

# chapter 6

## ENZYMES

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One way in which this condition might be fulfilled would be if the molecules when combined with the enzyme, lay slightly further apart than their equilibrium distance when [covalently joined], but nearer than their equilibrium distance when free. . . . Using Fischer's lock and key simile, the key does not fit the lock quite perfectly but exercises a certain strain on it.

—J. B. S. Haldane, *Enzymes*, 1930

Catalysis can be described formally in terms of a stabilization of the transition state through tight binding to the catalyst.

—William P. Jencks, *article in Advances in Enzymology*, 1975

**T**here are two fundamental conditions for life. First, the living entity must be able to self-replicate (a topic considered in Part III); second, the organism must be able to catalyze chemical reactions efficiently and selectively. The central importance of catalysis may surprise some beginning students of biochemistry, but it is easy to demonstrate. As described in Chapter 1, living systems make use of energy from the environment. Many of us, for example, consume substantial amounts of sucrose—common table sugar—as a kind of fuel, whether in the form of sweetened foods and drinks or as sugar itself. The conversion of sucrose to  $\text{CO}_2$  and

$\text{H}_2\text{O}$  in the presence of oxygen is a highly exergonic process, releasing free energy that we can use to think, move, taste, and see. However, a bag of sugar can remain on the shelf for years without any obvious conversion to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . Although this chemical process is thermodynamically favorable, it is very slow! Yet when sucrose is consumed by a human (or almost any other organism), it releases its chemical energy in seconds. The difference is catalysis. Without catalysis, chemical reactions such as sucrose oxidation could not occur on a useful time scale, and thus could not sustain life.

In this chapter, then, we turn our attention to the reaction catalysts of biological systems: the enzymes, the most remarkable and highly specialized proteins. Enzymes have extraordinary catalytic power, often far greater than that of synthetic or inorganic catalysts. They have a high degree of specificity for their substrates, they accelerate chemical reactions tremendously, and they function in aqueous solutions under very mild conditions of temperature and pH. Few non-biological catalysts have all these properties.

Enzymes are central to every biochemical process. Acting in organized sequences, they catalyze the hundreds of stepwise reactions that degrade nutrient molecules, conserve and transform chemical energy, and make biological macromolecules from simple precursors. Through the action of regulatory enzymes, metabolic pathways are highly coordinated to yield a harmonious interplay among the many activities necessary to sustain life.

The study of enzymes has immense practical importance. In some diseases, especially inheritable genetic disorders, there may be a deficiency or even a total absence of one or more enzymes. For other disease conditions, excessive activity of an enzyme may be the cause. Measurements of the activities of enzymes in blood plasma, erythrocytes, or tissue samples are important in diagnosing certain illnesses. Many drugs exert their biological effects through interactions with enzymes. And enzymes are important practical tools,

not only in medicine but in the chemical industry, food processing, and agriculture.

We begin with descriptions of the properties of enzymes and the principles underlying their catalytic power, then introduce enzyme kinetics, a discipline that provides much of the framework for any discussion of enzymes. Specific examples of enzyme mechanisms are then provided, illustrating principles introduced earlier in the chapter. We end with a discussion of how enzyme activity is regulated.

## 6.1 An Introduction to Enzymes

Much of the history of biochemistry is the history of enzyme research. Biological catalysis was first recognized and described in the late 1700s, in studies on the digestion of meat by secretions of the stomach, and research continued in the 1800s with examinations of the conversion of starch to sugar by saliva and various plant extracts. In the 1850s, Louis Pasteur concluded that fermentation of sugar into alcohol by yeast is catalyzed by “ferments.” He postulated that these ferments were inseparable from the structure of living yeast cells; this view, called vitalism, prevailed for decades. Then in 1897 Eduard Buchner discovered that yeast extracts could ferment sugar to alcohol, proving that fermentation was promoted by molecules that continued to function when removed from cells. Frederick W. Kühne called these molecules **enzymes**. As vitalistic notions of life were disproved, the isolation of new enzymes and the investigation of their properties advanced the science of biochemistry.

The isolation and crystallization of urease by James Sumner in 1926 provided a breakthrough in early enzyme studies. Sumner found that urease crystals consisted entirely of protein, and he postulated that all enzymes are proteins. In the absence of other examples, this idea remained controversial for some time. Only in the 1930s was Sumner’s conclusion widely accepted, after John Northrop and Moses Kunitz crystallized pepsin, trypsin, and other digestive enzymes and found them also to be proteins. During this period, J. B. S. Haldane wrote a treatise entitled *Enzymes*. Although the molecular nature of enzymes was not yet fully appreciated, Haldane made the remarkable suggestion that weak bonding interactions between an enzyme and its substrate might be used to catalyze a reaction. This insight lies at the heart of our current understanding of enzymatic catalysis.

Since the latter part of the twentieth century, research on enzymes has been intensive. It has led to the purification of thousands of enzymes, elucidation of the

structure and chemical mechanism of many of them, and a general understanding of how enzymes work.

### Most Enzymes Are Proteins

With the exception of a small group of catalytic RNA molecules (Chapter 26), all enzymes are proteins. Their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or dissociated into its subunits, catalytic activity is usually lost. If an enzyme is broken down into its component amino acids, its catalytic activity is always destroyed. Thus the primary, secondary, tertiary, and quaternary structures of protein enzymes are essential to their catalytic activity.

Enzymes, like other proteins, have molecular weights ranging from about 12,000 to more than 1 million. Some enzymes require no chemical groups for activity other than their amino acid residues. Others require an additional chemical component called a **cofactor**—either one or more inorganic ions, such as  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , or  $\text{Zn}^{2+}$  (Table 6-1), or a complex organic or metalloorganic molecule called a **coenzyme** (Table 6-2). Some enzymes require *both* a coenzyme

**TABLE 6-1** Some Inorganic Elements That Serve as Cofactors for Enzymes

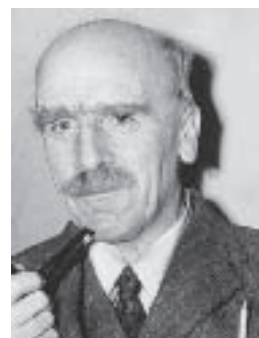
$\text{Cu}^{2+}$	Cytochrome oxidase
$\text{Fe}^{2+}$ or $\text{Fe}^{3+}$	Cytochrome oxidase, catalase, peroxidase
$\text{K}^{+}$	Pyruvate kinase
$\text{Mg}^{2+}$	Hexokinase, glucose 6-phosphatase, pyruvate kinase
$\text{Mn}^{2+}$	Arginase, ribonucleotide reductase
Mo	Dinitrogenase
$\text{Ni}^{2+}$	Urease
Se	Glutathione peroxidase
$\text{Zn}^{2+}$	Carbonic anhydrase, alcohol dehydrogenase, carboxypeptidases A and B



Eduard Buchner,  
1860–1917



James Sumner,  
1887–1955



J. B. S. Haldane,  
1892–1964

**TABLE 6–2** Some Coenzymes That Serve as Transient Carriers of Specific Atoms or Functional Groups

Coenzyme	Examples of chemical groups transferred	Dietary precursor in mammals
Biocytin	CO <sub>2</sub>	Biotin
Coenzyme A	Acyl groups	Pantothenic acid and other compounds
5'-Deoxyadenosylcobalamin (coenzyme B <sub>12</sub> )	H atoms and alkyl groups	Vitamin B <sub>12</sub>
Flavin adenine dinucleotide	Electrons	Riboflavin (vitamin B <sub>2</sub> )
Lipoate	Electrons and acyl groups	Not required in diet
Nicotinamide adenine dinucleotide	Hydride ion (:H <sup>−</sup> )	Nicotinic acid (niacin)
Pyridoxal phosphate	Amino groups	Pyridoxine (vitamin B <sub>6</sub> )
Tetrahydrofolate	One-carbon groups	Folate
Thiamine pyrophosphate	Aldehydes	Thiamine (vitamin B <sub>1</sub> )

Note: The structures and modes of action of these coenzymes are described in Part II.

and one or more metal ions for activity. A coenzyme or metal ion that is very tightly or even covalently bound to the enzyme protein is called a **prosthetic group**. A complete, catalytically active enzyme together with its bound coenzyme and/or metal ions is called a **holoenzyme**. The protein part of such an enzyme is called the **apoenzyme** or **apoprotein**. Coenzymes act as transient carriers of specific functional groups. Most are derived from vitamins, organic nutrients required in small amounts in the diet. We consider coenzymes in more detail as we encounter them in the metabolic pathways discussed in Part II. Finally, some enzyme proteins are modified covalently by phosphorylation, glycosylation, and other processes. Many of these alterations are involved in the regulation of enzyme activity.

### Enzymes Are Classified by the Reactions They Catalyze

Many enzymes have been named by adding the suffix “-ase” to the name of their substrate or to a word or phrase describing their activity. Thus urease catalyzes hydrolysis of urea, and DNA polymerase catalyzes the polymerization of nucleotides to form DNA. Other enzymes were named by their discoverers for a broad func-

tion, before the specific reaction catalyzed was known. For example, an enzyme known to act in the digestion of foods was named pepsin, from the Greek *pepsis*, “digestion,” and lysozyme was named for its ability to lyse bacterial cell walls. Still others were named for their source: trypsin, named in part from the Greek *tryein*, “to wear down,” was obtained by rubbing pancreatic tissue with glycerin. Sometimes the same enzyme has two or more names, or two different enzymes have the same name. Because of such ambiguities, and the ever-increasing number of newly discovered enzymes, biochemists, by international agreement, have adopted a system for naming and classifying enzymes. This system divides enzymes into six classes, each with subclasses, based on the type of reaction catalyzed (Table 6–3). Each enzyme is assigned a four-part classification number and a systematic name, which identifies the reaction it catalyzes. As an example, the formal systematic name of the enzyme catalyzing the reaction



is ATP:glucose phosphotransferase, which indicates that it catalyzes the transfer of a phosphoryl group from ATP to glucose. Its Enzyme Commission number (E.C. number) is 2.7.1.1. The first number (2) denotes the

**TABLE 6–3** International Classification of Enzymes

No.	Class	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer reactions
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to water)
4	Lyases	Addition of groups to double bonds, or formation of double bonds by removal of groups
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C—C, C—S, C—O, and C—N bonds by condensation reactions coupled to ATP cleavage

Note: Most enzymes catalyze the transfer of electrons, atoms, or functional groups. They are therefore classified, given code numbers, and assigned names according to the type of transfer reaction, the group donor, and the group acceptor.

class name (transferase); the second number (7), the subclass (phosphotransferase); the third number (1), a phosphotransferase with a hydroxyl group as acceptor; and the fourth number (1), D-glucose as the phosphoryl group acceptor. For many enzymes, a trivial name is more commonly used—in this case hexokinase. A complete list and description of the thousands of known enzymes is maintained by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology ([www.chem.qmul.ac.uk/iubmb/enzyme](http://www.chem.qmul.ac.uk/iubmb/enzyme)). This chapter is devoted primarily to principles and properties common to all enzymes.

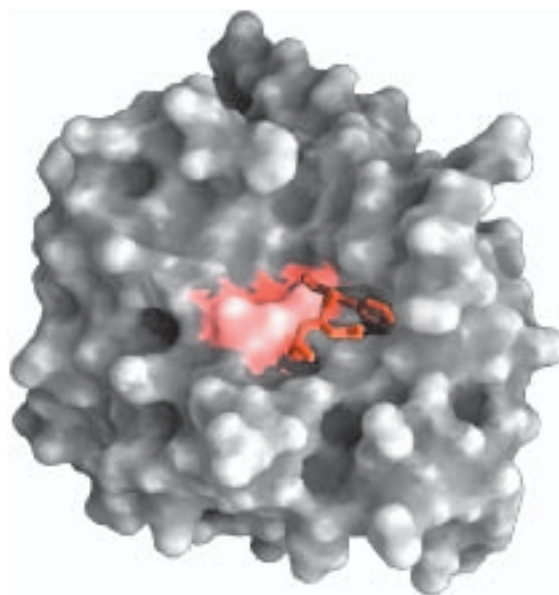
### SUMMARY 6.1 An Introduction to Enzymes

- Life depends on the existence of powerful and specific catalysts: the enzymes. Almost every biochemical reaction is catalyzed by an enzyme.
- With the exception of a few catalytic RNAs, all known enzymes are proteins. Many require nonprotein coenzymes or cofactors for their catalytic function.
- Enzymes are classified according to the type of reaction they catalyze. All enzymes have formal E.C. numbers and names, and most have trivial names.

## 6.2 How Enzymes Work

The enzymatic catalysis of reactions is essential to living systems. Under biologically relevant conditions, uncatalyzed reactions tend to be slow—most biological molecules are quite stable in the neutral-pH, mild-temperature, aqueous environment inside cells. Furthermore, many common reactions in biochemistry entail chemical events that are unfavorable or unlikely in the cellular environment, such as the transient formation of unstable charged intermediates or the collision of two or more molecules in the precise orientation required for reaction. Reactions required to digest food, send nerve signals, or contract a muscle simply do not occur at a useful rate without catalysis.

An enzyme circumvents these problems by providing a specific environment within which a given reaction can occur more rapidly. The distinguishing feature of an enzyme-catalyzed reaction is that it takes place within the confines of a pocket on the enzyme called the **active site** (Fig. 6–1). The molecule that is bound in the active site and acted upon by the enzyme is called the **substrate**. The surface of the active site is lined with amino acid residues with substituent groups that bind the substrate and catalyze its chemical transformation. Often, the active site encloses a substrate, sequestering it completely from solution. The enzyme-



**FIGURE 6-1** Binding of a substrate to an enzyme at the active site. The enzyme chymotrypsin, with bound substrate in red (PDB ID 7GCH). Some key active-site amino acid residues appear as a red splotch on the enzyme surface.

substrate complex, whose existence was first proposed by Charles-Adolphe Wurtz in 1880, is central to the action of enzymes. It is also the starting point for mathematical treatments that define the kinetic behavior of enzyme-catalyzed reactions and for theoretical descriptions of enzyme mechanisms.

### Enzymes Affect Reaction Rates, Not Equilibria

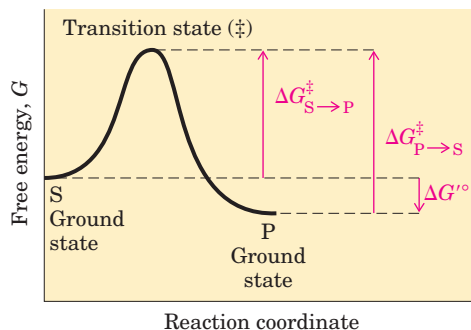
A simple enzymatic reaction might be written



where E, S, and P represent the enzyme, substrate, and product; ES and EP are transient complexes of the enzyme with the substrate and with the product.

To understand catalysis, we must first appreciate the important distinction between reaction equilibria and reaction rates. The function of a catalyst is to increase the *rate* of a reaction. Catalysts do not affect reaction *equilibria*. Any reaction, such as  $S \rightleftharpoons P$ , can be described by a reaction coordinate diagram (Fig. 6–2), a picture of the energy changes during the reaction. As discussed in Chapter 1, energy in biological systems is described in terms of free energy,  $G$ . In the coordinate diagram, the free energy of the system is plotted against the progress of the reaction (the reaction coordinate). The starting point for either the forward or the reverse reaction is called the **ground state**, the contribution to the free energy of the system by an average molecule (S or P) under a given set of conditions. To describe the free-energy changes for reactions, chemists define a standard set of conditions (temperature 298 K; partial pressure of each gas 1 atm, or 101.3 kPa; concentration





**FIGURE 6-2** Reaction coordinate diagram for a chemical reaction.

The free energy of the system is plotted against the progress of the reaction  $S \rightarrow P$ . A diagram of this kind is a description of the energy changes during the reaction, and the horizontal axis (reaction coordinate) reflects the progressive chemical changes (e.g., bond breakage or formation) as S is converted to P. The activation energies,  $\Delta G^\ddagger$ , for the  $S \rightarrow P$  and  $P \rightarrow S$  reactions are indicated.  $\Delta G^\circ$  is the overall standard free-energy change in the direction  $S \rightarrow P$ .

of each solute 1 M) and express the free-energy change for this reacting system as  $\Delta G^\circ$ , the **standard free-energy change**. Because biochemical systems commonly involve  $H^+$  concentrations far below 1 M, biochemists define a **biochemical standard free-energy change**,  $\Delta G'^\circ$ , the standard free-energy change at  $pH\ 7.0$ ; we employ this definition throughout the book. A more complete definition of  $\Delta G'^\circ$  is given in Chapter 13.

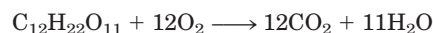
The equilibrium between S and P reflects the difference in the free energies of their ground states. In the example shown in Figure 6-2, the free energy of the ground state of P is lower than that of S, so  $\Delta G^\circ$  for the reaction is negative and the equilibrium favors P. The position and direction of equilibrium are *not* affected by any catalyst.

A favorable equilibrium does not mean that the  $S \rightarrow P$  conversion will occur at a detectable rate. The *rate* of a reaction is dependent on an entirely different parameter. There is an energy barrier between S and P: the energy required for alignment of reacting groups, formation of transient unstable charges, bond rearrangements, and other transformations required for the reaction to proceed in either direction. This is illustrated by the energy “hill” in Figures 6-2 and 6-3. To undergo reaction, the molecules must overcome this barrier and therefore must be raised to a higher energy level. At the top of the energy hill is a point at which decay to the S or P state is equally probable (it is downhill either way). This is called the **transition state**. The transition state is not a chemical species with any significant stability and should not be confused with a reaction intermediate (such as ES or EP). It is simply a fleeting molecular moment in which events such as bond breakage, bond formation, and charge development have proceeded to the precise point at which decay to

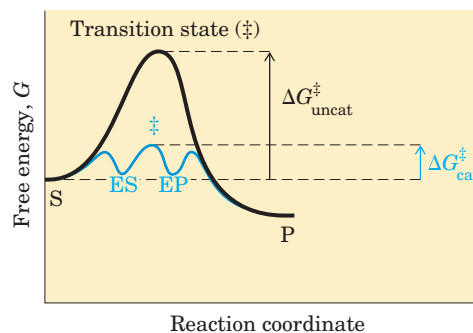
either substrate or product is equally likely. The difference between the energy levels of the ground state and the transition state is the **activation energy**,  $\Delta G^\ddagger$ . The rate of a reaction reflects this activation energy: a higher activation energy corresponds to a slower reaction. Reaction rates can be increased by raising the temperature, thereby increasing the number of molecules with sufficient energy to overcome the energy barrier. Alternatively, the activation energy can be lowered by adding a catalyst (Fig. 6-3). *Catalysts enhance reaction rates by lowering activation energies.*

Enzymes are no exception to the rule that catalysts do not affect reaction equilibria. The bidirectional arrows in Equation 6-1 make this point: any enzyme that catalyzes the reaction  $S \rightarrow P$  also catalyzes the reaction  $P \rightarrow S$ . The role of enzymes is to *accelerate* the interconversion of S and P. The enzyme is not used up in the process, and the equilibrium point is unaffected. However, the reaction reaches equilibrium much faster when the appropriate enzyme is present, because the rate of the reaction is increased.

This general principle can be illustrated by considering the conversion of sucrose and oxygen to carbon dioxide and water:



This conversion, which takes place through a series of separate reactions, has a very large and negative  $\Delta G'^\circ$ , and at equilibrium the amount of sucrose present is negligible. Yet sucrose is a stable compound, because the activation energy barrier that must be overcome before sucrose reacts with oxygen is quite high. Sucrose can be stored in a container with oxygen almost indefinitely without reacting. In cells, however, sucrose is readily broken down to  $CO_2$  and  $H_2O$  in a series of reactions catalyzed by enzymes. These enzymes not only accel-



**FIGURE 6-3** Reaction coordinate diagram comparing enzyme-catalyzed and uncatalyzed reactions.

In the reaction  $S \rightarrow P$ , the ES and EP intermediates occupy minima in the energy progress curve of the enzyme-catalyzed reaction. The terms  $\Delta G_{\text{uncat}}^\ddagger$  and  $\Delta G_{\text{cat}}^\ddagger$  correspond to the activation energy for the uncatalyzed reaction and the overall activation energy for the catalyzed reaction, respectively. The activation energy is lower when the enzyme catalyzes the reaction.

erate the reactions, they organize and control them so that much of the energy released is recovered in other chemical forms and made available to the cell for other tasks. The reaction pathway by which sucrose (and other sugars) is broken down is the primary energy-yielding pathway for cells, and the enzymes of this pathway allow the reaction sequence to proceed on a biologically useful time scale.

Any reaction may have several steps, involving the formation and decay of transient chemical species called **reaction intermediates**.<sup>\*</sup> A reaction intermediate is any species on the reaction pathway that has a finite chemical lifetime (longer than a molecular vibration,  $\sim 10^{-13}$  seconds). When the  $S \rightleftharpoons P$  reaction is catalyzed by an enzyme, the ES and EP complexes can be considered intermediates, even though S and P are stable chemical species (Eqn 6-1); the ES and EP complexes occupy valleys in the reaction coordinate diagram (Fig. 6-3). Additional, less stable chemical intermediates often exist in the course of an enzyme-catalyzed reaction. The interconversion of two sequential reaction intermediates thus constitutes a reaction step. When several steps occur in a reaction, the overall rate is determined by the step (or steps) with the highest activation energy; this is called the **rate-limiting step**. In a simple case, the rate-limiting step is the highest-energy point in the diagram for interconversion of S and P. In practice, the rate-limiting step can vary with reaction conditions, and for many enzymes several steps may have similar activation energies, which means they are all partially rate-limiting.

Activation energies are energy barriers to chemical reactions. These barriers are crucial to life itself. The rate at which a molecule undergoes a particular reaction decreases as the activation barrier for that reaction increases. Without such energy barriers, complex macromolecules would revert spontaneously to much simpler molecular forms, and the complex and highly ordered structures and metabolic processes of cells could not exist. Over the course of evolution, enzymes have developed lower activation energies *selectively* for reactions that are needed for cell survival.

### Reaction Rates and Equilibria Have Precise Thermodynamic Definitions

Reaction *equilibria* are inextricably linked to the standard free-energy change for the reaction,  $\Delta G'^{\circ}$ , and re-

action *rates* are linked to the activation energy,  $\Delta G^{\ddagger}$ . A basic introduction to these thermodynamic relationships is the next step in understanding how enzymes work.

An equilibrium such as  $S \rightleftharpoons P$  is described by an **equilibrium constant**,  $K'_{\text{eq}}$ , or simply  $K$  (p. 26). Under the standard conditions used to compare biochemical processes, an equilibrium constant is denoted  $K'_{\text{eq}}$  (or  $K'$ ):

$$K'_{\text{eq}} = \frac{[P]}{[S]} \quad (6-2)$$

From thermodynamics, the relationship between  $K'_{\text{eq}}$  and  $\Delta G'^{\circ}$  can be described by the expression

$$\Delta G'^{\circ} = -RT \ln K'_{\text{eq}} \quad (6-3)$$

where  $R$  is the gas constant,  $8.315 \text{ J/mol} \cdot \text{K}$ , and  $T$  is the absolute temperature,  $298 \text{ K}$  ( $25^{\circ}\text{C}$ ). Equation 6-3 is developed and discussed in more detail in Chapter 13. The important point here is that the equilibrium constant is directly related to the overall standard free-energy change for the reaction (Table 6-4). A large negative value for  $\Delta G'^{\circ}$  reflects a favorable reaction equilibrium—but as already noted, this does not mean the reaction will proceed at a rapid rate.

The rate of any reaction is determined by the concentration of the reactant (or reactants) and by a **rate constant**, usually denoted by  $k$ . For the unimolecular reaction  $S \rightarrow P$ , the rate (or velocity) of the reaction,  $V$ —representing the amount of S that reacts per unit time—is expressed by a **rate equation**:

$$V = k[S] \quad (6-4)$$

In this reaction, the rate depends only on the concentration of S. This is called a first-order reaction. The factor  $k$  is a proportionality constant that reflects the probability of reaction under a given set of conditions (pH, temperature, and so forth). Here,  $k$  is a first-order rate constant and has units of reciprocal time, such as  $\text{s}^{-1}$ . If a first-order reaction has a rate constant  $k$  of  $0.03 \text{ s}^{-1}$ ,

**TABLE 6-4** Relationship between  $K'_{\text{eq}}$  and  $\Delta G'^{\circ}$

$K'_{\text{eq}}$	$\Delta G'^{\circ}$ (kJ/mol)
$10^{-6}$	34.2
$10^{-5}$	28.5
$10^{-4}$	22.8
$10^{-3}$	17.1
$10^{-2}$	11.4
$10^{-1}$	5.7
1	0.0
$10^1$	-5.7
$10^2$	-11.4
$10^3$	-17.1

Note: The relationship is calculated from  $\Delta G'^{\circ} = -RT \ln K'_{\text{eq}}$  (Eqn 6-3).

<sup>\*</sup>In this chapter, *step* and *intermediate* refer to chemical species in the reaction pathway of a single enzyme-catalyzed reaction. In the context of metabolic pathways involving many enzymes (discussed in Part II), these terms are used somewhat differently. An entire enzymatic reaction is often referred to as a “step” in a pathway, and the product of one enzymatic reaction (which is the substrate for the next enzyme in the pathway) is referred to as an “intermediate.”

this may be interpreted (qualitatively) to mean that 3% of the available S will be converted to P in 1 s. A reaction with a rate constant of  $2,000 \text{ s}^{-1}$  will be over in a small fraction of a second. If a reaction rate depends on the concentration of two different compounds, or if the reaction is between two molecules of the same compound, the reaction is second order and  $k$  is a second-order rate constant, with units of  $\text{M}^{-1}\text{s}^{-1}$ . The rate equation then becomes

$$V = k[\text{S}_1][\text{S}_2] \quad (6-5)$$

From transition-state theory we can derive an expression that relates the magnitude of a rate constant to the activation energy:

$$k = \frac{kT}{h} e^{-\Delta G^\ddagger/RT} \quad (6-6)$$

where  $k$  is the Boltzmann constant and  $h$  is Planck's constant. The important point here is that the relationship between the rate constant  $k$  and the activation energy  $\Delta G^\ddagger$  is inverse and exponential. In simplified terms, this is the basis for the statement that a lower activation energy means a faster reaction rate.

Now we turn from *what* enzymes do to *how* they do it.

### A Few Principles Explain the Catalytic Power and Specificity of Enzymes

Enzymes are extraordinary catalysts. The rate enhancements they bring about are in the range of 5 to 17 orders of magnitude (Table 6-5). Enzymes are also very specific, readily discriminating between substrates with quite similar structures. How can these enormous and highly selective rate enhancements be explained? What is the source of the energy for the dramatic lowering of the activation energies for specific reactions?

The answer to these questions has two distinct but interwoven parts. The first lies in the rearrangements of covalent bonds during an enzyme-catalyzed reaction. Chemical reactions of many types take place between substrates and enzymes' functional groups (specific

amino acid side chains, metal ions, and coenzymes). Catalytic functional groups on an enzyme may form a transient covalent bond with a substrate and activate it for reaction, or a group may be transiently transferred from the substrate to the enzyme. In many cases, these reactions occur only in the enzyme active site. Covalent interactions between enzymes and substrates lower the activation energy (and thereby accelerate the reaction) by providing an alternative, lower-energy reaction path. The specific types of rearrangements that occur are described in Section 6.4.

The second part of the explanation lies in the *non-covalent* interactions between enzyme and substrate. Much of the energy required to lower activation energies is derived from weak, noncovalent interactions between substrate and enzyme. What really sets enzymes apart from most other catalysts is the formation of a specific ES complex. The interaction between substrate and enzyme in this complex is mediated by the same forces that stabilize protein structure, including hydrogen bonds and hydrophobic and ionic interactions (Chapter 4). Formation of each weak interaction in the ES complex is accompanied by release of a small amount of free energy that provides a degree of stability to the interaction. The energy derived from enzyme-substrate interaction is called **binding energy,  $\Delta G_B$** . Its significance extends beyond a simple stabilization of the enzyme-substrate interaction. *Binding energy is a major source of free energy used by enzymes to lower the activation energies of reactions.*

Two fundamental and interrelated principles provide a general explanation for how enzymes use noncovalent binding energy:

1. Much of the catalytic power of enzymes is ultimately derived from the free energy released in forming many weak bonds and interactions between an enzyme and its substrate. This binding energy contributes to specificity as well as to catalysis.
2. Weak interactions are optimized in the reaction transition state; enzyme active sites are complementary not to the substrates per se but to the transition states through which substrates pass as they are converted to products during an enzymatic reaction.

These themes are critical to an understanding of enzymes, and they now become our primary focus.

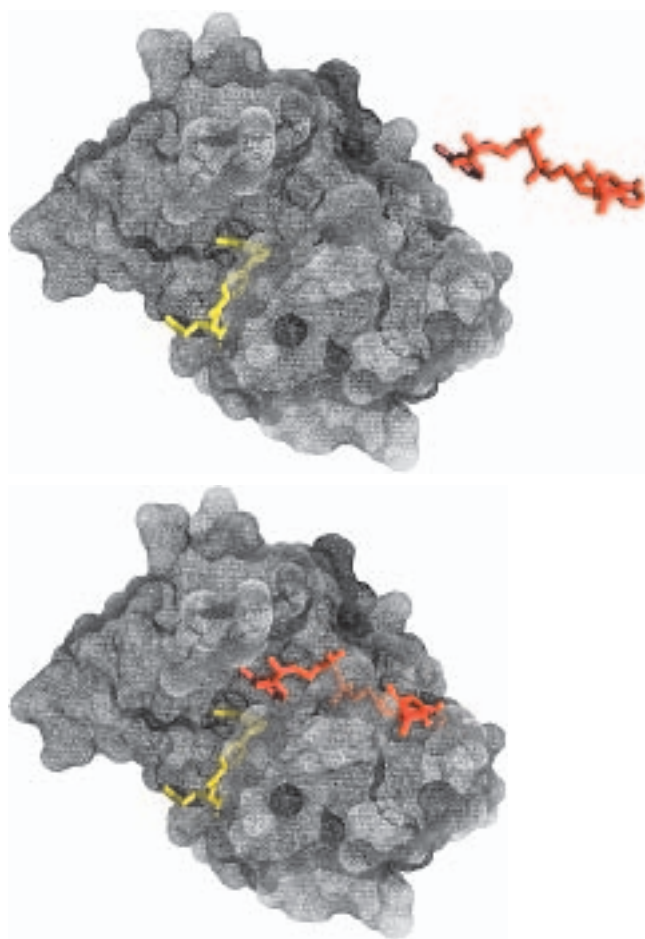
### Weak Interactions between Enzyme and Substrate Are Optimized in the Transition State

How does an enzyme use binding energy to lower the activation energy for a reaction? Formation of the ES complex is not the explanation in itself, although some

**TABLE 6-5** Some Rate Enhancements Produced by Enzymes

Cyclophilin	$10^5$
Carbonic anhydrase	$10^7$
Triose phosphate isomerase	$10^9$
Carboxypeptidase A	$10^{11}$
Phosphoglucosmutase	$10^{12}$
Succinyl-CoA transferase	$10^{13}$
Urease	$10^{14}$
Orotidine monophosphate decarboxylase	$10^{17}$

of the earliest considerations of enzyme mechanisms began with this idea. Studies on enzyme specificity carried out by Emil Fischer led him to propose, in 1894, that enzymes were structurally complementary to their substrates, so that they fit together like a lock and key (Fig. 6-4). This elegant idea, that a specific (exclusive) interaction between two biological molecules is mediated by molecular surfaces with complementary shapes, has greatly influenced the development of biochemistry, and such interactions lie at the heart of many biochemical processes. However, the “lock and key” hypothesis can be misleading when applied to enzymatic catalysis. An enzyme completely complementary to its substrate would be a very poor enzyme, as we can demonstrate.



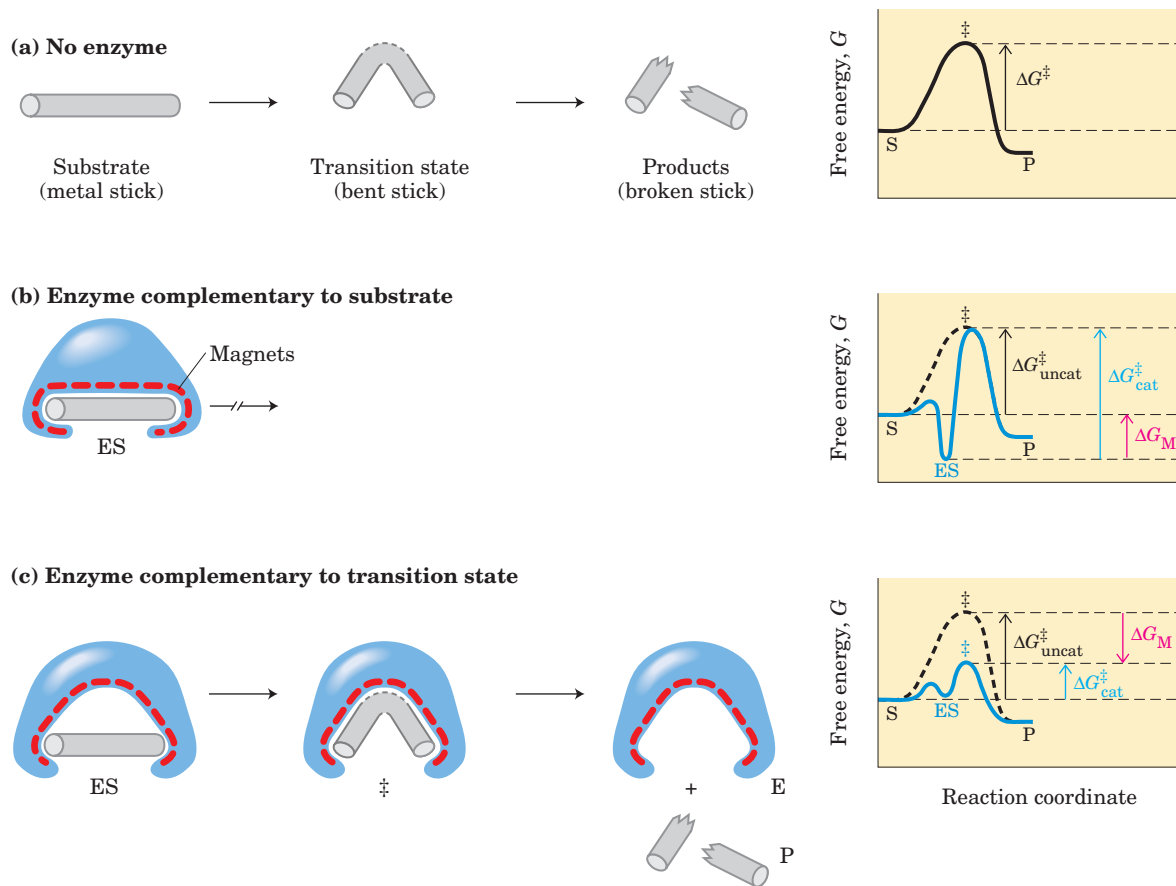
**FIGURE 6-4** Complementary shapes of a substrate and its binding site on an enzyme. The enzyme dihydrofolate reductase with its substrate  $\text{NADP}^+$  (red), unbound (top) and bound (bottom). Another bound substrate, tetrahydrofolate (yellow), is also visible (PDB ID 1RA2). The  $\text{NADP}^+$  binds to a pocket that is complementary to it in shape and ionic properties. In reality, the complementarity between protein and ligand (in this case substrate) is rarely perfect, as we saw in Chapter 5. The interaction of a protein with a ligand often involves changes in the conformation of one or both molecules, a process called induced fit. This *lack* of perfect complementarity between enzyme and substrate (not evident in this figure) is important to enzymatic catalysis.

Consider an imaginary reaction, the breaking of a magnetized metal stick. The uncatalyzed reaction is shown in Figure 6-5a. Let's examine two imaginary enzymes—two “stickases”—that could catalyze this reaction, both of which employ magnetic forces as a paradigm for the binding energy used by real enzymes. We first design an enzyme perfectly complementary to the substrate (Fig. 6-5b). The active site of this stickase is a pocket lined with magnets. To react (break), the stick must reach the transition state of the reaction, but the stick fits so tightly in the active site that it cannot bend, because bending would eliminate some of the magnetic interactions between stick and enzyme. Such an enzyme *impedes* the reaction, stabilizing the substrate instead. In a reaction coordinate diagram (Fig. 6-5b), this kind of ES complex would correspond to an energy trough from which the substrate would have difficulty escaping. Such an enzyme would be useless.

The modern notion of enzymatic catalysis, first proposed by Michael Polanyi (1921) and Haldane (1930), was elaborated by Linus Pauling in 1946: in order to catalyze reactions, an enzyme must be complementary to the *reaction transition state*. This means that optimal interactions between substrate and enzyme occur only in the transition state. Figure 6-5c demonstrates how such an enzyme can work. The metal stick binds to the stickase, but only a subset of the possible magnetic interactions are used in forming the ES complex. The bound substrate must still undergo the increase in free energy needed to reach the transition state. Now, however, the increase in free energy required to draw the stick into a bent and partially broken conformation is offset, or “paid for,” by the magnetic interactions (binding energy) that form between the enzyme and substrate in the transition state. Many of these interactions involve parts of the stick that are distant from the point of breakage; thus interactions between the stickase and nonreacting parts of the stick provide some of the energy needed to catalyze stick breakage. This “energy payment” translates into a lower net activation energy and a faster reaction rate.

Real enzymes work on an analogous principle. Some weak interactions are formed in the ES complex, but the full complement of such interactions between substrate and enzyme is formed only when the substrate reaches the transition state. The free energy (binding energy) released by the formation of these interactions partially offsets the energy required to reach the top of the energy hill. The summation of the unfavorable (positive) activation energy  $\Delta G^\ddagger$  and the favorable (negative) binding energy  $\Delta G_B$  results in a lower *net* activation energy (Fig. 6-6). Even on the enzyme, the transition state is not a stable species but a brief point in time that the substrate spends atop an energy hill. The enzyme-catalyzed reaction is much faster than the uncatalyzed process, however, because the hill is much smaller. The





**FIGURE 6-5** An imaginary enzyme (stickase) designed to catalyze breakage of a metal stick. (a) Before the stick is broken, it must first be bent (the transition state). In both stickase examples, magnetic interactions take the place of weak bonding interactions between enzyme and substrate. (b) A stickase with a magnet-lined pocket complementary in structure to the stick (the substrate) stabilizes the substrate. Bending is impeded by the magnetic attraction between stick and stickase. (c) An enzyme with a pocket complementary to the reaction transition state helps to destabilize the stick, contributing to catalysis of the reaction. The binding energy of the magnetic interactions

compensates for the increase in free energy required to bend the stick. Reaction coordinate diagrams (right) show the energy consequences of complementarity to substrate versus complementarity to transition state (EP complexes are omitted).  $\Delta G_M$ , the difference between the transition-state energies of the uncatalyzed and catalyzed reactions, is contributed by the magnetic interactions between the stick and stickase. When the enzyme is complementary to the substrate (b), the ES complex is more stable and has less free energy in the ground state than substrate alone. The result is an *increase* in the activation energy.

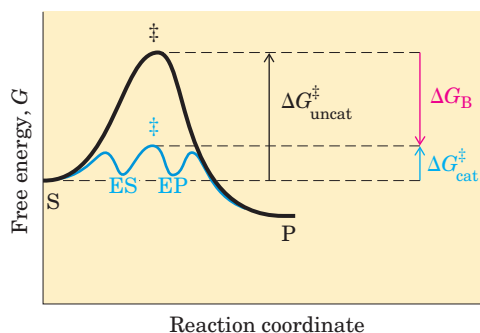
important principle is that *weak binding interactions between the enzyme and the substrate provide a substantial driving force for enzymatic catalysis*. The groups on the substrate that are involved in these weak interactions can be at some distance from the bonds that are broken or changed. The weak interactions formed only in the transition state are those that make the primary contribution to catalysis.

The requirement for multiple weak interactions to drive catalysis is one reason why enzymes (and some coenzymes) are so large. An enzyme must provide functional groups for ionic, hydrogen-bond, and other interactions, and also must precisely position these groups so that binding energy is optimized in the transition state. Adequate binding is accomplished most readily by positioning a substrate in a cavity (the active site) where it is effectively removed from water. The size of proteins

reflects the need for superstructure to keep interacting groups properly positioned and to keep the cavity from collapsing.

### Binding Energy Contributes to Reaction Specificity and Catalysis

Can we demonstrate quantitatively that binding energy accounts for the huge rate accelerations brought about by enzymes? Yes. As a point of reference, Equation 6-6 allows us to calculate that  $\Delta G^\ddagger$  must be lowered by about 5.7 kJ/mol to accelerate a first-order reaction by a factor of ten, under conditions commonly found in cells. The energy available from formation of a single weak interaction is generally estimated to be 4 to 30 kJ/mol. The overall energy available from a number of such interactions is therefore sufficient to lower activation en-

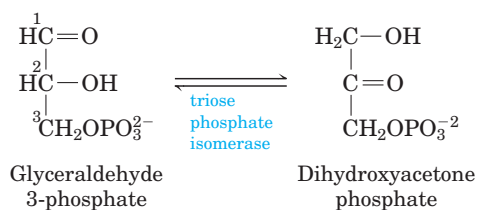


**FIGURE 6-6** Role of binding energy in catalysis. To lower the activation energy for a reaction, the system must acquire an amount of energy equivalent to the amount by which  $\Delta G^\ddagger$  is lowered. Much of this energy comes from binding energy ( $\Delta G_B$ ) contributed by formation of weak noncovalent interactions between substrate and enzyme in the transition state. The role of  $\Delta G_B$  is analogous to that of  $\Delta G_M$  in Figure 6-5.

ergies by the 60 to 100 kJ/mol required to explain the large rate enhancements observed for many enzymes.

The same binding energy that provides energy for catalysis also gives an enzyme its **specificity**, the ability to discriminate between a substrate and a competing molecule. Conceptually, specificity is easy to distinguish from catalysis, but this distinction is much more difficult to make experimentally, because catalysis and specificity arise from the same phenomenon. If an enzyme active site has functional groups arranged optimally to form a variety of weak interactions with a particular substrate in the transition state, the enzyme will not be able to interact to the same degree with any other molecule. For example, if the substrate has a hydroxyl group that forms a hydrogen bond with a specific Glu residue on the enzyme, any molecule lacking a hydroxyl group at that particular position will be a poorer substrate for the enzyme. In addition, any molecule with an extra functional group for which the enzyme has no pocket or binding site is likely to be excluded from the enzyme. In general, *specificity* is derived from the formation of many weak interactions between the enzyme and its specific substrate molecule.

The importance of binding energy to catalysis can be readily demonstrated. For example, the glycolytic enzyme triose phosphate isomerase catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate:



This reaction rearranges the carbonyl and hydroxyl groups on carbons 1 and 2. However, more than 80% of the enzymatic rate acceleration has been traced to enzyme-substrate interactions involving the phosphate group on carbon 3 of the substrate. This was determined by a careful comparison of the enzyme-catalyzed reactions with glyceraldehyde 3-phosphate and with glyceraldehyde (no phosphate group at position 3) as substrate.

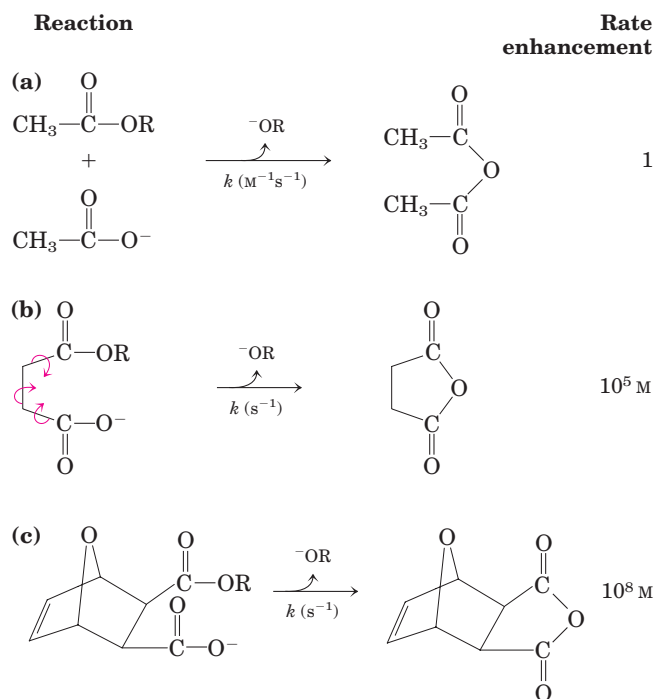
The general principles outlined above can be illustrated by a variety of recognized catalytic mechanisms. These mechanisms are not mutually exclusive, and a given enzyme might incorporate several types in its overall mechanism of action. For most enzymes, it is difficult to quantify the contribution of any one catalytic mechanism to the rate and/or specificity of a particular enzyme-catalyzed reaction.

As we have noted, binding energy makes an important, and sometimes the dominant, contribution to catalysis. Consider what needs to occur for a reaction to take place. Prominent physical and thermodynamic factors contributing to  $\Delta G^\ddagger$ , the barrier to reaction, might include (1) a reduction in entropy, in the form of decreased freedom of motion of two molecules in solution; (2) the solvation shell of hydrogen-bonded water that surrounds and helps to stabilize most biomolecules in aqueous solution; (3) the distortion of substrates that must occur in many reactions; and (4) the need for proper alignment of catalytic functional groups on the enzyme. Binding energy can be used to overcome all these barriers.

First, a large restriction in the relative motions of two substrates that are to react, or **entropy reduction**, is one obvious benefit of binding them to an enzyme. Binding energy holds the substrates in the proper orientation to react—a substantial contribution to catalysis, because productive collisions between molecules in solution can be exceedingly rare. Substrates can be precisely aligned on the enzyme, with many weak interactions between each substrate and strategically located groups on the enzyme clamping the substrate molecules into the proper positions. Studies have shown that constraining the motion of two reactants can produce rate enhancements of many orders of magnitude (Fig. 6-7).

Second, formation of weak bonds between substrate and enzyme also results in **desolvation** of the substrate. Enzyme-substrate interactions replace most or all of the hydrogen bonds between the substrate and water. Third, binding energy involving weak interactions formed only in the reaction transition state helps to compensate thermodynamically for any distortion, primarily electron redistribution, that the substrate must undergo to react.

Finally, the enzyme itself usually undergoes a change in conformation when the substrate binds, induced by multiple weak interactions with the substrate.



**FIGURE 6-7 Rate enhancement by entropy reduction.** Shown here are reactions of an ester with a carboxylate group to form an anhydride. The R group is the same in each case. (a) For this bimolecular reaction, the rate constant  $k$  is second order, with units of  $\text{M}^{-1}\text{s}^{-1}$ . (b) When the two reacting groups are in a single molecule, the reaction is much faster. For this unimolecular reaction,  $k$  has units of  $\text{s}^{-1}$ . Dividing the rate constant for (b) by the rate constant for (a) gives a rate enhancement of about  $10^5 \text{ M}$ . (The enhancement has units of molarity because we are comparing a unimolecular and a bimolecular reaction.) Put another way, if the reactant in (b) were present at a concentration of  $1 \text{ M}$ , the reacting groups would behave as though they were present at a concentration of  $10^5 \text{ M}$ . Note that the reactant in (b) has freedom of rotation about three bonds (shown with curved arrows), but this still represents a substantial reduction of entropy over (a). If the bonds that rotate in (b) are constrained as in (c), the entropy is reduced further and the reaction exhibits a rate enhancement of  $10^8 \text{ M}$  relative to (a).

This is referred to as **induced fit**, a mechanism postulated by Daniel Koshland in 1958. Induced fit serves to bring specific functional groups on the enzyme into the proper position to catalyze the reaction. The conformational change also permits formation of additional weak bonding interactions in the transition state. In either case, the new enzyme conformation has enhanced catalytic properties. As we have seen, induced fit is a common feature of the reversible binding of ligands to proteins (Chapter 5). Induced fit is also important in the interaction of almost every enzyme with its substrate.

### Specific Catalytic Groups Contribute to Catalysis

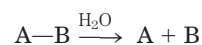
In most enzymes, the binding energy used to form the ES complex is just one of several contributors to the

overall catalytic mechanism. Once a substrate is bound to an enzyme, properly positioned catalytic functional groups aid in the cleavage and formation of bonds by a variety of mechanisms, including general acid-base catalysis, covalent catalysis, and metal ion catalysis. These are distinct from mechanisms based on binding energy, because they generally involve transient *covalent* interaction with a substrate or group transfer to or from a substrate.

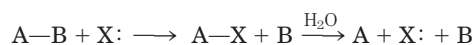
**General Acid-Base Catalysis** Many biochemical reactions involve the formation of unstable charged intermediates that tend to break down rapidly to their constituent reactant species, thus impeding the reaction (Fig. 6–8). Charged intermediates can often be stabilized by the transfer of protons to or from the substrate or intermediate to form a species that breaks down more readily to products. For nonenzymatic reactions, the proton transfers can involve either the constituents of water alone or other weak proton donors or acceptors. Catalysis of this type that uses only the  $\text{H}^+$  ( $\text{H}_3\text{O}^+$ ) or  $\text{OH}^-$  ions present in water is referred to as **specific acid-base catalysis**. If protons are transferred between the intermediate and water faster than the intermediate breaks down to reactants, the intermediate is effectively stabilized every time it forms. No additional catalysis mediated by other proton acceptors or donors will occur. In many cases, however, water is not enough. The term **general acid-base catalysis** refers to proton transfers mediated by other classes of molecules. For nonenzymatic reactions in aqueous solutions, this occurs only when the unstable reaction intermediate breaks down to reactants faster than protons can be transferred to or from water. Many weak organic acids can supplement water as proton donors in this situation, or weak organic bases can serve as proton acceptors.

In the active site of an enzyme, a number of amino acid side chains can similarly act as proton donors and acceptors (Fig. 6–9). These groups can be precisely positioned in an enzyme active site to allow proton transfers, providing rate enhancements of the order of  $10^2$  to  $10^5$ . This type of catalysis occurs on the vast majority of enzymes. In fact, proton transfers are the most common biochemical reactions.

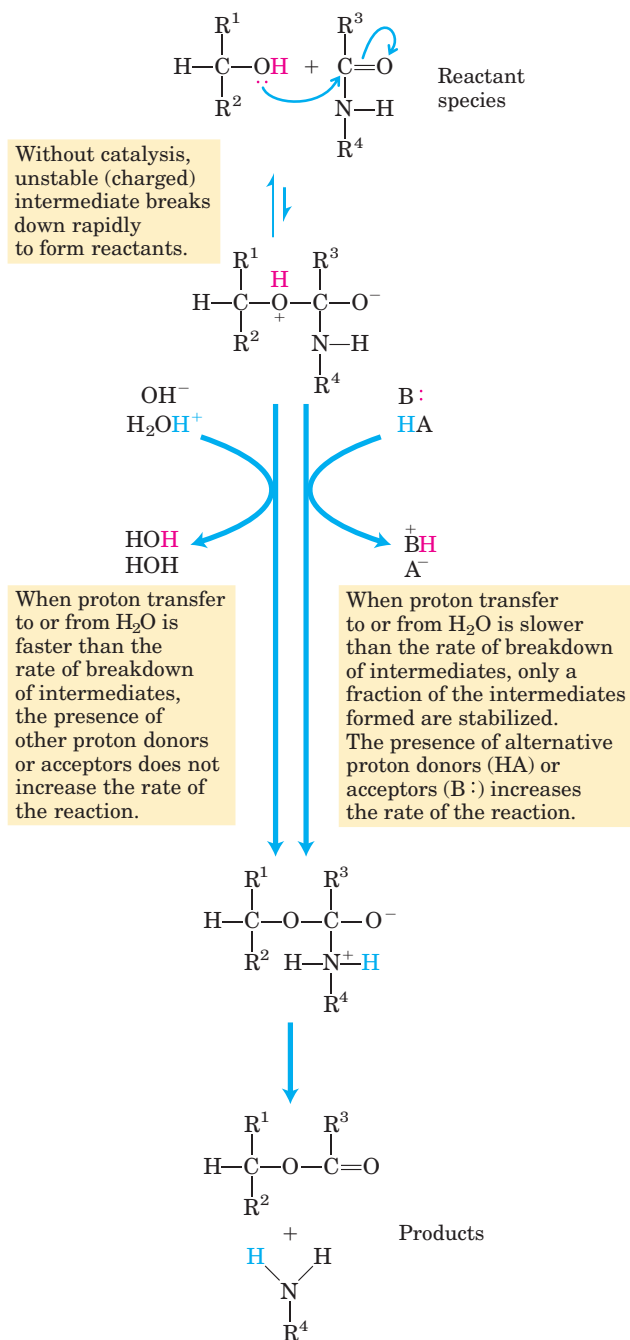
**Covalent Catalysis** In covalent catalysis, a transient covalent bond is formed between the enzyme and the substrate. Consider the hydrolysis of a bond between groups A and B:



In the presence of a covalent catalyst (an enzyme with a nucleophilic group X:) the reaction becomes



This alters the pathway of the reaction, and it results in catalysis only when the new pathway has a lower activation energy than the uncatalyzed pathway. Both of the new steps must be faster than the uncatalyzed



**FIGURE 6-8** How a catalyst circumvents unfavorable charge development during cleavage of an amide. The hydrolysis of an amide bond, shown here, is the same reaction as that catalyzed by chymotrypsin and other proteases. Charge development is unfavorable and can be circumvented by donation of a proton by  $H_3O^+$  (specific acid catalysis) or HA (general acid catalysis), where HA represents any acid. Similarly, charge can be neutralized by proton abstraction by  $OH^-$  (specific base catalysis) or  $B:$  (general base catalysis), where  $B:$  represents any base.

reaction. A number of amino acid side chains, including all those in Figure 6-9, and the functional groups of some enzyme cofactors can serve as nucleophiles in the formation of covalent bonds with substrates. These covalent complexes always undergo further reaction to regenerate the free enzyme. The covalent bond formed between the enzyme and the substrate can activate a substrate for further reaction in a manner that is usually specific to the particular group or coenzyme.

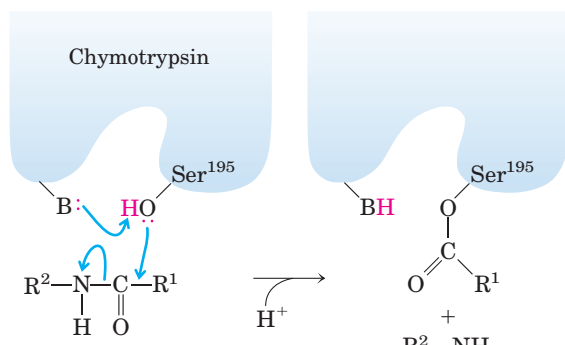
**Metal Ion Catalysis** Metals, whether tightly bound to the enzyme or taken up from solution along with the substrate, can participate in catalysis in several ways. Ionic interactions between an enzyme-bound metal and a substrate can help orient the substrate for reaction or stabilize charged reaction transition states. This use of weak bonding interactions between metal and substrate is similar to some of the uses of enzyme-substrate binding energy described earlier. Metals can also mediate oxidation-reduction reactions by reversible changes in the metal ion's oxidation state. Nearly a third of all known enzymes require one or more metal ions for catalytic activity.

Most enzymes employ a combination of several catalytic strategies to bring about a rate enhancement. A good example of the use of both covalent catalysis and general acid-base catalysis is the reaction catalyzed by chymotrypsin. The first step is cleavage of a peptide bond, which is accompanied by formation of a covalent linkage between a Ser residue on the enzyme and part

Amino acid residues	General acid form (proton donor)	General base form (proton acceptor)
<b>Glu, Asp</b>	$R-COOH$	$R-COO^-$
<b>Lys, Arg</b>	$R-NH_3^+$	$R-NH_2$
<b>Cys</b>	$R-SH$	$R-S^-$
<b>His</b>	$R-NH-C(=O)-NH_2$	$R-NH-C(=O)-NH^-$
<b>Ser</b>	$R-OH$	$R-O^-$
<b>Tyr</b>	$R-OH$	$R-O^-$

**FIGURE 6-9** Amino acids in general acid-base catalysis. Many organic reactions are promoted by proton donors (general acids) or proton acceptors (general bases). The active sites of some enzymes contain amino acid functional groups, such as those shown here, that can participate in the catalytic process as proton donors or proton acceptors.





**FIGURE 6-10** Covalent and general acid-base catalysis. The first step in the reaction catalyzed by chymotrypsin is the acylation step. The hydroxyl group of Ser<sup>195</sup> is the nucleophile in a reaction aided by general base catalysis (the base is the side chain of His<sup>57</sup>). This provides a new pathway for the hydrolytic cleavage of a peptide bond. Catalysis occurs only if each step in the new pathway is faster than the uncatalyzed reaction. The chymotrypsin reaction is described in more detail in Figure 6-21.

of the substrate; the reaction is enhanced by general base catalysis by other groups on the enzyme (Fig. 6-10). The chymotrypsin reaction is described in more detail in Section 6.4.

## SUMMARY 6.2 How Enzymes Work

- Enzymes are highly effective catalysts, commonly enhancing reaction rates by a factor of  $10^5$  to  $10^{17}$ .
- Enzyme-catalyzed reactions are characterized by the formation of a complex between substrate and enzyme (an ES complex). Substrate binding occurs in a pocket on the enzyme called the active site.
- The function of enzymes and other catalysts is to lower the activation energy,  $\Delta G^\ddagger$ , for a reaction and thereby enhance the reaction rate. The equilibrium of a reaction is unaffected by the enzyme.
- A significant part of the energy used for enzymatic rate enhancements is derived from weak interactions (hydrogen bonds and hydrophobic and ionic interactions) between substrate and enzyme. The enzyme active site is structured so that some of these weak interactions occur preferentially in the reaction transition state, thus stabilizing the transition state. The need for multiple interactions is one reason for the large size of enzymes. The binding energy,  $\Delta G_B$ , can be used to lower substrate entropy or to cause a conformational change in the enzyme (induced fit). Binding energy also accounts for the exquisite specificity of enzymes for their substrates.

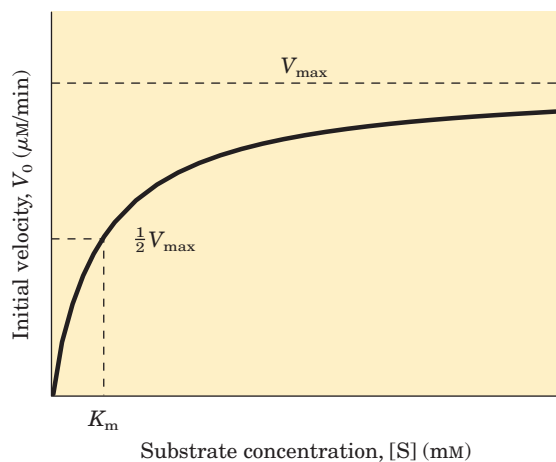
■ Additional catalytic mechanisms employed by enzymes include general acid-base catalysis, covalent catalysis, and metal ion catalysis. Catalysis often involves transient covalent interactions between the substrate and the enzyme, or group transfers to and from the enzyme, so as to provide a new, lower-energy reaction path.

## 6.3 Enzyme Kinetics as an Approach to Understanding Mechanism

Biochemists commonly use several approaches to study the mechanism of action of purified enzymes. A knowledge of the three-dimensional structure of the protein provides important information, and the value of structural information is greatly enhanced by classical protein chemistry and modern methods of site-directed mutagenesis (changing the amino acid sequence of a protein by genetic engineering; see Fig. 9-12). These technologies permit enzymologists to examine the role of individual amino acids in enzyme structure and action. However, the central approach to studying the mechanism of an enzyme-catalyzed reaction is to determine the *rate* of the reaction and how it changes in response to changes in experimental parameters, a discipline known as **enzyme kinetics**. This is the oldest approach to understanding enzyme mechanisms and remains the most important. We provide here a basic introduction to the kinetics of enzyme-catalyzed reactions. More advanced treatments are available in the sources cited at the end of the chapter.

### Substrate Concentration Affects the Rate of Enzyme-Catalyzed Reactions

A key factor affecting the rate of a reaction catalyzed by an enzyme is the concentration of substrate, [S]. However, studying the effects of substrate concentration is complicated by the fact that [S] changes during the course of an *in vitro* reaction as substrate is converted to product. One simplifying approach in kinetics experiments is to measure the **initial rate** (or **initial velocity**), designated  $V_0$ , when [S] is much greater than the concentration of enzyme, [E]. In a typical reaction, the enzyme may be present in nanomolar quantities, whereas [S] may be five or six orders of magnitude higher. If only the beginning of the reaction is monitored (often the first 60 seconds or less), changes in [S] can be limited to a few percent, and [S] can be regarded as constant.  $V_0$  can then be explored as a function of [S], which is adjusted by the investigator. The effect on  $V_0$  of varying [S] when the enzyme concentration is held constant is shown in Figure 6-11. At relatively low concentrations of substrate,  $V_0$  increases almost linearly



**FIGURE 6-11** Effect of substrate concentration on the initial velocity of an enzyme-catalyzed reaction.  $V_{\max}$  is extrapolated from the plot, because  $V_0$  approaches but never quite reaches  $V_{\max}$ . The substrate concentration at which  $V_0$  is half maximal is  $K_m$ , the Michaelis constant. The concentration of enzyme in an experiment such as this is generally so low that  $[S] \gg [E]$  even when  $[S]$  is described as low or relatively low. The units shown are typical for enzyme-catalyzed reactions and are given only to help illustrate the meaning of  $V_0$  and  $[S]$ . (Note that the curve describes *part* of a rectangular hyperbola, with one asymptote at  $V_{\max}$ . If the curve were continued below  $[S] = 0$ , it would approach a vertical asymptote at  $[S] = -K_m$ .)

with an increase in  $[S]$ . At higher substrate concentrations,  $V_0$  increases by smaller and smaller amounts in response to increases in  $[S]$ . Finally, a point is reached beyond which increases in  $V_0$  are vanishingly small as  $[S]$  increases. This plateau-like  $V_0$  region is close to the **maximum velocity,  $V_{\max}$** .

The ES complex is the key to understanding this kinetic behavior, just as it was a starting point for our discussion of catalysis. The kinetic pattern in Figure 6-11 led Victor Henri, following the lead of Wurtz, to propose in 1903 that the combination of an enzyme with its substrate molecule to form an ES complex is a necessary step in enzymatic catalysis. This idea was expanded into a general theory of enzyme action, particularly by Leonor Michaelis and Maud Menten in 1913. They postulated that the enzyme first combines reversibly with

its substrate to form an enzyme-substrate complex in a relatively fast reversible step:



The ES complex then breaks down in a slower second step to yield the free enzyme and the reaction product P:



Because the slower second reaction (Eqn 6-8) must limit the rate of the overall reaction, the overall rate must be proportional to the concentration of the species that reacts in the second step, that is, ES.

At any given instant in an enzyme-catalyzed reaction, the enzyme exists in two forms, the free or uncombined form E and the combined form ES. At low  $[S]$ , most of the enzyme is in the uncombined form E. Here, the rate is proportional to  $[S]$  because the equilibrium of Equation 6-7 is pushed toward formation of more ES as  $[S]$  increases. The maximum initial rate of the catalyzed reaction ( $V_{\max}$ ) is observed when virtually all the enzyme is present as the ES complex and  $[E]$  is vanishingly small. Under these conditions, the enzyme is “saturated” with its substrate, so that further increases in  $[S]$  have no effect on rate. This condition exists when  $[S]$  is sufficiently high that essentially all the free enzyme has been converted to the ES form. After the ES complex breaks down to yield the product P, the enzyme is free to catalyze reaction of another molecule of substrate. The saturation effect is a distinguishing characteristic of enzymatic catalysts and is responsible for the plateau observed in Figure 6-11. The pattern seen in Figure 6-11 is sometimes referred to as **saturation kinetics**.

When the enzyme is first mixed with a large excess of substrate, there is an initial period, the **pre-steady state**, during which the concentration of ES builds up. This period is usually too short to be easily observed, lasting just microseconds. The reaction quickly achieves a **steady state** in which  $[ES]$  (and the concentrations of any other intermediates) remains approximately constant over time. The concept of a steady state was introduced by G. E. Briggs and Haldane in 1925. The measured  $V_0$  generally reflects the steady state, even though  $V_0$  is limited to the early part of the reaction, and analysis of these initial rates is referred to as **steady-state kinetics**.

### The Relationship between Substrate Concentration and Reaction Rate Can Be Expressed Quantitatively

The curve expressing the relationship between  $[S]$  and  $V_0$  (Fig. 6-11) has the same general shape for most enzymes (it approaches a rectangular hyperbola), which can be expressed algebraically by the Michaelis-Menten



Leonor Michaelis,  
1875–1949



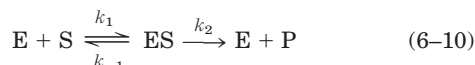
Maud Menten,  
1879–1960

equation. Michaelis and Menten derived this equation starting from their basic hypothesis that the rate-limiting step in enzymatic reactions is the breakdown of the ES complex to product and free enzyme. The equation is

$$V_0 = \frac{V_{\max} [S]}{K_m + [S]} \quad (6-9)$$

The important terms are  $[S]$ ,  $V_0$ ,  $V_{\max}$ , and a constant called the Michaelis constant,  $K_m$ . All these terms are readily measured experimentally.

Here we develop the basic logic and the algebraic steps in a modern derivation of the Michaelis-Menten equation, which includes the steady-state assumption introduced by Briggs and Haldane. The derivation starts with the two basic steps of the formation and breakdown of ES (Eqns 6-7 and 6-8). Early in the reaction, the concentration of the product,  $[P]$ , is negligible, and we make the simplifying assumption that the reverse reaction,  $P \rightarrow S$  (described by  $k_{-2}$ ), can be ignored. This assumption is not critical but it simplifies our task. The overall reaction then reduces to



$V_0$  is determined by the breakdown of ES to form product, which is determined by  $[ES]$ :

$$V_0 = k_2[ES] \quad (6-11)$$

Because  $[ES]$  in Equation 6-11 is not easily measured experimentally, we must begin by finding an alternative expression for this term. First, we introduce the term  $[E_t]$ , representing the total enzyme concentration (the sum of free and substrate-bound enzyme). Free or unbound enzyme can then be represented by  $[E_t] - [ES]$ . Also, because  $[S]$  is ordinarily far greater than  $[E_t]$ , the amount of substrate bound by the enzyme at any given time is negligible compared with the total  $[S]$ . With these conditions in mind, the following steps lead us to an expression for  $V_0$  in terms of easily measurable parameters.

**Step 1** The rates of formation and breakdown of ES are determined by the steps governed by the rate constants  $k_1$  (formation) and  $k_{-1} + k_2$  (breakdown), according to the expressions

$$\text{Rate of ES formation} = k_1([E_t] - [ES])[S] \quad (6-12)$$

$$\text{Rate of ES breakdown} = k_{-1}[ES] + k_2[ES] \quad (6-13)$$

**Step 2** We now make an important assumption: that the initial rate of reaction reflects a steady state in which  $[ES]$  is constant—that is, the rate of formation of ES is equal to the rate of its breakdown. This is called the **steady-state assumption**. The expressions in Equations 6-12 and 6-13 can be equated for the steady state, giving

$$k_1([E_t] - [ES])[S] = k_{-1}[ES] + k_2[ES] \quad (6-14)$$

**Step 3** In a series of algebraic steps, we now solve Equation 6-14 for  $[ES]$ . First, the left side is multiplied out and the right side simplified to give

$$k_1[E_t][S] - k_1[ES][S] = (k_{-1} + k_2)[ES] \quad (6-15)$$

Adding the term  $k_1[ES][S]$  to both sides of the equation and simplifying gives

$$k_1[E_t][S] = (k_1[S] + k_{-1} + k_2)[ES] \quad (6-16)$$

We then solve this equation for  $[ES]$ :

$$[ES] = \frac{k_1[E_t][S]}{k_1[S] + k_{-1} + k_2} \quad (6-17)$$

This can now be simplified further, combining the rate constants into one expression:

$$[ES] = \frac{[E_t][S]}{[S] + (k_2 + k_{-1})/k_1} \quad (6-18)$$

The term  $(k_2 + k_{-1})/k_1$  is defined as the **Michaelis constant**,  $K_m$ . Substituting this into Equation 6-18 simplifies the expression to

$$[ES] = \frac{[E_t][S]}{K_m + [S]} \quad (6-19)$$

**Step 4** We can now express  $V_0$  in terms of  $[ES]$ . Substituting the right side of Equation 6-19 for  $[ES]$  in Equation 6-11 gives

$$V_0 = \frac{k_2[E_t][S]}{K_m + [S]} \quad (6-20)$$

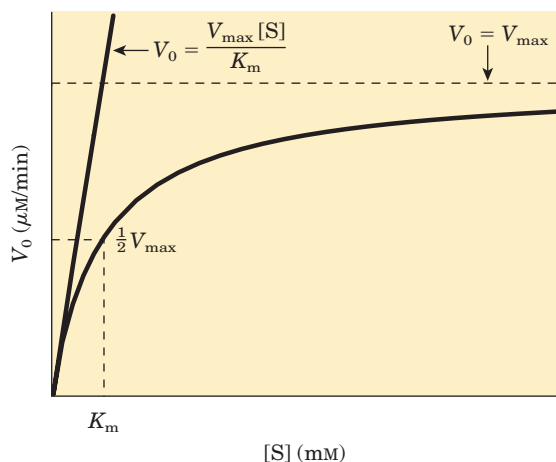
This equation can be further simplified. Because the maximum velocity occurs when the enzyme is saturated (that is, with  $[ES] = [E_t]$ )  $V_{\max}$  can be defined as  $k_2[E_t]$ . Substituting this in Equation 6-20 gives Equation 6-9:

$$V_0 = \frac{V_{\max} [S]}{K_m + [S]}$$

This is the **Michaelis-Menten equation**, the **rate equation** for a one-substrate enzyme-catalyzed reaction. It is a statement of the quantitative relationship between the initial velocity  $V_0$ , the maximum velocity  $V_{\max}$ , and the initial substrate concentration  $[S]$ , all related through the Michaelis constant  $K_m$ . Note that  $K_m$  has units of concentration. Does the equation fit experimental observations? Yes; we can confirm this by considering the limiting situations where  $[S]$  is very high or very low, as shown in Figure 6-12.

An important numerical relationship emerges from the Michaelis-Menten equation in the special case when  $V_0$  is exactly one-half  $V_{\max}$  (Fig. 6-12). Then

$$\frac{V_{\max}}{2} = \frac{V_{\max} [S]}{K_m + [S]} \quad (6-21)$$



**FIGURE 6-12** Dependence of initial velocity on substrate concentration. This graph shows the kinetic parameters that define the limits of the curve at high and low  $[S]$ . At low  $[S]$ ,  $K_m \gg [S]$  and the  $[S]$  term in the denominator of the Michaelis-Menten equation (Eqn 6-9) becomes insignificant. The equation simplifies to  $V_0 = V_{\max}[S]/K_m$  and  $V_0$  exhibits a linear dependence on  $[S]$ , as observed here. At high  $[S]$ , where  $[S] \gg K_m$ , the  $K_m$  term in the denominator of the Michaelis-Menten equation becomes insignificant and the equation simplifies to  $V_0 = V_{\max}$ ; this is consistent with the plateau observed at high  $[S]$ . The Michaelis-Menten equation is therefore consistent with the observed dependence of  $V_0$  on  $[S]$ , and the shape of the curve is defined by the terms  $V_{\max}/K_m$  at low  $[S]$  and  $V_{\max}$  at high  $[S]$ .

On dividing by  $V_{\max}$ , we obtain

$$\frac{1}{2} = \frac{[S]}{K_m + [S]} \quad (6-22)$$

Solving for  $K_m$ , we get  $K_m + [S] = 2[S]$ , or

$$K_m = [S], \text{ when } V_0 = \frac{1}{2}V_{\max} \quad (6-23)$$

This is a very useful, practical definition of  $K_m$ :  $K_m$  is equivalent to the substrate concentration at which  $V_0$  is one-half  $V_{\max}$ .

The Michaelis-Menten equation (Eqn 6-9) can be algebraically transformed into versions that are useful in the practical determination of  $K_m$  and  $V_{\max}$  (Box 6-1) and, as we describe later, in the analysis of inhibitor action (see Box 6-2 on page 210).

### Kinetic Parameters Are Used to Compare Enzyme Activities

It is important to distinguish between the Michaelis-Menten equation and the specific kinetic mechanism on which it was originally based. The equation describes the kinetic behavior of a great many enzymes, and all enzymes that exhibit a hyperbolic dependence of  $V_0$  on  $[S]$  are said to follow **Michaelis-Menten kinetics**. The practical rule that

$K_m = [S]$  when  $V_0 = \frac{1}{2}V_{\max}$  (Eqn 6-23) holds for all enzymes that follow Michaelis-Menten kinetics. (The most important exceptions to Michaelis-Menten kinetics are the regulatory enzymes, discussed in Section 6.5.) However, the Michaelis-Menten equation does not depend on the relatively simple two-step reaction mechanism proposed by Michaelis and Menten (Eqn 6-10). Many enzymes that follow Michaelis-Menten kinetics have quite different reaction mechanisms, and enzymes that catalyze reactions with six or eight identifiable steps often exhibit the same steady-state kinetic behavior. Even though Equation 6-23 holds true for many enzymes, both the magnitude and the real meaning of  $V_{\max}$  and  $K_m$  can differ from one enzyme to the next. This is an important limitation of the steady-state approach to enzyme kinetics. The parameters  $V_{\max}$  and  $K_m$  can be obtained experimentally for any given enzyme, but by themselves they provide little information about the number, rates, or chemical nature of discrete steps in the reaction. Steady-state kinetics nevertheless is the standard language by which biochemists compare and characterize the catalytic efficiencies of enzymes.

**Interpreting  $V_{\max}$  and  $K_m$**  Figure 6-12 shows a simple graphical method for obtaining an approximate value for  $K_m$ . A more convenient procedure, using a **double-reciprocal plot**, is presented in Box 6-1. The  $K_m$  can vary greatly from enzyme to enzyme, and even for different substrates of the same enzyme (Table 6-6). The term is sometimes used (often inappropriately) as an indicator of the affinity of an enzyme for its substrate. The actual meaning of  $K_m$  depends on specific aspects of the reaction mechanism such as the number and relative rates of the individual steps. For reactions with two steps,

$$K_m = \frac{k_2 + k_{-1}}{k_1} \quad (6-24)$$

When  $k_2$  is rate-limiting,  $k_2 \ll k_{-1}$  and  $K_m$  reduces to  $k_{-1}/k_1$ , which is defined as the **dissociation constant,  $K_d$** , of the ES complex. Where these conditions hold,  $K_m$  does represent a measure of the affinity of the enzyme

**TABLE 6-6**  $K_m$  for Some Enzymes and Substrates

Enzyme	Substrate	$K_m$ (mM)
Hexokinase (brain)	ATP	0.4
	D-Glucose	0.05
	D-Fructose	1.5
Carbonic anhydrase	$\text{HCO}_3^-$	26
Chymotrypsin	Glycyltyrosylglycine	108
	N-Benzoyltyrosinamide	2.5
$\beta$ -Galactosidase	D-Lactose	4.0
Threonine dehydratase	L-Threonine	5.0



## BOX 6-1 WORKING IN BIOCHEMISTRY

### Transformations of the Michaelis-Menten Equation: The Double-Reciprocal Plot

The Michaelis-Menten equation

$$V_0 = \frac{V_{\max} [S]}{K_m + [S]}$$

can be algebraically transformed into equations that are more useful in plotting experimental data. One common transformation is derived simply by taking the reciprocal of both sides of the Michaelis-Menten equation:

$$\frac{1}{V_0} = \frac{K_m + [S]}{V_{\max} [S]}$$

Separating the components of the numerator on the right side of the equation gives

$$\frac{1}{V_0} = \frac{K_m}{V_{\max} [S]} + \frac{[S]}{V_{\max} [S]}$$

which simplifies to

$$\frac{1}{V_0} = \frac{K_m}{V_{\max} [S]} + \frac{1}{V_{\max}}$$

This form of the Michaelis-Menten equation is called the **Lineweaver-Burk equation**. For enzymes obeying the Michaelis-Menten relationship, a plot of  $1/V_0$  versus  $1/[S]$  (the “double reciprocal” of the  $V_0$  versus  $[S]$  plot we have been using to this point) yields a straight line (Fig. 1). This line has a slope of  $K_m/V_{\max}$ , an intercept of  $1/V_{\max}$  on the  $1/V_0$  axis, and an intercept

of  $-1/K_m$  on the  $1/[S]$  axis. The double-reciprocal presentation, also called a Lineweaver-Burk plot, has the great advantage of allowing a more accurate determination of  $V_{\max}$ , which can only be *approximated* from a simple plot of  $V_0$  versus  $[S]$  (see Fig. 6-12).

Other transformations of the Michaelis-Menten equation have been derived, each with some particular advantage in analyzing enzyme kinetic data. (See Problem 11 at the end of this chapter.)

The double-reciprocal plot of enzyme reaction rates is very useful in distinguishing between certain types of enzymatic reaction mechanisms (see Fig. 6-14) and in analyzing enzyme inhibition (see Box 6-2).

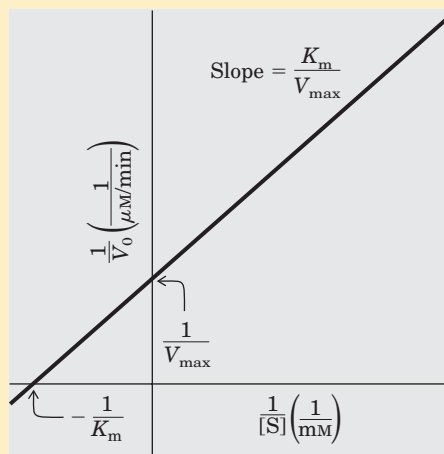
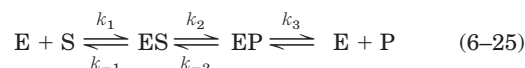


FIGURE 1 A double-reciprocal or Lineweaver-Burk plot.

for its substrate in the ES complex. However, this scenario does not apply for most enzymes. Sometimes  $k_2 \gg k_{-1}$ , and then  $K_m = k_2/k_1$ . In other cases,  $k_2$  and  $k_{-1}$  are comparable and  $K_m$  remains a more complex function of all three rate constants (Eqn 6-24). The Michaelis-Menten equation and the characteristic saturation behavior of the enzyme still apply, but  $K_m$  cannot be considered a simple measure of substrate affinity. Even more common are cases in which the reaction goes through several steps after formation of ES;  $K_m$  can then become a very complex function of many rate constants.

The quantity  $V_{\max}$  also varies greatly from one enzyme to the next. If an enzyme reacts by the two-step Michaelis-Menten mechanism,  $V_{\max} = k_2[E_t]$ , where  $k_2$  is rate-limiting. However, the number of reaction steps and the identity of the rate-limiting step(s) can vary from enzyme to enzyme. For example, consider the quite common situation where product release,  $EP \rightarrow E + P$ , is rate-limiting. Early in the reaction (when  $[P]$  is low), the overall reaction can be described by the scheme



In this case, most of the enzyme is in the EP form at saturation, and  $V_{\max} = k_3[E_t]$ . It is useful to define a more general rate constant,  $k_{\text{cat}}$ , to describe the limiting rate of any enzyme-catalyzed reaction at saturation. If the reaction has several steps and one is clearly rate-limiting,  $k_{\text{cat}}$  is equivalent to the rate constant for that limiting step. For the simple reaction of Equation 6-10,  $k_{\text{cat}} = k_2$ . For the reaction of Equation 6-25,  $k_{\text{cat}} = k_3$ . When several steps are partially rate-limiting,  $k_{\text{cat}}$  can become a complex function of several of the rate constants that define each individual reaction step. In the Michaelis-Menten equation,  $k_{\text{cat}} = V_{\max}/[E_t]$ , and Equation 6-9 becomes

$$V_0 = \frac{k_{\text{cat}} [E_t][S]}{K_m + [S]} \quad (6-26)$$

The constant  $k_{\text{cat}}$  is a first-order rate constant and hence has units of reciprocal time. It is also called the

**turnover number.** It is equivalent to the number of substrate molecules converted to product in a given unit of time on a single enzyme molecule when the enzyme is saturated with substrate. The turnover numbers of several enzymes are given in Table 6-7.

#### Comparing Catalytic Mechanisms and Efficiencies

The kinetic parameters  $k_{\text{cat}}$  and  $K_m$  are generally useful for the study and comparison of different enzymes, whether their reaction mechanisms are simple or complex. Each enzyme has values of  $k_{\text{cat}}$  and  $K_m$  that reflect the cellular environment, the concentration of substrate normally encountered in vivo by the enzyme, and the chemistry of the reaction being catalyzed.

The parameters  $k_{\text{cat}}$  and  $K_m$  also allow us to evaluate the kinetic efficiency of enzymes, but either parameter alone is insufficient for this task. Two enzymes catalyzing different reactions may have the same  $k_{\text{cat}}$  (turnover number), yet the rates of the uncatalyzed reactions may be different and thus the rate enhancements brought about by the enzymes may differ greatly. Experimentally, the  $K_m$  for an enzyme tends to be similar to the cellular concentration of its substrate. An enzyme that acts on a substrate present at a very low concentration in the cell usually has a lower  $K_m$  than an enzyme that acts on a substrate that is more abundant.

The best way to compare the catalytic efficiencies of different enzymes or the turnover of different substrates by the same enzyme is to compare the ratio  $k_{\text{cat}}/K_m$  for the two reactions. This parameter, sometimes called the **specificity constant**, is the rate constant for the conversion of  $E + S$  to  $E + P$ . When  $[S] \ll K_m$ , Equation 6-26 reduces to the form

$$V_0 = \frac{k_{\text{cat}}}{K_m} [E_t][S] \quad (6-27)$$

$V_0$  in this case depends on the concentration of two reactants,  $[E_t]$  and  $[S]$ ; therefore this is a second-order rate equation and the constant  $k_{\text{cat}}/K_m$  is a second-order rate

**TABLE 6-7** Turnover Numbers,  $k_{\text{cat}}$ , of Some Enzymes

Enzyme	Substrate	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )
Catalase	$\text{H}_2\text{O}_2$	40,000,000
Carbonic anhydrase	$\text{HCO}_3^-$	400,000
Acetylcholinesterase	Acetylcholine	14,000
$\beta$ -Lactamase	Benzylpenicillin	2,000
Fumarase	Fumarate	800
RecA protein (an ATPase)	ATP	0.4

constant with units of  $\text{M}^{-1}\text{s}^{-1}$ . There is an upper limit to  $k_{\text{cat}}/K_m$ , imposed by the rate at which E and S can diffuse together in an aqueous solution. This diffusion-controlled limit is  $10^8$  to  $10^9 \text{ M}^{-1}\text{s}^{-1}$ , and many enzymes have a  $k_{\text{cat}}/K_m$  near this range (Table 6-8). Such enzymes are said to have achieved catalytic perfection. Note that different values of  $k_{\text{cat}}$  and  $K_m$  can produce the maximum ratio.

#### Many Enzymes Catalyze Reactions with Two or More Substrates

We have seen how  $[S]$  affects the rate of a simple enzymatic reaction ( $S \rightarrow P$ ) with only one substrate molecule. In most enzymatic reactions, however, two (and sometimes more) different substrate molecules bind to the enzyme and participate in the reaction. For example, in the reaction catalyzed by hexokinase, ATP and glucose are the substrate molecules, and ADP and glucose 6-phosphate are the products:



The rates of such bisubstrate reactions can also be analyzed by the Michaelis-Menten approach. Hexokinase has a characteristic  $K_m$  for each of its substrates (Table 6-6).

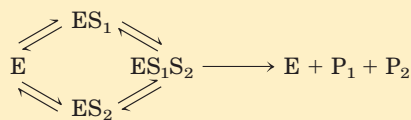
Enzymatic reactions with two substrates usually involve transfer of an atom or a functional group from one substrate to the other. These reactions proceed by one

**TABLE 6-8** Enzymes for Which  $k_{\text{cat}}/K_m$  Is Close to the Diffusion-Controlled Limit ( $10^8$  to  $10^9 \text{ M}^{-1}\text{s}^{-1}$ )

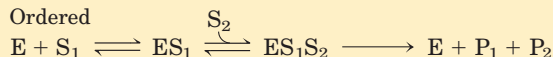
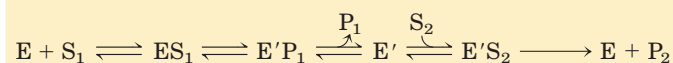
Enzyme	Substrate	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_m$ (M)	$k_{\text{cat}}/K_m$ ( $\text{M}^{-1}\text{s}^{-1}$ )
Acetylcholinesterase	Acetylcholine	$1.4 \times 10^4$	$9 \times 10^{-5}$	$1.6 \times 10^8$
Carbonic anhydrase	$\text{CO}_2$	$1.1 \times 10^6$	$1.2 \times 10^{-2}$	$8.3 \times 10^7$
	$\text{HCO}_3^-$	$1.4 \times 10^5$	$2.6 \times 10^{-2}$	$1.5 \times 10^7$
Catalase	$\text{H}_2\text{O}_2$	$1.4 \times 10^7$	$1.1 \times 10^0$	$4 \times 10^7$
Crotonase	Crotonyl-CoA	$5.7 \times 10^3$	$2 \times 10^{-5}$	$2.8 \times 10^8$
Fumarase	Fumarate	$1.8 \times 10^2$	$5 \times 10^{-6}$	$1.6 \times 10^8$
	Malate	$1.9 \times 10^2$	$2.5 \times 10^{-5}$	$3.6 \times 10^7$
	Benzylpenicillin	$2.0 \times 10^3$	$2 \times 10^{-5}$	$1 \times 10^8$

**(a) Enzyme reaction involving a ternary complex**

Random order



Ordered

**(b) Enzyme reaction in which no ternary complex is formed**

of several different pathways. In some cases, both substrates are bound to the enzyme concurrently at some point in the course of the reaction, forming a noncovalent ternary complex (Fig. 6-13a); the substrates bind in a random sequence or in a specific order. In other cases, the first substrate is converted to product and dissociates before the second substrate binds, so no ternary complex is formed. An example of this is the Ping-Pong, or double-displacement, mechanism (Fig. 6-13b). Steady-state kinetics can often help distinguish among these possibilities (Fig. 6-14).

### Pre-Steady State Kinetics Can Provide Evidence for Specific Reaction Steps

We have introduced kinetics as the primary method for studying the steps in an enzymatic reaction, and we have also outlined the limitations of the most common kinetic parameters in providing such information. The two most important experimental parameters obtained from steady-state kinetics are  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_{\text{m}}$ . Variation in  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_{\text{m}}$  with changes in pH or temperature can provide additional information about steps in a reaction pathway. In the case of bisubstrate reactions, steady-state kinetics can help determine whether a ternary complex is formed during the reaction (Fig. 6-14). A more complete picture generally requires more sophisticated kinetic methods that go beyond the scope of an introductory text. Here, we briefly introduce one of the most important kinetic approaches for studying reaction mechanisms, pre-steady state kinetics.

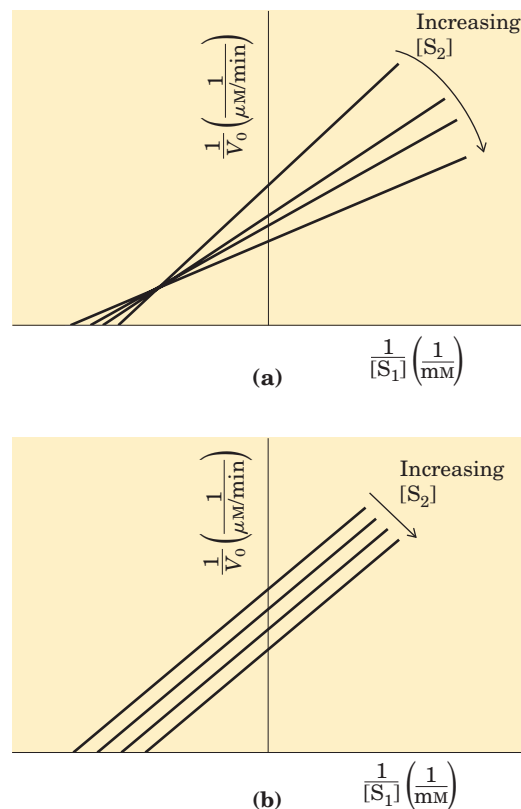
A complete description of an enzyme-catalyzed reaction requires direct measurement of the rates of individual reaction steps—for example, measurement of the association of enzyme and substrate to form the ES complex. It is during the pre-steady state that the rates of many reaction steps can be measured independently. Experimenters adjust reaction conditions so that they can observe events during reaction of a single substrate molecule. Because the pre-steady state phase is gener-

**FIGURE 6-13 Common mechanisms for enzyme-catalyzed bisubstrate reactions.**

(a) The enzyme and both substrates come together to form a ternary complex. In ordered binding, substrate 1 must bind before substrate 2 can bind productively. In random binding, the substrates can bind in either order.

(b) An enzyme-substrate complex forms, a product leaves the complex, the altered enzyme forms a second complex with another substrate molecule, and the second product leaves, regenerating the enzyme. Substrate 1 may transfer a functional group to the enzyme (to form the covalently modified E'), which is subsequently transferred to substrate 2. This is called a Ping-Pong or double-displacement mechanism.

ally very short, the experiments often require specialized techniques for very rapid mixing and sampling. One objective is to gain a complete and quantitative picture of the energy changes during the reaction. As we have already noted, reaction rates and equilibria are related to the free-energy changes during a reaction. Measur-

**FIGURE 6-14 Steady-state kinetic analysis of bisubstrate reactions.**

In these double-reciprocal plots (see Box 6-1), the concentration of substrate 1 is varied while the concentration of substrate 2 is held constant. This is repeated for several values of  $[S_2]$ , generating several separate lines. (a) Intersecting lines indicate that a ternary complex is formed in the reaction; (b) parallel lines indicate a Ping-Pong (double-displacement) pathway.

ing the rate of individual reaction steps reveals how energy is used by a specific enzyme, which is an important component of the overall reaction mechanism. In a number of cases investigators have been able to record the rates of every individual step in a multistep enzymatic reaction. Some examples of the application of pre-steady state kinetics are included in the descriptions of specific enzymes in Section 6.4.

### Enzymes Are Subject to Reversible or Irreversible Inhibition

Enzyme inhibitors are molecular agents that interfere with catalysis, slowing or halting enzymatic reactions. Enzymes catalyze virtually all cellular processes, so it should not be surprising that enzyme inhibitors are among the most important pharmaceutical agents known. For example, aspirin (acetylsalicylate) inhibits the enzyme that catalyzes the first step in the synthesis of prostaglandins, compounds involved in many processes, including some that produce pain. The study of enzyme inhibitors also has provided valuable information about enzyme mechanisms and has helped define some metabolic pathways. There are two broad classes of enzyme inhibitors: reversible and irreversible.

**Reversible Inhibition** One common type of **reversible inhibition** is called competitive (Fig. 6-15a). A **competitive inhibitor** competes with the substrate for the active site of an enzyme. While the inhibitor (I) occupies the active site it prevents binding of the substrate to the enzyme. Many competitive inhibitors are compounds that resemble the substrate and combine with the enzyme to form an EI complex, but without leading to catalysis. Even fleeting combinations of this type will reduce the efficiency of the enzyme. By taking into account the molecular geometry of inhibitors that resemble the substrate, we can reach conclusions about which parts of the normal substrate bind to the enzyme. Competitive inhibition can be analyzed quantitatively by steady-state kinetics. In the presence of a competitive inhibitor, the Michaelis-Menten equation (Eqn 6-9) becomes

$$V_0 = \frac{V_{\max} [S]}{\alpha K_m + [S]} \quad (6-28)$$

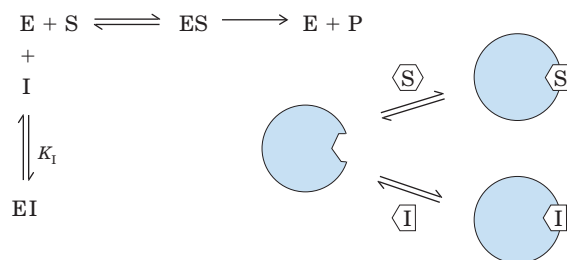
where

$$\alpha = 1 + \frac{[I]}{K_I} \quad \text{and} \quad K_I = \frac{[E][I]}{[EI]}$$

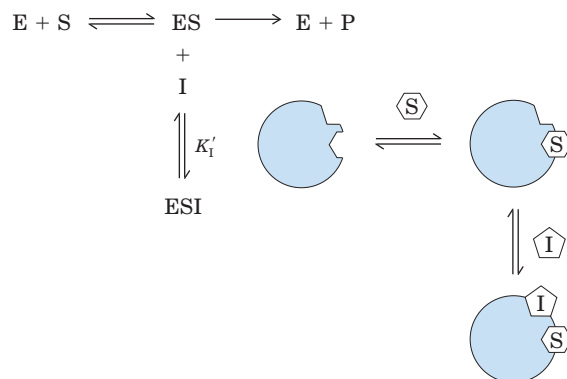
Equation 6-28 describes the important features of competitive inhibition. The experimentally determined variable  $\alpha K_m$ , the  $K_m$  observed in the presence of the inhibitor, is often called the “apparent”  $K_m$ .

Because the inhibitor binds reversibly to the enzyme, the competition can be biased to favor the substrate sim-

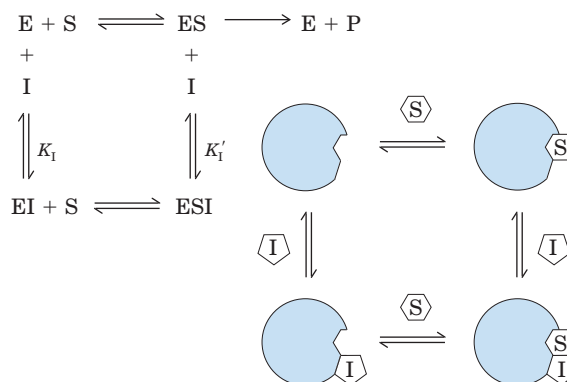
#### (a) Competitive inhibition



#### (b) Uncompetitive inhibition



#### (c) Mixed inhibition



**FIGURE 6-15 Three types of reversible inhibition.** (a) Competitive inhibitors bind to the enzyme’s active site. (b) Uncompetitive inhibitors bind at a separate site, but bind only to the ES complex.  $K_I$  is the equilibrium constant for inhibitor binding to E;  $K'_I$  is the equilibrium constant for inhibitor binding to ES. (c) Mixed inhibitors bind at a separate site, but may bind to either E or ES.

ply by adding more substrate. When  $[S]$  far exceeds  $[I]$ , the probability that an inhibitor molecule will bind to the enzyme is minimized and the reaction exhibits a normal  $V_{\max}$ . However, the  $[S]$  at which  $V_0 = \frac{1}{2} V_{\max}$ , the apparent  $K_m$ , increases in the presence of inhibitor by the factor  $\alpha$ . This effect on apparent  $K_m$ , combined with the absence of an effect on  $V_{\max}$ , is diagnostic of competitive inhibition and is readily revealed in a double-reciprocal plot (Box 6-2). The equilibrium constant for inhibitor binding,  $K_I$ , can be obtained from the same plot.



## BOX 6-2 WORKING IN BIOCHEMISTRY

## Kinetic Tests for Determining Inhibition Mechanisms

The double-reciprocal plot (see Box 6-1) offers an easy way of determining whether an enzyme inhibitor is competitive, uncompetitive, or mixed. Two sets of rate experiments are carried out, with the enzyme concentration held constant in each set. In the first set,  $[S]$  is also held constant, permitting measurement of the effect of increasing inhibitor concentration  $[I]$  on the initial rate  $V_0$  (not shown). In the second set,  $[I]$  is held constant but  $[S]$  is varied. The results are plotted as  $1/V_0$  versus  $1/[S]$ .

Figure 1 shows a set of double-reciprocal plots, one obtained in the absence of inhibitor and two at different concentrations of a competitive inhibitor. Increasing  $[I]$  results in a family of lines with a common intercept on the  $1/V_0$  axis but with different slopes. Because the intercept on the  $1/V_0$  axis equals  $1/V_{\max}$ , we know that  $V_{\max}$  is unchanged by the presence of a competitive inhibitor. That is, regardless of the concentration of a competitive inhibitor, a sufficiently high substrate concentration will always displace the inhibitor from the enzyme's active site. Above the graph is the rearrangement of Equation 6-28 on which the plot is based. The value of  $\alpha$  can be calculated

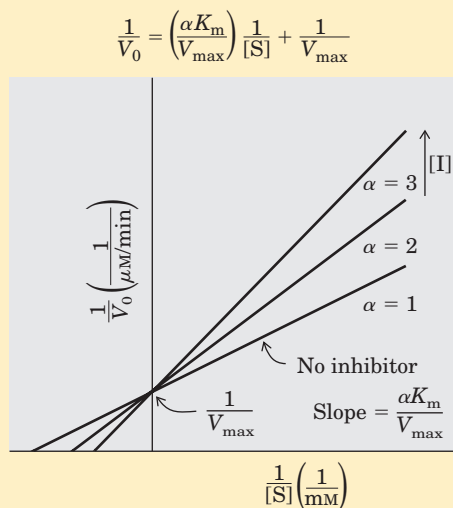


FIGURE 1 Competitive inhibition.

from the change in slope at any given  $[I]$ . Knowing  $[I]$  and  $\alpha$ , we can calculate  $K_I$  from the expression

$$\alpha = 1 + \frac{[I]}{K_I}$$

For uncompetitive and mixed inhibition, similar plots of rate data give the families of lines shown in Figures 2 and 3. Changes in axis intercepts signal changes in  $V_{\max}$  and  $K_m$ .

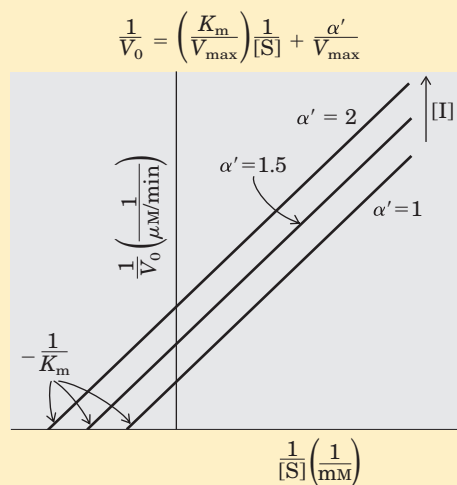


FIGURE 2 Uncompetitive inhibition.

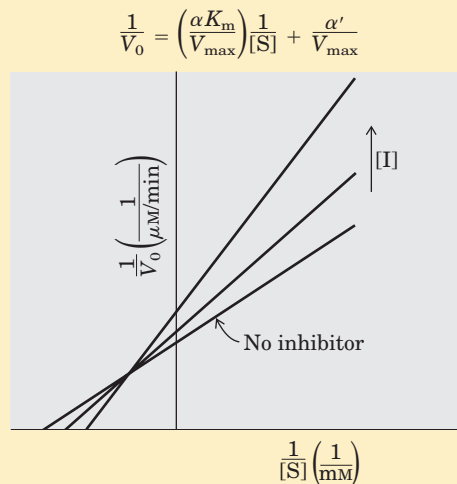


FIGURE 3 Mixed inhibition.



A medical therapy based on competition at the active site is used to treat patients who have ingested methanol, a solvent found in gas-line antifreeze. The liver enzyme alcohol dehydrogenase converts methanol to formaldehyde, which is damaging to many tissues. Blindness is a common result of methanol ingestion, because

the eyes are particularly sensitive to formaldehyde. Ethanol competes effectively with methanol as an alternative substrate for alcohol dehydrogenase. The effect of ethanol is much like that of a competitive inhibitor, with the distinction that ethanol is also a substrate for alcohol dehydrogenase and its concentration will decrease over

time as the enzyme converts it to acetaldehyde. The therapy for methanol poisoning is slow intravenous infusion of ethanol, at a rate that maintains a controlled concentration in the bloodstream for several hours. This slows the formation of formaldehyde, lessening the danger while the kidneys filter out the methanol to be excreted harmlessly in the urine. ■

Two other types of reversible inhibition, uncompetitive and mixed, though often defined in terms of one-substrate enzymes, are in practice observed only with enzymes having two or more substrates. An **uncompetitive inhibitor** (Fig. 6-15b) binds at a site distinct from the substrate active site and, unlike a competitive inhibitor, binds only to the ES complex. In the presence of an uncompetitive inhibitor, the Michaelis-Menten equation is altered to

$$V_0 = \frac{V_{\max} [S]}{K_m + \alpha' [S]} \quad (6-29)$$

where

$$\alpha' = 1 + \frac{[I]}{K_I} \quad \text{and} \quad K_I = \frac{[ES][I]}{[ESI]}$$

As described by Equation 6-29, at high concentrations of substrate,  $V_0$  approaches  $V_{\max}/\alpha'$ . Thus, an uncompetitive inhibitor lowers the measured  $V_{\max}$ . Apparent  $K_m$  also decreases, because the  $[S]$  required to reach one-half  $V_{\max}$  decreases by the factor  $\alpha'$ .

A **mixed inhibitor** (Fig. 6-15c) also binds at a site distinct from the substrate active site, but it binds to either E or ES. The rate equation describing mixed inhibition is

$$V_0 = \frac{V_{\max} [S]}{\alpha K_m + \alpha' [S]} \quad (6-30)$$

where  $\alpha$  and  $\alpha'$  are defined as above. A mixed inhibitor usually affects both  $K_m$  and  $V_{\max}$ . The special case of  $\alpha = \alpha'$ , rarely encountered in experiments, classically has been defined as **noncompetitive inhibition**. Examine Equation 6-30 to see why a noncompetitive inhibitor would affect the  $V_{\max}$  but not the  $K_m$ .

Equation 6-30 serves as a general expression for the effects of reversible inhibitors, simplifying to the expressions for competitive and uncompetitive inhibition when  $\alpha' = 1.0$  or  $\alpha = 1.0$ , respectively. From this expression we can summarize the effects of inhibitors on individual kinetic parameters. For all reversible inhibitors, apparent  $V_{\max} = V_{\max}/\alpha'$ , because the right side of Equation 6-30 always simplifies to  $V_{\max}/\alpha'$  at sufficiently high substrate concentrations. For competitive inhibitors,  $\alpha' = 1.0$  and can thus be ignored. Taking this expression for apparent  $V_{\max}$ , we can also derive a general expression for apparent  $K_m$  to show how this parameter changes in the presence of reversible inhibitors. Apparent  $K_m$ , as always, equals the  $[S]$  at which  $V_0$  is one-half apparent  $V_{\max}$  or, more generally, when  $V_0 = V_{\max}/2\alpha'$ . This condition is

**TABLE 6-9** Effects of Reversible Inhibitors on Apparent  $V_{\max}$  and Apparent  $K_m$

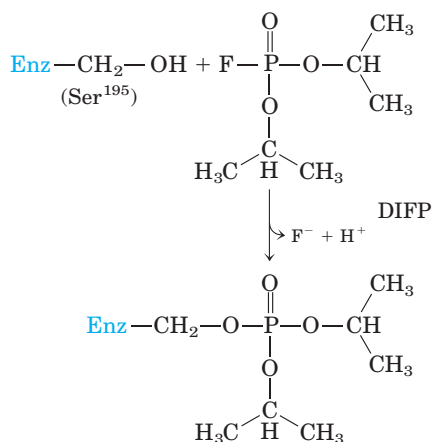
Inhibitor type	Apparent $V_{\max}$	Apparent $K_m$
None	$V_{\max}$	$K_m$
Competitive	$V_{\max}$	$\alpha K_m$
Uncompetitive	$V_{\max}/\alpha'$	$K_m/\alpha'$
Mixed	$V_{\max}/\alpha'$	$\alpha K_m/\alpha'$

met when  $[S] = \alpha K_m/\alpha'$ . Thus, apparent  $K_m = \alpha K_m/\alpha'$ . This expression is simpler when either  $\alpha$  or  $\alpha'$  is 1.0 (for uncompetitive or competitive inhibitors), as summarized in Table 6-9.

In practice, uncompetitive and mixed inhibition are observed only for enzymes with two or more substrates—say,  $S_1$  and  $S_2$ —and are very important in the experimental analysis of such enzymes. If an inhibitor binds to the site normally occupied by  $S_1$ , it may act as a competitive inhibitor in experiments in which  $[S_1]$  is varied. If an inhibitor binds to the site normally occupied by  $S_2$ , it may act as a mixed or uncompetitive inhibitor of  $S_1$ . The actual inhibition patterns observed depend on whether the  $S_1$ - and  $S_2$ -binding events are ordered or random, and thus the order in which substrates bind and products leave the active site can be determined. Use of one of the reaction products as an inhibitor is often particularly informative. If only one of two reaction products is present, no reverse reaction can take place. However, a product generally binds to some part of the active site, thus serving as an inhibitor. Enzymologists can use elaborate kinetic studies involving different combinations and amounts of products and inhibitors to develop a detailed picture of the mechanism of a bisubstrate reaction.

**Irreversible Inhibition** The **irreversible inhibitors** are those that bind covalently with or destroy a functional group on an enzyme that is essential for the enzyme's activity, or those that form a particularly stable noncovalent association. Formation of a covalent link between an irreversible inhibitor and an enzyme is common. Irreversible inhibitors are another useful tool for studying reaction mechanisms. Amino acids with key catalytic functions in the active site can sometimes be identified by determining which residue is covalently linked to an inhibitor after the enzyme is inactivated. An example is shown in Figure 6-16.

A special class of irreversible inhibitors is the **suicide inactivators**. These compounds are relatively unreactive until they bind to the active site of a specific enzyme. A suicide inactivator undergoes the first few chemical steps of the normal enzymatic reaction, but instead of being transformed into the normal product, the



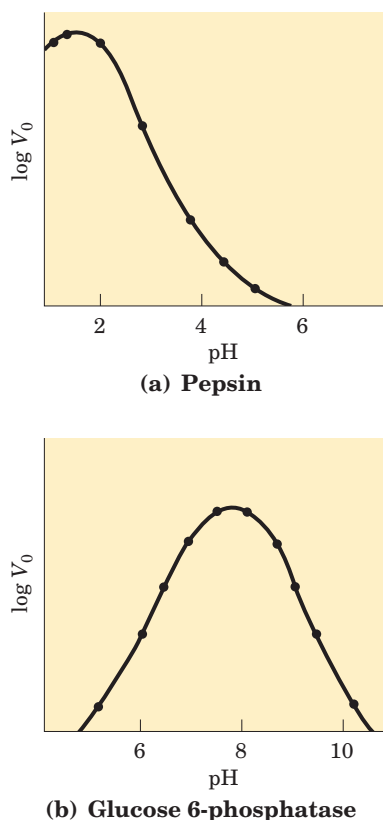
**FIGURE 6-16 Irreversible inhibition.** Reaction of chymotrypsin with diisopropylfluorophosphate (DIFP) irreversibly inhibits the enzyme. This has led to the conclusion that Ser<sup>195</sup> is the key active-site Ser residue in chymotrypsin.

inactivator is converted to a very reactive compound that combines irreversibly with the enzyme. These compounds are also called **mechanism-based inactivators**, because they hijack the normal enzyme reaction mechanism to inactivate the enzyme. Suicide inactivators play a significant role in *rational drug design*, a modern approach to obtaining new pharmaceutical agents in which chemists synthesize novel substrates based on knowledge of substrates and reaction mechanisms. A well-designed suicide inactivator is specific for a single enzyme and is unreactive until within that enzyme's active site, so drugs based on this approach can offer the important advantage of few side effects (see Box 22-2).

## Enzyme Activity Depends on pH

Enzymes have an optimum pH (or pH range) at which their activity is maximal (Fig. 6-17); at higher or lower pH, activity decreases. This is not surprising. Amino acid side chains in the active site may act as weak acids and bases with critical functions that depend on their maintaining a certain state of ionization, and elsewhere in the protein ionized side chains may play an essential role in the interactions that maintain protein structure. Removing a proton from a His residue, for example, might eliminate an ionic interaction essential for stabilizing the active conformation of the enzyme. A less common cause of pH sensitivity is titration of a group on the substrate.

The pH range over which an enzyme undergoes changes in activity can provide a clue to the type of amino acid residue involved (see Table 3-1). A change in activity near pH 7.0, for example, often reflects titration of a His residue. The effects of pH must be interpreted with some caution, however. In the closely packed environment of a protein, the  $pK_a$  of amino acid



**FIGURE 6-17 The pH-activity profiles of two enzymes.** These curves are constructed from measurements of initial velocities when the reaction is carried out in buffers of different pH. Because pH is a logarithmic scale reflecting tenfold changes in  $[H^+]$ , the changes in  $V_0$  are also plotted on a logarithmic scale. The pH optimum for the activity of an enzyme is generally close to the pH of the environment in which the enzyme is normally found. **(a)** Pepsin, which hydrolyzes certain peptide bonds of proteins during digestion in the stomach, has a pH optimum of about 1.6. The pH of gastric juice is between 1 and 2. **(b)** Glucose 6-phosphatase of hepatocytes (liver cells), with a pH optimum of about 7.8, is responsible for releasing glucose into the blood. The normal pH of the cytosol of hepatocytes is about 7.2.

side chains can be significantly altered. For example, a nearby positive charge can lower the  $\text{pK}_{\text{a}}$  of a Lys residue, and a nearby negative charge can increase it. Such effects sometimes result in a  $\text{pK}_{\text{a}}$  that is shifted by several pH units from its value in the free amino acid. In the enzyme acetoacetate decarboxylase, for example, one Lys residue has a  $\text{pK}_{\text{a}}$  of 6.6 (compared with 10.5 in free lysine) due to electrostatic effects of nearby positive charges.

### SUMMARY 6.3 Enzyme Kinetics As an Approach to Understanding Mechanism

- Most enzymes have certain kinetic properties in common. When substrate is added to an enzyme, the reaction rapidly achieves a steady state in which the rate at which the ES

complex forms balances the rate at which it reacts. As  $[S]$  increases, the steady-state activity of a fixed concentration of enzyme increases in a hyperbolic fashion to approach a characteristic maximum rate,  $V_{\max}$ , at which essentially all the enzyme has formed a complex with substrate.

- The substrate concentration that results in a reaction rate equal to one-half  $V_{\max}$  is the Michaelis constant  $K_m$ , which is characteristic for each enzyme acting on a given substrate. The Michaelis-Menten equation

$$V_0 = \frac{V_{\max} [S]}{K_m + [S]}$$

relates initial velocity to  $[S]$  and  $V_{\max}$  through the constant  $K_m$ . Michaelis-Menten kinetics is also called steady-state kinetics.

- $K_m$  and  $V_{\max}$  have different meanings for different enzymes. The limiting rate of an enzyme-catalyzed reaction at saturation is described by the constant  $k_{\text{cat}}$ , the turnover number. The ratio  $k_{\text{cat}}/K_m$  provides a good measure of catalytic efficiency. The Michaelis-Menten equation is also applicable to bisubstrate reactions, which occur by ternary-complex or Ping-Pong (double-displacement) pathways.
- Reversible inhibition of an enzyme is competitive, uncompetitive, or mixed. Competitive inhibitors compete with substrate by binding reversibly to the active site, but they are not transformed by the enzyme. Uncompetitive inhibitors bind only to the ES complex, at a site distinct from the active site. Mixed inhibitors bind to either E or ES, again at a site distinct from the active site. In irreversible inhibition an inhibitor binds permanently to an active site by forming a covalent bond or a very stable noncovalent interaction.
- Every enzyme has an optimum pH (or pH range) at which it has maximal activity.

## 6.4 Examples of Enzymatic Reactions

Thus far we have focused on the general principles of catalysis and on introducing some of the kinetic parameters used to describe enzyme action. We now turn to several examples of specific enzyme reaction mechanisms.

An understanding of the complete mechanism of action of a purified enzyme requires identification of all substrates, cofactors, products, and regulators. Moreover, it requires a knowledge of (1) the temporal sequence in which enzyme-bound reaction intermediates

form, (2) the structure of each intermediate and each transition state, (3) the rates of interconversion between intermediates, (4) the structural relationship of the enzyme to each intermediate, and (5) the energy contributed by all reacting and interacting groups to intermediate complexes and transition states. As yet, there is probably no enzyme for which we have an understanding that meets all these requirements. Many decades of research, however, have produced mechanistic information about hundreds of enzymes, and in some cases this information is highly detailed.

We present here the mechanisms for four enzymes: chymotrypsin, hexokinase, enolase, and lysozyme. These examples are not intended to cover all possible classes of enzyme chemistry. They are chosen in part because they are among the best understood enzymes, and in part because they clearly illustrate some general principles outlined in this chapter. The discussion concentrates on selected principles, along with some key experiments that have helped to bring these principles into focus. We use the chymotrypsin example to review some of the conventions used to depict enzyme mechanisms. Much mechanistic detail and experimental evidence is necessarily omitted; no one book could completely document the rich experimental history of these enzymes. Also absent from these discussions is the special contribution of coenzymes to the catalytic activity of many enzymes. The function of coenzymes is chemically varied, and we describe each as it is encountered in Part II.

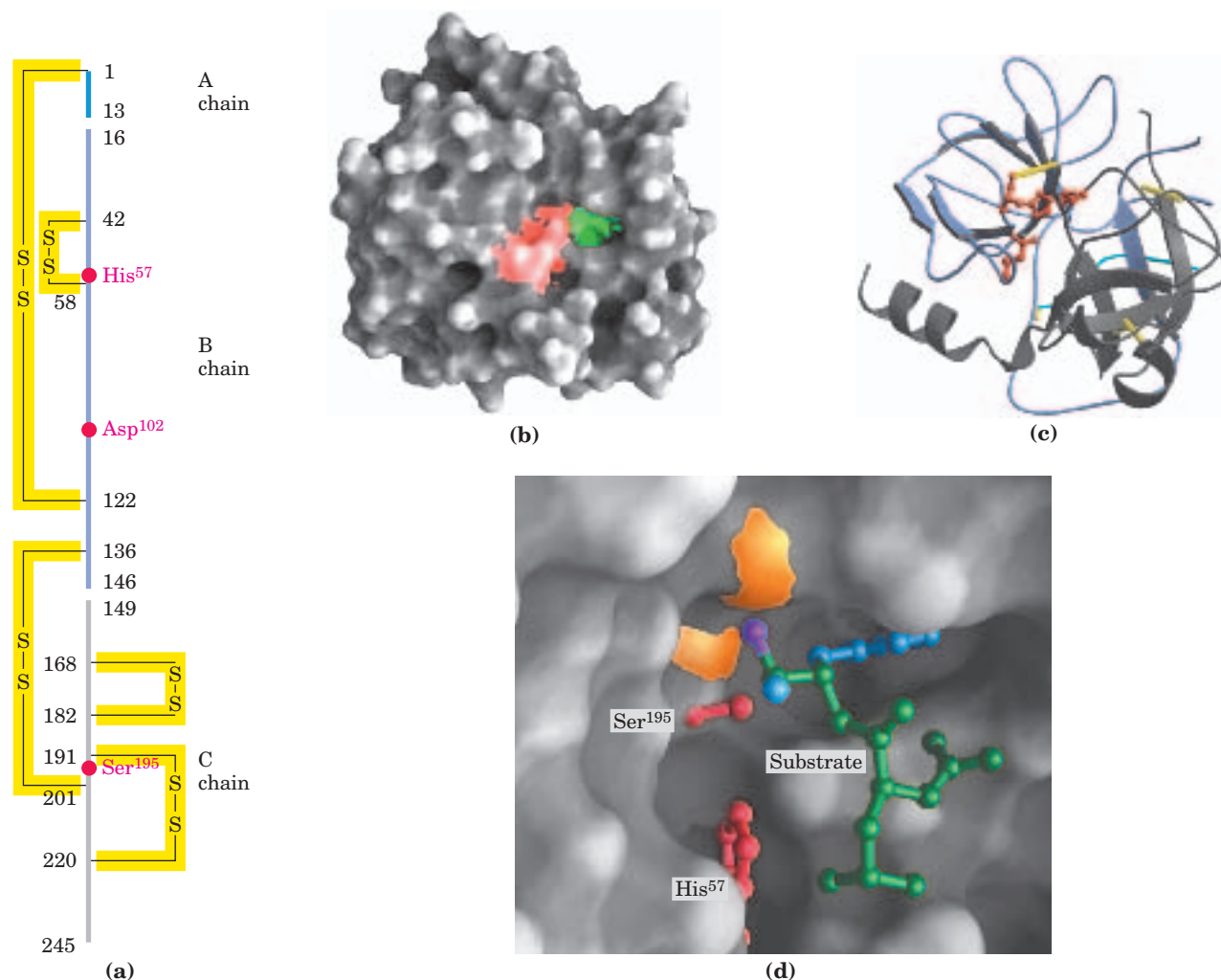
### The Chymotrypsin Mechanism Involves Acylation and Deacylation of a Ser Residue

Bovine pancreatic chymotrypsin ( $M_r$  25,191) is a protease, an enzyme that catalyzes the hydrolytic cleavage of peptide bonds. This protease is specific for peptide bonds adjacent to aromatic amino acid residues (Trp, Phe, Tyr). The three-dimensional structure of chymotrypsin is shown in Figure 6-18, with functional groups in the active site emphasized. The reaction catalyzed by this enzyme illustrates the principle of transition-state stabilization and also provides a classic example of general acid-base catalysis and covalent catalysis.

Chymotrypsin enhances the rate of peptide bond hydrolysis by a factor of at least  $10^9$ . It does not catalyze a direct attack of water on the peptide bond; instead, a transient covalent acyl-enzyme intermediate is formed. The reaction thus has two distinct phases. In the acylation phase, the peptide bond is cleaved and an ester linkage is formed between the peptide carbonyl carbon and the enzyme. In the deacylation phase, the ester linkage is hydrolyzed and the nonacylated enzyme regenerated.

The first evidence for a covalent acyl-enzyme intermediate came from a classic application of pre-steady state kinetics. In addition to its action on polypeptides,



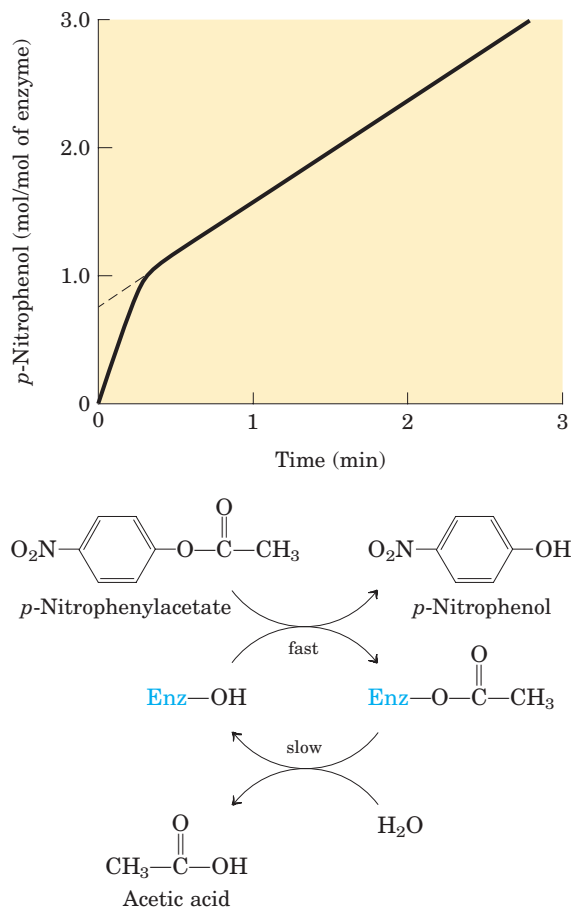


**FIGURE 6-18 Structure of chymotrypsin.** (PDB ID 7GCH) (a) A representation of primary structure, showing disulfide bonds and the amino acid residues crucial to catalysis. The protein consists of three polypeptide chains linked by disulfide bonds. (The numbering of residues in chymotrypsin, with “missing” residues 14, 15, 147, and 148, is explained in Fig. 6–33.) The active-site amino acid residues are grouped together in the three-dimensional structure. (b) A depiction of the enzyme emphasizing its surface. The pocket in which the aromatic amino acid side chain of the substrate is bound is shown in green. Key active-site residues, including Ser<sup>195</sup>, His<sup>57</sup>, and Asp<sup>102</sup>, are red. The roles of these residues in catalysis are illustrated in Fig-

ure 6–21. (c) The polypeptide backbone as a ribbon structure. Disulfide bonds are yellow; the three chains are colored as in part (a). (d) A close-up of the active site with a substrate (mostly green) bound. Two of the active-site residues, Ser<sup>195</sup> and His<sup>57</sup> (both red), are partly visible. Ser<sup>195</sup> attacks the carbonyl group of the substrate (the oxygen is purple); the developing negative charge on the oxygen is stabilized by the oxyanion hole (amide nitrogens in orange), as explained in Figure 6–21. In the substrate, the aromatic amino acid side chain and the amide nitrogen of the peptide bond to be cleaved (protruding toward the viewer and projecting the path of the rest of the substrate polypeptide chain) are in blue.

chymotrypsin also catalyzes the hydrolysis of small esters and amides. These reactions are much slower than hydrolysis of peptides because less binding energy is available with smaller substrates, and they are therefore easier to study. Investigations by B. S. Hartley and B. A. Kilby in 1954 found that chymotrypsin hydrolysis of the ester *p*-nitrophenylacetate, as measured by release of *p*-nitrophenol, proceeded with a rapid burst before leveling off to a slower rate (Fig. 6–19). By extrapolating back to zero time, they concluded that the

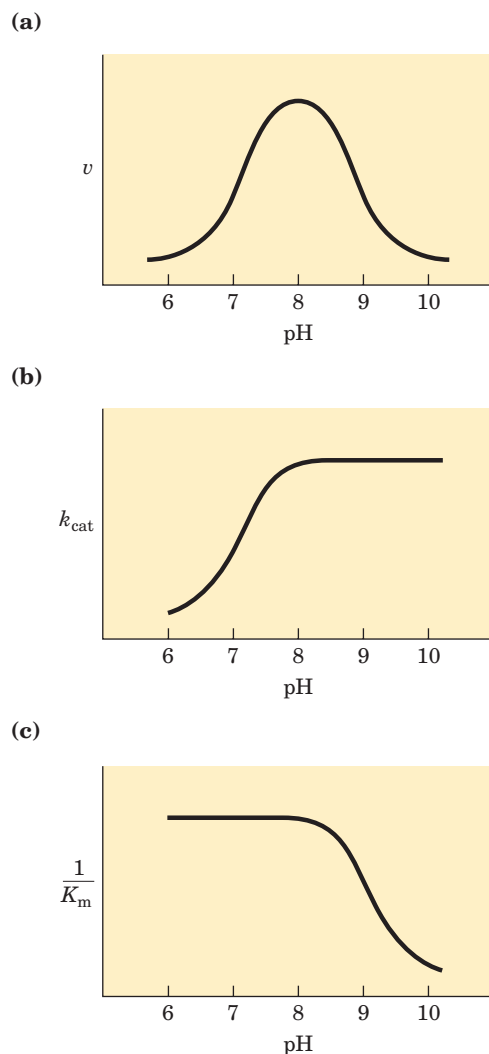
burst phase corresponded to just under one molecule of *p*-nitrophenol released for every enzyme molecule present. Hartley and Kilby suggested that this reflected a rapid acylation of all the enzyme molecules (with release of *p*-nitrophenol), with the rate for subsequent turnover of the enzyme limited by a slow deacylation step. Similar results have since been obtained with many other enzymes. The observation of a burst phase provides yet another example of the use of kinetics to break down a reaction into its constituent steps.



**FIGURE 6-19** Pre-steady state kinetic evidence for an acyl-enzyme intermediate. The hydrolysis of *p*-nitrophenylacetate by chymotrypsin is measured by release of *p*-nitrophenol (a colored product). Initially, the reaction releases a rapid burst of *p*-nitrophenol nearly stoichiometric with the amount of enzyme present. This reflects the fast acylation phase of the reaction. The subsequent rate is slower, because enzyme turnover is limited by the rate of the slower deacylation phase.

Additional features of the chymotrypsin mechanism have been elucidated by analyzing the dependence of the reaction on pH. The rate of chymotrypsin-catalyzed cleavage generally exhibits a bell-shaped pH-rate profile (Fig. 6-20). The rates plotted in Figure 6-20a are obtained at low (subsaturating) substrate concentrations and therefore represent  $k_{\text{cat}}/K_m$ . The plot can be dissected further by first obtaining the maximum rates at each pH, and then plotting  $k_{\text{cat}}$  alone versus pH (Fig. 6-20b); after obtaining the  $K_m$  at each pH, researchers can then plot  $1/K_m$  (Fig. 6-20c). Kinetic and structural analyses have revealed that the change in  $k_{\text{cat}}$  reflects the ionization state of His<sup>57</sup>. The decline in  $k_{\text{cat}}$  at low pH results from protonation of His<sup>57</sup> (so that it cannot extract a proton from Ser<sup>195</sup> in step ① of the reaction; see Fig. 6-21). This rate reduction illustrates the importance of general acid and general base catalysis in the mechanism for chymotrypsin. The changes in the

$1/K_m$  term reflect the ionization of the  $\alpha$ -amino group of Ile<sup>16</sup> (at the amino-terminal end of one of chymotrypsin's three polypeptide chains). This group forms a salt bridge to Asp<sup>194</sup>, stabilizing the active conformation of the enzyme. When this group loses its proton at high pH, the salt bridge is eliminated and a conformational change closes the hydrophobic pocket where the



**FIGURE 6-20** The pH dependence of chymotrypsin-catalyzed reactions. (a) The rates of chymotrypsin-mediated cleavage produce a bell-shaped pH-rate profile with an optimum at pH 8.0. The rate (*v*) being plotted is that at low substrate concentrations and thus reflects the term  $k_{\text{cat}}/K_m$ . The plot can be broken down to its components by using kinetic methods to determine the terms  $k_{\text{cat}}$  and  $K_m$  separately at each pH. When this is done (b and c), it becomes clear that the transition just above pH 7 is due to changes in  $k_{\text{cat}}$ , whereas the transition above pH 8.5 is due to changes in  $1/K_m$ . Kinetic and structural studies have shown that the transitions illustrated in (b) and (c) reflect the ionization states of the His<sup>57</sup> side chain (when substrate is not bound) and the  $\alpha$ -amino group of Ile<sup>16</sup> (at the amino terminus of the B chain), respectively. For optimal activity, His<sup>57</sup> must be unprotonated and Ile<sup>16</sup> must be protonated.

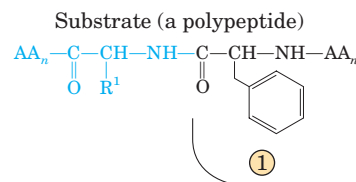
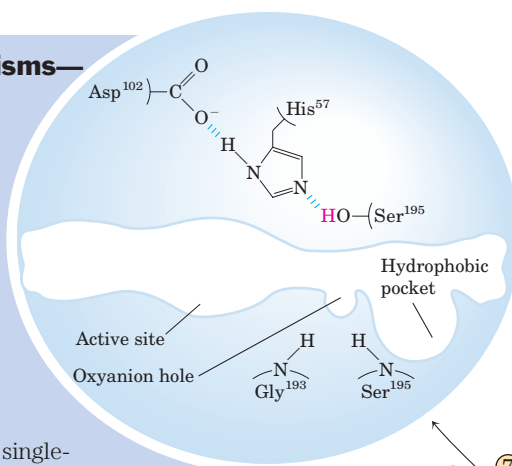
## How to Read Reaction Mechanisms—A Refresher

Chemical reaction mechanisms, which trace the formation and breakage of covalent bonds, are communicated with dots and curved arrows, a convention known informally as “electron pushing.” A covalent bond consists of a shared pair of electrons. Nonbonded electrons are designated by dots ( $\cdot\cdot$ ). Curved arrows ( $\curvearrowright$ ) represent the movement of electron pairs. For movement of a single electron (as in a free radical reaction), a single-headed (fishhook-type) arrow is used ( $\frown$ ). Most reaction steps involve an unshared electron pair (as in the chymotrypsin mechanism).

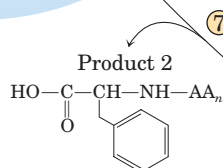
Some atoms are more electronegative than others; that is, they more strongly attract electrons. The relative electronegativities of atoms encountered in this text are  $F > O > N > C \approx S > P \approx H$ . For example, the two electron pairs making up a  $C=O$  (carbonyl) bond are not shared equally; the carbon is relatively electron-deficient as the oxygen draws away the electrons. Many reactions involve an electron-rich atom (a nucleophile) reacting with an electron-deficient atom (an electrophile). Some common nucleophiles and electrophiles in biochemistry are shown at right.

In general, a reaction mechanism is initiated at an unshared electron pair of a nucleophile. In mechanism diagrams, the base of the electron-pushing arrow originates near the electron-pair dots, and the head of the arrow points directly at the electrophilic center being attacked. Where the unshared electron pair confers a formal negative charge on the nucleophile, the negative charge symbol itself can represent the unshared electron pair, and serves as the base of the arrow. In the chymotrypsin mechanism, the nucleophilic electron pair in the ES complex between steps ① and ② is provided by the oxygen of the  $\text{Ser}^{195}$  hydroxyl group. This electron pair (2 of the 8 valence electrons of the hydroxyl oxygen) provides the base of the curved arrow. The electrophilic center under attack is the carbonyl carbon of the peptide bond to be cleaved. The C, O, and N atoms have a maximum of 8 valence electrons, and H has a maximum of 2. These atoms are occasionally found in unstable states with less than their maximum allotment of electrons, but C, O, and N cannot have more than 8. Thus, when the electron pair from chymotrypsin's  $\text{Ser}^{195}$  attacks the substrate's carbonyl carbon, an electron pair is displaced from the carbon valence shell (you cannot have 5 bonds to carbon!). These electrons move toward the more electronegative carbonyl oxygen. The oxygen has 8 valence electrons both before and after this chemical process, but the number shared with the carbon is reduced from 4 to 2, and the carbonyl oxygen acquires a negative charge. In the next step, the electron pair conferring the negative charge on the oxygen moves back to re-form a bond with carbon and reestablish the carbonyl linkage. Again, an electron pair must be displaced from the carbon, and this time it is the electron pair shared with the amino group of the peptide linkage. This breaks the peptide bond. The remaining steps follow a similar pattern.

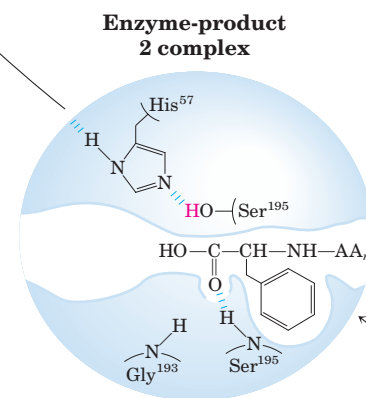
### Chymotrypsin (free enzyme)



When substrate binds, the side chain of the residue adjacent to the peptide bond to be cleaved nestles in a hydrophobic pocket on the enzyme, positioning the peptide bond for attack.



Diffusion of the second product from the active site regenerates free enzyme.



### Nucleophiles

$-\text{O}^-$   
Negatively charged oxygen (as in an unprotonated hydroxyl group or an ionized carboxylic acid)

$-\text{S}^-$   
Negatively charged sulfhydryl

$-\text{C}^-$   
Carbanion

$-\text{N}^-$   
Uncharged amine group

Imidazole

$\text{H}-\text{O}^-$   
Hydroxide ion

### Electrophiles

$\text{C}=\text{O}$   
Carbon atom of a carbonyl group (the more electronegative oxygen of the carbonyl group pulls electrons away from the carbon)

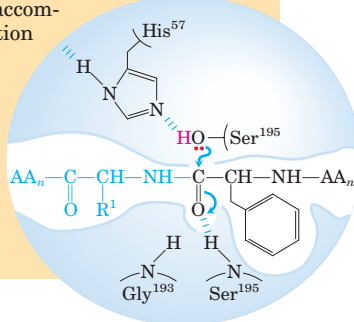
$\text{C}=\text{N}^+-\text{H}$   
Protonated imine group (activated for nucleophilic attack at the carbon by protonation of the imine)

$\text{P}=\text{O}$   
Phosphorus of a phosphate group

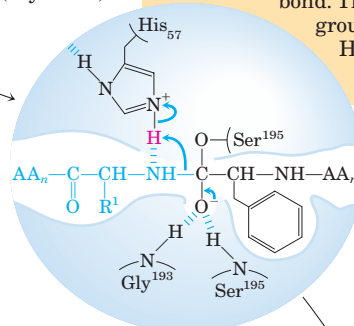
$\text{H}^+$   
Proton

Interaction of Ser<sup>195</sup> and His<sup>57</sup> generates a strongly nucleophilic alkoxide ion on Ser<sup>195</sup>; the ion attacks the peptide carbonyl group, forming a tetrahedral acyl-enzyme. This is accompanied by formation of a short-lived negative charge on the carbonyl oxygen of the substrate, which is stabilized by hydrogen bonding in the oxyanion hole.

### ES complex

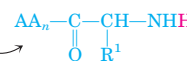


### Short-lived intermediate (acylation)



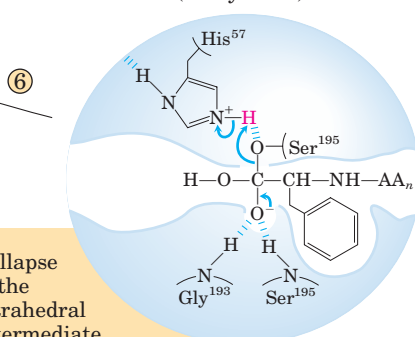
Instability of the negative charge on the substrate carbonyl oxygen leads to collapse of the tetrahedral intermediate; re-formation of a double bond with carbon displaces the bond between carbon and the amino group of the peptide linkage, breaking the peptide bond. The amino leaving group is protonated by His<sup>57</sup>, facilitating its displacement.

### Product 1



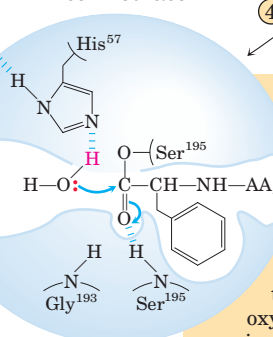
**MECHANISM FIGURE 6-21 Hydrolytic cleavage of a peptide bond by chymotrypsin.** The reaction has two phases. In the acylation phase (steps ① to ③), formation of a covalent acyl-enzyme intermediate is coupled to cleavage of the peptide bond. In the deacylation phase (steps ④ to ⑦), deacylation regenerates the free enzyme; this is essentially the reverse of the acylation phase, with water mirroring, in reverse, the role of the amine component of the substrate. **Chymotrypsin Mechanism**

### Short-lived intermediate (deacylation)



Collapse of the tetrahedral intermediate forms the second product, a carboxylate anion, and displaces Ser<sup>195</sup>.

### Acyl-enzyme intermediate



### Acyl-enzyme intermediate

An incoming water molecule is deprotonated by general base catalysis, generating a strongly nucleophilic hydroxide ion. Attack of hydroxide on the ester linkage of the acyl-enzyme generates a second tetrahedral intermediate, with oxygen in the oxyanion hole again taking on a negative charge.

\*The tetrahedral intermediate in the chymotrypsin reaction pathway, and the second tetrahedral intermediate that forms later, are sometimes referred to as transition states, which can lead to confusion. An *intermediate* is any chemical species with a finite lifetime, “finite” being defined as longer than the time required for a molecular vibration ( $\sim 10^{-13}$  seconds). A *transition state* is simply the maximum-energy species formed on the reaction coordinate and does not have a finite lifetime. The tetrahedral intermediates formed in the chymotrypsin reaction closely resemble, both energetically and structurally, the transition states leading to their formation and breakdown. However, the intermediate represents a committed stage of completed

bond formation, whereas the transition state is part of the process of reaction. In the case of chymotrypsin, given the close relationship between the intermediate and the actual transition state the distinction between them is routinely glossed over. Furthermore, the interaction of the negatively charged oxygen with the amide nitrogens in the oxyanion hole, often referred to as transition-state stabilization, also serves to stabilize the intermediate in this case. Not all intermediates are so short-lived that they resemble transition states. The chymotrypsin acyl-enzyme intermediate is much more stable and more readily detected and studied, and it is never confused with a transition state.



aromatic amino acid side chain of the substrate inserts (Fig. 6–18). Substrates can no longer bind properly, which is measured kinetically as an increase in  $K_m$ .

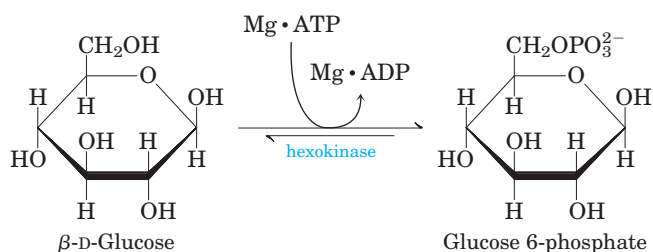
The nucleophile in the acylation phase is the oxygen of Ser<sup>195</sup>. (Proteases with a Ser residue that plays this role in reaction mechanisms are called serine proteases.) The  $pK_a$  of a Ser hydroxyl group is generally too high for the unprotonated form to be present in significant concentrations at physiological pH. However, in chymotrypsin, Ser<sup>195</sup> is linked to His<sup>57</sup> and Asp<sup>102</sup> in a hydrogen-bonding network referred to as the **catalytic triad**. When a peptide substrate binds to chymotrypsin, a subtle change in conformation compresses the hydrogen bond between His<sup>57</sup> and Asp<sup>102</sup>, resulting in a stronger interaction, called a low-barrier hydrogen bond. This enhanced interaction increases the  $pK_a$  of His<sup>57</sup> from ~7 (for free histidine) to >12, allowing the His residue to act as an enhanced general base that can remove the proton from the Ser<sup>195</sup> hydroxyl group. Deprotonation prevents development of a very unstable positive charge on the Ser<sup>195</sup> hydroxyl and makes the Ser side chain a stronger nucleophile. At later reaction stages, His<sup>57</sup> also acts as a proton donor, protonating the amino group in the displaced portion of the substrate (the leaving group).

As the Ser<sup>195</sup> oxygen attacks the carbonyl group of the substrate, a very short-lived tetrahedral intermediate is formed in which the carbonyl oxygen acquires a negative charge (Fig 6-21). This charge, forming within a pocket on the enzyme called the oxyanion hole, is stabilized by hydrogen bonds contributed by the amide groups of two peptide bonds in the chymotrypsin backbone. One of these hydrogen bonds (contributed by Gly<sup>193</sup>) is present only in this intermediate and in the transition states for its formation and breakdown; it reduces the energy required to reach these states. This is an example of the use of binding energy in catalysis.

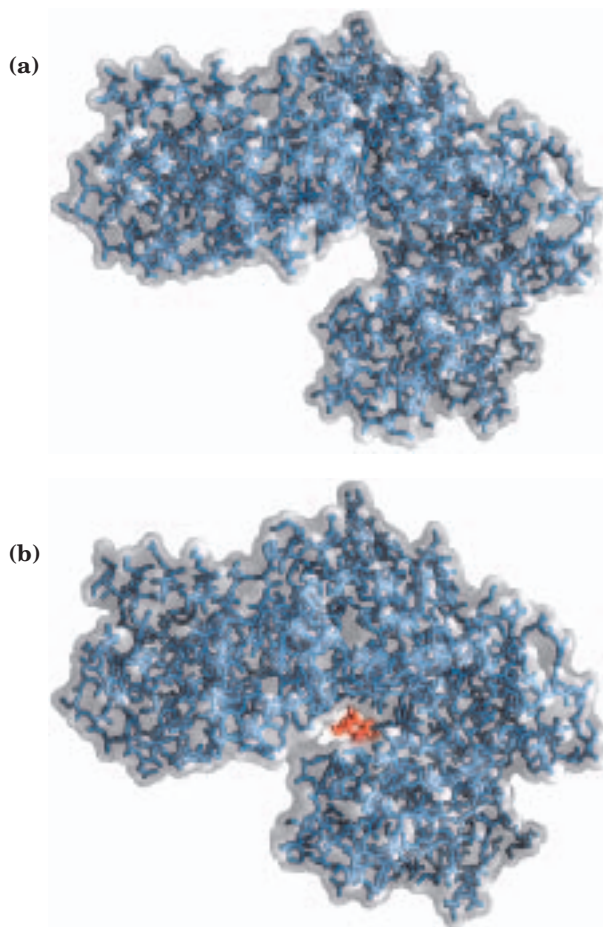
The role of transition state complementarity in enzyme catalysis is further explored in Box 6-3.

### Hexokinase Undergoes Induced Fit on Substrate Binding

Yeast hexokinase ( $M_r$  107,862) is a bisubstrate enzyme that catalyzes the reversible reaction



ATP and ADP always bind to enzymes as a complex with the metal ion  $\text{Mg}^{2+}$ .

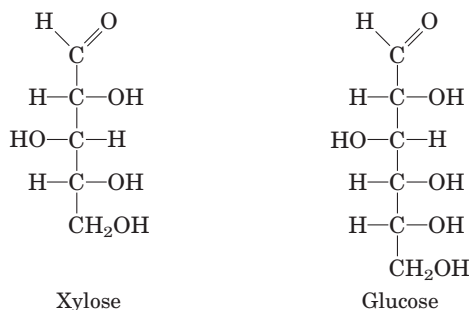


**FIGURE 6-22 Induced fit in hexokinase.** (a) Hexokinase has a U-shaped structure (PDB ID 2YHX). (b) The ends pinch toward each other in a conformational change induced by binding of  $\alpha$ -glucose (red) (derived from PDB ID 1HKG and PDB ID 1GLK).

The hydroxyl at C-6 of glucose (to which the  $\gamma$ -phosphoryl of ATP is transferred in the hexokinase reaction) is similar in chemical reactivity to water, and water freely enters the enzyme active site. Yet hexokinase favors the reaction with glucose by a factor of  $10^6$ . The enzyme can discriminate between glucose and water because of a conformational change in the enzyme when the correct substrates binds (Fig. 6–22). Hexokinase thus provides a good example of induced fit. When glucose is not present, the enzyme is in an inactive conformation with the active-site amino acid side chains out of position for reaction. When glucose (but not water) and  $\text{Mg} \cdot \text{ATP}$  bind, the binding energy derived from this interaction induces a conformational change in hexokinase to the catalytically active form.

This model has been reinforced by kinetic studies. The five-carbon sugar xylose, stereochemically similar to glucose but one carbon shorter, binds to hexokinase but in a position where it cannot be phosphorylated. Nevertheless, addition of xylose to the reaction mixture increases the rate of ATP hydrolysis. Evidently, the binding of xylose is sufficient to induce a change in

hexokinase to its active conformation, and the enzyme is thereby “tricked” into phosphorylating water. The hexokinase reaction also illustrates that enzyme specificity is not always a simple matter of binding one compound but not another. In the case of hexokinase, specificity is observed not in the formation of the ES complex but in the relative rates of subsequent catalytic steps. Water is not excluded from the active site, but reaction rates increase greatly in the presence of the functional phosphoryl group acceptor (glucose).

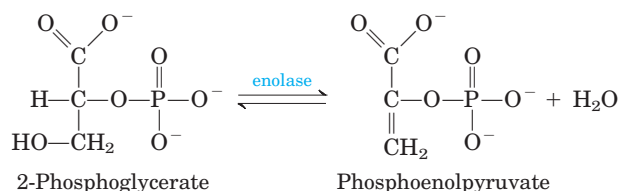


Induced fit is only one aspect of the catalytic mechanism of hexokinase—like chymotrypsin, hexokinase uses several catalytic strategies. For example, the active-

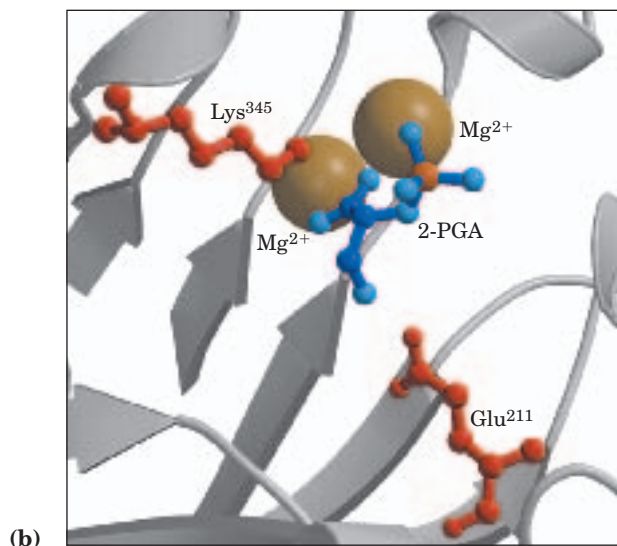
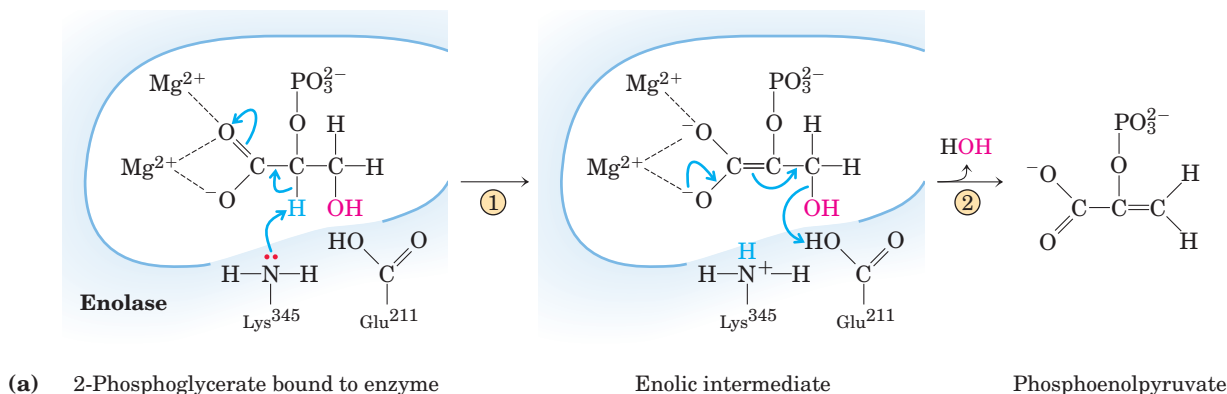
site amino acid residues (those brought into position by the conformational change that follows substrate binding) participate in general acid-base catalysis and transition-state stabilization.

### The Enolase Reaction Mechanism Requires Metal Ions

Another glycolytic enzyme, enolase, catalyzes the reversible dehydration of 2-phosphoglycerate to phosphoenolpyruvate:



Yeast enolase ( $M_r$  93,316) is a dimer with 436 amino acid residues per subunit. The enolase reaction illustrates one type of metal ion catalysis and provides an additional example of general acid-base catalysis and transition-state stabilization. The reaction occurs in two steps (Fig. 6-23a). First, Lys<sup>345</sup> acts as a general base catalyst,



**MECHANISM FIGURE 6-23 Two-step reaction catalyzed by enolase.** (a) The mechanism by which enolase converts 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate. The carboxyl group of 2-PGA is coordinated by two magnesium ions at the active site. A proton is abstracted in step ① by general base catalysis (Lys<sup>345</sup>), and the resulting enolic intermediate is stabilized by the two Mg<sup>2+</sup> ions. Elimination of the —OH in step ② is facilitated by general acid catalysis (Glu<sup>211</sup>). (b) The substrate, 2-PGA, in relation to the Mg<sup>2+</sup> ions, Lys<sup>345</sup>, and Glu<sup>211</sup> in the enolase active site. Hydrogen atoms are not shown. All the oxygen atoms of 2-PGA are light blue; phosphorus is orange (PDB ID 1ONE).

## Evidence for Enzyme-Transition State Complementarity

The transition state of a reaction is difficult to study because it is so short-lived. To understand enzymatic catalysis, however, we must dissect the interaction between the enzyme and this ephemeral moment in the course of a reaction. Complementarity between an enzyme and the transition state is virtually a requirement for catalysis, because the energy hill upon which the transition state sits is what the enzyme must lower if catalysis is to occur. How can we obtain evidence for enzyme-transition state complementarity? Fortunately, we have a variety of approaches, old and new, to address this problem, each providing compelling evidence in support of this general principle of enzyme action.

### Structure-Activity Correlations

If enzymes are complementary to reaction transition states, then some functional groups in both the substrate and the enzyme must interact preferentially in the transition state rather than in the ES complex. Changing these groups should have little effect on formation of the ES complex and hence should not affect kinetic parameters (the dissociation constant,  $K_d$ ; or sometimes  $K_m$ , if  $K_d = K_m$ ) that reflect the  $E + S \rightleftharpoons ES$  equilibrium. Changing these same groups should have a large effect on the overall rate ( $k_{cat}$  or  $k_{cat}/K_m$ ) of the reaction, however, because the bound substrate lacks potential binding interactions needed to lower the activation energy.

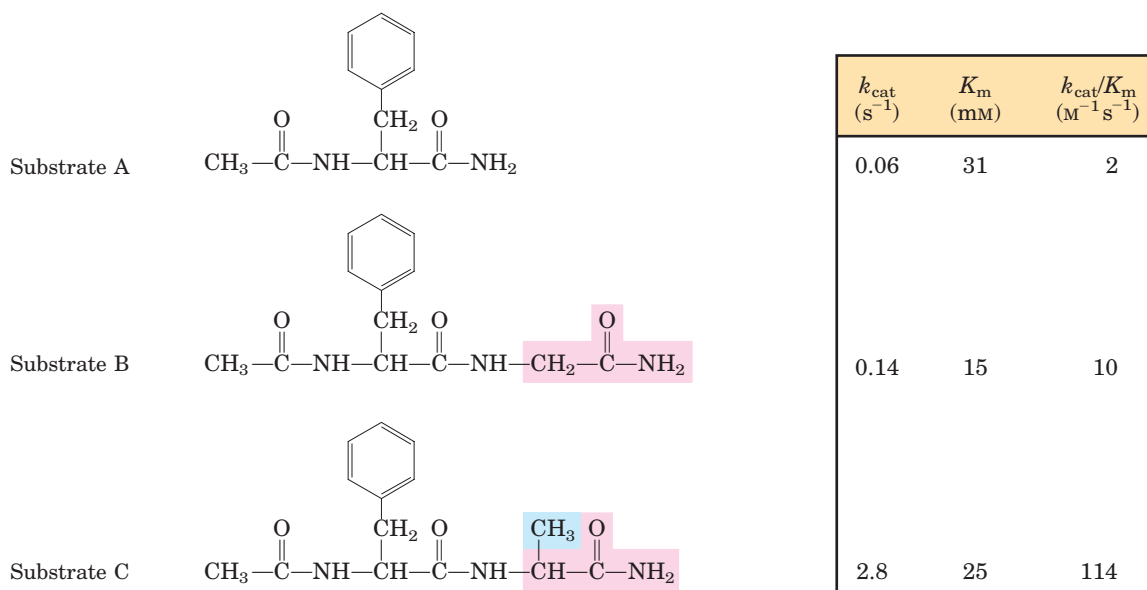
An excellent example of this effect is seen in the kinetics associated with a series of related substrates for the enzyme chymotrypsin (Fig. 1). Chymotrypsin

normally catalyzes the hydrolysis of peptide bonds next to aromatic amino acids. The substrates shown in Figure 1 are convenient smaller models for the natural substrates (long polypeptides and proteins). The additional chemical groups added in each substrate (A to B to C) are shaded. As the table shows, the interaction between the enzyme and these added functional groups has a minimal effect on  $K_m$  (taken here as a reflection of  $K_d$ ) but a large, positive effect on  $k_{cat}$  and  $k_{cat}/K_m$ . This is what we would expect if the interaction contributed largely to stabilization of the transition state. The results also demonstrate that the rate of a reaction can be affected greatly by enzyme-substrate interactions that are physically remote from the covalent bonds that are altered in the enzyme-catalyzed reaction. Chymotrypsin is described in more detail in the text.

A complementary experimental approach is to modify the enzyme, eliminating certain enzyme-substrate interactions by replacing specific amino acid residues through site-directed mutagenesis (see Fig. 9-12). Results from such experiments again demonstrate the importance of binding energy in stabilizing the transition state.

### Transition-State Analogs

Even though transition states cannot be observed directly, chemists can often predict the approximate structure of a transition state based on accumulated knowledge about reaction mechanisms. The transition state is by definition transient and so unstable that direct measurement of the binding interaction between this species and the enzyme is impossible. In some



**FIGURE 1** Effects of small structural changes in the substrate on kinetic parameters for chymotrypsin-catalyzed amide hydrolysis.

cases, however, stable molecules can be designed that resemble transition states. These are called **transition-state analogs**. In principle, they should bind to an enzyme more tightly than does the substrate in the ES complex, because they should fit the active site better (that is, form a greater number of weak interactions) than the substrate itself. The idea of transition-state analogs was suggested by Pauling in the 1940s, and it has been explored using a number of enzymes. These experiments have the limitation that a transition-state analog cannot perfectly mimic a transition state. Some analogs, however, bind an enzyme  $10^2$  to  $10^6$  times more tightly than does the normal substrate, providing good evidence that enzyme active sites are indeed complementary to transition states. The same principle is now used in the pharmaceutical industry to design new drugs. The powerful anti-HIV drugs called protease inhibitors were designed in part as tight-binding transition-state analogs directed at the active site of HIV protease.

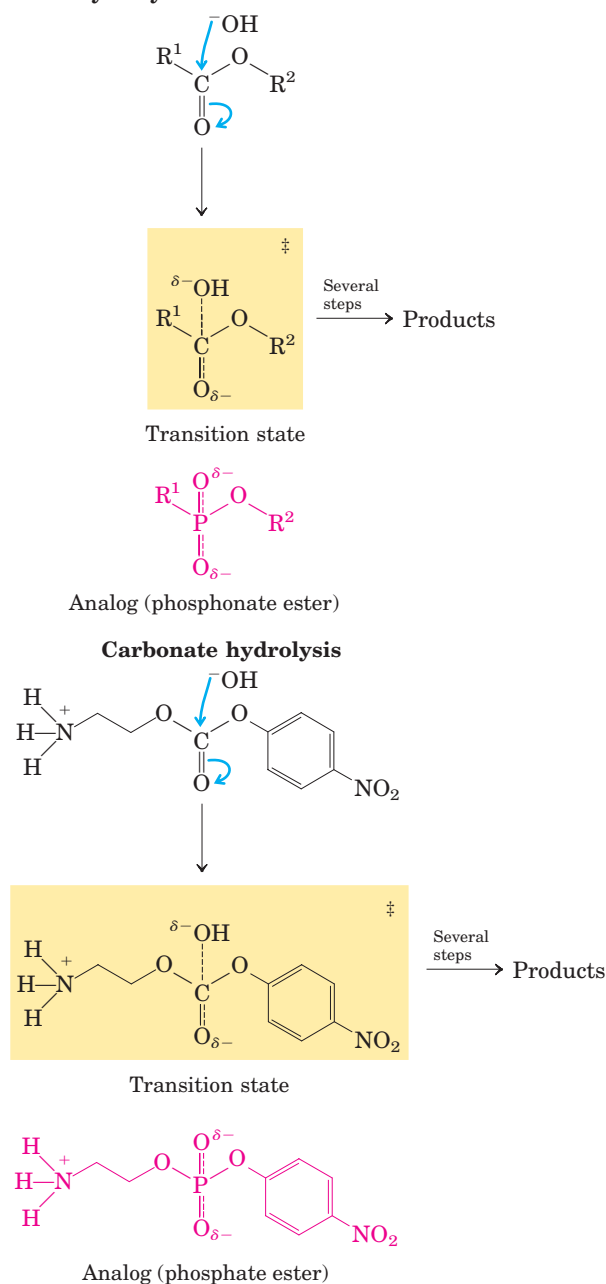
### Catalytic Antibodies

If a transition-state analog can be designed for the reaction  $S \rightarrow P$ , then an antibody that binds tightly to this analog might be expected to catalyze  $S \rightarrow P$ . Antibodies (immunoglobulins; see Fig. 5-23) are key components of the immune response. When a transition-state analog is used as a protein-bound epitope to stimulate the production of antibodies, the antibodies that bind it are potential catalysts of the corresponding reaction. This use of “catalytic antibodies,” first suggested by William P. Jencks in 1969, has become practical with the development of laboratory techniques to produce quantities of identical antibodies that bind one specific antigen (monoclonal antibodies; see Chapter 5).

Pioneering work in the laboratories of Richard Lerner and Peter Schultz has resulted in the isolation of a number of monoclonal antibodies that catalyze the hydrolysis of esters or carbonates (Fig. 2). In these reactions, the attack by water ( $\text{OH}^-$ ) on the carbonyl carbon produces a tetrahedral transition state in which a partial negative charge has developed on the carbonyl oxygen. Phosphonate ester compounds mimic the structure and charge distribution of this transition state in ester hydrolysis, making them good transition-state analogs; phosphate ester compounds are used for carbonate hydrolysis reactions. Antibodies that bind the phosphonate or phosphate compound tightly have been found to accelerate the corresponding ester or carbonate hydrolysis reaction by factors of  $10^3$  to  $10^4$ . Structural analyses of a few of these catalytic antibodies have shown that some catalytic amino acid side chains are arranged such that they could interact with the substrate in the transition state.

Catalytic antibodies generally do not approach the catalytic efficiency of enzymes, but medical and industrial uses for them are nevertheless emerging. For example, catalytic antibodies designed to degrade cocaine are being investigated as a potential aid in the treatment of cocaine addiction.

#### Ester hydrolysis



**FIGURE 2** The expected transition states for ester or carbonate hydrolysis reactions. Phosphonate ester and phosphate ester compounds, respectively, make good transition-state analogs for these reactions.



abstracting a proton from C-2 of 2-phosphoglycerate; then Glu<sup>211</sup> acts as a general acid catalyst, donating a proton to the —OH leaving group. The proton at C-2 of 2-phosphoglycerate is not very acidic and thus is not readily removed. However, in the enzyme active site, 2-phosphoglycerate undergoes strong ionic interactions with two bound Mg<sup>2+</sup> ions (Fig. 6-23b), making the C-2 proton more acidic (lowering the p*K*<sub>a</sub>) and easier to abstract. Hydrogen bonding to other active-site amino acid residues also contributes to the overall mechanism. The various interactions effectively stabilize both the enolate intermediate and the transition state preceding its formation.

### Lysozyme Uses Two Successive Nucleophilic Displacement Reactions

Lysozyme is a natural antibacterial agent found in tears and egg whites. The hen egg white lysozyme (*M*<sub>r</sub> 14,296) is a monomer with 129 amino acid residues. This was the first enzyme to have its three-dimensional structure determined, by David Phillips and colleagues in 1965. The structure revealed four stabilizing disulfide bonds and a cleft containing the active site (Fig. 6-24a; see also Fig. 4-18). More than five decades of lysozyme investigations have provided a detailed picture of the structure and activity of the enzyme, and an interesting story of how biochemical science progresses.

The substrate of lysozyme is peptidoglycan, a carbohydrate found in many bacterial cell walls (see Fig. 7-22). Lysozyme cleaves the (β1→4) glycosidic C—O bond between the two types of sugar residue in the molecule, *N*-acetylmuramic acid (Mur2Ac) and *N*-acetylglucosamine (GlcNAc), often referred to as NAM and NAG, respectively, in the research literature on enzymology (Fig. 6-24b). Six residues of the alternating Mur2Ac and GlcNAc in peptidoglycan bind in the active site, in binding sites labeled A through F. Model building has shown that the lactyl side chain of Mur2Ac cannot be accommodated in sites C and E, restricting Mur2Ac binding to sites B, D, and F. Only one of the bound glycosidic bonds is cleaved, that between a Mur2Ac residue in site D and a GlcNAc residue in site E. The key catalytic amino acid residues in the active site are Glu<sup>35</sup> and Asp<sup>52</sup> (Fig. 6-25a). The reaction is a nucleophilic substitution, with —OH from water replacing the GlcNAc at C-1 of Mur2Ac.

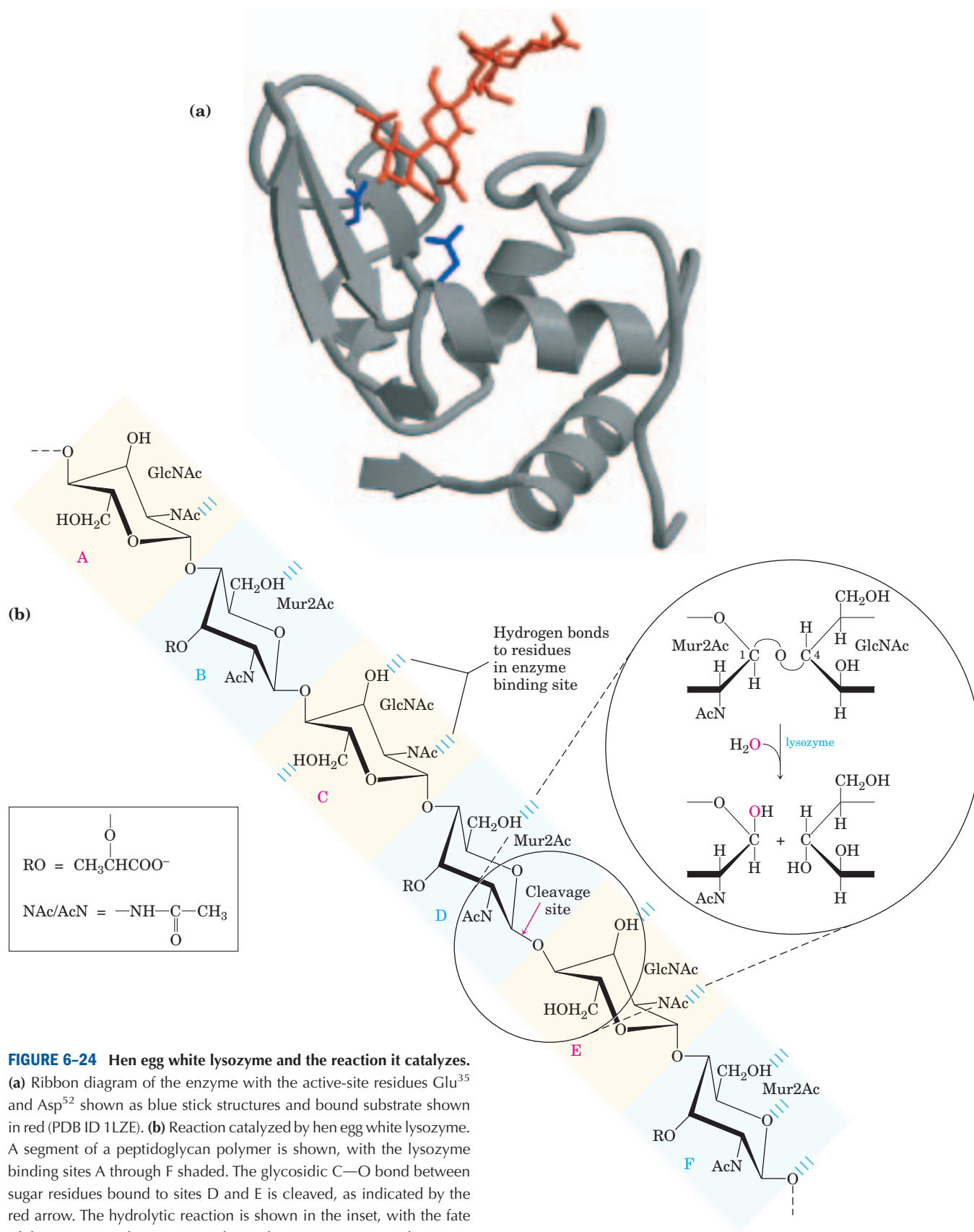
With the active site residues identified and a detailed structure of the enzyme available, the path to understanding the reaction mechanism seemed open in the 1960s. However, definitive evidence for a particular mechanism eluded investigators for nearly four decades. There are two chemically reasonable mechanisms that could generate the observed product of lysozyme-mediated cleavage of the glycosidic bond. Phillips and

colleagues proposed a dissociative (S<sub>N</sub>1-type) mechanism (Fig. 6-25a, left), in which the GlcNAc initially dissociates in step ① to leave behind a glycosyl cation (a carbocation) intermediate. In this mechanism, the departing GlcNAc is protonated by general acid catalysis by Glu<sup>35</sup>, located in a hydrophobic pocket that gives its carboxyl group an unusually high p*K*<sub>a</sub>. The carbocation is stabilized by resonance involving the adjacent ring oxygen, as well as by electrostatic interaction with the negative charge on the nearby Asp<sup>52</sup>. In step ②, water attacks at C-1 of Mur2Ac to yield the product. The alternative mechanism (Fig. 6-25a, right) involves two consecutive direct-displacement (S<sub>N</sub>2-type) steps. In step ①, Asp<sup>52</sup> attacks C-1 of Mur2Ac to displace the GlcNAc. As in the first mechanism, Glu<sup>35</sup> acts as a general acid to protonate the departing GlcNAc. In step ②, water attacks at C-1 of Mur2Ac to displace the Asp<sup>52</sup> and generate product.

The Phillips mechanism (S<sub>N</sub>1), based on structural considerations and bolstered by a variety of binding studies with artificial substrates, was widely accepted for more than three decades. However, some controversy persisted and tests continued. The scientific method sometimes advances an issue slowly, and a truly insightful experiment can be difficult to design. Some early arguments against the Phillips mechanism were suggestive but not completely persuasive. For example, the half-life of the proposed glycosyl cation was estimated to be 10<sup>-12</sup> seconds, just longer than a molecular vibration and not long enough for the needed diffusion of other molecules. More important, lysozyme is a member of a family of enzymes called “retaining glycosidases,” all of which catalyze reactions in which the product has the same anomeric configuration as the substrate (anomeric configurations of carbohydrates are examined in Chapter 7), and all of which are known to have reactive covalent intermediates like that envisioned in the alternative (S<sub>N</sub>2) pathway. Hence, the Phillips mechanism ran counter to experimental findings for closely related enzymes.

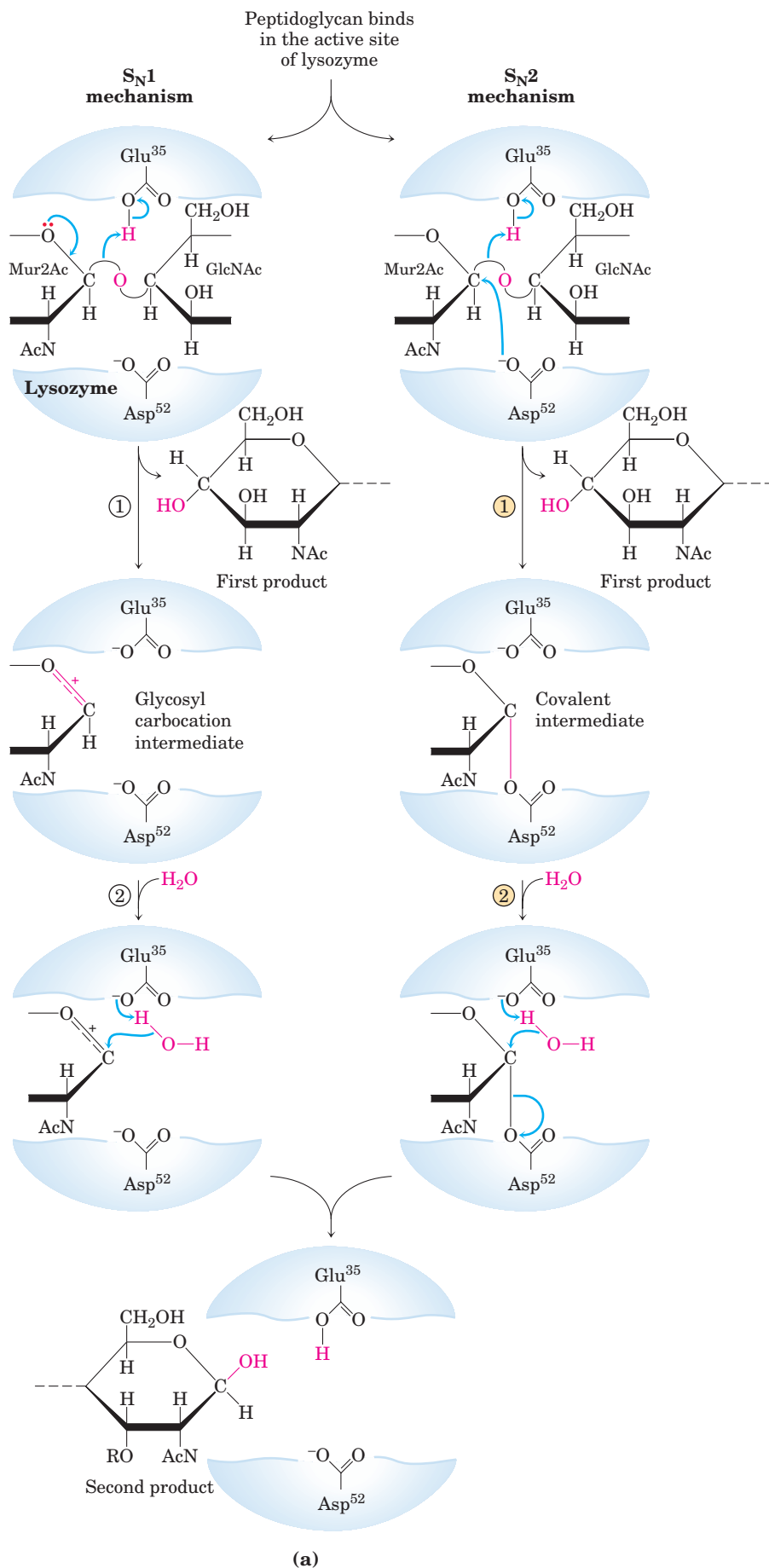
A compelling experiment tipped the scales decidedly in favor of the S<sub>N</sub>2 pathway, as reported by Stephen Withers and colleagues in 2001. Making use of a mutant enzyme (with residue 35 changed from Glu to Gln) and artificial substrates, which combined to slow the rate of key steps in the reaction, these workers were able to stabilize the elusive covalent intermediate. This in turn allowed them to observe the intermediate directly, using both mass spectrometry and x-ray crystallography (Fig. 6-25b).

Is the lysozyme mechanism now proven? No. A key feature of the scientific method, as Albert Einstein once summarized it, is “No amount of experimentation can ever prove me right; a single experiment can prove me wrong.” In the case of the lysozyme mechanism,

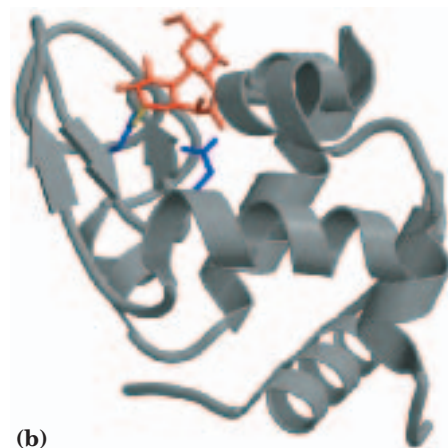


**FIGURE 6-24** Hen egg white lysozyme and the reaction it catalyzes.

(a) Ribbon diagram of the enzyme with the active-site residues Glu<sup>35</sup> and Asp<sup>52</sup> shown as blue stick structures and bound substrate shown in red (PDB ID 1LZE). (b) Reaction catalyzed by hen egg white lysozyme. A segment of a peptidoglycan polymer is shown, with the lysozyme binding sites A through F shaded. The glycosidic C—O bond between sugar residues bound to sites D and E is cleaved, as indicated by the red arrow. The hydrolytic reaction is shown in the inset, with the fate of the oxygen in the H<sub>2</sub>O traced in red. Mur2Ac is *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine. RO— represents a lactyl (lactic acid) group; —NAC and AcN—, an *N*-acetyl group (see key).

**MECHANISM FIGURE 6-25** Lysozyme

**reaction.** In this reaction (described on p. 222), the water introduced into the product at C-1 of Mur2Ac is in the same configuration as the original glycosidic bond. The reaction is thus a molecular substitution with retention of configuration. (a) Two proposed pathways potentially explain the overall reaction and its properties. The S<sub>N</sub>1 pathway (left) is the original Phillips mechanism. The S<sub>N</sub>2 pathway (right) is the mechanism most consistent with current data. (b) A ribbon diagram of the covalent enzyme-substrate intermediate with the active-site residues (blue) and bound substrate (red) shown as stick structures (PDB ID 1H6M).



one might argue (and some have) that the artificial substrates, with fluorine substitutions at C-1 and C-2, that were used to stabilize the covalent intermediate might have altered the reaction pathway. The highly electronegative fluorine could destabilize an already electron-deficient oxocarbenium ion in the glycosyl cation intermediate that might occur in an  $S_N1$  pathway. However, the  $S_N2$  pathway is now the mechanism most in concert with available data.

### SUMMARY 6.4 Examples of Enzymatic Reactions

- Chymotrypsin is a serine protease with a well-understood mechanism, featuring general acid-base catalysis, covalent catalysis, and transition-state stabilization.
- Hexokinase provides an excellent example of induced fit as a means of using substrate binding energy.
- The enolase reaction proceeds via metal ion catalysis.
- Lysozyme makes use of covalent catalysis and general acid catalysis as it promotes two successive nucleophilic displacement reactions.

## 6.5 Regulatory Enzymes

In cellular metabolism, groups of enzymes work together in sequential pathways to carry out a given metabolic process, such as the multireaction breakdown of glucose to lactate or the multireaction synthesis of an amino acid from simpler precursors. In such enzyme systems, the reaction product of one enzyme becomes the substrate of the next.

Most of the enzymes in each metabolic pathway follow the kinetic patterns we have already described. Each pathway, however, includes one or more enzymes that have a greater effect on the rate of the overall sequence. These **regulatory enzymes** exhibit increased or decreased catalytic activity in response to certain signals. Adjustments in the rate of reactions catalyzed by regulatory enzymes, and therefore in the rate of entire metabolic sequences, allow the cell to meet changing needs for energy and for biomolecules required in growth and repair.

In most multienzyme systems, the first enzyme of the sequence is a regulatory enzyme. This is an excellent place to regulate a pathway, because catalysis of even the first few reactions of a sequence that leads to an unneeded product diverts energy and metabolites from more important processes. Other enzymes in the sequence are usually present at levels that provide an excess of catalytic activity; they can generally promote

their reactions as fast as their substrates are made available from preceding reactions.

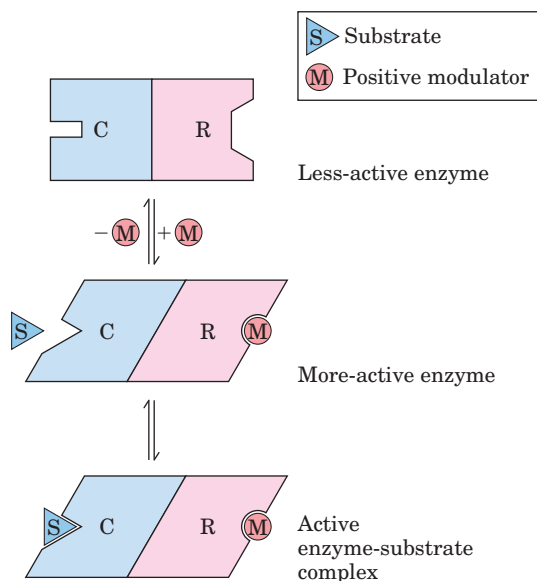
The activities of regulatory enzymes are modulated in a variety of ways. **Allosteric enzymes** function through reversible, noncovalent binding of regulatory compounds called **allosteric modulators** or **allosteric effectors**, which are generally small metabolites or cofactors. Other enzymes are regulated by reversible **covalent modification**. Both classes of regulatory enzymes tend to be multisubunit proteins, and in some cases the regulatory site(s) and the active site are on separate subunits. Metabolic systems have at least two other mechanisms of enzyme regulation. Some enzymes are stimulated or inhibited when they are bound by separate regulatory proteins. Others are activated when peptide segments are removed by proteolytic cleavage; unlike effector-mediated regulation, regulation by proteolytic cleavage is irreversible. Important examples of both mechanisms are found in physiological processes such as digestion, blood clotting, hormone action, and vision.

Cell growth and survival depend on efficient use of resources, and this efficiency is made possible by regulatory enzymes. No single rule governs the occurrence of different types of regulation in different systems. To a degree, allosteric (noncovalent) regulation may permit fine-tuning of metabolic pathways that are required continuously but at different levels of activity as cellular conditions change. Regulation by covalent modification may be all or none—usually the case with proteolytic cleavage—or it may allow for subtle changes in activity. Several types of regulation may occur in a single regulatory enzyme. The remainder of this chapter is devoted to a discussion of these methods of enzyme regulation.

### Allosteric Enzymes Undergo Conformational Changes in Response to Modulator Binding

As we saw in Chapter 5, allosteric proteins are those having “other shapes” or conformations induced by the binding of modulators. The same concept applies to certain regulatory enzymes, as conformational changes induced by one or more modulators interconvert more-active and less-active forms of the enzyme. The modulators for allosteric enzymes may be inhibitory or stimulatory. Often the modulator is the substrate itself; regulatory enzymes for which substrate and modulator are identical are called homotropic. The effect is similar to that of  $O_2$  binding to hemoglobin (Chapter 5): binding of the ligand—or substrate, in the case of enzymes—causes conformational changes that affect the subsequent activity of other sites on the protein. When the modulator is a molecule other than the substrate, the enzyme is said to be heterotropic. Note that allosteric modulators should not be confused with uncompetitive





**FIGURE 6-26** Subunit interactions in an allosteric enzyme, and interactions with inhibitors and activators. In many allosteric enzymes the substrate binding site and the modulator binding site(s) are on different subunits, the catalytic (C) and regulatory (R) subunits, respectively. Binding of the positive (stimulatory) modulator (M) to its specific site on the regulatory subunit is communicated to the catalytic subunit through a conformational change. This change renders the catalytic subunit active and capable of binding the substrate (S) with higher affinity. On dissociation of the modulator from the regulatory subunit, the enzyme reverts to its inactive or less active form.

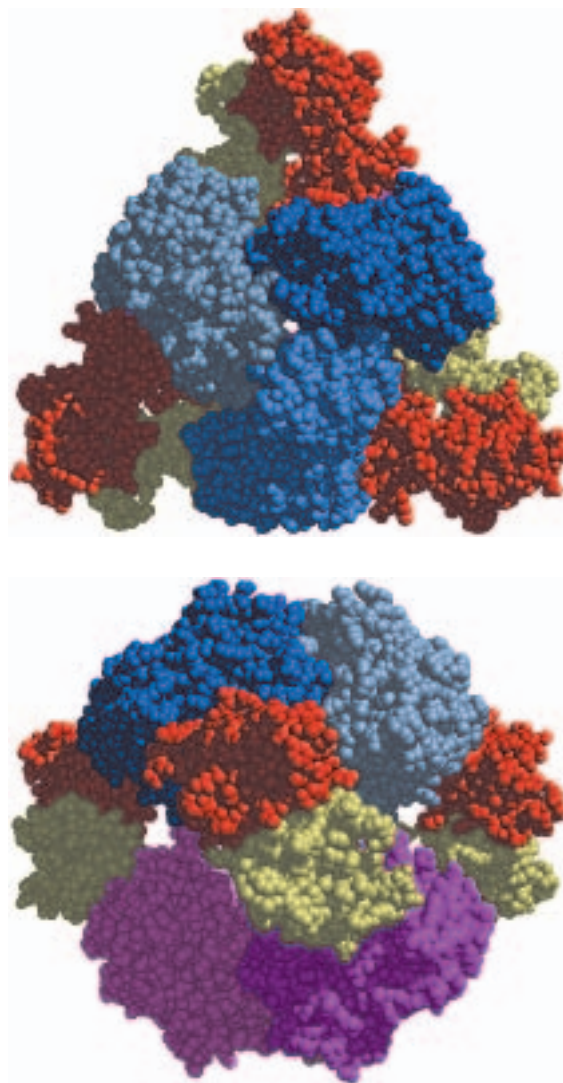
and mixed inhibitors. Although the latter bind at a second site on the enzyme, they do not necessarily mediate conformational changes between active and inactive forms, and the kinetic effects are distinct.

The properties of allosteric enzymes are significantly different from those of simple nonregulatory enzymes. Some of the differences are structural. In addition to active sites, allosteric enzymes generally have one or more regulatory, or allosteric, sites for binding the modulator (Fig. 6-26). Just as an enzyme's active site is specific for its substrate, each regulatory site is specific for its modulator. Enzymes with several modulators generally have different specific binding sites for each. In homotropic enzymes, the active site and regulatory site are the same.

Allosteric enzymes are generally larger and more complex than nonallosteric enzymes. Most have two or more subunits. Aspartate transcarbamoylase, which catalyzes an early reaction in the biosynthesis of pyrimidine nucleotides (see Fig. 22-36), has 12 polypeptide chains organized into catalytic and regulatory subunits. Figure 6-27 shows the quaternary structure of this enzyme, deduced from x-ray analysis.

### In Many Pathways a Regulated Step Is Catalyzed by an Allosteric Enzyme

In some multienzyme systems, the regulatory enzyme is specifically inhibited by the end product of the pathway whenever the concentration of the end product exceeds the cell's requirements. When the regulatory enzyme reaction is slowed, all subsequent enzymes operate at reduced rates as their substrates are depleted. The rate



**FIGURE 6-27** Two views of the regulatory enzyme aspartate transcarbamoylase. (Derived from PDB ID 2AT2.) This allosteric regulatory enzyme has two stacked catalytic clusters, each with three catalytic polypeptide chains (in shades of blue and purple), and three regulatory clusters, each with two regulatory polypeptide chains (in red and yellow). The regulatory clusters form the points of a triangle surrounding the catalytic subunits. Binding sites for allosteric modulators are on the regulatory subunits. Modulator binding produces large changes in enzyme conformation and activity. The role of this enzyme in nucleotide synthesis, and details of its regulation, are discussed in Chapter 22.

of production of the pathway's end product is thereby brought into balance with the cell's needs. This type of regulation is called **feedback inhibition**. Buildup of the end product ultimately slows the entire pathway.

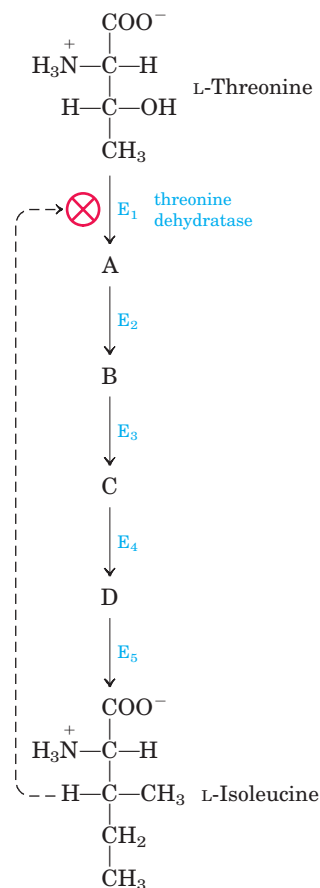
One of the first known examples of allosteric feedback inhibition was the bacterial enzyme system that catalyzes the conversion of L-threonine to L-isoleucine in five steps (Fig. 6–28). In this system, the first enzyme, threonine dehydratase, is inhibited by isoleucine, the product of the last reaction of the series. This is an example of heterotropic allosteric inhibition. Isoleucine is quite specific as an inhibitor. No other intermediate in this sequence inhibits threonine dehydratase, nor is any other enzyme in the sequence inhibited by isoleucine. Isoleucine binds not to the active site but to another specific site on the enzyme molecule, the regulatory site. This binding is noncovalent and readily reversible; if the isoleucine concentration decreases, the rate of threonine dehydration increases. Thus threonine dehydratase activity responds rapidly and reversibly to fluctuations in the cellular concentration of isoleucine.

### The Kinetic Properties of Allosteric Enzymes Diverge from Michaelis-Menten Behavior

Allosteric enzymes show relationships between  $V_0$  and  $[S]$  that differ from Michaelis-Menten kinetics. They do exhibit saturation with the substrate when  $[S]$  is sufficiently high, but for some allosteric enzymes, plots of  $V_0$  versus  $[S]$  (Fig. 6–29) produce a sigmoid saturation curve, rather than the hyperbolic curve typical of non-regulatory enzymes. On the sigmoid saturation curve we can find a value of  $[S]$  at which  $V_0$  is half-maximal, but we cannot refer to it with the designation  $K_m$ , because the enzyme does not follow the hyperbolic Michaelis-Menten relationship. Instead, the symbol  $[S]_{0.5}$  or  $K_{0.5}$  is often used to represent the substrate concentration giving half-maximal velocity of the reaction catalyzed by an allosteric enzyme (Fig. 6–29).

Sigmoid kinetic behavior generally reflects cooperative interactions between protein subunits. In other words, changes in the structure of one subunit are translated into structural changes in adjacent subunits, an effect mediated by noncovalent interactions at the interface between subunits. The principles are particularly well illustrated by a nonenzyme:  $O_2$  binding to hemoglobin. Sigmoid kinetic behavior is explained by the concerted and sequential models for subunit interactions (see Fig. 5–15).

Homotropic allosteric enzymes generally are multi-subunit proteins and, as noted earlier, the same binding site on each subunit functions as both the active site and the regulatory site. Most commonly, the substrate acts as a positive modulator (an activator), because the subunits act cooperatively: the binding of one molecule

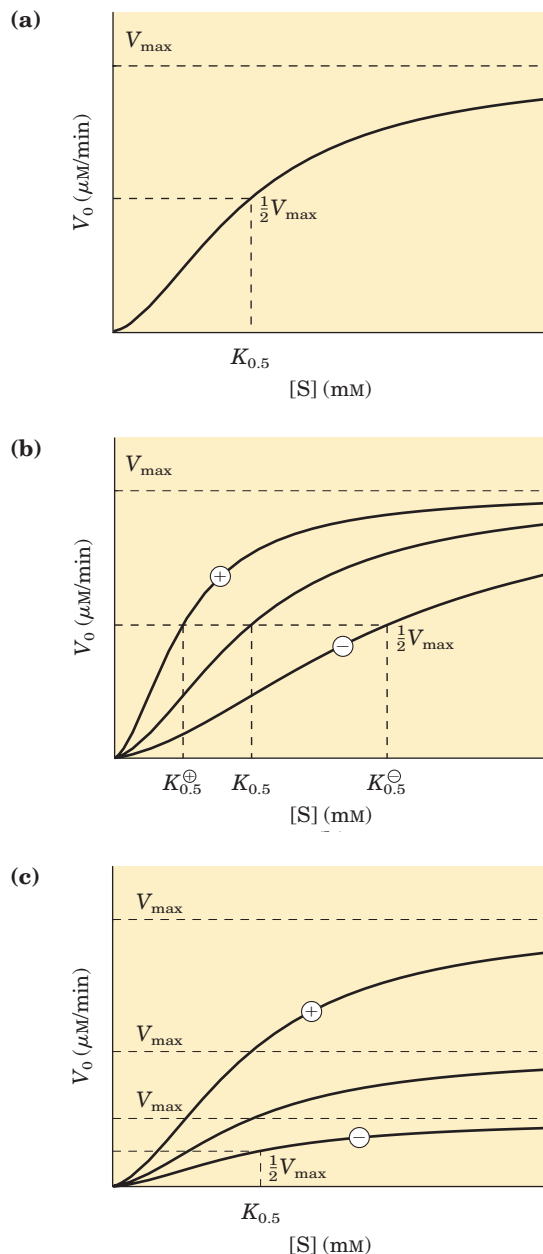


**FIGURE 6–28 Feedback inhibition.** The conversion of L-threonine to L-isoleucine is catalyzed by a sequence of five enzymes ( $E_1$  to  $E_5$ ). Threonine dehydratase ( $E_1$ ) is specifically inhibited allosterically by L-isoleucine, the end product of the sequence, but not by any of the four intermediates (A to D). Feedback inhibition is indicated by the dashed feedback line and the  $\otimes$  symbol at the threonine dehydratase reaction arrow, a device used throughout this book.

of substrate to one binding site alters the enzyme's conformation and enhances the binding of subsequent substrate molecules. This accounts for the sigmoid rather than hyperbolic change in  $V_0$  with increasing  $[S]$ . One characteristic of sigmoid kinetics is that small changes in the concentration of a modulator can be associated with large changes in activity. As is evident in Figure 6–29a, a relatively small increase in  $[S]$  in the steep part of the curve causes a comparatively large increase in  $V_0$ .

For heterotropic allosteric enzymes, those whose modulators are metabolites other than the normal substrate, it is difficult to generalize about the shape of the substrate-saturation curve. An activator may cause the curve to become more nearly hyperbolic, with a decrease in  $K_{0.5}$  but no change in  $V_{\max}$ , resulting in an increased reaction velocity at a fixed substrate concentration ( $V_0$  is higher for any value of  $[S]$ ; Fig. 6–29b, upper curve).

Other heterotropic allosteric enzymes respond to an activator by an increase in  $V_{\max}$  with little change in  $K_{0.5}$  (Fig. 6–29c). A negative modulator (an inhibitor) may produce a *more* sigmoid substrate-saturation curve,



**FIGURE 6-29** Substrate-activity curves for representative allosteric enzymes. Three examples of complex responses of allosteric enzymes to their modulators. **(a)** The sigmoid curve of a homotropic enzyme, in which the substrate also serves as a positive (stimulatory) modulator, or activator. Note the resemblance to the oxygen-saturation curve of hemoglobin (see Fig. 5–12). **(b)** The effects of a positive modulator (+) and a negative modulator (–) on an allosteric enzyme in which  $K_{0.5}$  is altered without a change in  $V_{\max}$ . The central curve shows the substrate-activity relationship without a modulator. **(c)** A less common type of modulation, in which  $V_{\max}$  is altered and  $K_{0.5}$  is nearly constant.

with an increase in  $K_{0.5}$  (Fig. 6–29b, lower curve). Heterotropic allosteric enzymes therefore show different kinds of responses in their substrate-activity curves, because some have inhibitory modulators, some have activating modulators, and some have both.

### Some Regulatory Enzymes Undergo Reversible Covalent Modification

In another important class of regulatory enzymes, activity is modulated by covalent modification of the enzyme molecule. Modifying groups include phosphoryl, adenylyl, uridylyl, methyl, and adenosine diphosphate ribosyl groups (Fig. 6–30). These groups are generally linked to and removed from the regulatory enzyme by separate enzymes.

An example of an enzyme regulated by methylation is the methyl-accepting chemotaxis protein of bacteria. This protein is part of a system that permits a bacterium to swim toward an attractant (such as a sugar) in solution and away from repellent chemicals. The methylating agent is *S*-adenosylmethionine (adoMet) (see Fig. 18–18b). ADP-ribosylation is an especially interesting reaction, observed in only a few proteins; the ADP-ribose is derived from nicotinamide adenine dinucleotide (NAD) (see Fig. 8–41). This type of modification occurs for the bacterial enzyme dinitrogenase reductase, resulting in regulation of the important process of biological nitrogen fixation. Diphtheria toxin and cholera toxin are enzymes that catalyze the ADP-ribosylation (and inactivation) of key cellular enzymes or proteins. Diphtheria toxin acts on and inhibits elongation factor 2, a protein involved in protein biosynthesis. Cholera toxin acts on a G protein that is part of a signaling pathway (see Fig. 12–39), leading to several physiological responses including a massive loss of body fluids and, sometimes, death.

Phosphorylation is the most common type of regulatory modification; one-third to one-half of all proteins in a eukaryotic cell are phosphorylated. Some proteins have only one phosphorylated residue, others have several, and a few have dozens of sites for phosphorylation. This mode of covalent modification is central to a large number of regulatory pathways, and we therefore discuss it in considerable detail.

### Phosphoryl Groups Affect the Structure and Catalytic Activity of Proteins

The attachment of phosphoryl groups to specific amino acid residues of a protein is catalyzed by **protein kinases**; removal of phosphoryl groups is catalyzed by **protein phosphatases**. The addition of a phosphoryl group to a Ser, Thr, or Tyr residue introduces a bulky, charged group into a region that was only moderately polar. The oxygen atoms of a phosphoryl group can hydrogen-bond with one or several groups in a protein, commonly the

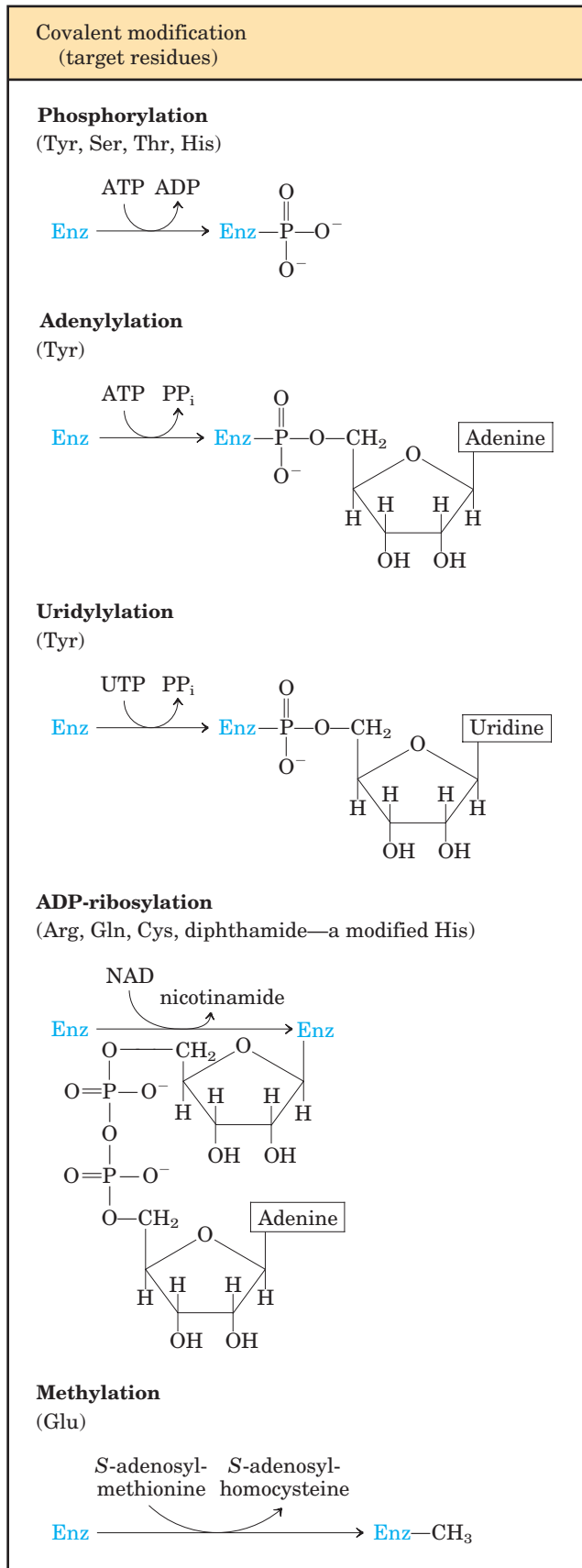
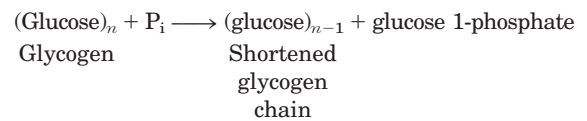


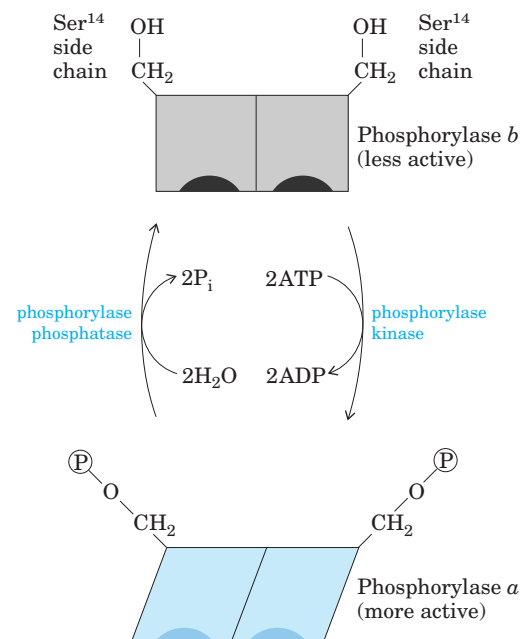
FIGURE 6-30 Some enzyme modification reactions.

amide groups of the peptide backbone at the start of an  $\alpha$  helix or the charged guanidinium group of an Arg residue. The two negative charges on a phosphorylated side chain can also repel neighboring negatively charged (Asp or Glu) residues. When the modified side chain is located in a region of the protein critical to its three-dimensional structure, phosphorylation can have dramatic effects on protein conformation and thus on substrate binding and catalysis.

An important example of regulation by phosphorylation is seen in glycogen phosphorylase ( $M_r$  94,500) of muscle and liver (Chapter 15), which catalyzes the reaction



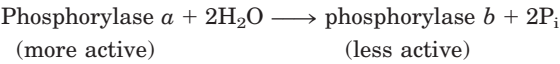
The glucose 1-phosphate so formed can be used for ATP synthesis in muscle or converted to free glucose in the liver. Glycogen phosphorylase occurs in two forms: the more active phosphorylase *a* and the less active phosphorylase *b* (Fig. 6-31). Phosphorylase *a* has two subunits, each with a specific Ser residue that is phosphorylated at its hydroxyl group. These serine phosphate residues are required for maximal activity of the enzyme.



**FIGURE 6-31 Regulation of glycogen phosphorylase activity by covalent modification.** In the more active form of the enzyme, phosphorylase *a*, specific Ser residues, one on each subunit, are phosphorylated. Phosphorylase *a* is converted to the less active phosphorylase *b* by enzymatic loss of these phosphoryl groups, promoted by phosphorylase phosphatase. Phosphorylase *b* can be reconverted (reactivated) to phosphorylase *a* by the action of phosphorylase kinase.

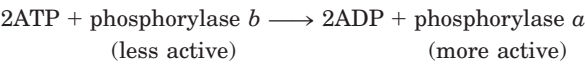


The phosphoryl groups can be hydrolytically removed by a separate enzyme called phosphorylase phosphatase:



In this reaction, phosphorylase *a* is converted to phosphorylase *b* by the cleavage of two serine phosphate covalent bonds, one on each subunit of glycogen phosphorylase.

Phosphorylase *b* can in turn be reactivated—covalently transformed back into active phosphorylase *a*—by another enzyme, phosphorylase kinase, which catalyzes the transfer of phosphoryl groups from ATP to the hydroxyl groups of the two specific Ser residues in phosphorylase *b*:



The breakdown of glycogen in skeletal muscles and the liver is regulated by variations in the ratio of the two forms of glycogen phosphorylase. The *a* and *b* forms differ in their secondary, tertiary, and quaternary structures; the active site undergoes changes in structure and, consequently, changes in catalytic activity as the two forms are interconverted.

The regulation of glycogen phosphorylase by phosphorylation illustrates the effects on both structure and catalytic activity of adding a phosphoryl group. In the unphosphorylated state, each subunit of this protein is folded so as to bring the 20 residues at its amino terminus, including a number of basic residues, into a region containing several acidic amino acids; this produces an electrostatic interaction that stabilizes the conformation. Phosphorylation of Ser<sup>14</sup> interferes with this interaction, forcing the amino-terminal domain out of the acidic environment and into a conformation that allows interaction between the  $\text{P}^-$ -Ser and several Arg side chains. In this conformation, the enzyme is much more active.

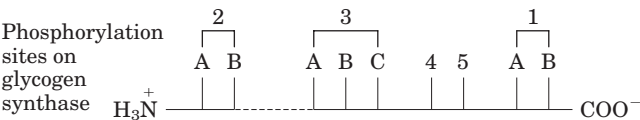
Phosphorylation of an enzyme can affect catalysis in another way: by altering substrate-binding affinity. For example, when isocitrate dehydrogenase (an enzyme of the citric acid cycle; Chapter 16) is phosphorylated, electrostatic repulsion by the phosphoryl group inhibits the binding of citrate (a tricarboxylic acid) at the active site.

Multiple Phosphorylations Allow Exquisite Regulatory Control

The Ser, Thr, or Tyr residues that are phosphorylated in regulated proteins occur within common structural motifs, called consensus sequences, that are recognized by specific protein kinases (Table 6-10). Some kinases are basophilic, preferring to phosphorylate a residue having basic neighbors; others have different substrate preferences, such as for a residue near a Pro residue.

Primary sequence is not the only important factor in determining whether a given residue will be phosphorylated, however. Protein folding brings together residues that are distant in the primary sequence; the resulting three-dimensional structure can determine whether a protein kinase has access to a given residue and can recognize it as a substrate. Another factor influencing the substrate specificity of certain protein kinases is the proximity of other phosphorylated residues.

Regulation by phosphorylation is often complicated. Some proteins have consensus sequences recognized by several different protein kinases, each of which can phosphorylate the protein and alter its enzymatic activity. In some cases, phosphorylation is hierarchical: a certain residue can be phosphorylated only if a neighboring residue has already been phosphorylated. For example, glycogen synthase, the enzyme that catalyzes the condensation of glucose monomers to form glycogen (Chapter 15), is inactivated by phosphorylation of specific Ser residues and is also modulated by at least four other protein kinases that phosphorylate four other sites in the protein (Fig. 6-32). The protein is not a substrate for glycogen synthase kinase 3, for example, until one site has been phosphorylated by casein kinase II. Some phosphorylations inhibit glycogen synthase more than



Kinase	Phosphorylation sites	Degree of synthase inactivation
Protein kinase A	1A, 1B, 2, 4	+
Protein kinase G	1A, 1B, 2	+
Protein kinase C	1A	+
Ca <sup>2+</sup> /calmodulin kinase	1B, 2	+
Phosphorylase <i>b</i> kinase	2	+
Casein kinase I	At least nine	+ + + +
Casein kinase II	5	0
Glycogen synthase kinase 3	3A, 3B, 3C	+ + +
Glycogen synthase kinase 4	2	+

**FIGURE 6-32 Multiple regulatory phosphorylations.** The enzyme glycogen synthase has at least nine separate sites in five designated regions susceptible to phosphorylation by one of the cellular protein kinases. Thus, regulation of this enzyme is a matter not of binary (on/off) switching but of finely tuned modulation of activity over a wide range in response to a variety of signals.

**TABLE 6-10** Consensus Sequences for Protein Kinases

Protein kinase	Consensus sequence and phosphorylated residue*
Protein kinase A	-X-R-(R/K)-X-( <b>S/T</b> )-B-
Protein kinase G	-X-R-(R/K)-X-( <b>S/T</b> )-X-
Protein kinase C	-(R/K)-(R/K)-X-( <b>S/T</b> )-B-(R/K)-(R/K)-
Protein kinase B	-X-R-X-( <b>S/T</b> )-X-K-
Ca <sup>2+</sup> /calmodulin kinase I	-B-X-R-X-X-( <b>S/T</b> )-X-X-X-B-
Ca <sup>2+</sup> /calmodulin kinase II	-B-X-(R/K)-X-X-( <b>S/T</b> )-X-X-
Myosin light chain kinase (smooth muscle)	-K-K-R-X-X- <b>S</b> -X-B-B-
Phosphorylase b kinase	-K-R-K-Q-I- <b>S</b> -V-R-
Extracellular signal-regulated kinase (ERK)	-P-X-( <b>S/T</b> )-P-P-
Cyclin-dependent protein kinase (cdc2)	-X-( <b>S/T</b> )-P-X-(K/R)-
Casein kinase I	-(Sp/Tp)-X-X-(X)-( <b>S/T</b> )-B
Casein kinase II	-X-( <b>S/T</b> )-X-X-(E/D/Sp/Yp)-X-
$\beta$ -Adrenergic receptor kinase	-(D/E) <sub>n</sub> -( <b>S/T</b> )-X-X-X-
Rhodopsin kinase	-X-X-( <b>S/T</b> )-(E) <sub>n</sub> -
Insulin receptor kinase	-X-E-E-E- <b>Y</b> -M-M-M-M-K-K-S-R-G-D- <b>Y</b> -M-T-M-Q-I-G-K-K-K- L-P-A-T-G-D- <b>Y</b> -M-N-M-S-P-V-G-D-
Epidermal growth factor (EGF) receptor kinase	-E-E-E-E- <b>Y</b> -F-E-L-V-

**Sources:** Pinna, L.A. & Ruzzene, M.H. (1996) How do protein kinases recognize their substrates? *Biochim. Biophys. Acta* **1314**, 191-225; Kemp, B.E. & Pearson, R.B. (1990) Protein kinase recognition sequence motifs. *Trends Biochem. Sci.* **15**, 342-346; Kennelly, P.J. & Krebs, E.G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**, 15,555-15,558.

\*Shown here are deduced consensus sequences (in roman type) and actual sequences from known substrates (italic). The Ser (S), Thr (T), or Tyr (Y) residue that undergoes phosphorylation is in red; all amino acid residues are shown as their one-letter abbreviations (see Table 3-1). X represents any amino acid; B, any hydrophobic amino acid; Sp, Tp, and Yp, already phosphorylated Ser, Thr, and Tyr residues.

others, and some combinations of phosphorylations are cumulative. These multiple regulatory phosphorylations provide the potential for extremely subtle modulation of enzyme activity.

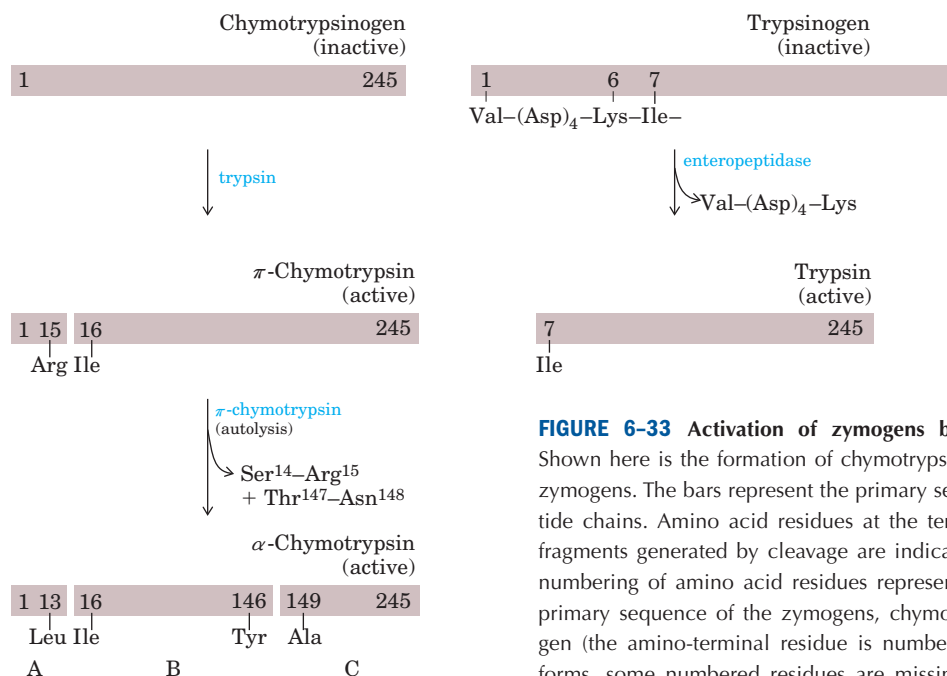
To serve as an effective regulatory mechanism, phosphorylation must be reversible. In general, phosphoryl groups are added and removed by different enzymes, and the processes can therefore be separately regulated. Cells contain a family of phosphoprotein phosphatases that hydrolyze specific  $\text{P-Ser}$ ,  $\text{P-Thr}$ , and  $\text{P-Tyr}$  esters, releasing  $\text{P}_i$ . The phosphoprotein phosphatases we know of thus far act only on a subset of phosphoproteins, but they show less substrate specificity than protein kinases.

### Some Enzymes and Other Proteins Are Regulated by Proteolytic Cleavage of an Enzyme Precursor

For some enzymes, an inactive precursor called a **zymogen** is cleaved to form the active enzyme. Many proteolytic enzymes (proteases) of the stomach and pancreas are regulated in this way. Chymotrypsin and trypsin are initially synthesized as chymotrypsinogen and trypsinogen (Fig. 6-33). Specific cleavage causes conformational changes that expose the enzyme active site. Because this type of activation is irreversible, other

mechanisms are needed to inactivate these enzymes. Proteases are inactivated by inhibitor proteins that bind very tightly to the enzyme active site. For example, pancreatic trypsin inhibitor ( $M_r$  6,000) binds to and inhibits trypsin;  $\alpha_1$ -antiprotease ( $M_r$  53,000) primarily inhibits neutrophil elastase (neutrophils are a type of leukocyte, or white blood cell; elastase is a protease acting on elastin, a component of some connective tissues). An insufficiency of  $\alpha_1$ -antiprotease, which can be caused by exposure to cigarette smoke, has been associated with lung damage, including emphysema.

Proteases are not the only proteins activated by proteolysis. In other cases, however, the precursors are called not zymogens but, more generally, **proproteins** or **proenzymes**, as appropriate. For example, the connective tissue protein collagen is initially synthesized as the soluble precursor procollagen. The blood clotting system provides many examples of the proteolytic activation of proteins. Fibrin, the protein of blood clots, is produced by proteolysis of fibrinogen, its inactive proprotein. The protease responsible for this activation is thrombin (similar in many respects to chymotrypsin), which itself is produced by proteolysis of a proprotein (in this case a zymogen), prothrombin. Blood clotting is mediated by a complicated cascade of proteolytic activations.



**FIGURE 6-33 Activation of zymogens by proteolytic cleavage.**

Shown here is the formation of chymotrypsin and trypsin from their zymogens. The bars represent the primary sequences of the polypeptide chains. Amino acid residues at the termini of the polypeptide fragments generated by cleavage are indicated below the bars. The numbering of amino acid residues represents their positions in the primary sequence of the zymogens, chymotrypsinogen or trypsinogen (the amino-terminal residue is number 1). Thus, in the active forms, some numbered residues are missing. Recall that the three polypeptide chains (A, B, and C) of chymotrypsin are linked by disulfide bonds (see Fig. 6-18).

### Some Regulatory Enzymes Use Several Regulatory Mechanisms

Glycogen phosphorylase catalyzes the first reaction in a pathway that feeds stored glucose into energy-yielding carbohydrate metabolism (Chapters 14 and 15). This is an important metabolic step, and its regulation is correspondingly complex. Although its primary regulation is through covalent modification, as outlined in Figure 6-31, glycogen phosphorylase is also modulated allosterically by AMP, which is an activator of phosphorylase *b*, and by several other molecules that are inhibitors.

Other complex regulatory enzymes are found at key metabolic crossroads. Bacterial glutamine synthetase, which catalyzes a reaction that introduces reduced nitrogen into cellular metabolism (Chapter 22), is among the most complex regulatory enzymes known. It is regulated allosterically (with at least eight different modulators); by reversible covalent modification; and by the association of other regulatory proteins, a mechanism examined in detail when we consider the regulation of specific metabolic pathways.

What is the advantage of such complexity in the regulation of enzymatic activity? We began this chapter by stressing the central importance of catalysis to the very existence of life. The *control* of catalysis is also critical to life. If all possible reactions in a cell were catalyzed simultaneously, macromolecules and metabolites would quickly be broken down to much simpler chem-

ical forms. Instead, cells catalyze only the reactions they need at a given moment. When chemical resources are plentiful, cells synthesize and store glucose and other metabolites. When chemical resources are scarce, cells use these stores to fuel cellular metabolism. Chemical energy is used economically, parceled out to various metabolic pathways as cellular needs dictate. The availability of powerful catalysts, each specific for a given reaction, makes the regulation of these reactions possible. This in turn gives rise to the complex, highly regulated symphony we call life.

### SUMMARY 6.5 Regulatory Enzymes

- The activities of metabolic pathways in cells are regulated by control of the activities of certain enzymes.
- In feedback inhibition, the end product of a pathway inhibits the first enzyme of that pathway.
- The activity of allosteric enzymes is adjusted by reversible binding of a specific modulator to a regulatory site. Modulators may be the substrate itself or some other metabolite, and the effect of the modulator may be inhibitory or stimulatory. The kinetic behavior of allosteric enzymes reflects cooperative interactions among enzyme subunits.

- Other regulatory enzymes are modulated by covalent modification of a specific functional group necessary for activity. The phosphorylation of specific amino acid residues is a particularly common way to regulate enzyme activity.
- Many proteolytic enzymes are synthesized as inactive precursors called zymogens, which are activated by cleavage of small peptide fragments.
- Enzymes at important metabolic intersections may be regulated by complex combinations of effectors, allowing coordination of the activities of interconnected pathways.

## Key Terms

*Terms in bold are defined in the glossary.*

<b>enzyme</b> 191	<b>rate constant</b> 195	$k_{\text{cat}}$ 206
<b>cofactor</b> 191	<b>binding energy (<math>\Delta G_{\text{B}}</math>)</b> 196	<b>turnover number</b> 207
<b>coenzyme</b> 191	<b>specificity</b> 199	reversible inhibition 209
<b>prosthetic group</b> 192	<b>induced fit</b> 200	<b>competitive inhibition</b> 209
<b>holoenzyme</b> 192	<b>specific acid-base catalysis</b> 200	<b>uncompetitive inhibition</b> 211
<b>apoenzyme</b> 192	<b>general acid-base catalysis</b> 200	<b>mixed inhibition</b> 211
<b>apoprotein</b> 192	covalent catalysis 200	noncompetitive inhibition 211
<b>active site</b> 193	enzyme kinetics 202	irreversible inhibitors 211
<b>substrate</b> 193	initial rate (initial velocity), $V_0$ 202	<b>suicide inactivator</b> 211
<b>ground state</b> 193	$V_{\text{max}}$ 203	transition state analogs 220
<b>standard free-energy change (<math>\Delta G^\circ</math>)</b> 194	pre-steady state 203	<b>regulatory enzyme</b> 225
<b>transition state</b> 194	<b>steady state</b> 203	<b>allosteric enzyme</b> 225
<b>activation energy (<math>\Delta G^\ddagger</math>)</b> 194	steady-state kinetics 203	allosteric modulator 225
<b>reaction intermediate</b> 195	<b>Michaelis constant (<math>K_{\text{m}}</math>)</b> 204	<b>feedback inhibition</b> 227
<b>rate-limiting step</b> 195	<b>Michaelis-Menten equation</b> 204	<b>protein kinases</b> 228
<b>equilibrium constant (<math>K_{\text{eq}}</math>)</b> 195	<b>dissociation constant (<math>K_{\text{d}}</math>)</b> 205	<b>zymogen</b> 231
	<b>Lineweaver-Burk equation</b> 206	

## Further Reading

### General

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A collection of excellent papers on fundamentals; continues to be very useful.

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A collection of classic papers on enzyme chemistry, with historical commentaries by the editor. Extremely interesting.

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An outstanding book on the subject. More advanced.

**Kornberg, A.** (1989) *For the Love of Enzymes: The Odyssey of a Biochemist*, Harvard University Press, Cambridge.

### Principles of Catalysis

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## Enzyme Examples

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A classic paper introducing the concept of allosteric regulation.

## Problems

**1. Keeping the Sweet Taste of Corn** The sweet taste of freshly picked corn (maize) is due to the high level of sugar in the kernels. Store-bought corn (several days after picking) is not as sweet, because about 50% of the free sugar is converted to starch within one day of picking. To preserve the sweetness of fresh corn, the husked ears can be immersed in boiling water for a few minutes (“blanched”) then cooled in cold water. Corn processed in this way and stored in a freezer maintains its sweetness. What is the biochemical basis for this procedure?

**2. Intracellular Concentration of Enzymes** To approximate the actual concentration of enzymes in a bacterial cell, assume that the cell contains equal concentrations of 1,000 different enzymes in solution in the cytosol and that each protein has a molecular weight of 100,000. Assume also that the bacterial cell is a cylinder (diameter 1.0  $\mu\text{m}$ , height 2.0  $\mu\text{m}$ ), that the cytosol (specific gravity 1.20) is 20% soluble protein by weight, and that the soluble protein consists entirely of enzymes. Calculate the *average* molar concentration of each enzyme in this hypothetical cell.

**3. Rate Enhancement by Urease** The enzyme urease enhances the rate of urea hydrolysis at pH 8.0 and 20 °C by a factor of  $10^{14}$ . If a given quantity of urease can completely hydrolyze a given quantity of urea in 5.0 min at 20 °C and pH 8.0, how long would it take for this amount of urea to be hydrolyzed under the same conditions in the absence of urease? Assume that both reactions take place in sterile systems so that bacteria cannot attack the urea.

**4. Protection of an Enzyme against Denaturation by Heat** When enzyme solutions are heated, there is a progressive loss of catalytic activity over time due to denaturation of the enzyme. A solution of the enzyme hexokinase incubated at 45 °C lost 50% of its activity in 12 min, but when incubated at 45 °C in the presence of a very large concentration of one of its substrates, it lost only 3% of its activity in 12 min. Suggest why thermal denaturation of hexokinase was retarded in the presence of one of its substrates.

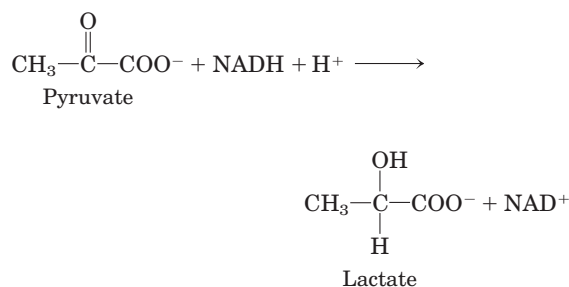
**5. Requirements of Active Sites in Enzymes** Carboxypeptidase, which sequentially removes carboxyl-terminal

amino acid residues from its peptide substrates, is a single polypeptide of 307 amino acids. The two essential catalytic groups in the active site are furnished by Arg<sup>145</sup> and Glu<sup>270</sup>.

(a) If the carboxypeptidase chain were a perfect  $\alpha$  helix, how far apart (in Å) would Arg<sup>145</sup> and Glu<sup>270</sup> be? (Hint: See Fig. 4-4b.)

(b) Explain how the two amino acid residues can catalyze a reaction occurring in the space of a few angstroms.

**6. Quantitative Assay for Lactate Dehydrogenase** The muscle enzyme lactate dehydrogenase catalyzes the reaction



NADH and NAD<sup>+</sup> are the reduced and oxidized forms, respectively, of the coenzyme NAD. Solutions of NADH, but *not* NAD<sup>+</sup>, absorb light at 340 nm. This property is used to determine the concentration of NADH in solution by measuring spectrophotometrically the amount of light absorbed at 340 nm by the solution. Explain how these properties of NADH can be used to design a quantitative assay for lactate dehydrogenase.

**7. Relation between Reaction Velocity and Substrate Concentration: Michaelis-Menten Equation** (a) At what substrate concentration would an enzyme with a  $k_{\text{cat}}$  of 30.0 s<sup>-1</sup> and a  $K_{\text{m}}$  of 0.0050 M operate at one-quarter of its maximum rate? (b) Determine the fraction of  $V_{\text{max}}$  that would be obtained at the following substrate concentrations:  $[S] = \frac{1}{2}K_{\text{m}}$ ,  $2K_{\text{m}}$ , and  $10K_{\text{m}}$ .

**8. Estimation of  $V_{\text{max}}$  and  $K_{\text{m}}$  by Inspection** Although graphical methods are available for accurate determination of the  $V_{\text{max}}$  and  $K_{\text{m}}$  of an enzyme-catalyzed reaction (see Box 6-1), sometimes these quantities can be quickly estimated by inspecting values of  $V_0$  at increasing  $[S]$ . Estimate the  $V_{\text{max}}$  and  $K_{\text{m}}$  of the enzyme-catalyzed reaction for which the following data were obtained.

[S] (M)	$V_0$ ( $\mu\text{M}/\text{min}$ )
$2.5 \times 10^{-6}$	28
$4.0 \times 10^{-6}$	40
$1 \times 10^{-5}$	70
$2 \times 10^{-5}$	95
$4 \times 10^{-5}$	112
$1 \times 10^{-4}$	128
$2 \times 10^{-3}$	139
$1 \times 10^{-2}$	140


**9. Properties of an Enzyme of Prostaglandin Synthesis** Prostaglandins are a class of eicosanoids, fatty acid derivatives with a variety of extremely potent actions on

vertebrate tissues. They are responsible for producing fever and inflammation and its associated pain. Prostaglandins are derived from the 20-carbon fatty acid arachidonic acid in a reaction catalyzed by the enzyme prostaglandin endoperoxide synthase. This enzyme, a cyclooxygenase, uses oxygen to convert arachidonic acid to PGG<sub>2</sub>, the immediate precursor of many different prostaglandins (prostaglandin synthesis is described in Chapter 21).

(a) The kinetic data given below are for the reaction catalyzed by prostaglandin endoperoxide synthase. Focusing here on the first two columns, determine the  $V_{\text{max}}$  and  $K_{\text{m}}$  of the enzyme.

[Arachidonic acid] (mM)	Rate of formation of PGG <sub>2</sub> (mM/min)	Rate of formation of PGG <sub>2</sub> with 10 mg/mL ibuprofen (mM/min)
0.5	23.5	16.67
1.0	32.2	25.25
1.5	36.9	30.49
2.5	41.8	37.04
3.5	44.0	38.91

(b) Ibuprofen is an inhibitor of prostaglandin endoperoxide synthase. By inhibiting the synthesis of prostaglandins, ibuprofen reduces inflammation and pain. Using the data in the first and third columns of the table, determine the type of inhibition that ibuprofen exerts on prostaglandin endoperoxide synthase.

 **10. Graphical Analysis of  $V_{\text{max}}$  and  $K_{\text{m}}$**  The following experimental data were collected during a study of the catalytic activity of an intestinal peptidase with the substrate glycylglycine:



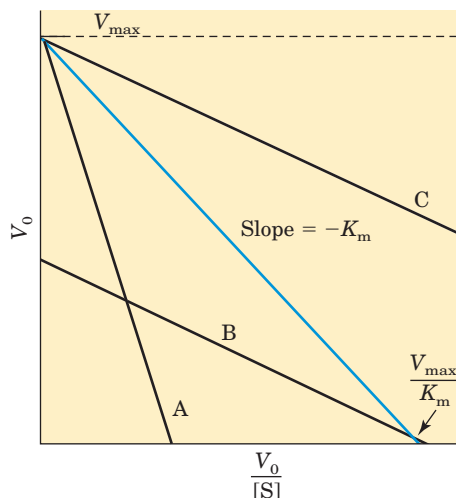
[S] (mM)	Product formed ( $\mu\text{mol}/\text{min}$ )
1.5	0.21
2.0	0.24
3.0	0.28
4.0	0.33
8.0	0.40
16.0	0.45

Use graphical analysis (see Box 6-1 and its associated Living Graph) to determine the  $K_{\text{m}}$  and  $V_{\text{max}}$  for this enzyme preparation and substrate.

**11. The Eadie-Hofstee Equation** One transformation of the Michaelis-Menten equation is the Lineweaver-Burk, or double-reciprocal, equation. Multiplying both sides of the Lineweaver-Burk equation by  $V_{\text{max}}$  and rearranging gives the Eadie-Hofstee equation:

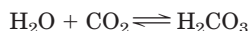
$$V_0 = (-K_{\text{m}}) \frac{V_0}{[S]} + V_{\text{max}}$$

A plot of  $V_0$  vs.  $V_0/[S]$  for an enzyme-catalyzed reaction is shown below. The blue curve was obtained in the absence of inhibitor. Which of the other curves (A, B, or C) shows the enzyme activity when a competitive inhibitor is added to the reaction mixture? Hint: See Equation 6–30.



## 12. The Turnover Number of Carbonic Anhydrase

Carbonic anhydrase of erythrocytes ( $M_r$  30,000) has one of the highest turnover numbers we know of. It catalyzes the reversible hydration of  $\text{CO}_2$ :



This is an important process in the transport of  $\text{CO}_2$  from the tissues to the lungs. If  $10.0 \mu\text{g}$  of pure carbonic anhydrase catalyzes the hydration of  $0.30 \text{ g}$  of  $\text{CO}_2$  in  $1 \text{ min}$  at  $37^\circ\text{C}$  at  $V_{\max}$ , what is the turnover number ( $k_{\text{cat}}$ ) of carbonic anhydrase (in units of  $\text{min}^{-1}$ )?

**13. Deriving a Rate Equation for Competitive Inhibition** The rate equation for an enzyme subject to competitive inhibition is

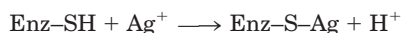
$$V_0 = \frac{V_{\max} [S]}{\alpha K_m + [S]}$$

Beginning with a new definition of total enzyme as

$$[E]_t = [E] + [EI] + [ES]$$

and the definitions of  $\alpha$  and  $K_I$  provided in the text, derive the rate equation above. Use the derivation of the Michaelis-Menten equation as a guide.

**14. Irreversible Inhibition of an Enzyme** Many enzymes are inhibited irreversibly by heavy metal ions such as  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$ , or  $\text{Ag}^+$ , which can react with essential sulfhydryl groups to form mercaptides:



The affinity of  $\text{Ag}^+$  for sulfhydryl groups is so great that  $\text{Ag}^+$  can be used to titrate  $-\text{SH}$  groups quantitatively. To  $10.0 \text{ mL}$  of a solution containing  $1.0 \text{ mg/mL}$  of a pure enzyme, an investigator added just enough  $\text{AgNO}_3$  to completely inactivate the enzyme. A total of  $0.342 \mu\text{mol}$  of  $\text{AgNO}_3$  was required.

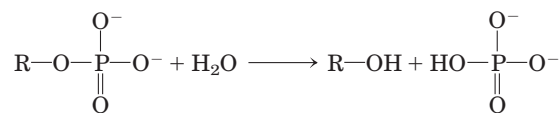
Calculate the minimum molecular weight of the enzyme. Why does the value obtained in this way give only the *minimum* molecular weight?



## 15. Clinical Application of Differential Enzyme

### Inhibition

Human blood serum contains a class of enzymes known as acid phosphatases, which hydrolyze biological phosphate esters under slightly acidic conditions (pH 5.0):

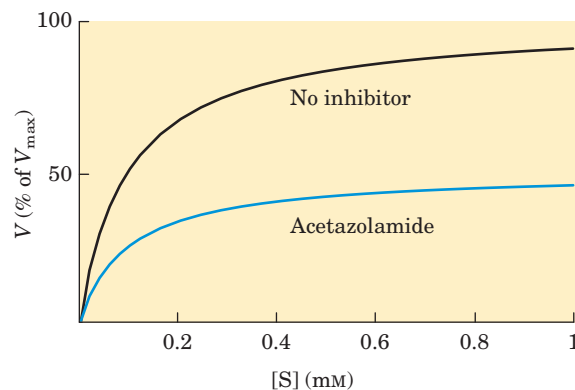


Acid phosphatases are produced by erythrocytes, the liver, kidney, spleen, and prostate gland. The enzyme of the prostate gland is clinically important, because its increased activity in the blood can be an indication of prostate cancer. The phosphatase from the prostate gland is strongly inhibited by tartrate ion, but acid phosphatases from other tissues are not. How can this information be used to develop a specific procedure for measuring the activity of the acid phosphatase of the prostate gland in human blood serum?



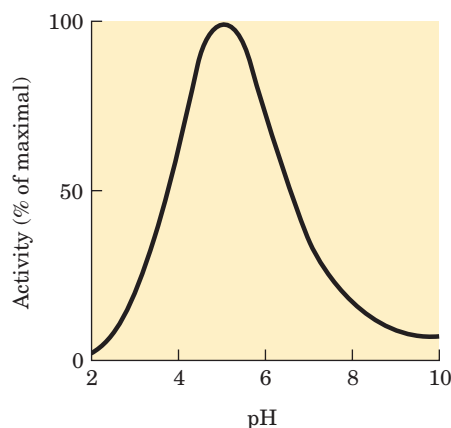
## 16. Inhibition of Carbonic Anhydrase by Acetazolamide

Carbonic anhydrase is strongly inhibited by the drug acetazolamide, which is used as a diuretic (i.e., to increase the production of urine) and to lower excessively high pressure in the eye (due to accumulation of intraocular fluid) in glaucoma. Carbonic anhydrase plays an important role in these and other secretory processes, because it participates in regulating the pH and bicarbonate content of several body fluids. The experimental curve of initial reaction velocity (as percentage of  $V_{\max}$ ) versus  $[S]$  for the carbonic anhydrase reaction is illustrated below (upper curve). When the experiment is repeated in the presence of acetazolamide, the lower curve is obtained. From an inspection of the curves and your knowledge of the kinetic properties of competitive and mixed enzyme inhibitors, determine the nature of the inhibition by acetazolamide. Explain your reasoning.



**17. The Effects of Reversible Inhibitors** Derive the expression for the effect of a reversible inhibitor on observed  $K_m$  (apparent  $K_m = \alpha K_m / \alpha'$ ). Start with Equation 6–30 and the statement that apparent  $K_m$  is equivalent to the  $[S]$  at which  $V_0 = V_{\max} / 2\alpha'$ .

**18. pH Optimum of Lysozyme** The active site of lysozyme contains two amino acid residues essential for catalysis: Glu<sup>35</sup> and Asp<sup>52</sup>. The  $pK_a$  values of the carboxyl side chains of these residues are 5.9 and 4.5, respectively. What is the ionization state (protonated or deprotonated) of each residue at pH 5.2, the pH optimum of lysozyme? How can the ionization states of these residues explain the pH-activity profile of lysozyme shown below?



**19. Working with Kinetics** Go to the Living Graphs for Chapter 6.

(a) Using the Living Graph for Equation 6-9, create a  $V$  versus  $[S]$  plot. Use  $V_{\max} = 100 \mu\text{M s}^{-1}$ , and  $K_m = 10 \mu\text{M}$ . How much does  $V_0$  increase when  $[S]$  is doubled, from  $0.2$  to  $0.4 \mu\text{M}$ ? What is  $V_0$  when  $[S] = 10 \mu\text{M}$ ? How much does the  $V_0$  increase when  $[S]$  increases from  $100$  to  $200 \mu\text{M}$ ? Observe how the graph changes when the values for  $V_{\max}$  or  $K_m$  are halved or doubled.

(b) Using the Living Graph for Equation 6-30 and the kinetic parameters in (a), create a plot in which both  $\alpha$  and  $\alpha'$  are  $1.0$ . Now observe how the plot changes when  $\alpha = 2.0$ ; when  $\alpha' = 3.0$ ; and when  $\alpha = 2.0$  and  $\alpha' = 3.0$ .

(c) Using the Living Graphs for Equation 6-30 and the Lineweaver-Burk equation in Box 6-1, create Lineweaver-Burk (double-reciprocal) plots for all the cases in (a) and (b). When  $\alpha = 2.0$ , does the  $x$  intercept move to the right or to the left? If  $\alpha = 2.0$  and  $\alpha' = 3.0$ , does the  $x$  intercept move to the right or to the left?