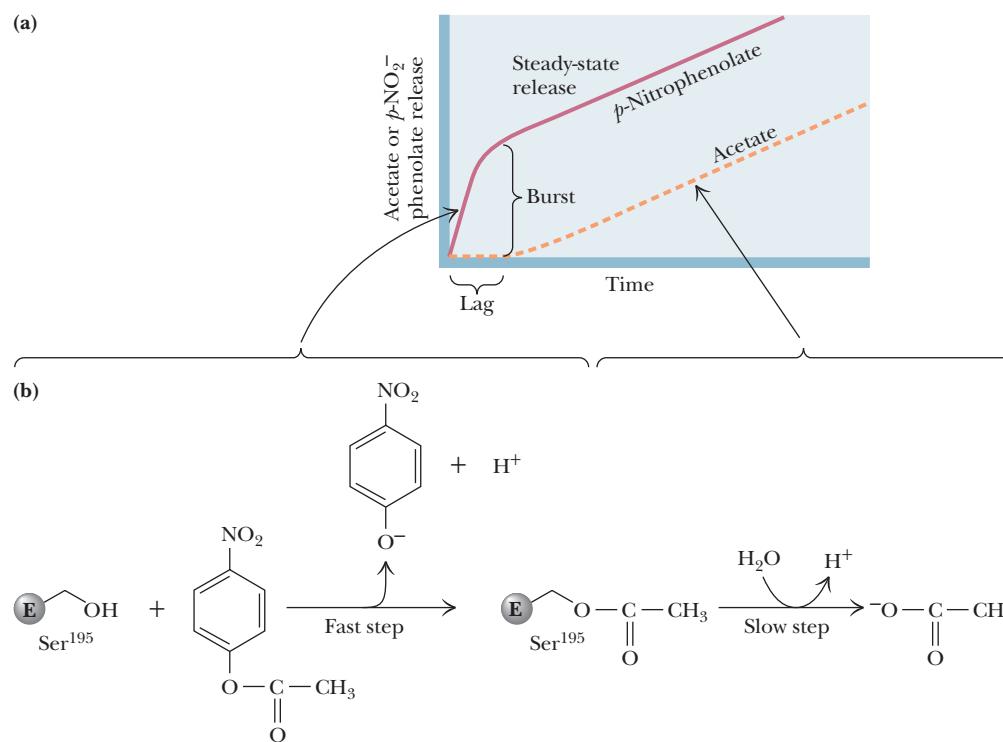
### The Chymotrypsin Mechanism in Detail: Kinetics

Much of what is known about the chymotrypsin mechanism is based on studies of the hydrolysis of artificial substrates—simple organic esters, such as *p*-nitrophenylacetate (Figure 14.19). *p*-Nitrophenylacetate is an especially useful model substrate, because the nitrophenolate product is easily observed, owing to its strong absorbance at 400 nm. When large amounts of chymotrypsin are used in kinetic studies with this substrate, a **rapid initial burst** of *p*-nitrophenolate is observed (in an amount approximately equal to the enzyme concentration), followed by a much slower, linear rate of nitrophenolate release (Figure 14.20). Observation of a burst, followed by slower, steady-state product release, is strong evidence for a multistep mechanism, with a fast first step and a slower second step.



*p*-Nitrophenylacetate

**FIGURE 14.19** Chymotrypsin cleaves simple esters, in addition to peptide bonds. *p*-Nitrophenylacetate has been used in studies of the chymotrypsin mechanism.



**FIGURE 14.20** Burst kinetics observed in the chymotrypsin reaction (a). A burst of nitrophenolate (b, first step) is followed by a slower, steady-state release. After an initial lag period, acetate release (b, second step) is observed. This kinetic pattern is consistent with rapid formation of an acyl-enzyme intermediate (and the burst of nitrophenolate). The slower, steady-state release of products corresponds to rate-limiting breakdown of the acyl-enzyme intermediate.

In the chymotrypsin mechanism, the nitrophenylacetate combines with the enzyme to form an ES complex. This is followed by a rapid step in which an **acyl-enzyme intermediate** is formed, with the acetyl group covalently bound to the very reactive Ser<sup>195</sup>. The nitrophenyl moiety is released as nitrophenolate (Figure 14.20), accounting for the burst of nitrophenolate product. Attack of a water molecule on the acyl-enzyme intermediate yields acetate as the second product in a subsequent, slower step. The enzyme is now free to bind another molecule of *p*-nitrophenylacetate, and the *p*-nitrophenolate product produced at this point corresponds to the slower, steady-state formation of product in the upper right portion of Figure 14.20. In this mechanism, the release of acetate is the **rate-limiting step** and accounts for the observation of **burst kinetics**—the pattern shown in Figure 14.20.

## The Serine Protease Mechanism in Detail: Events at the Active Site

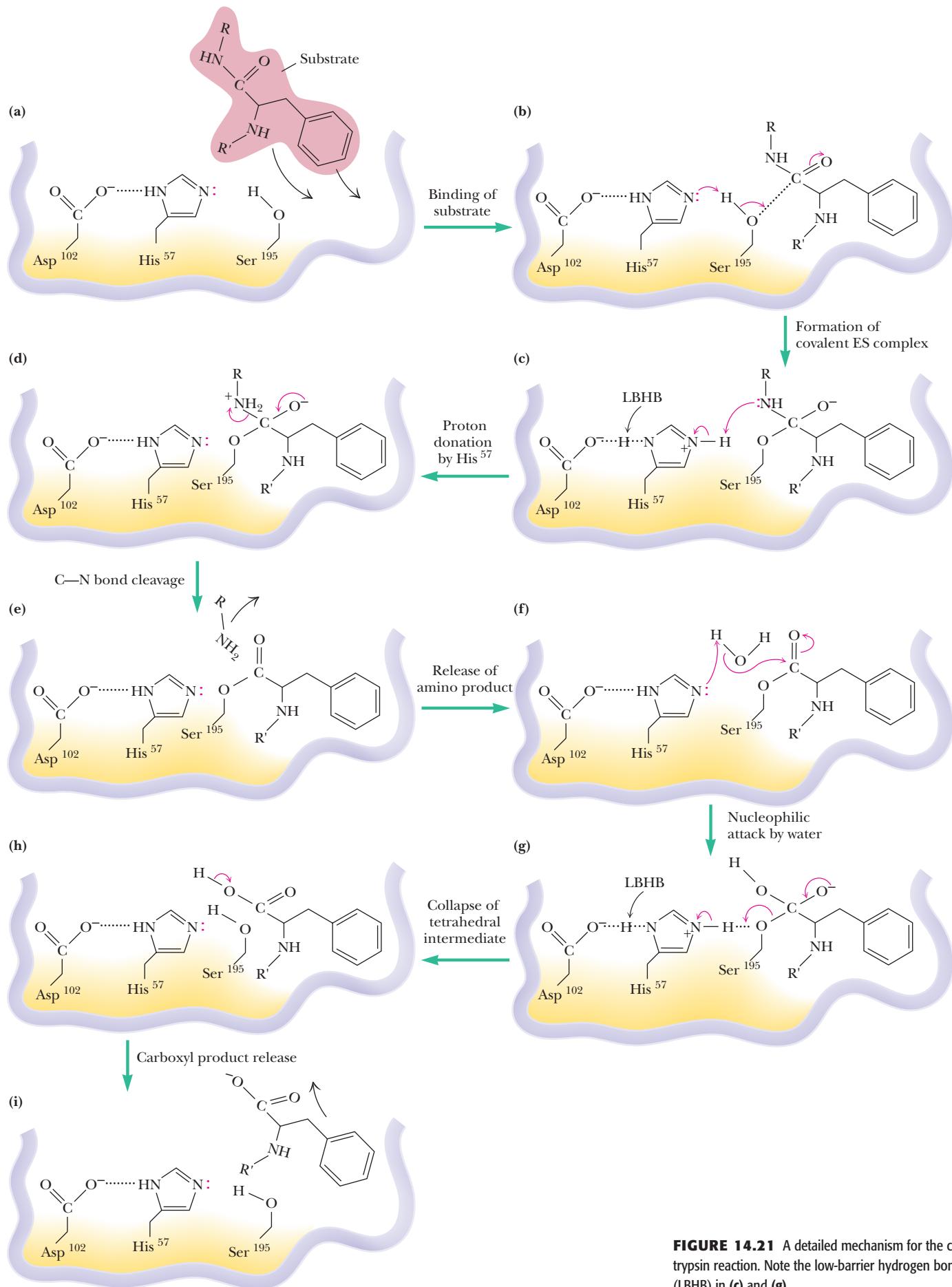
A likely mechanism for peptide hydrolysis is shown in Figure 14.21. As the backbone of the substrate peptide binds adjacent to the catalytic triad, the specific side chain fits into its pocket. Asp<sup>102</sup> of the catalytic triad positions His<sup>57</sup> and immobilizes it through a hydrogen bond as shown. In the first step of the reaction, His<sup>57</sup> acts as a general base to withdraw a proton from Ser<sup>195</sup>, facilitating nucleophilic attack by Ser<sup>195</sup> on the carbonyl carbon of the peptide bond to be cleaved. This is probably a *concerted step*, because proton transfer prior to Ser<sup>195</sup> attack on the acyl carbon would leave a relatively unstable negative charge on the serine oxygen. In the next step, donation of a proton from His<sup>57</sup> to the peptide's amide nitrogen creates a protonated amine on the covalent, tetrahedral intermediate, facilitating the subsequent bond breaking and dissociation of the amine product. The negative charge on the peptide oxygen is unstable; the tetrahedral intermediate is short lived and rapidly breaks down to expel the amine product. The acyl-enzyme intermediate that results is reasonably stable; it can even be isolated using substrate analogs for which further reaction cannot occur. With normal peptide substrates, however, subsequent nucleophilic attack at the carbonyl carbon by water generates another transient tetrahedral intermediate (Figure 14.21g). His<sup>57</sup> acts as a general base in this step, accepting a proton from the attacking water molecule. The subsequent collapse of the tetrahedral intermediate is assisted by proton donation from His<sup>57</sup> to the serine oxygen in a concerted manner. Deprotonation of the carboxyl group and its departure from the active site complete the reaction as shown.

Until recently, the catalytic role of Asp<sup>102</sup> in trypsin and the other serine proteases had been surmised on the basis of its proximity to His<sup>57</sup> in structures obtained from X-ray diffraction studies, but it had never been demonstrated with certainty in physical or chemical studies. As can be seen in Figure 14.16, Asp<sup>102</sup> is buried at the active site; it is normally inaccessible to chemical modifying reagents. In 1987, Charles Craik, William Rutter, and their colleagues used site-directed mutagenesis (see Chapter 12) to prepare a mutant trypsin with an asparagine in place of Asp<sup>102</sup>. This mutant trypsin possessed a hydrolytic activity with ester substrates only 1/10,000 that of native trypsin, demonstrating that Asp<sup>102</sup> is indeed essential for catalysis and that its ability to immobilize and orient His<sup>57</sup> by formation of a hydrogen bond is crucial to the function of the catalytic triad.

The serine protease mechanism relies in part on a low-barrier hydrogen bond. In the free enzyme, the pK<sub>a</sub> values of Asp<sup>102</sup> and His<sup>57</sup> are very different, and the H bond between them is a weak one. However, donation of the proton of Ser<sup>195</sup> to His<sup>57</sup> lowers the pK<sub>a</sub> of the protonated imidazole ring so it becomes a close match to that of Asp<sup>102</sup>, and the H bond between them becomes an LBHB. The energy released in the formation of this LBHB is used to facilitate the formation of the subsequent tetrahedral intermediate (Figure 14.21c, g).

## The Aspartic Proteases

Mammals, fungi, and higher plants produce a family of proteolytic enzymes known as **aspartic proteases**. These enzymes are active at acidic (or sometimes neutral) pH, and each possesses two aspartic acid residues at the active site. Aspartic proteases carry out



**FIGURE 14.21** A detailed mechanism for the chymotrypsin reaction. Note the low-barrier hydrogen bond (LBHB) in (c) and (g).

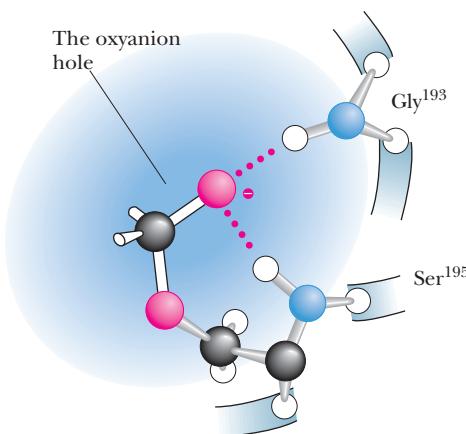
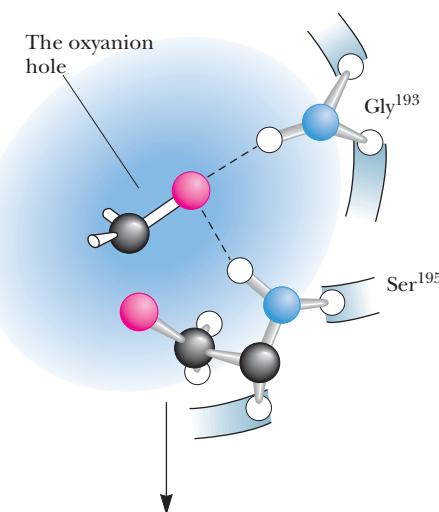
## A DEEPER LOOK

### Transition-State Stabilization in the Serine Proteases

X-ray crystallographic studies of serine protease complexes with transition-state analogs have shown how chymotrypsin stabilizes the **tetrahedral oxyanion transition states** [structures (c) and (g) in Figure 14.21] of the protease reaction. The amide nitrogens of Ser<sup>195</sup> and Gly<sup>193</sup> form an “oxyanion hole” in which the substrate carbonyl oxygen is hydrogen bonded to the amide N—H groups.

Formation of the tetrahedral transition state increases the interaction of the carbonyl oxygen with the amide N—H groups in two ways. Conversion of the carbonyl double bond to the longer tetrahedral single bond brings the oxygen atom closer to the amide hydrogens. Also, the hydrogen bonds between the charged oxygen and the amide hydrogens are significantly stronger than the hydrogen bonds with the uncharged carbonyl oxygen.

Transition-state stabilization in chymotrypsin also involves the side chains of the substrate. The side chain of the departing amine product forms stronger interactions with the enzyme upon formation of the tetrahedral intermediate. When the tetrahedral intermediate breaks down (Figure 14.21d and h), steric repulsion between the product amine group and the carbonyl group of the acyl-enzyme intermediate leads to departure of the amine product.



The “oxyanion hole” of chymotrypsin stabilizes the tetrahedral oxyanion intermediate of the mechanism in Figure 14.21.

a variety of functions (Table 14.3), including digestion (*pepsin* and *chymosin*), lysosomal protein degradation (*cathepsin D* and *E*), and regulation of blood pressure (*renin* is an aspartic protease involved in the production of *angiotensin*, a hormone that stimulates smooth muscle contraction and reduces excretion of salts and fluid). The aspartic proteases display a variety of substrate specificities, but normally they are most active in the cleavage of peptide bonds between two hydrophobic amino acid residues. The preferred substrates of pepsin, for example, contain aromatic residues on both sides of the peptide bond to be cleaved.

Most aspartic proteases are composed of 323 to 340 amino acid residues, with molecular weights near 35,000. Aspartic protease polypeptides consist of two homologous domains that fold to produce a tertiary structure composed of two similar lobes, with approximate twofold symmetry (Figure 14.22). Each of these lobes or domains consists of two  $\beta$ -sheets and two short  $\alpha$ -helices. The two domains are bridged and connected by a six-stranded, antiparallel  $\beta$ -sheet. The active site is a deep and extended cleft, formed by the two juxtaposed domains and large enough to accommodate about seven amino acid residues. The two catalytic aspartate residues, residues 32 and 215 in porcine pepsin, for example, are located deep in the center of the active site cleft. The N-terminal domain forms a “flap” that extends over the active site, which may help to immobilize the substrate in the active site.

**TABLE 14.3** Some Representative Aspartic Proteases

| Name          | Source                                       | Function   |
|---------------|--|--|
| Pepsin*       | Stomach                                      | Digestion of dietary protein   |
| Chymosin†     | Stomach                                      | Digestion of dietary protein   |
| Cathepsin D   | Spleen, liver, and many other animal tissues | Lysosomal digestion of proteins  |
| Renin‡        | Kidney                                       | Conversion of angiotensinogen to angiotensin I; regulation of blood pressure |
| HIV-protease§ | AIDS virus                                   | Processing of AIDS virus proteins  |

\*The second enzyme to be crystallized (by John Northrop in 1930). Even more than urease before it, pepsin study by Northrop established that enzyme activity comes from proteins.

†Also known as rennin, it is the major pepsinlike enzyme in gastric juice of fetal and newborn animals. It has been used for thousands of years, in a gastric extract called rennet, in the making of cheese.

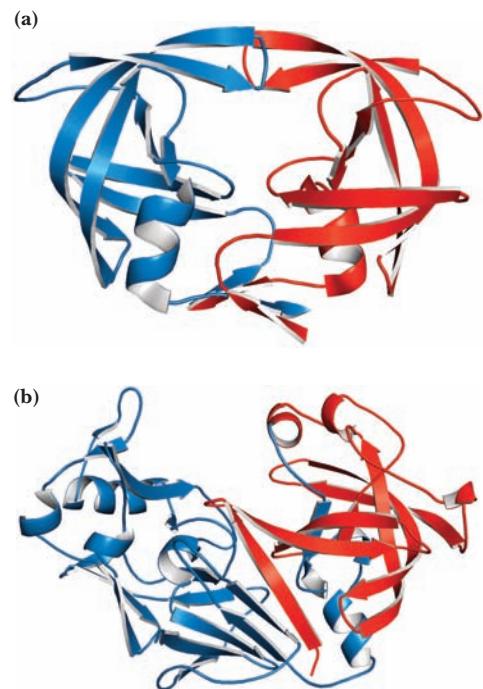
‡A drop in blood pressure causes release of renin from the kidneys, which converts more angiotensinogen to angiotensin.

§A dimer of identical monomers, homologous to pepsin

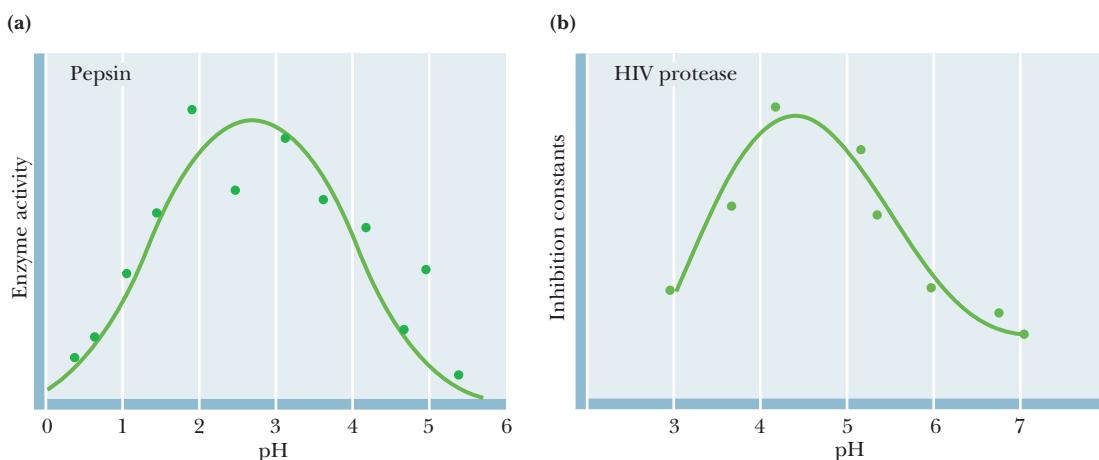
On the basis, in part, of comparisons with chymotrypsin, trypsin, and the other serine proteases, it was at first hypothesized that aspartic proteases might function by formation of covalent enzyme–substrate intermediates involving the active-site aspartate residues. However, all attempts to trap or isolate a covalent intermediate failed, and a mechanism (see following section) favoring noncovalent enzyme–substrate intermediates and general acid–general base catalysis is now favored for aspartic proteases.

## The Mechanism of Action of Aspartic Proteases

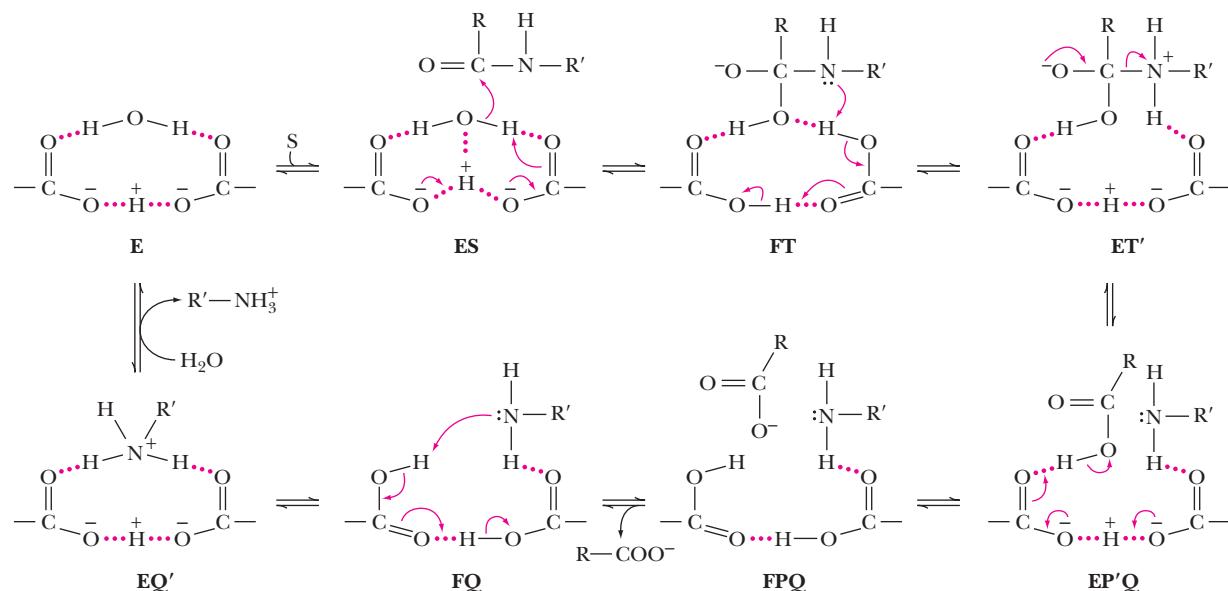
A crucial datum supporting the general acid–general base model is the pH dependence of protease activity (Figure 14.23). For many years, enzymologists hypothesized that the aspartate carboxyl groups functioned alternately as general acid and general base. This model requires that one of the aspartate carboxyls be protonated and one be deprotonated when substrate binds. (This made sense, because X-ray diffraction data on aspartic proteases had shown that the active-site structure in the vicinity of the two aspartates is highly symmetric.) However, Stefano Piana and Paolo Carloni reported in 2000 that molecular dynamics simulations of aspartic proteases were consistent with a



**FIGURE 14.22** Structures of (a) HIV-1 protease, a dimer (pdb id = 7HVP), and (b) pepsin, a monomer. Pepsin's N-terminal half is shown in red; C-terminal half is shown in blue (pdb id = 5PEP).



**FIGURE 14.23** pH-rate profiles for (a) pepsin and (b) HIV protease. (Adapted from Denburg, J., et al., 1968. The effect of pH on the rates of hydrolysis of three acylated dipeptides by pepsin. *Journal of the American Chemical Society* **90**:479–486; and Hyland, J., et al., 1991. Human immunodeficiency virus-1 protease. 2. Use of pH rate studies and solvent kinetic isotope effects to elucidate details of chemical mechanism. *Biochemistry* **30**:8454–8463.)

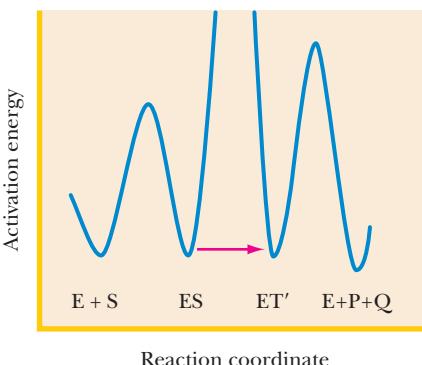


**FIGURE 14.24** Mechanism for the aspartic proteases. The letter titles describe the states as follows: E represents the enzyme form with a low-barrier hydrogen bond between the catalytic aspartates, F represents the enzyme form with both aspartates protonated and joined by a conventional hydrogen bond, S represents bound substrate, T represents a tetrahedral amide hydrate intermediate, P represents bound carboxyl product, and Q represents bound amine product. This mechanism is based in part on a mechanism proposed by Dexter Northrop, a distant relative of John Northrop, who had first crystallized pepsin in 1930. (Northrop, D. B., 2001. Follow the protons: A low-barrier hydrogen bond unifies the mechanisms of the aspartic proteases. *Accounts of Chemical Research* 34:790–797.) The mechanism is also based on data of Thomas Meek. (Meek, T. D., Catalytic mechanisms of the aspartic proteinases. In Sinnott, M., ed, *Comprehensive Biological Catalysis: A Mechanistic Reference*, San Diego: Academic Press, 1998.)

low-barrier hydrogen bond involving the two active-site aspartates. This led to a new mechanism for the aspartic proteases (Figure 14.24) that begins with Piana and Carloni's model of the LBHB structure of the free enzyme (state E). In this model, the LBHB holds the twin aspartate carboxyls in a coplanar conformation, with the catalytic water molecule on the opposite side of a ten-atom cyclic structure.

Following substrate binding, a counterclockwise flow of electrons moves two protons clockwise and creates a tetrahedral intermediate bound to a diprotonated enzyme form (FT). Then a clockwise movement of electrons moves two protons counterclockwise and generates the zwitterion intermediate bound to a monoprotonated enzyme form (ET'). Collapse of the zwitterion cleaves the C—N bond of the substrate. Dissociation of one product leaves the enzyme in the diprotonated FQ form. Finally, deprotonation and rehydration lead to regeneration of the ten-atom cyclic structure, E.

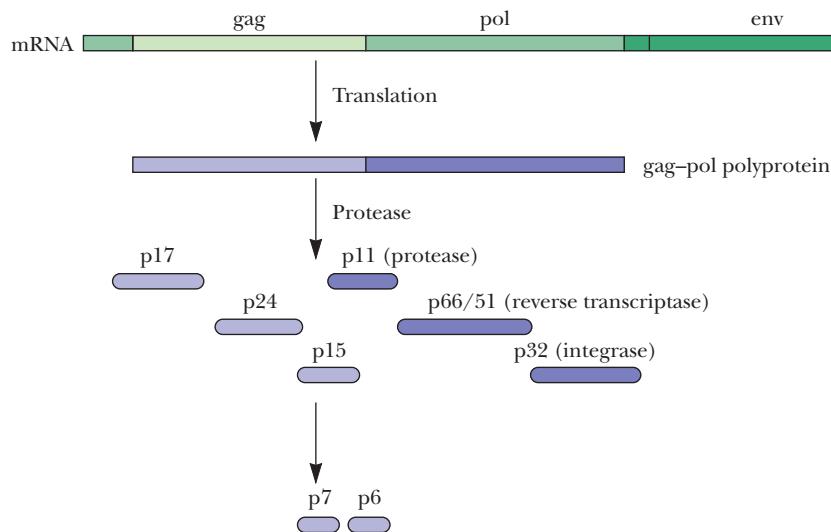
What is the purpose of the low-barrier hydrogen bond in the aspartic protease mechanism? It may be to disperse electron density in the ten-atom cyclic structure, accomplishing rate acceleration by means of “hydrogen tunneling” (Figure 14.25). The barrier between the ES and ET' states of Figure 14.24 is imagined to be large, and the state FT may not exist as a discrete intermediate but rather may exist transiently to facilitate conversion of ES and ET'.



**FIGURE 14.25** Energy level diagram for the aspartic protease reaction, showing ground-state hydrogen tunneling (arrow), with consequent rate acceleration.

## The AIDS Virus HIV-1 Protease Is an Aspartic Protease

Recent research on acquired immunodeficiency syndrome (AIDS) and its causative viral agent, the human immunodeficiency virus (HIV-1), has brought a new aspartic protease to light. **HIV-1 protease** cleaves the polyprotein products of the HIV-1 genome, producing several proteins necessary for viral growth and cellular infection (Figure 14.26). HIV-1 protease cleaves several different peptide linkages. For example, the protease cleaves between the Tyr and Pro residues of the sequence Ser-Gln-Asn-Tyr-Pro-Ile-Val, which joins the p17 and p24 HIV-1 proteins.

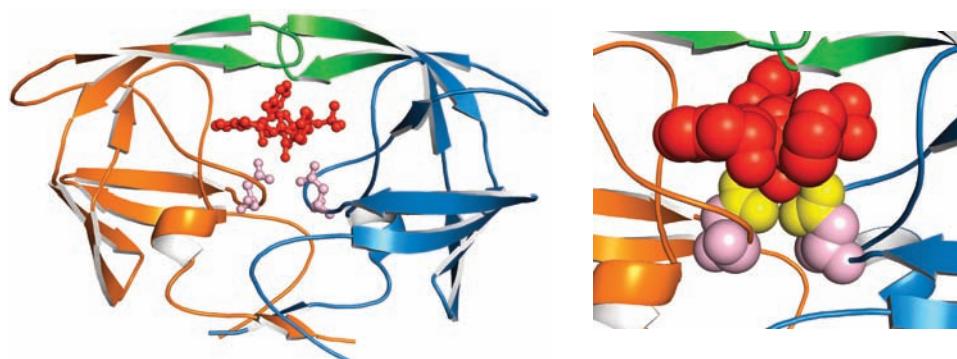


**FIGURE 14.26** HIV mRNA provides the genetic information for synthesis of a polyprotein. Proteolytic cleavage of this polyprotein by HIV protease produces the individual proteins required for viral growth and cellular infection.

The HIV-1 protease is a remarkable viral imitation of mammalian aspartic proteases: It is a **dimer of identical subunits** that mimics the two-lobed monomeric structure of pepsin and other aspartic proteases. The HIV-1 protease subunits are 99-residue polypeptides that are homologous with the individual domains of the monomeric proteases. Structures determined by X-ray diffraction studies reveal that the active site of HIV-1 protease is formed at the interface of the homodimer and consists of two aspartate residues, designated Asp<sup>25</sup> and Asp<sup>25'</sup>, one contributed by each subunit (Figure 14.27). In the homodimer, the active site is covered by two identical “flaps,” one from each subunit, in contrast to the monomeric aspartic proteases, which possess only a single active-site flap. Enzyme kinetic measurements by Thomas Meek and his collaborators at SmithKline Beecham Pharmaceuticals have shown that the mechanism of HIV-1 protease is very similar to those of other aspartic proteases.

## Chorismate Mutase: A Model for Understanding Catalytic Power and Efficiency

Direct comparison of an enzyme reaction with the analogous uncatalyzed reaction is usually difficult, if not impossible. There are several problems: First, many enzyme-catalyzed reactions do not proceed at measurable rates in the absence of the enzyme. Second, many enzyme-catalyzed reactions involve formation of a covalent intermediate between the enzyme and the substrate. Third, a reaction occurring in an enzyme active site might proceed through a different transition state than the corresponding solution reaction. **Chorismate mutase** is a happy exception to all these potential problems. First, although the rate of this reaction is more than a million times faster on the enzyme, the



**FIGURE 14.27** (left) HIV-1 protease complexed with the inhibitor Crixivan (red) made by Merck. The flaps (residues 46–55 from each subunit) covering the active site are shown in green, and the active-site aspartate residues involved in catalysis are shown in light purple. (right) The close-up of the active site shows the interaction of Crixivan with the carboxyl groups (yellow) of the essential aspartate residues (pdb id = 1HSG).

## HUMAN BIOCHEMISTRY

### Protease Inhibitors Give Life to AIDS Patients

Infection with HIV was once considered a death sentence, but the emergence of a new family of drugs called protease inhibitors has made it possible for some AIDS patients to improve their overall health and extend their lives. These drugs are all specific inhibitors of the HIV protease. By inhibiting the protease, they prevent the development of new virus particles in the cells of infected patients. A combination of drugs—including a protease inhibitor together with a reverse transcriptase inhibitor like AZT—can reduce the human immunodeficiency virus (HIV) to undetectable levels in about 40% to 50% of infected individuals. Patients who respond successfully to this combination therapy have experienced dramatic improvement in their overall health and a substantially lengthened life span.

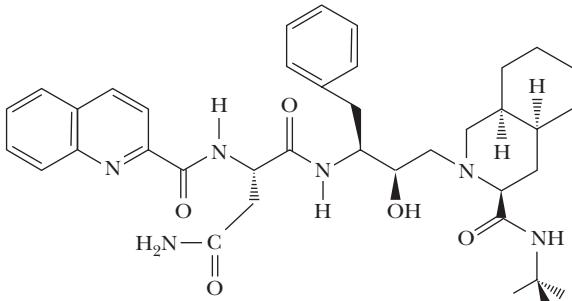
Four of the protease inhibitors approved for use in humans by the U.S. Food and Drug Administration are shown below: Crixivan by Merck, Invirase by Hoffman-LaRoche, Norvir by Abbott, and Viracept by Agouron. These drugs were all developed from a “structure-based” design strategy; that is, the drug molecules were designed to bind tightly to the active site of the HIV-1 protease. The backbone OH-group in all these substances inserts between the two active-site carboxyl groups of the protease.

In the development of an effective drug, it is not sufficient merely to show that a candidate compound can cause the desired biochemi-

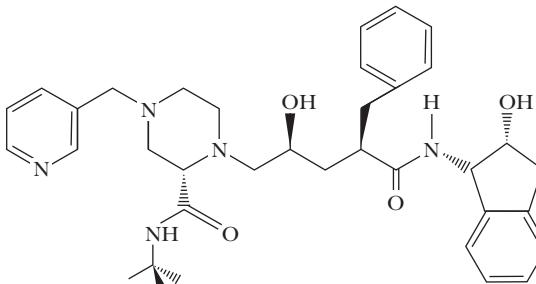
cal effect. It must also be demonstrated that the drug can be effectively delivered in sufficient quantities to the desired site(s) of action in the organism and that the drug does not cause undesirable side effects. The HIV-1 protease inhibitors shown here fulfill all of these criteria. Other drug candidates have been found that are even better inhibitors of HIV-1 protease in cell cultures, but many of these fail the test of bioavailability—the ability of a drug to be delivered to the desired site(s) of action in the organism.

Candidate protease inhibitor drugs must be relatively specific for the HIV-1 protease. Many other aspartic proteases exist in the human body and are essential to a variety of body functions, including digestion of food and processing of hormones. An ideal drug thus must strongly inhibit the HIV-1 protease, must be delivered effectively to the lymphocytes where the protease must be blocked, and should not adversely affect the activities of the essential human aspartic proteases.

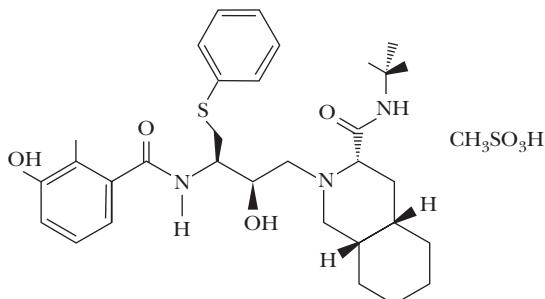
A final but important consideration is viral mutation. Certain mutant HIV strains are resistant to one or more of the protease inhibitors, and even for patients who respond initially to protease inhibitors it is possible that mutant viral forms may eventually arise and thrive in infected individuals. The search for new and more effective protease inhibitors is ongoing.



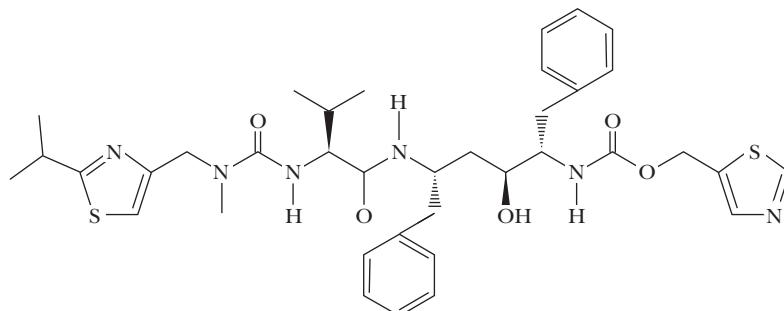
Invirase (saquinavir)



Crixivan (indinavir)



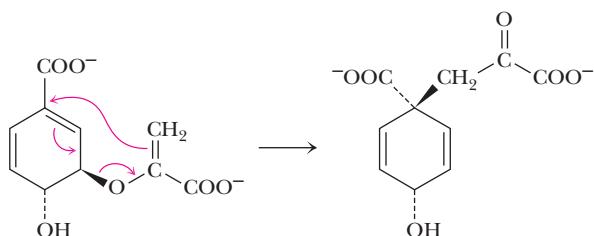
Viracept (nelfinavir mesylate)



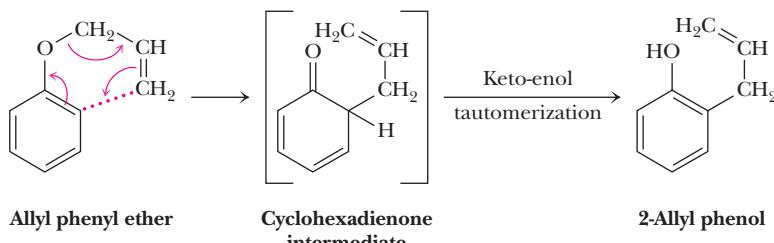
Norvir (ritonavir)

uncatalyzed solution reaction still proceeds at reasonable and measurable rates. Second, the enzyme reaction does not employ a covalent intermediate. What about the transition states for the catalyzed and uncatalyzed reactions? Chorismate mutase acts in the biosynthesis of phenylalanine and tyrosine in microorganisms and plants. It involves a single substrate and catalyzes a concerted intramolecular rearrangement of chorismate to prephenate. In this simple reaction, one carbon-oxygen bond is broken, and one

## (a) Chorismate mutase reaction



## (b) Classic Claisen rearrangement



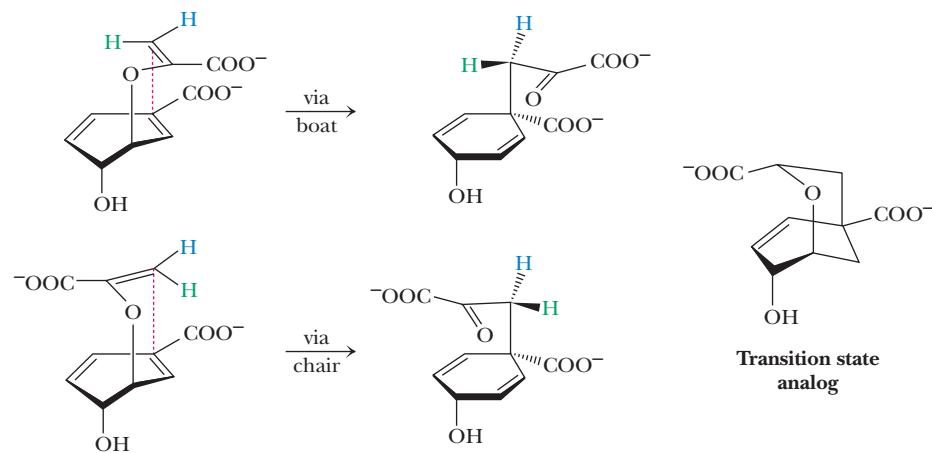
**FIGURE 14.28** (a) The chorismate mutase reaction converts chorismate to prephenate. (b) A classic Claisen rearrangement. Conversion of allyl phenyl ether to 2-allyl alcohol proceeds through a cyclohexadienone intermediate, which then undergoes a keto-enol tautomerization.

carbon–carbon bond is formed. It is an example of a **Claisen rearrangement**, familiar to any student of organic chemistry (Figure 14.28). There are two possible transition states, one involving a chair conformation and the other a boat (Figure 14.29). Jeremy Knowles and his co-workers have shown that both the enzymatic and the solution reactions take place via a chair transition state, and a transition-state analog of this state has been characterized (Figure 14.29).

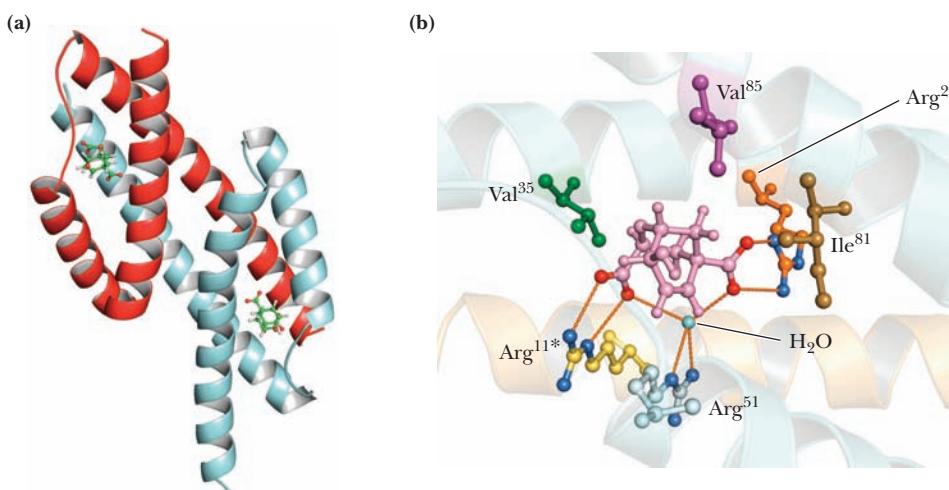
**The Chorismate Mutase Active Site Lies at the Interface of Two Subunits** The chorismate mutase from *E. coli* is the N-terminal portion (109 residues) of a bifunctional enzyme, termed the **P protein**, which also has a C-terminal prephenate dehydrogenase activity. The N-terminal portion of the P protein has been prepared as a separate protein by recombinant DNA techniques, and this engineered protein is a fully functional chorismate mutase. The structure shown in Figure 14.30 is a homodimer, each monomer consisting of three  $\alpha$ -helices (denoted H1, H2, and H3) connected by short loops. The two monomers are dovetailed in the dimer structure, with the H1 helices paired and the H3 helices overlapping significantly. The long, ten-turn H1 helices form an antiparallel coiled coil, with leucines at positions 10, 17, 24, and 31 in a classic 7-residue repeat pattern (see Chapter 6).

## Chorismate

## Prephenate



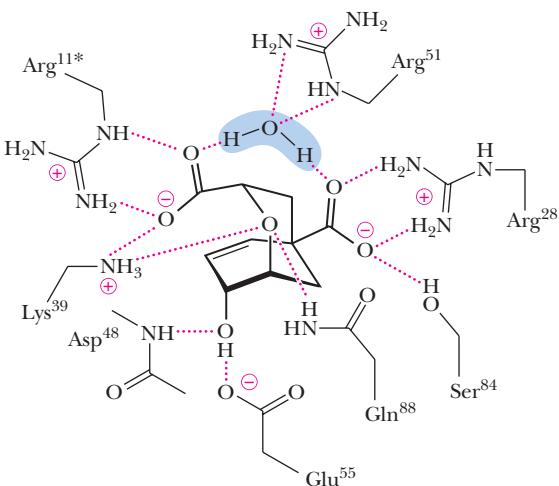
**FIGURE 14.29** The conversion of chorismate to prephenate could occur (in principle) through a boat transition state or a chair transition state. The difference can be understood by imagining two different isotopes of hydrogen (blue and green) at carbon 9 of chorismate and the products that would result in each case. Knowles and co-workers have shown that both the uncatalyzed reaction and the reaction on chorismate mutase occur through a chair transition state. The molecule shown at right is a transition state analog for the chorismate mutase reaction.



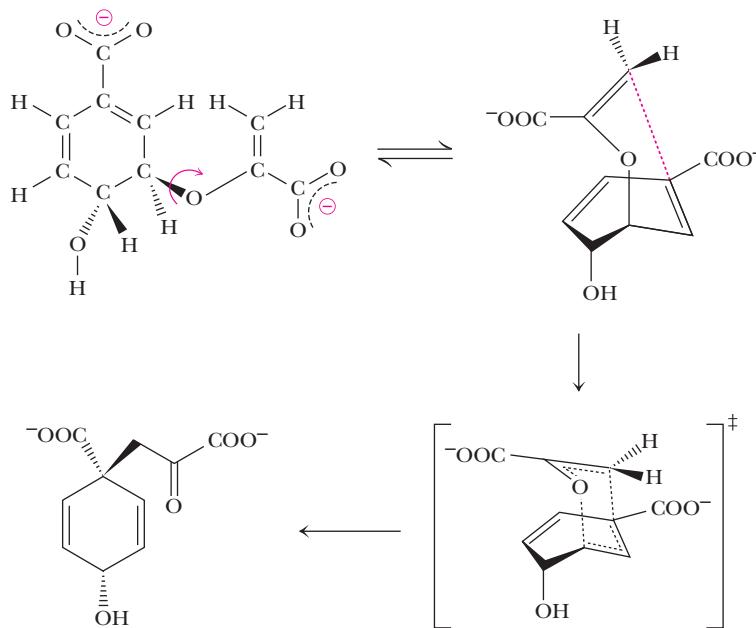
**FIGURE 14.30** Chorismate mutase is a symmetric homodimer, each monomer consisting of three  $\alpha$ -helices connected by short loops. (a) The dimer contains two equivalent active sites, each formed from portions of both monomers (pdb id = 4CSM). (b) A close-up of the active site, showing the bound transition-state analog (pink, see Figure 14.29).

The chorismate mutase dimer contains two equivalent active sites, each formed from portions of both monomers. The structure shown in Figure 14.20 contains a bound transition-state analog (Figure 14.29) stabilized by 12 electrostatic and hydrogen-bonding interactions (Figure 14.31). Arg<sup>28</sup> from one subunit and Arg<sup>11\*</sup> from the other coordinate the carboxyl groups of the analog, and a third arginine (Arg<sup>51</sup>) coordinates a water molecule, which in turn coordinates both carboxyls of the analog. Each oxygen of the analog is coordinated by two groups from the active site. In addition, there are hydrophobic residues surrounding the analog, especially Val<sup>35</sup> on one side and Ile<sup>81</sup> and Val<sup>85</sup> on the other.

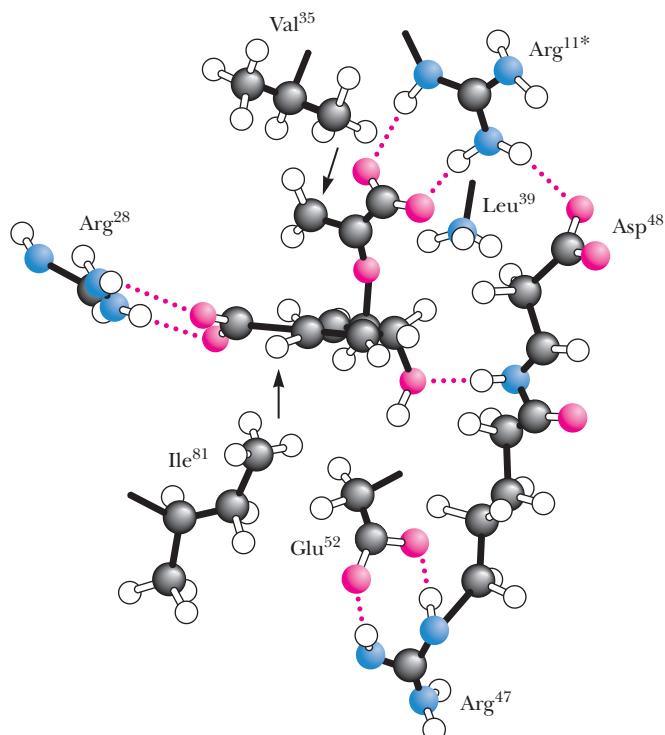
**The Chorismate Mutase Active Site Favors a Near-Attack Conformation** The chorismate mutase reaction mechanism requires that the carboxyvinyl group fold over the chorismate ring to facilitate the Claisen rearrangement (Figure 14.32). This implies the formation of a NAC on the way to the transition state. Bruice and his co-workers have carried out extensive molecular dynamics simulations of the chorismate mutase reaction. Their calculations show that, in the nonenzymatic reaction, only 0.0001% of chorismate in solution exists in the NAC required for reaction. Similar calculations show that, in the enzyme active site, chorismate adopts a NAC 30% of the time. The computer-simulated NAC in the chorismate mutase active site (Figure 14.33) is similar in many ways to the chorismate mutase-TSA complex, with Arg<sup>28</sup> and Arg<sup>11\*</sup> coordinating the two carboxylate groups of chorismate so as to position the carboxyvinyl group



**FIGURE 14.31** In the chorismate mutase active site, the transition-state analog is stabilized by 12 electrostatic and hydrogen-bonding interactions. (Adapted from Lee, A., et al., 1995. Atomic structure of the buried catalytic pocket of *Escherichia coli* chorismate mutase. *Journal of the American Chemical Society* 117:3627–3628.)

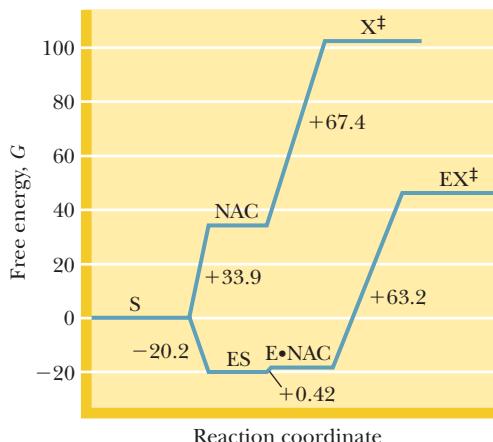


**FIGURE 14.32** The mechanism of the chorismate mutase reaction. The carboxyvinyl group folds up and over the chorismate ring, and the reaction proceeds via an internal rearrangement.



**FIGURE 14.33** Chorismate bound to the active site of chorismate mutase in a structure that resembles a NAC. Arrows indicate hydrophobic interactions, and red dotted lines indicate electrostatic interactions. (Adapted from Hur, S., and Bruice, T., 2003. The near attack conformation approach to the study of the chorismate to prephenate reaction. *Proceedings of the National Academy of Sciences USA* **100**:12015–12020.)

**FIGURE 14.34** The energetic profile of the chorismate mutase reaction. Computer simulations by Bruice and his co-workers show that the NAC and the E-S complex are separated by only 0.42 kJ/mol, meaning that the NAC forms much more readily in the enzyme active site than it does in the absence of enzyme. The NAC and the reaction transition state are separated by similar energy barriers in either the presence or the absence of the enzyme. Thus, the catalytic prowess of the enzyme lies in its ability to form the NAC at a very small energetic cost. (Adapted from Bruice, T., 2002. A view at the millennium: The efficiency of enzymatic catalysis. *Accounts of Chemical Reactions* 35:139–148.)



in the conformation required for transition-state formation. This conformation is also stabilized by Val<sup>135</sup> and Ile<sup>85</sup>, which are in van der Waals contact with the vinyl group and the chorismate ring, respectively. Thus, the NAC of chorismate is promoted by electrostatic and hydrophobic interactions with active-site residues.

The energetics of the chorismate mutase reaction are revealing (Figure 14.34). Computer simulations by Bruice and his co-workers show that formation of a NAC in the absence of the enzyme is energetically costly, whereas formation of the NAC in the enzyme active site is facile, with only a modest energy cost. On the other hand, the energy required to move from the NAC to the transition state is about the same for the solution and the enzyme reactions. Clearly, the catalytic advantage of chorismate mutase is the ease of formation of a NAC in the active site.

## SUMMARY



**OWL** Login to OWL to develop problem-solving skills and complete online homework assigned by your professor.

It is simply chemistry—the breaking and making of bonds—that gives enzymes their prowess. This chapter explores the unique features of this chemistry. The mechanisms of thousands have been studied in at least some detail.

**14.1 What Are the Magnitudes of Enzyme-Induced Rate Accelerations?** Enzymes are powerful catalysts. Enzyme-catalyzed reactions are typically  $10^7$  to  $10^{14}$  times faster than their uncatalyzed counterparts and may exceed  $10^{16}$ .

**14.2 What Role Does Transition-State Stabilization Play in Enzyme Catalysis?** The energy barrier for the uncatalyzed reaction is the difference in energies of the S and X<sup>‡</sup> states. Similarly, the energy barrier to be surmounted in the enzyme-catalyzed reaction, assuming that E is saturated with S, is the energy difference between ES and EX<sup>‡</sup>. Reaction rate acceleration by an enzyme means simply that the energy barrier between ES and EX<sup>‡</sup> is less than the energy barrier between S and X<sup>‡</sup>. In terms of the free energies of activation,  $\Delta G_e^{\ddagger} < \Delta G_u^{\ddagger}$ .

**14.3 How Does Destabilization of ES Affect Enzyme Catalysis?** The favorable interactions between the substrate and amino acid residues on the enzyme account for the intrinsic binding energy,  $\Delta G_b$ . The intrinsic binding energy ensures the favorable formation of the ES complex, but if uncompensated, it makes the activation energy for the enzyme-catalyzed reaction unnecessarily large and wastes some of the catalytic power of the enzyme. Because the enzymatic reaction rate is determined by the difference in energies between ES and EX<sup>‡</sup>,

the smaller this difference, the faster the enzyme-catalyzed reaction. Tight binding of the substrate deepens the energy well of the ES complex and actually lowers the rate of the reaction.

Destabilization of the ES complex can involve structural strain, desolvation, or electrostatic effects. Destabilization by strain or distortion is usually just a consequence of the fact that the enzyme has evolved to bind the transition state more strongly than the substrate.

**14.4 How Tightly Do Transition-State Analogs Bind to the Active Site?** Given a ratio  $k_a/k_u$  of  $10^{12}$  and a typical  $K_S$  of  $10^{-3}\text{ M}$ , the value of  $K_T$  would be  $10^{-15}\text{ M}$ . This is the dissociation constant for the transition-state complex from the enzyme, and this very low value corresponds to very tight binding of the transition state by the enzyme. It is unlikely that such tight binding in an enzyme transition state will ever be measured experimentally, however, because the lifetimes of transition states are typically  $10^{-14}$  to  $10^{-13}\text{ sec}$ .

**14.5 What Are the Mechanisms of Catalysis?** Enzymes facilitate formation of NACs (near-attack conformations). Enzyme reaction mechanisms involve covalent bond formation, general acid–base catalysis, low-barrier hydrogen bonds, metal ion effects, and proximity and favorable orientation of reactants. Most enzymes display involvement of two or more of these in any given reaction.

**14.6 What Can Be Learned from Typical Enzyme Mechanisms?** The enzymes examined in this chapter—serine proteases, aspartic proteases, and chorismate mutase—provide representative examples of catalytic mechanisms; all embody two or more of the rate enhancement contributions.

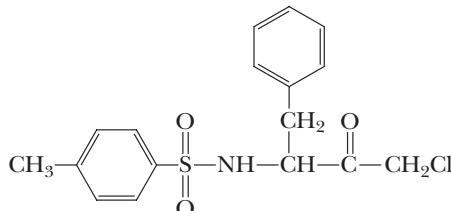
**FOUNDATIONAL BIOCHEMISTRY** Things You Should Know After Reading Chapter 14.

- The approximate magnitudes of catalyzed and uncatalyzed reactions described in Section 14.1.
- The role that transition-state stabilization plays in enzyme catalysis.
- The manner in which destabilization of ES affects enzyme catalysis.
- The nature, lifetime, and binding characteristics of the transition state of an enzyme reaction.
- The utility of transition state analogs as therapeutic agents.
- How to read and write chemical reaction mechanisms.
- The nature of near-attack complexes in enzyme reactions.
- The role of protein motions in enzyme catalysis.
- The role of covalent intermediates in enzyme catalysis.
- The role of general acid–base catalysis in enzyme reactions.
- The characteristic features of low-barrier hydrogen bonds and their role in enzyme catalysis.
- The role of metal ion catalysis in enzyme reactions.
- The essential features of the serine proteases.
- The mechanistic basis of burst kinetics in an enzyme reaction.
- The mechanism of serine proteases.
- The essential features of the aspartic proteases.
- The mechanism of aspartic proteases.
- The roles of low-barrier hydrogen bonds in the serine and aspartic proteases.
- The structural contrasts between mammalian aspartic proteases and the HIV-1 protease.
- The significance of protease inhibitors as AIDS drugs.
- The essential features of chorismate mutase.
- The mechanism of action of chorismate mutase.
- The role of a near-attack complex in the chorismate mutase reaction.

**PROBLEMS**

Answers to all problems are at the end of this book. Detailed solutions are available in the *Student Solutions Manual, Study Guide, and Problems Book*.

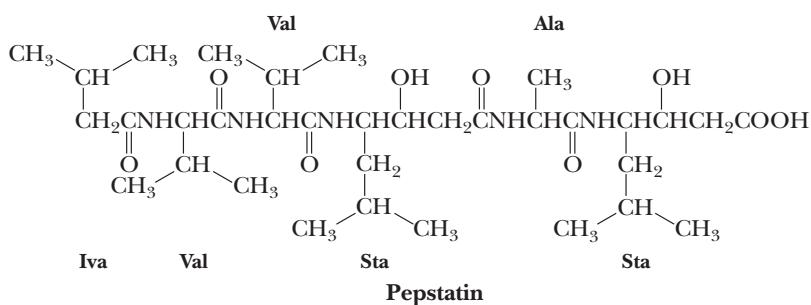
- 1. Characterizing a Covalent Enzyme Inhibitor** Tosyl-L-phenylalanine chloromethyl ketone (TPCK) specifically inhibits chymotrypsin by covalently labeling His<sup>57</sup>.

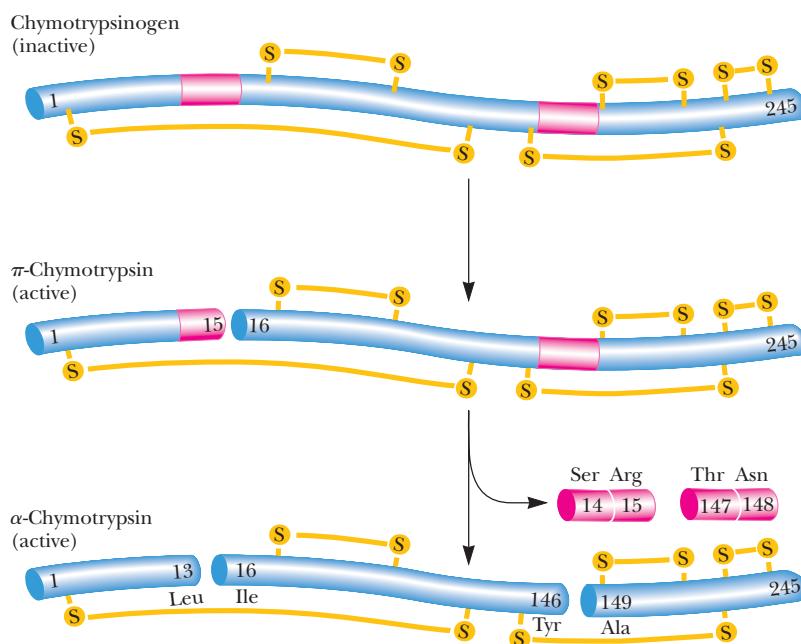


Tosyl-L-phenylalanine chloromethyl ketone (TPCK)

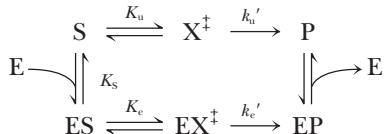
- Propose a mechanism for the inactivation reaction, indicating the structure of the product(s).
- State why this inhibitor is specific for chymotrypsin.
- Propose a reagent based on the structure of TPCK that might be an effective inhibitor of trypsin.

- 2. Using Site-Directed Mutants to Understand an Enzyme Mechanism** In this chapter, the experiment in which Craik and Rutter replaced Asp<sup>102</sup> with Asn in trypsin (reducing activity 10,000-fold) was discussed.
- On the basis of your knowledge of the catalytic triad structure in trypsin, suggest a structure for the “uncatalytic triad” of Asn-His-Ser in this mutant enzyme.
  - Explain why the structure you have proposed explains the reduced activity of the mutant trypsin.
  - See the original journal articles (Sprang, et al., 1987. *Science* 237: 905–909; and Craik, et al., 1987. *Science* 237:909–913) to see Craik and Rutter’s answer to this question.
- 3. Assessing the Action of an Aspartic Protease Inhibitor** Pepstatin (see below) is an extremely potent inhibitor of the monomeric aspartic proteases, with  $K_i$  values of less than 1 nM.
- On the basis of the structure of pepstatin, suggest an explanation for the strongly inhibitory properties of this peptide.
  - Would pepstatin be expected to also inhibit the HIV-1 protease? Explain your answer.
- 4. Deriving an Expression for Catalytic Power** Based on the following reaction scheme, derive an expression for  $k_c/k_u$ , the ratio of the rate constants for the catalyzed and uncatalyzed reactions,





respectively, in terms of the free energies of activation for the catalyzed ( $\Delta G_{\text{c}}^{\ddagger}$ ) and the uncatalyzed ( $\Delta G_{\text{u}}^{\ddagger}$ ) reactions.



#### 5. Comparison of Enzymatic and Nonenzymatic Rate Constants

The  $k_{\text{cat}}$  for alkaline phosphatase-catalyzed hydrolysis of methylphosphate is approximately 14/sec at pH 8 and 25°C. The rate constant for the uncatalyzed hydrolysis of methylphosphate under the same conditions is approximately  $10^{-15}/\text{sec}$ . What is the difference in the free energies of activation of these two reactions?

#### 6. Understanding the Actions of Proteolytic Enzymes

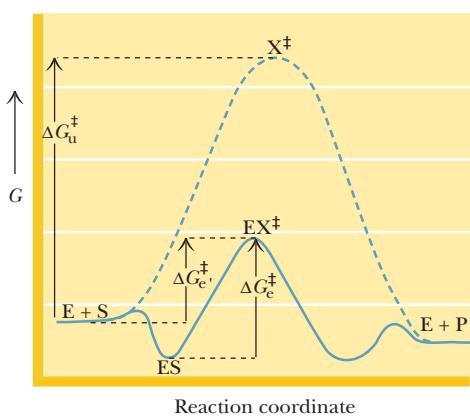
Active  $\alpha$ -chymotrypsin is produced from chymotrypsinogen, an inactive precursor, as shown in the color figure at the top of this page. The first intermediate— $\pi$ -chymotrypsin—displays chymotrypsin activity. Suggest proteolytic enzymes that might carry out these cleavage reactions effectively.

#### 7. Understanding the Mechanism of Carboxypeptidase A

Consult the classic paper by William Lipscomb (1982, *Accounts of Chemical Research* 15:232–238), and on the basis of this article write a simple mechanism for the enzyme carboxypeptidase A.

#### 8. Calculation of Rate Enhancement from Energies of Activation

The relationships between the free energy terms defined in the solution to Problem 4 above are shown in the following figure:



If the energy of the ES complex is 10 kJ/mol lower than the energy of  $\text{E} + \text{S}$ , the value of  $\Delta G_{\text{c}}^{\ddagger}$  is 20 kJ/mol, and the value of  $\Delta G_{\text{u}}^{\ddagger}$  is 90 kJ/mol, what is the rate enhancement achieved by an enzyme in this case?

#### 9. Understanding the Implications of Transition State Stabilization

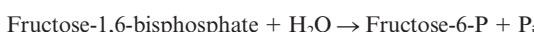
As noted on page 451, a true transition state can bind to an enzyme active site with a  $K_{\text{T}}$  as low as  $7 \times 10^{-26} \text{ M}$ . This is a remarkable number, with interesting consequences. Consider a hypothetical solution of an enzyme in equilibrium with a ligand that binds with a  $K_{\text{D}}$  of  $10^{-27} \text{ M}$ . If the concentration of free enzyme, [E], is equal to the concentration of the enzyme–ligand complex, [EL], what would [L], the concentration of free ligand, be? Calculate the volume of solution that would hold one molecule of free ligand at this concentration.

#### 10. Understanding the Very Tight Binding of Transition States

Another consequence of tight binding (problem 9) is the free energy change for the binding process. Calculate  $\Delta G^{\circ\prime}$  for an equilibrium with a  $K_{\text{D}}$  of  $10^{-27} \text{ M}$ . Compare this value to the free energies of the noncovalent and covalent bonds with which you are familiar. What are the implications of this number, in terms of the binding of a transition state to an enzyme active site?

#### 11. Assessing the Metabolic Consequences of Life Without Enzymes

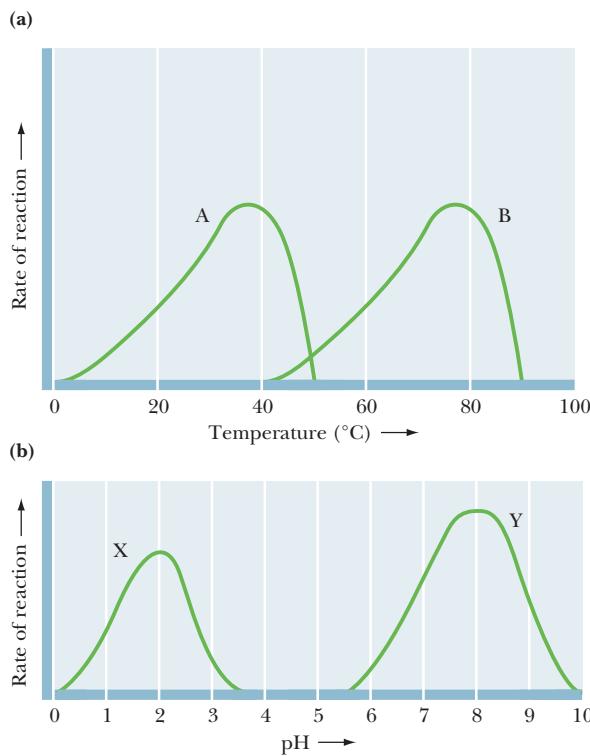
The incredible catalytic power of enzymes can perhaps best be appreciated by imagining how challenging life would be without just one of the thousands of enzymes in the human body. For example, consider life without fructose-1,6-bisphosphatase, an enzyme in the gluconeogenesis pathway in liver and kidneys (see Chapter 22), which helps produce new glucose from the food we eat:



The human brain requires glucose as its only energy source, and the typical brain consumes about 120 g (or 480 calories) of glucose daily. Ordinarily, two pieces of sausage pizza could provide more than enough potential glucose to feed the brain for a day. According to a national fast-food chain, two pieces of sausage pizza provide 1340 calories, 48% of which is from fat. Fats cannot be converted to glucose in gluconeogenesis, so that leaves 697 calories potentially available for glucose synthesis. The first-order rate constant for the hydrolysis of fructose-1,6-bisphosphate in the absence of enzyme is  $2 \times 10^{-20}/\text{sec}$ . Calculate how long it would take to provide enough glucose for one day of brain activity from two pieces of sausage pizza without the enzyme.

### Preparing for the MCAT® Exam

The following graphs show the temperature and pH dependencies of four enzymes, A, B, X, and Y. Problems 12 through 18 refer to these graphs.



12. Enzymes X and Y in the figure are both protein-digesting enzymes found in humans. Where would they most likely be at work?
  - a. X is found in the mouth, Y in the small intestine.
  - b. X in the small intestine, Y in the mouth.
  - c. X in the stomach, Y in the small intestine.
  - d. X in the small intestine, Y in the stomach.
13. Which statement is true concerning enzymes X and Y?
  - a. They could not possibly be at work in the same part of the body at the same time.
  - b. They have different temperature ranges at which they work best.
  - c. At a pH of 4.5, enzyme X works slower than enzyme Y.
  - d. At their appropriate pH ranges, both enzymes work equally fast.
14. What conclusion may be drawn concerning enzymes A and B?
  - a. Neither enzyme is likely to be a human enzyme.
  - b. Enzyme A is more likely to be a human enzyme.

### FURTHER READING

#### General

- Alegre-Cebollada, J., Perez-Jimenez, R., Kosuri, P., and Fernandez, J. M., 2010. Single-molecule force spectroscopy approach to enzyme catalysis. *Journal of Biological Chemistry* **285**:18961–18966.
- Allen, K. N., and Dunaway-Mariano, D., 2010. Markers of fitness in a successful enzyme superfamily. *Current Opinion in Structural Biology* **19**:658–665.
- Cleland, W. W., 2005. The use of isotope effects to determine enzyme mechanisms. *Archives of Biochemistry and Biophysics* **433**:2–12.
- Eigen, M., 1964. Proton transfer, acid–base catalysis, and enzymatic hydrolysis. *Angewandte Chemie International Edition* **3**:1–72.
- Hammes-Schiffer, S., and Stuchebrukhov, A. A., 2010. Coupled electron and proton transfer reactions. *Chemical Reviews* **110**:6939–6960.

- c. Enzyme B is more likely to be a human enzyme.
- d. Both enzymes are likely to be human enzymes.

15. At which temperatures might enzymes A and B both work?

- a. Above 40°C
- b. Below 50°C
- c. Above 50°C and below 40°C
- d. Between 40° and 50°C

16. An enzyme–substrate complex can form when the substrate(s) bind(s) to the active site of the enzyme. Which environmental condition might alter the conformation of an enzyme to the extent that its substrate is unable to bind?

- a. Enzyme A at 40°C
- b. Enzyme B at pH 2
- c. Enzyme X at pH 4
- d. Enzyme Y at 37°C

17. At 35°C, the rate of the reaction catalyzed by enzyme A begins to level off. Which hypothesis best explains this observation?

- a. The temperature is too far below optimum.
- b. The enzyme has become saturated with substrate.
- c. Both A and B.
- d. Neither A nor B.

18. In which of the following environmental conditions would digestive enzyme Y be unable to bring its substrate(s) to the transition state?

- a. At any temperature below optimum
- b. At any pH where the rate of reaction is not maximum
- c. At any pH lower than 5.5
- d. At any temperature higher than 37°C

19. Review the mechanisms of the serine and aspartic proteases, and compare these two mechanisms carefully. Are there steps in the mechanisms that are similar? How are they similar? How are they different? Suggest experiments that could support or refute your hypotheses.

#### ActiveModels Problems

20. Examine the ActiveModel for porcine pepsin, then identify and explain the purpose of the functionally important residues in this enzyme's active site.

21. Using specific examples from the ActiveModel for angiotensin converting enzyme (ACE), explain how N-glycosylation stabilizes ACE.

22. As noted in its ActiveModel, the flap domain of HIV-1 protease is responsible for ligand-binding interactions. Why do the flap domains have glycine-rich ends?

23. According to its ActiveModel, where is the HIV-1 protease cleavage site?

Kamerlin, S. C. L., and Warshel, A., 2010. At the dawn of the 21<sup>st</sup> century: is dynamics the missing link for understanding enzyme catalysis. *Proteins* **78**:1339–1375.

Ringe, D., and Petsko, G. A., 2008. How enzymes work. *Science* **320**:1428–1429.

Schramm, V., 2011. Chemical mechanisms in biochemical reactions. *Journal of the American Chemical Society* **133**:13207–13212.

Stockbridge, R. B., Lewis, Jr., C. A., Yuan, Y., and Wolfenden, R., 2010. Impact of structure on the time required for the establishment of primordial biochemistry, and for the evolution of enzymes. *Proceedings of the National Academy of Sciences* **107**:22102–22105.

Tang, Q., and Leyh, T. S., 2010. Precise, facile, initial rate measurements. *Journal of Physical Chemistry B* **114**:16131–16136.

- Warshel, A., Sharma, P. K., Kato, M., Xiang, Y., Liu, H., and Olsson, M., 2006. Electrostatic basis for enzyme catalysis. *Chemical Reviews* **106**:3210–3235.
- Wolfenden, R., 2006. Degree of difficulty of water-consuming reactions in the absence of enzymes. *Chemical Reviews* **106**:3379–3397.
- Wolfenden, R., and Snider, M. T., 2001. The depth of chemical time and the power of enzymes as catalysts. *Accounts of Chemical Research* **34**:938–945.
- Zhang, X., and Houk, K. N., 2005. Why enzymes are proficient catalysts: Beyond the Pauling paradigm. *Accounts of Chemical Research* **38**:379–385.

### Transition-State Stabilization and Transition-State Analogs

- Hopkins, A. L., and Groom, C. R., 2002. The druggable genome. *Nature Reviews Drug Discovery* **1**:727–730.
- Overington, J. P., Al-Lazikani, B., and Hopkins, A. L., 2006. How many drug targets are there? *Nature Reviews Drug Discovery* **5**:993–996.
- Schramm, V. L., 2007. Enzymatic transition state theory and transition state analogue design. *Journal of Biological Chemistry* **282**:28297–28300.
- Schwartz, S. D., and Schramm, V. L., 2010. Enzymatic transition states and dynamic motion in barrier crossing. *Nature Chemical Biology* **5**:551–558.
- Wogulis, M., Wheelock, C. E., et al., 2006. Structural studies of a potent insect maturation inhibitor bound to the juvenile hormone esterase of *Manduca sexta*. *Biochemistry* **45**:4045–4057.

### Near-Attack Conformations

- Bruice, T. C., 2002. A view at the millennium: The efficiency of enzymatic catalysis. *Accounts of Chemical Research* **35**:139–148.
- Bruice, T. C., 2006. Computational approaches: reaction trajectories, structures, and atomic motions. Enzyme reactions and proficiency. *Chemical Reviews* **106**:3119–3139.
- Hur, S., and Bruice, T., 2003. The near attack conformation approach to the study of the chorismate to prephenate reaction. *Proceedings of the National Academy of Sciences USA* **100**:12015–12020.
- Luo, J., and Bruice, T. C., 2007. Low frequency normal modes in horse liver alcohol dehydrogenase and motions of residues involved in the enzymatic reaction. *Biophysical Chemistry* **126**:80–85.
- Schowen, R. L., 2003. How an enzyme surmounts the activation energy barrier. *Proceedings of the National Academy of Sciences USA* **100**:11931–11932.

### Motion in Enzymes

- Baldwin, A. J., and Kay, L. E., 2009. NMR spectroscopy brings invisible protein states into focus. *Nature Chemical Biology* **5**:808–814.
- Benkovic, S. J., and Hammes-Schiffer, S., 2006. Enzyme motions inside and out. *Science* **312**:208–209.
- Boehr, D. D., Dyson, H. J., and Wright, P. E., 2006. An NMR perspective on enzyme dynamics. *Chemical Reviews* **106**:3055–3079.
- Hammes-Schiffer, S., and Benkovic, S. J., 2006. Relating protein motion to catalysis. *Annual Review of Biochemistry* **75**:519–541.
- Hirschi, J. S., Arora, K., Brooks III, C. L., and Schramm, V. L., 2010. Conformational dynamics in human purine nucleoside phosphorylase with reactants and transition-state analogues. *Journal of Physical Chemistry B* **114**:16263–16272.
- Nashine, V. C., Hammes-Schiffer, S., and Benkovic, S. J., 2010. Coupled motions in enzyme catalysis. *Current Opinion in Chemical Biology* **14**:644–651.

### Low-Barrier Hydrogen Bonds

- Cleland, W. W., 2000. Low barrier hydrogen bonds and enzymatic catalysis. *Archives of Biochemistry and Biophysics* **382**:1–5.
- Yamaguchia, S., Kamikubo, H., Kuriharab, K., Kurokib, R., et al., 2009. Low barrier hydrogen bond in photoactive yellow protein. *Proceedings of the National Academy of Sciences* **106**:440–444.

### Serine Proteases

- Bruice, T. C., and Bruice, P. Y., 2005. Covalent intermediates and enzyme proficiency. *Journal of the American Chemical Society* **127**:12478–12479.
- Craik, C. S., Rocznak, S., et al. 1987. The catalytic role of the active site aspartic acid in serine proteases. *Science* **237**:909–913.
- Sprang, S., and Standing, T., 1987. The three dimensional structure of Asn<sup>102</sup> mutant of trypsin: Role of Asp<sup>102</sup> in serine protease catalysis. *Science* **237**:905–909.

### Aspartic Proteases

- Northrop, D. B., 2001. Follow the protons: A low-barrier hydrogen bond unifies the mechanisms of the aspartic proteases. *Accounts of Chemical Research* **34**:790–797.
- Wolfe, M. S., 2010. Structure, mechanism and inhibition of  $\gamma$ -secretase and presenilin-like proteases. *Biology and Chemistry* **391**:839–847.

### HIV-1 Protease

- Hyland, L., et al., 1991. Human immunodeficiency virus-1 protease 1: Initial velocity studies and kinetic characterization of reaction intermediates by <sup>18</sup>O isotope exchange. *Biochemistry* **30**:8441–8453.
- Hyland, L., Tomaszek, T., and Meek, T., 1991. Human immunodeficiency virus-1 protease 2: Use of pH rate studies and solvent isotope effects to elucidate details of chemical mechanism. *Biochemistry* **30**:8454–8463.

### Chorismate Mutase

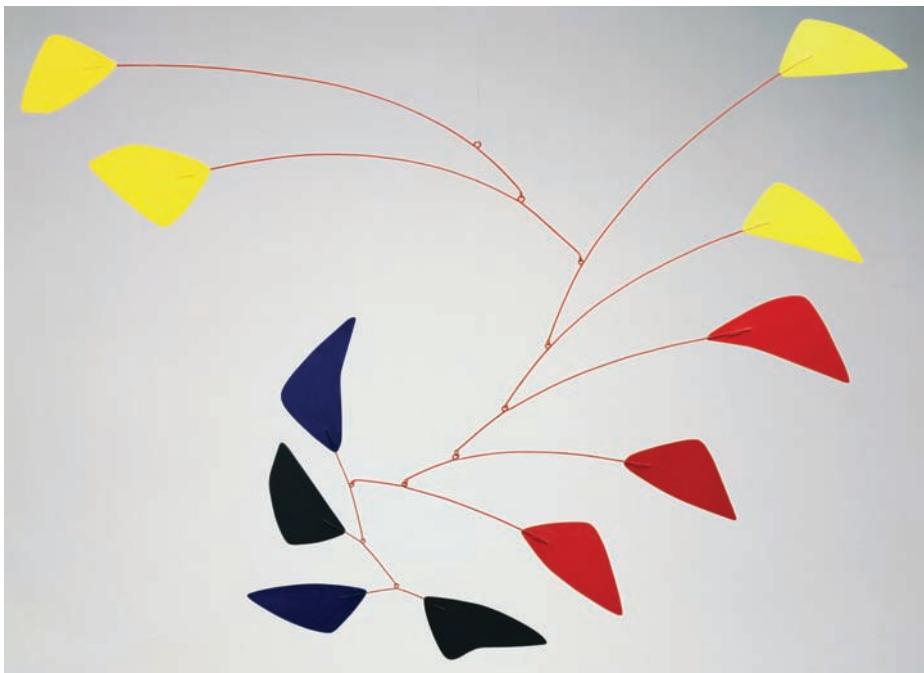
- Bartlett, P. A., and Johnson, C. R., 1985. An inhibitor of chorismate mutase resembling the transition state conformation. *Journal of the American Chemical Society* **107**:7792–7793.
- Copley, S. D., and Knowles, J. R., 1985. The uncatalyzed Claisen rearrangement of chorismate to prephenate prefers a transition state of chairlike geometry. *Journal of the American Chemical Society* **107**:5306–5308.
- Hur, S., and Bruice, T. C., 2003. The near attack conformation approach to the study of the chorismate to prephenate reaction. *Proceedings of the National Academy of Sciences USA* **100**:12015–12020.
- Lee, A., Karplus, A., et al., 1995. Atomic structure of the buried catalytic pocket of *Escherichia coli* chorismate mutase. *Journal of the American Chemical Society* **117**:3627–3628.
- Sasso, S., Okvist, M., Rodere, K., et al., 2009. Structure and function of a complex between chorismate mutase and DAHP synthase: efficiency boost for the junior partner. *The EMBO Journal* **28**:2128–2142.
- Sogo, S. G., Widlanski, T. S., et al., 1984. Stereochemistry of the rearrangement of chorismate to prephenate: Chorismate mutase involves a chair transition state. *Journal of the American Chemical Society* **106**:2701–2703.
- Zhang, X., Zhang, X., et al., 2005. A definitive mechanism for chorismate mutase. *Biochemistry* **44**:10443–10448.

### Tunneling in Enzyme Mechanisms

- Hammes-Schiffer, 2010. Introduction: Proton-Coupled Electron Transfer. *Chemical Reviews* **110**:6937–6938.
- Machleder, S. Q., Pineda, E. T., and Schwartz, S. D., 2010. On the origin of the chemical barrier and tunneling in enzymes. *Journal of Physical Organic Chemistry* **23**:690–695.
- Nagel, Z. D., and Klinman, J. P., 2009. A 21<sup>st</sup> century revisionist's view at a turning point in enzymology. *Nature Chemical Biology* **5**:543–550.
- Nagel, Z. D., and Klinman, J. P., 2010. Tunneling and dynamics in enzymatic hydride transfer. *Chemical Reviews* **110**:PR41–PR67.
- Pudney, C. R., Johannissen, L. O., Sutcliffe, M. J., Hay, S., and Scrutton, N. S., 2010. Direct analysis of donor-acceptor distance and relationship to isotope effects and the force constant for barrier compression in enzymatic H-tunneling reactions. *Journal of the American Chemical Society* **132**:11329–11335.

# Enzyme Regulation

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## ESSENTIAL QUESTION

Enzymes catalyze essentially all of the thousands of metabolic reactions taking place in cells. Many of these reactions are at cross-purposes: Some enzymes catalyze the breakdown of substances, whereas others catalyze synthesis of the same substances; many metabolic intermediates have more than one fate; and energy is released in some reactions and consumed in others. At key positions within the metabolic pathways, regulatory enzymes sense the momentary needs of the cell and adjust their catalytic activity accordingly. Regulation of these enzymes ensures the harmonious integration of the diverse and often divergent reactions of metabolism.

**What are the properties of regulatory enzymes? How do regulatory enzymes sense the momentary needs of cells? What molecular mechanisms are used to regulate enzyme activity?**

## 15.1 | What Factors Influence Enzymatic Activity?

The activity displayed by enzymes is affected by a variety of factors, some of which are essential to the harmony of metabolism. Two of the more obvious ways to regulate the amount of activity at a given time are (1) to increase or decrease the number of enzyme molecules and (2) to increase or decrease the activity of each enzyme molecule. Although these ways are obvious, the cellular mechanisms that underlie them are complex and varied, as we shall see. A general overview of factors influencing enzyme activity includes the following considerations.

◀ Metabolic regulation is achieved through an exquisitely balanced interplay among enzymes and small molecules.

*Allostery is a key chemical process that makes possible intracellular and intercellular regulation: “... the molecular interactions which ensure the transmission and interpretation of (regulatory) signals rest upon (allosteric) proteins endowed with discriminatory stereospecific recognition properties.”*

Jacques Monod  
Chance and Necessity

## KEY QUESTIONS

- 15.1** What Factors Influence Enzymatic Activity?
- 15.2** What Are the General Features of Allosteric Regulation?
- 15.3** Can Allosteric Regulation Be Explained by Conformational Changes in Proteins?
- 15.4** What Kinds of Covalent Modification Regulate the Activity of Enzymes?
- 15.5** Is the Activity of Some Enzymes Controlled by Both Allosteric Regulation and Covalent Modification?  
*Special Focus: Is There an Example in Nature That Exemplifies the Relationship Between Quaternary Structure and the Emergence of Allosteric Properties? Hemoglobin and Myoglobin—Paradigms of Protein Structure and Function*

 Online homework and a Student Self Assessment for this chapter may be assigned in OWL.