Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002.

Fractionation of Cells

Although biochemical analysis requires disruption of the anatomy of the cell, gentle fractionation techniques have been devised to separate the various cell components while preserving their individual functions. Just as a tissue can be separated into its living constituent cell types, so the cell can be separated into its functioning organelles and macromolecules. In this section we consider the methods that allow organelles and proteins to be purified and analyzed biochemically.

Organelles and Macromolecules Can Be Separated by Ultracentrifugation

Cells can be broken up in various ways: they can be subjected to osmotic shock or ultrasonic vibration, forced through a small orifice, or ground up in a blender. These procedures break many of the membranes of the cell (including the plasma membrane and membranes of the endoplasmic reticulum) into fragments that immediately reseal to form small closed vesicles. If carefully applied, however, the disruption procedures leave organelles such as nuclei, mitochondria, the Golgi apparatus, lysosomes, and peroxisomes largely intact. The suspension of cells is thereby reduced to a thick slurry (called a *homogenate* or *extract)* that contains a variety of membrane-enclosed organelles, each with a distinctive size, charge, and density. Provided that the homogenization medium has been carefully chosen (by trial and error for each organelle), the various components—including the vesicles derived from the endoplasmic reticulum, called microsomes—retain most of their original biochemical properties.

The different components of the homogenate must then be separated. Such cell fractionations became possible only after the commercial development in the early 1940s of an instrument known as the *preparative ultracentrifuge*, in which extracts of broken cells are rotated at high speeds (Figure 8-7). This treatment separates cell components by size and density: in general, the largest units experience the largest centrifugal force and move the most rapidly. At relatively low speed, large components such as nuclei sediment to form a pellet at the bottom of the centrifuge tube; at slightly higher speed, a pellet of mitochondria is deposited; and at even higher speeds and with longer periods of centrifugation, first the small closed vesicles and then the ribosomes can be collected (Figure 8-8). All of these fractions are impure, but many of the contaminants can be removed by resuspending the pellet and repeating the centrifugation procedure several times.

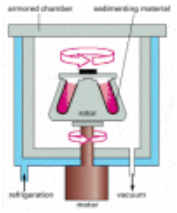

Figure 8-7

The preparative ultracentrifuge. The sample is contained in tubes that are inserted into a ring of cylindrical holes in a metal *rotor*. Rapid rotation of the rotor generates enormous centrifugal forces, which cause particles in the sample to sediment. (more...)

Figure 8-8

Cell fractionation by centrifugation. Repeated centrifugation at progressively higher speeds will fractionate homogenates of cells into their components. In general, the smaller the subcellular component, the greater is the centrifugal force required (more...)

Centrifugation is the first step in most fractionations, but it separates only components that differ greatly in size. A finer degree of separation can be achieved by layering the homogenate in a thin

band on top of a dilute salt solution that fills a centrifuge tube. When centrifuged, the various components in the mixture move as a series of distinct bands through the salt solution, each at a different rate, in a process called *velocity sedimentation* (Figure 8-9A). For the procedure to work effectively, the bands must be protected from convective mixing, which would normally occur whenever a denser solution (for example, one containing organelles) finds itself on top of a lighter one (the salt solution). This is achieved by filling the centrifuge tube with a shallow gradient of sucrose prepared by a special mixing device. The resulting density gradient—with the dense end at the bottom of the tube—keeps each region of the salt solution denser than any solution above it, and it thereby prevents convective mixing from distorting the separation.

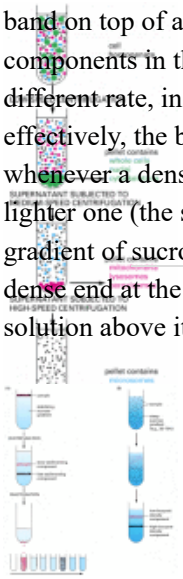

Figure 8-9

Comparison of velocity sedimentation and equilibrium sedimentation. In velocity sedimentation (A) subcellular components sediment at different speeds according to their size and shape when layered over a dilute solution containing sucrose. To stabilize (more...)

When sedimented through such dilute sucrose gradients, different cell components separate into distinct bands that can be collected individually. The relative rate at which each component sediments depends primarily on its size and shape—being normally described in terms of its sedimentation coefficient, or s value. Present-day ultracentrifuges rotate at speeds of up to 80,000 rpm and produce forces as high as 500,000 times gravity. With these enormous forces, even small macromolecules, such as tRNA molecules and simple enzymes, can be driven to sediment at an appreciable rate and so can be separated from one another by size. Measurements of sedimentation coefficients are routinely used to help in determining the size and subunit composition of the organized assemblies of macromolecules found in cells.

The ultracentrifuge is also used to separate cellular components on the basis of their buoyant density, independently of their size and shape. In this case the sample is usually sedimented through a steep density gradient that contains a very high concentration of sucrose or cesium chloride. Each cellular component begins to move down the gradient as in Figure 8-9A, but it eventually reaches a position where the density of the solution is equal to its own density. At this point the component floats and can move no farther. A series of distinct bands is thereby produced in the centrifuge tube, with the bands closest to the bottom of the tube containing the components of highest buoyant density (Figure 8-9B). This method, called *equilibrium sedimentation*, is so sensitive that it is capable of separating macromolecules that have incorporated heavy isotopes, such as ^{13}C or ^{15}N, from the same macromolecules that contain the lighter, common isotopes (^{12}C or ^{14}N). In fact, the cesium-chloride method was developed in 1957 to separate the labeled from the unlabeled DNA produced after exposure of a growing population of bacteria to nucleotide precursors containing ^{15}N; this classic experiment provided direct evidence for the semiconservative replication of DNA (see Figure 5-5).

The Molecular Details of Complex Cellular Processes Can Be Deciphered in Cell-Free Systems

Studies of organelles and other large subcellular components isolated in the ultracentrifuge have contributed enormously to our understanding of the functions of different cellular components. Experiments on mitochondria and chloroplasts purified by centrifugation, for example, demonstrated the central function of these organelles in converting energy into forms that the cell can use. Similarly, resealed vesicles formed from fragments of rough and smooth endoplasmic reticulum (microsomes) have been separated from each other and analyzed as functional models of these compartments of the intact cell.

An extension of this approach makes it possible to study many other biological processes free

from all of the complex side reactions that occur in a living cell, by using purified cell-free systems. In this case, cell homogenates are fractionated with the aim of purifying each of the individual macromolecules that are needed to catalyze a biological process of interest. For example, the mechanisms of protein synthesis were deciphered in experiments that began with a cell homogenate that could translate RNA molecules to produce proteins. Fractionation of this homogenate, step by step, produced in turn the ribosomes, tRNAs, and various enzymes that together constitute the protein-synthetic machinery. Once individual pure components were available, each could be added or withheld separately to define its exact role in the overall process. A major goal today is the reconstitution of every biological process in a purified cell-free system, so as to be able to define all of its components and their mechanism of action. Some landmarks in the development of this critical approach for understanding the cell are listed in Table 8-4.

Table 8-4

Some Major Events in the Development of Cell-Free Systems.

Much of what we know about the molecular biology of the cell has been discovered by studying cell-free systems. As a few of many examples, they have been used to decipher the molecular details of DNA replication and DNA transcription, RNA splicing, protein translation, muscle contraction, and particle transport along microtubules. Cell-free systems have even been used to study such complex and highly organized processes as the cell-division cycle, the separation of chromosomes on the mitotic spindle, and the vesicular-transport steps involved in the movement of proteins from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane.

Cell homogenates also provide, in principle, the starting material for the complete separation of all of the individual macromolecular components from the cell. We now consider how this separation is achieved, focusing on proteins.

Proteins Can Be Separated by Chromatography

Proteins are most often fractionated by **column chromatography**, in which a mixture of proteins in solution is passed through a column containing a porous solid matrix. The different proteins are retarded to different extents by their interaction with the matrix, and they can be collected separately as they flow out of the bottom of the column (Figure 8-10). Depending on the choice of matrix, proteins can be separated according to their charge *(ion-exchange chromatography),* their hydrophobicity *(hydrophobic chromatography),* their size *(gel-filtration chromatography),* or their ability to bind to particular small molecules or to other macromolecules *(affinity chromatography)*.

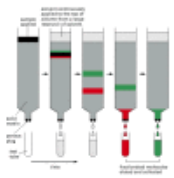

Figure 8-10

The separation of molecules by column chromatography. The sample, a mixture of different molecules, is applied to the top of a cylindrical glass or plastic column filled with a permeable solid matrix, such as cellulose, immersed in solvent. A large amount (more...)

Many types of matrices are commercially available (Figure 8-11). Ion-exchange columns are packed with small beads that carry either a positive or negative charge, so that proteins are fractionated according to the arrangement of charges on their surface. Hydrophobic columns are packed with beads from which hydrophobic side chains protrude, so that proteins with exposed hydrophobic regions are retarded. Gel-filtration columns, which separate proteins according to their size, are packed with tiny porous beads: molecules that are small enough to enter the pores linger inside successive beads as they pass down the column, while larger molecules remain in

the solution flowing between the beads and therefore move more rapidly, emerging from the column first. Besides providing a means of separating molecules, gel-filtration chromatography is a convenient way to determine their size.

Figure 8-11

Three types of matrices used for chromatography. In ion-exchange chromatography (A) the insoluble matrix carries ionic charges that retard the movement of molecules of opposite charge. Matrices used for separating proteins include diethylaminoethylcellulose (more...)

The resolution of conventional column chromatography is limited by inhomogeneities in the matrices (such as cellulose), which cause an uneven flow of solvent through the column. Newer chromatography resins (usually silica-based) have been developed in the form of tiny spheres (3 to 10 μm in diameter) that can be packed with a special apparatus to form a uniform column bed. A high degree of resolution is attainable on such **high-performance liquid chromatography (HPLC)** columns. Because they contain such tightly packed particles, HPLC columns have negligible flow rates unless high pressures are applied. For this reason these columns are typically packed in steel cylinders and require an elaborate system of pumps and valves to force the solvent through them at sufficient pressure to produce the desired rapid flow rates of about one column volume per minute. In conventional column chromatography, flow rates must be kept slow (often about one column volume per hour) to give the solutes being fractionated time to equilibrate with the interior of the large matrix particles. In HPLC the solutes equilibrate very rapidly with the interior of the tiny spheres, so solutes with different affinities for the matrix are efficiently separated from one another even at fast flow rates. This allows most fractionations to be carried out in minutes, whereas hours are required to obtain a poorer separation by conventional chromatography. HPLC has therefore become the method of choice for separating many proteins and small molecules.

Affinity Chromatography Exploits Specific Binding Sites on Proteins

If one starts with a complex mixture of proteins, these types of column chromatography do not produce very highly purified fractions: a single passage through the column generally increases the proportion of a given protein in the mixture no more than twentyfold. Because most individual proteins represent less than 1/1000 of the total cellular protein, it is usually necessary to use several different types of column in succession to attain sufficient purity (Figure 8-12). A more efficient procedure, known as affinity chromatography, takes advantage of the biologically important binding interactions that occur on protein surfaces. If a substrate molecule is covalently coupled to an inert matrix such as a polysaccharide bead, for example, the enzyme that operates on that substrate will often be specifically retained by the matrix and can then be eluted (washed out) in nearly pure form. Likewise, short DNA oligonucleotides of a specifically designed sequence can be immobilized in this way and used to purify DNA-binding proteins that normally recognize this sequence of nucleotides in chromosomes (see Figure 7-30). Alternatively, specific antibodies can be coupled to a matrix to purify protein molecules recognized by the antibodies. Because of the great specificity of all such affinity columns, 1000- to 10,000-fold purifications can sometimes be achieved in a single pass.

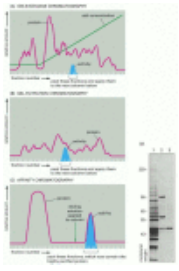

Figure 8-12

Protein purification by chromatography. Typical results obtained when three different chromatographic steps are used in succession to purify a protein. In this example a homogenate of cells was first fractionated by allowing it to percolate through an (more...)

Any gene can be modified, using the recombinant DNA methods discussed in the next section, to produce its protein with a molecular tag attached to it, making subsequent purification of the protein by affinity chromatography simple and rapid (see Figure 8-48, below). For example, the amino acid histidine binds to certain metal ions, including nickel and copper. If genetic engineering techniques are used to attach a short string of histidine residues to either end of a protein, the slightly modified protein can be retained selectively on an affinity column containing immobilized nickel ions. Metal affinity chromatography can thereby be used to purify that modified protein from a complex molecular mixture. In other cases, an entire protein is used as the molecular tag. When the small enzyme glutathione S-transferase (GST) is attached to a target protein, the resulting fusion protein can be purified using an affinity column containing glutathione, a substrate molecule that binds specifically and tightly to GST (see Figure 8-50, below).

As a further refinement of this last technique, an amino acid sequence that forms a cleavage site for a highly specific protease can be engineered between the protein of choice and the histidine or GST tag. The cleavage sites for the proteases that are used, such as factor X that functions during blood clotting, are very rarely found by chance in proteins. Thus, the tag can later be specifically removed by cleavage at the cleavage site without destroying the purified protein.

The Size and Subunit Composition of a Protein Can Be Determined by SDS Polyacrylamide-Gel Electrophoresis

Proteins usually possess a net positive or negative charge, depending on the mixture of charged amino acids they contain. When an electric field is applied to a solution containing a protein molecule, the protein migrates at a rate that depends on its net charge and on its size and shape. This technique, known as electrophoresis, was originally used to separate mixtures of proteins either in free aqueous solution or in solutions held in a solid porous matrix such as starch.

In the mid-1960s a modified version of this method—which is known as **SDS polyacrylamide-gel electrophoresis (SDS-PAGE)**—was developed that has revolutionized routine protein analysis. It uses a highly cross-linked gel of polyacrylamide as the inert matrix through which the proteins migrate. The gel is prepared by polymerization from monomers; the pore size of the gel can be adjusted so that it is small enough to retard the migration of the protein molecules of interest. The proteins themselves are not in a simple aqueous solution but in one that includes a powerful negatively charged detergent, sodium dodecyl sulfate, or SDS (Figure 8-13). Because this detergent binds to hydrophobic regions of the protein molecules, causing them to unfold into extended polypeptide chains, the individual protein molecules are released from their associations with other proteins or lipid molecules and rendered freely soluble in the detergent solution. In addition, a reducing agent such as β-mercapto-ethanol (see Figure 8-13) is usually added to break any S-S linkages in the proteins, so that all of the constituent polypeptides in multisubunit molecules can be analyzed separately.

Figure 8-13

The detergent sodium dodecyl sulfate (SDS) and the reducing agent β-mercaptoethanol. These two chemicals are used to solubilize proteins for SDS polyacrylamide-gel electrophoresis. The SDS is shown here in its ionized form.

What happens when a mixture of SDS-solubilized proteins is run through a slab of polyacrylamide gel? Each protein molecule binds large numbers of the negatively charged detergent molecules, which mask the protein's intrinsic charge and cause it to migrate toward the positive electrode when a voltage is applied. Proteins of the same size tend to move through the

gel with similar speeds because (1) their native structure is completely unfolded by the SDS, so that their shapes are the same, and (2) they bind the same amount of SDS and therefore have the same amount of negative charge. Larger proteins, with more charge, will be subjected to larger electrical forces and also to a larger drag. In free solution the two effects would cancel out, but in the mesh of the polyacryl-amide gel, which acts as a molecular sieve, large proteins are retarded much more than small ones. As a result, a complex mixture of proteins is fractionated into a series of discrete protein bands arranged in order of molecular weight (Figure 8-14). The major proteins are readily detected by staining the proteins in the gel with a dye such as Coomassie blue, and even minor proteins are seen in gels treated with a silver or gold stain (with which as little as 10 ng of protein can be detected in a band).

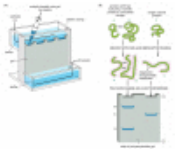

Figure 8-14

SDS polyacrylamide-gel electrophoresis (SDS-PAGE). (A) An electrophoresis apparatus. (B) Individual polypeptide chains form a complex with negatively charged molecules of sodium dodecyl sulfate (SDS) and therefore migrate as a negatively charged SDS-protein (more...)

SDS polyacrylamide-gel electrophoresis is a more powerful procedure than any previous method of protein analysis principally because it can be used to separate all types of proteins, including those that are insoluble in water. Membrane proteins, protein components of the cytoskeleton, and proteins that are part of large macromolecular aggregates can all be resolved. Because the method separates polypeptides by size, it also provides information about the molecular weight and the subunit composition of any protein complex. A photograph of a gel that has been used to analyze each of the successive stages in the purification of a protein is shown in Figure 8-15.

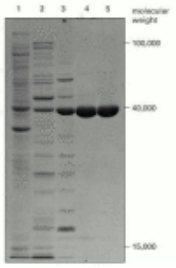

Figure 8-15

Analysis of protein samples by SDS polyacrylamide-gel electrophoresis. The photograph shows a Coomassie-stained gel that has been used to detect the proteins present at successive stages in the purification of an enzyme. The leftmost lane (lane 1) contains (more...)

More Than 1000 Proteins Can Be Resolved on a Single Gel by Two-dimensional Polyacrylamide-Gel Electrophoresis

Because closely spaced protein bands or peaks tend to overlap, one-dimensional separation methods, such as SDS polyacrylamide-gel electrophoresis or chromatography, can resolve only a relatively small number of proteins (generally fewer than 50). In contrast, two-dimensional gel electrophoresis, which combines two different separation procedures, can resolve up to 2000 proteins—the total number of different proteins in a simple bacterium—in the form of a two-dimensional protein map.

In the first step, the proteins are separated by their intrinsic charges. The sample is dissolved in a small volume of a solution containing a nonionic (uncharged) detergent, together with β-mercaptoethanol and the denaturing reagent urea. This solution solubilizes, denatures, and dissociates all the polypeptide chains but leaves their intrinsic charge unchanged. The polypeptide chains are then separated by a procedure called isoelectric focusing, which takes advantage of the fact that the net charge on a protein molecule varies with the pH of the surrounding solution. Every protein has a characteristic isoelectric point, the pH at which the protein has no net charge and therefore does not migrate in an electric field. In isoelectric focusing, proteins are separated electrophoretically in a narrow tube of polyacrylamide gel in which a gradient of pH is established by a mixture of special buffers. Each protein moves to a position in the gradient that

corresponds to its isoelectric point and stays there (Figure 8-16). This is the first dimension of two-dimensional gel electrophoresis.

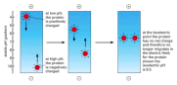

Figure 8-16

Separation of protein molecules by isoelectric focusing. At low pH (high H^+ concentration) the carboxylic acid groups of proteins tend to be uncharged (-COOH) and their nitrogen-containing basic groups fully charged (for example, $-NH_3^+$), giving most (more...)

In the second step the narrow gel containing the separated proteins is again subjected to electrophoresis but in a direction that is at a right angle to the direction that used in the first step. This time SDS is added, and the proteins are separated according to their size, as in one-dimensional SDS-PAGE: the original narrow gel is soaked in SDS and then placed on one edge of an SDS polyacryl-amide-gel slab, through which each polypeptide chain migrates to form a discrete spot. This is the second dimension of two-dimensional polyacrylamide-gel electrophoresis. The only proteins left unresolved are those that have both identical sizes and identical isoelectric points, a relatively rare situation. Even trace amounts of each polypeptide chain can be detected on the gel by various staining procedures—or by autoradiography if the protein sample was initially labeled with a radioisotope (Figure 8-17). The technique has such great resolving power that it can distinguish between two proteins that differ in only a single charged amino acid.

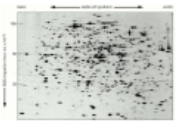

Figure 8-17

Two-dimensional polyacrylamide-gel electrophoresis. All the proteins in an *E. coli* bacterial cell are separated in this gel, in which each spot corresponds to a different polypeptide chain. The proteins were first separated on the basis of their isoelectric (more...)

A specific protein can be identified after its fractionation on either one-dimensional or two-dimensional gels by exposing all the proteins present on the gel to a specific antibody that has been coupled to a radioactive isotope, to an easily detectable enzyme, or to a fluorescent dye. For convenience, this is normally done after all the separated proteins present in the gel have been transferred (by "blotting") onto a sheet of nitrocellulose paper, as described later for nucleic acids (see Figure 8-27). This protein-detection method is called Western blotting (Figure 8-18).

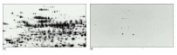

Figure 8-18

Western blotting. The total proteins from dividing tobacco cells in culture are first separated by two-dimensional polyacrylamide-gel electrophoresis and in (A) their positions are revealed by a sensitive protein stain. In (B) the separated proteins on (more...)

Some landmarks in the development of chromatography and electrophoresis are listed in Table 8-5.

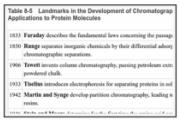

Table 8-5

Landmarks in the Development of Chromatography and Electrophoresis and their Applications to Protein Molecules.

Selective Cleavage of a Protein Generates a Distinctive Set of Peptide

Fragments

Although proteins have distinctive molecular weights and isoelectric points, unambiguous identification ultimately depends on determining their amino acid sequences. This can be most easily accomplished by determining the nucleotide sequence of the gene encoding the protein and using the genetic code to deduce the amino acid sequence of the protein, as discussed later in this chapter. It can also be done by directly analyzing the protein, although the complete amino acid sequences of proteins are rarely determined directly today.

There are several more rapid techniques that are used to reveal crucial information about the identity of purified proteins. For example, simply cleaving the protein into smaller fragments can provide information that helps to characterize the molecule. Proteolytic enzymes and chemical reagents are available that cleave proteins between specific amino acid residues (Table 8-6). The enzyme trypsin, for instance, cuts on the carboxyl side of lysine or arginine residues, whereas the chemical cyanogen bromide cuts peptide bonds next to methionine residues. Because these enzymes and chemicals cleave at relatively few sites, they tend to produce a few relatively large peptides when applied to a purified protein. If such a mixture of peptides is separated by chromatographic or electrophoretic procedures, the resulting pattern, or peptide map, is diagnostic of the protein from which the peptides were generated and is sometimes referred to as the protein's "fingerprint" (Figure 8-19).

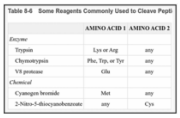

Table 8-6

Some Reagents Commonly Used to Cleave Peptide Bonds in Proteins.

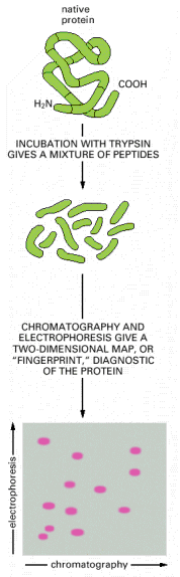

Figure 8-19

Production of a peptide map, or fingerprint, of a protein. Here, the protein was digested with trypsin to generate a mixture of polypeptide fragments, which was then fractionated in two dimensions by electrophoresis and partition chromatography. The latter (more...)

Protein fingerprinting was developed in 1956 to compare normal hemoglobin with the mutant form of the protein found in patients suffering from sickle-cell anemia. A single peptide difference was found and was eventually traced to a single amino acid change, providing the first demonstration that a mutation can change a single amino acid in a protein. Nowadays it is most often used to map the position of posttranslational modifications, such as phosphorylation sites.

Historically, cleaving a protein into a set of smaller peptides was an essential step in determining its amino acid sequence. This was ultimately accomplished through a series of repeated chemical reactions that removed one amino acid at a time from each peptide's N-terminus. After each cycle, the identity of the excised amino acid was determined by chromatographic methods. Now that the complete genome sequences for many organisms are available, mass spectrometry has become the method of choice for identifying proteins and matching each to its corresponding gene, thereby also determining its amino acid sequence as we discuss next.

Mass Spectrometry Can Be Used to Sequence Peptide Fragments and Identify Proteins

Mass spectrometry allows one to determine the precise mass of intact proteins and of peptides derived from them by enzymatic or chemical cleavage. This information can then be used to search genomic databases, in which the masses of all proteins and of all their predicted peptide fragments have been tabulated (Figure 8-20A). An unambiguous match to a particular open reading frame can often be made knowing the mass of only a few peptides derived from a given protein. Mass spectrometric methods are therefore critically important for the field of *proteomics*, the large-scale effort to identify and characterize all of the proteins encoded in an organism's genome, including their posttranslational modifications.

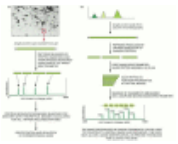

Figure 8-20

Mass-spectrometric approaches to identify proteins and sequence peptides. (A) Mass spectrometry can be used to identify proteins by determining their precise masses, and the masses of peptides derived from them, and using that information to search a (more...)

Mass spectrometry is an enormously sensitive technique that requires very little material. Masses can be obtained with great accuracy, often with an error of less than one part in a million. The most commonly used mass spectrometric method is called *matrix-assisted laser desorption ionization-time-of-flight spectrometry (MALDI-TOF).* In this method, peptides are mixed with an organic acid and then dried onto a metal or ceramic slide. The sample is then blasted with a laser, causing the peptides to become ejected from the slide in the form of an ionized gas in which each molecule carries one or more positive charges. The ionized peptides are then accelerated in an electric field and fly toward a detector. The time it takes them to reach the detector is determined by their mass and their charge: large peptides move more slowly, and more highly charged molecules move more quickly. The precise mass is readily determined by analysis of those peptides with a single charge. MALDI-TOF can even be used to measure the mass of intact proteins as large as 200,000 daltons, which corresponds to a polypeptide about 2000 amino acids in length.

Mass spectrometry is also used to determine the sequence of amino acids of individual peptide fragments. This method is particularly useful when the genome for the organism of interest has not yet been fully sequenced; the partial amino acid sequence obtained in this way can then be used to identify and clone the gene. Peptide sequencing is also important if proteins contain modifications, such as attached carbohydrates, phosphates, or methyl groups. In this case, the precise amino acids that are the sites of modifications can be determined.

To obtain such peptide sequence information, two mass spectrometers are required in tandem. The first separates peptides obtained after digestion of the protein of interest and allows one to zoom in on one peptide at a time. This peptide is then further fragmented by collision with high-energy gas atoms. This method of fragmentation preferentially cleaves the peptide bonds, generating a ladder of fragments, each differing by a single amino acid. The second mass spectrometer then separates these fragments and displays their masses. The amino acid sequence can be deduced from the differences in mass between the peptides (Figure 8-20B). Post-translational modifications are identified when the amino acid to which they are attached show a characteristically increased mass.

To learn more about the structure and function of a protein, one must obtain large amounts of the protein for analysis. This is most often accomplished by using the powerful recombinant DNA technologies discussed next.

Summary

Populations of cells can be analyzed biochemically by disrupting them and fractionating their contents by ultracentrifugation. Further fractionations allow functional cell-free systems to be developed; such systems are required to determine the molecular details of complex cellular processes. Protein synthesis, DNA replication, RNA splicing, the cell cycle, mitosis, and various types of intracellular transport can all be studied in this way. The molecular weight and subunit composition of even very small amounts of a protein can be determined by SDS polyacrylamide-gel electrophoresis. In two-dimensional gel electrophoresis, proteins are resolved as separate spots by isoelectric focusing in one dimension, followed by SDS polyacrylamide-gel electrophoresis in a second dimension. These electrophoretic separations can be applied even to proteins that are normally insoluble in water.

The major proteins in soluble cell extracts can be purified by column chromatography; depending on the type of column matrix, biologically active proteins can be separated on the basis of their molecular weight, hydrophobicity, charge characteristics, or affinity for other molecules. In a typical purification the sample is passed through several different columns in turn—the enriched fractions obtained from one column are applied to the next. Once a protein has been purified to homogeneity, its biological activities can be examined in detail. Using mass spectrometry, the masses of proteins and peptides derived from them can be rapidly determined. With this information one can refer to genome databases to deduce the remaining amino acid sequence of the protein from the nucleotide sequence of its gene.

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