Amgen Inc. v. F. Hoffmann-LaRoche LTD et al Doc. 637 Att. 8
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Hsp70-mediated folding of many proteins by stimulating the hydrolysis of ATP by Hsp70/DnaK (see Figure 3-16). An additional protein called GrpE in bacteria (similar activity of BAG1 in mammals) also interacts with the Hsp70/DnaK, promoting the exchange of ATP for ADP. Multiple molecular chaperones are thought to bind all nascent polypeptide chains as they are being synthesized on ribosomes. In bacteria, 85 percent of the proteins are released from their chaperones and proceed to fold normally; an even higher percentage of proteins in eukaryotes follow this pathway.

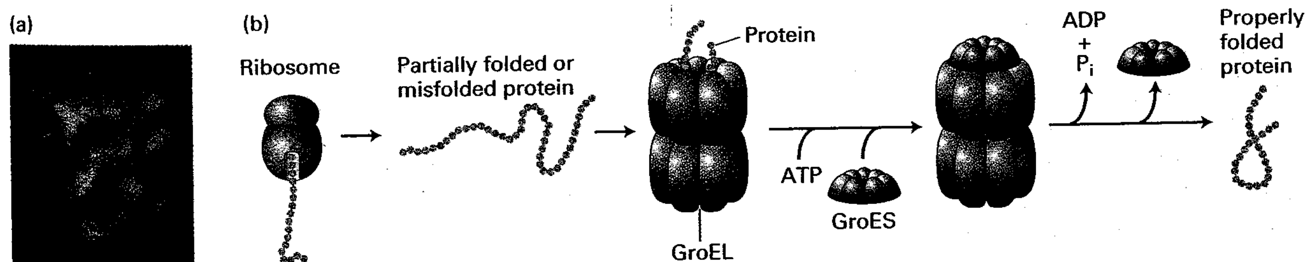
Chaperonins The proper folding of a large variety of newly synthesized proteins also requires the assistance of another class of proteins, the chaperonins. These huge cylindrical macromolecular assemblies are formed from two rings of oligomers, which can exist in a "tight" peptide-binding state and a "relaxed" peptide-releasing state. The eukaryotic chaperonin TriC consists of eight subunits per ring. In the bacterial, mitochondrial, and chloroplast chaperonin, known as GroEL, each ring contains seven identical subunits (Figure 3-17a). The GroEL folding mechanism, which is better understood than TriC-mediated folding, serves as a general model (Figure 3-17b). A partly folded or misfolded polypeptide is inserted into the cavity of the barrel-like GroEL, where it binds to the inner wall and folds into its native conformation. In an ATP-dependent step, GroEL undergoes a conformational change and releases the folded protein, a process assisted by a co-chaperonin, GroES, which caps the ends of GroEL. The binding of ATP and the co-chaperonin GroES to one of the rings in the tight state of GroEL causes a twofold expansion of its cavity, shifting the equilibrium toward the relaxed peptide-folding state. There is a striking similarity between the capped-barrel design of GroEL/GroES, in which proteins are sequestered for folding, and the structure of the 26S proteasome that participates in protein degradation (discussed in Section 3.4).

Alternatively Folded Proteins Are Implicated in Diseases

As noted earlier, each protein normally folds into a single, energetically favorable conformation that is specified by its amino acid sequence. Recent evidence suggests, however, that a protein may fold into an alternative three-dimensional structure as the result of mutations, inappropriate covalent modifications made after the protein is synthesized, or other as-yet-unidentified reasons. Such "misfolding" not only leads to a loss of the normal function of the protein but often marks it for proteolytic degradation. However, when degradation isn't complete or doesn't keep pace with misfolding, the subsequent accumulation of the misfolded protein or its proteolytic fragments contributes to certain degenerative diseases characterized by the presence of insoluble protein plaques in various organs, including the liver and brain.

Some neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease in humans and transmissible spongiform encephalopathy ("mad cow" disease) in cows and sheep, are marked by the formation of tangled filamentous plaques in a deteriorating brain (Figure 3-18). The *amyloid filaments* composing these structures derive from abundant natural proteins such as amyloid precursor protein, which is embedded in the plasma membrane; Tau, a microtubule-binding protein; and prion protein, an "infectious" protein. Influenced by unknown causes, these α helix–containing proteins or their proteolytic fragments fold into alternative β sheet–containing structures that polymerize into very stable filaments. Whether the extracellular deposits of these filaments or the soluble alternatively folded proteins are toxic to the cell is unclear. ■

Video: GroEl ATPase Cycle



▲ **FIGURE 3-17 Chaperonin-mediated protein folding.** Proper folding of some proteins depends on chaperonins such as the prokaryotic GroEL. (a) GroEL is a hollow, barrel-shaped complex of 14 identical 60,000-MW subunits arranged in two stacked rings. (b) In the absence of ATP or presence of ADP, GroEL exists in a "tight" conformational state that binds partly folded or misfolded proteins. Binding of ATP shifts GroEL to a more open, "relaxed" state, which releases the folded protein. During this process, one end of GroEL is transiently blocked by the co-chaperonin GroES, an assembly of 10,000-MW subunits. [Part (a) from A. Roseman et al., 1996, *Cell* **87**:241; courtesy of H. Saibil.]

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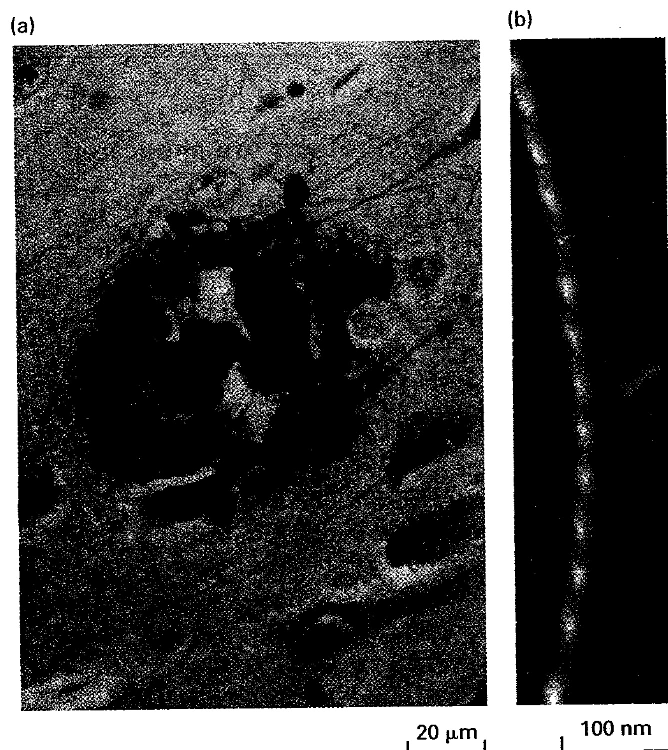


▲ **FIGURE 3-18 Alzheimer's disease is characterized by the formation of insoluble plaques composed of amyloid protein.** (a) At low resolution, an amyloid plaque in the brain of an Alzheimer's patient appears as a tangle of filaments. (b) The regular structure of filaments from plaques is revealed in the atomic force microscope. Proteolysis of the naturally occurring amyloid precursor protein yields a short fragment, called *β-amyloid protein,* that for unknown reasons changes from an α-helical to a β-sheet conformation. This alternative structure aggregates into the highly stable filaments (amyloid) found in plaques. Similar pathologic changes in other proteins cause other degenerative diseases. [Courtesy of K. Kosik.]

KEY CONCEPTS OF SECTION 3.2

Protein Folding

- The sequence of a protein determines its three-dimensional structure, which determines its function. In short, function derives from structure; structure derives from sequence.
- Because protein function derives from protein structure, newly synthesized proteins must fold into the correct shape to function properly.
- The planar structure of the peptide bond limits the number of conformations a polypeptide can have.
- The amino acid sequence of a protein dictates its folding into a specific three-dimensional conformation, the native state. Proteins will unfold, or denature, if treated under conditions that disrupt the noncovalent interactions stabilizing their three-dimensional structures.
- Protein folding in vivo occurs with assistance from chaperones, which bind to nascent polypeptides emerging from ribosomes and prevent their misfolding.
- Some neurodegenerative diseases are caused by aggregates of proteins that are stably folded in an alternative conformation.

3.3 Protein Function

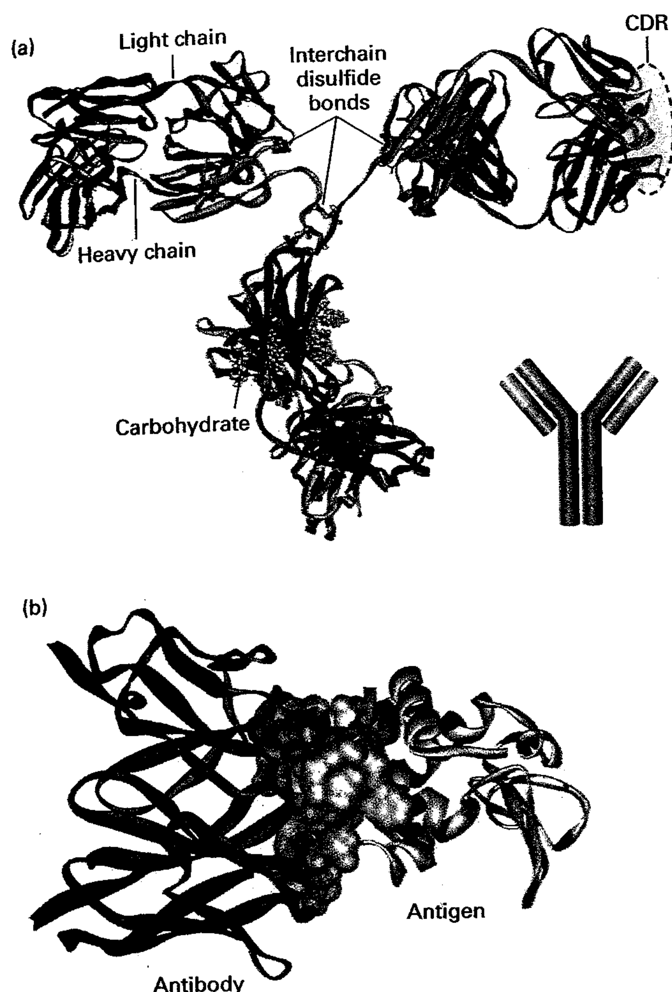
Although proteins have many different shapes and sizes and mediate an extraordinarily diverse array of activities both inside and outside of cells, most of these diverse functions are based on the ability of proteins to engage in a common activity, the binding to themselves, other macromolecules, small molecules, and ions. Here we will describe some of the key features underlying protein binding, and then turn to look at one group of proteins, enzymes, in greater detail. The activities of the other functional classes of proteins (structural, scaffold, transport, regulatory, motor) will be described in other chapters.

Specific Binding of Ligands Underlies the Functions of Most Proteins

The molecule to which a protein binds is often called its **ligand.** In some cases ligand binding causes a change in the shape of a protein. Ligand-binding-driven conformational changes are integral to the mechanism of action of many proteins and are important in regulating protein activity.

Two properties of a protein characterize how it binds ligands. *Specificity* refers to the ability of a protein to bind one molecule in preference to other molecules. *Affinity* refers to the tightness or strength of binding, usually expressed as the dissociation constant (K_d). The K_d for a protein–ligand complex, which is the inverse of the equilibrium constant K_{eq} for the binding reaction, is the most common quantitative measure of affinity (Chapter 2). The stronger the interaction between a protein and ligand, the lower the value of K_d. Both the specificity and the affinity of a protein for a ligand depend on the structure of the *ligand-binding site.* For high-affinity and highly specific interactions to take place, the shape and chemical properties of the binding site must be complementary to that of the ligand molecule, a property termed **molecular complementarity.** As we saw in Chapter 2, molecular complementarity allows molecules to form multiple noncovalent interactions at close range and thus stick together.

One of the best-studied examples of protein–ligand binding, involving high affinity and exquisite specificity, is that of **antibodies** binding to **antigens.** Antibodies are proteins that circulate in the blood and are made by the immune system in response to the invasion by antigens, which are usually macromolecules present in infectious agents (e.g., a bacterium or a virus) or other foreign substances (e.g., proteins or polysaccharides in pollens). Different antibodies are generated in response to different antigens, and these antibodies have the remarkable characteristic of binding specifically to ("recognizing") a portion of the antigen, called an **epitope,** which initially induced the production of the antibody, and not to other molecules. Antibodies act as specific sensors for antigens, forming antibody–antigen complexes that initiate a cascade of protective reactions in cells of the immune system.



▲ **FIGURE 3-19 Protein–ligand binding of antibodies.** (a) Ribbon model of an antibody. Every antibody molecule of the immunoglobulin IgG class consists of two identical heavy chains (light and dark red) and two identical light chains (blue) covalently linked by disulfide bonds. The inset shows a diagram of the overall structure containing the two heavy and two light chains. (b) The hand-in-glove fit between an antibody and the site to which it binds (epitope) on its target antigen—in this case, chicken egg-white lysozyme. Regions where the two molecules make contact are shown as surfaces. The antibody contacts the antigen with residues from all its complementarity-determining regions (CDRs). In this view, the molecular complementarity of the antigen and antibody is especially apparent where "fingers" extending from the antigen surface are opposed to "clefts" in the antibody surface.

All antibodies are Y-shaped molecules formed from two identical heavy chains and two identical light chains (Figure 3-19a). Each arm of an antibody molecule contains a single light chain linked to a heavy chain by a disulfide bond. Near the end of each arm are six highly variable loops, called *complementarity-determining regions (CDRs),* which form the antigen-binding sites. The sequences of the six loops are highly variable among antibodies, generating unique complementary ligand-binding sites that make them specific for different epitopes (Figure 3-19b). The intimate contact between these two surfaces, stabilized by numerous noncovalent interactions, is responsible for the extremely precise binding specificity exhibited by an antibody.

The specificity of antibodies is so precise that they can distinguish between the cells of individual members of a species and in some cases can distinguish between proteins that differ by only a single amino acid. Because of their specificity and the ease with which they can be produced, antibodies are highly useful reagents used in many of the experiments discussed in subsequent chapters.

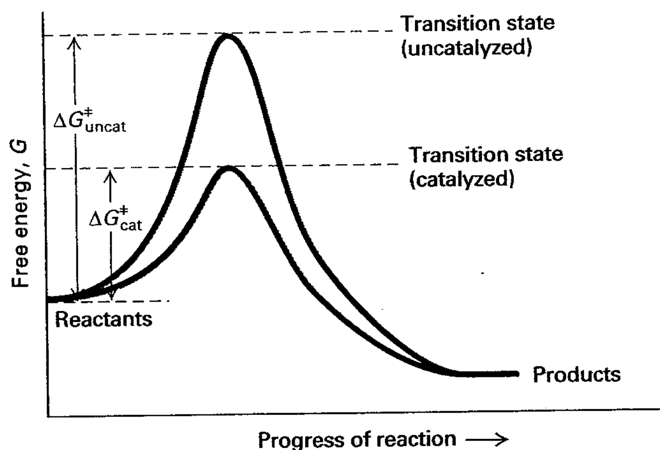
We will see many examples of protein–ligand binding throughout this book, including hormones binding to receptors (Chapter 15), regulatory molecules binding to DNA (Chapter 7), cell-adhesion molecules binding to extracellular matrix (Chapter 19), to name just a few. Next we will consider how the binding of one class of proteins, enzymes, to their ligands results in the catalysis of the chemical reactions essential for the survival and function of cells.

Enzymes Are Highly Efficient and Specific Catalysts

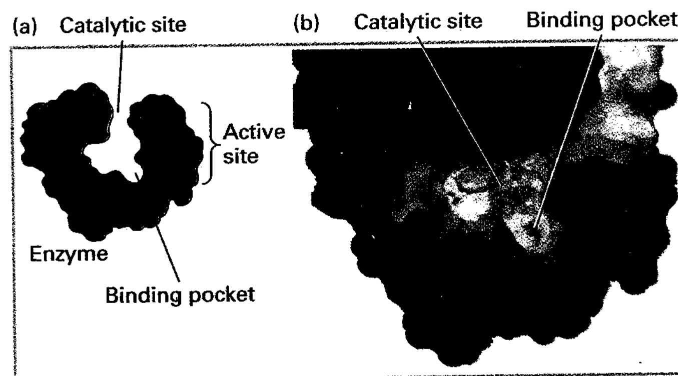
Proteins that catalyze chemical reactions, the making and breaking of covalent bonds, are called **enzymes,** and their ligands are called **substrates.** While not all proteins are enzymes, enzymes make up a large and very important class of protein—indeed, almost every chemical reaction in the cell is catalyzed by a specific enzyme. In many ways, enzymes are the cell's chemists, performing many of a cell's chemical reactions. (An additional form of catalytic macromolecule in cells is made from RNA. These RNAs are called **ribozymes.**)

Thousands of different types of enzymes, each of which catalyzes a single chemical reaction or set of closely related reactions, have been identified. Certain enzymes are found in the majority of cells because they catalyze the synthesis of common cellular products (e.g., proteins, nucleic acids, and phospholipids) or take part in the production of energy (e.g., by the conversion of glucose and oxygen into carbon dioxide and water). Other enzymes are present only in a particular type of cell because they catalyze chemical reactions unique to that cell type (e.g., the enzymes in nerve cells that convert tyrosine into dopamine, a neurotransmitter). Although most enzymes are located within cells, some are secreted and function at extracellular sites such as the blood, the digestive tract, or even outside the organism (e.g., toxic enzymes in the venom of poisonous snakes).

Like all **catalysts,** enzymes increase the rate of a reaction but do not affect the extent of a reaction, which is determined by the change in free energy ΔG between reactants and products, and are not themselves permanently changed as a consequence of the reaction they catalyze (Chapter 2). Enzymes increase the reaction rate by lowering the energy of the **transition state,** and therefore the **activation energy** (Figure 3-20). In the test tube, catalysts such as charcoal and platinum facilitate reactions but usually only at high temperatures or pressures, at extremes of high or low pH, or in organic solvents. As the cell's protein catalysts, however, enzymes must function effectively in aqueous environment at 37 °C and 1 atmosphere pressure, and at physiologic pH values, usually 6.5–7.5, but sometimes lower. Remarkably, enzymes exhibit immense catalytic power, accelerating the



▲ **FIGURE 3-20 Effect of an enzyme on the activation energy of a chemical reaction.** This hypothetical reaction pathway depicts the changes in free energy G as a reaction proceeds. A reaction will take place spontaneously only if the total G of the products is less than that of the reactants (negative ΔG). However, all chemical reactions proceed through one or more high-energy transition states, and the rate of a reaction is inversely proportional to the activation energy ($\Delta G^{\ddagger}$), which is the difference in free energy between the reactants and the transition state (highest point along the pathway). Enzymes and other catalysts accelerate the rate of a reaction by reducing the free energy of the transition state and thus $\Delta G^{\ddagger}$.



▲ **FIGURE 3-21 Active site of the enzyme trypsin.** (a) An enzyme's active site is composed of a binding pocket, which binds specifically to a substrate, and a catalytic site, which carries out catalysis. (b) A surface representation of the serine protease trypsin. Active site clefts containing the catalytic site (side chains of the catalytic triad Ser-195, Asp-102, and His-57 shown as stick figures) and the substrate side chain specificity binding pocket are clearly visible. [Part (b) courtesy of P. Teesdale-Spittle.]

rates of reactions 10^6–10^{12} times that of the corresponding uncatalyzed reactions under otherwise similar conditions.

An Enzyme's Active Site Binds Substrates and Carries Out Catalysis

Certain amino acids of an enzyme are particularly important in determining its specificity and catalytic power. In the native conformation of an enzyme, these side chains (which usually come from different parts of the linear sequence of the polypeptide) are brought into proximity, forming a cleft in the surface called the **active site** (Figure 3-21). Most active sites make up only a small fraction of the total protein, with the rest involved in folding of the polypeptide, regulation of the active site, and interactions with other molecules.

Active sites consist of two functionally important regions: the *substrate-binding site* that recognizes and binds the substrate (or substrates) and the *catalytic site* whose catalytic groups (amino acid side chains and backbone carbonyl and amino groups) mediate the chemical reaction once the substrate has bound. In some enzymes, the catalytic and substrate-binding sites overlap; in others, the two regions are structurally as well as functionally distinct.

The substrate-binding site is responsible for the remarkable specificity of enzymes—their ability to act selectively on one substrate or a small number of chemically similar substrates. The alteration of the structure of an enzyme's substrate by only one or a few atoms, or a subtle change in the geometry (e.g., stereochemistry) of the substrate, can result in a variant molecule that is no longer a substrate of the enzyme. As noted above, this specificity of enzymes is a consequence of the precise molecular complementarity between its substrate-binding site and the substrate, which is mediated by multiple weak noncovalent interactions and is very sensitive to the shapes of substrates. Usually only one or a few substrates can fit precisely into a binding site.

The idea that enzymes might function by binding to their substrates in the manner of a key fitting into a lock was suggested first by Emil Fischer in 1894. In 1913 Leonor Michaelis and Maud Leonora Menten provided crucial evidence supporting this hypothesis. They showed that the rate of an enzymatic reaction was proportional to the substrate concentration at low substrate concentrations, but that as the substrate concentrations increased, the rate reached a **maximal velocity** V_{max} and became substrate concentration–independent, with the value of V_{max} being directly proportional to the amount of enzyme present in the reaction mixture (Figure 3-22).

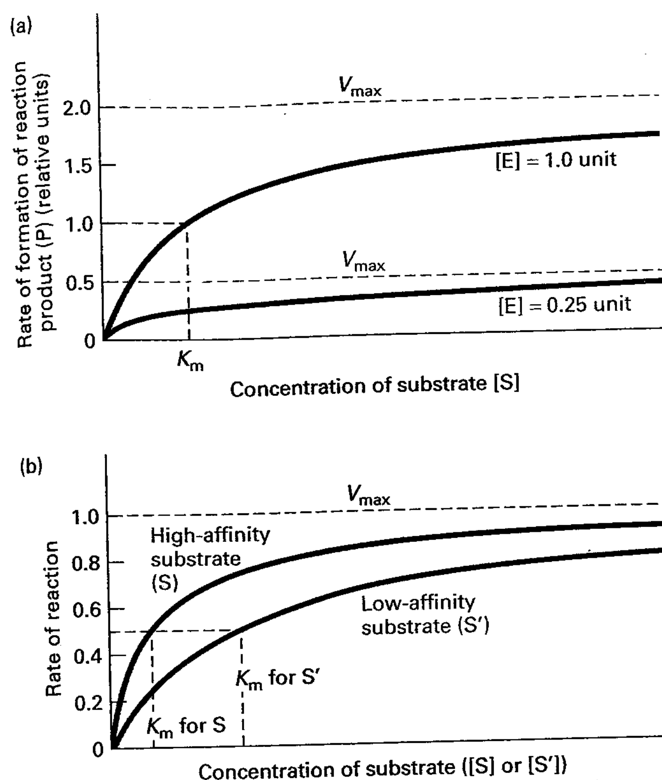
They deduced that this saturation at high substrate concentrations was due to the binding of substrate molecules (S) to a fixed and limited number of sites on the enzymes (E), and they called the bound species the enzyme-substrate (ES) complex. They proposed that the ES complex is in equilibrium with the unbound enzyme and substrate and is an intermediate step in the ultimately irreversible conversion of substrate to product (P) (Figure 3-23):

$$E + S \rightleftharpoons ES \rightarrow E + P$$

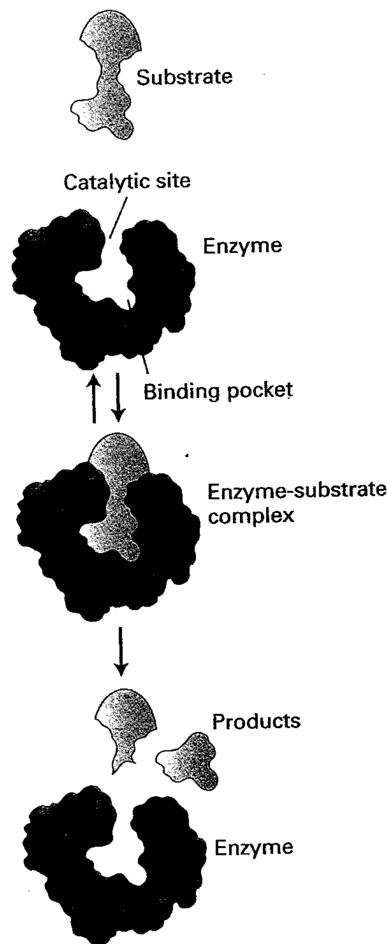
and that the rate V_0 of formation of product at a particular substrate concentration [S] is given by what is now called the *Michaelis-Menten equation:*

$$V_0 = V_{max}\frac{[S]}{[S] + K_m} \qquad (3\text{-}1)$$

where the **Michaelis constant** K_m, a measure of the affinity of an enzyme for its substrate (see Figure 3-22), is the substrate concentration that yields a half-maximal reaction rate



▲ **FIGURE 3-22 K_m and V_{max} for an enzyme-catalyzed reaction.** K_m and V_{max} are determined from analysis of the dependence of the initial reaction velocity on substrate concentration. The shape of these hypothetical kinetic curves is characteristic of a simple enzyme-catalyzed reaction in which one substrate (S) is converted into product (P). The initial velocity is measured immediately after addition of enzyme to substrate before the substrate concentration changes appreciably. (a) Plots of the initial velocity at two different concentrations of enzyme [E] as a function of substrate concentration [S]. The [S] that yields a half-maximal reaction rate is the Michaelis constant K_m, a measure of the affinity of E for turning S into P. Quadrupling the enzyme concentration causes a proportional increase in the reaction rate, and so the maximal velocity V_{max} is quadrupled; the K_m, however, is unaltered. (b) Plots of the initial velocity versus substrate concentration with a substrate S for which the enzyme has a high affinity and with a substrate S′ for which the enzyme has a lower affinity. Note that the V_{max} is the same with both substrates, because [E] is the same, but that K_m is higher for S′, the low-affinity substrate.

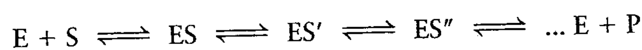


▲ **FIGURE 3-23 Schematic model of an enzyme's reaction mechanism.** Enzyme kinetics suggest that enzymes (E) bind substrate molecules (S) through a fixed and limited number of sites on the enzymes (the active sites). The bound species is known as an enzyme-substrate (ES) complex. The ES complex is in equilibrium with the unbound enzyme and substrate and is an intermediate step in the conversion of substrate to products (P).

(i.e., 1/2 V_{max}), and thus is analogous to the dissociation constant K_d (Chapter 2). The smaller the value of K_m, the more effective the enzyme is at making product from dilute solutions of substrate and the smaller the substrate concentration needed to reach half-maximal velocity. The concentrations of the various small molecules in a cell vary widely, as do the K_m values for the different enzymes that act on them. A good rule of thumb is that the intracellular concentration of a substrate is approximately the same as or somewhat greater than the K_m value of the enzyme to which it binds.

The rates of reaction at substrate saturation vary enormously among enzymes. The maximum number of substrate molecules converted to product at a single enzyme active site per second is called the *turnover number*, which can be less than 1 for very slow enzymes. The turnover number for carbonic anhydrase, one of the fastest enzymes, is 6×10^5 molecules/s.

Many enzymes catalyze the conversion of substrates to products by dividing the process into multiple, discrete chemical reactions that involve multiple, distinct enzyme substrate complexes (ES, ES′, ES″, etc.) generated prior to the final release of the products:

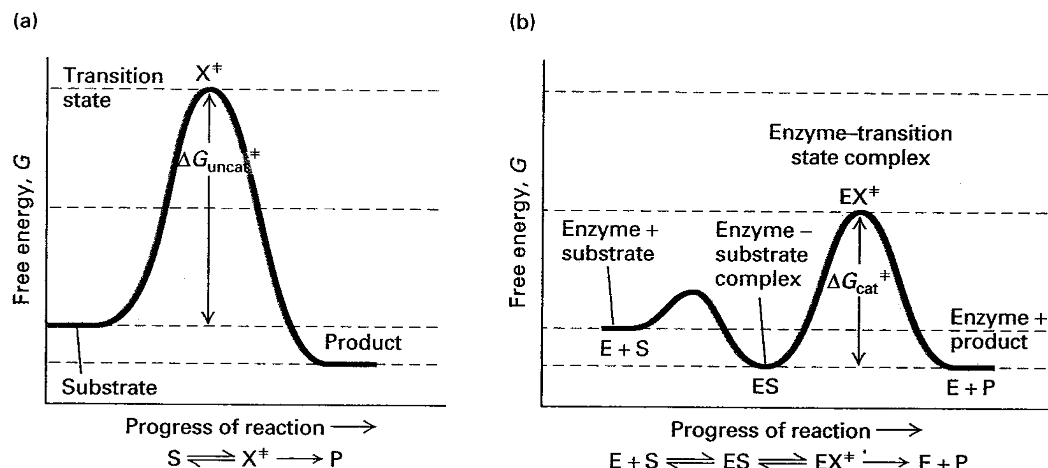


$$E + S \rightleftharpoons ES \rightleftharpoons ES' \rightleftharpoons ES'' \rightleftharpoons \ldots E + P$$

The energy profiles for such multistep reactions involve multiple hills and valleys (Figure 3-24), and methods have been developed to trap the intermediates in such reactions to learn more about the details of how enzymes catalyze reactions.

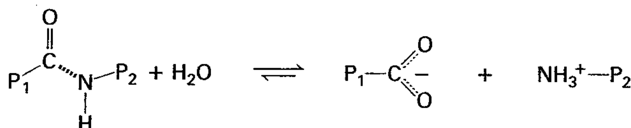
Serine Proteases Demonstrate How an Enzyme's Active Site Works

Serine proteases, a large family of proteolytic enzymes, are used throughout the biological world—to digest meals (the



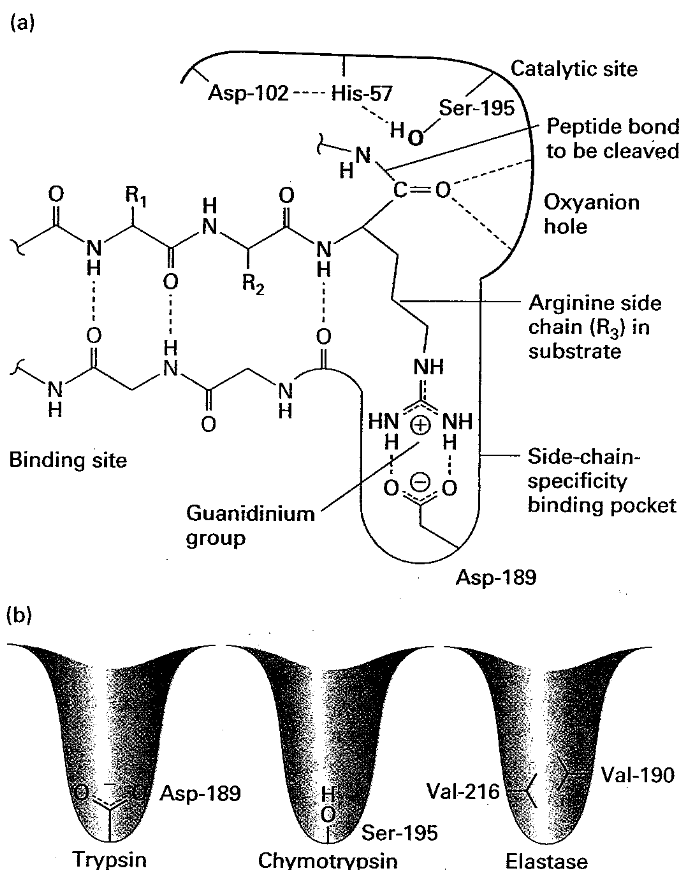
▲ **FIGURE 3-24 Free-energy reaction profiles of uncatalyzed and multistep enzyme-catalyzed reactions.** (a) The free-energy reaction profile of a hypothetical simple uncatalyzed reaction converting substrate (S) to product (P) via a single high-energy transition state. (b) Many enzymes catalyze such reactions by dividing the process into multiple discrete steps, in this case the initial formation of an ES complex followed by conversion via a single transition state ($EX^{‡}$) to the free enzyme (E) and P. The activation energy for each of these steps is significantly less than the activation energy for the uncatalyzed reaction; thus the enzyme dramatically enhances the reaction rate.

pancreatic enzymes trypsin, chymotrypsin, and elastase), to control blood clotting (the enzyme thrombin), even to help silk moths chew their way out of their cocoons (cocoonase). This class of enzymes usefully illustrates how an enzyme's substrate-binding site and catalytic site cooperate in multistep reactions to convert substrates to products. Here we will consider how trypsin and its two evolutionarily closely related pancreatic proteases, chymotrypsin and elastase, catalyze cleavage of a peptide bond:



where P_1 is the portion of the protein on the N-terminal side of the peptide bond, and P_2 is the portion on the C-terminal side. We first consider how serine proteases bind specifically to their substrates and then show in detail how catalysis takes place.

Figure 3-25a shows how a substrate polypeptide binds to the substrate-binding site in the active site of trypsin. There are two key binding interactions. First, the substrate and enzyme form hydrogen bonds that resemble a β sheet. Second, a key side chain of the substrate that determines which peptide in the substrate is to be cleaved extends into the enzyme's *side-chain-specificity binding pocket,* at the bottom of which resides the negatively charged side chain of the



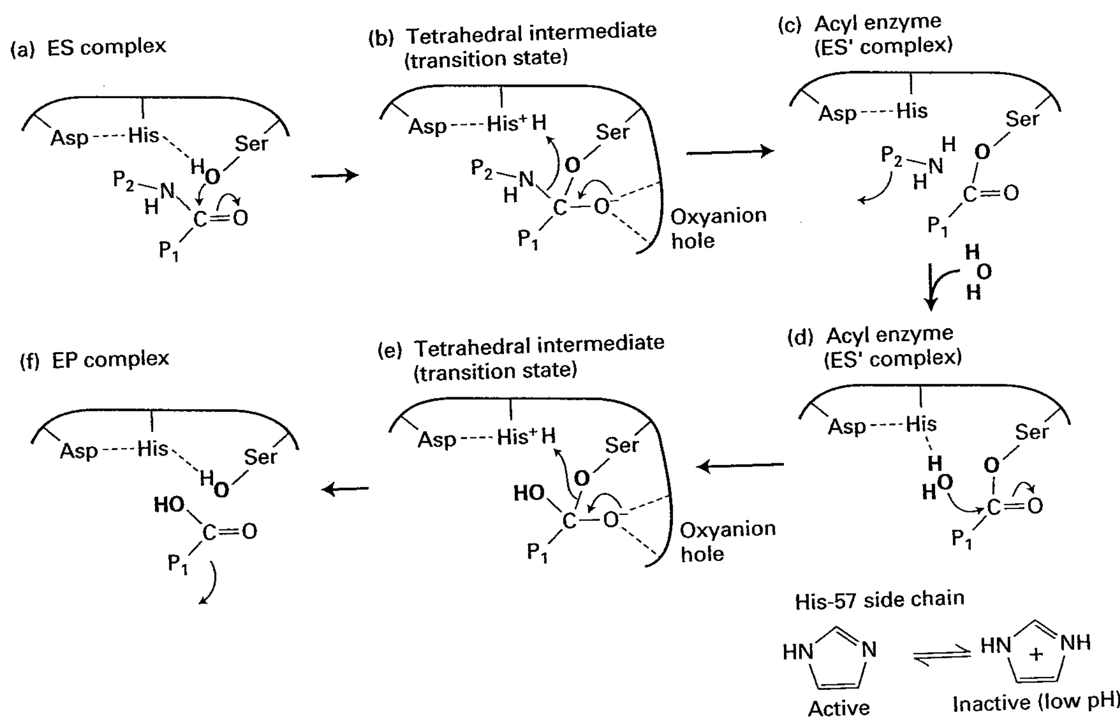
► **FIGURE 3-25 Substrate binding in the active site of typsin-like serine proteases.** (a) The active site of trypsin (blue molecule) with a bound substrate (black molecule). The substrate forms a two-stranded β sheet with the binding site, and the side chain of an arginine (R_3) in the substrate is bound in the side-chain-specificity binding pocket. Its positively charged guanidinium group is stabilized by the negative charge on the side chain of the enzyme's Asp-189. This binding aligns the peptide bond of the arginine appropriately for hydrolysis catalyzed by the enzyme's active-site catalytic triad (side chains of Ser-195, His-57 and Asp-102). (b) The amino acids lining the side-chain-specificity binding pocket determine its shape and charge, and thus its binding properties. Trypsin accommodates the positively charged side chains of arginine and lysine; chymotrypsin, large, hydrophobic side chains such as phenylalanine; and elastase, small side chains such as glycine and alanine. [Part (a) modified from J. J. Perona and C. S. Craik, 1997, *J. Biol. Chem.* **272**(48):29987–29990.]

enzyme's Asp-189. Trypsin has a marked preference for hydrolyzing proteins (black in Figure 3-25a) at the carboxyl side of a residue with a long positively charged side chain (arginine or lysine), because the side chain is stabilized in the specificity binding pocket by the negative Asp-189.

Slight differences in the structures of otherwise similar specificity pockets help explain the differing substrate specificities of the two related serine proteases: chymotrypsin prefers large aromatic groups (as in Phe, Tyr, Trp), and elastase prefers the small side chains of Gly and Ala (Figure 3-25b). The uncharged Ser-189 in chymotrypsin allows large, uncharged, hydrophobic side chains to bind stably in the pocket. The branched aliphatic side chains of valine and threonine in elastase replace glycines in the sides of the pocket in trypsin and thus prevent large side chains in substrates from binding, but allow stable binding of the short alanine or glycine side chain.

In the catalytic site, all three enzymes use the hydroxyl group on the side chain of a serine in position 195 to catalyze the hydrolysis of peptide bonds in protein substrates. A catalytic triad formed by the three side chains of Ser-195, His-57, Asp-102 participates in what is essentially a two-step reaction. Figure 3-26 shows how the catalytic triad cooperates in breaking the peptide bond, with Asp-102 and His-57 supporting the attack of the hydroxyl oxygen of Ser-195 on the carbonyl carbon in the substrate. This attack initially forms an unstable transition state with four groups attached to this carbon (tetrahedral intermediate). Breaking of the C—N peptide bond then releases one part of the protein (NH_3—P_2), while the other part remains covalently attached to the enzyme via an ester bond to the serine's oxygen, forming a relatively stable intermediate (the acyl enzyme). The subsequent replacement of this oxygen by one from water, in a reaction involving another unstable tetrahedral intermediate, leads to release of the final product (P_1—COOH). The tetrahedral intermediates are partially stabilized by hydrogen bonding from the enzyme's backbone amino groups in what is called the *oxyanion hole*. The large family of serine proteases and related enzymes with an active-site serine illustrates how an efficient reaction mechanism is used over and over by distinct enzymes to catalyze similar reactions.

The serine protease mechanism points out several general key features of enzymatic catalysis: (1) enzyme catalytic sites are designed to stabilize the binding of a transition state, thus lowering the activation energy and accelerating the overall



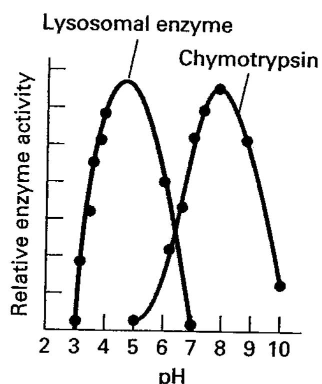
▲ **FIGURE 3-26 Mechanism of serine protease–mediated hydrolysis of peptide bonds.** The catalytic triad of Ser-195, His-57, and Asp-102 in the active sites of serine proteases employs a multistep mechanism to hydrolyze peptide bonds in target proteins. (a) After a polypeptide substrate binds to the active site (see Figure 3-23) forming an ES complex, the hydroxyl oxygen of Ser-195 attacks the carbonyl carbon of the substrate's targeted peptide bond (yellow). Movements of electrons are indicated by arrows. (b) This attack results in the formation of a transition state called the *tetrahedral intermediate*, in which the negative charge on the substrate's oxygen is stabilized by hydrogen bonds formed with the enzyme's *oxyanion hole*. (c) Additional electron movements result in the breaking of the peptide bond, release of one of the reaction products (NH_2-P_2), and formation of the acyl enzyme (ES′ complex). (d) An oxygen from a solvent water molecule then attacks the carbonyl carbon of the acyl enzyme. (e) This attack results in the formation of a second tetrahedral intermediate. (f) Additional electron movements result in the breaking of the Ser-195–substrate bond (formation of the EP complex) and release of the final reaction product (P_1-COOH). The side chain of His-57, which is held in the proper orientation by hydrogen bonding to the side chain of Asp-102, facilitates catalysis by withdrawing and donating protons throughout the reaction (*inset*). If the pH is too low and the side chain of His-57 is protonated, it cannot participate in catalysis and the enzyme is inactive.

reaction, (2) multiple side chains, together with the polypeptide backbone, carefully organized in three dimensions, work together to chemically transform substrate into product, often by multistep reactions, and (3) acid-base catalysis mediated by one or more amino acid side chains is often used by enzymes, as when the imidazole group of His-57 in serine proteases acts as a base to remove the hydrogen from Ser-195's hydroxyl group. As a consequence, often only a particular ionization state (protonated or nonprotonated) of one or more amino acid side chains in the catalytic site is compatible with catalysis, and thus the enzyme's activity is pH-dependent.

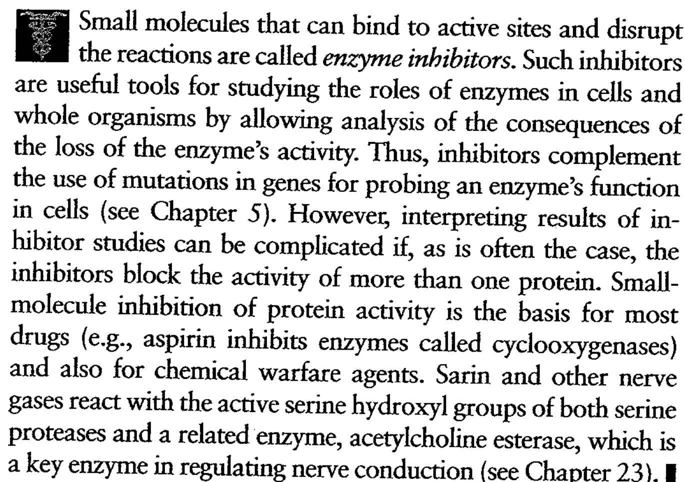
For example, the imidazole of His-57 in serine proteases, whose pK_a is ≈6.8, can help the Ser-195 hydroxyl attack the substrate only if it is not protonated. Thus, the activity of the protease is low at pH < 6.8, and the shape of the pH activity profile in the pH range 4–8 matches the titration of the His-57 side chain, which is governed by the Henderson-Hasselbalch equation, with an inflection near pH 6.8 (see Figure 3-27, *right*, and Chapter 2). The activity drops at higher pH values, generating a bell-shaped curve, because the proper folding of the protein is disrupted when the amino group at the protein's amino terminus is deprotonated ($pK_a \approx 9$); the conformation near the active site changes as a consequence.

The pH sensitivity of an enzyme's activity can be due to changes in the ionization of catalytic groups, groups that participate directly in substrate binding, or groups that influence the conformation of the protein. Pancreatic proteases evolved to function in the neutral or slightly basic conditions in the intestines; hence, their pH optima are ≈8. Proteases and other hydrolytic enzymes that function in acidic conditions must employ a different catalytic mechanism. This is the case for enzymes within the stomach (pH ≈ 1) such as the protease pepsin or those within lysosomes (pH ≈ 4.5), which play a key role in degrading macromolecules within cells (see Figure 3-27, *left*). Indeed, lysosomal hydrolases that degrade a wide variety of biomolecules (proteins, lipids, etc.) are relatively inactive at the pH in the cytosol (≈7), and that helps protect a cell from self-digestion should these enzymes escape the confines of the membrane-bound lysosome.

One key feature of enzymatic catalysis not seen in serine proteases, but found in many other enzymes, is a *cofactor,* or *prosthetic* (helper) *group.* This is a nonpolypeptide small molecule or ion (e.g., iron, zinc, copper, manganese) that is bound in the active site and plays an essential role in the reaction mechanism. Small organic prosthetic groups in enzymes are also called *coenzymes.* Some of these are chemically modified during the reaction and thus need to be replaced or regenerated after each reaction; others are not. Examples of the former include NAD^+ (nicotinamide adenine dinucleotide) and FAD (flavin adenine dinucleotide) (see Figure 2-33), whereas heme groups that bind oxygen in hemoglobin or transfer electrons in some cytochromes are examples of the latter (Figure 12-14). Thus, the chemistry catalyzed by enzymes is not restricted by the limited number of amino acids in polypeptide chains. Many vitamins—e.g., the B vitamins, thiamine (B_1), riboflavin (B_2), niacin (B_3), and pyridoxine (B_6), and vitamin C—which cannot be synthesized in higher animal cells, function as or are used to generate coenzymes. That is why supplements of vitamins must be added to the liquid medium in which animals cells are grown in the laboratory (Chapter 9).



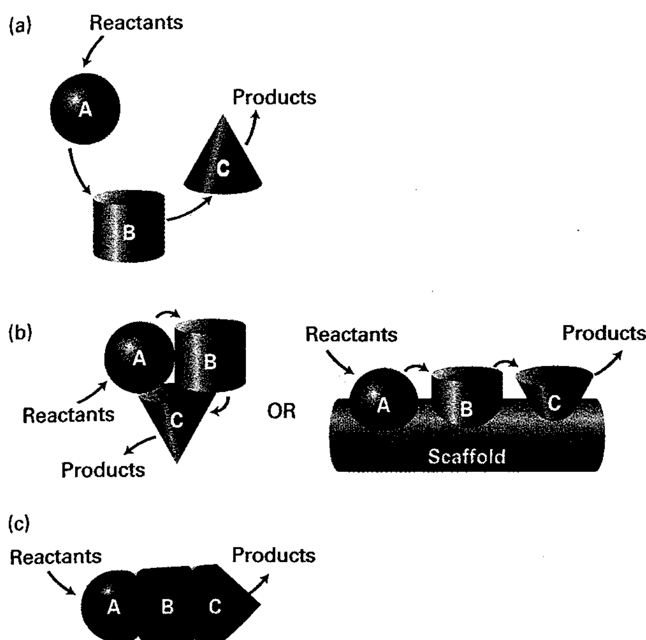
▲ **FIGURE 3-27 pH dependence of enzyme activity.** Ionizable (pH-titratable) groups in the active sites or elsewhere in enzymes often must be either protonated or deprotonated to permit proper substrate binding or catalysis or to permit the enzyme to adopt the correct conformation. Measurement of enzyme activity as a function of pH can be used to identify the pKa's of these groups. The pancreatic serine proteases, such as chymotrypsin (*right curve*), exhibit maximum activity around pH 8 because of titration of the active site His-57 (required for catalysis, $pK_a \approx 6.8$) and of the amino terminus of the protein (required for proper conformation, $pK_a \approx 9$). Many lysosomal hydrolases have evolved to exhibit a lower pH optimum (≈4.5, *left curve*) to match the low internal pH in lysosomes in which they function. [Adapted from P. Lozano, T. De Diego, and J. L. Iborra, 1997, *Eur. J. Biochem.* **248**(1):80–85, and W. A. Judice et al., 2004, *Eur. J. Biochem.* **271**(5):1046–1053.]



Small molecules that can bind to active sites and disrupt the reactions are called *enzyme inhibitors.* Such inhibitors are useful tools for studying the roles of enzymes in cells and whole organisms by allowing analysis of the consequences of the loss of the enzyme's activity. Thus, inhibitors complement the use of mutations in genes for probing an enzyme's function in cells (see Chapter 5). However, interpreting results of inhibitor studies can be complicated if, as is often the case, the inhibitors block the activity of more than one protein. Small-molecule inhibition of protein activity is the basis for most drugs (e.g., aspirin inhibits enzymes called cyclooxygenases) and also for chemical warfare agents. Sarin and other nerve gases react with the active serine hydroxyl groups of both serine proteases and a related enzyme, acetylcholine esterase, which is a key enzyme in regulating nerve conduction (see Chapter 23). ■

Enzymes in a Common Pathway Are Often Physically Associated with One Another

Enzymes taking part in a common metabolic process (e.g., the degradation of glucose to pyruvate) are generally located in the same cellular compartment (e.g., in the cytosol, at a membrane, or within a particular organelle). Within a compartment, products from one reaction can move by diffusion to the next enzyme in the pathway. However, diffusion



▲ **FIGURE 3-28 Assembly of enzymes into efficient multienzyme complexes.** In the hypothetical reaction pathways illustrated here the initial reactants are converted into final products by the sequential action of three enzymes: A, B, and C. (a) When the enzymes are free in solution or even constrained within the same cellular compartment, the intermediates in the reaction sequence must diffuse from one enzyme to the next, an inherently slow process. (b) Diffusion is greatly reduced or eliminated when the individual enzymes associate into multisubunit complexes, either by themselves or with the aid of a scaffold protein. (c) The closest integration of different catalytic activities occurs when the enzymes are fused at the genetic level, becoming domains in a single polypeptide chain.

entails random movement and can be a slow, relatively inefficient process for moving molecules between widely dispersed enzymes (Figure 3-28a). To overcome this impediment, cells have evolved mechanisms for bringing enzymes in a common pathway into close proximity.

In the simplest such mechanism, polypeptides with different catalytic activities cluster closely together as subunits of a multimeric enzyme or assemble on a common "scaffold" that holds them together (Figure 3-28b). This arrangement allows the products of one reaction to be channeled directly to the next enzyme in the pathway. In some cases, independent proteins have been fused together at the genetic level to create a single multidomain, multifunctional enzyme (Figure 3-28c).

Enzymes Called Molecular Motors Convert Energy into Motion

At the nanoscale of cells and molecules, movement is influenced by forces that differ from those in the macroscopic world. For example, the high protein concentration (200–300 mg/ml) of the cytoplasm prevents organelles and vesicles from diffusing faster than 100 μm/3 hours. Even a micrometer-sized bacterium experiences a drag force from water that stops its forward movement within a fraction of a nanometer when it stops actively swimming. To generate the forces necessary for many cellular movements, cells depend on specialized enzymes commonly called **molecular motors,** or **motor proteins.** These mechanochemical enzymes convert energy released by the hydrolysis of ATP or contained within ion gradients into a mechanical force, usually generating either linear or rotary motion.

From the observed activities of motor proteins, we can infer three general properties that they possess:

- The ability to transduce a source of energy, either ATP or an ion gradient, into linear or rotary movement
- The ability to bind and translocate along a substrate
- Net movement in a given direction

We will see many examples of such motors in subsequent chapters.

KEY CONCEPTS OF SECTION 3.3

Protein Function

- The functions of nearly all proteins depend on their ability to bind other molecules (ligands).
- The specificity of a protein for a particular ligand refers to the preferential binding of one or a few closely related ligands.
- The affinity of a protein for a particular ligand refers to the strength of binding, usually expressed as the dissociation constant K_d.
- Ligand-binding sites on proteins and the corresponding ligands themselves are chemically and spatially complementary.
- Enzymes are catalytic proteins that accelerate the rate of cellular reactions by lowering the activation energy and stabilizing transition-state intermediates (see Figure 3-20).
- An enzyme active site, which is usually only a small part of the protein, comprises two functional parts: a substrate-binding site and a catalytic site. The amino acids composing the active site are not necessarily adjacent in the amino acid sequence but are brought into proximity in the native conformation.
- The substrate-binding site is responsible for the exquisite specificity of enzymes owing to its molecular complementarity with the substrate and the transition state.
- The initial binding of substrates (S) to enzymes (E) results in the formation of an enzyme-substrate complex (ES), which then undergoes one or more reactions catalyzed by the catalytic groups in the active site until the products (P) are formed and diffuse away from the enzyme.
- From plots of reaction rate versus substrate concentration, two characteristic parameters of an enzyme can be determined: the Michaelis constant K_m, a rough measure of

the enzyme's affinity for converting substrate into product, and the maximal velocity V_{max}, a measure of its catalytic power (see Figure 3-22).

- The rates of enzyme-catalyzed reactions vary enormously, with the turnover numbers (number of substrate molecules converted to products at a single active site at substrate saturation) ranging between <1 to 6×10^5 molecules/s.
- Many enzymes catalyze the conversion of substrates to products by dividing the process into multiple discrete chemical reactions that involve multiple distinct enzyme substrate complexes (ES′, ES″, etc.).
- Serine proteases hydrolyze peptide bonds in protein substrates using as catalytic groups the side chains of Ser-195, His-57, and Asp-102.
- Amino acids lining the specificity binding pocket in the binding site of serine proteases determine the residue in a protein substrate that will be hydrolyzed and account for differences in the specificity of trypsin, chymotrypsin, and elastase.
- Enzymes often use acid-base catalysis mediated by one or more amino acid side chains, such as the imidazole group of His-57 in serine proteases, to catalyze reactions.
- The pH dependence of protonation of catalytic groups (pK_a) is often reflected in the pH-rate profile of the enzyme's activity. The pH sensitivity of an enzyme's activity can be due to changes in the ionization of catalytic groups, of groups that participate directly in substrate binding, or of groups that influence the conformation of the protein.
- In some enzymes, nonpolypeptide small molecules or ions, called *cofactors* or *prosthetic groups,* can bind to the active site and play an essential role in enzymatic catalysis. Small organic prosthetic groups in enzymes are also called *coenzymes;* vitamins, which cannot be synthesized in higher animal cells, function as or are used to generate coenzymes.
- Enzymes in a common pathway are located within specific cell compartments and may be further associated as domains of a monomeric protein, subunits of a multimeric protein, or components of a protein complex assembled on a common scaffold (see Figure 3-28).
- Motor proteins are mechanochemical enzymes that convert energy released by ATP hydrolysis into either linear or rotary movement.

3.4 Regulating Protein Function I: Protein Degradation

Most processes in cells do not take place independently of one another or at a constant rate. The activities of all proteins and other biomolecules are regulated to integrate their functions for optimal performance for survival. For example, the catalytic activity of enzymes is regulated so that the amount of reaction product is just sufficient to meet the needs of the cell. As a result, the steady-state concentrations of substrates and products will vary, depending on cellular conditions. Regulation of nonenzymatic proteins—the opening or closing of membrane channels or the assembly of a macromolecular complex, for example—is also essential.

In general, there are three ways to regulate protein activity. First, cells can increase or decrease the steady-state level of the protein by altering its rate of synthesis, its rate of degradation, or both. Second, cells can change the intrinsic activity, as distinct from the amount, of the protein (e.g., the affinity of substrate binding, the fraction of time the protein is in an active versus inactive conformation). Third, there can be a change in location or concentration within the cell of the protein itself, the target of the protein's activity (e.g., an enzyme's substrate), or some other molecule required for the protein's activity (e.g., an enzyme's cofactor). All three types of regulation play essential roles in the lives and functions of cells.

Regulated Synthesis and Degradation of Proteins Is a Fundamental Property of Cells

Control of Protein Synthesis The rate of synthesis of proteins is determined by the rate at which the DNA encoding the protein is converted to mRNA (transcription), the steady-state amount of the active mRNA in the cell, and the rate at which the mRNA is converted into newly synthesized protein (translation). These important pathways are described in detail in Chapter 4.

Control of Protein Degradation The life span of intracellular proteins varies from as short as a few minutes for mitotic cyclins, which help regulate passage through the mitotic stage of cell division, to as long as the age of an organism for proteins in the lens of the eye. Protein life span is controlled primarily by regulated protein degradation.

There are two especially important roles for protein degradation. First, degradation removes proteins that are potentially toxic, improperly folded or assembled, or damaged—including the products of mutated genes and proteins damaged by chemically active cell metabolites. Despite the existence of chaperone-mediated protein folding, it is estimated that as many as 30 percent of newly made proteins are rapidly degraded because they are misfolded, their assembly into complexes is defective, or they are otherwise unsuitable. Most other proteins are degraded more slowly, about 1–2 percent degradation per hour in mammalian cells. Second, the controlled destruction of otherwise normal proteins provides a powerful mechanism for maintaining the appropriate levels of the proteins and their activities, and for permitting rapid changes in these levels to help the cells respond to changing conditions.

Eukaryotic cells have several pathways for degrading proteins. One major pathway is degradation by enzymes within lysosomes, membrane-limited organelles whose acidic interior (pH $\approx$4.5) is filled with a host of hydrolytic enzymes. Lysosomal degradation is directed primarily toward aged or defective organelles of the cell—a process