3.6 PROPERTIES OF PROTEINS

Molecular weight

Proteins are macromolecules. They have high molecular weights (e.g., hemoglobin 64500, serum albumin – 69000, γ-globulin – 156000). MW is estimated by molecular sieve chromatography or from sedimentation rate, osmotic pressure, freezing point, light scattering, X-ray diffraction or turbidity. Large molecules give them colloidal properties.

Amphoteric nature and isoelectric pH

Proteins carry many ionizable groups in the charged polar sidechains of amino acid residues. The free α-NH_2 at one end and the free α-COOH group at the other end of the peptide chain may also ionize. Depending on the pH, some of these

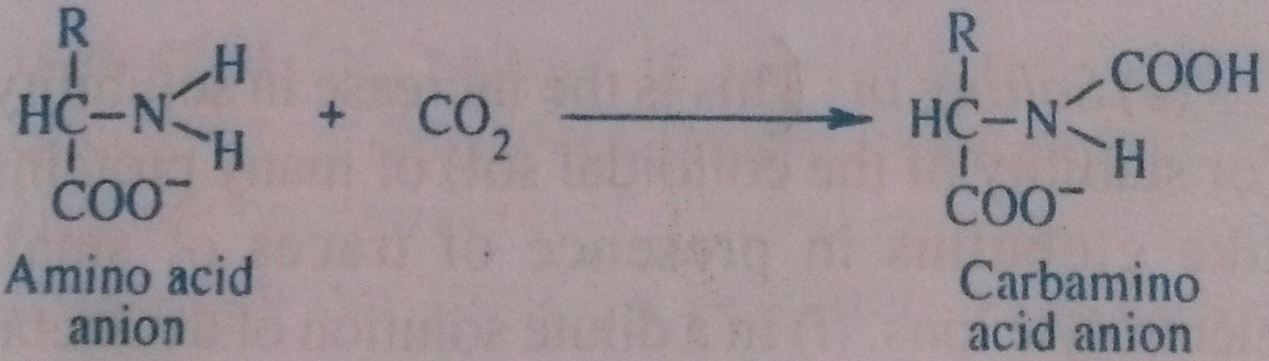

Fig. 3.19. Siegfried's carbamino reaction.

Depending on the pH, the α-NH_2 group and the sidechain groups such as NH_2, imidazole and guanido groups may accept protons (H^+) to form cations while the α-COOH and sidechain COOH groups may donate H^+ to form anions. An amino acid consequently behaves as an *ampholyte*, acting both as donor and acceptor of H^+. At a specific pH called the *isoelectric pH* (pI), the amino acid exists as a *dipolar ion* or *zwitterion* carrying equal numbers of positive and negative charges on its ionizable groups so that the net charge is minimum or zero (Fig. 3.10). So, the zwitterion moves neither to the cathode nor to the anode in an electric field. If the pH of the solution is lower than the pI, the amino acid exists as a cation by accepting H^+ from the acidic solution. If the pH exceeds the pI, the same amino acid forms an anion by donating H^+ to the alkaline solution.

The immobility of protein zwitterions in an electric field is used in separating proteins by *isoelectrophoresis*, depending on their pI values.

The isoelectric pH depends on relative numbers of cationic and anionic sidechains in the amino acid residues, and their ionization exponents (pK). If the basic amino acids carrying cationic sidechains (e.g., lysine or arginine) predominate in a protein, its pI is in the alkaline range. Such a protein forms a cation and behaves as a base at the physiological pH of 7.4, and is called a *basic protein*; e.g., histones and protamines. If the acidic amino acids carrying anionic sidechains (e.g., glutamate or aspartate) predominate in a protein, it has the isoelectric pH in the acidic range. Such a protein forms an anion at the physiological pH of 7.4 and is called an *acidic protein*; e.g., pepsin. As the normal pH of blood plasma is on the alkaline side of the pIs of plasma proteins, the latter exist as anions in the plasma. Thus, the ionic form of a protein in a solution depends on its pI as well as the pH of the solution.

Hydration

Polar groups on the surface of a protein particle bind to the polar molecules of water by hydrogen bonds or ion-dipole interactions. Thus, a relatively immobile shell-like layer of water, called the *solvation layer* or *hydration layer*, is held around the protein particle in aqueous media.

Solubility

Solubility depends on many factors.

(*a*) *Molecular forms and sizes* : Globular proteins such as albumins have higher solubilities than elongated fibrous proteins like keratins; moreover, smaller protein molecules are more soluble than larger ones. Higher solubility of globular proteins results from their compactly folded forms. These folds have many polar amino acids sidechains on the surface exposed to the surrounding water; most nonpolar amino acid sidechains lie hidden in the nonaqueous interior of the folds. Many polar groups on its surface make the globular protein more soluble in water.

(*b*) *pH of the medium* : Protein zwitterions at the isoelectric pH have minimum net charges and so, possess minimum electrostatic repulsive forces which normally prevent the aggregation and precipitation of protein molecules. So, at the isoelectric pH, a protein has the minimum solubility. If dilute acids or alkalies are added to such solutions, the protein forms cations or anions respectively; all its ions now being similarly charged, electrostatic repulsive forces increase between them and raise the solubility.

(*c*) *Hydration of protein* : The relatively immobile hydration layer of water, held around polar protein particles by their surface polar groups, prevents aggregation and precipitation of the particles and stabilizes them in a *lyophilic colloidal sol* in the aqueous medium.

(*d*) *Dielectric constant of solvent* : High dielectric constant of water reduces the electrostatic attraction between charged polar groups of different protein molecules. This decreases their aggregation and helps to keep them in solution. Nonpolar solvents like chloroform and benzene have poor dielectric constants. So, their addition lowers the dielectric constant of an aqueous solution of proteins and decreases their solubility.

(*e*) *Salting in* : This is the increase in solubility (or stability of the colloidal sol) of many proteins like globulins in presence of traces of small electrolyte ions. (*i*) In a dilute solution of a mineral salt, the low concentration of its ions may lead to the preferential adsorption of only a particular type of electrolyte ion on the protein particles; this increases the like charges on protein particles and keeps them dispersed in the aqueous medium. (*ii*) Adsorbed electrolyte ions may interact with surface counterionic groups of protein particles to suppress antagonistic surface charges, lowering thereby mutual electrostatic attractions and aggregations of the particles. But more concentrated mineral salt solutions may precipitate a protein (*salting out*).

Viscosity

Viscosity of a protein solution results from the restriction in free movements of water molecules, due to (*i*) immobilization of significant volumes of solvent in the solvation layer around protein particles, (*ii*) large sizes of the protein particles, (*iii*) their elongated, coiled, three-dimensional forms, and (*iv*) their surface ionic groups. So, higher the molecular weight, elongated shape or concentration of the protein, greater is the viscosity. (*v*) Viscosity is the lowest at the isoelectric pH because protein zwitterions at this pH carry little net charges and suffer minimum resistance against their flow due to electrostatic attractions.

Precipitation

(*a*) *Isoelectric precipitation* : If the pH of a protein solution is adjusted to the pI of that protein, its molecules form zwitterions. These carry minimum net charges and exert minimum electrostatic repulsion. So, the zwitterions aggregate and precipitate without denaturation.

(*b*) *Salting out* : Concentrated solutions of neutral mineral salts such as $MgSO_4$, Na_2SO_4 and $(NH_4)_2SO_4$ may precipitate a protein from its solution. This 'salting out' of protein requires a higher concentration of the mineral salt than what is needed for 'salting in'. Salting out is most effective near the *isoelectric pH* of the protein. (*i*) Higher concentration of mineral ions osmotically removes water from the solvation layer around protein particles. (*ii*) At relatively high concentrations, both cations and anions from the mineral salt may bind to the respective counterionic groups on their protein particles to reduce their surface charges. Both actions enhance aggregation and precipitation of protein particles. Salting out rises with the concentration, valency and charge of that electrolyte ion which bears a charge opposite to the surface ionic groups of protein particles. Indeed, if a protein solution containing a low concentration of mineral salts is frozen, the protein is *salted out* from it because

the formation of pure ice crystals raises the concentration of mineral ions in the remaining solution.

(*c*) *By nonpolar organic solvents* : Nonpolar solvents such as chloroform possess lower dielectric constants than water. Their addition lowers the dielectric constant of an aqueous protein solution. This enhances the electrostatic attraction between the antagonistic ions of proteins and facilitates their aggregation and precipitation.

(*d*) *By heavy negative ions* : On the acidic side of its pI, a protein remains as cation and may then be precipitated by heavy anions like tungstate, trichloroacetate and picrate. To precipitate plasma proteins for estimating blood sugar (*Folin-Wu method*), $^2/_3$N H_2SO_4 and 10% sodium tungstate are added to blood. H_2SO_4 renders the medium far more acidic than the isoelectric pHs of plasma proteins. Protein cations, formed consequently, bind to tungstate anions to get precipitated as protein tungstate. Similarly, for estimating blood sugar by *Dubowski's ortho-toluidine method*, 6% trichloroacetic acid (TCA) solution is added to blood. TCA makes the medium more acidic than the pI of plasma proteins which consequently change to protein cations, bind to trichloroacetate anions and get precipitated.

(*e*) *By heavy positive ions* : On the alkaline side of its pI, a protein exists as anion and may then be precipitated as metal proteinates by heavy metal ions like Zn^{2+}. For example, to precipitate plasma proteins in the *Nelson-Somogyi method* for estimation of blood glucose, $Ba(OH)_2$ and $ZnSO_4$ solutions are mixed with the blood. $Ba(OH)_2$ makes the medium more alkaline than the isoelectric pHs of the plasma proteins; the plasma protein anions, formed consequently, bind to Zn^{2+} to get precipitated as zinc proteinates. *Clinically*, patients poisoned by the oral intake of metal compounds are orally administered milk or eggwhite solution; eggwhite or milk proteins exist as anions as their pI values are lower than the pH

(f) *By specific antibodies* : Antibodies are γ-globulins released on exposure to foreign proteins called *antigens*. Each type of antibody may bind to and precipitate the specific antigen which induces its production. This is utilised clinically in treating diseases like diptheria and tetanus with specific *antisera* containing antibodies against the respective bacterial antigens. Similarly, vaccination with an antigen like an inactivated toxin (toxoid) or dead or attenuated microbes induces the production of specific antibodies which may precipitate the same specific antigen in case of a subsequent infection. A protein may also be separated from a biological sample by *immuno-electrophoresis*, using antisera containing specific antibodies which precipitate that particular protein.

Denaturation and coagulation

Various physical and chemical agents such as heat, X-rays, UV rays, ultrasound, high pressure, violent agitation, concentrated mineral acids, strong alkalies, acetone, alcohols, urea and even dilution may rupture weak noncovalent bonds such as electrostatic, hydrophobic and hydrogen bonds, and van der Waals forces between different groups or amino acid sidechains of a native protein. This unfolds the natural three-dimensional structure of the protein into a disordered and randomly coiled form, bringing about changes in many of its physicochemical properties and biological activities. Such a change in the natural three-dimensional form of a protein is called its *denaturation*.

Effects of denaturation :

(*a*) Denaturation involves changes in the native *secondary, tertiary and quaternary structures* of a protein by breaking weak noncovalent bonds stabilizing those higher orders of structure; but the primary structure, i.e., the amino acid sequence,

of the protein is not changd because covalent peptide bonds are not cleaved in denaturation. The "free energy" of denaturation mostly ranges from about 7 to 17 kcal per mole.

(*b*) Because of no change in the amino acid sequence, *molecular weight* and *osmotic pressure* of the denatured protein do not differ significantly from those of the original native protein.

(*c*) *Isolectric pH*, *levorotation* and *viscosity* are increased.

(*d*) Denaturation decreases the *diffusion coefficient* and *crystallization* of proteins.

(*e*) It also affects the *surface tension*, *absorption spectrum* and *extinction coefficient* of protein solutions.

(*f*) As the uncoiling of the protein exposes many nonpolar amino acid sidechains, *solubility* and *hydration* of the protein decline considerably, reaching their lowest at its isoelectric pH.

(*g*) Large proteins such as albumins, globulins and glutelins are precipitated as floccules (*flocculation*) at the pI on denaturation, but may still be dissolved at higher or lower pHs. However, denaturation by some agents like heat converts the floccules further into a dense solid mass (*coagulum*) which cannot be dissolved even at very low or high pH (*coagulation*).

(*h*) *Biological activities* such as enzymic actions, immunological reactions, contractility, carrier functions, redox activities and informational or signal roles are lost on denaturation.

(*i*) Denaturation enhances the *digestibility* of proteins by uncoiling the peptide chain to expose a larger number of peptide bonds to protease action. This is one of the ways by which gastric HCl enhances protein digestion.

(*j*) If the native form of a protein has a lower free energy than its denatured form, the latter may *spontaneously refold* itself into its original native form on removal of the denaturing agent. But where the native form has a higher free energy

Color reactions

Many color reactions are due to sidechains of specific amino acids and are given by those amino acids and proteins bearing them.

Xanthoproteic reaction : Proteins containing phenolic or indolic amino acids (phenylalanine, tyrosine and tryptophan) give yellow precipitate on being boiled with conc. HNO_3. On adding an alkali, the precipitate turns orange. The precipitation and color changes result from the nitration of aromatic sidechains in proteins and the consequent formation of nitro compounds involving those amino acid residues. Collagens are deficient in these amino acids; so, gelatins derived from collagens cannot give this test.

Millon's reaction : Proteins containing the phenolic amino acid tyrosine give a white precipitate on being mixed with Millon's reagent which contains mercuric nitrate, mercurous nitrate, nitric acid and a small amount of nitrous acid. The reagent causes mercuration of the phenolic sidechain of tyrosine; then, heating

produces a red phenolic complex of mercury, turning the precipitate red. Peptones give a red solution. Gelatins, deficient in tyrosine, do not give this test.

Hopkins-Cole reaction : A protein solution is mixed with Hopkins-Cole reagent (containing glyoxylic acid). Conc. H_2SO_4 is then added gently to that mixture to form a separate lower layer. A viotet or purple ring appears at the junction of the two layers if the protein contains tryptophan.

Adamkiewcz reaction : A protein solution is first mixed with glacial acetic acid which contains glyoxylic acid as impurity. The mixture is then layered over conc. H_2SO_4. A purple ring appears at the junction of the two layers if the protein contains tryptophan.

Acree-Rosenheim reaction : A protein solution is mixed with dilute formaldehyde solution. The mixture is then layered over conc. H_2SO_4. A violet ring appears at the junction of the two layers if the protein contains tryptophan.

Gelatins are deficient in tryptophan. So, they do not respond to Hopkins-Cole, Adamkiewcz and Acree-Rosenheim tests.

Sakaguchi reaction : Proteins containing arginine give this reaction. On boiling the protein solution with Sakaguchi's reagent containing sodium hypochlorite and α-naphthol, a red color is produced.

Pauly reaction : This reaction is positive for proteins containing either tyrosine or histidine. On treating such proteins with diazotized sulfanilic acid in alkaline solution, a red color is produced by a coupling reaction.

Sullivan reaction : On heating a protein with sodium 1,2-naphthoquinone 4-sulfonate and $Na_2S_2O_4$ in an alkaline solution, a red color appears if the protein contains cysteine or cystine.

Nitroprusside reaction : If the solution of a cysteine-containing protein is treated with sodium nitroprusside in dilute NH_4OH solution, a red color develops.