3

Amino Acids, Peptides, and Proteins

Proteins mediate virtually every process that takes place in a cell, exhibiting an almost endless diversity of functions. To explore the molecular mechanism of a biological process, a biochemist almost inevitably studies one or more proteins. Proteins are the most abundant biological macromolecules, occurring in all cells and all parts of cells. Proteins also occur in great variety; thousands of different kinds may be found in a single cell. As the arbiters of molecular function, proteins are the most important final products of the information pathways discussed in Part III of this book. Proteins are the molecular instruments through which genetic information is expressed.

Relatively simple monomeric subunits provide the key to the structure of the thousands of different proteins. The proteins of every organism, from the simplest of bacteria to human beings, are constructed from the same ubiquitous set of 20 amino acids. Because each of these amino acids has a side chain with distinctive chemical properties, this group of 20 precursor molecules may be regarded as the alphabet in which the language of protein structure is written.

To generate a particular protein, amino acids are covalently linked in a characteristic linear sequence. What is most remarkable is that cells can produce proteins with strikingly different properties and activities by joining the same 20 amino acids in many different combinations and sequences. From these building blocks different organisms can make such widely diverse products as enzymes, hormones, antibodies, transporters, muscle fibers, the lens protein of the eye, feathers, spider webs, rhinoceros horn, milk proteins, antibiotics, mushroom poisons, and myriad other substances having distinct biological activities **(Fig. 3–1)**. Among these protein products, the enzymes are the most varied and specialized. As the catalysts of virtually all cellular reactions, enzymes are one of the keys to understanding the chemistry of life and thus provide a focal point for any course in biochemistry.

Protein structure and function are the topics of this and the next three chapters. Here, we begin with a description of the fundamental chemical properties of amino acids, peptides, and proteins. We also consider how a biochemist works with proteins.

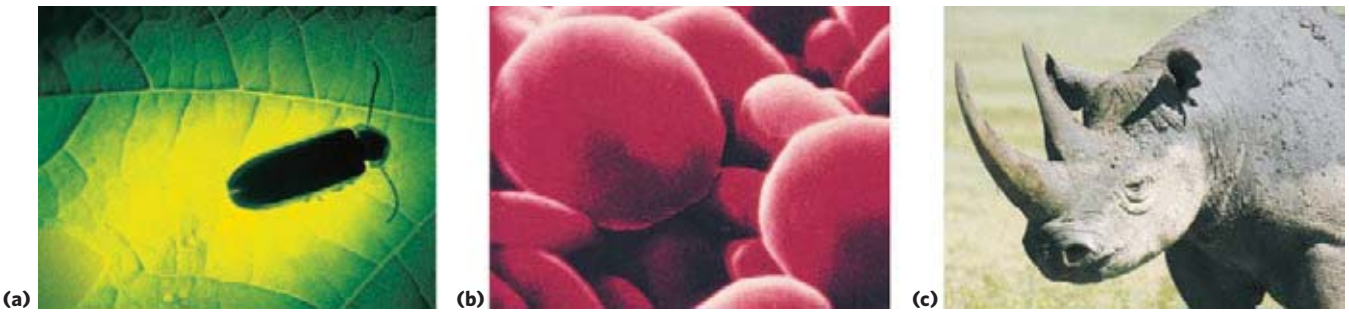


FIGURE 3–1 Some functions of proteins. (a) The light produced by fireflies is the result of a reaction involving the protein luciferin and ATP, catalyzed by the enzyme luciferase (see Box 13–1). **(b)** Erythrocytes contain large amounts of the oxygen-transporting protein hemoglobin. **(c)** The protein keratin, formed by all vertebrates, is the chief structural component of hair, scales, horn, wool, nails, and feathers. The black rhinoceros is nearing extinction in the wild because of the belief prevalent in some parts of the world that a powder derived from its horn has aphrodisiac properties. In reality, the chemical properties of powdered rhinoceros horn are no different from those of powdered bovine hooves or human fingernails.

3.1 Amino Acids

Protein Architecture—Amino Acids Proteins are polymers of amino acids, with each **amino acid residue** joined to its neighbor by a specific type of covalent bond. (The term "residue" reflects the loss of the elements of water when one amino acid is joined to another.) Proteins can be broken down (hydrolyzed) to their constituent amino acids by a variety of methods, and the earliest studies of proteins naturally focused on the free amino acids derived from them. Twenty different amino acids are commonly found in proteins. The first to be discovered was asparagine, in 1806. The last of the 20 to be found, threonine, was not identified until 1938. All the amino acids have trivial or common names, in some cases derived from the source from which they were first isolated. Asparagine was first found in asparagus, and glutamate in wheat gluten; tyrosine was first isolated from cheese (its name is derived from the Greek *tyros*, "cheese"); and glycine (Greek *glykos*, "sweet") was so named because of its sweet taste.

Amino Acids Share Common Structural Features

All 20 of the common amino acids are α-amino acids. They have a carboxyl group and an amino group bonded to the same carbon atom (the α carbon) **(Fig. 3–2)**. They differ from each other in their side chains, or **R groups**, which vary in structure, size, and electric charge, and which influence the solubility of the amino acids in water. In addition to these 20 amino acids there are many less common ones. Some are residues modified after a protein has been synthesized; others are amino acids present in living organisms but not as constituents of proteins. The common amino acids of proteins have been assigned three-letter abbreviations and one-letter symbols (Table 3–1), which are used as shorthand to indicate the composition and sequence of amino acids polymerized in proteins.

KEY CONVENTION: The three-letter code is transparent, the abbreviations generally consisting of the first three letters of the amino acid name. The one-letter code was devised by Margaret Oakley Dayhoff, considered by many to be the founder of the field of bioinformatics. The one-letter code reflects an attempt to reduce the size of the data files (in an era of punch-card computing) used to describe amino acid sequences. It was designed to be easily memorized, and understanding its origin can help students do just that. For six amino acids (CHIMSV), the first letter of the amino acid name is unique and thus is used as the symbol. For five others (AGLPT), the first letter is not

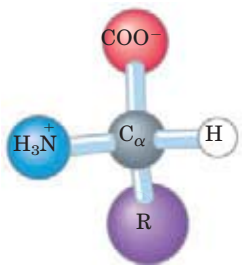


FIGURE 3–2 General structure of an amino acid. This structure is common to all but one of the α-amino acids. (Proline, a cyclic amino acid, is the exception.) The R group, or side chain (purple), attached to the α carbon (gray) is different in each amino acid.

Margaret Oakley Dayhoff, 1925–1983

unique but is assigned to the amino acid that is most common in proteins (for example, leucine is more common than lysine). For another four, the letter used is phonetically suggestive (RFYW: aRginine, Fenyl-alanine, tYrosine, tWiptophan). The rest were harder to assign. Four (DNEQ) were assigned letters found within or suggested by their names (asparDic, asparagiNe, glutamEke, Q-tamine). That left lysine. Only a few letters were left in the alphabet, and K was chosen because it was the closest to L. ■

For all the common amino acids except glycine, the α carbon is bonded to four different groups: a carboxyl group, an amino group, an R group, and a hydrogen atom (Fig. 3–2; in glycine, the R group is another hydrogen atom). The α-carbon atom is thus a **chiral center** (p. 17). Because of the tetrahedral arrangement of the bonding orbitals around the α-carbon atom, the four different groups can occupy two unique spatial arrangements, and thus amino acids have two possible stereoisomers. Since they are nonsuperposable mirror images of each other **(Fig. 3–3)**, the two forms

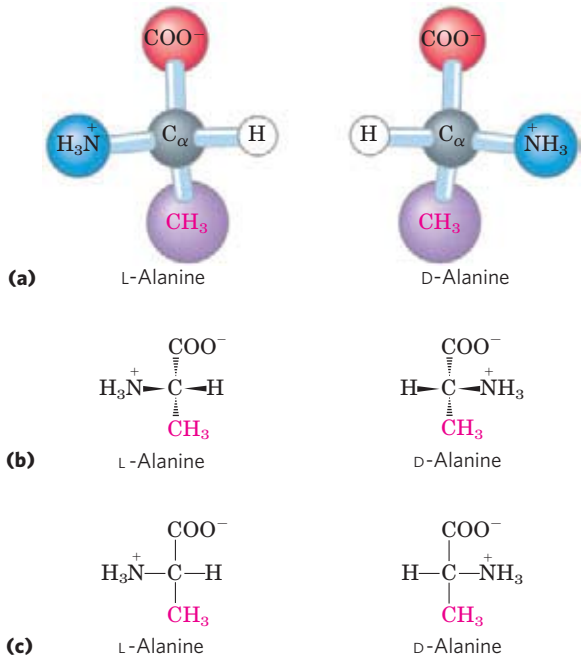


FIGURE 3–3 Stereoisomerism in α-amino acids. (a) The two stereoisomers of alanine, L- and D-alanine, are nonsuperposable mirror images of each other (enantiomers). **(b, c)** Two different conventions for showing the configurations in space of stereoisomers. In perspective formulas **(b)**, the solid wedge-shaped bonds project out of the plane of the paper, the dashed bonds behind it. In projection formulas **(c)**, the horizontal bonds are assumed to project out of the plane of the paper, the vertical bonds behind. However, projection formulas are often used casually and are not always intended to portray a specific stereochemical configuration.

TABLE 3–1 Properties and Conventions Associated with the Common Amino Acids Found in Proteins

Amino acid	Abbreviation/ symbol	M_r*	pK_a values: pK_1 (—COOH)	pK_2 ($—NH_3^+$)	pK_R (R group)	pI	Hydropathy index†	Occurrence in proteins (%)‡
Nonpolar, aliphatic R groups								
Glycine	Gly G	75	2.34	9.60		5.97	−0.4	7.2
Alanine	Ala A	89	2.34	9.69		6.01	1.8	7.8
Proline	Pro P	115	1.99	10.96		6.48	−1.6	5.2
Valine	Val V	117	2.32	9.62		5.97	4.2	6.6
Leucine	Leu L	131	2.36	9.60		5.98	3.8	9.1
Isoleucine	Ile I	131	2.36	9.68		6.02	4.5	5.3
Methionine	Met M	149	2.28	9.21		5.74	1.9	2.3
Aromatic R groups								
Phenylalanine	Phe F	165	1.83	9.13		5.48	2.8	3.9
Tyrosine	Tyr Y	181	2.20	9.11	10.07	5.66	−1.3	3.2
Tryptophan	Trp W	204	2.38	9.39		5.89	−0.9	1.4
Polar, uncharged R groups								
Serine	Ser S	105	2.21	9.15		5.68	−0.8	6.8
Threonine	Thr T	119	2.11	9.62		5.87	−0.7	5.9
Cysteine¶	Cys C	121	1.96	10.28	8.18	5.07	2.5	1.9
Asparagine	Asn N	132	2.02	8.80		5.41	−3.5	4.3
Glutamine	Gln Q	146	2.17	9.13		5.65	−3.5	4.2
Positively charged R groups								
Lysine	Lys K	146	2.18	8.95	10.53	9.74	−3.9	5.9
Histidine	His H	155	1.82	9.17	6.00	7.59	−3.2	2.3
Arginine	Arg R	174	2.17	9.04	12.48	10.76	−4.5	5.1
Negatively charged R groups								
Aspartate	Asp D	133	1.88	9.60	3.65	2.77	−3.5	5.3
Glutamate	Glu E	147	2.19	9.67	4.25	3.22	−3.5	6.3

*M_r values reflect the structures as shown in Figure 3–5. The elements of water (M_r 18) are deleted when the amino acid is incorporated into a polypeptide.

†A scale combining hydrophobicity and hydrophilicity of R groups. The values reflect the free energy (ΔG) of transfer of the amino acid side chain from a hydrophobic solvent to water. This transfer is favorable ($\Delta G < 0$; negative value in the index) for charged or polar amino acid side chains, and unfavorable ($\Delta G > 0$; positive value in the index) for amino acids with nonpolar or more hydrophobic side chains. See Chapter 11. From Kyte, J. & Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157,** 105–132.

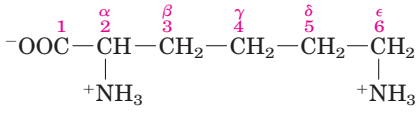
‡Average occurrence in more than 1,150 proteins. From Doolittle, R.F. (1989) Redundancies in protein sequences. In *Prediction of Protein Structure and the Principles of Protein Conformation* (Fasman, G.D., ed.), pp. 599–623, Plenum Press, New York.

¶Cysteine is generally classified as polar despite having a positive hydropathy index. This reflects the ability of the sulfhydryl group to act as a weak acid and to form a weak hydrogen bond with oxygen or nitrogen.

represent a class of stereoisomers called **enantiomers** (see Fig. 1–20). All molecules with a chiral center are also **optically active**—that is, they rotate plane-polarized light (see Box 1–2).

KEY CONVENTION: Two conventions are used to identify the carbons in an amino acid—a practice that can be confusing. The additional carbons in an R group are commonly designated β, γ, δ, ε, and so forth, proceeding out from the α carbon. For most other organic molecules, carbon atoms are simply numbered from one end, giving highest priority (C-1) to the carbon with the substituent containing the atom of highest atomic number. Within this latter convention, the carboxyl

carbon of an amino acid would be C-1 and the α carbon would be C-2.



Lysine

In some cases, such as amino acids with heterocyclic R groups (such as histidine), the Greek lettering system is ambiguous and the numbering convention is therefore used. For branched amino acid side chains, equivalent carbons are given numbers after the Greek letters. Leucine thus has $\delta 1$ and $\delta 2$ carbons (see the structure in Fig. 3–5). ■

Special nomenclature has been developed to specify the **absolute configuration** of the four substituents of asymmetric carbon atoms. The absolute configurations of simple sugars and amino acids are specified by the **D, L system (Fig. 3–4)**, based on the absolute configuration of the three-carbon sugar glyceraldehyde, a convention proposed by Emil Fischer in 1891. (Fischer knew what groups surrounded the asymmetric carbon of glyceraldehyde but had to guess at their absolute configuration; he guessed right, as was later confirmed by x-ray diffraction analysis.) For all chiral compounds, stereoisomers having a configuration related to that of L-glyceraldehyde are designated L, and stereoisomers related to D-glyceraldehyde are designated D. The functional groups of L-alanine are matched with those of L-glyceraldehyde by aligning those that can be interconverted by simple, one-step chemical reactions. Thus the carboxyl group of L-alanine occupies the same position about the chiral carbon as does the aldehyde group of L-glyceraldehyde, because an aldehyde is readily converted to a carboxyl group via a one-step oxidation. Historically, the similar L and D designations were used for levorotatory (rotating plane-polarized light to the left) and dextrorotatory (rotating light to the right). However, not all L-amino acids are levorotatory, and the convention shown in Figure 3–4 was needed to avoid potential ambiguities about absolute configuration. By Fischer's convention, L and D refer *only* to the absolute configuration of the four substituents around the chiral carbon, not to optical properties of the molecule.

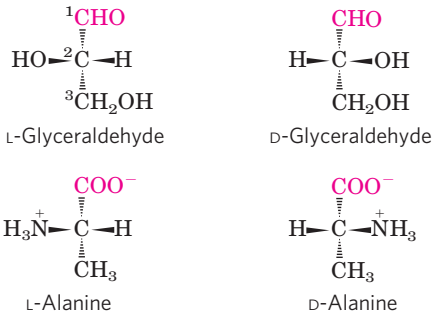


FIGURE 3–4 Steric relationship of the stereoisomers of alanine to the absolute configuration of L- and D-glyceraldehyde. In these perspective formulas, the carbons are lined up vertically, with the chiral atom in the center. The carbons in these molecules are numbered beginning with the terminal aldehyde or carboxyl carbon (red), 1 to 3 from top to bottom as shown. When presented in this way, the R group of the amino acid (in this case the methyl group of alanine) is always below the α carbon. L-Amino acids are those with the α-amino group on the left, and D-amino acids have the α-amino group on the right.

Another system of specifying configuration around a chiral center is the **RS system**, which is used in the systematic nomenclature of organic chemistry and describes more precisely the configuration of molecules with more than one chiral center (p. 18).

The Amino Acid Residues in Proteins Are L Stereoisomers

Nearly all biological compounds with a chiral center occur naturally in only one stereoisomeric form, either D or L. The amino acid residues in protein molecules are exclusively L stereoisomers. D-Amino acid residues have been found in only a few, generally small peptides, including some peptides of bacterial cell walls and certain peptide antibiotics.

It is remarkable that virtually all amino acid residues in proteins are L stereoisomers. When chiral compounds are formed by ordinary chemical reactions, the result is a racemic mixture of D and L isomers, which are difficult for a chemist to distinguish and separate. But to a living system, D and L isomers are as different as the right hand and the left. The formation of stable, repeating substructures in proteins (Chapter 4) generally requires that their constituent amino acids be of one stereochemical series. Cells are able to specifically synthesize the L isomers of amino acids because the active sites of enzymes are asymmetric, causing the reactions they catalyze to be stereospecific.

Amino Acids Can Be Classified by R Group

Knowledge of the chemical properties of the common amino acids is central to an understanding of biochemistry. The topic can be simplified by grouping the amino acids into five main classes based on the properties of their R groups (Table 3–1), particularly their **polarity**, or tendency to interact with water at biological pH (near pH 7.0). The polarity of the R groups varies widely, from nonpolar and hydrophobic (water-insoluble) to highly polar and hydrophilic (water-soluble). A few amino acids are somewhat difficult to characterize or do not fit perfectly in any one group, particularly glycine, histidine, and cysteine. Their assignments to particular groupings are the results of considered judgments rather than absolutes.

The structures of the 20 common amino acids are shown in **Figure 3–5**, and some of their properties are listed in Table 3–1. Within each class there are gradations of polarity, size, and shape of the R groups.

Nonpolar, Aliphatic R Groups The R groups in this class of amino acids are nonpolar and hydrophobic. The side

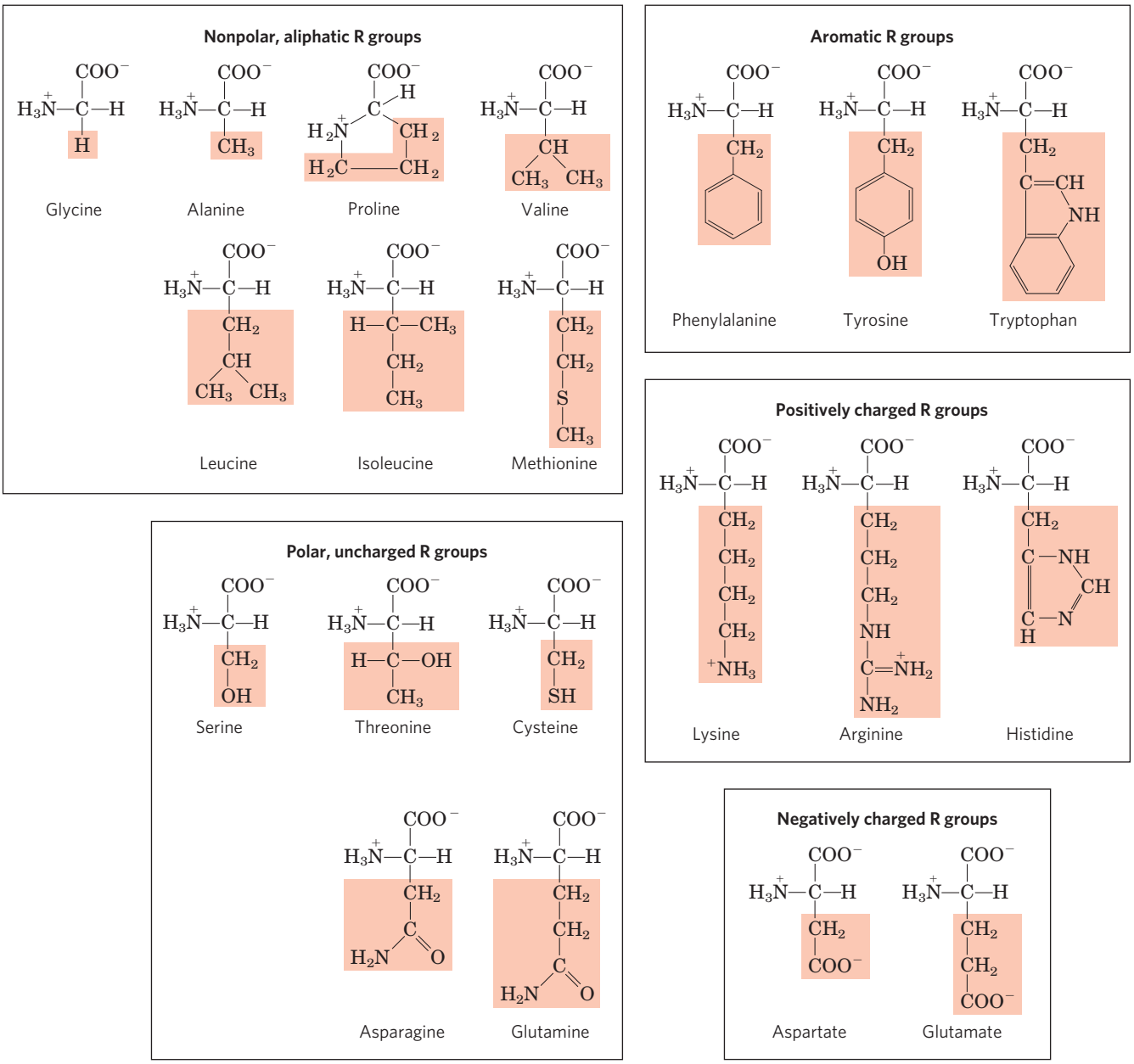


FIGURE 3–5 The 20 common amino acids of proteins. The structural formulas show the state of ionization that would predominate at pH 7.0. The unshaded portions are those common to all the amino acids; the shaded portions are the R groups. Although the R group of histidine is shown uncharged, its pK_a (see Table 3–1) is such that a small but significant fraction of these groups are positively charged at pH 7.0. The protonated form of histidine is shown above the graph in Figure 3–12b.

chains of **alanine**, **valine**, **leucine**, and **isoleucine** tend to cluster together within proteins, stabilizing protein structure by means of hydrophobic interactions. **Glycine** has the simplest structure. Although it is most easily grouped with the nonpolar amino acids, its very small side chain makes no real contribution to hydrophobic interactions. **Methionine**, one of the two sulfur-containing amino acids, has a slightly nonpolar thioether group in its side chain. **Proline** has an aliphatic side chain with a distinctive cyclic structure. The secondary amino (imino) group of proline residues is held in a rigid conformation that reduces the structural flexibility of polypeptide regions containing proline.

Aromatic R Groups **Phenylalanine**, **tyrosine**, and **tryptophan**, with their aromatic side chains, are relatively nonpolar (hydrophobic). All can participate in hydrophobic interactions. The hydroxyl group of tyrosine can form hydrogen bonds, and it is an important functional group in some enzymes. Tyrosine and tryptophan are significantly more polar than phenylalanine, because of the tyrosine hydroxyl group and the nitrogen of the tryptophan indole ring.

Tryptophan and tyrosine, and to a much lesser extent phenylalanine, absorb ultraviolet light (**Fig. 3–6**; see also Box 3–1). This accounts for the characteristic strong absorbance of light by most proteins at a wavelength of

280 nm, a property exploited by researchers in the characterization of proteins.

Polar, Uncharged R Groups The R groups of these amino acids are more soluble in water, or more hydrophilic, than those of the nonpolar amino acids, because they contain functional groups that form hydrogen bonds

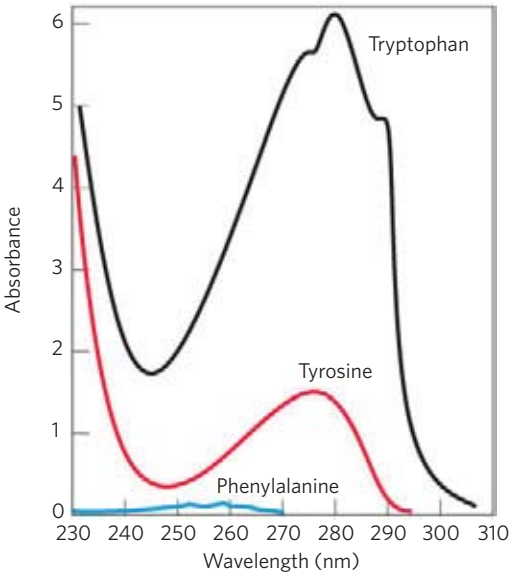


FIGURE 3–6 Absorption of ultraviolet light by aromatic amino acids. Comparison of the light absorption spectra of the aromatic amino acids tryptophan, tyrosine, and phenylalanine at pH 6.0. The amino acids are present in equimolar amounts (10^{-3} M) under identical conditions. The measured absorbance of tryptophan is more than four times that of tyrosine at a wavelength of 280 nm. Note that the maximum light absorption for both tryptophan and tyrosine occurs near 280 nm. Light absorption by phenylalanine generally contributes little to the spectroscopic properties of proteins.

BOX 3–1 METHODS Absorption of Light by Molecules: The Lambert-Beer Law

A wide range of biomolecules absorb light at characteristic wavelengths, just as tryptophan absorbs light at 280 nm (see Fig. 3–6). Measurement of light absorption by a spectrophotometer is used to detect and identify molecules and to measure their concentration in solution. The fraction of the incident light absorbed by a solution at a given wavelength is related to the thickness of the absorbing layer (path length) and the concentration of the absorbing species (Fig. 1). These two relationships are combined into the Lambert-Beer law,

$$\log \frac{I_0}{I} = \varepsilon cl$$

where I_0 is the intensity of the incident light, I is the intensity of the transmitted light, the ratio I/I_0 (the inverse of the ratio in the equation) is the transmittance, ε is the molar extinction coefficient (in units of liters per mole-centimeter), c is the concentration of the absorbing species (in moles per liter), and l is the path length of the light-absorbing sample (in centimeters). The Lambert-Beer law assumes that the incident light is parallel and monochromatic (of a single wavelength) and that the solvent and solute molecules are randomly oriented. The expression log (I_0/I) is called the **absorbance**, designated A.

It is important to note that each successive millimeter of path length of absorbing solution in a 1.0 cm cell absorbs not a constant amount but a constant fraction of the light that is incident upon it. However, with an absorbing layer of fixed path length, *the absorbance,* A, *is directly proportional to the concentration of the absorbing solute.*

The molar extinction coefficient varies with the nature of the absorbing compound, the solvent, and the wavelength, and also with pH if the light-absorbing species is in equilibrium with an ionization state that has different absorbance properties.

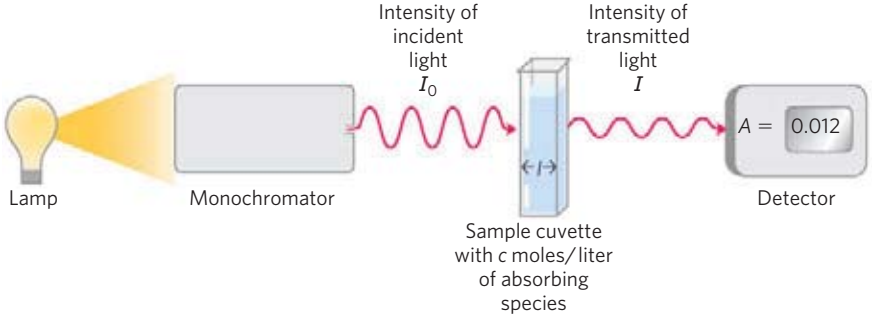


FIGURE 1 The principal components of a spectrophotometer. A light source emits light along a broad spectrum, then the monochromator selects and transmits light of a particular wavelength. The monochromatic light passes through the sample in a cuvette of path length l. The absorbance of the sample, log (I_0/I), is proportional to the concentration of the absorbing species. The transmitted light is measured by a detector.

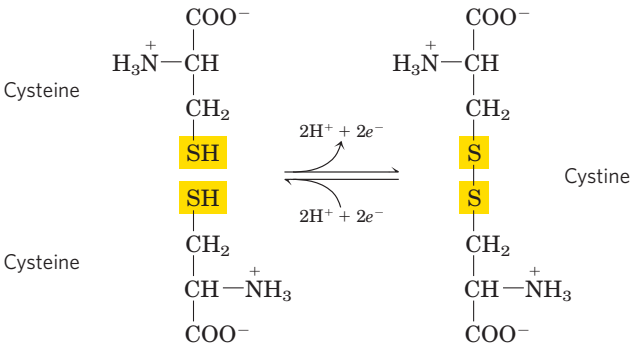


FIGURE 3–7 Reversible formation of a disulfide bond by the oxidation of two molecules of cysteine. Disulfide bonds between Cys residues stabilize the structures of many proteins.

with water. This class of amino acids includes **serine**, **threonine**, **cysteine**, **asparagine**, and **glutamine**. The polarity of serine and threonine is contributed by their hydroxyl groups, and that of asparagine and glutamine by their amide groups. Cysteine is an outlier here because its polarity, contributed by its sulfhydryl group, is quite modest. Cysteine is a weak acid and can make weak hydrogen bonds with oxygen or nitrogen.

Asparagine and glutamine are the amides of two other amino acids also found in proteins—aspartate and glutamate, respectively—to which asparagine and glutamine are easily hydrolyzed by acid or base. Cysteine is readily oxidized to form a covalently linked dimeric amino acid called **cystine**, in which two cysteine molecules or residues are joined by a disulfide bond **(Fig. 3–7)**. The disulfide-linked residues are strongly hydrophobic (nonpolar). Disulfide bonds play a special role in the structures of many proteins by forming covalent links between parts of a polypeptide molecule or between two different polypeptide chains.

Positively Charged (Basic) R Groups The most hydrophilic R groups are those that are either positively or negatively charged. The amino acids in which the R groups have significant positive charge at pH 7.0 are **lysine**, which has a second primary amino group at the ε position on its aliphatic chain; **arginine**, which has a positively charged guanidinium group; and **histidine**, which has an aromatic imidazole group. As the only common amino acid having an ionizable side chain with pK_a near neutrality, histidine may be positively charged (protonated form) or uncharged at pH 7.0. His residues facilitate many enzyme-catalyzed reactions by serving as proton donors/acceptors.

Negatively Charged (Acidic) R Groups The two amino acids having R groups with a net negative charge at pH 7.0 are **aspartate** and **glutamate**, each of which has a second carboxyl group.

Uncommon Amino Acids Also Have Important Functions

In addition to the 20 common amino acids, proteins may contain residues created by modification of common residues already incorporated into a polypeptide **(Fig. 3–8a)**. Among these uncommon amino acids are **4-hydroxyproline**, a derivative of proline, and **5-hydroxylysine**, derived from lysine. The former is found in plant cell wall proteins, and both are found in collagen, a fibrous protein of connective tissues. **6-*N*-Methyllysine** is a constituent of myosin, a contractile protein of muscle. Another important uncommon amino acid is **γ-carboxyglutamate**, found in the blood-clotting protein prothrombin and in certain other proteins that bind Ca^{2+} as part of their biological function. More complex is **desmosine**, a derivative of four Lys residues, which is found in the fibrous protein elastin.

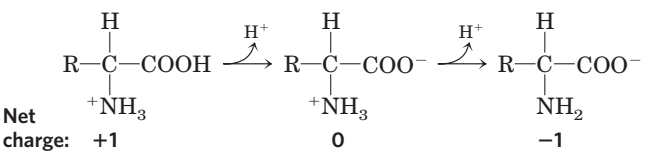
Selenocysteine is a special case. This rare amino acid residue is introduced during protein synthesis rather than created through a postsynthetic modification. It contains selenium rather than the sulfur of cysteine. Actually derived from serine, selenocysteine is a constituent of just a few known proteins.

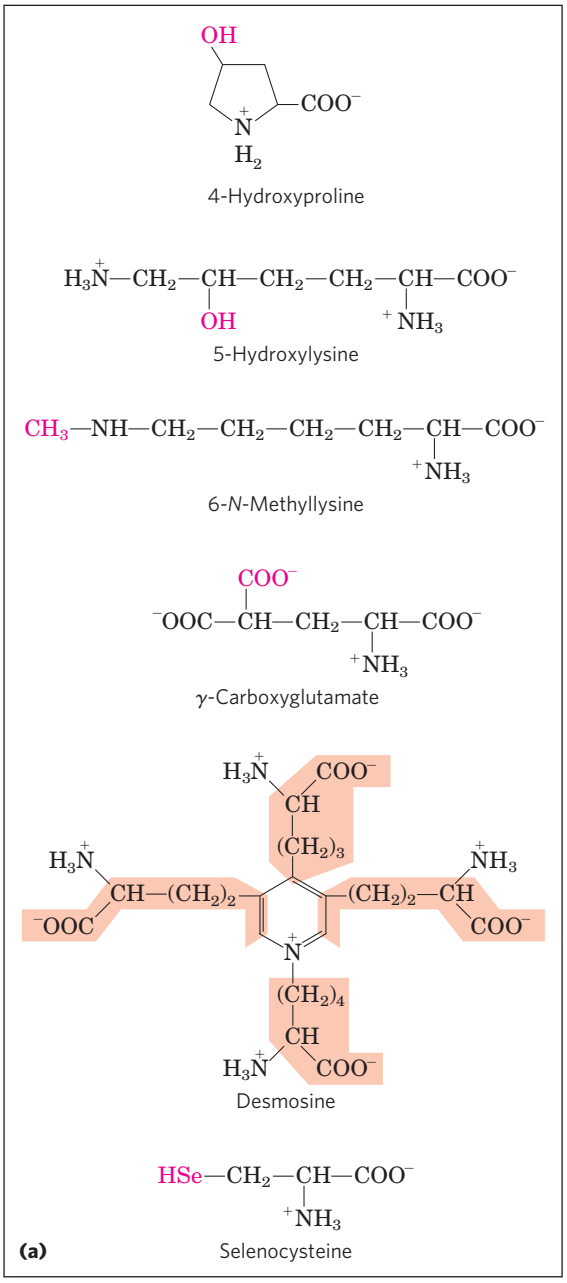
Some amino acid residues in a protein may be modified transiently to alter the protein's function. The addition of phosphoryl, methyl, acetyl, adenylyl, ADP-ribosyl, or other groups to particular amino acid residues can increase or decrease a protein's activity (Fig. 3–8b). Phosphorylation is a particularly common regulatory modification. Covalent modification as a protein regulatory strategy is discussed in more detail in Chapter 6.

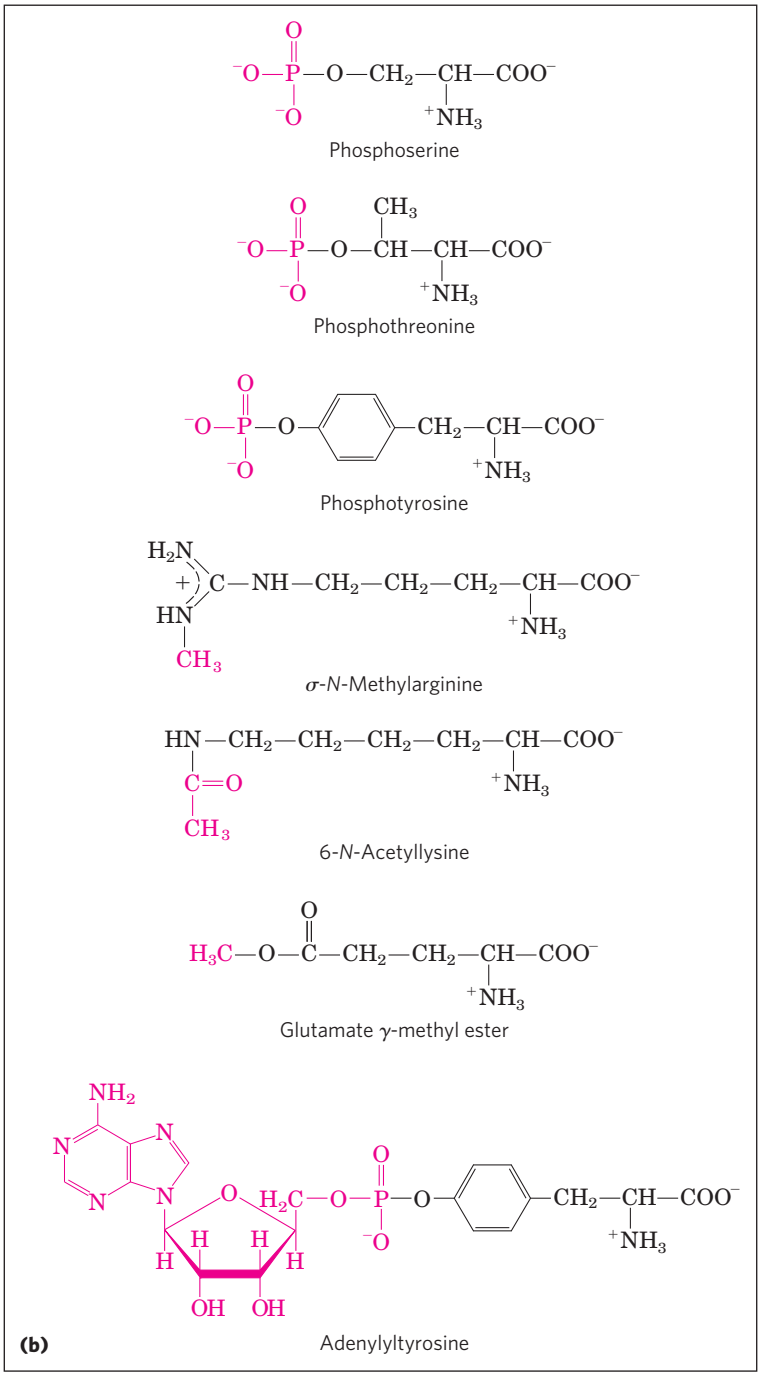
Some 300 additional amino acids have been found in cells. They have a variety of functions but are not all constituents of proteins. **Ornithine** and **citrulline** (Fig. 3–8c) deserve special note because they are key intermediates (metabolites) in the biosynthesis of arginine (Chapter 22) and in the urea cycle (Chapter 18).

Amino Acids Can Act as Acids and Bases

The amino and carboxyl groups of amino acids, along with the ionizable R groups of some amino acids, function as weak acids and bases. When an amino acid lacking an ionizable R group is dissolved in water at neutral pH, it exists in solution as the dipolar ion, or **zwitterion** (German for "hybrid ion"), which can act as either an acid or a base **(Fig. 3–9)**. Substances having this dual (acid-base) nature are **amphoteric** and are often called **ampholytes** (from "amphoteric electrolytes"). A simple monoamino monocarboxylic α-amino acid, such as alanine, is a diprotic acid when fully protonated; it has two groups, the —COOH group and the —NH_3^+ group, that can yield protons:







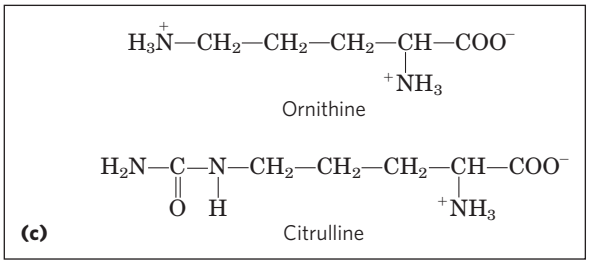


FIGURE 3–8 Uncommon amino acids. (a) Some uncommon amino acids found in proteins. All are derived from common amino acids. Extra functional groups added by modification reactions are shown in red. Desmosine is formed from four Lys residues (the carbon backbones are shaded in light red). Note the use of either numbers or Greek letters in the names of these structures to identify the altered carbon atoms. **(b)** Reversible amino acid modifications involved in regulation of protein activity. Phosphorylation is the most common type of regulatory modification. **(c)** Ornithine and citrulline, which are not found in proteins, are intermediates in the biosynthesis of arginine and in the urea cycle.

Amino Acids Have Characteristic Titration Curves

Acid-base titration involves the gradual addition or removal of protons (Chapter 2). **Figure 3–10** shows the titration curve of the diprotic form of glycine. The two ionizable groups of glycine, the carboxyl group and the amino group, are titrated with a strong base such as NaOH. The plot has two distinct stages, corresponding to deprotonation of two different groups on glycine. Each of the two stages resembles in shape the titration

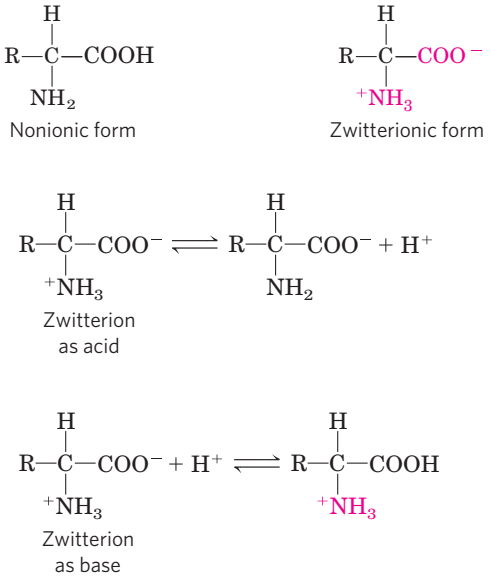


FIGURE 3–9 **Nonionic and zwitterionic forms of amino acids.** The nonionic form does not occur in significant amounts in aqueous solutions. The zwitterion predominates at neutral pH. A zwitterion can act as either an acid (proton donor) or a base (proton acceptor).

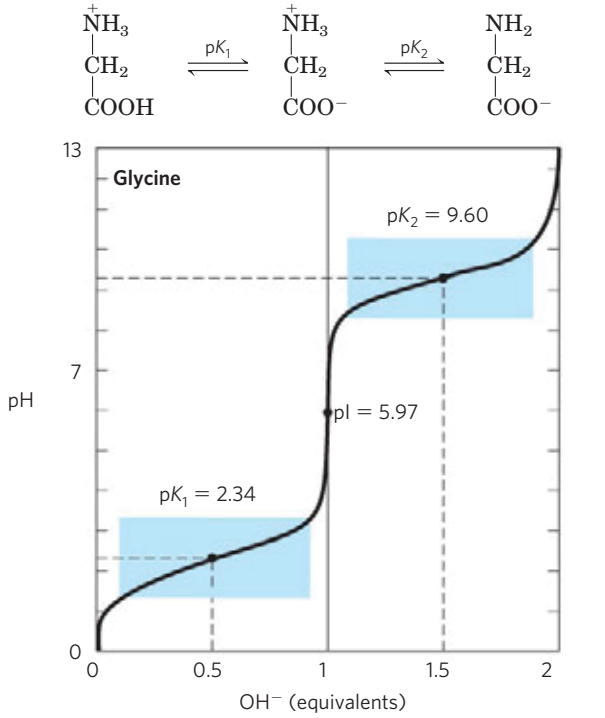


FIGURE 3–10 **Titration of an amino acid.** Shown here is the titration curve of 0.1 M glycine at 25°C. The ionic species predominating at key points in the titration are shown above the graph. The shaded boxes, centered at about $pK_1 = 2.34$ and $pK_2 = 9.60$, indicate the regions of greatest buffering power. Note that 1 equivalent of OH^- = 0.1 M NaOH added.

curve of a monoprotic acid, such as acetic acid (see Fig. 2–17), and can be analyzed in the same way. At very low pH, the predominant ionic species of glycine is the fully protonated form, $^+H_3N—CH_2—COOH$. In the first stage of the titration, the —COOH group of glycine loses its proton. At the midpoint of this stage, equimolar concentrations of the proton-donor ($^+H_3N—CH_2—COOH$) and proton-acceptor ($^+H_3N—CH_2—COO^-$) species are present. As in the titration of any weak acid, a point of inflection is reached at this midpoint where the pH is equal to the pK_a of the protonated group being titrated (see Fig. 2–18). For glycine, the pH at the midpoint is 2.34, thus its —COOH group has a pK_a (labeled pK_1 in Fig. 3–10) of 2.34. (Recall from Chapter 2 that pH and pK_a are simply convenient notations for proton concentration and the equilibrium constant for ionization, respectively. The pK_a is a measure of the tendency of a group to give up a proton, with that tendency decreasing tenfold as the pK_a increases by one unit.) As the titration of glycine proceeds, another important point is reached at pH 5.97. Here there is another point of inflection, at which removal of the first proton is essentially complete and removal of the second has just begun. At this pH glycine is present largely as the dipolar ion (zwitterion) $^+H_3N—CH_2—COO^-$. We shall return to the significance of this inflection point in the titration curve (labeled pI in Fig. 3–10) shortly.

The second stage of the titration corresponds to the removal of a proton from the $—NH_3^+$ group of glycine. The pH at the midpoint of this stage is 9.60, equal to the pK_a (labeled pK_2 in Fig. 3–10) for the $—NH_3^+$ group. The titration is essentially complete at a pH of about 12, at which point the predominant form of glycine is $H_2N—CH_2—COO^-$.

From the titration curve of glycine we can derive several important pieces of information. First, it gives a quantitative measure of the pK_a of each of the two ionizing groups: 2.34 for the —COOH group and 9.60 for the $—NH_3^+$ group. Note that the carboxyl group of glycine is over 100 times more acidic (more easily ionized) than the carboxyl group of acetic acid, which, as we saw in Chapter 2, has a pK_a of 4.76—about average for a carboxyl group attached to an otherwise unsubstituted aliphatic hydrocarbon. The perturbed pK_a of glycine is caused by repulsion between the departing proton and the nearby positively charged amino group on the α-carbon atom, as described in **Figure 3–11**. The opposite charges on the resulting zwitterion are stabilizing. Similarly, the pK_a of the amino group in glycine is perturbed downward relative to the average pK_a of an amino group. This effect is due partly to the electronegative oxygen atoms in the carboxyl groups, which tend to pull electrons toward them, increasing the tendency of the amino group to give up a proton. Hence, the α-amino group has a pK_a that is lower than that of an aliphatic amine such as methylamine (Fig. 3–11). In short, the pK_a of any functional group is greatly affected by its chemical environment, a phenomenon sometimes exploited in the active sites of enzymes to promote exquisitely adapted reaction mechanisms that depend

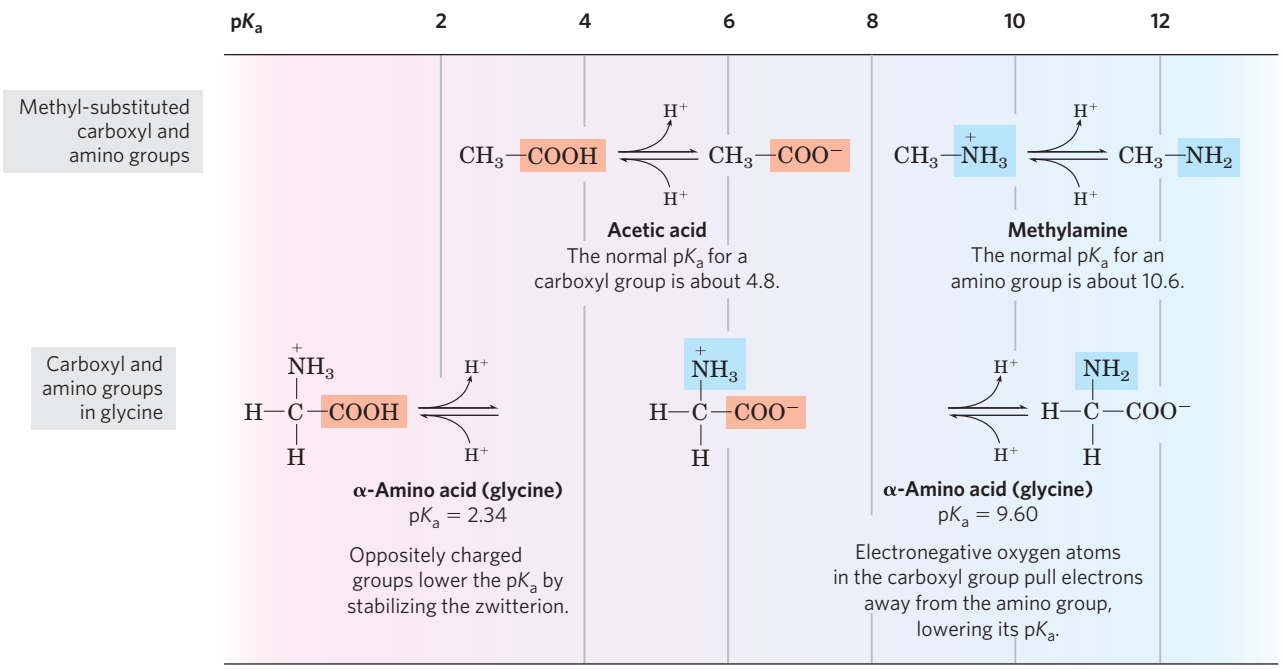


FIGURE 3–11 Effect of the chemical environment on pK_a. The pK_a values for the ionizable groups in glycine are lower than those for simple, methyl-substituted amino and carboxyl groups. These downward perturbations of pK_a are due to intramolecular interactions. Similar effects can be caused by chemical groups that happen to be positioned nearby—for example, in the active site of an enzyme.

on the perturbed pK_a values of proton donor/acceptor groups of specific residues.

The second piece of information provided by the titration curve of glycine is that this amino acid has two regions of buffering power. One of these is the relatively flat portion of the curve, extending for approximately 1 pH unit on either side of the first pK_a of 2.34, indicating that glycine is a good buffer near this pH. The other buffering zone is centered around pH 9.60. (Note that glycine is not a good buffer at the pH of intracellular fluid or blood, about 7.4.) Within the buffering ranges of glycine, the Henderson-Hasselbalch equation (p. 64) can be used to calculate the proportions of proton-donor and proton-acceptor species of glycine required to make a buffer at a given pH.

Titration Curves Predict the Electric Charge of Amino Acids

Another important piece of information derived from the titration curve of an amino acid is the relationship between its net charge and the pH of the solution. At pH 5.97, the point of inflection between the two stages in its titration curve, glycine is present predominantly as its dipolar form, fully ionized but with no *net* electric charge (Fig. 3–10). The characteristic pH at which the *net* electric charge is zero is called the **isoelectric point** or **isoelectric pH**, designated **pI**. For glycine, which has no ionizable group in its side chain, the isoelectric point is simply the arithmetic mean of the two pK_a values:

$$\text{pI} = \frac{1}{2}(\text{p}K_1 + \text{p}K_2) = \frac{1}{2}(2.34 + 9.60) = 5.97$$

As is evident in Figure 3–10, glycine has a net negative charge at any pH above its pI and will thus move toward the positive electrode (the anode) when placed in an electric field. At any pH below its pI, glycine has a net positive charge and will move toward the negative electrode (the cathode). The farther the pH of a glycine solution is from its isoelectric point, the greater the net electric charge of the population of glycine molecules. At pH 1.0, for example, glycine exists almost entirely as the form $^{+}H_3N—CH_2—COOH$ with a net positive charge of 1.0. At pH 2.34, where there is an equal mixture of $^{+}H_3N—CH_2—COOH$ and $^{+}H_3N—CH_2—COO^{-}$, the average or net positive charge is 0.5. The sign and the magnitude of the net charge of any amino acid at any pH can be predicted in the same way.

Amino Acids Differ in Their Acid-Base Properties

The shared properties of many amino acids permit some simplifying generalizations about their acid-base behaviors. First, all amino acids with a single α-amino group, a single α-carboxyl group, and an R group that does not ionize have titration curves resembling that of glycine (Fig. 3–10). These amino acids have very similar, although not identical, pK_a values: pK_a of the —COOH group in the range of 1.8 to 2.4, and pK_a of the $—NH_3^+$

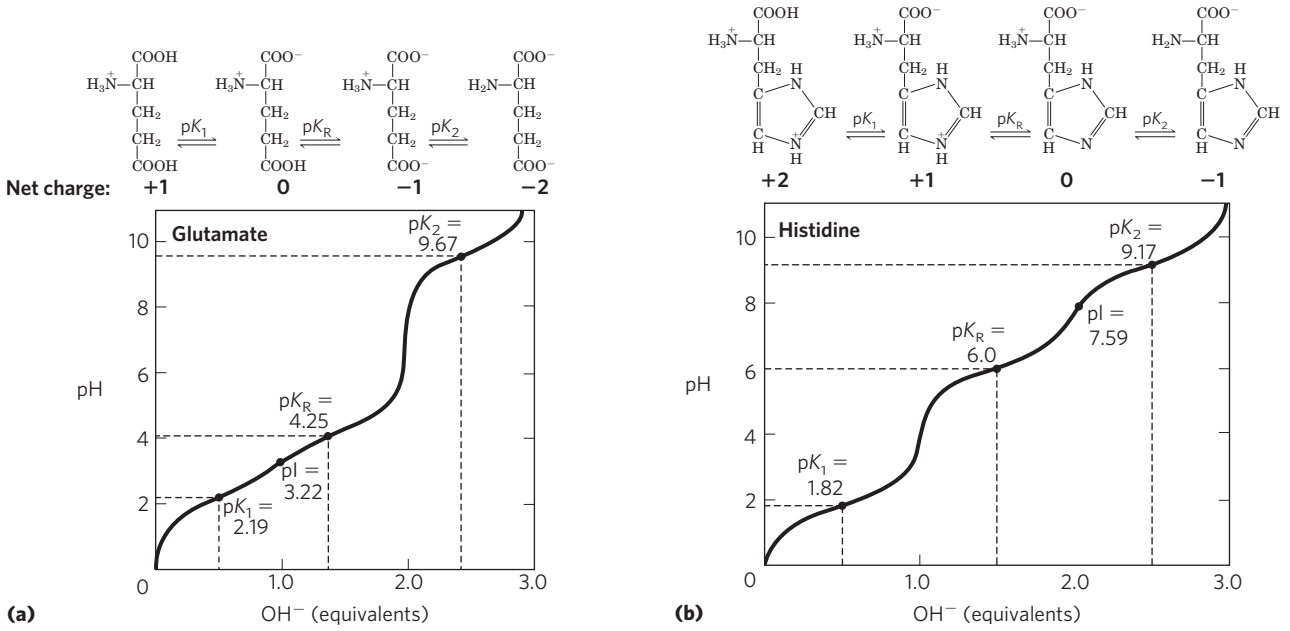


FIGURE 3–12 Titration curves for **(a)** glutamate and **(b)** histidine. The pK_a of the R group is designated here as pK_R.

group in the range of 8.8 to 11.0 (Table 3–1). The differences in these pK_a values reflect the chemical environments imposed by their R groups. Second, amino acids with an ionizable R group have more complex titration curves, with *three* stages corresponding to the three possible ionization steps; thus they have three pK_a values. The additional stage for the titration of the ionizable R group merges to some extent with that for the titration of the α-carboxyl group, the titration of the α-amino group, or both. The titration curves for two amino acids of this type, glutamate and histidine, are shown in **Figure 3–12**. The isoelectric points reflect the nature of the ionizing R groups present. For example, glutamate has a pI of 3.22, considerably lower than that of glycine. This is due to the presence of two carboxyl groups, which, at the average of their pK_a values (3.22), contribute a net charge of −1 that balances the +1 contributed by the amino group. Similarly, the pI of histidine, with two groups that are positively charged when protonated, is 7.59 (the average of the pK_a values of the amino and imidazole groups), much higher than that of glycine.

Finally, as pointed out earlier, under the general condition of free and open exposure to the aqueous environment, only histidine has an R group (pK_a = 6.0) providing significant buffering power near the neutral pH usually found in the intracellular and extracellular fluids of most animals and bacteria (Table 3–1).

SUMMARY 3.1 Amino Acids

- The 20 amino acids commonly found as residues in proteins contain an α-carboxyl group, an α-amino group, and a distinctive R group substituted on the α-carbon atom. The α-carbon atom of all amino acids except glycine is asymmetric, and thus amino acids can exist in at least two stereoisomeric forms. Only the L stereoisomers, with a configuration related to the absolute configuration of the reference molecule L-glyceraldehyde, are found in proteins.
- Other, less common amino acids also occur, either as constituents of proteins (through modification of common amino acid residues after protein synthesis) or as free metabolites.
- Amino acids can be classified into five types on the basis of the polarity and charge (at pH 7) of their R groups.
- Amino acids vary in their acid-base properties and have characteristic titration curves. Monoamino monocarboxylic amino acids (with nonionizable R groups) are diprotic acids ($^+H_3NCH(R)COOH$) at low pH and exist in several different ionic forms as the pH is increased. Amino acids with ionizable R groups have additional ionic species, depending on the pH of the medium and the pK_a of the R group.

3.2 Peptides and Proteins

We now turn to polymers of amino acids, the **peptides** and **proteins**. Biologically occurring polypeptides range in size from small to very large, consisting of two or three to thousands of linked amino acid residues. Our focus is on the fundamental chemical properties of these polymers.

Peptides Are Chains of Amino Acids

Two amino acid molecules can be covalently joined through a substituted amide linkage, termed a **peptide**

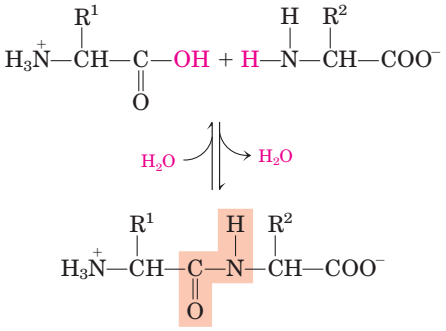


FIGURE 3–13 Formation of a peptide bond by condensation. The α-amino group of one amino acid (with R^2 group) acts as a nucleophile to displace the hydroxyl group of another amino acid (with R^1 group), forming a peptide bond (shaded in light red). Amino groups are good nucleophiles, but the hydroxyl group is a poor leaving group and is not readily displaced. At physiological pH, the reaction shown here does not occur to any appreciable extent.

bond, to yield a dipeptide. Such a linkage is formed by removal of the elements of water (dehydration) from the α-carboxyl group of one amino acid and the α-amino group of another **(Fig. 3–13)**. Peptide bond formation is an example of a condensation reaction, a common class of reactions in living cells. Under standard biochemical conditions, the equilibrium for the reaction shown in Figure 3–13 favors the amino acids over the dipeptide. To make the reaction thermodynamically more favorable, the carboxyl group must be chemically modified or activated so that the hydroxyl group can be more readily eliminated. A chemical approach to this problem is outlined later in this chapter. The biological approach to peptide bond formation is a major topic of Chapter 27.

Three amino acids can be joined by two peptide bonds to form a tripeptide; similarly, four amino acids can be linked to form a tetrapeptide, five to form a pentapeptide, and so forth. When a few amino acids are joined in this fashion, the structure is called an **oligopeptide**. When many amino acids are joined, the product is called a **polypeptide**. Proteins may have thousands of amino acid residues. Although the terms "protein" and "polypeptide" are sometimes used interchangeably, molecules referred to as polypeptides generally have molecular weights below 10,000, and those called proteins have higher molecular weights.

Figure 3–14 shows the structure of a pentapeptide. As already noted, an amino acid unit in a peptide is often called a residue (the part left over after losing the elements of water—a hydrogen atom from its amino group and the hydroxyl moiety from its carboxyl group). In a peptide, the amino acid residue at the end with a free α-amino group is the **amino-terminal** (or *N*-terminal) residue; the residue at the other end, which has a free carboxyl group, is the **carboxyl-terminal** (*C*-terminal) residue.

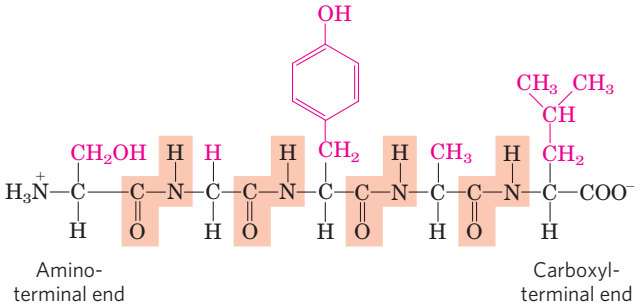


FIGURE 3–14 The pentapeptide serylglycyltyrosylalanylleucine, Ser-Gly-Tyr-Ala-Leu, or SGYAL. Peptides are named beginning with the amino-terminal residue, which by convention is placed at the left. The peptide bonds are shaded in light red; the R groups are in red.

KEY CONVENTION: When an amino acid sequence of a peptide, polypeptide, or protein is displayed, the amino-terminal end is placed on the left, the carboxyl-terminal end on the right. The sequence is read left to right, beginning with the amino-terminal end. ■

Although hydrolysis of a peptide bond is an exergonic reaction, it occurs only slowly because it has a high activation energy (p. 27). As a result, the peptide bonds in proteins are quite stable, with an average half-life ($t_{1/2}$) of about 7 years under most intracellular conditions.

Peptides Can Be Distinguished by Their Ionization Behavior

Peptides contain only one free α-amino group and one free α-carboxyl group, at opposite ends of the chain **(Fig. 3–15)**. These groups ionize as they do in free amino acids, although the ionization constants are different because an oppositely charged group is no longer

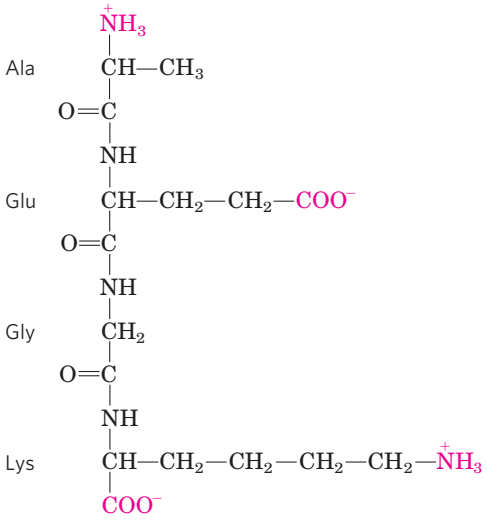


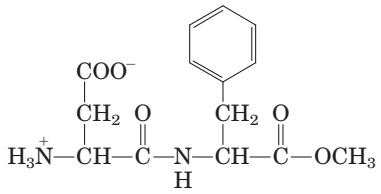
FIGURE 3–15 Alanylglutamylglycyllysine. This tetrapeptide has one free α-amino group, one free α-carboxyl group, and two ionizable R groups. The groups ionized at pH 7.0 are in red.

linked to the α carbon. The α-amino and α-carboxyl groups of all nonterminal amino acids are covalently joined in the peptide bonds, which do not ionize and thus do not contribute to the total acid-base behavior of peptides. However, the R groups of some amino acids can ionize (Table 3–1), and in a peptide these contribute to the overall acid-base properties of the molecule (Fig. 3–15). Thus the acid-base behavior of a peptide can be predicted from its free α-amino and α-carboxyl groups combined with the nature and number of its ionizable R groups.

Like free amino acids, peptides have characteristic titration curves and a characteristic isoelectric pH (pI) at which they do not move in an electric field. These properties are exploited in some of the techniques used to separate peptides and proteins, as we shall see later in the chapter. It should be emphasized that the pK_a value for an ionizable R group can change somewhat when an amino acid becomes a residue in a peptide. The loss of charge in the α-carboxyl and α-amino groups, the interactions with other peptide R groups, and other environmental factors can affect the pK_a. The pK_a values for R groups listed in Table 3–1 can be a useful guide to the pH range in which a given group will ionize, but they cannot be strictly applied to peptides.

Biologically Active Peptides and Polypeptides Occur in a Vast Range of Sizes and Compositions

No generalizations can be made about the molecular weights of biologically active peptides and proteins in relation to their functions. Naturally occurring peptides range in length from two to many thousands of amino acid residues. Even the smallest peptides can have biologically important effects. Consider the commercially synthesized dipeptide L-aspartyl-L-phenylalanine methyl ester, the artificial sweetener better known as aspartame or NutraSweet.



L-Aspartyl-L-phenylalanine methyl ester (aspartame)

Many small peptides exert their effects at very low concentrations. For example, a number of vertebrate hormones (Chapter 23) are small peptides. These include oxytocin (nine amino acid residues), which is secreted by the posterior pituitary gland and stimulates uterine contractions, and thyrotropin-releasing factor (three residues), which is formed in the hypothalamus and stimulates the release of another hormone, thyrotropin, from the anterior pituitary gland. Some extremely toxic mushroom poisons, such as amanitin, are also small peptides, as are many antibiotics.

How long are the polypeptide chains in proteins? As Table 3–2 shows, lengths vary considerably. Human cytochrome *c* has 104 amino acid residues linked in a single chain; bovine chymotrypsinogen has 245 residues. At the extreme is titin, a constituent of vertebrate muscle, which has nearly 27,000 amino acid residues and a molecular weight of about 3,000,000. The vast majority of naturally occurring proteins are much smaller than this, containing fewer than 2,000 amino acid residues.

Some proteins consist of a single polypeptide chain, but others, called **multisubunit** proteins, have two or more polypeptides associated noncovalently (Table 3–2). The individual polypeptide chains in a multisubunit

TABLE 3–2 Molecular Data on Some Proteins

	Molecular weight	Number of residues	Number of polypeptide chains
Cytochrome *c* (human)	12,400	104	1
Ribonuclease A (bovine pancreas)	13,700	124	1
Lysozyme (chicken egg white)	14,300	129	1
Myoglobin (equine heart)	16,700	153	1
Chymotrypsin (bovine pancreas)	25,200	241	3
Chymotrypsinogen (bovine)	25,700	245	1
Hemoglobin (human)	64,500	574	4
Serum albumin (human)	66,000	609	1
Hexokinase (yeast)	107,900	972	2
RNA polymerase (*E. coli*)	450,000	4,158	5
Apolipoprotein B (human)	513,000	4,536	1
Glutamine synthetase (*E. coli*)	619,000	5,628	12
Titin (human)	2,993,000	26,926	1

protein may be identical or different. If at least two are identical the protein is said to be **oligomeric**, and the identical units (consisting of one or more polypeptide chains) are referred to as **protomers**. Hemoglobin, for example, has four polypeptide subunits: two identical α chains and two identical β chains, all four held together by noncovalent interactions. Each α subunit is paired in an identical way with a β subunit within the structure of this multisubunit protein, so that hemoglobin can be considered either a tetramer of four polypeptide subunits or a dimer of $\alpha\beta$ protomers.

A few proteins contain two or more polypeptide chains linked covalently. For example, the two polypeptide chains of insulin are linked by disulfide bonds. In such cases, the individual polypeptides are not considered subunits but are commonly referred to simply as chains.

The amino acid composition of proteins is also highly variable. The 20 common amino acids almost never occur in equal amounts in a protein. Some amino acids may occur only once or not at all in a given type of protein; others may occur in large numbers. Table 3–3 shows the amino acid composition of bovine cytochrome *c* and chymotrypsinogen, the inactive precursor of the digestive enzyme chymotrypsin. These two proteins, with very different functions, also differ significantly in the relative numbers of each kind of amino acid residue.

We can calculate the approximate number of amino acid residues in a simple protein containing no other chemical constituents by dividing its molecular weight by 110. Although the average molecular weight of the 20 common amino acids is about 138, the smaller amino acids predominate in most proteins. If we take into account the proportions in which the various amino acids occur in an average protein (Table 3–1; the averages are determined by surveying the amino acid compositions of more than 1,000 different proteins), the average molecular weight of protein amino acids is nearer to 128. Because a molecule of water (M_r 18) is removed to create each peptide bond, the average molecular weight of an amino acid residue in a protein is about 128 − 18 = 110.

TABLE 3–3 Amino Acid Composition of Two Proteins

Amino acid	Bovine cytochrome *c*		Bovine chymotrypsinogen	
	Number of residues per molecule	Percentage of total*	Number of residues per molecule	Percentage of total*
Ala	6	6	22	9
Arg	2	2	4	1.6
Asn	5	5	14	5.7
Asp	3	3	9	3.7
Cys	2	2	10	4
Gln	3	3	10	4
Glu	9	9	5	2
Gly	14	13	23	9.4
His	3	3	2	0.8
Ile	6	6	10	4
Leu	6	6	19	7.8
Lys	18	17	14	5.7
Met	2	2	2	0.8
Phe	4	4	6	2.4
Pro	4	4	9	3.7
Ser	1	1	28	11.4
Thr	8	8	23	9.4
Trp	1	1	8	3.3
Tyr	4	4	4	1.6
Val	3	3	23	9.4
Total	104	102	245	99.7

Note: In some common analyses, such as acid hydrolysis, Asp and Asn are not readily distinguished from each other and are together designated Asx (or B). Similarly, when Glu and Gln cannot be distinguished, they are together designated Glx (or Z). In addition, Trp is destroyed by acid hydrolysis. Additional procedures must be employed to obtain an accurate assessment of complete amino acid content.

*Percentages do not total to 100%, due to rounding.

TABLE 3–4 Conjugated Proteins

Class	Prosthetic group	Example
Lipoproteins	Lipids	β_1-Lipoprotein of blood
Glycoproteins	Carbohydrates	Immunoglobulin G
Phosphoproteins	Phosphate groups	Casein of milk
Hemoproteins	Heme (iron porphyrin)	Hemoglobin
Flavoproteins	Flavin nucleotides	Succinate dehydrogenase
Metalloproteins	Iron Zinc Calcium Molybdenum Copper	Ferritin Alcohol dehydrogenase Calmodulin Dinitrogenase Plastocyanin

Some Proteins Contain Chemical Groups Other Than Amino Acids

Many proteins, for example the enzymes ribonuclease A and chymotrypsin, contain only amino acid residues and no other chemical constituents; these are considered simple proteins. However, some proteins contain permanently associated chemical components in addition to amino acids; these are called **conjugated proteins**. The non–amino acid part of a conjugated protein is usually called its **prosthetic group**. Conjugated proteins are classified on the basis of the chemical nature of their prosthetic groups (Table 3–4); for example, **lipoproteins** contain lipids, **glycoproteins** contain sugar groups, and **metalloproteins** contain a specific metal. Some proteins contain more than one prosthetic group. Usually the prosthetic group plays an important role in the protein's biological function.

SUMMARY 3.2 Peptides and Proteins

- Amino acids can be joined covalently through peptide bonds to form peptides and proteins. Cells generally contain thousands of different proteins, each with a different biological activity.
- Proteins can be very long polypeptide chains of 100 to several thousand amino acid residues. However, some naturally occurring peptides have only a few amino acid residues. Some proteins are composed of several noncovalently associated polypeptide chains, called subunits.
- Simple proteins yield only amino acids on hydrolysis; conjugated proteins contain in addition some other component, such as a metal or organic prosthetic group.

3.3 Working with Proteins

Biochemists' understanding of protein structure and function has been derived from the study of many individual proteins. To study a protein in detail, the researcher must be able to separate it from other proteins in pure form and must have the techniques to determine its properties. The necessary methods come from protein chemistry, a discipline as old as biochemistry itself and one that retains a central position in biochemical research.

Proteins Can Be Separated and Purified

A pure preparation is essential before a protein's properties and activities can be determined. Given that cells contain thousands of different kinds of proteins, how can one protein be purified? Classical methods for separating proteins take advantage of properties that vary from one protein to the next, including size, charge, and binding properties. These have been supplemented in recent decades by methods involving DNA cloning and genome sequencing that can simplify the process of protein purification. The newer methods, presented in Chapter 9, often artificially modify the protein being purified, adding a few or many amino acid residues to one or both ends. Convenience thus comes at the price of potentially altering the activity of the purified protein. The purification of proteins in their native state (the form in which they function in the cell) usually relies on methods described here.

The source of a protein is generally tissue or microbial cells. The first step in any protein purification procedure is to break open these cells, releasing their proteins into a solution called a **crude extract**. If necessary, differential centrifugation can be used to prepare subcellular fractions or to isolate specific organelles (see Fig. 1–8).

Once the extract or organelle preparation is ready, various methods are available for purifying one or more of the proteins it contains. Commonly, the extract is subjected to treatments that separate the proteins into different **fractions** based on a property such as size or charge, a process referred to as **fractionation**. Early fractionation steps in a purification utilize differences in protein solubility, which is a complex function of pH, temperature, salt concentration, and other factors.

The solubility of proteins is lowered in the presence of some salts, an effect called "salting out." The addition of certain salts in the right amount can selectively precipitate some proteins, while others remain in solution. Ammonium sulfate ($(NH_4)_2SO_4$) is particularly effective and is often used to salt out proteins. The proteins thus precipitated are removed from those remaining in solution by low-speed centrifugation.

A solution containing the protein of interest usually must be further altered before subsequent purification steps are possible. For example, **dialysis** is a procedure that separates proteins from small solutes by taking advantage of the proteins' larger size. The partially purified extract is placed in a bag or tube made of a semipermeable membrane. When this is suspended in a much larger volume of buffered solution of appropriate ionic strength, the membrane allows the exchange of salt and buffer but not proteins. Thus dialysis retains large proteins within the membranous bag or tube while allowing the concentration of other solutes in the protein preparation to change until they come into equilibrium with the solution outside the membrane. Dialysis might be used, for example, to remove ammonium sulfate from the protein preparation.

The most powerful methods for fractionating proteins make use of **column chromatography**, which takes advantage of differences in protein charge, size, binding affinity, and other properties **(Fig. 3–16)**. A porous solid material with appropriate chemical properties (the stationary phase) is held in a column, and a buffered solution (the mobile phase) migrates through it. The protein, dissolved in the same buffered solution that was used to establish the mobile phase, is layered on the top of the column. The protein then percolates through the solid matrix as an ever-expanding band within the larger mobile phase. Individual proteins migrate faster or more slowly through the column depending on their properties.

Ion-exchange chromatography exploits differences in the sign and magnitude of the net electric charge of proteins at a given pH **(Fig. 3–17a)**. The column matrix is a synthetic polymer (resin) containing bound charged groups; those with bound anionic groups are called **cation exchangers**, and those with bound cationic groups are called **anion exchangers**. The affinity of each protein for the charged groups on the column is affected by the pH (which determines the ionization state of the molecule) and the concentration of competing free salt ions in the surrounding solution. Separation can be optimized by gradually changing the pH and/or salt concentration of the mobile phase so as to create a pH or salt gradient. In **cation-exchange chromatography**, the solid matrix has negatively charged groups. In the mobile phase, proteins with a net positive charge migrate through the matrix more slowly than those with a net negative charge, because the migration of the former is retarded more by interaction with the stationary phase.

In ion-exchange columns, the expansion of the protein band in the mobile phase (the protein solution) is caused both by separation of proteins with different properties and by diffusional spreading. As the length of the column increases, the resolution of two types of protein with different net charges generally improves. However, the rate at which the protein solution can flow through

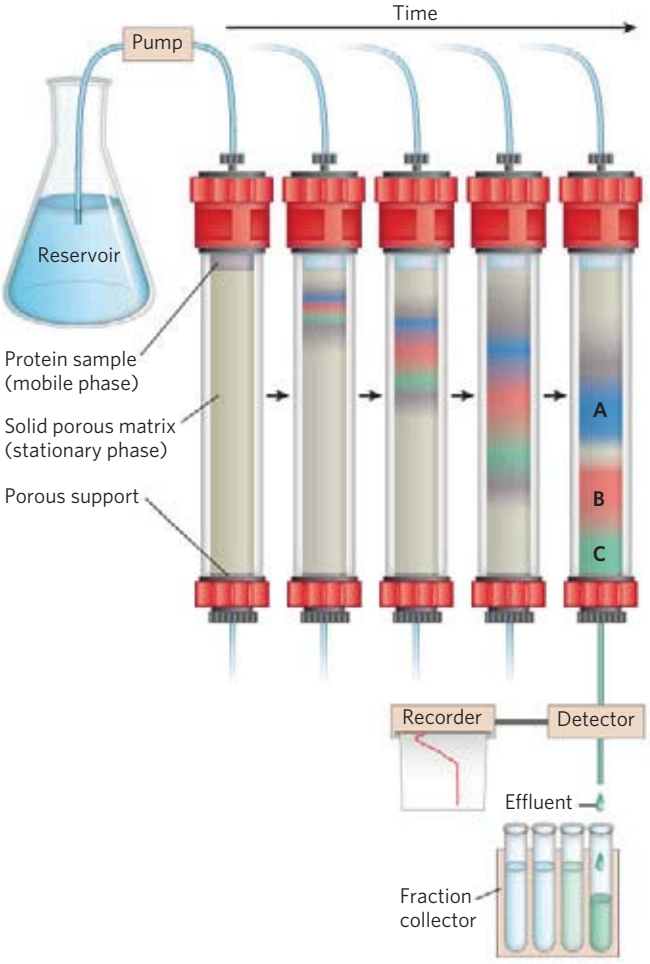


FIGURE 3–16 Column chromatography. The standard elements of a chromatographic column include a solid, porous material (matrix) supported inside a column, generally made of plastic or glass. A solution, the mobile phase, flows through the matrix, the stationary phase. The solution that passes out of the column at the bottom (the effluent) is constantly replaced by solution supplied from a reservoir at the top. The protein solution to be separated is layered on top of the column and allowed to percolate into the solid matrix. Additional solution is added on top. The protein solution forms a band within the mobile phase that is initially the depth of the protein solution applied to the column. As proteins migrate through the column (shown here at five different times), they are retarded to different degrees by their different interactions with the matrix material. The overall protein band thus widens as it moves through the column. Individual types of proteins (such as A, B, and C, shown in blue, red, and green) gradually separate from each other, forming bands within the broader protein band. Separation improves (i.e., resolution increases) as the length of the column increases. However, each individual protein band also broadens with time due to diffusional spreading, a process that decreases resolution. In this example, protein A is well separated from B and C, but diffusional spreading prevents complete separation of B and C under these conditions.

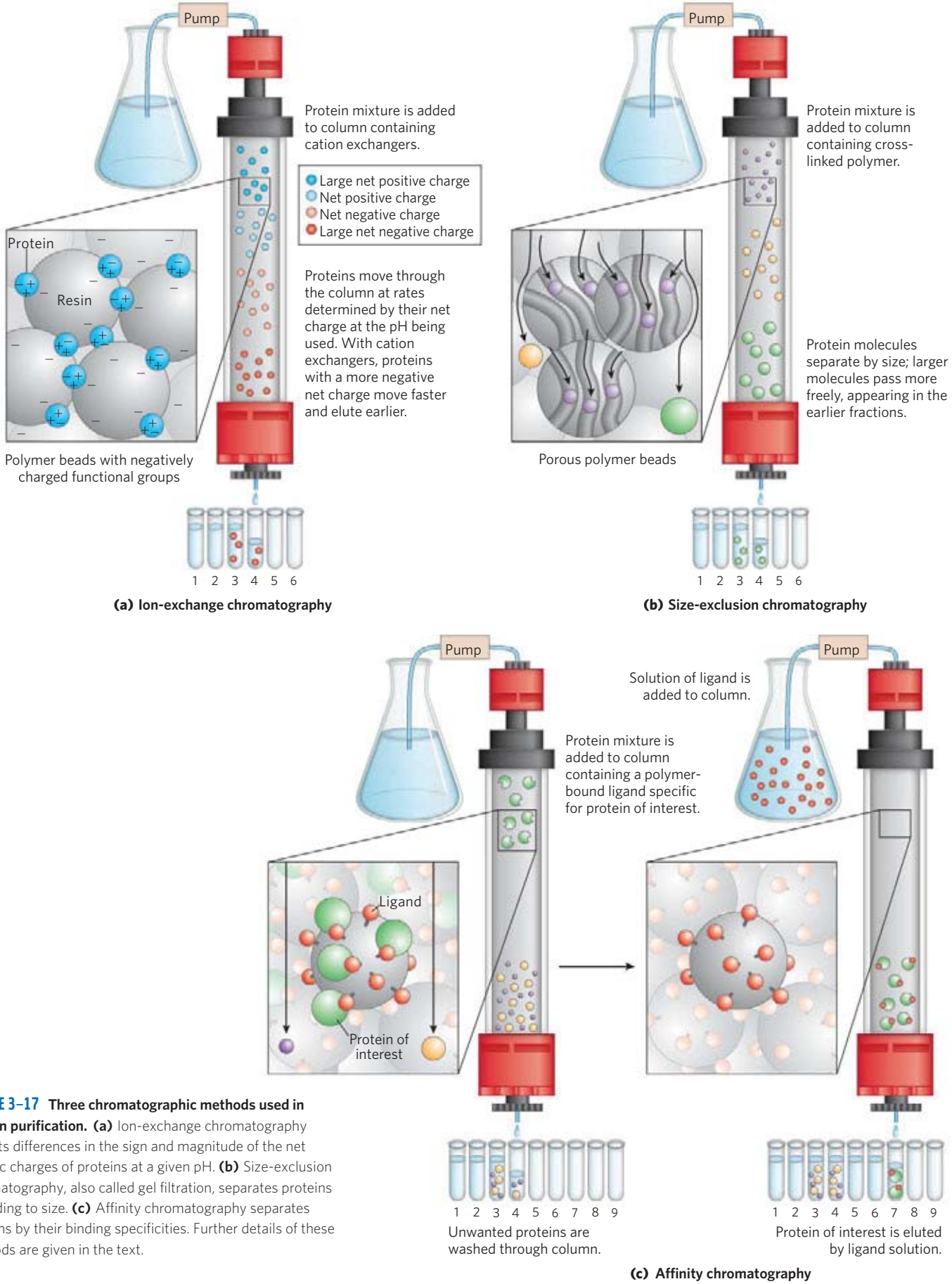


FIGURE 3–17 Three chromatographic methods used in protein purification. (a) Ion-exchange chromatography exploits differences in the sign and magnitude of the net electric charges of proteins at a given pH. **(b)** Size-exclusion chromatography, also called gel filtration, separates proteins according to size. **(c)** Affinity chromatography separates proteins by their binding specificities. Further details of these methods are given in the text.

the column usually decreases with column length. And as the length of time spent on the column increases, the resolution can decline as a result of diffusional spreading within each protein band. As the protein-containing solution exits a column, successive portions (fractions) of this effluent are collected in test tubes. Each fraction can be tested for the presence of the protein of interest as well as other properties, such as ionic strength or total protein concentration. All fractions positive for the protein of interest can be combined as the product of this chromatographic step of the protein purification.

WORKED EXAMPLE 3–1 Ion Exchange of Peptides

A biochemist wants to separate two peptides by ion-exchange chromatography. At the pH of the mobile phase to be used on the column, one peptide (A) has a net charge of −3, due to the presence of more Glu and Asp residues than Arg, Lys, and His residues. Peptide B has a net charge of +1. Which peptide would elute first from a cation-exchange resin? Which would elute first from an anion-exchange resin?

Solution: A cation-exchange resin has negative charges and binds positively charged molecules, retarding their progress through the column. Peptide B, with its net positive charge, will interact more strongly than peptide A with the cation-exchange resin, and thus peptide A will elute first. On the anion-exchange resin, peptide B will elute first. Peptide A, being negatively charged, will be retarded by its interaction with the positively charged resin.

Figure 3–17 shows two other variations of column chromatography in addition to ion exchange. **Size-exclusion chromatography**, also called gel filtration (Fig. 3–17b), separates proteins according to size. In this method, large proteins emerge from the column sooner than small ones—a somewhat counterintuitive result. The solid phase consists of cross-linked polymer beads with engineered pores or cavities of a particular size. Large proteins cannot enter the cavities and so take a shorter (and more rapid) path through the column, around the beads. Small proteins enter the cavities and are slowed by their more labyrinthine path through the column. Size-exclusion chromatography can also be used to approximate the size of a protein being purified, using methods similar to those described in Figure 3–19.

Affinity chromatography is based on binding affinity (Fig. 3–17c). The beads in the column have a covalently attached chemical group called a ligand—a group or molecule that binds to a macromolecule such as a protein. When a protein mixture is added to the column, any protein with affinity for this ligand binds to the beads, and its migration through the matrix is retarded. For example, if the biological function of a protein involves binding to ATP, then attaching a molecule that resembles ATP to the beads in the column creates an affinity matrix that can help purify the protein. As the protein solution moves through the column, ATP-binding proteins (including the protein of interest) bind to the matrix. After proteins that do not bind are washed through the column, the bound protein is eluted by a solution containing either a high concentration of salt or free ligand—in this case, ATP or an analog of ATP. Salt weakens the binding of the protein to the immobilized ligand, interfering with ionic interactions. Free ligand competes with the ligand attached to the beads, releasing the protein from the matrix; the protein product that elutes from the column is often bound to the ligand used to elute it.

Chromatographic methods are typically enhanced by the use of **HPLC**, or **high-performance liquid chromatography**. HPLC makes use of high-pressure pumps that speed the movement of the protein molecules down the column, as well as higher-quality chromatographic materials that can withstand the crushing force of the pressurized flow. By reducing the transit time on the column, HPLC can limit diffusional spreading of protein bands and thus greatly improve resolution.

The approach to purification of a protein that has not previously been isolated is guided both by established precedents and by common sense. In most cases, several different methods must be used sequentially to purify a protein completely, each method separating proteins on the basis of different properties. For example, if one step separates ATP-binding proteins from those that do not bind ATP, then the next step must separate the various ATP-binding proteins on the basis of size or charge to isolate the particular protein that is wanted. The choice of methods is somewhat empirical, and many strategies may be tried before the most effective one is found. Trial and error can often be minimized by basing the new procedure on purification techniques developed for similar proteins. Published purification protocols are available for many thousands of proteins. Common sense dictates that inexpensive procedures such as salting out be used first, when the total volume and the number of contaminants are greatest. Chromatographic methods are often impractical at early stages, because the amount of chromatographic medium needed increases with sample size. As each purification step is completed, the sample size generally becomes smaller (Table 3–5), making it feasible to use more sophisticated (and expensive) chromatographic procedures at later stages.

Proteins Can Be Separated and Characterized by Electrophoresis

Another important technique for the separation of proteins is based on the migration of charged proteins in an electric field, a process called **electrophoresis**. These procedures are not generally used to purify proteins, because simpler alternatives are usually available and electrophoretic methods often adversely affect the

TABLE 3–5 A Purification Table for a Hypothetical Enzyme

Procedure or step	Fraction volume (mL)	Total protein (mg)	Activity (units)	Specific activity (units/mg)
1. Crude cellular extract	1,400	10,000	100,000	10
2. Precipitation with ammonium sulfate	280	3,000	96,000	32
3. Ion-exchange chromatography	90	400	80,000	200
4. Size-exclusion chromatography	80	100	60,000	600
5. Affinity chromatography	6	3	45,000	15,000

Note: All data represent the status of the sample *after* the designated procedure has been carried out. Activity and specific activity are defined on page 95.

structure and thus the function of proteins. However, as an analytical method, electrophoresis is extremely important. Its advantage is that proteins can be visualized as well as separated, permitting a researcher to estimate quickly the number of different proteins in a mixture or the degree of purity of a particular protein preparation. Also, electrophoresis can be used to determine crucial properties of a protein such as its isoelectric point and approximate molecular weight.

Electrophoresis of proteins is generally carried out in gels made up of the cross-linked polymer polyacrylamide **(Fig. 3–18)**. The polyacrylamide gel acts as a molecular sieve, slowing the migration of proteins approximately in proportion to their charge-to-mass ratio. Migration may also be affected by protein shape. In electrophoresis, the force moving the macromolecule is the electrical potential, E. The electrophoretic mobility, μ, of a molecule is the ratio of its velocity, V,

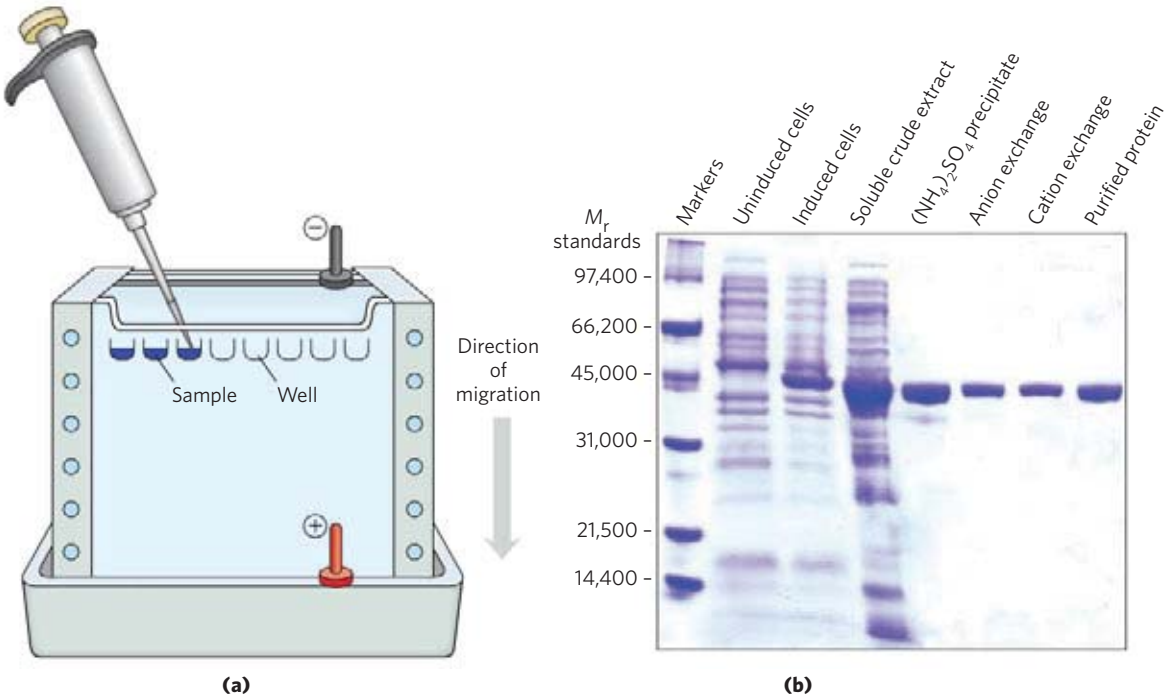


FIGURE 3–18 Electrophoresis. (a) Different samples are loaded in wells or depressions at the top of the SDS–polyacrylamide gel. The proteins move into the gel when an electric field is applied. The gel minimizes convection currents caused by small temperature gradients, as well as protein movements other than those induced by the electric field. **(b)** Proteins can be visualized after electrophoresis by treating the gel with a stain such as Coomassie blue, which binds to the proteins but not to the gel itself. Each band on the gel represents a different protein (or protein subunit); smaller proteins move through the gel more rapidly than larger proteins and therefore are found nearer the bottom of the gel. This gel illustrates purification of the RecA protein of *Escherichia coli* (described in Chapter 25). The gene for the RecA protein was cloned (Chapter 9) so that its expression (synthesis of the protein) could be controlled. The first lane shows a set of standard proteins (of known M_r), serving as molecular weight markers. The next two lanes show proteins from *E. coli* cells before and after synthesis of RecA protein was induced. The fourth lane shows the proteins in a crude cellular extract. Subsequent lanes (left to right) show the proteins present after successive purification steps. The purified protein is a single polypeptide chain (M_r ~38,000), as seen in the rightmost lane.

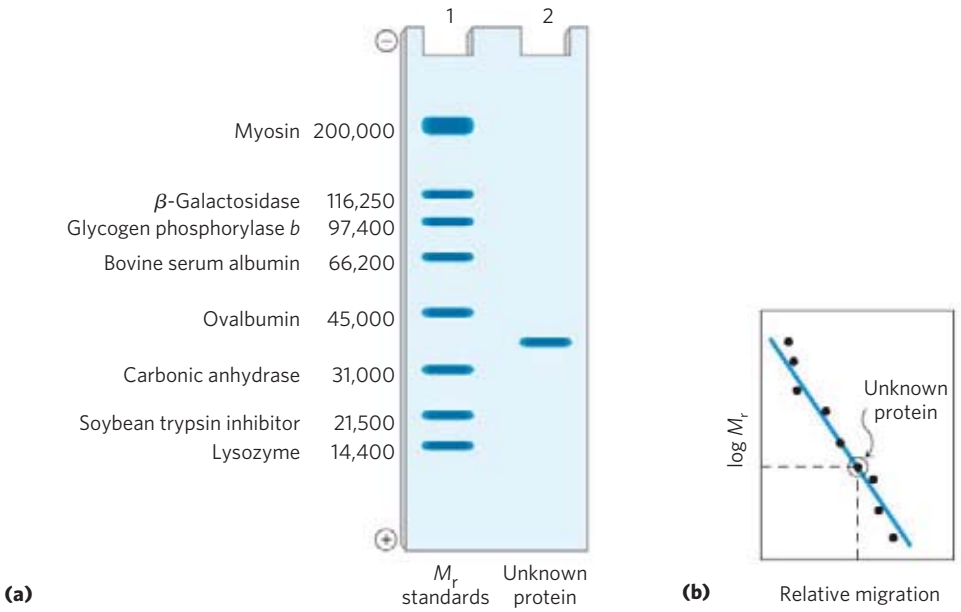


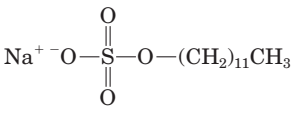
FIGURE 3–19 Estimating the molecular weight of a protein. The electrophoretic mobility of a protein on an SDS polyacrylamide gel is related to its molecular weight, M_r. **(a)** Standard proteins of known molecular weight are subjected to electrophoresis (lane 1). These marker proteins can be used to estimate the molecular weight of an unknown protein (lane 2). **(b)** A plot of log M_r of the marker proteins versus relative migration during electrophoresis is linear, which allows the molecular weight of the unknown protein to be read from the graph. (In similar fashion, a set of standard proteins with reproducible retention times on a size-exclusion column can be used to create a standard curve of retention time versus log M_r. The retention time of an unknown substance on the column can be compared with this standard curve to obtain an approximate M_r.)

to the electrical potential. Electrophoretic mobility is also equal to the net charge, Z, of the molecule divided by the frictional coefficient, f, which reflects in part a protein's shape. Thus:

$$\mu = \frac{V}{E} = \frac{Z}{f}$$

The migration of a protein in a gel during electrophoresis is therefore a function of its size and its shape.

An electrophoretic method commonly employed for estimation of purity and molecular weight makes use of the detergent **sodium dodecyl sulfate (SDS)** ("dodecyl" denoting a 12-carbon chain).



Sodium dodecyl sulfate (SDS)

A protein will bind about 1.4 times its weight of SDS, nearly one molecule of SDS for each amino acid residue. The bound SDS contributes a large net negative charge, rendering the intrinsic charge of the protein insignificant and conferring on each protein a similar charge-to-mass ratio. In addition, SDS binding partially unfolds proteins, such that most SDS-bound proteins assume a similar rodlike shape. Electrophoresis in the presence of SDS therefore separates proteins almost exclusively on the basis of mass (molecular weight), with smaller polypeptides migrating more rapidly. After electrophoresis, the proteins are visualized by adding a dye such as Coomassie blue, which binds to proteins but not to the gel itself (Fig. 3–18b). Thus, a researcher can monitor the progress of a protein purification procedure as the number of protein bands visible on the gel decreases after each new fractionation step. When compared with the positions to which proteins of known molecular weight migrate in the gel, the position of an unidentified protein can provide a good approximation of its molecular weight **(Fig. 3–19)**. If the protein has two or more different subunits, the subunits are generally separated by the SDS treatment, and a separate band appears for each. **SDS Gel Electrophoresis**

Isoelectric focusing is a procedure used to determine the isoelectric point (pI) of a protein **(Fig. 3–20)**. A pH gradient is established by allowing a mixture of low molecular weight organic acids and bases (ampholytes; p. 81) to distribute themselves in an electric field generated across the gel. When a protein mixture is applied, each protein migrates until it reaches the pH that matches its pI. Proteins with different isoelectric points are thus distributed differently throughout the gel.

Combining isoelectric focusing and SDS electrophoresis sequentially in a process called **two-dimensional electrophoresis** permits the resolution of complex mixtures of proteins **(Fig. 3–21)**. This is a more sensitive analytical method than either electrophoretic method alone. Two-dimensional electrophoresis separates proteins of identical molecular weight that differ

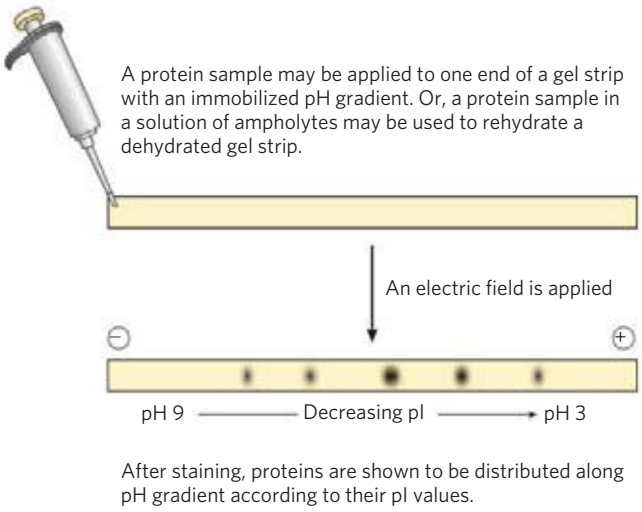


FIGURE 3–20 Isoelectric focusing. This technique separates proteins according to their isoelectric points. A protein mixture is placed on a gel strip containing an immobilized pH gradient. With an applied electric field, proteins enter the gel and migrate until each reaches a pH equivalent to its pI. Remember that when pH = pI, the net charge of a protein is zero.

in pI, or proteins with similar pI values but different molecular weights.

Unseparated Proteins Can Be Quantified

To purify a protein, it is essential to have a way of detecting and quantifying that protein in the presence of many other proteins at each stage of the procedure. Often, purification must proceed in the absence of any information about the size and physical properties of the protein or about the fraction of the total protein mass it represents in the extract. For proteins that are enzymes, the amount in a given solution or tissue extract can be measured, or assayed, in terms of the catalytic effect the enzyme produces—that is, the *increase* in the rate at which its substrate is converted to reaction products when the enzyme is present. For this purpose the researcher must know (1) the overall equation of the reaction catalyzed, (2) an analytical procedure for determining the disappearance of the substrate or the appearance of a reaction product, (3) whether the enzyme requires cofactors such as metal ions or coenzymes, (4) the dependence of the enzyme activity on substrate concentration, (5) the optimum pH, and (6) a temperature zone in which the enzyme is stable and has high activity. Enzymes are usually assayed at their optimum pH and at some convenient temperature within the range 25 to 38°C. Also, very high substrate concentrations are generally used so that the initial reaction rate, measured experimentally, is proportional to enzyme concentration (Chapter 6).

By international agreement, 1.0 unit of enzyme activity for most enzymes is defined as the amount of enzyme causing transformation of 1.0 μmol of substrate to product per minute at 25°C under optimal conditions of measurement (for some enzymes, this definition is

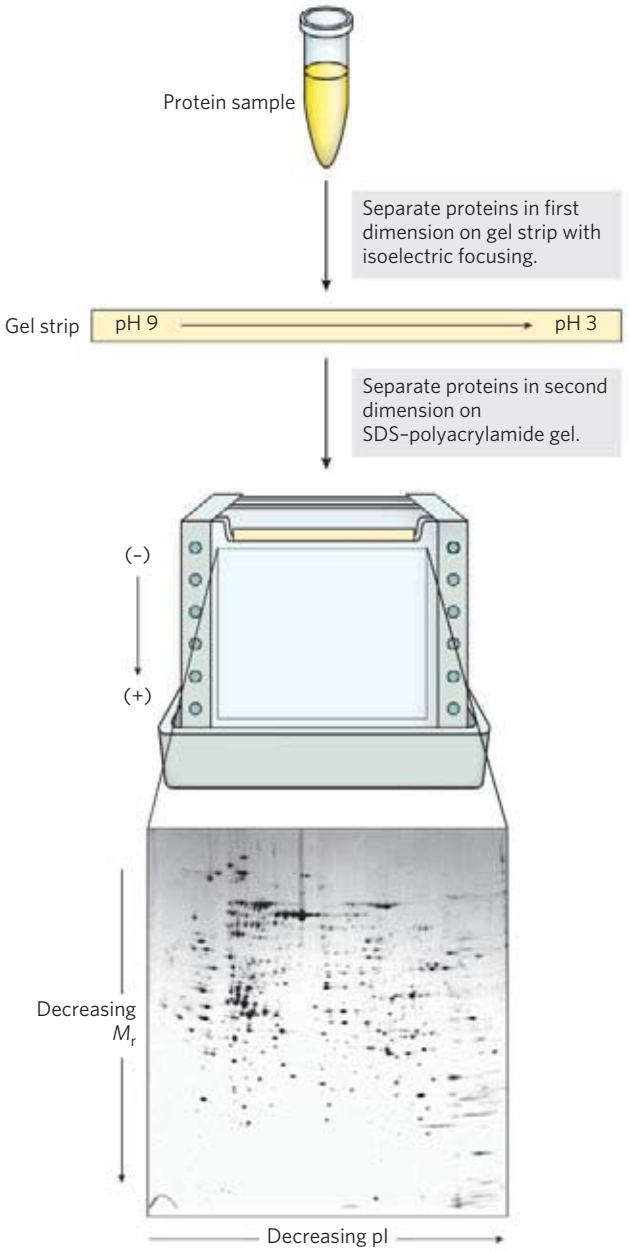


FIGURE 3–21 Two-dimensional electrophoresis. Proteins are first separated by isoelectric focusing in a thin strip gel. The gel is then laid horizontally on a second, slab-shaped gel, and the proteins are separated by SDS polyacrylamide gel electrophoresis. Horizontal separation reflects differences in pI; vertical separation reflects differences in molecular weight. The original protein complement is thus spread in two dimensions. Thousands of cellular proteins can be resolved using this technique. Individual protein spots can be cut out of the gel and identified by mass spectrometry (see Figs 3–30 and 3–31).

inconvenient, and a unit may be defined differently). The term **activity** refers to the total units of enzyme in a solution. The **specific activity** is the number of enzyme units per milligram of total protein **(Fig. 3–22)**. The specific activity is a measure of enzyme purity: it increases during purification of an enzyme and becomes maximal and constant when the enzyme is pure (Table 3–5, p. 93).

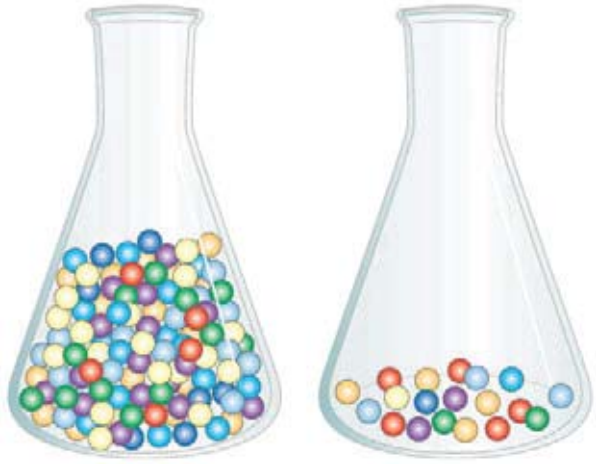


FIGURE 3–22 Activity versus specific activity. The difference between these terms can be illustrated by considering two flasks containing marbles. The flasks contain the same number of red marbles, but different numbers of marbles of other colors. If the marbles represent proteins, both flasks contain the same *activity* of the protein represented by the red marbles. The second flask, however, has the higher *specific activity* because red marbles represent a higher fraction of the total.

After each purification step, the activity of the preparation (in units of enzyme activity) is assayed, the total amount of protein is determined independently, and the ratio of the two gives the specific activity. Activity and total protein generally decrease with each step. Activity decreases because there is always some loss due to inactivation or nonideal interactions with chromatographic materials or other molecules in the solution. Total protein decreases because the objective is to remove as much unwanted or nonspecific protein as possible. In a successful step, the loss of nonspecific protein is much greater than the loss of activity; therefore, specific activity increases even as total activity falls. The data are assembled in a purification table similar to Table 3–5. A protein is generally considered pure when further purification steps fail to increase specific activity and when only a single protein species can be detected (for example, by electrophoresis).

For proteins that are not enzymes, other quantification methods are required. Transport proteins can be assayed by their binding to the molecule they transport, and hormones and toxins by the biological effect they produce; for example, growth hormones will stimulate the growth of certain cultured cells. Some structural proteins represent such a large fraction of a tissue mass that they can be readily extracted and purified without a functional assay. The approaches are as varied as the proteins themselves.

SUMMARY 3.3 Working with Proteins

- Proteins are separated and purified on the basis of differences in their properties. Proteins can be selectively precipitated by changes in pH or temperature, and particularly by the addition of certain salts. A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties. These include ion-exchange, size-exclusion, affinity, and high-performance liquid chromatography.
- Electrophoresis separates proteins on the basis of mass or charge. SDS gel electrophoresis and isoelectric focusing can be used separately or in combination for higher resolution.
- All purification procedures require a method for quantifying or assaying the protein of interest in the presence of other proteins. Purification can be monitored by assaying specific activity.

3.4 The Structure of Proteins: Primary Structure

Purification of a protein is usually only a prelude to a detailed biochemical dissection of its structure and function. What is it that makes one protein an enzyme, another a hormone, another a structural protein, and still another an antibody? How do they differ chemically? The most obvious distinctions are structural, and to protein structure we now turn.

The structure of large molecules such as proteins can be described at several levels of complexity, arranged in a kind of conceptual hierarchy. Four levels of protein structure are commonly defined **(Fig. 3–23)**. A description

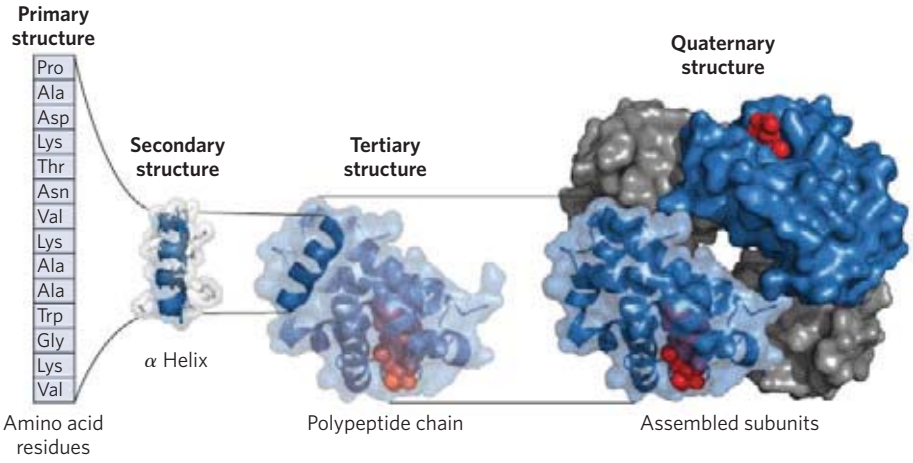


FIGURE 3–23 Levels of structure in proteins. The *primary structure* consists of a sequence of amino acids linked together by peptide bonds and includes any disulfide bonds. The resulting polypeptide can be arranged into units of *secondary structure*, such as an α helix. The helix is a part of the *tertiary structure* of the folded polypeptide, which is itself one of the subunits that make up the *quaternary structure* of the multisubunit protein, in this case hemoglobin.

of all covalent bonds (mainly peptide bonds and disulfide bonds) linking amino acid residues in a polypeptide chain is its **primary structure**. The most important element of primary structure is the *sequence* of amino acid residues. **Secondary structure** refers to particularly stable arrangements of amino acid residues giving rise to recurring structural patterns. **Tertiary structure** describes all aspects of the three-dimensional folding of a polypeptide. When a protein has two or more polypeptide subunits, their arrangement in space is referred to as **quaternary structure**. Our exploration of proteins will eventually include complex protein machines consisting of dozens to thousands of subunits. Primary structure is the focus of the remainder of this chapter; the higher levels of structure are discussed in Chapter 4.

Differences in primary structure can be especially informative. Each protein has a distinctive number and sequence of amino acid residues. As we shall see in Chapter 4, the primary structure of a protein determines how it folds up into its unique three-dimensional structure, and this in turn determines the function of the protein. We first consider empirical clues that amino acid sequence and protein function are closely linked, then describe how amino acid sequence is determined; finally, we outline the many uses to which this information can be put.

The Function of a Protein Depends on Its Amino Acid Sequence

The bacterium *Escherichia coli* produces more than 3,000 different proteins; a human has ~25,000 genes encoding a much larger number of proteins (through genetic processes discussed in Part III of this text). In both cases, each type of protein has a unique amino acid sequence that confers a particular three-dimensional structure. This structure in turn confers a unique function.

Some simple observations illustrate the importance of primary structure, or the amino acid sequence of a protein. First, as we have already noted, proteins with different functions always have different amino acid sequences. Second, thousands of human genetic diseases have been traced to the production of defective proteins. The defect can range from a single change in the amino acid sequence (as in sickle cell anemia, described in Chapter 5) to deletion of a larger portion of the polypeptide chain (as in most cases of Duchenne muscular dystrophy: a large deletion in the gene encoding the protein dystrophin leads to production of a shortened, inactive protein). Finally, on comparing functionally similar proteins from different species, we find that these proteins often have similar amino acid sequences. Thus, a close link between protein primary structure and function is evident.

Is the amino acid sequence absolutely fixed, or invariant, for a particular protein? No; some flexibility is possible. An estimated 20% to 30% of the proteins in humans are **polymorphic**, having amino acid sequence variants in the human population. Many of these variations in sequence have little or no effect on the function of the protein. Furthermore, proteins that carry out a broadly similar function in distantly related species can differ greatly in overall size and amino acid sequence.

Although the amino acid sequence in some regions of the primary structure might vary considerably without affecting biological function, most proteins contain crucial regions that are essential to their function and whose sequence is therefore conserved. The fraction of the overall sequence that is critical varies from protein to protein, complicating the task of relating sequence to three-dimensional structure, and structure to function. Before we can consider this problem further, however, we must examine how sequence information is obtained.

The Amino Acid Sequences of Millions of Proteins Have Been Determined

Two major discoveries in 1953 were of crucial importance in the history of biochemistry. In that year, James D. Watson and Francis Crick deduced the double-helical structure of DNA and proposed a structural basis for its precise replication (Chapter 8). Their proposal illuminated the molecular reality behind the idea of a gene. In the same year, Frederick Sanger worked out the sequence of amino acid residues in the polypeptide chains of the hormone insulin **(Fig. 3–24)**, surprising many researchers who had long thought that determining the amino acid sequence of a polypeptide would be a hopelessly difficult task. It quickly became evident that the nucleotide sequence in DNA and the amino acid sequence in proteins were somehow related. Barely a decade after these discoveries, the genetic code relating the nucleotide sequence of DNA to the amino acid sequence of protein molecules was elucidated (Chapter 27). The amino acid sequences of proteins are now most often derived indirectly from the DNA sequences in genome databases. However, an array of techniques derived from traditional methods of polypeptide sequencing still command an important place in protein chemistry. Below, we summarize the traditional method and mention a few of the techniques derived from it.

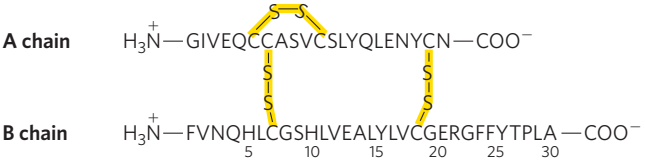
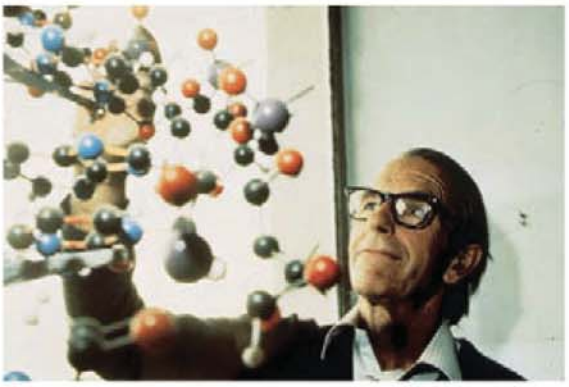


FIGURE 3–24 Amino acid sequence of bovine insulin. The two polypeptide chains are joined by disulfide cross-linkages (yellow). The A chain of insulin is identical in human, pig, dog, rabbit, and sperm whale insulins. The B chains of the cow, pig, dog, goat, and horse are identical.

Protein Chemistry Is Enriched by Methods Derived from Classical Polypeptide Sequencing

The methods used in the 1950s by Fred Sanger to determine the sequence of the protein insulin are summarized, in their modern form, in **Figure 3–25**. Few proteins are sequenced in this way now, at least in their entirety. However, these traditional sequencing protocols have provided a rich array of tools for biochemists, and almost every step in Figure 3–25 makes use of methods that are widely used, sometimes in quite different contexts.



Frederick Sanger

In the traditional scheme for sequencing large proteins, the amino-terminal amino acid residue was first labeled and its identity determined. The amino-terminal α-amino group can be labeled with 1-fluoro-2,4-dinitrobenzene (FDNB), dansyl chloride, or dabsyl chloride **(Fig. 3–26)**.

The chemical sequencing process itself is based on a two-step process developed by Pehr Edman **(Fig. 3–27)**. The **Edman degradation** procedure labels and removes only the amino-terminal residue from a peptide, leaving all other peptide bonds intact. The peptide is reacted with phenylisothiocyanate under mildly alkaline conditions, which converts the amino-terminal amino acid to a phenylthiocarbamoyl (PTC) adduct. The peptide bond next to the PTC adduct is then cleaved in a step carried out in anhydrous trifluoroacetic acid, with removal of the amino-terminal amino acid as an anilinothiazolinone derivative. The derivatized amino acid is extracted with organic solvents, converted to the more stable phenylthiohydantoin derivative by treatment with aqueous acid, and then identified. The use of sequential reactions carried out under first basic and then acidic conditions provides a means of controlling the entire process. Each reaction with the amino-terminal amino acid can go essentially to completion without affecting any of the other peptide bonds in the peptide. The process is repeated until, typically, as many as 40 sequential amino acid residues are identified. The reactions of the Edman degradation have been automated.

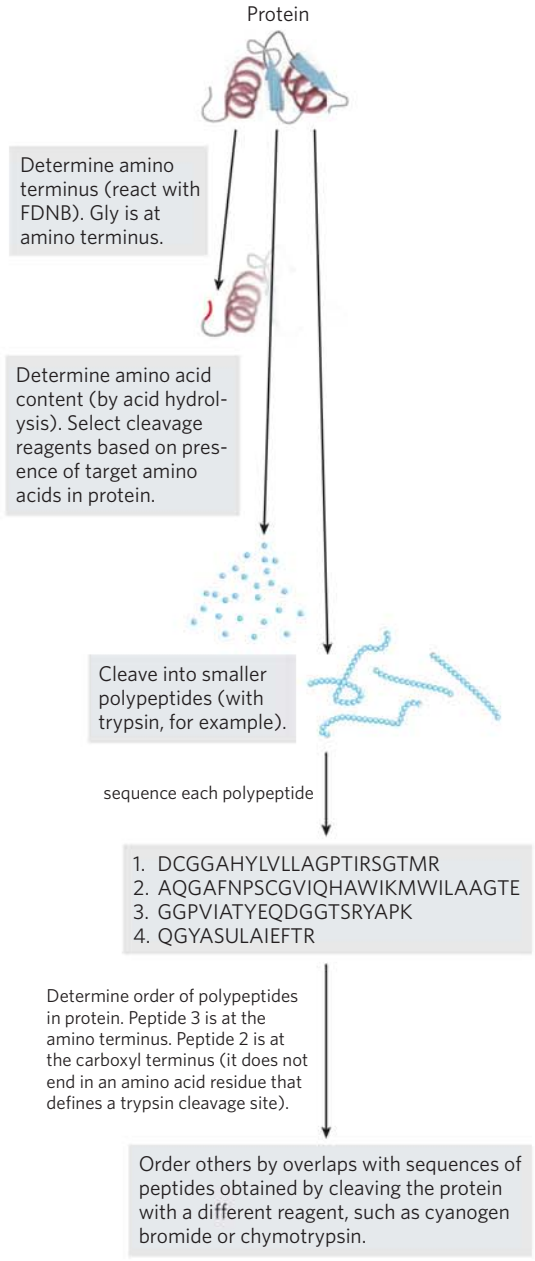
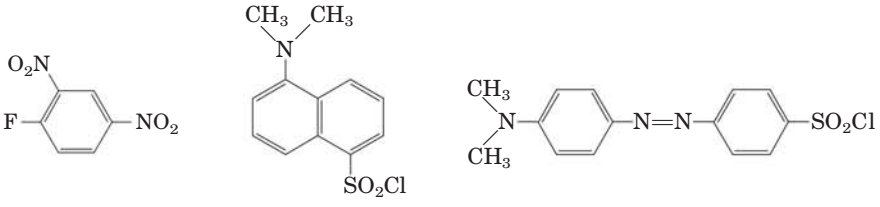


FIGURE 3–25 **Direct protein sequencing.** The procedures shown here are those developed by Fred Sanger to sequence insulin, and they have been used subsequently for many additional proteins. FDNB is 1-fluoro-2,4-dinitrobenzene (see text and Fig. 3–26).



FDNB Dansyl chloride Dabsyl chloride

FIGURE 3–26 **Reagents used to modify the α-amino group at the amino terminus.**

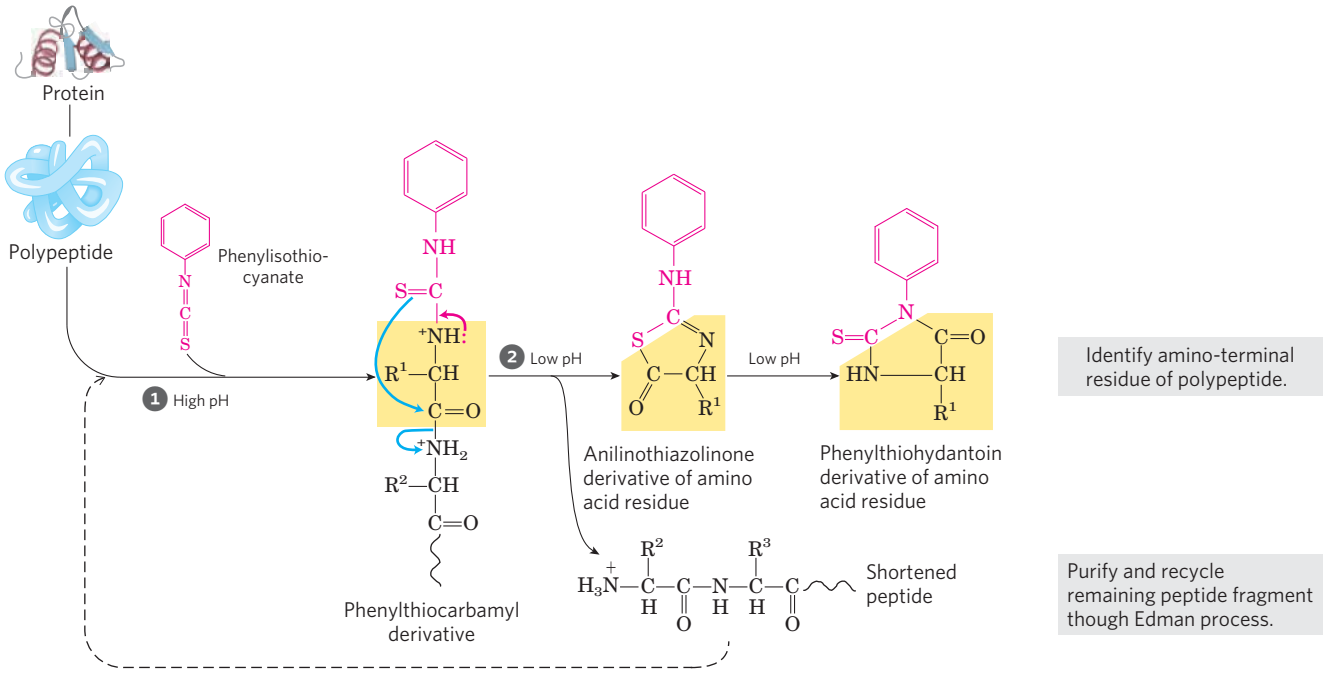


FIGURE 3–27 The protein sequencing chemistry devised by Pehr Edman. The peptide bond nearest to the amino terminus of the protein or polypeptide is cleaved in two steps. The two steps are carried out under very different reaction conditions (basic conditions in step 1, acidic in step 2), allowing one step to proceed to completion before the second is initiated.

To determine the sequence of large proteins, early developers of sequencing protocols had to devise methods to eliminate disulfide bonds and to cleave proteins precisely into smaller polypeptides. Two approaches to irreversible breakage of disulfide bonds are outlined in **Figure 3–28**. Enzymes called **proteases** catalyze the hydrolytic cleavage of peptide bonds. Some proteases cleave only the peptide bond adjacent to particular amino acid residues (Table 3–6) and thus fragment a polypeptide chain in a predictable and reproducible way. A few chemical reagents also cleave the peptide bond adjacent to specific residues. Among proteases, the digestive enzyme trypsin catalyzes the hydrolysis of only those peptide bonds in

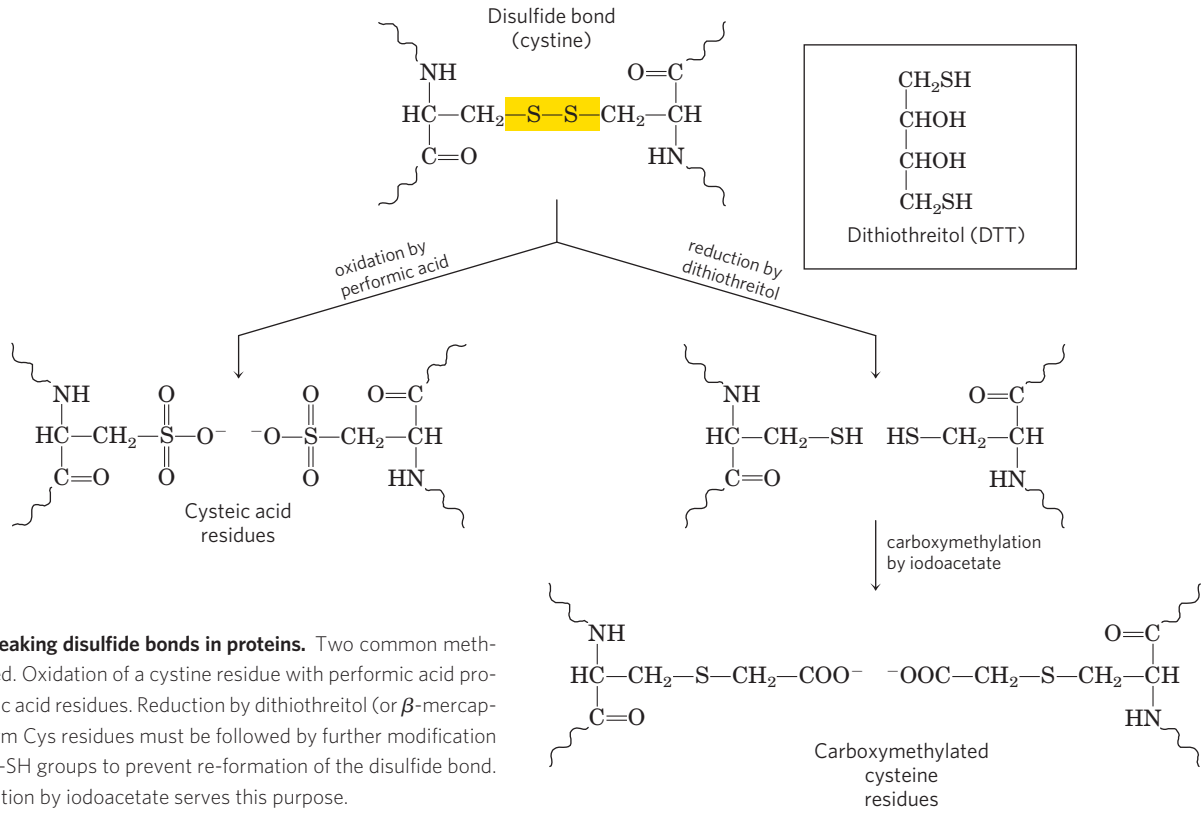


FIGURE 3–28 Breaking disulfide bonds in proteins. Two common methods are illustrated. Oxidation of a cystine residue with performic acid produces two cysteic acid residues. Reduction by dithiothreitol (or β-mercaptoethanol) to form Cys residues must be followed by further modification of the reactive —SH groups to prevent re-formation of the disulfide bond. Carboxymethylation by iodoacetate serves this purpose.

TABLE 3–6 The Specificity of Some Common Methods for Fragmenting Polypeptide Chains

Reagent (biological source)*	Cleavage points†
Trypsin (bovine pancreas)	Lys, Arg (C)
Submaxillary protease (mouse submaxillary gland)	Arg (C)
Chymotrypsin (bovine pancreas)	Phe, Trp, Tyr (C)
Staphylococcus aureus V8 protease (bacterium *S. aureus*)	Asp, Glu (C)
Asp-*N*-protease (bacterium *Pseudomonas fragi*)	Asp, Glu (N)
Pepsin (porcine stomach)	Leu, Phe, Trp, Tyr (N)
Endoproteinase Lys C (bacterium *Lysobacter enzymogenes*)	Lys (C)
Cyanogen bromide	Met (C)

*All reagents except cyanogen bromide are proteases. All are available from commercial sources.

†Residues furnishing the primary recognition point for the protease or reagent; peptide bond cleavage occurs on either the carbonyl (C) or the amino (N) side of the indicated amino acid residues.

which the carbonyl group is contributed by either a Lys or an Arg residue, regardless of the length or amino acid sequence of the chain. A polypeptide with three Lys and/or Arg residues will usually yield four smaller peptides on cleavage with trypsin. Moreover, all except one of these will have a carboxyl-terminal Lys or Arg. The choice of a reagent to cleave the protein into smaller peptides can be aided by first determining the amino acid content of the entire protein, employing acid to reduce the protein to its constituent amino acids. Trypsin would be used only on proteins that have an appropriate number of Lys or Arg residues.

In classical sequencing, a large protein would be cleaved into fragments twice, using a different protease or cleavage reagent each time so that the fragment endpoints would be different. Both sets of fragments would be purified and sequenced. The order in which the fragments appeared in the original protein could then be determined by examining the overlaps in sequence between the two sets of fragments.

Even if no longer used to sequence entire proteins, the traditional sequencing methods are still valuable in the lab. The sequencing of some amino acids from the amino terminus using the Edman chemistry is often sufficient to confirm the identity of a known protein that has just been purified, or to identify an unknown protein purified on the basis of an unusual activity. Techniques employed in individual steps of the traditional sequencing method are also useful for other purposes. For example, the methods used to break disulfide bonds can also be used to denature proteins when that is required. Furthermore, the effort to label the amino-terminal amino acid residue led eventually to the development of an array of reagents that could react with specific groups on a protein. The same reagents used to label the amino-terminal α-amino group can be used to label the primary–amines of Lys residues (Fig. 3–26). The sulfhydryl group on Cys residues can be modified with iodoacetamides, maleimides, benzyl halides, and bromomethyl ketones **(Fig. 3–29)**. Other amino acid residues can be modified

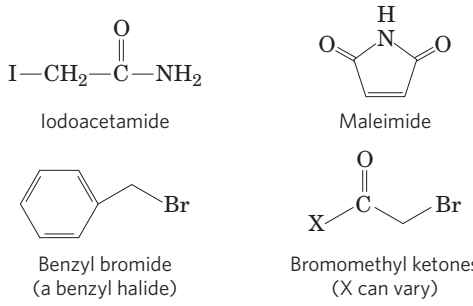


FIGURE 3–29 Reagents used to modify the sulfhydryl groups of Cys residues. (See also Fig. 3–28.)

by reagents linked to a dye or other molecule to aid in protein detection or functional studies.

Mass Spectrometry Offers an Alternative Method to Determine Amino Acid Sequences

Modern adaptations of **mass spectrometry** provide an important alternative to the sequencing methods described above. Mass spectrometry can provide a highly accurate measure of the molecular weight of a protein, but can also do much more. In particular, some variants of mass spectrometry can provide the sequences of multiple short polypeptide segments (20 to 30 amino acid residues) in a protein sample quite rapidly.

The mass spectrometer has long been an indispensable tool in chemistry. Molecules to be analyzed, referred to as **analytes**, are first ionized in a vacuum. When the newly charged molecules are introduced into an electric and/or magnetic field, their paths through the field are a function of their mass-to-charge ratio, m/z. This measured property of the ionized species can be used to deduce the mass (m) of the analyte with very high precision.

Although mass spectrometry has been in use for many years, it could not be applied to macromolecules such as proteins and nucleic acids. The m/z measurements are made on molecules in the gas phase, and the heating or other treatment needed to transfer a macromolecule to the gas phase usually caused its rapid

decomposition. In 1988, two different techniques were developed to overcome this problem. In one, proteins are placed in a light-absorbing matrix. With a short pulse of laser light, the proteins are ionized and then desorbed from the matrix into the vacuum system. This process, known as **matrix-assisted laser desorption/ionization mass spectrometry**, or **MALDI MS**, has been successfully used to measure the mass of a wide range of macromolecules. In a second and equally successful method, macromolecules in solution are forced directly from the liquid to gas phase. A solution of analytes is passed through a charged needle that is kept at a high electrical potential, dispersing the solution into a fine mist of charged microdroplets. The solvent surrounding the macromolecules rapidly evaporates, leaving multiply charged macromolecular ions in the gas phase. This technique is called **electrospray ionization mass spectrometry**, or **ESI MS**. Protons added during passage through the needle give additional charge to the macromolecule. The *m/z* of the molecule can be analyzed in the vacuum chamber.

Mass spectrometry provides a wealth of information for proteomics research, enzymology, and protein chemistry in general. The techniques require only miniscule amounts of sample, so they can be readily applied to the small amounts of protein that can be extracted from a two-dimensional electrophoretic gel. The accurately measured molecular mass of a protein is critical to its identification. Once the mass of a protein is accurately known, mass spectrometry is a convenient and accurate method for detecting changes in mass due to the presence of bound cofactors, bound metal ions, covalent modifications, and so on.

The process for determining the molecular mass of a protein with ESI MS is illustrated in **Figure 3–30**. As it is injected into the gas phase, a protein acquires a variable number of protons, and thus positive charges, from the solvent. The variable addition of these charges creates a spectrum of species with different mass-to-charge ratios. Each successive peak corresponds to a species that differs from that of its neighboring peak by a charge difference of 1 and a mass difference of 1 (1 proton). The mass of the protein can be determined from any two neighboring peaks.

Mass spectrometry can also be used to sequence short stretches of polypeptide, an application that has emerged as an invaluable tool for quickly identifying unknown proteins. Sequence information is extracted using a technique called **tandem MS**, or **MS/MS**. A solution containing the protein under investigation is first treated with a protease or chemical reagent to hydrolyze it to a mixture of shorter peptides. The mixture is then injected into a device that is essentially two mass spectrometers in tandem (**Fig. 3–31a**, top). In the first, the peptide mixture is sorted so that only one of the several types of peptides produced by cleavage emerges at the other end. The sample of the selected peptide, each molecule of which has a charge some-

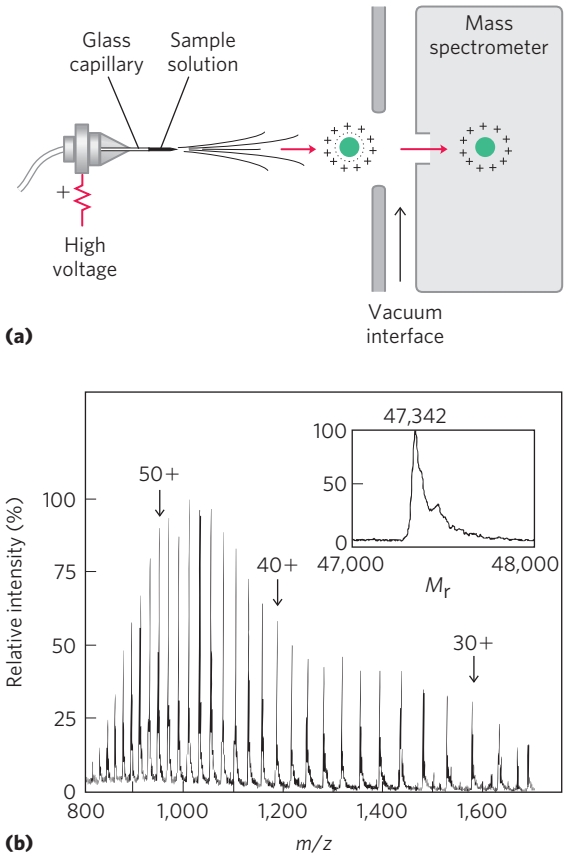


FIGURE 3–30 **Electrospray ionization mass spectrometry of a protein.** **(a)** A protein solution is dispersed into highly charged droplets by passage through a needle under the influence of a high-voltage electric field. The droplets evaporate, and the ions (with added protons in this case) enter the mass spectrometer for *m/z* measurement. The spectrum generated **(b)** is a family of peaks, with each successive peak (from right to left) corresponding to a charged species increased by 1 in both mass and charge. The inset shows a computer-generated transformation of this spectrum.

where along its length, then travels through a vacuum chamber between the two mass spectrometers. In this collision cell, the peptide is further fragmented by high-energy impact with a "collision gas" such as helium or argon that is bled into the vacuum chamber. Each individual peptide is broken in only one place, on average. Although the breaks are not hydrolytic, most occur at the peptide bonds.

The second mass spectrometer then measures the *m/z* ratios of all the charged fragments. This process generates one or more sets of peaks. A given set of peaks (Fig. 3–31b) consists of all the charged fragments that were generated by breaking the same type of bond (but at different points in the peptide). One set of peaks includes only the fragments in which the charge was retained on the amino-terminal side of the broken bonds; another includes only the fragments in which the charge was retained on the carboxyl-terminal side of the broken bonds. Each successive peak in a given set has one less amino acid than the peak before. The difference in mass from peak to peak identifies the amino

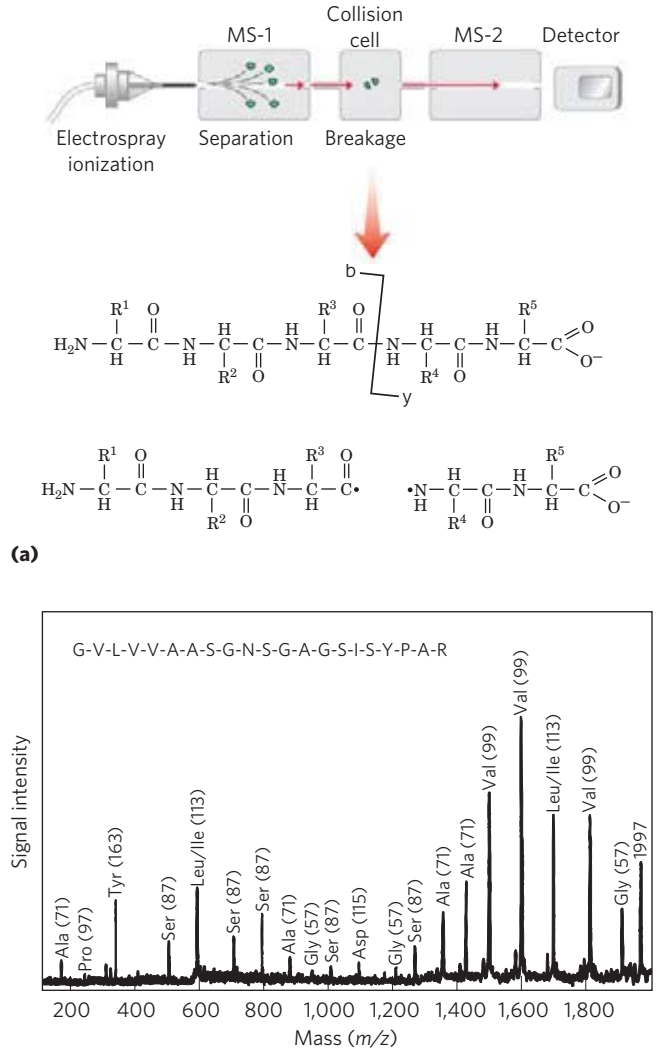


FIGURE 3–31 Obtaining protein sequence information with tandem MS. (a) After proteolytic hydrolysis, a protein solution is injected into a mass spectrometer (MS-1). The different peptides are sorted so that only one type is selected for further analysis. The selected peptide is further fragmented in a chamber between the two mass spectrometers, and *m/z* for each fragment is measured in the second mass spectrometer (MS-2). Many of the ions generated during this second fragmentation result from breakage of the peptide bond, as shown. These are called b-type or y-type ions, depending on whether the charge is retained on the amino- or carboxyl-terminal side, respectively. **(b)** A typical spectrum with peaks representing the peptide fragments generated from a sample of one small peptide (21 residues). The labeled peaks are y-type ions derived from amino acid residues. The number in parentheses over each peak is the molecular weight of the amino acid ion. The successive peaks differ by the mass of a particular amino acid in the original peptide. The deduced sequence is shown at the top.

acid that was lost in each case, thus revealing the sequence of the peptide. The only ambiguities involve leucine and isoleucine, which have the same mass. Although multiple sets of peaks are usually generated, the two most prominent sets generally consist of charged fragments derived from breakage of the peptide bonds. The amino acid sequence derived from one set can be confirmed by the other, improving the confidence in the sequence information obtained.

The various methods for obtaining protein sequence information complement one another. The Edman degradation procedure is sometimes convenient to get sequence information uniquely from the amino terminus of a protein or peptide. However, it is relatively slow and requires a larger sample than does mass spectrometry. Mass spectrometry can be used for small amounts of sample and for mixed samples. It provides sequence information, but the fragmentation processes can leave unpredictable sequence gaps. Although most protein sequences are now extracted from genomic DNA sequences (Chapter 9) by employing our understanding of the genetic code (Chapter 27), direct protein sequencing is often necessary to identify unknown protein samples. Both protein sequencing methods permit the unambiguous identification of newly purified proteins. Mass spectrometry is the method of choice to identify proteins that are present in small amounts. For example, the technique is sensitive enough to analyze the few hundred nanograms of protein that might be extracted from a single protein band on a polyacrylamide gel. Direct sequencing by mass spectrometry also can reveal the addition of phosphoryl groups or other modifications (Chapter 6). Sequencing by either method can reveal changes in protein sequence that result from the editing of messenger RNA in eukaryotes (Chapter 26). Thus, these methods are all part of a robust toolbox used to investigate proteins and their functions.

Small Peptides and Proteins Can Be Chemically Synthesized

Many peptides are potentially useful as pharmacologic agents, and their production is of considerable commercial importance. There are three ways to obtain a peptide: (1) purification from tissue, a task often made difficult by the vanishingly low concentrations of some peptides; (2) genetic engineering (Chapter 9); or (3) direct chemical synthesis. Powerful techniques now make direct chemical synthesis an attractive option in many cases. In addition to commercial applications, the synthesis of specific peptide portions of larger proteins is an increasingly important tool for the study of protein structure and function.

The complexity of proteins makes the traditional synthetic approaches of organic chemistry impractical for peptides with more than four or five amino acid residues. One problem is the difficulty of purifying the product after each step.

The major breakthrough in this technology was provided by R. Bruce Merrifield in 1962. His innovation was to synthesize a peptide while keeping it attached at one end to a solid support. The support is an insoluble polymer (resin) contained within a column, similar to that used for chromatographic procedures. The peptide is built up on this support one amino acid at a time,

through a standard set of reactions in a repeating cycle **(Fig. 3–32)**. At each successive step in the cycle, protective chemical groups block unwanted reactions.

The technology for chemical peptide synthesis is now automated. An important limitation of the process (a limitation shared by the Edman degradation sequencing process) is the efficiency of each chemical cycle, as can be seen by calculating the overall yields of peptides of various lengths when the yield for addition of each new amino acid is 96.0% versus 99.8% (Table 3–7).

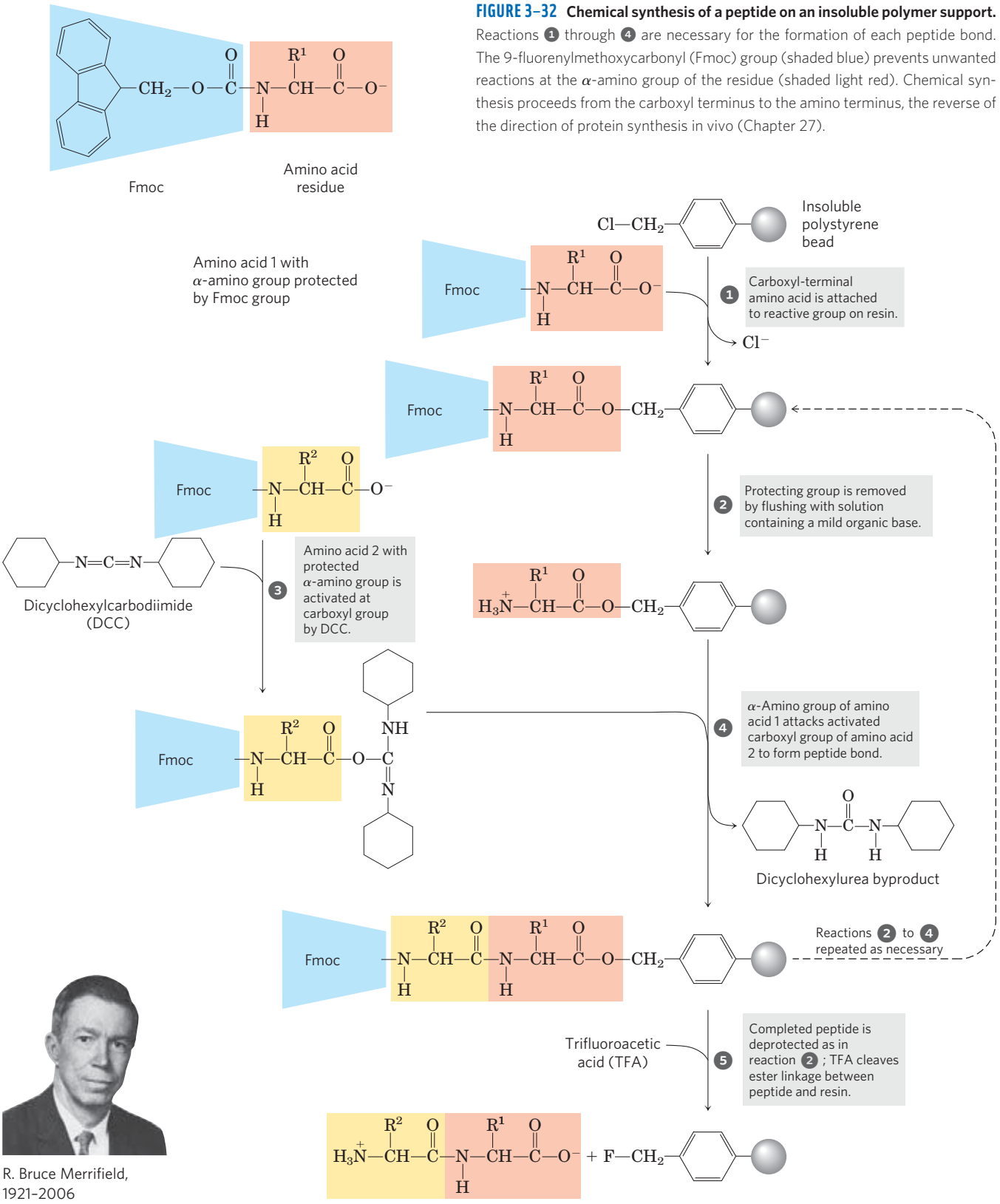


FIGURE 3–32 Chemical synthesis of a peptide on an insoluble polymer support. Reactions 1 through 4 are necessary for the formation of each peptide bond. The 9-fluorenylmethoxycarbonyl (Fmoc) group (shaded blue) prevents unwanted reactions at the α-amino group of the residue (shaded light red). Chemical synthesis proceeds from the carboxyl terminus to the amino terminus, the reverse of the direction of protein synthesis in vivo (Chapter 27).

R. Bruce Merrifield, 1921–2006

TABLE 3–7 Effect of Stepwise Yield on Overall Yield in Peptide Synthesis

Number of residues in the final polypeptide	Overall yield of final peptide (%) when the yield of each step is:	
	96.0%	99.8%
11	66	98
21	44	96
31	29	94
51	13	90
100	1.8	82

Incomplete reaction at one stage can lead to formation of an impurity (in the form of a shorter peptide) in the next. The chemistry has been optimized to permit the synthesis of proteins of 100 amino acid residues in a few days in reasonable yield. A very similar approach is used to synthesize nucleic acids (see Fig. 8–35). It is worth noting that this technology, impressive as it is, still pales when compared with biological processes. The same 100-residue protein would be synthesized with exquisite fidelity in about 5 seconds in a bacterial cell.

A variety of new methods for the efficient ligation (joining together) of peptides has made possible the assembly of synthetic peptides into larger polypeptides and proteins. With these methods, novel forms of proteins can be created with precisely positioned chemical groups, including those that might not normally be found in a cellular protein. These novel forms provide new ways to test theories of enzyme catalysis, to create proteins with new chemical properties, and to design protein sequences that will fold into particular structures. This last application provides the ultimate test of our increasing ability to relate the primary structure of a peptide to the three-dimensional structure that it takes up in solution.

Amino Acid Sequences Provide Important Biochemical Information

Knowledge of the sequence of amino acids in a protein can offer insights into its three-dimensional structure and its function, cellular location, and evolution. Most of these insights are derived by searching for similarities between a protein of interest and previously studied proteins. Thousands of sequences are known and available in databases accessible through the Internet. A comparison of a newly obtained sequence with this large bank of stored sequences often reveals relationships both surprising and enlightening.

Exactly how the amino acid sequence determines three-dimensional structure is not understood in detail, nor can we always predict function from sequence. However, protein families that have some shared structural or functional features can be readily identified on the basis of amino acid sequence similarities. Individual proteins are assigned to families based on the degree of similarity in amino acid sequence. Members of a family are usually identical across 25% or more of their sequences, and proteins in these families generally share at least some structural and functional characteristics. Some families are defined, however, by identities involving only a few amino acid residues that are critical to a certain function. A number of similar substructures, or "domains" (to be defined more fully in Chapter 4), occur in many functionally unrelated proteins. These domains often fold into structural configurations that have an unusual degree of stability or that are specialized for a certain environment. Evolutionary relationships can also be inferred from the structural and functional similarities within protein families.

Certain amino acid sequences serve as signals that determine the cellular location, chemical modification, and half-life of a protein. Special signal sequences, usually at the amino terminus, are used to target certain proteins for export from the cell; other proteins are targeted for distribution to the nucleus, the cell surface, the cytosol, or other cellular locations. Other sequences act as attachment sites for prosthetic groups, such as sugar groups in glycoproteins and lipids in lipoproteins. Some of these signals are well characterized and are easily recognized in the sequence of a newly characterized protein (Chapter 27).

KEY CONVENTION: Much of the functional information encapsulated in protein sequences comes in the form of **consensus sequences**. This term is applied to such sequences in DNA, RNA, or protein. When a series of related nucleic acid or protein sequences are compared, a consensus sequence is the one that reflects the most common base or amino acid at each position. Parts of the sequence that have particularly good agreement often represent evolutionarily conserved functional domains. A range of mathematical tools available on the Internet can be used to generate consensus sequences or identify them in sequence databases. Box 3–2 illustrates common conventions for displaying consensus sequences. ■

Protein Sequences Can Elucidate the History of Life on Earth

The simple string of letters denoting the amino acid sequence of a protein holds a surprising wealth of information. As more protein sequences have become available, the development of more powerful methods for extracting information from them has become a major biochemical enterprise. Analysis of the information available in the many, ever-expanding biological databases, including gene and protein sequences and macromolecular structures, has given rise to the new field of **bioinformatics**. One outcome of this discipline is a growing suite of computer programs, many readily available on the Internet, that can be used by any scientist,

BOX 3–2 Consensus Sequences and Sequence Logos

Consensus sequences can be represented in several ways. To illustrate two types of conventions, we use two examples of consensus sequences, shown in Figure 1: (a) an ATP-binding structure called a P loop (see Box 12–2) and (b) a Ca^{2+}-binding structure called an EF hand (see Fig. 12–11). The rules described here are adapted from those used by the sequence comparison website PROSITE (expasy.org/prosite); they use the standard one-letter codes for the amino acids.

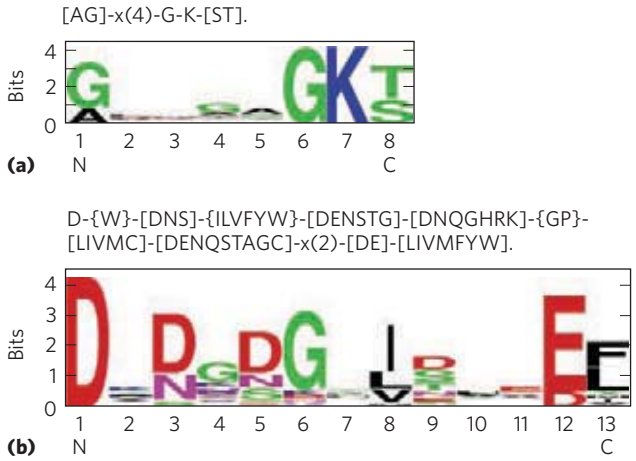


FIGURE 1 **Pepresentations of two consensus sequences. (a)** P loop, an ATP-binding structure; **(b)** EF hand, a Ca^{2+}-binding structure.

In one type of consensus sequence designation (shown at the top of (a) and (b)), each position is separated from its neighbor by a hyphen. A position where any amino acid is allowed is designated x. Ambiguities are indicated by listing the acceptable amino acids for a given position between square brackets. For example, in (a) [AG] means Ala or Gly. If all but a few amino acids are allowed at one position, the amino acids that are *not* allowed are listed between curly brackets. For example, in (b) {W} means any amino acid except Trp. Repetition of an element of the pattern is indicated by following that element with a number or range of numbers between parentheses. In (a), for example, x(4) means x-x-x-x; x(2,4) would mean x-x, or x-x-x, or x-x-x-x. When a pattern is restricted to either the amino or carboxyl terminus of a sequence, that pattern starts with < or ends with >, respectively (not so for either example here). A period ends the pattern. Applying these rules to the consensus sequence in (a), either A or G can be found at the first position. Any amino acid can occupy the next four positions, followed by an invariant G and an invariant K. The last position is either S or T.

Sequence logos provide a more informative and graphic representation of an amino acid (or nucleic acid) multiple sequence alignment. Each logo consists of a stack of symbols for each position in the sequence. The overall height of the stack (in bits) indicates the degree of sequence conservation at that position, while the height of each symbol in the stack indicates the relative frequency of that amino acid (or nucleotide). For amino acid sequences, the colors denote the characteristics of the amino acid: polar (G, S, T, Y, C, Q, N) green; basic (K, R, H) blue; acidic (D, E) red; and hydrophobic (A, V, L, I, P, W, F, M) black. The classification of amino acids in this scheme is somewhat different from that in Table 3–1 and Figure 3–5. The amino acids with aromatic side chains are subsumed into the nonpolar (F, W) and polar (Y) classifications. Glycine, always hard to group, is assigned to the polar group. Note that when multiple amino acids are acceptable at a particular position, they rarely occur with equal probability. One or a few usually predominate. The logo representation makes the predominance clear, and a conserved sequence in a protein is made obvious. However, the logo obscures some amino acid residues that may be allowed at a position, such as the Cys that occasionally occurs at position 8 of the EF hand in (b).

student, or knowledgeable layperson. Each protein's function relies on its three-dimensional structure, which in turn is determined largely by its primary structure. Thus, the biochemical information conveyed by a protein sequence is limited only by our own understanding of structural and functional principles. The constantly evolving tools of bioinformatics make it possible to identify functional segments in new proteins and help establish both their sequence and their structural relationships to proteins already in the databases. On a different level of inquiry, protein sequences are beginning to tell us how the proteins evolved and, ultimately, how life evolved on this planet.

The field of molecular evolution is often traced to Emile Zuckerkandl and Linus Pauling, whose work in the mid-1960s advanced the use of nucleotide and protein sequences to explore evolution. The premise is deceptively straightforward. If two organisms are closely related, the sequences of their genes and proteins should be similar. The sequences increasingly diverge as the evolutionary distance between two organisms increases. The promise of this approach began to be realized in the 1970s, when Carl Woese used ribosomal RNA sequences to define the Archaea as a group of living organisms distinct from the Bacteria and Eukarya (see Fig. 1–4). Protein sequences offer an opportunity to greatly refine the available information. With the advent of genome projects investigating organisms from bacteria to humans, the number of available sequences is growing at an enormous rate. This information can be

used to trace biological history. The challenge is in learning to read the genetic hieroglyphics.

Evolution has not taken a simple linear path. Complexities abound in any attempt to mine the evolutionary information stored in protein sequences. For a given protein, the amino acid residues essential for the activity of the protein are conserved over evolutionary time. The residues that are less important to function may vary over time—that is, one amino acid may substitute for another—and these variable residues can provide the information to trace evolution. Amino acid substitutions are not always random, however. At some positions in the primary structure, the need to maintain protein function may mean that only particular amino acid substitutions can be tolerated. Some proteins have more variable amino acid residues than others. For these and other reasons, different proteins can evolve at different rates.

Another complicating factor in tracing evolutionary history is the rare transfer of a gene or group of genes from one organism to another, a process called **horizontal gene transfer**. The transferred genes may be quite similar to the genes they were derived from in the original organism, whereas most other genes in the same two organisms may be quite distantly related. An example of horizontal gene transfer is the recent rapid spread of antibiotic-resistance genes in bacterial populations. The proteins derived from these transferred genes would not be good candidates for the study of bacterial evolution, because they share only a very limited evolutionary history with their "host" organisms.

The study of molecular evolution generally focuses on families of closely related proteins. In most cases, the families chosen for analysis have essential functions in cellular metabolism that must have been present in the earliest viable cells, thus greatly reducing the chance that they were introduced relatively recently by horizontal gene transfer. For example, a protein called EF-1α (elongation factor 1α) is involved in the synthesis of proteins in all eukaryotes. A similar protein, EF-Tu, with the same function, is found in bacteria. Similarities in sequence and function indicate that EF-1α and EF-Tu are members of a family of proteins that share a common ancestor. The members of protein families are called **homologous proteins**, or **homologs**. The concept of a homolog can be further refined. If two proteins in a family (that is, two homologs) are present in the same species, they are referred to as **paralogs**. Homologs from different species are called **orthologs**. The process of tracing evolution involves first identifying suitable families of homologous proteins and then using them to reconstruct evolutionary paths.

Homologs are identified through the use of increasingly powerful computer programs that can directly compare two or more chosen protein sequences, or can search vast databases to find the evolutionary relatives of one selected protein sequence. The electronic search process can be thought of as sliding one sequence past the other until a section with a good match is found. Within this sequence alignment, a positive score is assigned for each position where the amino acid residues in the two sequences are identical—the value of the score varying from one program to the next—to provide a measure of the quality of the alignment. The process has some complications. Sometimes the proteins being compared match well at, say, two sequence segments, and these segments are connected by less related sequences of different lengths. Thus the two matching segments cannot be aligned at the same time. To handle this, the computer program introduces "gaps" in one of the sequences to bring the matching segments into register **(Fig. 3–33)**. Of course, if a sufficient number of gaps are introduced, almost any two sequences could be brought into some sort of alignment. To avoid uninformative alignments, the programs include penalties for each gap introduced, thus lowering the overall alignment score. With electronic trial and error, the program selects the alignment with the optimal score that maximizes identical amino acid residues while minimizing the introduction of gaps.

Finding identical amino acids is often inadequate to identify related proteins or, more importantly, to determine how closely related the proteins are on an evolutionary time scale. A more useful analysis also considers the chemical properties of substituted amino acids. Many of the amino acid differences within a protein family may be conservative—that is, an amino acid residue is replaced by a residue having similar chemical properties. For example, a Glu residue may substitute in one family member for the Asp residue found in another; both amino acids are negatively charged. Such a conservative substitution should logically receive a higher score in a sequence alignment than does a nonconservative substitution, such as the replacement of the Asp residue with a hydrophobic Phe residue.

For most efforts to find homologies and explore evolutionary relationships, protein sequences (derived either directly from protein sequencing or from the

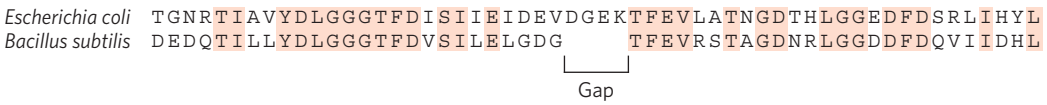


FIGURE 3–33 Aligning protein sequences with the use of gaps. Shown here is the sequence alignment of a short section of the Hsp70 proteins (a widespread class of protein-folding chaperones) from two well-studied bacterial species, *E. coli* and *Bacillus subtilis*. Introduction of a gap in the *B. subtilis* sequence allows a better alignment of amino acid residues on either side of the gap. Identical amino acid residues are shaded.

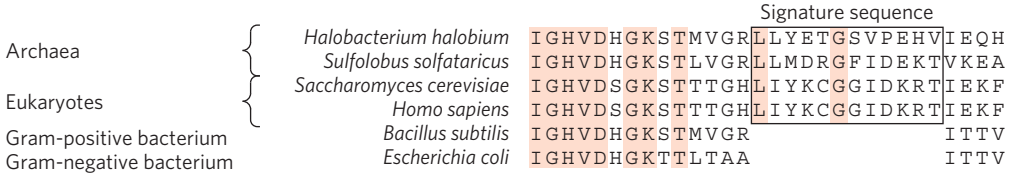


FIGURE 3–34 A signature sequence in the EF-1α/EF-Tu protein family. The signature sequence (boxed) is a 12-residue insertion near the amino terminus of the sequence. Residues that align in all species are shaded. Both archaea and eukaryotes have the signature, although the sequences of the insertions are quite distinct for the two groups. The variation in the signature sequence reflects the significant evolutionary divergence that has occurred at this site since it first appeared in a common ancestor of both groups.

sequencing of the DNA encoding the protein) are superior to nongenic nucleic acid sequences (those that do not encode a protein or functional RNA). For a nucleic acid, with its four different types of residues, random alignment of nonhomologous sequences will generally yield matches for at least 25% of the positions. Introduction of a few gaps can often increase the fraction of matched residues to 40% or more, and the probability of chance alignment of unrelated sequences becomes quite high. The 20 different amino acid residues in proteins greatly lower the probability of uninformative chance alignments of this type.

The programs used to generate a sequence alignment are complemented by methods that test the reliability of the alignments. A common computerized test is to shuffle the amino acid sequence of one of the proteins being compared to produce a random sequence, then to instruct the program to align the shuffled sequence with the other, unshuffled one. Scores are assigned to the new alignment, and the shuffling and alignment process is repeated many times. The original alignment, before shuffling, should have a score significantly higher than any of those within the distribution of scores generated by the random alignments; this increases the confidence that the sequence alignment has identified a pair of homologs. Note that the absence of a significant alignment score does not necessarily mean that no evolutionary relationship exists between two proteins. As we shall see in Chapter 4, three-dimensional structural similarities sometimes reveal evolutionary relationships where sequence homology has been wiped away by time.

To use a protein family to explore evolution, researchers identify family members with similar molecular functions in the widest possible range of organisms. Information from the family can then be used to trace the evolution of those organisms. By analyzing the sequence divergence in selected protein families, investigators can segregate organisms into classes based on their evolutionary relationships. This information must be reconciled with more classical examinations of the physiology and biochemistry of the organisms.

Certain segments of a protein sequence may be found in the organisms of one taxonomic group but not in other groups; these segments can be used as **signature sequences** for the group in which they are found. An example of a signature sequence is an insertion of 12 amino acids near the amino terminus of the EF-1α/EF-Tu proteins in all archaea and eukaryotes but not in bacteria **(Fig. 3–34)**. This particular signature is one of many biochemical clues that can help establish the evolutionary relatedness of eukaryotes and archaea. Signature sequences have been used to establish evolutionary relationships among groups of organisms at many different taxonomic levels.

By considering the entire sequence of a protein, researchers can now construct more elaborate evolutionary trees with many species in each taxonomic group. **Figure 3–35** presents one such tree for bacteria, based on sequence divergence in the protein GroEL (a protein present in all bacteria that assists in the proper folding of proteins). The tree can be refined by basing it on the sequences of multiple proteins and by supplementing the sequence information with data on the unique biochemical and physiological properties of each species. There are many methods for generating trees, each method with its own advantages and shortcomings, and many ways to represent the resulting evolutionary relationships. In Figure 3–35, the free end points of lines are called “external nodes”; each represents an extant species, and each is so labeled. The points where two lines come together, the “internal nodes,” represent extinct ancestor species. In most representations (including Fig. 3–35), the lengths of the lines connecting the nodes are proportional to the number of amino acid substitutions separating one species from another. If we trace two extant species to a common internal node (representing the common ancestor of the two species), the length of the branch connecting each external node to the internal node represents the number of amino acid substitutions separating one extant species from this ancestor. The sum of the lengths of all the line segments that connect an extant species to another extant species through a common ancestor reflects the number of substitutions separating the two extant species. To determine how much time was needed for the various species to diverge, the tree must be calibrated by comparing it with information from the fossil record and other sources.

As more sequence information is made available in databases, we can generate evolutionary trees based on multiple proteins. And we can refine these trees as

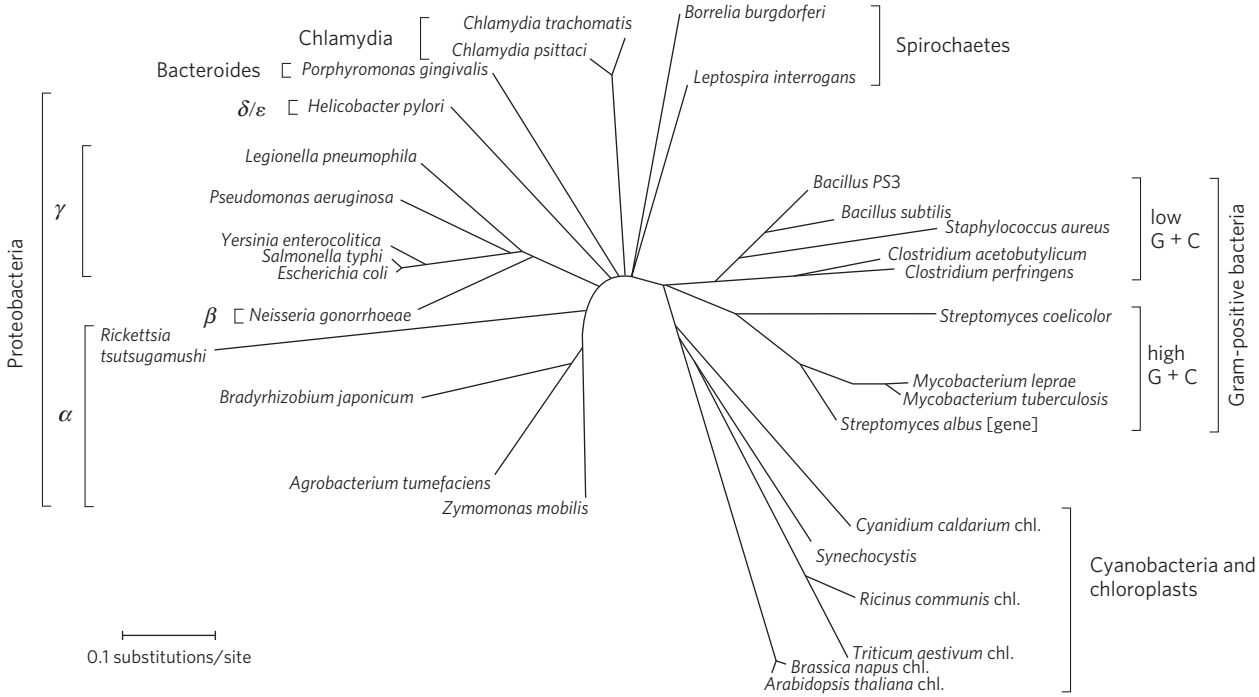


FIGURE 3–35 Evolutionary tree derived from amino acid sequence comparisons. A bacterial evolutionary tree, based on the sequence divergence observed in the GroEL family of proteins. Also included in this tree (lower right) are the chloroplasts (chl.) of some nonbacterial species.

additional genomic information emerges from increasingly sophisticated methods of analysis. All of this work moves us toward the goal of creating a detailed tree of life that describes the evolution and relationship of every organism on Earth. The story is a work in progress, of course **(Fig. 3–36)**. The questions being asked and answered are fundamental to how humans view themselves and the world around them. The field of molecular evolution promises to be among the most vibrant of the scientific frontiers in the twenty-first century.

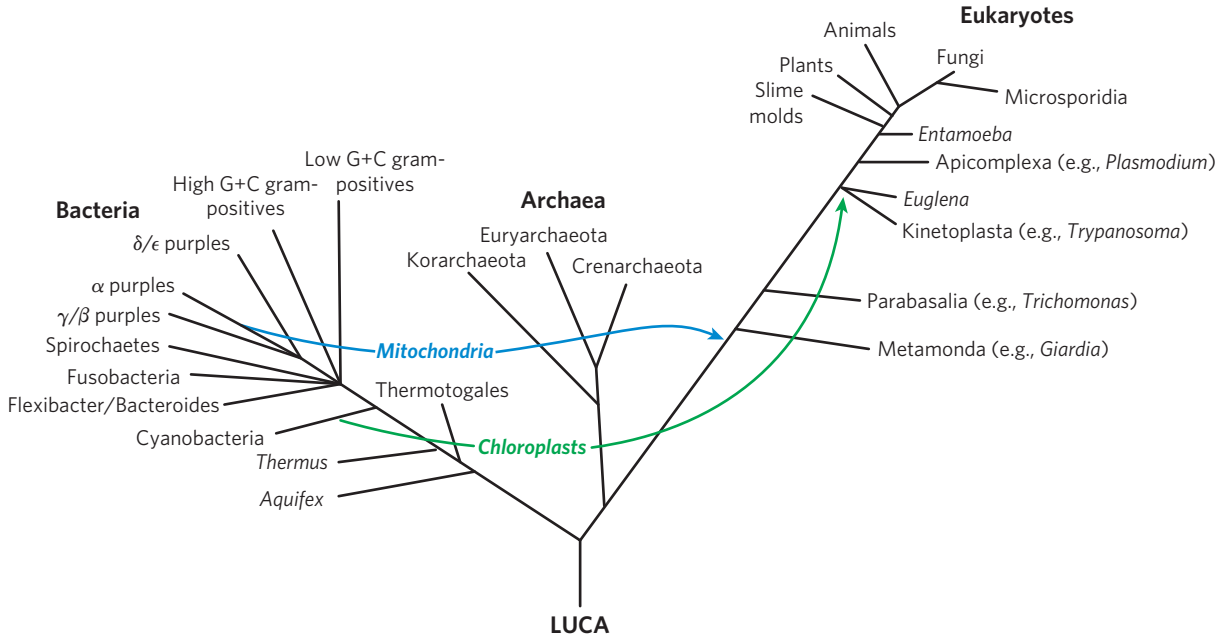


FIGURE 3–36 A consensus tree of life. The tree shown here is based on analyses of many different protein sequences and additional genomic features. The tree presents only a fraction of the available information, as well as only a fraction of the issues remaining to be resolved. Each extant group shown is a complex evolutionary story unto itself. LUCA is the last universal common ancestor from which all other life forms evolved. The blue and green arrows indicate the endosymbiotic assimilation of particular types of bacteria into eukaryotic cells to become mitochondria and chloroplasts, respectively (see Fig. 1–38).

SUMMARY 3.4 The Structure of Proteins: Primary Structure

- Differences in protein function result from differences in amino acid composition and sequence. Some variations in sequence may occur in a particular protein, with little or no effect on its function.
- Amino acid sequences are deduced by fragmenting polypeptides into smaller peptides with reagents known to cleave specific peptide bonds; determining the amino acid sequence of each fragment by the automated Edman degradation procedure; then ordering the peptide fragments by finding sequence overlaps between fragments generated by different reagents. A protein sequence can also be deduced from the nucleotide sequence of its corresponding gene in DNA, or by mass spectrometry.
- Short proteins and peptides (up to about 100 residues) can be chemically synthesized. The peptide is built up, one amino acid residue at a time, while tethered to a solid support.
- Protein sequences are a rich source of information about protein structure and function, as well as the evolution of life on Earth. Sophisticated methods are being developed to trace evolution by analyzing the resultant slow changes in amino acid sequences of homologous proteins.

Key Terms

Terms in bold are defined in the glossary.

amino acids 76
residue 76
R group 76
chiral center 76
enantiomers 76
absolute configuration 78
D, L system 78
polarity 78
absorbance, *A* 80
zwitterion 81
isoelectric pH (isoelectric point, pI) 84
peptide 85
protein 85
peptide bond 85
oligopeptide 86
polypeptide 86
oligomeric protein 88
protomer 88
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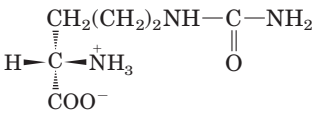
Many consider this the founding paper in the field of molecular evolution.

Problems

1. Absolute Configuration of Citrulline The citrulline isolated from watermelons has the structure shown below. Is it a D- or L-amino acid? Explain.



2. Relationship between the Titration Curve and the Acid-Base Properties of Glycine A 100 mL solution of 0.1 M glycine at pH 1.72 was titrated with 2 M NaOH solution. The pH was monitored and the results were plotted as shown in the graph. The key points in the titration are designated I to V. For each of the statements (a) to (o), *identify* the appropriate key point in the titration and *justify* your choice.

(a) Glycine is present predominantly as the species $^{+}H_3N—CH_2—COOH$.

(b) The *average* net charge of glycine is $+\frac{1}{2}$.

(c) Half of the amino groups are ionized.

(d) The pH is equal to the pK_a of the carboxyl group.

(e) The pH is equal to the pK_a of the protonated amino group.

(f) Glycine has its maximum buffering capacity.

(g) The *average* net charge of glycine is zero.

(h) The carboxyl group has been completely titrated (first equivalence point).

(i) Glycine is completely titrated (second equivalence point).

(j) The predominant species is $^{+}H_3N—CH_2—COO^{-}$.

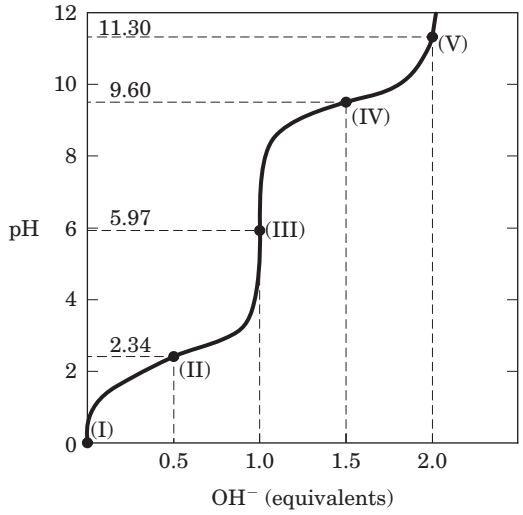
(k) The *average* net charge of glycine is -1.

(l) Glycine is present predominantly as a 50:50 mixture of $^{+}H_3N—CH_2—COOH$ and $^{+}H_3N—CH_2—COO^{-}$.

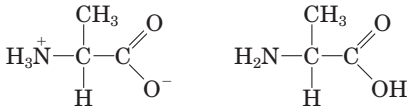
(m) This is the isoelectric point.

(n) This is the end of the titration.

(o) These are the *worst* pH regions for buffering power.



3. How Much Alanine Is Present as the Completely Uncharged Species? At a pH equal to the isoelectric point of alanine, the *net* charge on alanine is zero. Two structures can be drawn that have a net charge of zero, but the predominant form of alanine at its pI is zwitterionic.



(a) Why is alanine predominantly zwitterionic rather than completely uncharged at its pI?

(b) What fraction of alanine is in the completely uncharged form at its pI? Justify your assumptions.

4. Ionization State of Histidine Each ionizable group of an amino acid can exist in one of two states, charged or neutral. The electric charge on the functional group is determined by the relationship between its pK_a and the pH of the solution. This relationship is described by the Henderson-Hasselbalch equation.

(a) Histidine has three ionizable functional groups. Write the equilibrium equations for its three ionizations and assign the proper pK_a for each ionization. Draw the structure of histidine in each ionization state. What is the net charge on the histidine molecule in each ionization state?

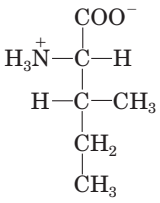
(b) Draw the structures of the predominant ionization state of histidine at pH 1, 4, 8, and 12. Note that the ionization state can be approximated by treating each ionizable group independently.

(c) What is the net charge of histidine at pH 1, 4, 8, and 12? For each pH, will histidine migrate toward the anode (+) or cathode (–) when placed in an electric field?

5. Separation of Amino Acids by Ion-Exchange Chromatography Mixtures of amino acids can be analyzed by first separating the mixture into its components through ion-exchange chromatography. Amino acids placed on a cation-exchange resin (see Fig. 3–17a) containing sulfonate ($—SO_3^-$) groups flow down the column at different rates because of two factors that influence their movement: (1) ionic attraction between the sulfonate residues on the column and positively charged functional groups on the amino acids, and (2) hydrophobic interactions between amino acid side chains and the strongly hydrophobic backbone of the polystyrene resin. For each pair of amino acids listed, determine which will be eluted first from the cation-exchange column by a pH 7.0 buffer.

(a) Asp and Lys
(b) Arg and Met
(c) Glu and Val
(d) Gly and Leu
(e) Ser and Ala

6. Naming the Stereoisomers of Isoleucine The structure of the amino acid isoleucine is



(a) How many chiral centers does it have?
(b) How many optical isomers?
(c) Draw perspective formulas for all the optical isomers of isoleucine.

7. Comparing the pK_a Values of Alanine and Polyalanine The titration curve of alanine shows the ionization of two functional groups with pK_a values of 2.34 and 9.69, corresponding to the ionization of the carboxyl and the protonated amino groups, respectively. The titration of di-, tri-, and larger oligopeptides of alanine also shows the ionization of only two functional groups, although the experimental pK_a values are different. The trend in pK_a values is summarized in the table.

Amino acid or peptide	pK_1	pK_2
Ala	2.34	9.69
Ala–Ala	3.12	8.30
Ala–Ala–Ala	3.39	8.03
Ala–$(Ala)_n$–Ala, $n \geq 4$	3.42	7.94

(a) Draw the structure of Ala–Ala–Ala. Identify the functional groups associated with pK_1 and pK_2.
(b) Why does the value of pK_1 *increase* with each additional Ala residue in the oligopeptide?
(c) Why does the value of pK_2 *decrease* with each additional Ala residue in the oligopeptide?

8. The Size of Proteins What is the approximate molecular weight of a protein with 682 amino acid residues in a single polypeptide chain?

9. The Number of Tryptophan Residues in Bovine Serum Albumin A quantitative amino acid analysis reveals that bovine serum albumin (BSA) contains 0.58% tryptophan (M_r 204) by weight.

(a) Calculate the *minimum* molecular weight of BSA (i.e., assume there is only one Trp residue per protein molecule).
(b) Size-exclusion chromatography of BSA gives a molecular weight estimate of 70,000. How many Trp residues are present in a molecule of serum albumin?

10. Subunit Composition of a Protein A protein has a molecular mass of 400 kDa when measured by size-exclusion chromatography. When subjected to gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), the protein gives three bands with molecular masses of 180, 160, and 60 kDa. When electrophoresis is carried out in the presence of SDS and dithiothreitol, three bands are again formed, this time with molecular masses of 160, 90, and 60 kDa. Determine the subunit composition of the protein.

11. Net Electric Charge of Peptides A peptide has the sequence

Glu–His–Trp–Ser–Gly–Leu–Arg–Pro–Gly

(a) What is the net charge of the molecule at pH 3, 8, and 11? (Use pK_a values for side chains and terminal amino and carboxyl groups as given in Table 3–1.)
(b) Estimate the pI for this peptide.

12. Isoelectric Point of Pepsin Pepsin is the name given to a mix of several digestive enzymes secreted (as larger precursor proteins) by glands that line the stomach. These glands also secrete hydrochloric acid, which dissolves the particulate matter in food, allowing pepsin to enzymatically cleave individual protein molecules. The resulting mixture of food, HCl, and digestive enzymes is known as chyme and has a pH near 1.5. What pI would you predict for the pepsin proteins? What functional groups must be present to confer this pI on pepsin? Which amino acids in the proteins would contribute such groups?

13. Isoelectric Point of Histones Histones are proteins found in eukaryotic cell nuclei, tightly bound to DNA, which has many phosphate groups. The pI of histones is very high, about 10.8. What amino acid residues must be present in relatively large numbers in histones? In what way do these residues contribute to the strong binding of histones to DNA?

14. Solubility of Polypeptides One method for separating polypeptides makes use of their different solubilities. The solubility of large polypeptides in water depends on the relative polarity of their R groups, particularly on the number of ionized groups: the more ionized groups there are, the more soluble the polypeptide. Which of each pair of polypeptides that follow is more soluble at the indicated pH?

(a) $(Gly)_{20}$ or $(Glu)_{20}$ at pH 7.0
(b) $(Lys–Ala)_3$ or $(Phe–Met)_3$ at pH 7.0
(c) $(Ala–Ser–Gly)_5$ or $(Asn–Ser–His)_5$ at pH 6.0
(d) $(Ala–Asp–Gly)_5$ or $(Asn–Ser–His)_5$ at pH 3.0

15. Purification of an Enzyme A biochemist discovers and purifies a new enzyme, generating the purification table below.

Procedure	Total protein (mg)	Activity (units)
1. Crude extract	20,000	4,000,000
2. Precipitation (salt)	5,000	3,000,000
3. Precipitation (pH)	4,000	1,000,000
4. Ion-exchange chromatography	200	800,000
5. Affinity chromatography	50	750,000
6. Size-exclusion chromatography	45	675,000

(a) From the information given in the table, calculate the specific activity of the enzyme after each purification procedure.

(b) Which of the purification procedures used for this enzyme is most effective (i.e., gives the greatest relative increase in purity)?

(c) Which of the purification procedures is least effective?

(d) Is there any indication based on the results shown in the table that the enzyme after step 6 is now pure? What else could be done to estimate the purity of the enzyme preparation?

16. Dialysis A purified protein is in a Hepes (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)) buffer at pH 7 with 500 mM NaCl. A sample (1 mL) of the protein solution is placed in a tube made of dialysis membrane and dialyzed against 1 L of the same Hepes buffer with 0 mM NaCl. Small molecules and ions (such as Na^+, Cl^-, and Hepes) can diffuse across the dialysis membrane, but the protein cannot.

(a) Once the dialysis has come to equilibrium, what is the concentration of NaCl in the protein sample? Assume no volume changes occur in the sample during the dialysis.

(b) If the original 1 mL sample were dialyzed twice, successively, against 100 mL of the same Hepes buffer with 0 mM NaCl, what would be the final NaCl concentration in the sample?

17. Peptide Purification At pH 7.0, in what order would the following three peptides be eluted from a column filled with a cation-exchange polymer? Their amino acid compositions are:

Peptide A: Ala 10%, Glu 5%, Ser 5%, Leu 10%, Arg 10%, His 5%, Ile 10%, Phe 5%, Tyr 5%, Lys 10%, Gly 10%, Pro 5%, and Trp 10%.

Peptide B: Ala 5%, Val 5%, Gly 10%, Asp 5%, Leu 5%, Arg 5%, Ile 5%, Phe 5%, Tyr 5%, Lys 5%, Trp 5%, Ser 5%, Thr 5%, Glu 5%, Asn 5%, Pro 10%, Met 5%, and Cys 5%.

Peptide C: Ala 10%, Glu 10%, Gly 5%, Leu 5%, Asp 10%, Arg 5%, Met 5%, Cys 5%, Tyr 5%, Phe 5%, His 5%, Val 5%, Pro 5%, Thr 5%, Ser 5%, Asn 5%, and Gln 5%.

18. Sequence Determination of the Brain Peptide Leucine Enkephalin A group of peptides that influence nerve transmission in certain parts of the brain has been isolated from normal brain tissue. These peptides are known as opioids, because they bind to specific receptors that also bind opiate drugs, such as morphine and naloxone. Opioids thus mimic some of the properties of opiates. Some researchers consider these peptides to be the brain's own painkillers. Using the information below, determine the amino acid sequence of the opioid leucine enkephalin. Explain how your structure is consistent with each piece of information.

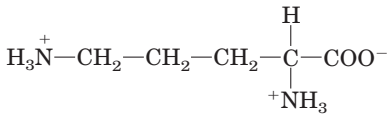
(a) Complete hydrolysis by 6 M HCl at 110 °C followed by amino acid analysis indicated the presence of Gly, Leu, Phe, and Tyr, in a 2:1:1:1 molar ratio.

(b) Treatment of the peptide with 1-fluoro-2,4-dinitrobenzene followed by complete hydrolysis and chromatography indicated the presence of the 2,4-dinitrophenyl derivative of tyrosine. No free tyrosine could be found.

(c) Complete digestion of the peptide with chymotrypsin followed by chromatography yielded free tyrosine and leucine, plus a tripeptide containing Phe and Gly in a 1:2 ratio.

19. Structure of a Peptide Antibiotic from *Bacillus brevis* Extracts from the bacterium *Bacillus brevis* contain a peptide with antibiotic properties. This peptide forms complexes with metal ions and seems to disrupt ion transport across the cell membranes of other bacterial species, killing them. The structure of the peptide has been determined from the following observations.

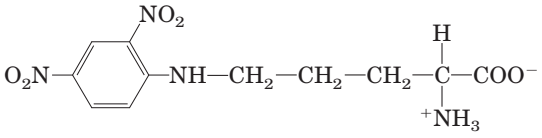
(a) Complete acid hydrolysis of the peptide followed by amino acid analysis yielded equimolar amounts of Leu, Orn, Phe, Pro, and Val. Orn is ornithine, an amino acid not present in proteins but present in some peptides. It has the structure



(b) The molecular weight of the peptide was estimated as about 1,200.

(c) The peptide failed to undergo hydrolysis when treated with the enzyme carboxypeptidase. This enzyme catalyzes the hydrolysis of the carboxyl-terminal residue of a polypeptide unless the residue is Pro or, for some reason, does not contain a free carboxyl group.

(d) Treatment of the intact peptide with 1-fluoro-2,4-dinitrobenzene, followed by complete hydrolysis and chromatography, yielded only free amino acids and the following derivative:



(Hint: The 2,4-dinitrophenyl derivative involves the amino group of a side chain rather than the α-amino group.)

(e) Partial hydrolysis of the peptide followed by chromatographic separation and sequence analysis yielded the following di- and tripeptides (the amino-terminal amino acid is always at the left):

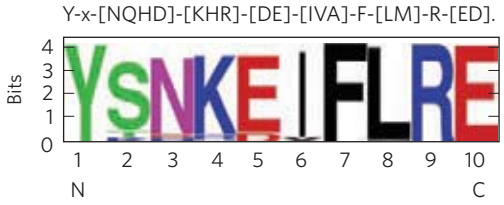
Leu–Phe Phe–Pro Orn–Leu Val–Orn

Val–Orn–Leu Phe–Pro–Val Pro–Val–Orn

Given the above information, deduce the amino acid sequence of the peptide antibiotic. Show your reasoning. When you have arrived at a structure, demonstrate that it is consistent with *each* experimental observation.

20. Efficiency in Peptide Sequencing A peptide with the primary structure Lys–Arg–Pro–Leu–Ile–Asp–Gly–Ala is sequenced by the Edman procedure. If each Edman cycle is 96% efficient, what percentage of the amino acids liberated in the fourth cycle will be leucine? Do the calculation a second time, but assume a 99% efficiency for each cycle.

21. Sequence Comparisons Proteins called molecular chaperones (described in Chapter 4) assist in the process of protein folding. One class of chaperone found in organisms from bacteria to mammals is heat shock protein 90 (Hsp90). All Hsp90 chaperones contain a 10 amino acid "signature sequence," which allows for ready identification of these proteins in sequence databases. Two representations of this signature sequence are shown below.



(a) In this sequence, which amino acid residues are invariant (conserved across all species)?

(b) At which position(s) are amino acids limited to those with positively charged side chains? For each position, which amino acid is more commonly found?

(c) At which positions are substitutions restricted to amino acids with negatively charged side chains? For each position, which amino acid predominates?

(d) There is one position that can be any amino acid, although one amino acid appears much more often than any other. What position is this, and which amino acid appears most often?

22. Chromatographic Methods Three polypeptides, the sequences of which are represented below using the one-letter code for their amino acids, are present in a mixture:

1. ATKNRASCLVPKHGALMFWRHKQLVSDPILQKR-QHILVCRNAAG
2. GPYFGDEPLDVHDEPEEG
3. PHLLSAWKGMEGVGKSQSFAALIVILA

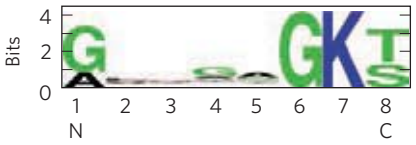
Of the three, which one would migrate most slowly during chromatography through:

(a) an ion-exchange resin; beads coated with positively charged groups?

(b) an ion-exchange resin; beads coated with negatively charged groups?

(c) a size-exclusion (gel-filtration) column designed to separate small peptides such as these?

(d) Which peptide contains the ATP-binding motif shown in the following sequence logo?

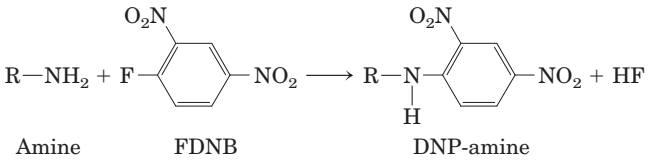


Data Analysis Problem

23. Determining the Amino Acid Sequence of Insulin Figure 3–24 shows the amino acid sequence of bovine insulin. This structure was determined by Frederick Sanger and his coworkers. Most of this work is described in a series of articles published in the *Biochemical Journal* from 1945 to 1955.

When Sanger and colleagues began their work in 1945, it was known that insulin was a small protein consisting of two or four polypeptide chains linked by disulfide bonds. Sanger and his coworkers had developed a few simple methods for studying protein sequences.

Treatment with FDNB. FDNB (1-fluoro-2,4-dinitrobenzene) reacted with free amino (but not amido or guanidino) groups in proteins to produce dinitrophenyl (DNP) derivatives of amino acids:



Acid Hydrolysis. Boiling a protein with 10% HCl for several hours hydrolyzed all of its peptide and amide bonds. Short treatments produced short polypeptides; the longer the treatment, the more complete the breakdown of the protein into its amino acids.

Oxidation of Cysteines. Treatment of a protein with performic acid cleaved all the disulfide bonds and converted all Cys residues to cysteic acid residues (see Fig. 3–28).

Paper Chromatography. This more primitive version of thin-layer chromatography (see Fig. 10–25) separated compounds based on their chemical properties, allowing identification of single amino acids and, in some cases, dipeptides. Thin-layer chromatography also separates larger peptides.

As reported in his first paper (1945), Sanger reacted insulin with FDNB and hydrolyzed the resulting protein. He found many free amino acids, but only three DNP–amino acids: α-DNP-glycine (DNP group attached to the α-amino group); α-DNP-phenylalanine; and ε-DNP-lysine (DNP attached to the ε-amino group). Sanger interpreted these results as showing that insulin had two protein chains: one with Gly at its amino terminus and one with Phe at its amino terminus. One of the two chains also contained a Lys residue, not at the amino terminus. He named the chain beginning with a Gly residue "A" and the chain beginning with Phe "B."

(a) Explain how Sanger's results support his conclusions.

(b) Are the results consistent with the known structure of bovine insulin (see Fig. 3–24)?

In a later paper (1949), Sanger described how he used these techniques to determine the first few amino acids (amino-terminal end) of each insulin chain. To analyze the B chain, for example, he carried out the following steps:

1. Oxidized insulin to separate the A and B chains.
2. Prepared a sample of pure B chain with paper chromatography.
3. Reacted the B chain with FDNB.
4. Gently acid-hydrolyzed the protein so that some small peptides would be produced.
5. Separated the DNP-peptides from the peptides that did not contain DNP groups.
6. Isolated four of the DNP-peptides, which were named B1 through B4.
7. Strongly hydrolyzed each DNP-peptide to give free amino acids.
8. Identified the amino acids in each peptide with paper chromatography.

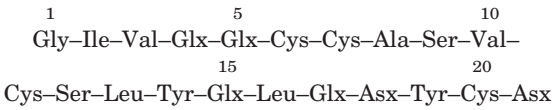
The results were as follows:

B1: α-DNP-phenylalanine only
B2: α-DNP-phenylalanine; valine
B3: aspartic acid; α-DNP-phenylalanine; valine
B4: aspartic acid; glutamic acid; α-DNP-phenylalanine; valine

(c) Based on these data, what are the first four (amino-terminal) amino acids of the B chain? Explain your reasoning.

(d) Does this result match the known sequence of bovine insulin (see Fig. 3–24)? Explain any discrepancies.

Sanger and colleagues used these and related methods to determine the entire sequence of the A and B chains. Their sequence for the A chain was as follows (amino terminus on left):



Because acid hydrolysis had converted all Asn to Asp and all Gln to Glu, these residues had to be designated Asx and Glx, respectively (exact identity in the peptide unknown). Sanger solved this problem by using protease enzymes that cleave peptide bonds, but not the amide bonds in Asn and Gln residues, to prepare short peptides. He then determined the number of amide groups present in each peptide by measuring the NH_4^+ released when the peptide was acid-hydrolyzed. Some of the results for the A chain are shown below. The peptides may not have been completely pure, so the numbers were approximate—but good enough for Sanger's purposes.

Peptide name	Peptide sequence	Number of amide groups in peptide
Ac1	Cys–Asx	0.7
Ap15	Tyr–Glx–Leu	0.98
Ap14	Tyr–Glx–Leu–Glx	1.06
Ap3	Asx–Tyr–Cys–Asx	2.10
Ap1	Glx–Asx–Tyr–Cys–Asx	1.94
Ap5pa1	Gly–Ile–Val–Glx	0.15
Ap5	Gly–Ile–Val–Glx–Glx–Cys–Cys–Ala–Ser–Val–Cys–Ser–Leu	1.16

(e) Based on these data, determine the amino acid sequence of the A chain. Explain how you reached your answer. Compare it with Figure 3–24.

References

Sanger, F. (1945) The free amino groups of insulin. *Biochem. J.* **39**, 507–515.

Sanger, F. (1949) The terminal peptides of insulin. *Biochem. J.* **45**, 563–574.