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FORMULATION CONCERNS OF PROTEIN DRUGS

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I. INTRODUCTION

Due to the advances in genetic engineering and biotechnology, peptides/proteins have become very important therapeutic agents. The clinical applications extend to the management of cardiovascular diseases, dwarfism, diabetes, ovulation inducement, digestive disorders, AIDS, cancers, and wound healing (Blohm *et al.*, 1988; Der Marderosian, 1990). The Pharmaceutical Manufacturers Association reports that 80 genetically engineered drugs and vaccines are in human clinical tests or at the FDA for review (Sterling, 1990). To date, there are nine products approved by FDA as listed in Table 1. Economic forecasts predict a bright future for this new industry.

The structural complexity of proteins offers new challenges for manufacturers whose well-established procedures for formulating conventional chemical entities may not be applicable. New strategies and new analytical methodologies may be involved in preformulation studies and formulation development. Review articles by Wang and Hanson (1988), and Manning *et al.*, (1989) provide an overview of protein formulation development. This review will include more recent data and will focus on the formulation aspects of protein drugs: the basic physicochemical properties of proteins; the degradation pathways; the modes of denaturation that may occur during the manufacture/formulation processes and the available analytical tools to monitor protein characteristics. In discussing these formulation concerns, strategies to prevent protein denaturation/degradation will be outlined when possible.

TABLE 1. Recombinant Biological Products Approved for Marketing in the U.S.

Product	Indication	Company
Erythropoietin	Kidney disease; anemia	Amgen(Epogen) Ortho Biotech.(Procrit)
Factor VIII C	Hemophilia	Armour (Monoclate-P)
Granulocyte/Colony stimulating factor	Chemotherapyeffects, AIDS	Amgen (Nupogen) Hoechst (Prokine)
Hepatitis B vaccine	Vaccination	Merck(Recombivax HB) Smith Kline & French (Engerix-B)
Human growth hormone	Dwarfism	Eli Lilly (Humatrope) Genentech (Protropin)
Insulin	Diabetes	Eli Lilly (Humulin)
α-Interferon	Hairy cell leukemia AIDS-related Kaposi's sarcoma	Hoffmann-La Roch (Roferon-A) Schering Plough (Intron A)
Murine monoclonal antibody to CD3 antigen	Kidney transplant rejection	Ortho Pharmaceutical (Orthoclone OKT®3)
Tissue plasminogen activator	Acute myocardial infarction	Genentech

II. PROTEIN CONFORMATION

Proteins are linear condensation polymers of amino acids that are linked by peptide bonds formed by the α-carboxyl and α-amino groups of the adjacent amino acid residues. The structural sequence of these covalent-bonded amino acids is known as the **primary structure**. By varying the permitted backbone bond angles of the primary structure, a polypeptide forms turns, loops, and random coils, i.e., the **secondary structure**. α-helices and β-sheets typically make up about half the conformation of globular protein. **Tertiary structure** refers to the overall three-dimensional architecture of the polypeptide chain and is the native or functional state of the protein *in vivo*. If dimers, trimers, tetramers, or other oligomers of proteins are formed, the phrase '**quaternary structure**' is used. The three-dimensional arrangements of the amino acid residues are generally described as **conformations** (Creighton, 1984; Franks, 1988). While the primary structure involves the high covalent bond energies, the secondary structure

FIGURE 1. Illustration of protein structure levels. Shown are secondary (α-helix shown as an example), tertiary, and quaternary structures (Havel *et al.*, 1989).

relies on weaker, hydrogen bonding interactions. The most common secondary structures in polypeptides are: **right-hand α-helices**; **parallel or antiparallel β-pleated sheets**; and **turns**.

Through the studies of folding/unfolding phenomena, it is recognized that protein conformation are mainly stabilized by electrostatics, hydrogen bonding, van der Waals interactions, and hydrophobic interactions (Dill, 1990; Cohen and Parry, 1986). The covalent bond that significantly contributes to protein conformation is the disulfide bond if protein contains two or more cysteine residues. These covalent cross-links are able to stabilize folded conformations by between 2 and 5 Kcal/mol for each disulfide bond (Creighton, 1988a). Disulfide formation and protein folding are cooperative. It is a mistake to consider disulfide bonds as determinants of protein folded conformation. Upon protein unfolding, disulfide bonds may be maintained, rearranged, or broken reversibly into thiol groups.(Creighton, 1988a; Wetzel, 1987; Manning *et al.*, 1989). After examining 22 proteins, Srinivasan *et al.* (1990) concluded that a large fraction of S-S bridges is involved in linking two segments of protein secondary structures, e.g., connecting helical segments to β-strands.

The interactions that maintain protein tertiary structures are weak (5-20 Kcal/mol), short-range, and sensitive to environmental factors, such as pressure, temperature, pH, ionic strength, and the composition of the solvent medium (Pace, 1990; Dill, 1990). The tertiary structure is therefore labile and easily disrupted.

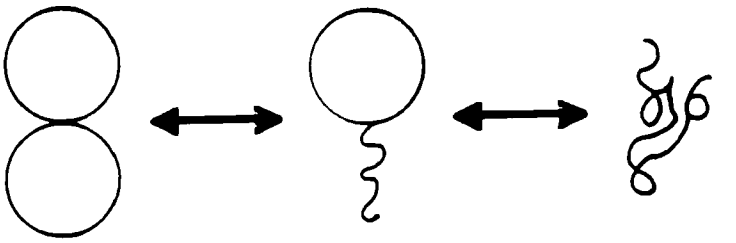


FIGURE 2. Simplified Folding Model (Matthews, 1990).

The conformation of crystalline protein, as determined by X-ray crystallography, is generally retained when the protein is in solution. In this conformation, protein is said to be in its *native* or *folded* state. When the tertiary or secondary structure is destroyed, protein is *denatured* and in its *unfolded* state. If rules of folding/unfolding were fully understood, a denatured protein could be re-constructed back to its native state by controlling its environment. Experimental elucidation of the folding mechanisms has been difficult since the interactions involved are complex and usually cooperative with other mechanism such as disulfide formation and ligand binding. The following statements regarding folding, however, are generally agreed upon and illustrated in Figure 2 (Franks, 1988; Creighton, 1988b; King, 1989; Van Brunt, 1989). (1) Protein can either exist in the native (N) state or in the unfolded (U) state with no intermediate state present. (2) The so called *'partially folded'* proteins or *'intermediates'* are proteins with incorrect folding, or with various domains in either N or U state. (3) These *'intermediate'* species do not accumulate and are difficult to isolate during folding/unfolding.

Through the studies of protein folding/unfolding mechanisms, interactions between proteins and their environment have been better understood. Protein conformation is described, in general, as follows: charged amino residues are located preferentially at the protein surface, where they can interact with water or polar solvent molecules; residues in the interior are close-packed, with the solvent essentially excluded. Upon unfolding, these hydrophobic domains are then exposed to solvents. The hydrophobic groups in the protein interior are a major source of entropic stabilization.

III. PROTEIN DEGRADATION

For a protein molecule to retain its biological functions, it usually must maintain its conformation, i.e., protein must be in the native state. Due to the hierarchical nature of protein structure, it is very rare that one single mechanism of degradation is involved. Rather, a striking feature of protein degradation is its multiplicity. Both physical and chemical reactions are involved in protein degradation.

Proteins can be degraded by disturbing the physical and chemical forces that maintain their conformational stability. If protein degradation is caused by changes in the non-covalent interactions such as hydrogen bonding, electrostatic forces, and hydrophobic interactions, the phase *'protein denaturation'* is generally used. Chemical degradation involves the breakage or formation of covalent bonds. Denaturation may be reversible whereas chemical degradation is not.

A. Non-covalent Denaturation Pathways

The physical properties of a protein do not change in a continuous manner as the environment is altered, e. g., by changing the temperature, pH, or pressure. Instead, they generally exhibit little or no change until a point is reached where there is a dramatic change in physical properties and, probably, a loss of biological function. Thus, an *'all-or-none* ' law seems to control the denaturation process. The unfolded proteins, however, can refold back to its native state if the environment becomes favorable. If denaturation is not reversed, these conformational changes frequently result in aggregation and/or chemical degradation which leads to loss of biological activity, particulate formation, and precipitation (Mitraki and King, 1989).

Through the studies of folding/unfolding phenomena, it is recognized that protein conformation is stabilized by electrostatics, hydrogen bonding, van der Waals interactions, and hydrophobic interactions (Dill, 1990; Cohen and Parry, 1986). Instead of a theoretical discussion of all the forces involved in protein stability, this review focuses on the physical interactions that concern a formulator most, i.e., electrostatic forces, hydrogen bonding and protein hydration, and hydrophobic interactions.

(1) Electrostatic Forces

Only those amino acids with acidic or basic side chains will be actively involved in such interactions. They are Asp, Glu, His, Cys, Tyr, Lys, and Arg. As discussed before, a majority of these ionizable residues are located on the protein surface. Electrostatic forces, also called salt bridges, are present intramolecularly as well as intermolecularly. Since protein is a polyelectrolyte, a pH titration of a protein provides a useful fingerprint of the number of ionic groups that are accessible to the solvent medium. But the end point of a titration curve depends on the ionic strength which reflects ion binding or shielding effects on electrostatic interactions (Matthew and Gurd, 1986).

Proteins are generally least prone to denaturation by temperature and pH at their isoelectric point (pI). pI of a protein is the unique pH at which the net charge of all the ionizable species is zero. At the extremes of the pH scale, the net charge on the protein increases and the resultant charge repulsion will tend to unfold the protein (Stigter and Dill, 1990). For some proteins, however, the pH of maximum stability does not coincide with the isoelectric pH due to the buried charged groups in the hydrophobic core of the protein. An example is T4 lysozyme (pI>10, yet most stable near pH 5) (Anderson *et al.*, 1990).

Thus, an environment conducive to the maintenance of electrostatic interactions should enhance protein stability. A single salt bridge between Asp70 and His31 of T4 lysozyme contributes 3-5 Kcal/mol to the folding free energy (Anderson *et al.*, 1990). It has also been shown that enzymes of some thermophile bacteria owe their extra stability mostly to additional salt bridges (Perutz, 1978).

(2) Hydrogen Bonds and Protein Hydration

The conformational stability of almost all naturally occurring globular proteins is in the range of 5-20 Kcal/mol. This is equivalent to only 3-5 hydrogen bonds. In native proteins, hydrogen bonds are abundant in the secondary structures and are responsible for the formation of such structures. It is unlikely that the breakage of 3 among 150 hydrogen bonds (for a hypothetical protein of 100 amino acids) could unfold the protein. In addition, mutation studies and thermodynamic studies of solvent denaturation indicate that intramolecular

hydrogen bonds, though important for protein stability, are not the dominant factor in maintaining conformational integrity (Creighton, 1984; Franks, 1988; Dill, 1990).

Among the phenomena involving hydrogen bonding, hydration of protein plays an important role in maintaining the conformational stability. Indeed, all biological activities of proteins are performed in aqueous medium, and crystalline proteins can contain almost 40% water. The positions of these so called *'bound water molecules'* have been identified in chymotrysin, papain, and collagen (Franks, 1988; Perutz, 1978). Rupley and Careri (1991) have recently reviewed in detail the effects of protein hydration on its biological functions, thermodynamic properties, spectroscopic activities, and protein conformation.

These *'bound water molecules'* affect the protein conformation. By using NMR, calorimetry, and IR techniques, thermodynamic properties of hemoglobin and lysozyme changed in accordance with the degree of hydration (Colombo and Sanches, 1990; Rupley *et al.*, 1983; Rupley and Careri, 1991). Three major stages of lysozyme hydration were accordingly identified. Hydration began with charged groups on the surface, then polar surface groups, and finally a monolayer of water covered the surface. Enzymatic activity was not observed until the polar surface groups of the lysozyme were fully hydrated. Stability of globular protein is strongly related to its degree of hydration. The water content (per g protein) of some proteins will stabilize them against heating at 100° C; e.g., 8% for myoglobin, 11% for hemoglobin, and 15% for lysozyme (Franks, 1988). The control of protein hydration is extremely important in developing lyophilization processes.

(3) Hydrophobic Interactions

Hydrophobic interactions are often considered as the driving force for protein denaturation. Many reactions such as ion-specific denaturation (salting in/salting out), aggregation, thermal denaturation are the results of changes in hydrophobic interactions (Creighton, 1984; Franks, 1988; King, 1989). Proteins exhibit the following characteristics due to these types of interactions: (1) Protein stability not only decreases at high temperatures but also may decrease at low temperatures. (2) For most of globular proteins, nonpolar residues are sequestered into a core where they largely avoid contact with water. (3) Protein stability is

affected by different salt species in the same rank order as the lyotropic (Hofmeister) series.

Hydrophobic interactions also play a significant role in protein aggregation, a common pathway of protein denaturation. The partially or fully unfolded protein exposes the interior hydrophobic region to the solvent, usually water. The insertion of such a hydrophobic material into aqueous solution is thermodynamically unfavorable. The decrease in entropy from structuring water molecules around the hydrophobic region forces the denatured proteins to aggregate, primarily through interaction of the exposed hydrophobic regions. Thus, aggregate formation can lead to a decrease in the apparent solubility of the protein (Franks, 1988, Whitaker and Feeney, 1982).

Protein aggregation can be observed *in vivo*. When cloning genes of mammalian origin into high-expression systems, microcrystalline granules or amorphous aggregates were observed in the cytoplasm of the cells. These so-called *inclusion bodies* were observed in cases involving human complement C_{3a}, interferon γ, interleukin-1α, interleukin-1β, angiogenin, renin, and urokinase (Hammond *et al.*, 1991). Mitraki and King (1989) proposed the following mechanisms in Figure 3 for the formation of inclusion bodies.

As illustrated in Figure 3, aggregation can be a side reaction of protein oligomerization, i.e., the assembly of quaternary structure. The major distinction is that aggregates cannot form native protein by simple dilution while oligomeric protein can (Mitraki and King, 1989; Jaenicke and Rudolph, 1986). Thus, oligomeric proteins consist of native protein monomers and the term *'aggregation'* is used when denaturation may have occurred. The processes of oligomerization or aggregate formation are strongly dependent on environmental conditions such as temperature, pH, ionic strength, protein concentration, and the presence of a ligand.

It appears that the most effective way to avoid aggregation is to prevent the generation of partially folded intermediates, i.e., to maintain protein in the native state or to promote protein oligomerization. However, the formation of aggregates or inclusion bodies can be advantageous during protein purification. Inorganic salts and organic solvents are routinely used to form protein aggregates in the early

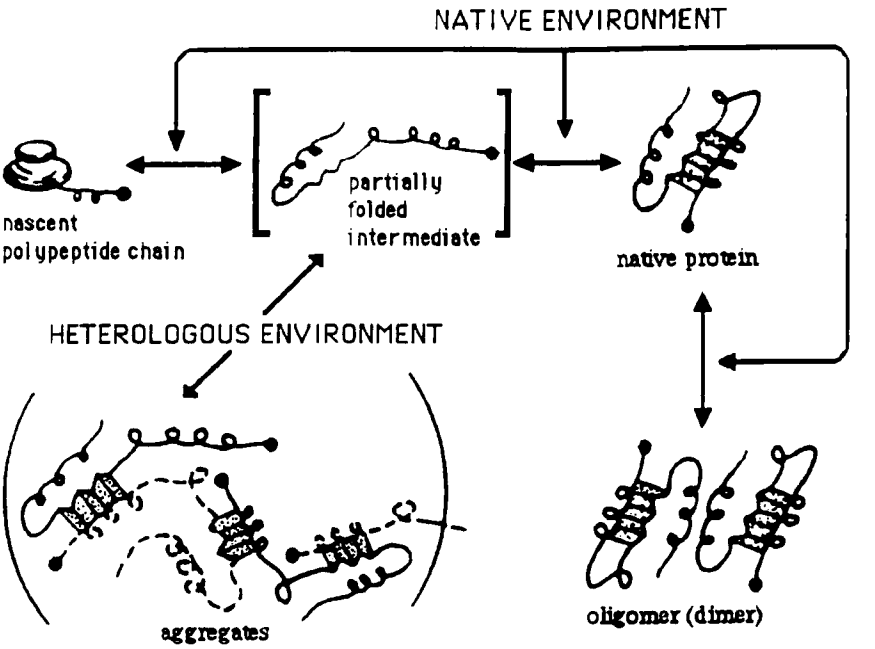
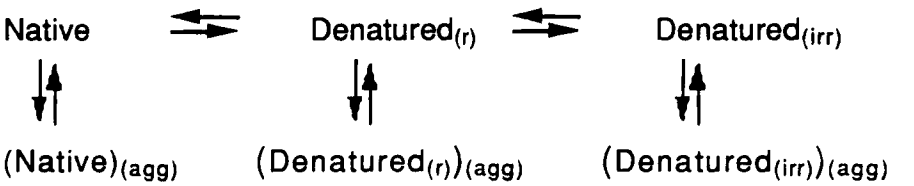


FIGURE 3. In vivo hypothetical folding and maturation pathways of an oligomeric protein (Mitraki and King, 1989).

stages of purification. Dissolution of aggregates by strong denaturants and subsequent dilution (or dialysis) can re-generate these aggregates back to their native state. Denaturants such as urea and guanidine HCl are often used in these re-naturation processes (Utsumi *et al.*, 1989; Jaenicke and Rudolph, 1986; Kohno *et al.*, 1990). Sodium dodecyl sulfate is discouraged in such an application due to the difficulty in removing it. Aggregate formation can be induced by moisture (Hoshi and Yamauchi, 1983; Liu *et al.*, 1991), inorganic salts (Franks, 1988), organic solvent (Przybycien and Bailey, 1989), high pressure (Wong and Heremans, 1988), high temperature (Mulkerrin and Wetzel, 1989), and surface adsorption (Lenk *et al.*, 1989). Przybycien and Bailey (1989) proposed the following detailed mechanisms for aggregate formation (r=reversible, irr=irreversible, agg=aggregate).



The proposed mechanism can explain the following observations: (1) Na_2SO_4 induced α-chymotrpsin aggregates could be dissolved in glycine buffer and have 100% activity (Przybycien and Bailey, 1989); (2) after treatment with denaturant and dialysis, only partial activity of trypotophanase was restored (Mitraki and King, 1989) while full activity was observed for interferon-β1 (Utsumi *et al.*, 1989).

From the above discussion, the best strategy to avoid aggregation is to inhibit the formation of denatured protein and/or to encourage the formation of oligomers or $(Native)_{(agg)}$. The use of low protein concentration and the addition of suitable ligands have also been successfully used to increase protein stability. Bauer *et al.* (1990) demonstrated that ADP-ribosyl-transferase is inactive at 2 μM, but active at 30 nM. Citrate ions induce the oligomer form of acetyl CoA carboxylase and activate the enzyme (Frieden, 1989). In the presence of zinc ions, a more stable form of insulin, the hexamer, is formed (Derewenda *et al.*, 1989).

B. Covalent Degradation Pathways

A variety of chemical reactions is known to affect proteins. Usually the conformational denaturation of proteins accelerate these chemical reactions. The major ones are deamidation (with isomerization or transpeptidation sometimes being a side-chain reaction), disulfide bond exchange, oxidation, β-elimination, and hydrolysis. These chemical reactions and their mechanisms are well documented (Geigert, 1989; Creighton, 1989). Detailed information regarding the covalent degradation mechanisms of protein drugs can be found in a review by Manning *et al.* (1989).

For proteins containing two or more cysteine residues, the most concerned chemical stability problem is to maintain the disulfide bonds. Under some conditions, disulfide can be very labile. Reducing agents such as dithiothreitol and mercaptoethanol will cleave the disulfide reversibly to individual thiol groups. Oxidants can cleave disulfides to the sulfinic (RSO_2H) and sulfonic (RSO_3H) acids. Various nucleophiles (e.g., cyanide, sulfites, and hydroxide ions) and electrophiles (e.g., protons and metal ions such as Hg^{2+} and Ag^{+}) are able to break disulfides reversibly (Creighton, 1988a; Wetzel, 1987; Srinivasan *et al.*, 1990). The thiol groups that generated can then produce disulfide exchange. Such

reactions can be very significant in proteins at elevated temperatures or extremes of pH and refolding usually does not occur (Creighton, 1988a; Wetzel, 1987;.Manning *et al.*, 1989).

Examples of aggregates formed through disulfide exchange are plentiful. The precipitation of interleukin-1β solution upon storage is the result of the intermolecular disulfide bond formation of the cysteine residue in the hydrophobic region (i.e., protein interior) (Gu *et al.*, 1991). The moisture-induced aggregation of lyophilized proteins such as ovalbumin, glucose oxidase, BSA, and β-lactoglobulin are the results of disulfide exchange in combination with conformational changes (Liu *et al.*, 1991).

Table 2 summarizes some of these chemical reactions and the conditions favorable for their occurrences. The rates of these reactions are also dependent on the three dimensional conformation of protein molecules. Wearne and Creighton (1989) have demonstrated that unfolded ribonuclease A degraded 30-fold faster than the native protein.

IV. DENATURATION INDUCED DURING FORMULATION DEVELOPMENT

Changes in temperature, pH, salt, pressure, shear, and interfaces are likely to be involved when manufacturing a protein formulation. By understanding how these variables affect protein stability, manipulation to minimize protein degradation may be achievable. Due to the multiplicity of protein degradation mechanisms, it is rare that only one degradation pathway is involved.

A. Thermal Denaturation

A change in temperature, higher or lower, may cause proteins to lose conformational stability and, consequently, biological functions. Different degradation pathways are involved in heat and cold treatment (Franks, 1988; Privalov, 1989). Irreversible thermal denaturation of many proteins is thought to proceed via the preliminary unfolding of the native structure to an unfolded state (Mulkerrin and Wetzel, 1989).

Thermo-stability in proteins is a result of several forces such as electrostatic effects, hydrophobic interactions and hydrogen bonds. Through studies of proteins

TABLE 2. Summary of Chemical Degradation Reactions of Proteins.

Chemical Reaction	Amino acid involved	Degradation conditions	Examples of Proteins
Deamidation,	Asn(→Asp), Gln(→Glu)	amino acid sequence (esp. Asn-Gly), neutral or alkaline pH, high temperature, buffer species, high ionic strength	Hemoglobulin (Asn-Gly) Human growth hormone (Asn-Asp)[1-3]
Oxidation	Met(→Met sulfoxide or Met sulfone)	acidic pH, oxygen, oxidizing agents	Corticortropin (Met) Calcitonin (Met) Human Growth hormone (Met)[3]
	Cys(→Sulfenic acid, disulfide, sulfinic acid, sulfonic acid)	metal ions, neutral or alkaline pH, high temperature, oxygen, oxidizing agents	α-amylase from *Bacillus* [4]
Disulfide bond exchange	Cys	neutral and alkaline pH	Interleukin-2, Interferon-β[3,4]
Hydrolysis	Asp (esp. Asp-Pro)	acidic pH	Lysozyme, Ribonuclease A[5]
Maillard reaction	interaction with dissacharides (browning)	acidic pH	Albumin, Hemoglobin[6]
β-elimination	Cys	alkaline pH, high temperature, metal ions	Lysozyme[5,7]

1: Geiger and Clarke, 1987 2: Stepheson and Clarke, 1989 3: Manning, *et al.*, 1989 4: Creighton, 1988a
5: Volkin and Klibanov, 1989 6: Cerami, 1986 7: Whitaker and Feeney, 1982

in mesophiles and thermophiles, it is suggested that decreased flexibility and increased hydrophobicity in α-helical regions enhance thermo-stability (Menendez-Arias and Argos, 1989; Pakula and Sauer, 1989; Nguyen *et al.* , 1989).

(1) Heat Denaturation

Heat denaturation of proteins is an important consideration in formulation development since elevated temperatures are used in accelerated stability studies. As the temperature increases, all proteins eventually lose their biological activity. This thermal inactivation may be either reversible or irreversible. Reversible thermal denaturation of proteins is caused by temperature-induced conformational transitions in the protein molecules, whereas irreversible thermal denaturation usually involves chemical reactions which occur especially at extremes of pH (Geigert, 1989; Ahern and Klibanov, 1985).

The temperature that limits reversibility depends on the nature of the protein, pH, buffering salts, and ionic strength of the medium. For human interferon γ, this temperature is 30.5° C at pH 4.75 and 41.8° C at pH 6.25 in the presence of 0.25 M guanidine HCl (Mulkerrin and Wetzel, 1989). Another example is the reversibility of the structural changes of bovine serum albumin (BSA) in the temperature range of 2-65° C at pH 2.8, while this temperature range narrows to 2-45° C at pH 7.0 (Takeda *et al.*, 1989). The rate of thermal degradation can also be influenced by the same parameters. Tomazic and Klibanov (1988a) demonstrated that the T_{50} of *B. licheniformis* α-amylase changed from 270 to 7 minutes after the addition of 1 M KCl at 90° C (pH 6.5).

Heat denaturation induces conformational changes in the secondary structure with an increase in β–sheets accompanied by a decrease in random coils and α-helices (Wong and Heremans, 1988; Takeda *et al.*, 1989). The denatured protein can subsequently form aggregates or lead to further chemical degradations. For γ-interferon, aggregates were formed as a result of heat denaturation (Mulkerrin and Wetzel, 1989). The major chemical reactions involved in thermal degradation are deamidation, racemization, disulfide bond exchange, and β-elimination. The relative incidence of each mechanism depends on the nature of the protein, temperature, pH, and the solutes in the reaction medium (Tomazic and Klibanov, 1988a & b; Mulkerrin and Wetzel, 1989). Table 3 illustrates such a relationship (Ahern and Klibanov, 1985; Zale and Klibanov, 1986).

TABLE 3. Rate constants of irreversible inactivation of Lysozyme at 100° C and Ribonuclease A at 90° C.

Mechanism	Rate of Degradation ($hour^{-1}$)					
	pH 4		pH 6		pH 8	
	Lysozyme	Ribonu-cleas A	Lysozyme	Ribonu-cleas A	Lysozyme	Ribonu-cleas A
Overall	**0.49**	**0.13**	**4.1**	**0.56**	**50**	**23.4**
Deamidation	0.45	0.024	4.1	0.15	18	0.8
Hydrolysis	0.12	0.10	0	0	0	0
Destruction of Cysteine residues	0	-	0	-	6	-
Disulfide bond exchange	-	0	-	0.31	-	19.4
β-elimination	-	0	-	0.054	-	2.8
Formation of incorrect structure	0	-	0	-	32	-

Due to the multiple degradation pathways that may take place in the heat-denatured protein, stability studies at accelerated temperature may not be practical and may be misleading. For interleukin-1β, the difference in degradation mechanisms precludes the prediction of formulation shelf life from accelerated temperature data (Gu *et al.*, 1991); whereas, the inactivation kinetics of α-chymotrypsin, kallikrein, and bromelain conform to the Arrhenius relationship between 30° and 45° C (Yoshioka *et al.*, 1991).

(2) Cold Denaturation

Freezing is the most effective means for the preservation of biological materials. It has long been known, however, that some damage to the structure and functions of various biological materials (tissues, cells, *etc.*) occurs with storage at low temperature. Cold denaturation of lyophilized proteins should be carefully monitored since the lyophilization process involves freezing. Cold denaturation in solution probably occurs as a results of weakened hydrophobic interactions at decreasing temperature. The conformation changes occurring at low temperature have been identified as partial or total destruction of tertiary structure while secondary (hydrogen-bonded) domains are maintained intact (Franks *et al.*, 1988).

Although cold denaturation can be a reversible process, it does occur with irreversible loss of biological activity for some proteins. The main injurious effect of freezing may not be the low temperature, but the concentration effect. The local concentrations of all solutes are very high when ice separates from the mixture as a pure phase (Franks, 1988). The residual activity of lactate dehydrogenase (LDH) after being frozen at -23° C for 24 hours was closely correlated with the solubility of the buffer species (Seguro *et al.*, 1990). For 67 mM phosphate buffer, the residual activity of LDH was 25% in K-K_2PO_4 buffer but <5% activity remained in K-Na_2PO_4 buffer (K_2HPO_4 is very soluble while 1 part of Na_2HPO_4 is soluble in 8 parts of water). A pH shift from pH 8 to 4 was simultaneously observed for both buffers. This relationship between buffer solubility and freeze damages, however, is less apparent at low buffer concentrations. A higher degree of degradation in human growth hormone was not observed by Pikal *et al.* (1991) in the highest Na_2HPO_4 concentration used of 3.2 mM. The precipitation of phosphate buffer salts also depends on the cooling rate. Murase and Franks (1989) observed that slow cooling (0.62° C/min) promoted the precipitation of Na_2HPO_4 at 0.2 M. The relationship between freeze denaturation and incubation temperature is not clear at present. Ovalbumin is shown to have the greatest conformational changes when frozen for 1 hour at -40° C, but with minor changes at -10°, -80°, and -196° C (Koseki *et al.*, 1990).

Some substances such as carbohydrates, some amino acids, and polyhydric alcohols, are both effective stabilizers and cryoprotectants. The mechanism of such stabilization in solution and during freezing involves solutes being excluded from the protein molecules. Thus, protein is preferentially hydrated by water molecules. Due to this exclusion effect, these stabilizers enhance self-association of proteins, protein-protein interactions, interactions of proteins with other ligands, and decrease protein solubility. In addition, they might enhance the binding of proteins to glass surfaces or lead to protein precipitation.

Although the exclusion effect is the predominant mechanism in these stabilizers, these solutes can also bind to proteins through hydrophobic interactions, hydrogen bonding, or electrostatic interactions. The net interaction observed between proteins and stabilizers is the balance between binding to the protein and exclusion (Timasheff and Arakawa, 1989; Arakawa *et al.*, 1990; Arakawa *et al.*, 1991; Geisow, 1991; Pikal, M. J., 1990; Carpenter and Crowe, 1989). Timasheff

TABLE 4. Nature of co-solvent interactions with proteins.

Co-solvent	Observation of exclusion mechanism	Observation of binding mechanism
Class I - independent of conditions		
Sugars	surface tension increase (exclusion in effect)	weak binding
Some amino acids (glycine, alanine, glutamic and aspartic acids)	surface tension increase (exclusion in effect)	weak binding
Salting-out salts (Na_2SO_4, NaCl, $MgSO_4$)	surface tension increase (exclusion in effect)	weak binding
Glycerol	solvophobicity (exclusion in effect)	to polar regions
Class II - dependent on conditions		
Weakly acting salts ($MgCl_2$, NaCl, $MgSO_4$)	surface tension increase (exclusion in effect)	to charged groups or peptide bonds
Arginine-HCl, Lysine-HCl	surface tension increase (exclusion in effect)	to peptide bonds and negative charges
Valine (possibly other non-polar amino acids)	surface tension increase (exclusion in effect)	to hydrophobic regions
Class III - destabilizer under unfolding conditions		
Polyethylene glycol (PEG)	steric exclusion	to hydrophobic regions
2-Methyl-2,4-pentanediol (MPD)	repulsion from charges	to hydrophobic regions

and Arakawa (1989) classified these cosolvents into three categories as listed in Table 4.

In choosing a stabilizing co-solvent, compounds of class I should be considered first. Since this type of cryoprotectants stabilize proteins via preferential exclusion, concentrations exceeding 0.2 M are usually required. However, compounds in class II can stabilize proteins in low concentrations due to specific binding. The combination of certain divalent cations and organic cosolvents may provide synergistic protection. For example, full activity of phosphofructokinase is recovered when the enzyme is frozen in the presence of 0.6 mM $ZnSO_4$ and 5 mM trehalose. With either additive alone, no enzyme activity is measurable after freeze-thawing (Arakawa *et al.*, 1991).

In addition to the concentration effect, the degree of supercooling and ice crystal formation are closely related to freeze damage (Heinz *et al.*, 1990; Seguro *et al.*, 1990; Franks, 1990). Seguro *et al.* (1990) demonstrated that the cryoprotection effect of monosodium glutamate (MSG) and lysine hydrochloride

(Lys-HCl) on lactate dehydrogenase is a function of freezing point depression and supercooling phenomenon at a cooling rate of 2° C/min. The degree of freezing unpredictable and sample-dependent event if the sample size is larger than droplets (Angell, 1979). The degree of supercooling depends on nature of the adjacent phase (e.g., container surface), purity of the sample and cooling mode (Gutmann *et al.*, 1989). More experimental data are needed before a conclusion on the relationship between cryoprotection and supercooling can be made.

By understanding the nature of freeze injuries in proteins, the following strategies can be used for prevention; (1) Avoid using Na_2HPO_4 in the buffer system, especially at high concentrations. (2) Use a higher concentration of protein. (3) Use cryoprotectants such as carbohydrates, amino acids, or polyhydric alcohols. (4) Carefully control the freezing process, i.e., cooling rate and incubation temperature.

B. pH Denaturation

As stated previously, proteins are generally most stable and least soluble at their isoelectric point (pI) due to electrostatic interactions. For pharmaceutical protein, solubility of proteins is rarely a challenge since product concentration is usually in the μg-mg range. Since buried non-titratable amino residues may contribute to protein stability significantly, it is necessary to establish a conventional pH-rate profile for protein drugs. The pH-rate profile of a protein is usually established in the presence of a denaturant such as guanidine HCl or high temperature. Outside the optimal pH range, the proteins will denature and may lead to aggregation and/or chemical degradations such as deamidation, β-elimination, disulfide exchange, and oxidation (Table 2) (Franks, 1988, Whitaker and Feeney, 1982).

Since a protein formulation may be a complex medium, the effects of pH on additives should also be considered. Higher pH usually fragments monosaccharides whereas lower pH will promote Maillard reactions. Low pH can also hydrolyze some dissacharides. Higher pH will also promote the oxidation of unsaturated fatty acid and phenols (used as preservative). The catalytic ability of metal ions such as copper increases at higher pH (Cerami, 1986; Townsend and DeLuca, 1988; Whitaker and Feeney, 1982). The chelating power of carboxylate-containing chelating agents (e.g., Na_2EDTA) also increases with increasing pH.

point depression and supercooling increased when a better cryoprotection was offered by Lys-HCl. The same observation is made in studies of the antifreeze effects of glycoproteins present in cold-water fishes (Harrison *et al.*, 1987; Raymond *et al.*, 1989). However, supercooling has been reported to be a highly

C. Salt Denaturation

The most significant effect of salts on proteins is the change in protein solubility; 'salting-in' refers to an increase whereas 'salting-out' refers to a decrease in solubility. In the lyotropic series (also known as Hofmeister series), the solubility of proteins varies dramatically with the nature of the salt, but independent of the nature of the protein (Franks, 1988; Arakawa and Timasheff, 1985). This type of non-specific interaction occurs only at high salt concentration, i.e., $\geq$0.15 M.

Salting out Neutral Salting in

$$SO_4^- > HPO_4^{2-} > OAc^- > F^- > \text{Citrate} > Cl^- < NO_3^- < I^- < CNS^- < ClO_4^-$$

$$Li^+ > Na^+ < K^+ < Rb^+ < NH_4^+$$

$$Me_4N^+ < Et_4N^+ < Pr_4N^+ << Bu_4N^+$$

The addition of these inorganic salts (at $\geq$0.15 M) can drastically change protein solubility. This implies that protein conformation has been changed and aggregates are consequently formed, as described previously. These ions also affect the stability of the native states of proteins and their degree of aggregation. The addition of 0.15 M NaCl in human interferon-β1 leads to a 60% loss of antiviral activity (Utsumi *et al.*, 1989). Human growth hormone in the presence of 0.1% NaCl (W/V) resulted in severe aggregation and precipitation during the freeze-drying process as well as acceleration of oxidation and/or deamidation (Pikal *et al.*, 1991). Salting-out ions enhance the stability of native state, whereas salting-in ions give rise to denaturation (Franks, 1988; Przybacien and Bailey, 1989). Using α-chymotrypsin and lysozyme as models, the denatured aggregates induced by inorganic salts such as KSCN, KBr, and NaBr have increased β-sheet contents and decreased α-helix contents whereas aggregates induced by Na_2SO_4 retained the secondary structures. The degree of such changes in secondary structure correlates quantitatively with the loss of biological activity (Przybacien and Bailey, 1989). Thermally induced globular protein gels of α-chymotrypsin, bovine serum albumin, insulin, glucagon and β-lactoglobin were also found to have increased

sheet and decreased helix contents. It appears that the formation of β-sheet strands may be a fundamental phenomenon in self-associating protein systems.

The mechanisms by which a salt influences the physical properties such as conformation and solubility are not yet completely understood. It is suggested that two mechanisms are involved: one deals with the preferential salt hydration and the other with specific ion binding. If preferential hydration is the dominant force, the interactions between proteins and salts are independent of the solution conditions, i.e., pH or the presence of other denaturants. However, interactions that involve specific ion binding are sensitive to solution environment (Arakawa *et al.*, 1990). As in the case of cryoprotection, higher salt concentrations are needed in preferential hydration whereas low concentrations of salts may be sufficient to initiate ion specific binding. For urinary plasminogen activator, the concentration that exerts 50% activity inhibition through specific binding is 32 mM for Ca^{2+}, 330 mM for Mg^{2+}, and 7 mM for Mn^{2+} (Stack *et al.*, 1991).

The interactions between salts and proteins have been widely applied in protein purification, e.g., salting-out, hydrophobic interaction chromatography (HIC) and metal-chelate chromatography (Hammond *et al.*, 1991; Porath, 1990). The salt selected for a purification process should precipitate the largest fraction of protein with minimal loss of conformation stability.

D. Pressure and Shear Denaturation

Proteins such as albumin, factor VIII, and insulin have been formulated by the drug industry for about thirty years and are routinely sterile filtered. The application of pressure to increase the rate of filtration is also used in manufacturing processes. Therefore, three kinds of protein denaturation may occur: (1) denaturation caused by pressure; (2) denaturation caused by shearing (i.e., pushing protein through small orifices of the filter); and (3) denaturation caused by adsorption to the filter (i.e., surface denaturation).

Limited information is available on the high-pressure denaturation of proteins. Present data indicate that irreversible denaturation of protein is seen only at extremely high pressure. Wong and Heremans (1988) observed that denaturation of chymotrypsinogen in aqueous solutions began at 3.7 Kbar with a pressurization rate of ≈4.5 Kbar/hour and 5.5 Kbar with a rate of ≈0.3 Kbar/hour. Therefore, pressure denaturation depends also on the rate of pressurization. Ca^{2+}-ATPase of

sarcoplasmic reticulum is irreversibly inactivated under 1.5-2.0 Kbar pressure for 30-60 minutes in a Ca^{2+}-free medium (Buchet *et al.*, 1990). Using glucose isomerase as a model protein, Visuri *et al.* (1990) used pressure up to 2 Kbar to obtain small and uniform protein crystals without enzyme inactivation.

Using infrared spectroscopy, it has been confirmed that high pressure enhances hydrogen bonding and restricts molecular fluctuations (Wong and Heremans, 1988; Buchet *et al.*, 1990). Structural changes are consistent in lysozyme, chymotrypsinogen, and Ca^{2+}-ATPase with a conversion of α-helix into β-sheet structures. Some gel-type aggregates were observed after irreversible denaturation. These conformational changes are consistent with those observed by Przybacien and Bailey (1989) that increased β-sheet strands are a common feature in self-associating protein systems. Some protection against pressure-induced inactivation can be provided by sucrose, glycerol, and KCl at relatively high concentrations. Through the specific binding, 5 mM of vanadate or 2-20 mM of Ca^{2+} can prevent the irreversible pressure-induced inactivation of Ca^{2+}-ATPase (Buchet *et al.*, 1990).

Shear denaturation of proteins such as fibrinogen, catalase, rennet, and carboxypeptidase was observed with a loss of activity (Charm and Wong, 1970a & b). This shear denaturation is a function of both shear rate and exposure time. When [shear rate (sec^{-1}) X exposure time (sec)] is less than 10^4, the denaturation was reversible with little or no inactivation. Shear denaturation could occur in mixing, flow in tubes, sterile filtration and ultrafiltration. Both pressure-induced and shear-induced denaturation are time-dependent. It is unlikely that proteins would be denatured by high shear rate and pressure during sterile filtration since the exposure time is short and the pressure used is moderate (< 1 Kbar). A study by Truskey *et al.* (1987) on bovine alkaline phosphatase, bovine pancreatic zinc insulin, and human immunoglobulin G indicates that no conformational change upon filtration was observed. Hsu *et al.* (1988) also concluded that shearing energy generated in a 0.22 μm sterile filter was not intensive enough to denature human recombinant growth hormone. A more recent study by Pikal *et al.* (1991), using capillary tubes of different length, also reported no shear-induced denaturation was observed for human growth hormone. Rather, the major concern of sterile filtration is the possible adsorptive loss of proteins (Truskey *et al.*,, 1987; Martin and Manteuffel, 1988; Hsu *et al.*, 1988; Pikal *et al.*, 1991).

E. Surface Denaturation

Since proteins are amphiphilic polyelectrolytes, they exhibit some degree of surface activity, i.e., they adsorb to surfaces. Hence, proteins act as emulsifying/dispersing agents to stabilize systems such as fat in blood/milk, or air bubbles in ice cream. Adsorptive denaturazation processes take place in three main stages: (1) diffusion of the native protein molecules to the interface and their adsorption; (2) uncoiling of the polypeptide chains at the interface (surface denaturation); (3) aggregation of the surface denatured protein into coagulum largely devoid of surface activity (coagulation) (Franks, 1988; Kalischewski and Schugerl, 1979).

For protein drugs, surface denaturation usually occurs at liquid/solid and liquid/air interfaces. Conformational studies of adsorbed proteins indicated a loss of α-helices to β-sheets and unordered structures (Lenk *et al.*, 1989). These structural changes vary with the nature of interfaces and are similar to those observed in protein self-associating systems induced by heat, chemical denaturants, and high pressure.

The most likely sources of surface-induced denaturation in pharmaceutical proteins are the polymer of the membrane/capsule filter, the container, the administration setup, and the agitation that might occur during purification and manufacturing procedures. Under static conditions, the variation in adsorption between proteins and solid surfaces reflects differences in the distribution of charged and hydrophobic regions on the protein molecule's surface and the nature of the solid surfaces. Thus, protein adsorption decreases for all solids in sequence from fibrinogen (the most hydrophobic) to IgG, human serum albumin (HSA), and bovine serum albumin (BSA) (the most hydrophilic) (Absolom *et al.*, 1987). The amount of protein adsorbed is always higher on the more hydrophobic solid surface, i. e., correlated with the wettability (Uyen *et al.*,1990; Wilkins *et al.*, 1989; Pitt, 1987).

Under high shear, the nature of the solid surface (usually a polymer) has a less significant effect on protein adsorption. Sato *et al.* (1983) observed that the morphology of the adsorbed insulin appears independent of the polymer nature. At a shear rate of 420 s^{-1}, the conformational changes in BSA are relatively insensitive to surface structure (Lenk *et al.*, 1989). The amount adsorbed on a polymeric surface increased with increasing shear rate at higher protein concentration but decreased if the concentration was lower (< 0.2 mg/mL) (Uyen *et al.*, 1990).

In general, the surface denaturation/adsorption of proteins depends on the nature of the interface, protein-protein interactions, time, temperature, pH and ionic strength of the medium. The most common strategy to minimize protein adsorption is to increase the protein concentration during filtration or in the dosage form. Other strategies are to modify the solid surface of containers (e.g. siliconization of the glass); to decrease agitation/mixing rate; and to add excipients such as salts, surfactants (of higher surface activity) and other macromolecules (e.g., albumin and gelatin) (Wang and Hanson, 1987; Franks, 1988; Oshima, 1989; Kurtzhals *et al.*, 1988). To prevent the surface denaturation of chymotrypsin, the addition of 0.1 M NaCl and the coating of lecithin or BSA on the container surface were effective (Oshima, 1989). Kurtzhals *et al.* (1988) reported the addition of 0.25% gelatin prevented the adsorption of urokinase to glass. To administer interleukin-1β at a low concentration of 100 ng/mL via a syringe-pump system, the addition of 1% human serum albumin (HSA) was necessary to prevent significant adsorptive loss (the delivered concentration was 80 ng/mL with 1% HSA and 17 ng/mL without) (Visor *et al.*, 1990).

F. Freeze-drying Denaturation

Many protein drugs are provided as freeze-dried products to ensure adequate shelf-life. As listed in Table 5, half the recombinant derived biological products available on the US market are freeze-dried. Since many proteins are inactivated during freeze-drying but not during freeze-thawing, it is obvious that different degradation mechanisms come into play during lyophilization. Through the studies of cryoprotectants, many features of cold temperature and freeze-drying-induced denaturation have been elucidated (Arakawa *et al.*, 1991; Geisow, 1991; Carpenter and Crowe, 1989). Using phosphofructokinase and lysozyme as models, Carpenter and Crowe (1989) demonstrated a clear distinction between freeze-thawing and freeze-drying damage. There was an optimal concentration for stabilizers such as trehalose, lactose, or inositol during freeze-drying but not during freeze-thawing.

During freeze-thawing, the presence of increasing concentrations of carbohydrates leads to increased recovery of activity. It is postulated that cryoprotection is due to preferential exclusion of the stabilizing solute from the surface of the protein. Even at very high concentrations of sugar, there should still

TABLE 5. Formulation of the Recombinant Biological Products Approved for Marketing in the U.S (PDR, 1991).

Product	Dosage Form	Stability	Composition
Epogen (Erythropoietin)	Solution	Product is stable at 2°-8° C within expiration date.	**Each vial** - 2,000-10,000 IU protein with 2.5 mg HSA, 5.8 mg Na citrate, 5,8 mg NaCl, 0.06 mg citric acid.
Monoclate-P	Lyophilized product with diluent	Lyophilized product is stable at 2°-8° C within expiration date or < 30° C for up to 6 months.	**Each vial** - 450 mM NaCl, 2-5 mM $CaCl_2$, 1-2% HSA, 0.8% mannitol, 1.2 mM Histidine.
Recombivax HB (Hepatitis B vaccine)	Suspension	Product is stable at 2°-8° C within expiration date.	**Each mL** - 10 mcg Hepatitis B surface antigen adsorbed onto ≈ 0.5 mg Al provided as $Al(OH)_3$.
Humatrope (Human growth hormone)	Lyophilized product with diluent (0.3% m-cresol and 1.7% glycerin)	Stable for 14 days at 2°-8° C after reconstitution.	**Each vial** - 5mg protein with 1.13 mg Na_2HPO_4, 25 mg glycine, 25 mg mannitol.
Protropin (Human growth hormone)	Lyophilized product with bacteriostatic water for injection as diluent	Stable for 14 days at 2°-8° C after reconstitution.	**5 mg vial** - 1.6 mg Na_2HPO_4, 0.1 mg NaH_2PO_4 (pH 7), and 40 mg mannitol. **10 mg vial** - 3.2mg Na_2HPO_4, 0.2 mg NaH_2PO_4 (pH 7), and 80 mg mannitol.
Humulin family (Insulin)	Solution or suspension	Product is stable within expiration date, preferable under refrigeration.	**Each vial** - with or without Na_2HPO_4 as the buffer.
Intron A (Interferon-α-2β)	Lyophilized product with bacteriostatic water for injection as diluent	Stable for 1 month at 2°-8° C after reconstitution.	**Each vial** - 5mg protein with 9 mg Na_2HPO_4, 2.25 mg NaH_2PO_4 (pH 7), 43 mg NaCl, and 1.0 mg Tween 80.
Roferon-A (Interferon-α-2a)	Solution (3,6, 36 million IU per vial) or lyophilized product (18 million IU per vial)	Stable for 1 month at 2°-8° C after reconstitution.	**Each mL** - 3, 6,36 million IU protein with 9 mg NaCl, 5 mg HSA, and 3 mg phenol
Orthoclone OKT®3 (murine MonoAb-CD3)	Solution	Product is stable at 2°-8° C within expiration date.	**Each 5 mL** - 0.015-0.24 mg protein with 20 mg glycine, 2.3 mg Na_2HPO_4, 0.55 mg NaH_2PO_4, and 1.0 mg HSA.
Activase (Tissue Plasminogen Activator)	Lyophilized product with water for injection as diluent	Stable for up to 8 hours at room temperature after reconstitution. Light sensitive product.	**20 mg vial** - 0.7 g L-Arg, 0.2 g H_3PO_4, <1.6 mg polysorbate 80 **50 mg vial** - 1.7 g L-Arg, 0.5 g H_3PO_4, <4 mg polysorbate 80

be preferential exclusion and hence cryoprotection. Using IR spectroscopy, it was demonstrated that, when carbohydrates are dried in the presence of protein, intermolecular H-bonding of carbohydrate molecules decreased and H-bonds were formed between carbohydrate and protein molecules. This observation implies that carbohydrates serve as water substitutes for dried proteins by satisfying the H-bonding requirements for the proteins, i.e., by preventing protein dehydration. At higher sugar concentrations (i.e., above the crystallization concentration), there is more H-bonding between sugar molecules than H-bonding between sugar and protein molecules, consequently, less protection Thus, the observation, '*protein stabilization offered by the additives is the net balance between binding to the protein and the exclusion effects*' (Table 4, Timasheff and Arakawa. 1989), can also be applied to the freezing-drying systems.

In addition to the interactions between lyoprotectant and protein, the individual stability of these two ingredients in the lyophilization formulation should be monitored. Townsend and DeLuca (1988) proposed that degradation of sucrose may be the cause of it being an ineffective lyoprotectant when ribonuclease A was freeze-dried under acidic conditions. When air was present in the vial headspace, there was accelerated degradation of the freeze-dried ribonuclease (Townsend *et al.*, 1990a).

Lyophilization can also alter the three-dimensional structure of proteins. Yu (1974) observed that the lyophilized α-lactalbumin has less uniform hydrogen bonds and disulfide bonds compared to those of the crystalline form. An increased content of disulfide bonds in freeze-dried proteins was also observed by Hoshi and Yamauchi (1983) and Liu *et al.* (1991).

As stated before, protein hydration is an important factor during lyophilization. It is also a major factor in the stability of freeze-dried products. Stability of the lyophilized products of hemoglobin (Pikal, 1990) and ribonuclease A (Townsend *et al.*,1990b) is closely related to their residual moisture content. Even moisture migration from or through rubber stoppers became a major discussion topic in a recent meeting, the International Symposium on Biological Product: Freeze-Drying and Formulation, Oct. 1990 (Franks, 1990). Patel (1990) reported that the lyophilized urokinase degraded faster in vials having stoppers containing higher moisture and suggested a longer drying process for stoppers.

Patel *et al.* (1991) recommanded a careful evaluation of miosutre content of different stopper formulations for moisture-sensitive lyophilized biologicals.

If a protein is labile toward freeze-drying, it may be desirable to adopt the following strategies: (1) add cryoprotectants and/or lyoprotectants, usually > 0.2 M or >1:6 ratio (protein:additive); (2) freeze-dry protein under acidic conditions if disulfide exchange is a possibility; (3) add an oxygen scavenger if either or both the protein and excipients are susceptible to oxidative degradation; (4) minimize the moisture content and headspace oxygen content in the freeze-dried products.

Preformulation studies of proteins which include solubility studies, pH-rate profile generation and investigation of buffer catalysis are similar to those of small molecule drug entities. Cold destabilization, surface denaturation/adsorption, and (freeze-)drying denaturation are unique characteristics of protein drugs and must also be considered in formulation studies. Although numerous articles have discussed the increased stability offered by additives, protein formulations should be as simple as possible, as shown in Table 5. With these guidelines, a more scientific and systematic approach can be used to solve formulation problems encountered in developing protein drugs.

V. ANALYTICAL METHODS TO MONITOR PROTEIN STABILITY

To ensure efficacy and safety of a drug product, the active compound and its degraded products must be quantitated to determine the expiry period of the product. Due to the multiplicity of protein degradation pathways, no single test method can be guaranteed to be stability indicating. Only the combined information from various methods can lead to the assurance that the physicochemical integrity and biological activities of a protein are retained throughout manufacturing and shelf life.

It is not the intention of this review to discuss every analytical method in detail. Only the major advantages and limitations of some analytical methods, such as optical spectroscopy, electrophoresis, and high performance liquid chromatography, will be discussed. Other methods such as 2-D NMR, amino acid

sequencing, and calorimetry, are informative but may not be suitable for routine analysis.

Many analytical techniques such as DNA sequencing, Edman sequencing, amino acid content analysis, and peptide mapping are frequently used to determine the primary structure of a protein. These techniques are used to monitor changes in protein primary structure that can be caused by genetic mutation or chemical degradation. Although no evidence of genetic mutations has been reported for any biotechnology product or process, the theoretical potential for producing aberrant protein is a concern. PMA's (Pharmaceutical Manufacturers Association) Committee on Process Development and Manufacturing (CPDM), Biotechnology Division, has recently outlined some approaches to evaluate the genetic stability of recombinant protein (CPDM, PMA, 1991; Garnick *et al.*, 1988; Riggin and Farid, 1990).

Among these techniques, peptide mapping is perhaps the most powerful and universally used. When a peptide map of a protein is fully developed and well defined (usually with the help of mass spectrometry), it can be used to monitor the genetic stability, the homogeneity of production lots, and protein stability during fermentation, purification, dosage form manufacture, and storage. However, the development of a useful peptide map requires a large amount of effort (Garnick *et al.*, 1988).

In addition to monitoring the integrity of the protein primary structure, protein conformational stability at secondary, tertiary, and quaternary levels must also be verified to assure maintenance of its biological functions.

A. Spectroscopy

In biochemistry, optical spectroscopy is usually confined to the observation of absorption spectra (except for fluorescence spectroscopy). Usually, the determination of absorption spectra is also confined to dilute solutions, where the Beer-Lambert law applies.

(1) Visible and U.V. Spectroscopy

Visible and near UV spectroscopy are used to monitor two types of chromophores: metalloproteins (>400 nm) and proteins that contains Phe, Trp, Tyr

TABLE 6. Absorbance and fluorescence properties of the aromatic amino acids (Schmid, 1989)[a]

	Absorbance		Fluorescence		
amino acid	λ_{max} (nm)	ε_{max} ($M^{-1}cm^{-1}$)	λ_{max} (nm)	Φ_F [b]	Sensitivity $\varepsilon_{max} \cdot \Phi_F$ [b]
Tryptophan	280	5600	348	0.20	1100
Tyrosine	274	1400	303	0.14	200
Phenylalanine	257	200	282	0.04	8

[a] In water at neutral pH.

[b] Φ_F = fluorescence quantum yield

residues (260-280 nm) (Table 6). This technique has been routinely used to determine protein conformational stability and protein concentration. For most proteins, UV spectra at 240-310 nm are most informative, i.e., the absorbance of Phe, Trp, Tyr residues. Solutions containing only proteins should not show absorbance above 310 nm. A sloping base line in the 310-400 nm region usually originates from light scattering when large particles, such as aggregates, are present in the solution (Schmid, 1989). In addition, if these residues are buried within the protein interior, the UV absorbance is no longer sensitive to changes in solvent and, consequently, not a good indicator of either conformational stability or protein concentration.

Using ribonuclease T1 as model, the difference in UV absorbance between folded and unfolded states was less than 0.05 (Creighton, 1984; Thomson *et al.*, 1989; Honore and Pedersen, 1989). The success in using spectral derivatives, up to the 4th derivative, to increase sensitivity has been limited (Metzler *et al.*, 1986). UV spectroscopy is most frequently used to detect proteins eluted from a LC (liquid chromatography) system or to determine protein concentration.

(2) Fluorescence Spectroscopy

Fluorescence spectroscopy measures the emission energy after the molecule has been irradiated into an excited state. Only proteins having Phe, Trp, Tyr residues can be measured. Trp fluorescence is generally observed since the order of intensity is Trp >> Tyr >> Phe (Table 6). Fluorescence is much more sensitive

to the molecular environment that UV absorbance. A 4-fold increase in fluorescence intensity was observed in the unfolded state as compared to the native (Havel *et al.*, 1989). In addition, the fluorescence of a protein depends on the solvent conditions even in the absence of conformational changes. This characteristic is very useful in studying solvent-protein interactions and solvent accessibility to the native protein. The electrostatic effects of salt and polylysine on flavodoxin can only be observed in fluorescence intensity, not in optical absorption or circular dichroism changes (Cheddar and Tollin, 1990). Using fluorophores and lifetime fluorescence (monitoring emission intensity on a nanosecond time scale), dynamics of protein structural fluctuations can be observed (Franks, 1988; Lakowicz, 1986).

Another useful application of UV and fluorescence spectroscopy is with pre- or post-column derivatization in HPLC to increase sensitivity of protein or peptide detection. Proteins with functional groups such as amino, alcoholic or phenolic hydroxyl, carbonyl, carboxyl, or thiol can be converted to strong chromophores. The most often used chemicals for such a derivatization are dansyl chloride, *o*-phthaladehyde and the ninhydrin reagents (Ohkura and Nohta, 1989).

(3) Circular Dichroism Spectroscopy

The responses of dissolved molecules to circularly polarized radiation depend explicitly on their structural asymmetry. The far-UV or amide region (170-250 nm) is dominated by contributions of peptide bonds, whereas CD bands in the near-UV region (250-300 nm) originate from aromatic amino acids. In addition, disulfide bonds give rise to minor CD bands around 250 nm.

Circular dichroism (CD) spectra of a protein are sensitive to its conformation, especially the secondary structure. The relative proportion among α-helices, β-sheets and disordered structure can be calculated from CD spectra at 190, 207 and 222 nm. The detailed calculation of CD data was reviewed by Yang *et al.* (1986). Recent reviews by Johnson (1990) and Schmid (1989) provide the following guidelines to determine the secondary structure more accurately: (1) measure a CD spectrum down to 180 nm; (2) flush cells with adequate and pure nitrogen; (3) use shorter pathlength cells, e.g., 0.05 mm to increase solvent transparency; (4) determine the protein concentration accurately. With these

TABLE 7. Comparison of secondary structure estimates for bovine growth hormone (Havel *et al.*, 1989).

secondary structure	X-ray	CD	IR/Raman
α-helix	54%	55%	45%
β-pleated sheet	3%	10%	15%
disordered	43%	35%	40%

precautions, the correlation coefficients were 0.97 for α-helix, 0.78 for antiparallel β-sheet, 0.68 for parallel β-sheet, and 0.49 for turns.

Thus, CD spectra can provide both qualitative and quantitative information about protein conformation. CD spectroscopy is also a useful probe for studying the folding and unfolding of protein secondary structure either at equilibrium or kinetically. Even in the presence of high concentrations of denaturant, it is possible to monitor the protein conformational stability by CD spectra (Villanueva *et al.*, 1989; Davis *et al.*, 1987).

(4) Infrared and Raman Spectroscopy

Both infrared and Raman spectroscopy measure the vibrations of bond lengths and angles. Due to the interferences from water and scattering light, FTIR and Raman spectroscopy are most often used instead of simple IR.

The most informative IR bands for protein analysis are amide I (1620-1700 cm^{-1}) , amide II (1520-1580 cm^{-1}) and amide III (1220-1350 cm^{-1}). IR spectra can provide qualitative and quantitative information of the secondary structures of proteins such as α-helix, β-sheet, β-turn, and disordered structure. Mathematical manipulation of the scanned spectra is necessary to resolve overlapping bands (Havel *et al.*, 1989; Susi and Byler, 1986; Bussian and Sander, 1989). The agreement between the IR results and values derived from X-ray crystallography was shown to be quite good but less accurate than those from CD results as shown in Table 7 (Havel *et al.*, 1989). However, IR has been demonstrated to be sensitive enough to detect conformational changes in proteins induced by freeze-drying, pressure, heat, and moisture as discussed before.

The most valuable feature of IR spectroscopy is its ability to examine proteins in different physical states. This capacity provides a means to monitor secondary structure in different media or formulations. The usefulness of IR spectroscopy to detect conformational changes caused by freeze-drying has been demonstrated by Havel *et al.* (1989) in human growth hormone, by Yu (1974) in α-lactalbumin, by Bussian and Sander (1989) in DNAase I, and by Carpenter and Crowe (1989) in lysozyme and phosphofructokinase.

Raman spectroscopy can also provide information regarding the disulfide bonds in protein molecules. The presence of an S-S stretch at about 500 cm^{-1} indicates an intact bridge, while an S-H stretch at about 2550 cm^{-1}.indicates the reduction of disulfide bonds (Havel *et al.*, 1989). Bussian and Sander (1989) have addressed the techniques to overcome the inherently weak signal from the Raman effect.

(5) Light Scattering Spectroscopy

Light scattering spectroscopy can be used to estimate the molecular weight of a protein and is a convenient tool to monitor protein quaternary structure or protein aggregation. While the degree of protein aggregation can be obtained by simple turbidity measurement, the precise determination of protein oligomerization is usually determined by dynamic light scattering. The advantages of this technique are ease of use, non-destruction of samples, the speed of determination, and minimal disruption of the sample environment (Phillies, 1990; Wu *et al.*, 1990).

Dynamic light scattering, also called photon correlation spectroscopy (PCS) or quasielastic light scattering (QELS) is used to calculate the molecular weight of a protein via its diffusion behavior. However, information regarding protein oligomerization or molecular weight may not be accurate if protein is highly denatured (Phillies, 1990). Another error may arise from using too concentrated protein samples. High concentrations of protein can cause aggregation and non-ideal diffusion, which can result in misleading data.

B. Electrophoresis

Electrophoresis is the migration of charged particles or molecules in a liquid medium under the influence of an applied field. The rate at which the molecules

migrate depends on factors such as the net charge, size and shape of the molecules and ionic strength, viscosity and temperature of the medium. The most often used techniques in biotechnology are PAGE, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and IEF (Isoelectric focusing) (Blackshear, 1984; Radola, 1984; Friz *et al.*, 1989; Hennessey and Scarborough, 1989).

IEF concentrates and separates protein in a stable pH gradient according to differences in charge. It is useful to monitor product quality and is especially capable of demonstrating heterogeneity in glycoproteins or deamidated proteins. The capability of IEF to distinguish glycosylation variants may make the IEF patterns too complicated to be analyzed (Garnick *et al.*, 1988). Larger proteins or their oligomers (i.e., dimers, trimers, tetramers, etc.) can be focused by using agarose gels as the matrix (Radola, 1984; Friz *et al.*, 1989).

Proteins can be separated on the basis of size alone by SDS-PAGE. In this method, proteins are first denatured by adding SDS and a reducing agent such as dithiothreitol or 2-mercaptoethanol, followed by incubation at elevated temperature, usually 100° C, for a short period of time. Optimal separation can be achieved by controlling the heating time, the pH of resolving gel, and the degree of gel polymerization (Blackshear, 1984; Friz *et al.*, 1989; Hennessey and Scarborough, 1989). Since protein samples in SDS-PAGE are denatured, its use in detecting conformational changes is limited unless proteolytic samples are used. This so-called peptide mapping technique has recently been used more extensively with the combination of reversed-phase HPLC, not with SDS-PAGE whose resolving power is limited unless a two-dimensional gel is used.

The separated protein bands can be silver- or Coomassie Blue-stained for visualization and quantification. The difficulty of Coomassie Blue binding to the carbohydrate moieties may lead to errors in analyzing glycoproteins (Osset *et al.*, 1989). Generally,the silver staining method is more sensitive and quantitative but a modified method should be used in detecting low molecular weight proteins in SDS-PAGE (Fan *et al.*, 1990). Electrophoresis was once a labor-intensive technique, however, currently available instruments and ready-made gels have been developed to achieve semi-automation.

The advantages of electrophoresis (IEF or SDS-PAGE) are efficient separation, multiple sample analysis, sensitivity, and versatility. Another unique

feature of electrophoresis is that the separated fragments can be transferred by blotting for further analyses such as immunoassay and DNA hybridization (Stott, 1989; Bennahmias, 1989).

With the recent advances in capillary electrophoresis, sample size of 2 ng can be separated and analyzed. Capillary electrophoresis uses the same basic principles for separation. High resolution in capillary electrophoresis can be obtained because the enhanced heat dissipation of capillary tubes permits the use of high potentials for separation. This technique may eventually replace the slab gels (Ewing *et al.*,1989; Cobb and Novotny, 1989).

C. High Performance Liquid Chromatography

Due to the versatility and possibility for total automation, HPLC has been widely used in bio-analysis. HPLC methods are classified by the stationary phase. The most often used ones are size exclusion (also called gel filtration), affinity, ion-exchange, hydrophobic interaction, and reversed-phase chromatography.

Size exclusion chromatography (SEC) separates proteins by size. Packing materials of different pore size are available for rapid molecular weight screening. SEC can be either preparative or analytical scale. This method gives low resolution and is usually used to detect protein polymerization and aggregation.

Separation by affinity chromatography (AC) is based on biological recognition and is highly selective. It is often used for large-scale purification, however, there is a limited selection of commercially available HPLC columns of silica-bound ligands. Depending on the affinity between ligand and sample, the resolution may be high. Artifacts having the same ligand affinity should be carefully monitored in data interpretation (Franks, 1988; Wilchek *et al.*,1984).

Protein retention on an ion exchange column is the result of the relative electrostatic interactions between the ion exchange matrix and the protein. Separation should be performed with the pH of the mobile phase close to pI of the protein. Fractions are then eluted by increasing the concentration of the counterion. However, even at the pI (isoelectric point) of the protein, all the charged groups will not interact simultaneously with the ion exchange matrix because of (1) the three dimensional nature of the protein, and (2) the non-uniformity of charge

distribution on protein surface. It is likely that proteins with different pI's and net charges could co-chromatographed if they have the same surface characteristics. By manipulating column selection, pH of the mobile phase, and concentration gradient of the counterion, high resolution separation can be achieved (Franks, 1988; Regnier, 1984). Affinity and ion exchange LC are often used for preparative scale purification.

Reversed-phase chromatography (RPC) is the most popular HPLC method and is usually suitable for proteins of MW < 25,000. Generally, RPC induces protein denaturation due to the organic solvent or hydrophobic column packing surfaces. With careful selection of stationary and mobile phases, good separation can be achieved. RPC has been used to study the kinetics of protein unfolding (Benedek *et al.*, 1989).

The technique 'peptide mapping', which is the separation of proteolytically digested samples by RPC, is very useful in quality control and stability studies. A map or 'fingerprint' is obtained with information regarding the primary structure of proteins. Thus, modifications of amino acid residues caused by lot-to-lot variation or protein degradation can be closely monitored. Proteolytic enzymes used are trypsin, V8 protease, chymotrypsin, subtilisin, and clostripain (Franks, 1988; Hearn, 1984; Dong *et al.*, 1989).

Hydrophobic Interaction chromatography (HIC) is performed using a reversed-phase column, identical to RPC but with a larger nominal pore size (≥ 300Å). The eluting solvent, however, consists of an aqueous salt solution, in contrast to aqueous organic mobile phases normally used in RPC. High resolution separation can be achieved by manipulating the nature of the salt used and salt concentration. The order of sample elution by HIC is reversed from that obtained by RPC. HIC is preferred to RPC at times because the mobile phase is less likely to denature proteins (Franks, 1988; Shaltiel, 1984).

Since each analytical method has its limitations, it is important to choose the appropriate methods to monitor protein stability. A detailed inspection of the protein structure (at primary, secondary, tertiary and quaternary levels) and the identification of possible denaturation/degradation that might occur during purification and manufacturing processes should provide guidelines in the selection of analytical methods. A summary of the typical analytical methods used in determining protein integrity is presented in Table 8.

TABLE 8. Advantages and limitations of the analytical methods used for determining protein integrity (Havel, *et. al.*, 1989; Garnick, *et. al.*, 1988; Middaugh, 1990; Riggin and Farid, 1990).

Method	Protein Structure Level[a]				Sample Requirement	Advantages	Limitations
	1°	2°	3°	4°			
Spectroscopic Methods							
UV		X	X		1-3%,, solution	Ease of use	Spectral responses to changes may be small, spectral derivatization may be necessary
Fluorescence steady state		X	X		1-3%,, solution	Sensitive to environment	Dependent on the content of trytophan
time-resolved			X	X			
CD		X	X		1-3%, solution	Accurate determination of secondary structure	Guard against deviation from light scattering of the sample
FTIR		X			0.1-3%, solution or solid	Information regarding amide and S-H bonds; applicable to solid samples	Quantitative information of the secondary structure may be less reliable
Raman		X	X		0.1-3%, solution or solid	Information regarding amide and S-S bonds; applicable to solid samples	Signal is weak; quantitative information of the secondary structure may be less reliable
Light scattering			X	X	3-5%, solution	Determine molecular weight and radius of gyration	Limit to globular proteins; error may arise due to denaturation
X-ray	X	X	X	X	> 50% , crystal	Complete 3-D structure determination	Difficult to obtain protein crystals, large amount of sample
Mass spectroscopy	X				0.1-0.5%, solution	Amino acid sequencing; LC-MS tryptic digest maps	Destructive to sample, limited to MW < 5000
Nuclear magnetic spectroscopy	X	X	X		2-10%, solution	Complete 3-D structure determination possible	Data is complicate to interpret; limit to MW < 20,000

[a]: 1°, 2°, 3°, and 4° are referred to the primary, secondary, tertiary, and quaternary structures of protein molecule.

TABLE 8. Continued.

Method	Protein Structure Level				Sample Requirement	Advantages	Limitations
	1°	2°	3°	4°			
High performance liquid chromatography					0.1-0.5%, solution	Fast, reproducible, computer compatible, high resolution	Protein may be denatured due to the interaction with packing material or with mobile phase
RP & HIC		X	X			N-terminal variant, glycosylation variants, disulfide isomers, proteolytic clips	
Size exclusion				X		Determine the degree of protein oligomerization or aggregation	
Ion-exchanger		X	X			Deamidated forms, glycosylation variants	
Electrophoresis					solution	Multiple sample application, separated species can be transferred for further analysis such immunoassay	Labor-intensive
SDS-PAGE							Denaturing assay system, resolution limited to MW< 500, artifacts in sample preparation
Coomassie Blue		X	X		0.5-1%	Information regarding molecular weight	
Silver stained		X	X		0.005%		
IEF							Resolution limited to 0.1 pH unit, difficulty in of result interpretation especially if glycoproteins are involved
Coomassie Blue		X	X		0.5-1%	Deamidated forms, glycosylation variants	
Silver stained		X	X		0.005%		
Capillary zone	X	X	X		ng levels	Efficient and fast separation, great resolving power, deamidated forms, N-terminal variants, proteolytic clips	Band broadening due to protein adsorption and heat accumulation
Differential scanning calorimetry		X	X		solution or solid	Denatured species 1-10% for DSC and 0.1-0.3% for microcalorimetry	Useful in protein formulation only if microcalorimetry is used

[a]: 1°, 2°, 3°, and 4° are referred to the primary, secondary, tertiary, and quaternary structures of protein molecule.

The above discussion of analytical methods did not address the importance of carbohydrate analysis of glycoproteins. Among therapeutic proteins, tissue plasminogen activator, erythropoietin, granulocyte colony stimulating factor, interferon-α Type II, interferon-β, interferon-γ are glycoproteins (Goddard, 1991). The carbohydrate portions of these proteins are thought to be as recognition determinants in immunological responses, in cell-cell interactions, and in enzyme recognition (i.e., determining pharmacokinetic half-life). Since the addition of carbohydrates to proteins is not gene-regulated, a small amount of heterogeneity in the resulting glycoproteins is the rule. The carbohydrate synthesis is also dependent on the physiological state of the cells and batch to batch variation in product quality may arise. Geisow (1991) and Hodgson (1991) have addressed the technical difficulties in analyzing glycoproteins. The analyses usually include the identification of the linkage sites, and a qualitative and quantitative profile of carbohydrate content. The detail of analysis required depends on the biological importance of the carbohydrates. Even with recent advances in analytical technology, a complete characterization of the carbohydrate portion of glycoproteins is a very formidable task due to the heterogeneity; therefore, it is usually not included for routine analysis.

VI. CONCLUSION

Obviously, formulation development of protein drugs requires monitoring of their biological, physical and chemical properties. Optimal stability can be achieved by manipulating protein environment and/or by genetically changing its primary structure. Due to the difficulty in predicting the tertiary structure of a protein molecule from its primary structure, the strategy of controlling protein environment is more accessible and more successful in increasing protein stability.

Strategies of formulation development for conventional chemical entities are adopted for protein drugs. A well characterized protein stability profile must be established first. The conditions of optimal protein stability regarding pH, ionic strength, buffering salt, and moisture content should be determined with more than one analytical method. Protein conformation stability and potency should also be critically monitored in selecting mixing rate and time, sterile filtration devices, containers, I. V. admixtures for dilution, and I. V. administration setup. When significant protein denaturation/degradation occurs, identification of the causes and

mechanisms is necessary in developing strategies to increase protein stability. For lyophilized products, possible denaturation/degradation of protein drugs caused by freezing/drying and moisture content of both stopper and product should be evaluated.

From the above discussion, it is obvious that formulation development of a protein drug requires extensive teamwork among analytical, preformulation and formulation staff. Awareness of the possible pitfalls during manufacturing processes and the limitations of the analytical methods will also help in developing a stable protein formulation.

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