

Review

# Instability, stabilization, and formulation of liquid protein pharmaceuticals

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## Abstract

One of the most challenging tasks in the development of protein pharmaceuticals is to deal with physical and chemical instabilities of proteins. Protein instability is one of the major reasons why protein pharmaceuticals are administered traditionally through injection rather than taken orally like most small chemical drugs. Protein

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*Abbreviations:* ADA, adenosine deaminase; ADH, alcohol dehydrogenase; AcP, acylphosphatase; BDNF, brain-derived neurotrophic factor; BGG, bovine  $\gamma$ -globulin; BSA, bovine serum albumin; BSF, bovine serum fetuin; CD, circular dichroism spectroscopy; CE, capillary electrophoresis; CMC-Na, carboxymethyl cellulose sodium; rhCNTF, recombinant human ciliary neurotrophic factor; rConIFN, recombinant consensus  $\alpha$ -interferon; DGK, diacylglycerol kinase; DMSO, dimethylsulfoxide; rhDNase, recombinant human deoxyribonuclease; DSC, differential scanning calorimetry; hEGF, human epidermal growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; rFVIII, recombinant factor VIII; pdFIX, plasma-derived factor IX; rFIX, recombinant factor IX; rFXIII, recombinant factor XIII; FTIR, Fourier transform infrared spectroscopy; GA, glucoamylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-CSF, granulocyte colony-stimulating factor; GDH, glutamate dehydrogenase; met-hGH, methionyl human growth hormone; pGH, porcine growth hormone; GRF, growth hormone releasing factor; GdnHCl, guanidine hydrochloride; HP- $\beta$ -CD, hydroxypropyl- $\beta$ -cyclodextrin; IFN- $\beta$ , interferon- $\beta$ ; IFN- $\gamma$ , interferon- $\gamma$ ; hIGF-I, recombinant human insulin-like growth factor I; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-1R, interleukin-1 receptor; IL-2, interleukin-2; rhIL-1ra, recombinant human interleukin-1 receptor antagonist; IR, infrared spectroscopy; rhKGF, recombinant human keratinocyte growth factor; LDH, lactate dehydrogenase; *phm*-MDH, pig heart mitochondrial malate dehydrogenase; LMW-UK, low molecular weight urokinase; rhM-CSF, recombinant human macrophage colony-stimulating factor; rhMGDF, recombinant human megakaryocyte growth and development factor; MS, mass spectroscopy; rhNGF, recombinant human nerve growth factor; NMR, nuclear magnetic resonance spectroscopy; tPA, tissue plasminogen activator; PAGE, polyacrylamide gel electrophoresis; hPAH, human phenylalanine hydroxylase; PE40, 40 kD segment of *Pseudomonas* exotoxin (PE); PEG, polyethylene glycol; *ml*-PEPC, maize leaf phosphoenolpyruvate carboxylase; PFK, phosphofructokinase; huPrP(90-231), recombinant protein corresponding to the human prion protein domain (residues 90-231); PVA, polyvinylalcohol; PVP, polyvinylpyrrolidone; rhPTH, recombinant human parathyroid hormone; RP-HPLC, reversed phase HPLC; RNase A, ribonuclease A; SDS, sodium dodecyl sulfate; SEC-HPLC, size exclusion HPLC; TGF, transforming growth factor; TMAO, trimethylamine *N*-oxide; TP40, Cys-replaced (with Ala in PE40) mutant of TGF- $\alpha$ -PE40; rhTPO, recombinant human thrombopoietin; YEI, yeast external invertase.

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pharmaceuticals usually have to be stored under cold conditions or freeze-dried to achieve an acceptable shelf life. To understand and maximize the stability of protein pharmaceuticals or any other usable proteins such as catalytic enzymes, many studies have been conducted, especially in the past two decades. These studies have covered many areas such as protein folding and unfolding/denaturation, mechanisms of chemical and physical instabilities of proteins, and various means of stabilizing proteins in aqueous or solid state and under various processing conditions such as freeze-thawing and drying. This article reviews these investigations and achievements in recent years and discusses the basic behavior of proteins, their instabilities, and stabilization in aqueous state in relation to the development of liquid protein pharmaceuticals. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Denaturation; Unfolding; Melting; Aggregation; Degradation; Preferential interaction

## 1. Introduction

The advent of recombinant DNA technology has led to a worldwide zeal to develop protein pharmaceuticals in the past two decades. These protein pharmaceuticals or pharmaceutical candidates include functional regulators and supplements, enzyme activators and inhibitors, poly- and monoclonal antibodies, and various vaccines. In comparison with small chemical drugs, protein pharmaceuticals have high specificity and activity at relatively low concentrations. These features have made protein pharmaceuticals indispensable in combating human diseases.

Due to advances in analytical separation technology, recombinant proteins can now be purified to an unprecedented level (Bond et al., 1998). Highly purified protein pharmaceuticals significantly reduce the known and unknown potential side or even toxic effects. However, one of the most challenging tasks remains in the development of protein pharmaceuticals: dealing with physical and chemical instabilities of proteins. Protein instability is one of the two major reasons why protein pharmaceuticals are administered traditionally through injection rather than taken orally like most small chemical drugs (Wang, 1996). Protein pharmaceuticals usually have to be stored under cold conditions or even freeze-dried to a solid form to achieve an acceptable shelf life.

In search for ways of stabilizing proteins, scientists turned their attention to nature for an answer. It is well known that certain natural organisms can grow well at extreme temperatures. Hyperthermophilic organisms (hyperthermophiles) such as anaerobic, methanogenic, or sulfate-reducing

archaeobacteria grow at temperatures near or above 100°C (Huber et al., 1989; Adams, 1993, 1994). Proteins in these organisms function normally at high temperatures. For example, enolase and  $\alpha$ -glucosidase in hyperthermophilic *Pyrococcus furiosus* have optimum activity, at  $>90$  and  $>105^\circ\text{C}$ , respectively (Costantino et al., 1990; Peak et al., 1994). The most thermostable proteins found so far have half-lives in excess of 10 min at 130°C (Daniel et al., 1996). The mechanisms responsible for the high molecular stability of thermophilic proteins include increased hydrophobic interactions, greater molecular packing, more H-bonds, more salt-bridging, loss of surface loops, more helix-forming amino acids, restricted N-terminus mobility, etc. (Vieille and Zeikus, 1996; Cowan, 1997; Vogt and Argos, 1997). Extrinsic factors (not primary structure-related) have also contributed to protein stabilization. One of these is the high cellular content of sugars, salts, or other organic solutes/osmolytes, such as  $\alpha$ -glutamate, di-myoinositol-phosphate and its isomer,  $\beta$ -mannosylglycerate, and diglycerol-phosphate (Huber et al., 1989; Rupley and Careri, 1991; Martins and Santos, 1995; Martins et al., 1997; Ramakrishnan et al., 1997).

The identification of intrinsic and extrinsic factors that contribute to the stabilization of thermophilic proteins has provided valuable information for stabilizing protein pharmaceuticals and for designing more stable mutant proteins. Yet the structural differences among different proteins are so significant that generalization of universal stabilization strategies has not been successful. Very often, proteins have to be evaluated individually and stabilized on a trial-and-error basis.

To understand and maximize the stability of protein pharmaceuticals or any other usable proteins such as various catalytic enzymes, many studies have been conducted in the past few decades. These studies have been reviewed with emphasis on general protein stability (Jaenicke, 1991; Kristjánsson and Kinsella, 1991), mechanisms of chemical and physical instabilities of proteins (Manning et al., 1989), mechanisms and prevention of major protein degradation pathways (Cleland et al., 1993), and various means of stabilizing proteins in aqueous or solid state and under various processing conditions such as freeze-thawing or drying (Gianfreda and Scarfi, 1991; Arakawa et al., 1993; Timasheff, 1993; Manning et al., 1995; Wong and Parascrampuria, 1997). This article reviews these investigations and achievements in recent years and discusses the basic behavior of proteins, their instabilities, and stabilization in aqueous state in relation to the development of liquid protein pharmaceuticals.

## 2. Basic protein behavior and properties

Protein pharmaceuticals, unlike small drug molecules, have high molecular weight (> 5 kD). Their large size, compositional variety, and amphipathic characteristics constitute specific behavior such as folding, conformational stability, and unfolding/denaturation. Understanding proteins' basic behavior may help toward their stabilization.

### 2.1. Protein folding and its related forces and stability

Biologically active proteins are properly folded. The number of possible conformations of a folded polypeptide chain with an average domain size is about  $10^{80}$  (Jaenicke, 1991). The three-dimensional folded state of a protein is a fluctuating state of a limited number of preferred conformations (Tang and Dill, 1998). The most stable (least energy) conformation of a protein is usually the native state (Darnell et al., 1986). Under native conditions the vast majority of protein molecules exist in their unique native state (N). A tiny

fraction must also occupy all possible higher energy states as dictated by the Boltzmann relationship (Bai and Englander, 1996).

#### 2.1.1. Protein Folding Process

Folding of newly-synthesized polypeptides in cells requires the assistance of so-called molecular chaperone proteins (Hendrick and Hartl, 1995). These proteins bind unfolded or partially folded polypeptides in their central cavity and promote folding by ATP-dependent cycles of release and rebinding. The molecular chaperone GroEL facilitates protein folding by preventing protein aggregation and correcting protein misfolding (Golbik et al., 1998). Protein folding is generally a highly cooperative process, in which only the native and unfolded states are stable (Goto and Fink, 1989).

When a protein folds, about 80% of nonpolar side chains (Ala, Val, Ile, Leu, Met, Phe, Trp, Cys) are buried in the interior of protein molecules out of contact with water. For example, folding of RNase T1, a compact globular protein (104 aa), removes about 85% of nonpolar residues from contact with water (Thomson et al., 1989). More than 80% of amino acids in globular proteins exist in  $\alpha$ -helix/ $\beta$ -sheet or in the turns connecting them (Pace et al., 1996).

The rate of protein folding is usually high. Many small proteins can fold in milliseconds or less (Dobson and Hore, 1998). Some may fold at a slower rate. For example, the folding of a bacteria protein MerP (72 aa) by diluting the protein solution containing 3 M guanidine hydrochloride (GdnHCl) shows two exponential phases. The initial phase is fast with a rate constant of 1.2/s, but the second phase is slow with a rate constant of 0.053/s, accounting for about 20% of the folding signal (Aronsson et al., 1997). Folding of proteins involving Pro isomerization is also relatively slow.

#### 2.1.2. Major forces involved in protein folding

Many forces are involved in protein folding. These include hydrophobic interactions, electrostatic interactions (charge repulsion and ion pairing), hydrogen bonding, intrinsic propensities, and van der Waals forces. Hydrophobic interactions are repulsive interactions between water and

non-polar residues in proteins, leading to minimal hydration of the hydrophobic core. These interactions are strongly disfavored and associated with a large increase in heat capacity (Dill, 1990). A hydrogen bond is the strong dipole–dipole attraction between covalently-bonded hydrogen atoms and other strongly electronegative atoms such as oxygen and nitrogen. It is primarily a linear arrangement of donor, hydrogen, and acceptor. Hydrogen bonds between amide hydrogen and carbonyl oxygen make up 68% of the total hydrogen bonds in globular proteins (Pace et al., 1996).

Among all the forces involved in protein folding, the apparent dominant force is hydrophobic interaction (Jaenicke, 1990; Kristjánsson and Kinsella, 1991). The dominant opposing force is the loss of non-local conformational entropy (Dill et al., 1989; Dill, 1990). The difference in heat capacity of a protein in the native folded and denatured states ( $\Delta C_p$ ) is a measure of hydrophobic stabilization of the protein, and the large  $\Delta C_p$  on thermal denaturation of proteins supports the major role of hydrophobic interactions in protein stabilization (Wang et al., 1996a). Recently, it was found that the contribution of electrostatic interactions in proteins at neutral conditions to the free energy difference between the folded and unfolded states is close to 0, indicating that the main driving forces for protein folding under these conditions are hydrophobic and hydrogen-bonding interactions (Dimitrov and Crichton, 1997).

Hydrophobic interaction, albeit dominant, needs to be balanced to maintain protein activity. In studying the stability of mutant Rop proteins, Munson et al. (1996) demonstrated that underpacking the hydrophobic core with small amino acids like Ala not only loses protein activity but also decreases protein stability. On the other hand, overpacking the hydrophobic core with only large amino acids like Leu stabilizes proteins but protein activity is lost. This indicates that both favorable steric interaction and burial of sufficient hydrophobic volume and surface area are important to stabilize a protein.

Protein molecular packing may be the most applicable factor that leads to the unique structures of most globular proteins (Richards, 1997).

The packing in native proteins is so well arranged that all solvent molecules are essentially excluded and the protein interior is more like a crystalline solid than a non-polar liquid (Jaenicke, 1991). Glu, Lys, and Arg, with three or four rotatable bonds, are almost invariably located on the protein surface and use their flexibility to help ensure exposure of their charged groups to the solvent. However, buried polar residues do not necessarily destabilize a protein if they can form stable hydrogen bonds (Pace et al., 1996) and/or form stable intramolecular salt bridges, enhancing the structural rigidity (Vieille and Zeikus, 1996).

Interaction forces in proteins can also be divided into two types: local (short-ranged or secondary) and non-local (long-ranged or tertiary) (Chan and Dill, 1991). Long-range interactions happen between two residues that are separated by at least ten residues (Dosztányi et al., 1997). Long-range interactions such as parallel and antiparallel sheets are mainly responsible for polymer collapse to compact states whereas local forces are mainly responsible for helix formation such as hydrogen bonding. It seems that long-range interactions (non-local forces) control overall protein stability. The dominant role of long-range interactions in protein stability is supported by recent identification of so-called elements of stabilization center (SC) in proteins. By long-range interactions, these SCs, mainly consisting of hydrophobic residues that are less flexible, tend to connect more sheets to each other, and are primarily responsible for stabilization of protein structures (Dosztányi et al., 1997). If different proteins have similar structural folds, their conformational stability may still be very different due to the difference in their primary sequences (Chiti et al., 1998).

### 2.1.3. Protein folding and free energy change

The folded state of proteins has conformational stability, which is defined as the free energy change,  $\Delta G_{f \rightarrow u}$  (or simply  $\Delta G$ ), for the unfolding/denaturation reaction under physiological conditions (Pace, 1990). The larger the  $\Delta G_{f \rightarrow u}$ , the more stable the protein. The folded state of proteins is only marginally more stable than the unfolded state since its  $\Delta G_{f \rightarrow u}$  is small. The  $\Delta G_{f \rightarrow u}$

for proteins has been reported in the following ranges: 21–63 kJ/mol (Kristjánsson and Kinsella, 1991); 5–20 kcal/mol (Volkin and Klibanov, 1989); or 5–10 kcal/mol (Jaenicke, 1990; Pace et al., 1996).

The low  $\Delta G_{f \rightarrow u}$  values indicate that conformational stability of a protein in aqueous solution is equivalent to a few H-bonds or ion pairs. A single hydrogen bond can lower the protein's free energy by 0.5–2 kcal/mol and an ion pair by 0.4–1.0 kcal/mol (Vogt and Argos, 1997). After analyzing more than a dozen mutant proteins, Pace et al. (1996) found that average free energy gain is 1.1–1.6 kcal/mol per hydrogen bond and 1.18 kcal/mol per  $-\text{CH}_2-$  for hydrophobic effect. These values suggest significant contribution of hydrogen bonding to  $\Delta G_{f \rightarrow u}$  relative to that from hydrophobic effect, however, if the polar groups must be buried to form intramolecular hydrogen bonds, the net gain is only about 0.6 kcal/mol per hydrogen bond. In comparison, the strength of a hydrogen bond in water is about 5 kcal/mol, and the energy of van der Waals interaction amounts to about 1 kcal/mol at 25°C (Darnell et al., 1986).

## 2.2. Protein unfolding/denaturation

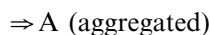
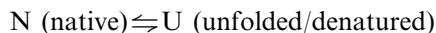
In solution, the folded state of any protein is not infinitely stable (Shortle, 1996). It may unfold/denature into an inactive form—a process of protein conformational changes. These conformational changes are due to predominant intermolecular protein–solvent interactions over intramolecular interactions, which keeps the folded state (Jaenicke, 1990). On the other hand, solvent-induced inactivation of proteins may occur without disruption of protein conformation (tertiary structure) (Cowan, 1997).

### 2.2.1. Protein unfolding process

Protein unfolding can be described generally by a single transition step between the completely folded and unfolded states since any intermediate state is highly unstable and only exists in negligible amounts (Chan and Dill, 1991; Jaenicke, 1991). This is true at least for small globular proteins (Kristjánsson and Kinsella, 1991). Others may have more than one unfolding process such

as human placental alkaline phosphatase (Hung and Chang, 1998).

The following equation describes the two-state model:



or



For most proteins, the unfolded state (U) is insoluble and favors aggregation. Under certain conditions, a particular U state exists, which is highly compact and has significant amounts of residual secondary structures such as thermally-unfolded RNase T1 (Pancoska et al., 1996) and acid-unfolded apomyoglobin (Staniforth et al., 1998). This particular U state is termed molten globule (Goto and Fink, 1989; Shortle, 1996).

The N state may unfold reversibly or, depending on the condition, irreversibly to the U state. For example, unfolding of wild-type barnase and some of its mutants are reversible based on the repeatability of DSC endotherms or CD transitions on rescanning (Johnson et al., 1997). Thermal treatment of IFN- $\beta$ -1a (Runkel et al., 1998) and  $\beta$ -galactosidase (Yoshioka et al., 1994a) causes irreversible denaturation of both proteins and eventually leads to formation of aggregates (A).

Proteins unfold locally and globally. Local and global unfolding occur concurrently and independently. Increasing denaturant concentration or temperature can selectively promote global unfolding because global unfolding exposes more surface and gives rise to higher chain entropy and enthalpy (at high temperatures) than local unfolding (Bai et al., 1994).

An important thermodynamic parameter in two-state transition is the change in heat capacity,  $\Delta C_p$ . The large and positive  $\Delta C_p$  observed in protein denaturation is due primarily to exposure of nonpolar groups (Pace et al., 1996; Johnson et al., 1997). The molar enthalpy of protein denaturation,  $\Delta H$ , at low temperatures may be either positive or negative but increases markedly with temperature. Since the driving force for protein unfolding is the increased conformational entropy

in aqueous solution,  $\Delta S_{f \rightarrow u}$  is expected to be positive.

### 2.2.2. Protein unfolding/melting temperature

Proteins unfold above certain temperatures. During a thermal unfolding process, the temperature at which 50% of protein molecules are unfolded ( $\Delta G = 0$  at this time) is defined as the unfolding (or melting/denaturation/transition) temperature ( $T_m$ ). Thermal unfolding of a protein is usually endothermic. The  $T_m$  of many proteins have been determined (Table 1) and appear mostly in the range of 40–80°C. When the level of protein hydration decreases,  $T_m$  may increase sharply due to destabilization of the unfolded state (Rupley and Careri, 1991).

The higher the  $T_m$ , the greater the thermal resistance of a protein. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from hyperthermophilic bacterium *Thermotoga maritima* has a melting temperature of 109°C (Jaenicke, 1996). However, there is no particular relationship between  $T_m$  and protein stability as measured by  $\Delta G_{f \rightarrow u}$  (Dill et al., 1989). For example, the  $T_m$  of serum albumin from different sources has the following order: human (59.7°C) > dog (59.5°C) > rabbit (57.8°C) > rat (57.6°C) > bovine (56.8°C), yet their temperatures of maximum stability ( $\Delta G$ ) are all similar at about 20°C (Kosa et al., 1998). The  $\Delta G$ s of three SH3 domains of the Tec family of tyrosine kinases are below 12–16 kJ/mol, but their melting temperatures are relatively high between 69 and 80°C (Knapp et al., 1998).

A protein may have two or more melting temperatures, depending on the experimental conditions and analytical techniques used (Table 1). For example, Kosa et al. (1998) demonstrated that dog and rabbit albumin have one transition, but human, bovine, and rat albumin have two transitions, suggesting the presence of a stable intermediate. The apical domain (residues 191–376) of GroEL protein shows two reversible melting temperatures at 35 and 67°C by far UV CD, which are attributed to unfolding of the C-terminal helices and the domain core, respectively. Protein huPrP(90–231) has one melting temperature in the presence of GdnHCl at pH 5.0 and 7.2,

but shows two transition temperatures at pH 3.6 and 4.0 with a stable unfolding intermediate (Swietnicki et al., 1997). By DSC, interleukin-1 receptor (IL-1R, 50 kD) shows two unfolding transitions near 48°C and 65°C, but after deconvolution, three melting transition peaks are identified, representing three different domains in the protein (Remmele et al., 1998).

Multimeric, chimeric, or modular proteins often have more than one melting temperature. For example, native recombinant human placental factor XIII (rFXIII) is a non-covalent dimer and each subunit consists of 3 thermolabile domains (56 kD, N-terminal) and two thermostable domains (24 kD, C-terminal). The intact protein melts in two distinct temperature regions by DSC: 69°C at pH 8.6, representing three thermolabile domains; and 90°C in 2 M GdnHCl, representing two thermostable domains (Kurochkin et al. 1995). The chimeric protein toxin sCD4(178)-PE40 (sCD4-PE40), consisting of HIV binding domains of the T-cell membrane protein (CD4) and the cytotoxic domains of *Pseudomonas* exotoxin A (PE-40), has two transition temperatures originating from both components. In addition, unfolding of the less stable PE-40 induces unfolding of the more stable CD4 component because the free form of CD4 denatures at a higher temperature (56°C) than that in the protein complex (46°C) at pH 6.5 (Davio et al., 1995).

### 2.3. Mesophilic versus thermophilic proteins

In describing basic properties of proteins, it is necessary to mention thermophilic proteins, a different class of proteins having much higher thermostability than that of mesophilic counterparts like those in humans. Mesophilic proteins usually retain their native structures over a narrow range of temperatures from about 5 to 50°C. For thermophilic proteins, the upper limit of thermostability is usually 20 to 30°C higher, corresponding to an increase in protein stability by 5–7 kcal/mol. A  $\Delta G$  of this order may be achieved by 1 or 2 additional salt bridges inside the protein globule, several additional hydrogen bonds, or 7–10 additional  $\text{CH}_3$ - groups in the hydrophobic nucleus of the protein (Mozhaev and Martinek, 1984).

Table 1  
Melting temperatures ( $T_m$ ) of proteins

Proteins	Protein solution compositions	Methods	$T_m$ (°C)	References
AcP (98 aa)	0.4 mg/ml in 50 mM acetate, pH 5.5	CD (222 nm)	57	Chiti et al., 1998
HSA (66 kD)	20 $\mu$ M in 100 mM phosphate, pH 7.4	Fluorescence ( $\lambda_{em}$ = 345 nm)	62	Farruggia et al., 1997
	88 $\mu$ M in 100 mM phosphate, pH 7.4	DSC	63	Picó, 1995
	0.1 mM in 67 mM phosphate, pH 7.4	DSC	60	Kosa et al., 1998
BSF (48 kD)	2.5 mg/ml in 50 mM phosphate, pH 6	DSC	60	Wang et al., 1996a
	2.5 mg/ml in 50 mM phosphate, pH 7	DSC	54	Wang et al., 1996a
	2.5 mg/ml in 50 mM phosphate, pH 8	DSC	48	Wang et al., 1996a
$\alpha$ -Chymotrypsin	0.3 mg/ml in 10 mM phosphate, pH 6.0	UV (281 nm)	47	Lozano et al., 1997
	0.3 mg/ml in 10 mM phosphate, pH 7.0	UV (281 nm)	44	Lozano et al., 1997
	0.3 mg/ml in 10 mM phosphate, pH 8.0	UV (281 nm)	42	Lozano et al., 1997
Cytochrome c	2 mg/ml in 600 mM NaCl	DSC	77	Lo and Rahman, 1998
rhDNase	10 mg/ml in water, pH 6.8	DSC	67	Chan et al., 1996
Elastase	20 mg/ml in 10 mM acetate, pH 5.0	DSC	66	Chang et al., 1993
aFGF	100 $\mu$ g/ml in PBS, pH 7.2	CD (228 nm)	45	Volkin et al., 1993
bFGF	1 mg/ml in phosphate-citrate-borate, pH 4	DSC	50	Wang et al., 1996b
	1 mg/ml in phosphate-citrate-borate, pH 9	DSC	64	Wang et al., 1996b
GA (82 kD)	1.8 mg/ml in 50 mM phosphate, pH 6	DSC	64	Wang et al., 1996a
	1.8 mg/ml in 50 mM phosphate, pH 7	DSC	62	Wang et al., 1996a
	1.8 mg/ml in 50 mM phosphate, pH 8	DSC	58	Wang et al., 1996a
rhGH (22 kD)	0.18 mg/ml in 10 mM citrate, pH 6.0	CD (222 nm)	89	Bam et al., 1998
	1 mg/ml in 10 mM citrate, pH 6.0	CD (222 nm)	81	Bam et al., 1998
	3 mg/ml in 10 mM citrate, pH 6.0	CD (222 nm)	79	Bam et al., 1998
IgG (mouse)	3.3 mg/ml in 5 mM phosphate, pH 6.0	DSC	74	Vermeer et al., 1998
Recombinant IFN- $\beta$ -1a	100 $\mu$ g/ml in 100 mM Na <sub>2</sub> HPO <sub>4</sub> , 200 mM NaCl, pH 7.2	UV (280 nm)	67	Runkel et al., 1998
Recombinant IFN- $\beta$ -1a, deglycosylated	100 $\mu$ g/ml in 100 mM Na <sub>2</sub> HPO <sub>4</sub> , 200 mM NaCl, pH 7.2	UV (280 nm)	63	Runkel et al., 1998
Lysozyme	In 0.1 M NaCl, 0.1 M acetate, pH 5.4	CD	66	Shoichet et al., 1995
	In 50 mM citrate, pH 4.0	DSC	74	Liu and Sturtevant, 1996
	In 50 mM citrate and 1 M sucrose, pH 4.0	DSC	80	Liu and Sturtevant, 1996

Table 1 (continued)

Proteins	Protein solution compositions	Methods	$T_m$ (°C)	References
M-CSF	1.2 mg/ml in 20 mM PolyB buffer	DSC	87	Schrier et al., 1993
wt-hPAH (452 aa)	14–18 mg/ml in 20 mM HEPES buffer, pH 7.4	IR, peak ratio of 1619 to 1650 $\text{cm}^{-1}$	57	Chehin et al. 1998
RNase A	In 0.04 M glycine, pH 2.8	UV (287 nm)	41	Lin and Timasheff, 1996
	In 0.03 M MES, pH 5.8	UV (287 nm)	60	Lin and Timasheff, 1996
	In 0.03 potassium phosphate, pH 6.7	UV (287 nm)	64	Lin and Timasheff, 1996
	2 mg/ml in 600 mM NaCl	DSC	62	Lo and Rahman, 1998
	0.1 mg/ml in water	CD (222 nm)	65	Tsai et al., 1998a
	0.2 mg/ml in 20 mM citric acid, pH 2.3	CE	36	McIntosh et al., 1998
	0.2 mg/ml in 20 mM citric acid, pH 3.1	CE	49	McIntosh et al., 1998
	0.2 mg/ml in 20 mM citric acid, pH 3.1	CD (222 nm)	50	McIntosh et al., 1998
	About 2 mg/ml in 50 mM glycine, pH 2.8	DSC	48	Liu and Sturtevant, 1996
	About 2 mg/ml in 50 mM citrate, pH 6.0	DSC	65	Liu and Sturtevant, 1996
	About 2 mg/ml in 50 mM citrate and 1 M sucrose, pH 6.0	DSC	70	Liu and Sturtevant, 1996
	About 2 mg/ml in 50 mM citrate and 1 M glycine, pH 6.0	DSC	68	Liu and Sturtevant, 1996
RNsae H	25 $\mu\text{g/ml}$ in 5 mM HEPES and 0.8 M GdnHCl, pH 8	CD (222 nm)	30	Goedken and Marqusee, 1998
	25 $\mu\text{g/ml}$ in 5 mM HEPES, 0.8 M GdnHCl, and 1 mM $\text{MnCl}_2$ , pH 8	CD (222 nm)	41	Goedken and Marqusee, 1998
wt-RNase T1	About 1 mg/ml in 30 mM MOPS, pH 7	Optical rotation (295 nm)	48	Shirley et al., 1989
	About 0.2 mg/ml in 30 mM MOPS, pH 7	UV (286 nm)	48	Thomson et al. 1989
	About 0.2 mg/ml in 30 mM MOPS, pH 7	CD (238 nm)	47	Thomson et al., 1989
	About 0.2 mg/ml in 30 mM MOPS, pH 7	CD (284 nm)	48	Thomson et al., 1989
Thrombin	1 mg/ml in 25 mM phosphate and 400 mM NaCl, pH 6.5	DSC	57	Boctor and Mehta, 1992
	1 mg/ml in 25 mM phosphate and 400 mM NaCl, pH 7.9	DSC	54	Boctor and Mehta, 1992
	1 mg/ml in 25 mM phosphate and 400 mM NaCl, pH 9.9	DSC	48	Boctor and Mehta, 1992
TP40 (40kD)	0.1mg/ml, pH 7.2	Fluorescence	42	Sanyal et al., 1996
	0.1mg/ml, pH 7.2	CD	55	Sanyal et al., 1996
	1 mg/ml, pH 7.2	DSC	48	Sanyal et al., 1996
Trypsin	1.5 mg/ml in HCl solution, pH 2.5	DSC	52	Boctor and Mehta, 1992
YEI (240 kD)	2 mg/ml in 50 mM phosphate, pH 6.0	DSC	63	Wang et al., 1996a
	2 mg/ml in 50 mM phosphate, pH 7.0	DSC	52	Wang et al., 1996a
	2 mg/ml in 50 mM phosphate, pH 8.0	DSC	48	Wang et al., 1996a



There are seemingly two basic mechanisms responsible for enhanced thermal stability of thermophilic proteins. First, thermophilic proteins may possess structural characteristics different from those of mesophilic proteins. These structural characteristics include (1) increased hydrophobic interactions; (2) formation of extra hydrogen bonds and/or salt bridges; (3) more compact protein structures (may have crystal-like density); and (4) presence of few Cys, high content of Arg, and low content of Lys (Mozhaev and Martinek, 1984; Vieille and Zeikus, 1996; Vogt and Argos, 1997). The second mechanism of stabilization is favorable interactions of proteins with other cellular components or accumulated low-molecular-weight compounds/osmolytes as thermoprotectants. The interaction reduces the contact area of nonpolar fragments in proteins with water, leading to a decrease in free energy of the system and stabilization of proteins. These osmolytes include potassium salt, sugars,  $\alpha$ -glutamate, cyclic 2, 3-diphosphoglycerate (DPG), 2-*O*- $\beta$ -mannosylglycerate, di-glycerol-phosphate, and di-myo-inositol-1,1'(3,3')-phosphate (Mozhaev and Martinek, 1984; Huber et al., 1989; Rupley and Careri, 1991; Martins and Santos, 1995; Martins et al., 1997).

Is there a major force that contributes the most to stability of thermophilic proteins? Many thermophilic proteins show good correlation between stability and high hydrophobicity as measured by the hydrophobic index, a ratio of the volumes of polar to nonpolar amino acids (Mozhaev and Martinek, 1984). Detailed analysis indicates that protein stability depends not on total content of hydrophobic amino acid residues but on those inside the protein. Recent findings suggest that hydrogen bonding may play a major role in stabilizing thermophilic proteins. By comparing a group of thermostable proteins, Vogt and Argos (1997) recently concluded that increasing hydrogen bonding density at the protein surface is a major factor for increased thermal stability. After analyzing 16 protein families containing a total of 56 proteins from thermophilic, mesophilic, and thermophobic sources, Vogt et al. (1997) found that hydrogen bonding can provide the most general explanation for thermal stability in proteins

because over 80% of the protein families show correlation between thermostability and an increase in the number of hydrogen bonds.

Secondary structure also contributes to enhanced stability of thermophilic proteins (Vogt and Argos, 1997). The  $\alpha$ -helix and loops/turns are stabilized in thermophilic proteins. Ala residues (with high helix propensity) are prevalent in helical structures, and loops are often shorter in thermozymes (Vieille and Zeikus, 1996). Two facets of intrahelical interactions—the intrinsic helical propensities of amino acids and side chain interactions, are found to be the main contributors to protein thermostability, and an enhanced total stability of  $\alpha$ -helices is a general feature of many thermophilic proteins (Petukhov et al., 1997).

### 3. Protein instability and its influencing factors and analytical monitoring

One of the most troubling and challenging tasks in the development of liquid protein pharmaceuticals is to deal with their physical and chemical instabilities. The most common physical instability is protein aggregation, which can be induced and/or affected by a variety of factors and chemical transformations. Careful examination of these stability-influencing factors may help to prevent or mitigate certain stability problems. In addition, selection of proper and adequate analytical methods for efficient and accurate monitoring of protein instability may ensure successful development of quality protein products.

#### 3.1. Protein aggregation—a major event of physical instability

Under certain conditions (or simply with time), the secondary, tertiary, and quaternary structure of a protein may change and lead to protein unfolding and/or aggregation, a major event of physical instability. Protein aggregates may have no or reduced activity, reduced solubility, and altered immunogenicity. Presence of any insoluble aggregates in a protein pharmaceutical is generally not acceptable for product release.

### 3.1.1. Mechanisms of protein aggregation

Protein aggregation in many cases results from intermolecular association of partially denatured protein chains (Fields et al., 1992). Recent evidence suggests that aggregation may occur by specific interaction of certain conformations of protein intermediates rather than by nonspecific coaggregation (Speed et al., 1996). The aggregation process can be roughly divided into three steps: initiation, propagation, and termination (Roefs and De Kruif, 1994).

Proteins aggregate to minimize thermodynamically unfavorable interactions between solvent and exposed hydrophobic residues of proteins. Hydrophobic interaction, the reluctance of non-polar groups to be exposed to water, is considered to be the major driving force for both protein folding and aggregation. Both protein aggregation and folding represent a balance of exposed and buried hydrophobic surface areas (Patro and Przybycien, 1996). The balance is so delicate that a change of one amino acid in a protein may substantially change its aggregation behavior (Fields et al., 1992).

Protein aggregation may be induced by a variety of physical factors, such as temperature, ionic strength, vortexing, surface/interface adsorption, etc. These factors can increase the hydrophobic surface area of proteins, causing aggregation. For example, recombinant human keratinocyte growth factor (rhKGF) undergoes slow unfolding at elevated temperatures, leading to immediate aggregation and precipitation (Chen et al., 1994a). Recombinant porcine growth hormone (pGH) at 0.5 mg/ml precipitates with time at 63°C (Charman et al., 1993). Vortexing hGH (22 kD) solutions (0.5 mg/ml at pH 7.4) for 1 min denature 67% as insoluble aggregates (Katakam et al., 1995). These influencing factors will be discussed in detail in Section 3.3.

Protein aggregation may result from chemical degradations or modifications and subsequent exposure of the hydrophobic surface(s). Proteins can directly form covalent aggregates such as insulin (Strickley and Anderson, 1997) or aggregate indirectly such as human relaxin after oxidation of His and Met residues (Li et al., 1995a). Both physical and chemical aggregations may occur

simultaneously. For example, bFGF in citrate buffer (pH 5) forms intact and truncated dimers and trimers after storage at 25°C for 7 weeks (Shahrokh et al., 1994a). Freeze-dried  $\beta$ -galactosidase aggregates and forms insoluble precipitates via covalent disulfide bonding during storage, while non-covalent interaction causes aggregation but forms soluble precipitates in solution (Yoshioka et al., 1993). Protein aggregates, like proteins, may have isomeric forms such as IL-1ra dimers (Chang et al., 1996a).

Protein aggregation may start from a single protein molecule (unimolecular/intramolecular process) or more than one protein molecule (multimolecular/intermolecular process). Unimolecular processes include  $\beta$ -elimination and intrachain disulfide scrambling or formations such as IL-1ra in aqueous solution (Chang et al., 1996a). Examples of multimolecular aggregation include thiol-disulfide interchange for bovine serum albumin, thiol-catalyzed disulfide exchange for insulin, other covalent aggregation such as ribonuclease A, and non-covalent aggregation such as tetanus toxoid (Costantino et al., 1994a). If the percentage of precipitates/aggregates increases with increasing protein concentrations, multimolecular aggregation/precipitation processes may be involved such as aggregation of hIGF-I (Charman et al., 1993; Fransson et al., 1996).

Protein aggregation may or may not follow first-order kinetics (Pikal et al., 1991). The formation of IL-1ra dimers in aqueous solution during storage follows first-order kinetics (Chang et al., 1996a). Both heat and denaturant-induced aggregations of recombinant human interferon- $\gamma$  (IFN- $\gamma$ ) apparently follow first-order kinetics (Kendrick et al., 1998a,b). At 60°C, the aggregation/precipitation of IL-1 $\beta$  follows apparent first-order behavior to 30% drug remaining, but at or below 55°C, it deviates and becomes biphasic (slow and fast) (Gu et al., 1991). Biphasic aggregation has also been observed for aFGF in phosphate buffer (pH 7.4) at 50°C.

Many agents can be used to probe possible mechanisms of protein aggregation. Denaturants such as sodium dodecyl sulfate (SDS), GdnHCl, and urea can be used to determine whether protein aggregation is covalent in nature. If

protein aggregates can be dissolved in these agents, formation of these aggregates is probably non-covalent. Otherwise, covalent/chemical aggregation is suggested. Examples of non-covalent aggregation evidenced by this method include chymotrypsinogen (Allison et al., 1996), insulin (Sluzky et al., 1991), and IL-1 $\beta$  (Gu et al., 1991). Similarly, reducing agents such as DTT or  $\beta$ -mercaptoethanol can be used to determine any involvement of disulfide bonds in protein aggregation. Since these reducing agents may have limited dissolution capability, denaturants are often included for complete dissolution of protein aggregates.

Nevertheless, there are cases in which experimental results may lead to inaccurate or even incorrect interpretation. For example, the insoluble aggregates of hGH can be completely dissolved in either 1 or 2% SDS. SEC-HPLC analysis of the solubilized aggregates indicates presence of both protein monomers and dimers in 1% SDS, suggesting possible covalent aggregation, but only monomers are found in 2% SDS, suggesting non-covalent aggregation (Katakam et al., 1995). This example illustrates that denaturants may not be able to dissolve non-covalent protein aggregates depending on their concentrations. As a matter of fact, non-covalent insulin fibrils cannot be dissolved in 7 M urea from pH 2 to 8, 5 M GdnHCl, 50% acetonitrile, or detergents (Brange et al., 1997). The failure of these agents to dissolve insulin fibrils suggests that these substances do not have access to the interfaces of insulin molecules due to strong hydrophobic interactions in fibrils.

Protein aggregation may be probed by computer simulation. Agitation or hydrophobic surface-induced aggregation of insulin has been modeled by computer simulation and the model predicts rather reliably the effect of protein concentration, agitation rate, and hydrophobic surface area on insulin aggregation (Sluzky et al., 1991, 1992). Recently, the colloid aggregation process has been simulated by a cluster–cluster aggregation (CCA) process. The heat-induced aggregation of BSA at pH 7.0 seems to fit the reaction-limited CCA model; at pH 5.1, it fits diffusion-limited CCA model (Hagiwara et al., 1996).

### 3.1.2. Reversibility of Protein Aggregation

Protein aggregation can be reversible or irreversible. Generally speaking, aggregation is irreversible if the aggregates cannot be resolubilized in denaturing and reducing agents. The aggregation and precipitation of apomyoglobin formed in 0.5–4 M urea is reversible, as the precipitates can be dissolved in 8 M urea and further dilution leads to protein refolding (De Young et al., 1993). Thermally-induced protein aggregation is often irreversible. Thrombin in solution aggregates and loses its clotting activity irreversibly when heated to 85°C (Boctor and Mehta, 1992). Thermally-induced precipitation of recombinant pGH is irreversible (Charman et al., 1993).

What determines reversibility of protein aggregation? Patro and Przybycien (1996) simulated the structure of reversible protein aggregates as a function of protein surface characteristics and protein–protein interactions. They demonstrated that the structure and morphology of protein aggregates are profoundly affected by the surface characteristics of protein monomers. Both the extent and distribution of hydrophilic and hydrophobic sites on protein surface affect aggregate properties kinetically and thermodynamically. Although physical properties of reversible and irreversible aggregates are similar, reversible aggregates are more stable energetically, more ordered (or crystal-like), and less dense than irreversible counterparts. An increase in the extent of monomer hydrophobic surface area can result in decreased solvent-exposed hydrophobic surface area and lower free energy, forming preferably reversible aggregates.

### 3.1.3. Activity of protein aggregates

Protein aggregates usually have no or reduced activity. Bovine pancreas ribonuclease A (RNase A, 13.7 kD) at 1.5 mg/ml forms soluble aggregates in 0.1 M phosphate buffer (pH 10) during storage at 45°C, and the amount of aggregates corresponds qualitatively to loss of enzyme activity (Townsend and DeLuca, 1990). The specific antiviral activity of interferon- $\beta$ -1b (IFN- $\beta$ -1b) aggregates is three times lower than that of the intact protein (Runkel et al., 1998).

Table 2  
Favored pH conditions for some degradation reactions in proteins/peptides

Degradations	Proteins/peptides	Reaction sites	Favored pH conditions	References
Cleavage	bFGF	Asp-X, esp.X = Pro	Very acidic	Shahrokh, et al., 1994a
Deamidation	hEGF	Asn <sup>1</sup>	Neutral to alkaline	Son and Kwon, 1995
Deamidation	bFGF	Asn-X	Neutral to alkaline	Shahrokh, et al., 1994a
Deamidation	Insulin	Asn <sup>A21</sup>	pH < 5	Strickley and Anderson, 1997
Deamidation	RNase A Lysozyme	Asn-X, Gln-X	High pHs	Kristjánsson and Kinsella, 1991
Oxidation (by H <sub>2</sub> O <sub>2</sub> )	rhPTH	Met <sup>8</sup> and Met <sup>18</sup>	10	Nabuchi et al., 1995
Oxidation (by ascorbic acid/CuCl <sub>2</sub> /O <sub>2</sub> )	Relaxin	His <sup>A12</sup> , Met <sup>B4</sup> , Met <sup>B25</sup> , etc.	5 > 6 > 7 > 8	Li et al., 1995a
Oxidation (by ascorbic acid/FeCl <sub>3</sub> /O <sub>2</sub> )	His-Met	Met	7–8	Li et al., 1993
Succinimidation	bFGF	Asx-Gly	4–5	Shahrokh et al., 1994a

Protein aggregation may or may not accompany major conformational changes. Interleukin-1 receptor antagonist (IL-1ra) in solution can form non-covalent but irreversible dimers during storage, and the activity of the non-covalent dimer is about one-third of the native monomer, even though the dimer has almost the same structure as the native form by CD and IR spectroscopy (Chang et al., 1996a).

### 3.2. Chemical instabilities of proteins

Many chemical reactions are responsible for inactivation of protein drugs. In many cases, a couple of reactions can happen simultaneously in proteins, making separation and identification of protein degradation products very difficult. For example, bFGF undergoes multiple degradations in solution, including multimerization, succinimidation, hydrolysis, and aggregation (Wang et al., 1996b). Lyophilized insulin, after forming a cyclic anhydride intermediate at low pH, may react with water to form desamido insulin or react with other amino acids to form two different dimers (Asp<sup>A21</sup>-Phe<sup>B1</sup> and Asp<sup>A21</sup>-Gly<sup>A1</sup>) (Strickley and Anderson, 1997). The eventual degradation product distribution (between desamido insulin or covalent dimers) may depend on water content of

the formulation, pH of the reconstituted solution, and nature of the excipients in the formulation. Many proteins show changes in degradation pathways at different pHs and temperatures. The degradation products (aggregates and C-terminal fragments) of recombinant human macrophage colony-stimulating factor (M-CSF) change significantly at different pHs (Schrier et al., 1993). Recombinant human thrombopoietin (rhTPO) shows different shapes of  $k_{\text{obs}}$ -pH profiles and different patterns of bands on SDS-PAGE at 5, 30, and 37°C (Senderoff et al., 1996). Therefore, to prevent proteins from chemical inactivation, the dominant reaction should first be identified and inhibited. This can be achieved to a certain degree by adjusting the formulation pH away from favorable ranges (Table 2).

The location of labile amino acids in a protein is critical in determining their chemical reactivity. Since a very limited number of water molecules exist inside a protein, presence of chemically labile amino acids may not be a problem if they are localized inside and have limited mobility (Mozhaev and Martinek, 1984). Chemical reactions of many amino acids in proteins require certain local molecular flexibility; and thus the rate of reaction may be higher in denatured proteins or small peptides with high flexibility

than in native proteins. For example, the reactivity (disulfide exchange) of the sulphhydryl group in  $\beta$ -lactoglobulin-A is very slow in its native state but increases significantly when the protein gradually unfolds with increasing urea concentration from 4 to 8 M (Apten, 1998). Native protein conformation, therefore, needs to be protected to prevent or inhibit potential chemical degradations.

Powell (1996) tried to correlate the reactivity of protein deamidation, hydrolysis, and oxidation in aqueous solutions with protein sequence structure by analyzing the hydrophathy/flexibility (hydroflex) plots for 73 proteins. The analysis shows that the primary reaction in proteins at pH 4.5–7.5 occurs largely at Asn and Asp within these motifs: -Asn-Gly-, -Asn-Ser-, -Asp-Gly-, and, to a lesser extent, -Gln-Gly-, -Asp-Pro-, and -Met- (the hot-spot sites). Reactions at non-hot-spot sites can also occur if the site conformation is favorable (hydrophilicity and flexibility) or if the reaction is catalyzed by contaminating proteases. Hydrophathy appears to be a better predictor of protein degradation than does calculated flexibility.

Chemical reactions may not always affect protein conformation or activity depending on the location of transformed residues. Due to the terminal location of Met<sup>B4</sup> and Met<sup>B25</sup> in recombinant human relaxin, oxidation at these two residues does not change the protein bioactivity (Nguyen et al., 1993). The Met<sup>1</sup> mono-oxidized recombinant human leptin (16 kD) does not show any detectable changes in tertiary structure and retains its full potency as compared to native form (Liu et al., 1998). Similarly, when 63% of human epidermal growth factor (hEGF, 6 kD) is deamidated, its mitogenic activity is completely unchanged, indicating that the deamidation site is not critical in determining the mitogenic activity. Other deamidated products having essentially the same biological activity as the intact proteins include insulin (Brange et al., 1992b) and rIL-2 (Sasaoki et al., 1992). In some cases, degraded proteins may have even higher activities such as rhTPO during storage at 5, 30, and 37°C (Senderoff et al., 1996).

### 3.2.1. Deamidation

Deamidation appears to be the most common degradation in protein pharmaceuticals. In many cases, it is a major degradation pathway in proteins, such as recombinant human deoxyribonuclease (rhDNase) (Shire, 1996; Chen et al., 1998) and rhVEGF at pH 5–6 (Goolcharran et al., 1998). The deamidated rhDNase has only about 40–50% of the original activity.

Several deamidation mechanisms have been reported and discussed (Cleland et al., 1993; Xie and Schowen, 1999). Asn and Gln are the two amino acids susceptible to deamidation in proteins and Asn is much more labile (Powell, 1994; Li et al., 1995b; Daniel et al., 1996). Deamidation of Asn in proteins and peptides in an aqueous solution can proceed at a much higher rate than hydrolysis of a peptide bond (Daniel et al., 1996).

The rate, mechanism, and location of deamidation in peptides or proteins are pH-dependent. Deamidation of Asn-X appears favored mostly at neutral or alkaline conditions (Son and Kwon, 1995; Daniel et al., 1996). Maximum stability of Asn residues within peptides is found between pH 2–5. Between pH 5 and 12, the reaction proceeds rapidly and entirely through a cyclic imide (succinimide) intermediate, while slow deamidation at pH 1–2 seems to bypass the succinimide intermediate (Daniel et al., 1996). The rate of deamidation at Asn<sup>74</sup> in bovine pancreatic DNase increases with increasing pH from 5.0 to 7.7 (Shire, 1996). In insulin, however, deamidation originates from a rate-limiting intramolecular nucleophilic attack at the C-terminal Asn<sup>A-21</sup> with a cyclic anhydride intermediate to form desamido insulin (Brange et al., 1992b; Darrington and Anderson, 1995; Strickley and Anderson, 1997). Since the unionized carboxyl group at the C terminal is the catalyzing group, increasing solution pH inhibits deamidation of Asn<sup>A-21</sup>. Therefore, the reaction is favored at low pH (< 5). In neutral solutions, deamidation in insulin takes place predominantly at Asn<sup>B-3</sup> residue (Brange et al., 1992b; Darrington and Anderson, 1995).

The relative position of Asn and/or Gln in proteins may determine their relative rate of deamidation. hEGF has three deamidation

residues (Asn<sup>1</sup>, Asn<sup>32</sup>, and Gln<sup>43</sup>) and Asn<sup>1</sup> is the most labile one in neutral and alkaline pH due to its relatively high mobility in the protein (Son and Kwon, 1995). In tPA, only three Asn-X sequences (Asn<sup>58</sup>-Gly<sup>59</sup>, Asn<sup>77</sup>-Ser<sup>178</sup>, and Asn<sup>37</sup>-Ser<sup>38</sup>) are deamidated; and all three sites are located on the protein surface and have more-than-average local sequence mobility (Paranandi et al., 1994).

Neighboring amino acids at deamidation sites in peptides or proteins may affect the rate of deamidation. Cross and Schirch (1991) have studied the rate of non-enzymatic deamidation of a series of pentapeptides. They have found that in neutral and alkaline solutions, amino acid residues on the amino side of Asn have little or no effect on the rate of deamidation regardless of its charge or size, but residues on the carboxyl side of Asn affect the rate significantly. Increasing the size and branching in the side chain of the residue decreases the rate of deamidation as much as 70-fold relative to Gly. Therefore, the most labile sequence seems to be Asn-Gly (Powell, 1994, 1996). However, under acidic conditions, the rate of deamidation of Asn residue is not affected by neighboring amino acids in these pentapeptides (Cross and Schirch, 1991). In tPA, 7 Asn-X sequences are identified to have more-than-average local sequence mobility, but only three of them show detectable deamidation, suggesting possible influence of neighboring amino acids on the rate of deamidation in addition to local flexibility (Paranandi et al., 1994). Local flexibility/mobility of proteins can be plotted for comparison based on hydrophobicity–volume product for consecutive quintuplets of amino acid residues (Ragone et al., 1989).

The rate of deamidation in proteins is also influenced by secondary structure of proteins. Xie and Schowen (1999) recently reviewed secondary structure and protein deamidation and indicated that both  $\alpha$ -helical and  $\beta$ -turn secondary structures tend to stabilize Asn residues against deamidation. A good example is the deamidation of Asn<sup>8</sup> in several growth hormone releasing factor (GRF) analogues, which decreases with increasing helical content in aqueous methanol solutions (Stevenson et al., 1993).

### 3.2.2. Oxidation

The side chains of His, Met, Cys, Trp, and Tyr residues are potential sites of oxidation (Manning et al., 1989; Li et al., 1995b,c; Daniel et al., 1996). Oxidation at these sites can be catalyzed by trace amount of transition metal ions (site-specific process) or enhanced by oxidants or upon exposure to light (non-site-specific process). The site specificity is due to generation of and oxidation by reactive oxygen species at specific metal-binding sites (Li et al., 1995a; Zhao et al., 1997). The complicated oxidation mechanisms and related major oxidation products have been reviewed elsewhere (Cleland et al., 1993; Stadtman, 1993; Li et al., 1995c).

The most easily oxidizable sites are thio groups on Met and Cys. Met residues in proteins can be easily oxidized by atmospheric oxygen such as hGH in a vial containing only 0.4% oxygen. The easy oxidation of Met residue occurs probably because Met (also Gln) is one of the most flexible of nonpolar or mildly polar residues in proteins (Richards, 1997). Nevertheless, it was concluded that oxidation at Met residues in most proteins is not as significant as deamidation and hydrolysis (Powell, 1996).

The thioether group of Met can be oxidized into two possible forms: sulfoxide and sulfone. Met is first oxidized reversibly to sulfoxide, which can be further oxidized irreversibly to sulfone under harsh conditions. Like deamidation, the rate of oxidation depends on the position of oxidizable groups in a protein. For example, Met sulfoxides can form at both Met<sup>18</sup> and Met<sup>8</sup> in recombinant human parathyroid hormone (rhPTH) in the presence of H<sub>2</sub>O<sub>2</sub>, but Met<sup>18</sup> is more susceptible to oxidation (Nabuchi et al., 1995). There are four oxidizable Met residues in recombinant human leptin, and their relative reactivity is Met<sup>1</sup> > Met<sup>69</sup> > > Met<sup>55</sup>  $\cong$  Met<sup>137</sup> (Liu et al., 1998).

The formulation pH may affect the rate of oxidation by changing the oxidation potential of oxidants, the affinity of binding between catalytic metal ions and the ionizable amino acids, and the stability of oxidation intermediates. In a few cases, the rate of oxidation is enhanced at alkaline pHs. rhPTH is oxidized most at pH 10 by H<sub>2</sub>O<sub>2</sub>

(Nabuchi et al., 1995). The relative rate of oxidation of human relaxin in ascorbate/Cu (II) solution is  $\text{pH } 8 > 7 > 6 > 5$  (Li et al., 1995a). The oxidation rate of human insulin-like growth factor I (hIGF-I, 7.65 kD) in solution increases as the solution pH is increased from 6.0 to 7.5. The pH dependence of hIGF-I oxidation was attributed to formation of a phosphorylated sulfonium ion between Met and phosphate as an oxidation intermediate (Fransson and Hagman, 1996). In a different case, changing the solution pH in the range of 3–8 fails to change significantly the rate of oxidation of recombinant human relaxin by  $\text{H}_2\text{O}_2$  (Nguyen et al., 1993).

Oxidation could dominate other degradation pathways in a protein. There are several potential degradation reactions in hIGF-I, including oxidation of Met<sup>59</sup>, deamidation of Asn<sup>26</sup> and Gln<sup>15</sup>, and reduction of three disulfide bridges (Cys<sup>6</sup>-Cys<sup>48</sup>, Cys<sup>47</sup>-Cys<sup>52</sup>, and Cys<sup>18</sup>-Cys<sup>61</sup>) and oxidation of Met is found to be the major degradation pathway (Fransson et al., 1996). Similarly, oxidation is the major degradation pathway for KGF-2 (Kaushal et al., 1998).

### 3.2.3. Disulfide bond breakage and formation

Disulfide bonds are often critical in controlling both protein activity and stability.  $\alpha$ -Interferon has two disulfide crosslinks between 1 and 98 and between 29 and 138. Selective breaking of the 1–98 crosslink results in no loss of activity, while breaking of the other results in almost complete loss of activity (Evans and Grassam, 1986). Ribonuclease T1 has two disulfide bonds, which stabilize the protein. When the two disulfide bonds are broken, its  $T_m$  is reduced by about 40°C and its conformational stability is decreased by over 9 kcal/mol (Thomson et al., 1989).

Free Cys residues in proteins can be oxidized easily to form disulfide bond linkages or cause thio-disulfide exchanges, causing protein aggregation or polymerization, such as basic fibroblast growth factor (bFGF) (Shahrokh, et al. 1994a; Wang et al., 1996b). However, if these cysteinyl residues are buried within the tertiary structure of proteins, they are much less reactive without much concern. Thio-disulfide exchange in a

protein is a reaction between an ionized thiol group (thiolate anion) and a disulfide bond. The rate of thiol-disulfide exchange depends on the extent of ionization of the nucleophilic thiol, and therefore, generally increases as the reaction pH increases until pK of the nucleophilic thiol group is exceeded (Darby and Creighton, 1997).

Even though a protein does not have free Cys residues, disulfide bond scrambling may still occur, causing protein aggregation. This is the case for lyophilized insulin, which has only three disulfide bonds but still forms disulfide-scrambled aggregates via  $\beta$ -elimination of an intact disulfide during storage (Costantino et al., 1994b).  $\beta$ -Elimination was also observed for IL-1ra in aqueous solution (Chang et al., 1996a).

### 3.2.4. Hydrolysis

Amino acids, the components of proteins, are subject to acid and base hydrolysis. Most peptide bonds are stable except those in -X-Asp-Y- sequence (Manning et al. 1989; Li et al., 1995b; Vieille and Zeikus, 1996). The Asp-Y bond may be at least 100 times more labile than other peptide bonds in dilute acid (Li et al., 1995b). Cleavage is particularly rapid at Asp-Gly and Asp-Pro (Powell, 1994, 1996). A major degradation pathway of rhM-CSF in an acidic solution is peptide cleavage at two sites: Asp<sup>169</sup>-Pro<sup>170</sup> and Asp<sup>213</sup>-Pro<sup>214</sup> (Schrier et al., 1993). During hydrolysis, Asp forms succinimide intermediate, which is similar to that for Asn (Daniel et al., 1996). In addition, formation of cyclic anhydride intermediate is also possible, especially when the C-flanking residue of Asp is Pro such as bFGF (Shahrokh et al., 1994a).

In many cases, hydrolysis is a continuation after deamidation of Asn residues. Insulin first forms *iso*-Asp and Asp derivatives at Asn<sup>A-21</sup> and/or Asn<sup>B-3</sup> depending on the solution pH during storage. These derivatives are further hydrolyzed and the rate of hydrolysis at B3 position is independent of the insulin strength between 40 and 400 IU/ml (Brange et al., 1992b).

### 3.2.5. Isomerization

All amino acids have the potential of racemization except Gly. Natural or deamidation-derived

Asp-X peptide bonds can easily undergo a reversible isomerization between Asp and *iso*-Asp via a cyclic imide (succinimide) intermediate. The succinimide intermediate is usually not stable, and significant hydrolysis may occur in hours such as tissue plasminogen activator (tPA) (Paranandi et al., 1994).

Many cases of protein isomerization have been reported, such as bFGF (Shahrokh et al., 1994a), insulin (Brange et al., 1992b), and tPA (Paranandi et al., 1994). Incubation of tPA at pH 7.3 and 37°C for 14 days can result in formation of 0.77 mol of *iso*-aspartate derivative per mol of tPA due to multiple deamidations at Asn<sup>58</sup>-Gly<sup>59</sup>, Asn<sup>177</sup>-Ser<sup>178</sup>, and Asn<sup>37</sup>-Ser<sup>38</sup> (Paranandi et al., 1994).

Like deamidation, the rate of Asp isomerization is strongly influenced by its location and mobility in a protein. The *iso*-aspartate formation is most likely to occur in relatively unstructured domains of intact proteins or in domains susceptible to transient unfolding (Johnson et al., 1989). Methionyl recombinant human growth hormone (met-hGH) at pH 7.4 forms isoaspartate derivatives only at Asp<sup>130</sup> (Asp-Gly) and Asn<sup>149</sup> (Asn-Ser) during storage at 37°C, even though a couple of Asn and Asp residues exist in the protein. The selective *iso*-aspartate formation at Asp<sup>130</sup> occurs because Asp<sup>130</sup> resides in a domain, which has conformational flexibility similar to that of a short synthetic peptide (Johnson et al., 1989).

### 3.2.6. Succinimidation

Formation of succinimide intermediates may precede deamidation of Asn and isomerization of Asp in proteins and peptides. In fact, formation of succinimide is the cause of *iso*-aspartate derivative formation in proteins. Asn deamidates via succinimide formation at neutral and alkaline conditions, but formation of succinimide at Asp-Gly linkages in proteins may occur at an optimum pH of 4–5, such as bFGF (Shahrokh et al., 1994a).

Like other chemical degradations, the rate of succinimide formation is strongly influenced by neighboring groups of the labile residue(s) and protein conformation. Stephenson and Clarke

(1989) studied the rate of succinimide formation in a series of synthetic peptides (Val-Tyr-Pro-X-Y-Ala) at pH 7.4. They demonstrated that the rate of succinimide formation of the asparaginyl (X) peptides is 13.1–35.6 times faster than those of the aspartyl peptides when Y is glycyl, seryl, or alanyl residue. The relative rate of succinimide formation is glycyl > seryl > alanyl peptides. Recently, Gietz et al. (1998) identified two degradation products in hirudin, which are succinimides at Asp<sup>53</sup> and Asp<sup>33</sup>. Both of these two sites are located in two flexible segments of the molecule.

### 3.2.7. Non-disulfide crosslinking

Proteins may form covalent dimers or polymers by non-disulfide pathways. Insulin has been shown to form such dimers and polymers during storage (Brange et al., 1992a). The rate-limiting generation of a cyclic anhydride intermediate is involved in formation of both dimeric and deamidated insulin (Darrington and Anderson, 1995). The covalent insulin dimer appears to form mainly through transamidation reactions involving Asn<sup>A-21</sup> and Phe<sup>B-1</sup> residues. The relative amounts of the two insulin reaction products (deamidated and dimeric insulin) changes with pH between 2.0 and 5.5. Increasing the pH decreases the formation of [desamido<sup>A-21</sup>] insulin with a concurrent increase in desamido<sup>A-21</sup>-Phe<sup>B-1</sup> dimer. The formation of insulin dimer is negligible below pH 4 at insulin concentration of about 10 μM, increases sharply with increasing pH, and becomes dominant near pH 5. Recently, it has been found that the C-terminal Asn in insulin, after forming a cyclic anhydride intermediate, may form two different dimers with either Phe (Asp<sup>A21</sup>-Phe<sup>B1</sup>) or Gly (Asp<sup>A21</sup>-Gly<sup>A1</sup>) in both aqueous and lyophilized formulations (Strickley and Anderson, 1997).

Another non-disulfide pathway is the formaldehyde-mediated crosslinking proposed by Schwendeman et al. (1995). This crosslinking pathway apparently causes significant aggregation of lyophilized tetanus and diphtheria toxoids during storage and thus raises concerns for storing formalin-treated vaccines in both liquid and solid forms.



### 3.2.8. Deglycosylation

One of the functions of carbohydrate moieties in proteins is to protect proteins from thermal and hydrolytic inactivation (Lis and Sharon, 1993). The effect of glycosylation on stability of proteins is highly protein-dependent. Wang et al. (1996a) studied the effect of deglycosylation on the stability of five glycoproteins, including yeast external invertase, bovine serum fetuin, glucoamylase from *Aspergillus niger*, chicken egg white ovotransferrin, and avidin. The amounts of saccharides in these proteins vary from 2.2 to 50%. Deglycosylation of the five model glycoproteins decreases the protein stability as evidenced by decreased  $T_m$  and denaturation enthalpy ( $\Delta H$ ), even though protein conformations are not significantly affected.

Natural IFN- $\beta$  is a 166 amino acid glycoprotein and has multiple functions. Runkel et al. (1998) demonstrated that deglycosylation of recombinant IFN- $\beta$ -1a with PNGase F decreases the protein activity, and the loss of activity is primarily due to formation of insoluble aggregates upon carbohydrate removal. Deglycosylation also makes the protein more sensitive to thermal denaturation. At a protein concentration of 100  $\mu\text{g/ml}$ , deglycosylated IFN- $\beta$ -1a denatures at 63°C while glycosylated IFN- $\beta$ -1a denatures at 67°C. Similarly, RNase A at 85  $\mu\text{M}$  in 50 mM Tris-HCl buffer (pH 8.0) unfolds at 60.4°C while RNase B, a carbohydrate derivative at Asn<sup>34</sup>, unfolds at 61.9°C under the same condition (Arnold and Ulbrich-Hofmann, 1997).

### 3.2.9. Maillard reaction

Sugars are often used as protein stabilizers in both liquid and solid formulations (see Section 4.2). Unfortunately, reducing sugars may react with amino groups in proteins forming carbohydrate adduct, especially at high temperatures. This extremely complex browning pathway is known as the Maillard reaction (Paulsen and Pflughaupt, 1980).

Maillard reactions are widely present in the food industry (Chuyen, 1998). Thermal treatment of milk easily leads to formation of  $\beta$ -lactoglobulin-lactose adduct via Lys residues (Fogliano et al., 1998). Although there have not been many reports on Maillard reactions in the development

of liquid protein pharmaceuticals, this reaction may present potential formulation issues. Tarelli et al. (1994) demonstrated that lysine vasopressin undergoes rapid glycation in the presence of reducing sugars in both aqueous and solid formulations and that the N-terminal adduct can form rapidly even at  $-20^\circ\text{C}$ .

## 3.3. Factors affecting protein stability

Protein stability is a result of balancing between destabilizing and stabilizing forces. The destabilizing forces are mainly due to the large increase in entropy of unfolding, and the stabilizing forces are provided by a few non-covalent interactions as discussed in Section 2.1. Disruption of any of these interactions will shift the balance and destabilize a protein. Many factors can disrupt this delicate balance.

### 3.3.1. Temperature

The most important factor affecting protein stability is temperature. Unfortunately, there is no general mechanism to describe the effect of temperature on the structure and function of proteins due to their structural complications. In general, the higher the temperature, the lower the protein stability.

Proteins are usually stable in a certain temperature range. Several globular proteins, including basic pancreatic trypsin inhibitor,  $\alpha$ -chymotrypsin, cytochrome c, lysozyme, metmyoglobin, RNase A, and trypsin, have maximum free energy change  $\Delta G_{f \rightarrow u}$  (8–17 kcal/mol) in a temperature range between 10 and 40°C, indicating that both high and low temperatures outside this range may destabilize or denature the proteins (Jaenicke, 1991). Under 3000 bar (to prevent freezing), RNase undergoes both cold ( $-22^\circ\text{C}$ ) and heat (40°C) denaturation (Zhang et al., 1995). Similarly, two extreme denaturation temperatures, lower than 0°C and higher than 50°C, exist for serum albumin in solution (Kosa et al., 1998).

Thermodynamically, protein stability comes from two large but opposing forces: enthalpic and entropic forces. Both are temperature-dependent. The enthalpic forces (the intramolecular interac-

tions) are stabilizing, and the entropic forces (the dissipative forces, loss of conformational entropy of native state) are destabilizing (Kristjánsson and Kinsella, 1991). High temperature-induced loss of protein stability ( $\Delta\Delta G < 0$ ) seems to result from a decrease in free energy of the denatured state instead of an increase in free energy of the native state (Shortle, 1996). The free energy of the denatured state decreases rapidly with increasing temperature mainly due to increased entropy ( $-T\Delta S$ ). When temperature increases, electrostatic interactions are not affected much, hydrogen bonding is weakened, and hydrophobic interactions are strengthened (to a certain point) due to its entropic origin (Jaenicke, 1990). Hydrophobic interaction increases until the temperature reaches about 110°C, when water does not solvate non-polar groups in proteins (Jaenicke, 1990; Kristjánsson and Kinsella, 1991). The  $\Delta H$  of protein unfolding also increases with increasing temperature and changes from negative to positive (Jaenicke, 1990). Therefore, low temperature denaturation is mainly enthalpy-driven (Jaenicke, 1991; Shortle, 1996).

High temperatures cause denaturation of many protein pharmaceuticals. Examples include the rapid formation of bovine insulin fibrils (Brange et al., 1997), aggregation/precipitation of rhKGF (Chen et al., 1994a), and precipitation of pGH (Charman et al., 1993). High temperature is also responsible for almost complete aggregation of recombinant consensus  $\alpha$ -interferon (rConIFN) at 0.5 mg/ml after ultrasonic nebulization in a Microstat device for 10 min (Ip et al., 1995).

Protein denaturation at high temperatures can be reversible depending on experimental conditions. Both thermal and chemical (urea) denaturation of muscle acylphosphatase (AcP, 98 aa) are reversible (Chiti et al., 1998). Patatin, the major potato tuber protein (40 kD), unfolds at a high temperature (91°C) but refolds partially upon cooling (20°C). Heating native serum albumin to 85°C causes reversible denaturation (approximately 80–90% recovery), but heating for a longer period (10 h) at 60°C (pasteurization condition) causes irreversible denaturation (Kosa et al., 1998).

High temperatures also accelerate chemical degradations, such as increased hydrolysis of Asp residues, deamidation of Asn or Gln residues in RNase A and lysozyme at high temperatures (Kristjánsson and Kinsella, 1991), and formation of covalent insulin oligo- and polymers at temperatures  $\geq 25^\circ\text{C}$  (Brange et al., 1992a). Degradation mechanisms in proteins easily change with temperature. At 60°C, the aggregation of interleukin 1 $\beta$  (IL-1 $\beta$ ) in aqueous solution follows apparent first-order behavior to 30% drug remaining, but at or below 55°C, the aggregation deviates from apparent first-order and becomes biphasic (slow and fast) (Gu et al., 1991).

### 3.3.2. Formulation pH

At extreme pHs, far away from  $pI$  of proteins, electrostatic repulsions between like charges in proteins increase, resulting in a tendency to unfold (Goto and Fink, 1989; Volkin and Klibanov, 1989; Dill, 1990). The process of unfolding leads to a reduction of charge density, thus lowering electrostatic free energy (Chan and Dill, 1991). A minor factor contributing to the unfolding tendency is the decreased capability of salt bridge formation at extreme pHs. In addition, the  $pK_a$  values of charged groups in the folded state are different from those in the unfolded state. Changes in pH lead to titration of groups only in the unfolded form, which causes destabilization of overall native protein structure (Chiti et al., 1998). pH-dependent unfolding has been found to be due only to a small number of groups with anomalous  $pK_a$ 's (Dimitrov and Crichton, 1997). These ionizable groups with anomalous  $pK_a$ 's may stabilize or destabilize a protein in different pH ranges, as those in hen egg-white lysozyme (Yang and Honig, 1993).

Proteins are often stable in a narrow pH range such as pH 6.5–7.0 for recombinant factor VIII SQ (FVIII SQ) (Fatouros et al. 1997a); 6–7 for low molecular weight urokinase (LMW-UK, 33 kD) (Vrkljan et al., 1994); and 4.5–5 for relaxin (Nguyen and Shire, 1996). Protein refolding to a wrong conformation is often seen at a pH close to its  $pI$  (Kristjánsson and Kinsella, 1991). pH-induced denaturation of proteins can be reversible, such as porcine pancreatic elastase, which dena-

tures on lowering the pH to less than 5.0 but renatures completely by immediately adjusting the pH back to 5.0 (Chang et al., 1993).

Certain proteins may have stable folded structures at extreme pHs. Apomyoglobin has five conformational states: native (N), acidic (A), acid-unfolded ( $U_A$ ), basic (B), and base-unfolded ( $U_B$ ) (Goto and Fink, 1989). Apomyoglobin starts to unfold at pH 5 with loss of helical structure and exposure of Trp residues as monitored by CD and fluorescence. At pH 2, the protein is maximally unfolded ( $U_A$ ) to an extent similar to that unfolded in 6 M GdnHCl. Further increase in HCl concentration results in refolding to A state. The conformation of  $U_A$  state varies from fully unfolded to a partially folded structure (Goto and Fink, 1994). The acidic transition under low salt conditions is reversible ( $N \rightleftharpoons U \rightleftharpoons N$ ), while reversibility of the alkaline transition depends on the length of time under high-pH conditions (Goto and Fink, 1989).

The rate of protein aggregation can be strongly affected by pH, such as the aggregation of bFGF (Wang et al., 1996b), deoxy hemoglobin (Kerwin et al., 1998), IL-1 $\beta$  (Gu et al., 1991), human relaxin (Li et al., 1995a), and bovine pancreas RNase A (Townsend and DeLuca, 1990; Tsai et al., 1998a). At pH 2.5, bovine insulin forms a significant amount of fibrils within a few hours at 37°C but is very stable without agitation for many months at pH 7. As pH increases from <2 to 7, monomeric insulins gradually form dimeric, tetrameric, and eventually hexameric insulins. The monomeric form has the highest tendency to form fibrils due to its large exposed hydrophobic area as compared with other forms (Brange et al., 1997).

Formulation pH, like temperature, may affect both physical and chemical stability of proteins. Different chemical degradations may be facilitated at different pHs (Table 2). This explains why degradation products are different at different pHs for the same protein, such as hirudin (Gietz et al., 1998). Hydrolysis can easily occur at Asp residues under mild acidic condition (Volkin and Klivanov, 1989). Deamidation of Asn and Gln residues readily takes place under strongly acidic, neutral, and basic conditions. Under basic

conditions, many reactions can occur, such as peptide bond hydrolysis, deamidation, hydrolysis of Arg to ornithine,  $\beta$ -elimination and racemization, and double bond formation. The pH effect on chemical stability may depend on the presence of other excipients. For example, a V-shaped chemical (oxidation and deamidation) degradation-pH profile has been demonstrated for hGH with minimum degradation at a pH of about 7.5, but the minimum is shifted to a pH of about 7 when formulated with dextran 40 (Pikal et al., 1991).

The destabilizing effect of pH on proteins can be estimated by determining of a variety of parameters. These parameters include (1)  $\Delta G_{f \rightarrow u}$  of proteins such as RNase T1 (Thomson et al., 1989), (2) hydrodynamic radius of proteins such as  $\beta$ -lactamase (28.5 kD) (Goto and Fink, 1989), and (3)  $T_m$  of proteins such as yeast external invertase (YEI), bovine serum fetuin, glucoamylase (Wang et al., 1996a), myoglobin (Chan and Dill, 1991), rhDNase (Chan et al., 1996), bFGF (Wang et al., 1996b), RNase A (Liu and Sturtevant, 1996; McIntosh et al., 1998), and thrombin (Boctor and Mehta, 1992). The pH effect on protein function and stability can also be probed by computerized molecular dynamics simulation (Baptista et al., 1997).

### 3.3.3. Adsorption

Proteins can be adsorbed to many surfaces and interfaces, such as container surfaces, and air/water interfaces. Protein adsorption in air/water interfaces starts with creation of an area for anchoring the protein molecule, followed by subsequent reorientation and rearrangement of the adsorbed molecules at the interface (Damodaran and Song, 1988). The severity of adsorption is protein-dependent and does not seem to depend on the size and pI of proteins (Burke et al., 1992). Evidence suggests that secondary structure of a protein may change significantly at the adsorption surface such as IgG (Vermeer et al., 1998). Therefore, surface adsorption may result in loss and/or destabilization of proteins. At 1  $\mu$ g/ml, the adsorptive loss of aFGF to surfaces of several containers (untreated glass, siliconized glass, sulfur-treated glass, Purcoat glass, polyester,

polypropylene, and nylon) are in the range of 20–40% or 0.2–0.8  $\mu\text{g protein}/\text{cm}^2$  (Volkin and Middaugh, 1996). Interleukin-2 (IL-2), at 0.1 mg/ml in 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl, has been shown to lose about 90% of its activity after being pumped through silicone rubber catheter tubing ( $60 \times 0.1$  cm) in 24 h (Tzannis et al., 1996). The majority ( $> 80\%$ ) of the activity loss is due to adsorption-induced protein denaturation (Tzannis et al., 1997).

Protein surface adsorption is usually concentration-dependent and may reach a maximum (at least for certain proteins) above certain protein concentrations. The adsorption of bovine serum albumin (BSA) is linearly proportional to its concentration (up to 0.5%) in membrane filters made of pleated nylon, polyvinylidene difluoride, or cellulose acetate. Burke et al. (1992) examined surface adsorption of six proteins, including alcohol dehydrogenase (ADH),  $\beta$ -amylase, lactate dehydrogenase (LDH),  $\alpha$ -chymotrypsinogen A, thyroglobulin, and IgG. Except LDH, adsorption of all other proteins to surfaces of containers (untreated glass, nylon, polyester + 0.3%, and polypropylene) appears saturated at approximately 5  $\mu\text{g}/\text{ml}$  at a surface/solution volume ratio of 2.4  $\text{cm}^2/\text{ml}$ . At this concentration, the amount of protein adsorbed is less than 0.5–1  $\mu\text{g}/\text{ml}$  (about 10–15% of the total protein).

The type of container or membrane significantly influences protein adsorption to its surface. The adsorption of 14 model proteins has been examined in a variety of containers made of untreated glass, siliconized glass, sulfur-treated glass, Purcoat-treated glass, nylon, polypropylene, polyester 5X0, and polyester + 0.3%. Among these different containers, the untreated glass, siliconized glass, and polypropylene seem to have the least protein binding to their surfaces, and sulfur-treated glass and polyester containers seem to have higher adsorption for certain proteins (Brose and Waibel, 1996). Storing 1 ml of transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) at 1.0  $\mu\text{g}/\text{ml}$  in 30 mM citrate buffer (pH 2.5) at 4°C for 11 days drops the protein concentration to 0.39, 0.37, and  $< 0.10$   $\mu\text{g}/\text{ml}$ , respectively, in polypropylene, siliconized glass, and untreated glass containers due to surface adsorption (Gom-

botz et al., 1996). BSA can be adsorbed to pleated nylon filter at 220  $\mu\text{g}/\text{cm}^2/\text{wt}\%$ , but at 120  $\mu\text{g}/\text{cm}^2/\text{wt}\%$  to filters made of polyvinylidene difluoride (PVDF) and cellulose acetate (CA).

The rate of protein adsorption can be very rapid. At 22 and 45°C, the percentages of recombinant human granulocyte colony-stimulating factor (rhG-CSF) adsorbed to PVC surface are, respectively, 93 and 97% of maximum after 10 min of equilibration (Johnston, 1996).

#### 3.3.4. Salts

The effect of salts on protein stability is complex, partly because of the complex ionic interactions on fully exposed surfaces, and in fully or partially buried interior of proteins. Salts may stabilize, destabilize, or have no effect on protein stability depending on the type and concentration of salt, nature of ionic interactions, and charged residues in proteins (Kohn et al., 1997). For example, the  $T_m$  (67.4°C) of recombinant human deoxyribonuclease (rhDNase) decreases to 65.3°C in the presence of 75 mg/ml of NaCl but increases to 70.1°C at 200 mg/ml (Chan et al., 1996). Increasing  $(\text{NH}_4)_2\text{SO}_4$  concentration from 50 to 200 mM increases aggregation of KGF during storage at 45°C, but NaCl has the opposite effect within the same concentration range (Zhang et al., 1995).

Salts can affect electrostatics in a protein in two ways: by non-specific (Debye-Hückel) electrostatic shielding, and by specific ion binding to the protein. At low concentrations, salts affect electrostatic shielding and weaken ionic repulsion/attractions as counterions. Therefore, this shielding effect may be either stabilizing when there are major repulsive interactions leading to protein unfolding, or destabilizing when there are major stabilizing salt bridges or ion pairs in the protein. At high concentrations, electrostatic shielding is saturated; the dominant effect of salt, like other additives, is on solvent properties of the solution. The net effect on stability of a protein would be determined by the relative effect on free energy of unfolded and native states (Goto and Fink, 1989). The stabilizing salts seem to increase surface tension at water-protein interface and strengthen hydrophobic interaction by keeping hydrophobic groups away from water molecules, inducing pref-

erential hydration of proteins (Kristjánsson and Kinsella, 1991; Kohn et al., 1997).

The salt effect strongly depends on the solution pH, which dictates charged state of ionizable groups. Recently, in a study on stability of de novo designed two-stranded  $\alpha$ -helical coiled coils, it has been found that stability of a designed protein (QQx), which has no intra or interhelical ionic interaction but contains 5 Lys residues for maintaining solubility, increases gradually with increasing KCl concentration (up to 1 M) against urea-induced denaturation (Kohn et al., 1997). The effect is the same at both pH 3 and 7, since there is no ionized state in this pH range. However, stability of another designed protein (KEx), which contains Glu and Lys at positions that form interhelical ionic interactions, increases drastically with increasing KCl concentration at pH 3. This is because at this pH, Lys is the only charged residue at interaction positions, and KCl can electrostatically shield the destabilizing repulsion by interacting with charged Lys. Addition of 50 mM  $\text{LaCl}_3$  increases slightly the stability of QQx at both pH 3 and 7 but decreases the stability of KEx due to  $\text{La}^{3+}$  binding to glutamate residues and disrupting interhelical ion pairs at pH 7. However, at pH 3,  $\text{LaCl}_3$  stabilizes KEx since Glu residues are largely protonated at this pH and can not bind to  $\text{La}^{3+}$ . The destabilizing effect of salts on proteins has been reported in many cases. Increasing ionic strength of insulin solution from 0.04 to 0.11 by addition of NaCl increases insulin fibrillation, possibly by shielding repulsive forces between similarly charged groups (Brange et al., 1997). Probably by similar shielding effect, NaCl causes thermal aggregation of RNase at pH 3 (Tsai et al., 1998b). Increasing NaCl concentration from 120 to 360 mM also increases the mean particle (aggregate) size of hepatitis A virus (HAV) in solution from about 30 nm to greater than 120 nm in 24 h at room temperature (Volkin et al., 1996).

In many cases, salts do not play a major role in altering chemical instability of proteins or peptides based on a limited number of studies. It has been found that the rate of deamidation of Asn residues in a series of pentapeptides is not affected by ionic strength (Cross and Schirch, 1991). The

rate of oxidation of Met in hIGF-I at 0.13 mM does not change whether or not sodium chloride (145 mM) is included at pH 6.0 (Fransson and Hagman, 1996). Similarly, the rate of oxidation of recombinant human relaxin by hydrogen peroxide does not change significantly at NaCl concentrations ranging from 0.007 to 0.21 M in 0.01 M acetate buffer at pH 5.0 (Nguyen et al., 1993). However, addition of 0.1 M KCl inhibits intracellular SH and S-S exchange in bovine mercaptalbumin at pH 8.6 (Kuwata et al., 1994).

### 3.3.5. Metal ions

Depending on the type and concentration, metal ions may destabilize or stabilize a protein. Since the negative counter ions may also significantly affect protein stability either positively or negatively, contribution of metal ions to protein stability should be carefully interpreted.

Some proteins are stable only in the presence of certain metal ions. The number of stabilizing metal ions required in each protein molecule is protein-dependent, and the metal ions may or may not be mutually replaceable for protein stability. It has been found that maximum activity of murine adenosine deaminase (ADA) is at 1:1 molar ratio of zinc ( $\text{Zn}^{2+}$ ) or cobalt ( $\text{Co}^{2+}$ ) to ADA. Both higher and lower ratios can inhibit the protein activity, and no measurable activity of ADA is left in the presence of other metals ions (at 1:1, 10:1 or 100:1 ratio), including  $\text{Ca}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Cu}^+$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Pb}^{2+}$ , or  $\text{Mg}^{2+}$  (Cooper et al., 1997). While  $\text{Ca}^{2+}$  (up to 100 mM) stabilizes rhDNase, other metal ions such as  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$  destabilize it (Chan et al., 1996). The stabilizing effect of  $\text{Ca}^{2+}$  on rhDNase is presumably due to direct binding of  $\text{Ca}^{2+}$  to the protein and preventing breaking of a disulfide bridge (Chen et al., 1998). A number of metal ions have been tested for stabilization of FVIII SQ, including  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Mn}^{2+}$ . While  $\text{Ca}^{2+}$  or  $\text{Sr}^{2+}$  increases the stability of the protein,  $\text{Fe}^{+2}$  at 1 or 10 mM and  $\text{Cu}^{+2}$  or  $\text{Zn}^{+2}$  at 10 mM sharply destabilize FVIII SQ (Fatouros et al., 1997a).

Metal ions may significantly affect protein stability without affecting much of its secondary structure. Although ADA (apoenzyme) is not ac-

tive (less than 0.1% of the holoenzyme activity) in the absence of  $Zn^{2+}$  or  $Co^{2+}$ , CD analysis of the protein indicates that secondary structures are the same with or without these metal ions (Cooper et al., 1997). Similarly, removal of exogenous  $Ca^{2+}$  by EGTA-treatment has little effect on the secondary structure of rhDNase based on IR determination in the amide III region in either aqueous or lyophilized state (Chen et al., 1998).

Trace amount of metal ions in protein formulation may catalyze oxidation in proteins mainly via the Fenton pathway (Stadtman, 1993). These labile residues include Met, Cys, His, Trp, Tyr, Pro, Arg, Lys, or Thr (Stadtman, 1993; Li et al., 1995b,c). The catalysis depends on concentration of the metal ions. For example, addition of 0.15 ppm (w/w) chloride salts of  $Fe^{3+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$ ,  $Mg^{2+}$ , or  $Zn^{2+}$  does not affect the oxidation rate of Met in hIGF-I at 0.13 mM in 50 mM sodium phosphate (pH 6.0) at room temperature, but when the salt concentration increases to 1 ppm,  $Fe^{3+}$  causes a significant increase in oxidation (Fransson and Hagman, 1996). The metal-catalyzed oxidation can be facilitated in the presence of a reducing agent such as ascorbate or RSH (Stadtman, 1993; Li et al., 1995c). Metal ions, oxygen, and reducing agents can generate reactive oxygen species (ROS) to oxidize proteins. The ascorbate/ $Cu(II)/O_2$  system easily oxidizes papain (Kanazawa et al., 1994), brain-derived neurotrophic factor (BDNF) (Jensen et al., 1998), His in hGH (Zhao et al., 1997), His and Met in human relaxin (Li et al., 1995a), and the His-containing fragment of relaxin (Khosravi and Borchardt, 1998). The dual effect of ascorbic acid might explain why it slightly inhibits oxidation of recombinant human nerve growth factor (rhNGF) but destabilizes FVIII SQ (Österberg and Fatouros, 1994) and increases oxidation of recombinant human ciliary neurotrophic factor (rhCNTF) in the presence of peroxides (Knepp et al., 1996).

### 3.3.6. Chelating agents

In close relation to metal ions, chelating agents such as EDTA and citric acid may destabilize a protein by binding to the protein and/or its critical metal ions or stabilize the protein by binding to any harmful metal ions.

Since transition metal ions can catalyze protein oxidation, metal ion-chelating agents should be able to protect a protein from metal-catalyzed oxidation. EDTA at 0.1 mM can completely inhibit oxidation of relaxin induced by the ascorbic acid/ $CuCl_2/O_2$  system (Li et al., 1995a). At 0.15 mM and 1 mM, it also effectively inhibits oxidation of Cys in aFGF (Wang et al., 1996b) and bFGF (Volkin and Middaugh, 1996).

In many cases, however, the effect of chelating agents is more complex. The net effect depends on the metal ions, oxidation mechanism, and type and concentration of the chelating agent (Stadtman, 1993). It has been found that EDTA at 0.02 mM suppresses formation of Met sulfoxide in His-Met catalysed by the ascorbic acid/ $FeCl_3/O_2$  system at pH 7.4 but increases the overall degradation (Li et al., 1993). In a recent study, some polyaminocarboxylate metal chelators including EDTA failed to inhibit oxidation of Met- or His-containing heptapeptides induced by the dithiothreitol/ $Fe_3Cl/O_2$  system but significantly changed the distribution of oxidation products (Zhao et al., 1996).

### 3.3.7. Shaking and shearing

Proteins can be denatured by shaking or shearing. Shaking creates hydrophobic air/water interface, which results in alignment of protein molecules at the interface, unfolding to maximize exposure of hydrophobic residues to air and to initiate aggregation (Volkin and Klivanov, 1989). The hydrophobic surfaces causing protein aggregation during shaking can be either gaseous or solid (Sluzky et al., 1991, 1992). Similarly, shearing also exposes hydrophobic areas of proteins, initiating aggregation.

Examples of shaking-induced protein instability include aggregation of rFXIII (Kreilgaard et al., 1998), hGH (Katakam et al., 1995; Katakam and Banga, 1997; Bam et al., 1998), hemoglobin (Kerwin et al., 1998), and insulin (Thurow and Geisen, 1984; Sluzky et al., 1991; Brange et al., 1997). More than 50% of urease activity is lost at 0.33 mg/ml in phosphate buffer (pH 7.0) after shaking for 24 h, and 77% of rIL-2 activity is lost at 0.2  $\mu$ g/ml in PBS (pH 7.0) after shaking for 8 h at 4°C (Wang and Johnston, 1993a). Air-jet nebu-

lization of rConIFN at pH 6.3 for 25 min at 40 psig causes 70% aggregation (Ip et al., 1995).

Proteins may tolerate shearing inactivation to a different degree. Catalase loses about 50% of its activity after exposure to a shear of  $7 \times 10^6$ , but plasma fibrinogen loses the same amount of its clotting activity after exposure to a shear of  $5 \times 10^8$  (Charm and Wong, 1970a,b). Similarly, about 30% of rhGH forms aggregates at a shear of  $10^5$ , while rhDNase is stable at a shear of  $2 \times 10^7$  when these proteins are homogenized (Maa and Hsu, 1997). This is because rhGH is more surface active and may easily accumulate at the homogenization-induced air/water interface, accelerating aggregation. Other factors such as the rigidity of protein structure and the number of hydrophobic residues exposed on the protein surface might have contributed to the different level of shear tolerance.

The effect of shear can be evaluated by pumping a protein solution through a capillary tube. The amount of shear experienced by the protein can be expressed as the product of shear rate ( $S$ ) and residence time ( $t$ ):  $S \cdot t = (8/3)(L/r)$ , where  $L$  is the length and  $r$  is the radius of the tube (Charm and Wong, 1970a).

### 3.3.8. Protein denaturants

GdnHCl, urea, NaSCN (thiocyanate), and SDS are often-used denaturants. These denaturants bind preferentially to proteins, disrupting both hydrophobic interaction and hydrogen bonds, and reducing protein chemical potential and free energy of denaturation (Darnell et al., 1986; Volkin and Klibanov, 1989; Timasheff, 1993; Allison et al., 1996). While GdnHCl and urea may interact weakly with the hydrophobic surface of a protein, SDS probably interacts strongly with it, causing protein denaturation, since a denatured state exposes more hydrophobic surface for binding (Shortle, 1996). The denaturant concentration for complete protein denaturation is usually 6 M for GdnHCl and 8 M for urea, although lower concentrations can be as effective depending on the protein.

GdnHCl is a more potent denaturant than urea. RNase H can be completely denatured in 2 M GdnHCl, but 4 M urea is needed for complete

denaturation (Goedken and Marqusee, 1998). For rhDNase, 0.5 M GdnHCl lowers its  $T_m$  from 67.4 to 61°C, but 2 M urea is needed to achieve the same effect (Chan et al., 1996). The denatured proteins by GdnHCl or urea may be reversible, such as diacylglycerol kinase (DGK) (Clarke and Waltho, 1997).

Denatured proteins are usually soluble in a denaturant solution because of increased solvation of nonpolar amino acids (Chan and Dill, 1991). Exceptions do exist. For example, the rate and extent of horse apomyoglobin aggregation increase with increasing urea concentrations (up to 2.4 M) (De Young et al., 1993). This may be due to the possible effect of protein-dependent preferential exclusion of urea at low concentrations (Timasheff, 1998). In fact, protein activation or stabilization by low concentrations of a denaturant has been reported. GdnHCl at concentrations up to 0.6 M stimulates activity of human placental alkaline phosphatase (Hung and Chang, 1998) and at concentrations up to 0.3 M stabilizes RNase T1 against thermal and urea-induced unfolding (Mayr and Schmid, 1993). The stabilizing effect of GdnHCl is apparently due to the contribution of stabilizing effect of  $\text{Cl}^-$  as observed for apomyoglobin (Staniforth et al., 1998).

It should be noted that cyanate may spontaneously form from urea, and an 8 M urea solution contains approximately 0.02 M cyanate, which can react with both amino and sulphhydryl groups in proteins, causing irreversible inactivation (Volkin and Klibanov, 1989). Therefore, urea should be prepared fresh or cyanate ions should be removed when used in probing protein stability. In IR studies NaSCN is a preferable denaturant since it does not absorb in the amide I region (Allison et al., 1996).

### 3.3.9. Non-aqueous solvents

A folded protein in an aqueous solution has hydrophobic regions sequestered from and hydrophilic areas in contact with the aqueous environment. When the polarity of an aqueous solvent decreases by adding a non-aqueous solvent, protein hydrophobic cores tend to dissipate in contact with the solvent, and the protein hydration shell may be disrupted, leading to destabiliza-

tion and unfolding of the protein. For example, addition of 60% (v/v) glycerol in 10 mM Tris-HCl buffer (pH 7.4) decreases  $\Delta G_{r \rightarrow u}$  of ferric horse heart myoglobin by about 10 kcal/mol (Barteri et al., 1996). Therefore, non-aqueous solvents reduce free energy of the unfolded state by solvating the exposed nonpolar residues (Dill, 1990). In two-phase organic/aqueous systems, proteins unfold/denature predominantly at the interface, which depends on both the interfacial tension and interfacial surface area (Cowan, 1997).

Non-aqueous solvent-induced inactivation of proteins by disruption of water residues at the active site may occur without disruption of the tertiary structure. Therefore, protein inactivation by non-aqueous solvents may be reversible. For instance, inactivation of certain proteins by dimethylsulfoxide (DMSO) is fully reversible upon immediate dilution with aqueous solution (Chang et al., 1991).

While non-aqueous solvents generally destabilize proteins, some at low concentrations may have the opposite (stabilizing) effect, such as polyhydric alcohol and some polar, aprotic solvents like DMSO and dimethylformamide (DMF) (Volkin and Klivanov, 1989). Four organic solvent additives, polyethylene glycol (PEG), 2-methyl-2,4-pentanediol (MPD), DMSO, and trimethylamine N-oxide (TMAO), have been found to induce preferential hydration of lysozyme and BSA (Kita et al., 1994). The  $T_m$  of IL-1R is increased from 48.1 to 48.6°C in the presence of 5.1 mg/ml ethanol but decreased to 43.8°C at 50 mg/ml (Remmele et al., 1998). The stabilization effect by low concentrations of non-aqueous solvents is probably due to the mixed solvent system more resembling the protein's cellular environment (Butler, 1979a,b).

Proteins suspended in certain non-aqueous solvents may be as stable as in solid state. Recently, it has been found that plasma-derived factor IX (pdFIX) suspended in perfluorodecalin with 0.0003% moisture does not lose protein activity within 24 weeks as observed in a lyophilized formulation at 37°C, while that suspended in soybean oil, methoxyflurane, octanol, or PEG 400 rapidly loses activity to a different degree within the same period (Knepp et al., 1998). When

pdFIX powder containing 10% moisture is suspended in perfluorodecalin, the resultant suspension is not as stable. This may be explained as the strong dependency of non-aqueous solvent-induced denaturation on availability of free water. In the absence of free water, protein conformation is highly rigid and compact, and resists unfolding processes (Cowan, 1997). Due to the inflexibility, certain proteins in non-aqueous solvents can resist thermal denaturation as well as chemical degradation (Tuena de Gómez-Puyou and Gómez-Puyou, 1998).

### 3.3.10. Protein concentration

Protein aggregation is generally concentration-dependent. The mean-field lattice model predicts that proteins will aggregate/precipitate at sufficiently high concentrations (Fields et al., 1992). It has been suggested that increasing protein concentration to higher than 0.02 mg/ml may facilitate potential protein aggregation (Ruddon and Bedows, 1997).

Accelerated aggregation of proteins at high concentrations has been reported in many cases. Examples include aFGF (Won et al., 1998),  $\beta$ -lactoglobulin (Roefs and De Kruif, 1994), IL-1 $\beta$  in the range of 0.1–0.5 mg/ml (Gu et al., 1991), LMW-UK in the range of 0.2–0.9 mg/ml (Vrkljan et al., 1994), and apomyoglobin in the range of 4–12 mg/ml in the presence of 2.4 M urea (De Young et al., 1993). The size of protein aggregates may also increase with increasing protein concentrations such as  $\beta$ -lactoglobulin (Roefs and De Kruif, 1994).

The effect of protein concentration on its aggregation may depend on the mechanism of aggregation and the experimental conditions. The formation of bovine insulin fibrils increases with increasing protein concentrations from 0.75 to 5% during storage at pH 2.5 and 21°C, and it takes less than 10 h to produce the same amount of fibrils at 5% as that in 15 days at 0.75% (Brange et al., 1997). In contrast, bovine insulin at 0.1 mg/ml at pH 7.4 aggregates more readily than at 0.6 mg/ml during shaking (Sluzky et al., 1991, 1992). This is attributable to the more favorable formation of insulin hexamers at 0.6 mg/ml, which are less susceptible to hydrophobic surface-



induced adsorption/aggregation than insulin monomers.

In some cases, protein concentration also affects chemical degradations to a certain degree. Increasing insulin concentration increases formation of covalent insulin oligomers and polymers in neutral solution at 37 or 45°C (Brange et al., 1992a). However, the rate constant for oxidation of Met in hIGF-I ( $k_{ox}$ ) decreases when the protein concentration is increased from 0.26 to 1.4 mM at either 30 or 50°C, presumably due to the limited amount of oxygen available for oxidation in the solution.

On the other hand, concentrated protein solutions can be more resistant against freezing-induced protein aggregation and loss of activity such as labile LDH (Carpenter et al., 1990, 1997). There are at least three explanations. First, the amount of protein accumulated at ice-water interface is finite and therefore, interface-induced protein denaturation is limited in concentrated protein solutions. Second, protein unfolding may be inhibited by steric repulsion of neighboring protein molecules (Allison et al., 1996). Last, protein–protein interactions may change monomers to active and more stable dimers or multimers (Mozhaev and Martinek, 1984).

### 3.3.11. Source and purity of proteins

Stability of proteins may be significantly different depending on the manufacturer and source of proteins. The rate of formation of high-molecular-weight products in human insulin preparations in neutral solutions can differ up to 100% among different manufacturers (Brange et al., 1992a). One of the major influencing factors is the purity of proteins. Any presence of trace amount of enzymes, metal ions, or other contaminants can potentially affect protein stability. Thus, protein purification is one of the most critical steps in controlling protein quality.

Very often, the extent and mechanism of protein degradation is different depending on the purification scheme such as rhTPO (Senderoff et al., 1996). Recombinant and plasma-derived proteins may require different purification schemes, which may influence protein stability. Recombinant factor IX (rFIX) is essentially free

of contamination from non-factor IX products, but many high-purity pdFIX preparations exhibit significant amounts of non-factor IX contaminants, including other coagulation proteins, which may potentially affect the protein stability (Bond et al., 1998).

### 3.3.12. Protein morphism

Protein morphism is also a factor influencing protein stability. Crystalline drugs are generally more stable chemically than amorphous forms, especially for small molecules. The formation of high-molecular-weight insulin in a suspension of amorphous zinc insulin appears faster than that in crystalline zinc insulin at 37°C (Brange et al., 1992a). The total hydrolytic transformation at B3 position of the amorphous (or soluble) insulin is considerably higher than that of the crystalline form (Brange et al., 1992b). On the contrary, freeze-dried amorphous insulin is far more stable than crystalline insulin against deamidation and dimerization at all water content up to 15%. While the rate of degradation of crystalline insulin increases with increasing water content, the rate of amorphous form is essentially independent of water content change in the same range (Pikal and Rigsbee, 1997).

### 3.3.13. High pressure

High pressure can cause protein unfolding because the volume of protein–solvent systems is smaller in the unfolded state. In other words, unfolded proteins are more compressible than folded ones. For example, 40% of staphylococcal nuclease at  $< 10 \mu\text{M}$  unfolds under 1000 bar in 10 mM Bis–Tris buffer (pH 4.5) containing 1.25% xylose (Frye and Royer, 1997). Bovine pancreas RNase A unfolds completely under 4000 bar (Zhang et al., 1995; Prehoda et al., 1998). Increasing the pressure from 0 to 2000 bar significantly inhibits activity of  $\text{H}^+$ -ATPase and increases its aggregation (Tsai et al., 1998c).

## 3.4. Analytical techniques in monitoring protein instability

To monitor protein instability, a protein activity assay is indispensable. Often, a bioassay is

needed based on the protein's function, such as the clot lysis assay for tissue plasminogen activator (tPA) (Hsu et al. 1995). A good activity assay should be reproducible and sensitive enough to detect small changes in protein activity. In reality, a protein activity assay is often variable, unable to detect small activity changes, labor-intensive, and unable to reveal any intricate changes in the structure of proteins. Therefore, other analytical techniques are used, preferably in conjunction with an activity assay, to characterize a protein and monitor its instability. The available analytical methods for assessing protein formulations have been discussed elsewhere (Jones, 1994). Table 3 lists the major analytical techniques and their applications in monitoring protein instability.

### 3.4.1. Monitoring the protein unfolding process

Proteins can be unfolded chemically or thermally. The often-used chemicals for protein unfolding studies include urea and GdnHCl. The denaturant-induced unfolding process can be slow depending on the protein, and on the type and concentration of denaturant. It has been reported that at least 6 h are needed to unfold human placental alkaline phosphatase (Hung and Chang, 1998). Different denaturants may also unfold a protein differently. Apomyoglobin from *Aplysia limacina* shows two transitions on titration with GdnHCl but only one with urea as monitored by far-UV CD (Staniforth et al., 1998).

Many physical techniques can be used to monitor an unfolding event, including circular dichroism (CD), fluorescence, optical rotation, or UV (Thomson et al., 1989; Lau and Bowie, 1997; Swietnicki et al., 1997). The signal of hydrogen exchange (HX) in proteins, as monitored by nuclear magnetic resonance spectroscopy (NMR), can also be used in these studies (Bai et al., 1994; Zhang et al., 1995). These techniques are based on signal differences between the folded and unfolded states. Two useful parameters in chemical unfolding studies can be determined:  $C_{\text{half}}$ , the concentration of a denaturant causing unfolding of 50% protein molecules, and  $\Delta G_{f \rightarrow u}$ , the free energy change of unfolding. Both parameters can be used to examine the effect of formulation excipients on protein stability (Barteri et al., 1996;

Foord and Leatherbarrow, 1998). To obtain  $\Delta G_{f \rightarrow u}$ , the fraction of unfolded protein,  $F_u$ , is first calculated using the following equation, assuming a two-state transition:

$$F_u = (y_f - y_{\text{obs}})/(y_f - y_u).$$

In this equation,  $y_{\text{obs}}$  is the observed variable signal in the presence of different denaturant concentrations, and  $y_f$  and  $y_u$  are, respectively, the signal of completely folded and unfolded conformations.  $\Delta G$  at the equilibrium state can be calculated using the following equation (Thomson et al., 1989; Pace, 1990):

$$\begin{aligned} \Delta G &= -RT \ln[(F_u)/(1 - F_u)] \\ &= -RT \ln[(y_f - y_{\text{obs}})/(y_{\text{obs}} - y_u)], \end{aligned}$$

where  $R$  is the gas constant and  $T$  is the absolute temperature. The  $\Delta G_{f \rightarrow u}$  is obtained by extrapolating the unfolding curve ( $\Delta G$  versus denaturant concentration) at 0 denaturant concentration.

What causes the signal difference between the folded and unfolded states of proteins? Protein unfolding exposes hydrophobic amino acid residues. Solvent exposure of these residues such as Tyr or Trp changes their environment and often results in a signal change. The change in UV or fluorescence signal can be negative or positive depending on protein sequence and solution properties. For example, unfolding of  $\alpha$ -chymotrypsin at 0.3 mg/ml at pH 7.0 decreases its UV absorption by about 10% at 281 nm and fluorescence intensity by about 70% with a shift of fluorescence  $\lambda_{\text{max}}$  from 331 to 347 nm (Lozano et al., 1997). The signal decrease results from an increase in polarity of the environment of Trp in the protein upon unfolding as observed for apomyoglobin (Staniforth et al., 1998). The intrinsic fluorescence of human albumin decreases substantially upon unfolding due to loss of energy transfer (several Tyr residues transferring excitation energy to Trp residues in the native state) (Farruggia et al., 1997). However, exposure of buried Tyr to solvent upon unfolding of cutinase increases its UV absorbance significantly at 250 nm at pH 9.6 due to ionization of Tyr (Melo et al., 1997). Positive change in fluorescence signal has also been reported. The fluorescence intensity of soluble protein MerP (72 aa) increases about 10 times at

Table 3  
Analytical techniques in protein characterization and instability monitoring

Analytical techniques	Major applications	Protein examples	References
Analytical centrifugation	Protein aggregation	Rop proteins	Munson et al., 1996
CE	Protein degradation Determination of $T_m$	Hirudin RNase	Gietz et al., 1998 McIntosh et al., 1998
CD	Estimation of secondary structures Determination of $T_m$ Probing protein conformation  Determination of multimers	$\alpha$ -Spectrin AcP $\beta$ -lactoglobulin  Fragment of ribosomal protein L9	Prieto et al., 1997 Chiti et al. 1998 Matsuura and Manning, 1994 Kuhlman et al., 1997
DSC	Determination of $T_g$ Determination of $T_m$ Protein unfolding	hGH aFGF BSA	Pikal et al., 1991 Tsai et al., 1993 Giancola et al., 1997
Electron paramagnetic resonance (EPR)	Ligand-protein interactions	rhGH, rhIFN- $\gamma$	Bam et al., 1995
Fluorescence	Protein unfolding/interaction Determination of $T_m$ Probing protein conformation	MerP HSA $\alpha$ -Antitrypsin	Aronsson et al., 1997 Farruggia et al., 1997 Kwon and Yu, 1997
HPLC-ion exchange	Protein degradation and aggregation	hGH	Zhao et al., 1997
HPLC-reversed phase	Protein degradation and aggregation Estimation of contamination	hGH rhPTH	Pikal et al., 1991 Nabuchi et al., 1995
HPLC-size exclusion	Protein degradation and aggregation Estimation of contamination	hGH $\beta$ -Galactosidase	Pikal et al., 1991 Yoshioka et al., 1993
IR	Estimation of secondary structures Determination of $T_m$ Probing protein confirmation	Chymotrypsinogen hPAH IL-1ra	Allison et al., 1996 Chehin et al. 1998 Chang et al., 1996b
Karl Fischer	Water determination	Insulin formulation	Strickley and Anderson, 1996, 1997
Light scattering	Protein aggregation	Human relaxin	Li et al., 1995a
MS	Determination of molecular weight, degradation products and contaminants	bFGF	Shahrokh et al., 1994a
NMR	Determination of 3-D and secondary structures Protein relaxation and softening  Protein unfolding	IL-6 BSA, BGG  RNase	Xu et al., 1997 Yoshioka et al., 1997, 1998 Zhang et al., 1995
Raman spectroscopy	Determination of secondary structures	Insulin	Yeo et al. 1994
Refractometry	Ligand-protein interactions	rhGH, rhIFN- $\gamma$	Bam et al., 1995
UV/visible spectroscopy	Determination of $T_m$ Protein aggregation Estimation of contamination  Probing protein conformation	RNase aFGF hGH  IL-2, Insulin	Thomson et al., 1989 Tsai et al., 1993 Perlman and Nguyen, 1992 Butler, 1979a,b; Brewster et al., 1991

300 nm upon unfolding (Aronsson et al., 1997). Similar increase in fluorescence signal was also observed for Trp RNase A mutant at 350 nm (Sendak et al., 1996). Effective quenching of Tyr and Trp in the folded proteins causes the large signal increase upon unfolding. Sometime the signal change is due to a simple solute effect rather than conformational change. A 30% drop in fluorescence intensity of DGK at 330 nm is observed upon SDS denaturation, and the same extent of signal change is also seen for free Trp (Lau and Bowie, 1997). To maximize detection sensitivity, a signal ratio may be used, such as the ratio of fluorescence intensity at 350 nm to that at 330 nm in the study of rFXIII unfolding (Kurochkin et al., 1995).

Proteins may have more than one unfolding process if there is a stable protein intermediate, which may not be detectable depending on the techniques used. Two unfolding processes are observed for human, bovine, rat, dog, and rabbit albumin when they are denatured by GdnHCl and monitored by CD, but only human, bovine, or rat albumin shows two transitions when monitored by fluorescence (Kosa et al., 1998). Similarly, the UV absorption of DGK drops biphasically with increasing concentration of denaturant SDS, suggesting two unfolding transitions, but CD measurement at 222 nm only shows a single denaturation phase (Lau and Bowie, 1997). The failure of CD to detect an intermediate is probably due to a variety of factors affecting CD determination, such as protein concentration (causing impropotional changes in secondary structure), pH, solvent property, and ionic strength (Farnsworth et al., 1997; Hu et al., 1997).

Fluorescence spectroscopy is often used to probe protein unfolding (Eftink, 1994). A red shift in fluorescence spectroscopy is commonly observed during protein unfolding because Trp or Tyr residues are in less hydrophobic environments (De Young et al., 1993; Volkin et al., 1996). An often-used agent in the investigation of protein unfolding is Bis-ANS, whose fluorescence is pH-independent and very weak in water but increases greatly upon binding to unfolding-exposed hydrophobic sites in proteins. Due to this property,

Bis-ANS has been used recently to probe the mechanism of  $\alpha_1$ -antitrypsin polymerization (James and Bottomley, 1998).

Both primary and second-derivative amide I IR spectra can be used to probe pH or temperature-induced protein unfolding or conformational changes in proteins such as hemoglobin (Dong and Caughey, 1994) and insulin (Pikal and Rigsbee, 1997). By monitoring changes in band intensity of native  $\beta$ -sheet structure at  $1638\text{ cm}^{-1}$  and an aggregate band at  $1619\text{ cm}^{-1}$ , human FXIII has been found to denature irreversibly as the temperature increases from 25 to  $85^\circ\text{C}$  (Dong et al., 1997). IR spectra of proteins in solution can be significantly different from those obtained in solid state. Insulin in solution shows a sharper and stronger IR helix band than solid insulin, and the spectrum of denatured insulin (spectrum at  $85^\circ\text{C}$ ) resembles that of solid insulin (Pikal and Rigsbee, 1997).

#### 3.4.2. Determination of protein melting/unfolding temperature ( $T_m$ )

Those physical methods used to determine  $\Delta G_{f \rightarrow u}$  can be used similarly to determine protein melting/unfolding temperature ( $T_m$ ), such as CD, fluorescence, optical rotation, or UV.  $T_m$  can be easily located at  $\Delta G = 0$  on the  $\Delta G$  versus  $T$  curve or directly determined from the signal-temperature curve (Farruggia et al., 1997).

Differential scanning calorimetry (DSC) is one of the frequently-used techniques to determine protein  $T_m$ . Proteins unfold with an endothermic peak, but aggregation is exothermic by DSC. If the two events happen approximately at the same time, a peak would be the sum of the two events. The first event may be overwhelmed by the second, such as the single exothermic peak observed for rhKGF unfolding and aggregation (Chen et al., 1994a). In this case, modulated DSC may be considered as a way to differentiate overlapping thermal events due to its higher resolution (Craig and Royall, 1998; Royall et al., 1998).

There are several other factors that can potentially affect determination of  $T_m$ . The first factor is the determination method to be used. Different methods can give significantly different  $T_m$  values

for the same protein. Detailed examples are listed in Table 1. Temperature-ramping rate during determination of  $T_m$  may have a significant effect. For example, lowering the temperature-ramping rate reduces the  $T_m$  of rhKGF, suggesting a kinetically controlled denaturation process (Chen et al., 1994a). Vermeer et al. (1998) recently demonstrated that at 0.5°C/min, mouse IgG shows both a melting and an aggregation peak by DSC but at 0.1°C/min, only the melting peak can be observed. Protein concentration may or may not affect determination of  $T_m$ . While the initial unfolding temperature of rhKGF is independent of protein concentration (Chen et al., 1994a), that of IFN- $\beta$ -1a decreases from about 77 to 68°C when the protein concentration is increased from 10 to 100  $\mu\text{g/ml}$  (Runkel et al., 1998). The  $T_m$  independence on protein concentration may imply a rate-limiting protein unfolding.

Recently, infrared spectroscopy (IR) and capillary electrophoresis (CE) have also found their application in determination of  $T_m$ . By IR, the  $T_m$  of human phenylalanine hydroxylase (hPAH) has been estimated based on temperature-induced changes in the intensity ratio of the amide I band at 1619 and 1650  $\text{cm}^{-1}$  (Chehin et al., 1998). Similarly, the  $T_m$  of RNase has been determined by CE based on changes in the protein's electrophoretic mobility at different temperatures (McIntosh et al., 1998).

### 3.4.3. Estimation of protein secondary structures

IR is one of oldest methods and remains the most extensively-used technique today for studying secondary structure of proteins (Susi and Byler, 1986; Dong et al., 1995b; Allison et al., 1996; D'Auria et al., 1997). The commonly-used band for determination of protein secondary structure is the amide I region (1620–1690  $\text{cm}^{-1}$ ), although the amide III band (1200–1330  $\text{cm}^{-1}$ ) has also been used in this regard. The amide I region covers different C=O stretching frequencies arising from different secondary structures ( $\alpha$ -helix,  $\beta$ -sheet, turn, and unordered structures) and has no major interfering bands from other structures in proteins, whereas the amide III band has better resolution for secondary structures and no water interference (Cooper and Knutson, 1995). A

good example of using the amide III band is the estimation of secondary structure of some dozen different proteins in both liquid and solid states by Griebenow and Klibanov (1995).

IR absorption bands are generally broad and consist of overlapping components representing different structures. Two methods have been used for resolution enhancement: Fourier self-deconvolution and second or fourth derivation (Goormaghtigh et al., 1994b). Both techniques work equally well in resolving overlapping components (Dong et al., 1995b). However, some believe that analysis of derivative spectra should be discouraged because derivation does not preserve the integrated areas of individual components (Surewicz et al., 1993). Nevertheless, the two resolution-enhancing methods can be conveniently used on modern instruments of Fourier transform infrared spectroscopy (FTIR), which also offer high sensitivity (high  $S/N$  ratio), greater accuracy, and higher speed (Susi and Byler, 1986; Surewicz et al. 1993). Secondary structure estimation after resolution enhancement seems rather accurate. Dong et al. (1990) determined the second-derivative IR spectra of 12 globular proteins in aqueous solutions, and the relative amounts of different secondary structures in all proteins were nearly identical (mostly  $\pm 5\%$ ) to those obtained by crystallography.

Unfortunately, water molecules absorb strongly in the amide I region (about 1640  $\text{cm}^{-1}$ ), interfering with data interpretation. There are two ways to overcome this obstacle: careful subtraction of water absorption or use of  $\text{D}_2\text{O}$  as the solvent (Goormaghtigh et al., 1994a). To make reliable subtraction, higher protein concentrations ( $> 10$  mg/ml) are recommended to increase the protein signal and a  $\text{CaF}_2$  (or  $\text{BaF}_2$ ) cell with a path length of 10  $\mu\text{m}$  or less should be used to control the total sample absorbance within 1 unit (Cooper and Knutson, 1995). Replacing water with  $\text{D}_2\text{O}$  as the solvent not only eliminates water interference in the amide I region but also stabilizes proteins in some cases (Bai et al., 1994). For example, human FXIII is apparently more stable in  $\text{D}_2\text{O}$  than in  $\text{H}_2\text{O}$  (Dong et al., 1997). The higher stability of proteins in  $\text{D}_2\text{O}$  is believed to be due to stronger hydrogen bonds formed by deuterium

than those formed by hydrogen. This suggests that IR spectra of proteins in D<sub>2</sub>O may not be the same as those in water. As a matter of fact, subtle differences in the secondary structure in bovine  $\beta$ -lactoglobulin variants are found in the two solvents (Dong et al., 1995a).

Another often-used method to determine the relative composition and changes of secondary structure in a protein is CD. A broad negative peak at about 218 nm is characteristic of a  $\beta$ -sheet structure, and two negative peaks at 208 and 222 nm are characteristic of a  $\alpha$ -helix structure (Chen et al., 1994a; Farnsworth et al., 1997; Hu et al., 1997; Swietnicki et al., 1997). Although CD is generally less sensitive than IR, the two methods are highly complementary and, if possible, should be used together (Surewicz et al., 1993). Their relative contribution can be illustrated in the following examples. In the comparison of the secondary structures of two generic bovine  $\beta$ -lactoglobulin variants in D<sub>2</sub>O solution, the CD spectra of the two proteins are nearly identical, but the IR spectra are significantly different (Dong et al., 1995a). In a different investigation, about 20–35% random or unordered elements in human FXIII in an aqueous solution are detected by CD but not observed by FTIR, although detailed analysis indicates that the secondary structures determined by the two methods are very similar (Dong et al., 1997).

Several factors can affect the appearance of the characteristic peaks in CD spectra, such as protein concentration, temperature, pH, and ionic strength. Increasing protein concentration may be associated with increased content of  $\beta$ -sheet (Matsuura and Manning, 1994). High ionic strength may reduce helicity of a protein (destabilization), if charge–charge interactions make a net favorable contribution to helix stability (Kuhlman et al., 1997). Although a double ellipticity minima at 208 and 222 nm is of a typical helical conformation, this may not be obvious if the helical content is very low and/or there are interfering aromatic residues such as Tyr (Prieto et al., 1997).

Lastly, NMR can also be used to determine secondary structures of proteins. It can provide accurate estimation of both the extent and location of the secondary structures (Reid et al.,

1997). A particular use of NMR is the determination of secondary structural propensities of residues in unfolded or partially-folded proteins (Dyson and Wright, 1998).

#### 3.4.4. Protein aggregation

In addition to polyacrylamide gel electrophoresis (PAGE) and analytical centrifugation, size exclusion HPLC (SEC-HPLC) is an often-used method in monitoring protein aggregation. Unfortunately, the size of a protein or its aggregates can be overestimated by SEC-HPLC if the protein is not spherical (such as highly-coiled proteins) and its Stokes radius is greater than that of a globular protein (Kuhlman et al., 1997). In addition, if a protein has carbohydrates or interacts with the column, the elution profile of the protein may change, leading to erroneous estimation of its molecular weight. To solve these issues, SEC-HPLC can be coupled with both light scattering and refractive index detectors to determine the size of a protein or its aggregates. The size calculated by the signal ratio of the two detectors is not affected by the above factors (Wen et al., 1996).

While SEC-HPLC can only determine the total amount of aggregates based on size, reversed phase HPLC (RP-HPLC) may detect both covalent and non-covalent aggregates such as hGH (Perlman and Nguyen, 1992) or different isoforms of protein aggregates such as bFGF dimers (Shahrokh et al. 1994b). Since a protein sample is usually filtered through a 0.2  $\mu$ m filter to remove any particulates before analysis, the HPLC method is limited for determination of soluble aggregates. In addition, a protein may unfold or refold during HPLC analysis depending on the protein and the nature of the mobile phase, which may change the retention time of the protein. For example, denatured bFGF partially refolds into native protein during analysis, which changes the retention time and peak shape of the denatured protein (Shahrokh et al. 1994b). Therefore, prevention of unfolding/refolding by addition of certain modifiers in the mobile phase may be needed in these cases.

The turbidimetric method is often used to estimate the amount of protein aggregates by measuring optical density of samples based on light scattering in near UV or visible region, where

proteins do not have any absorption (Eckhardt et al., 1994). A variety of wavelengths has been chosen and used, including 300 nm for urokinase (Vrkljan et al., 1994), 350 nm for aFGF (Tsai et al., 1993; Volkin et al., 1993), average optical density between 340 and 360 nm for met-hGH and tPA (Hsu et al. 1991, 1995), 450 nm for pGH (Charman et al. 1993), and 500 nm for IL-1ra (Chang et al., 1996b). This method has been proved to be more sensitive than HPLC in detecting early formation of aggregates (Wang et al., 1996b). In addition, the turbidity of a protein solution can be linear in a narrow concentration range for accurate quantitation, such as IL-1ra in the range of 0.1–0.3 mg/ml (Chang et al., 1996b). On the other hand, this method can be variable. For example, it has been found that the turbidimetric variation in determination of LMW-UK aggregation in phosphate buffer (pH 6.5) upon heat treatment is usually less than 10% but increases up to 30% when the LMW-UK solution contains other polymer additives (Vrkljan et al., 1994).

A simpler method for estimation of the extent of protein aggregation is to compare visually the level of sample cloudiness using seven categories of visual appearance: clear, slightly opalescent, opalescent, very opalescent, slightly cloudy, cloudy, and very cloudy (Eckhardt et al., 1994). The requirement of using this method is a uniform distribution of all the aggregates.

Recently, IR has been used extensively in protein denaturation/aggregation studies. A common feature of thermally-induced (or lyophilization-induced) protein aggregation is the formation of an intermolecular hydrogen-bonded antiparallel  $\beta$ -sheet structure, which is represented by a low-frequency band around  $1620\text{ cm}^{-1}$  and an associated weaker high-frequency band around  $1685\text{ cm}^{-1}$  (Dong et al., 1995b). For example, an aggregation band has been found at  $1619\text{ cm}^{-1}$  for human FXIII (Dong et al., 1997) and hPAH (Chelin et al., 1998), and at  $1620\text{ cm}^{-1}$  for recombinant human IFN- $\gamma$  (Kendrick et al., 1998b). This structural transition occurs regardless of the initial composition of the secondary structure of native proteins. Therefore, these bands can be used to monitor and quantify aggre-

gation in both aqueous and solid states. Similarly, reversibility of protein aggregation may be determined by monitoring an aggregate band. By this method, the temperature-induced aggregation of human FXIII in both  $\text{D}_2\text{O}$  and  $\text{H}_2\text{O}$  was found irreversible (Dong et al., 1997).

Another IR method for estimating protein aggregation is to compare overall spectral change of proteins, as the change may correlate with protein aggregation. The overall spectral change can be measured by fractional area overlapping of the second-derivative spectrum of a protein sample with that of a reference. Using this method, Allison et al. (1996) demonstrated that any decrease in fractional spectral area overlap of freeze-dried chymotrypsinogen directly correlates with the amount of aggregates formed after rehydration of the solid.

#### 3.4.5. Determination of protein degradation products

A variety of analytical methods is available to determine protein degradation products. Both SEC-HPLC and PAGE can detect protein degradation products or certain fragments but these methods are usually inadequate in detecting subtle structural changes in proteins such as oxidation and deamidation. Generally, RP-HPLC is the preferred method in these cases. RP-HPLC has been widely used to separate and quantitate deamidated proteins such as GRF (Stevenson et al., 1993), insulin (Darrington and Anderson, 1995), and tPA (Paranandi et al., 1994), oxidized proteins such as hIGF-1 (Fransson et al., 1996), human leptin (Liu et al., 1998), and rhPTH (Nabuchi et al., 1995), hydrolyzed proteins such as bFGF (Shahrokh et al., 1994a), insulin (Brange et al. 1992b) and rhM-CSF (Schrier et al., 1993), isomerized and oxidized (Met<sup>125</sup>) hGH (Johnson et al., 1989; Perlman and Nguyen, 1992), and succinimidated bFGF (Shahrokh et al., 1994a).

However, RP-HPLC itself is usually not capable of identifying protein degradation products. To achieve identification, RP-HPLC-separated degradation products have to be collected and further analyzed by other analytical methods such as mass spectroscopy (MS), peptide mapping and sequencing, and amino acid analysis (Johnson et

al., 1989; Schrier et al., 1993; Shahrokh et al., 1994a; Nabuchi et al., 1995; Liu et al., 1998). A more efficient technique in use is the coupled RP-HPLC and MS (LC-MS). This technique has been used in the identification of hydrolyzed bFGF (Shahrokh et al., 1994a), and different mono-, di- and tri-oxidized human leptin (Liu et al., 1998). In addition to HPLC, capillary electrophoresis has also been coupled to MS (CE-MS) for highly sensitive analysis of proteins and peptides (Figeys and Aebersold, 1998). MS alone has been a very powerful tool in identification of protein degradation products as well as molecular weight determination (Nguyen et al., 1995). The use of tandem MS (MS–MS) may allow direct identification of partial to complete sequence for peptides up to 25 amino acids, sites of deamidation, and isomerization (Carr et al., 1991).

#### 3.4.6. Probing protein tertiary/quaternary structure

The ultimate goal of structural studies on proteins is to gain insight into the protein's three-dimensional structure at a high-resolution level. Two delicate techniques can be used to probe protein structures: X-ray crystallography and NMR. Both methods have advantages and shortcomings. X-ray crystallography has more accuracy but needs highly-ordered crystals and a large amount of material in solid state. NMR can be applied for solutions containing an adequate amount of protein (typically millimolar) but is usually limited to small proteins (< 20 kD) (Midgough, 1990). Recent advances in multidimensional NMR methodology can now permit structural determination of larger proteins up to 60 kD (Clare and Gronenborn, 1998). Other indirect techniques for probing structures of large proteins by NMR include determination of the rate of hydrogen exchange (Raschke and Marqusee, 1998) or structures of 'dissected' protein subunits or domains (Campbell and Downing, 1998).

Other methods of probing protein tertiary structure include CD in the near UV region, fluorescence spectroscopy, and second-derivative UV spectroscopy. However, these methods generally do not provide detailed information about structure. Nevertheless, CD has been used to demonstrate destabi-

lization of methionyl pGH in the presence of either poloxamer 407 (Pluronic F127) or 188 (Pluronic F68) (Charman et al., 1995), and to monitor changes in the tertiary structure of  $\beta$ -lactoglobulin upon heating (Matsuura and Manning, 1994). Second-derivative UV spectroscopy in combination of SEC-HPLC has been used to monitor the relative solvent exposure of Tyr residues in IL-2 mutants (Ackland et al., 1991).

## 4. Stabilization and formulation of liquid protein pharmaceuticals

Proteins in extremophilic organisms can tolerate one or more of the following stresses: low or high temperatures, high hydrostatic pressure, high salinity, and extreme pHs, even though their building blocks are exclusively the canonical 20 natural amino acids. Therefore, significant room exists to stabilize any unstable protein pharmaceuticals. The central issue in protein stabilization is preservation of the functional state of proteins under various stressful conditions.

### 4.1. Means and mechanisms of protein stabilization

Proteins may be stabilized either by changing their structural characteristics (internally) or by changing the properties of solvent in contact with them (externally). Structural changes may increase the protein's unfolding free energy change ( $\Delta G_{\text{mut}}$ ), leading to its stabilization with a free energy change  $\Delta\Delta G = \Delta G_{\text{mut}} - \Delta G_{\text{nat}}$ . Since the difference between mesophilic and thermophilic proteins is small in terms of  $\Delta\Delta G$  (5–7 kcal/mol), stabilization of mesophilic proteins to the level of thermophilic proteins is feasible (Querol et al., 1996). To estimate the degree of internal or external stabilization, the change in the protein's unfolding temperature ( $T_m$ ) is often used as an indicator. A minimum increase of 1°C in  $T_m$  is considered as the threshold of thermostability increase (Querol et al., 1996).

#### 4.1.1. Mechanisms of internal protein stabilization

As discussed in Section 2.1, native proteins are stabilized by many forces. Any structural change



that increases any of the stabilizing forces without changing the overall protein conformation generally stabilizes proteins (Jaenicke, 1991). Protein thermal stability usually increases if amino acid substitution/modification results in increased internal and decreased external (or surface) hydrophobicity (Kristjánsson and Kinsella, 1991). Increasing internal hydrophobicity often correlates with increased packing efficiency and protein stability (Mozhaev and Martinek, 1984; Vieille and Zeikus, 1996). This positive correlation was demonstrated recently in a study on a series of hydrophobic mutants of human lysozyme (Takano et al., 1998). Protein stability also positively correlates with the content of hydrogen bond-forming amino acids and the ability of these amino acids to maintain these bonds, especially at critical locations (Kristjánsson and Kinsella, 1991). However, no direct correlation has been found between protein thermal stability and protein volume (Querol et al., 1996). Increasing charge density apparently destabilizes a protein (Sindelar et al., 1998).

Local interactions of amino acids are indispensable in native proteins. The interactions may come from residues close in sequence and contribute to the secondary structure propensities. Enhancement of native local interactions makes proteins more resistant to thermal and chemical denaturation, and introduction of non-native local interactions destabilizes proteins because non-native/denatured conformations are stabilized (Prieto et al., 1997). The  $\alpha$ -helix can be stabilized by introducing residues of high helix propensity (such as Ala), which eventually stabilizes proteins (Querol et al., 1996; Vieille and Zeikus, 1996).

Thermostable proteins are less flexible than thermolabile ones (Daniel et al., 1996). Protein stability inversely correlates with its flexibility (Tang and Dill, 1998). The flexible regions in proteins are typically the labile areas (Vieille and Zeikus, 1996). Some inactivation processes, such as oxidation of Cys and sensitive Met, hydrolysis of peptide bonds at Asp residues, and deamidation of Asn and Gln residues, are more likely to occur in flexible (or solvent-accessible) areas of the protein (Powell, 1996; Querol et al., 1996). These areas should be considered first for structural modification/stabilization.

#### 4.1.2. Mechanisms of external protein stabilization

There are two mechanisms of solvent-induced stabilization of proteins: (1) strengthening protein-stabilizing forces; and (2) destabilizing the denatured state. This is to say that any change in solvent property that enhances a native interaction or perturbs a non-native interaction increases stability of proteins (Wrabl and Shortle, 1996). Under non-native conditions, proteins may still be stabilized as long as the condition favors strengthening of non-covalent interactions, especially hydrophobic interactions. These non-native conditions, such as concentrated solutions of salt, polyols, or certain organic solvents can enhance hydrophobic interactions and stabilize proteins by altering the structure of water and reducing protein interaction with the solutes (Mozhaev and Martinek, 1984; Kristjánsson and Kinsella, 1991; Brange et al. 1997). Although an excipient(s) stabilizes a protein mostly by changing solvent property, some may interact favorably with charged sites on the protein surface and minimize electrostatic protein–protein interactions to achieve protein stabilization (Remmele et al., 1998).

#### 4.1.3. Preferential Interaction—a major stabilization mechanism of excipients

The most tenable and widely-accepted mechanism of protein stabilization in aqueous solution is preferential interaction of proteins. Preferential interaction means that a protein prefers to interact with either water or an excipient (co-solute/co-solvent). In the presence of a stabilizing excipient, a protein prefers to interact with water (preferential hydration) and the excipient is preferentially excluded from the protein domain (preferential exclusion). In this case, proportionally more water molecules and fewer excipient molecules are found at the surface of the protein than in the bulk (Arakawa et al., 1991, 1993; Timasheff, 1993, 1998; Lin and Timasheff, 1996).

Preferential interaction of a protein with an excipient has been defined as:

$$(\partial g_3 / \partial g_2)_{T, \mu_1, \mu_3} \text{ or } (\partial m_3 / \partial m_2)_{T, \mu_1, \mu_3}$$

in which 1, 2, and 3 represent water, protein, and excipient, respectively, and  $\mu$  is the chemical po-

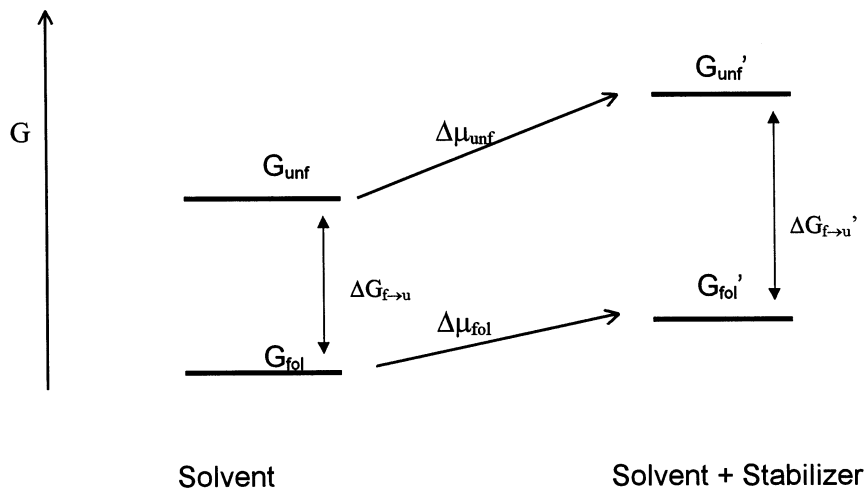


Fig. 1. Diagram of free energy change of a protein in the presence of a protein stabilizer

tential (Timasheff, 1992, 1993, 1998). The interaction can be expressed on a gram basis ( $g$ ) or on a molar basis ( $m$ ). Preferential interaction of a protein with either water or an excipient is the consequence of a difference in affinity of a protein for water or an excipient. If there is a preference for an excipient, the measured interaction is positive, preferential binding. In the opposite case, the measured interaction is negative, preferential hydration and the protein is stabilized. Sucrose is a commonly-used protein stabilizer, and the preferential interaction of rhIL-1ra and  $\alpha$ -chymotrypsin in 1 M sucrose is, respectively,  $-0.164$  g sucrose/g protein (Kendrick et al., 1997) and  $-7.6$  mol sucrose/mol protein (Timasheff, 1993). In a recent study on stabilization of RNase by sucrose, Liu and Bolen (1995) have found that the protein side chains actually favor exposure to sucrose relative to water, facilitating protein unfolding, and that it is the highly unfavorable exposure of polypeptide backbone on unfolding that leads to protein stabilization by sucrose. In contrast, peptide backbone exposure is favorable in the presence of strong denaturants such as urea and GdnHCl because they preferentially bind to the backbone.

Stabilizing preferential interaction of a protein with an excipient is usually associated with an increase in chemical potential of the protein, which is thermodynamically unfavorable (Carpen-

ter et al., 1990; Timasheff, 1993; Kendrick et al., 1997). This means that protein interaction with the excipient is unfavorable relative to water; hence, the protein is preferentially hydrated and the excipient is preferentially excluded from the protein surface. The positive change in chemical potential is marginal at low excipient concentrations and becomes more positive at higher concentrations. The increase in chemical potential is directly proportional to the surface area of the protein exposed to solvent. Since a denatured protein has a greater surface area than a native protein, preferential exclusion of the excipient from the surface of the denatured protein is more unfavorable than from that of the native protein. Therefore, protein stabilization arises from destabilization of the denatured state by the excipient, leading to a larger free energy change ( $\Delta G_{f \rightarrow u}$ ). Fig. 1 shows the free energy changes associated with this type of protein stabilization; a stabilizer destabilizes the unfolded state to a greater degree than the native state.

Stabilization of proteins in solution by a variety of excipients appears to correlate positively with their capability of increasing the surface tension of water. Introduction of a protein molecule into water must form additional protein–water interface, and the interfacial tension is equal to the surface tension of water after proper correction

for curvature of the protein surface (Kita et al., 1994). Substances lowering the surface tension of water accumulate at the protein surface; those raising it are kept away from the protein surface. It has been found that most stabilizers for lysozyme and BSA increase the surface tension of water, which also correlates with the increase in chemical potentials of the two proteins (Kita et al., 1994).

However, the surface tension effect of an excipient is not the sole factor involved in the protein–solvent interactions. Increasing the surface tension of water by an excipient does not necessarily mean automatic protein stabilization. Surface tension-derived stabilization can be overcome by weak binding of these excipients to proteins. For example, Arg hydrochloride (ArgHCl) increases the surface tension of water but destabilizes RNase A because ArgHCl binds to the protein, as indicated by a decreasing preferential hydration,  $(\partial g_1/\partial g_2)_{T, \mu_1, \mu_3}$ , with increasing ArgHCl concentration (Lin and Timasheff, 1996). Urea and  $MgCl_2$  increase the surface energy of water but are preferentially bound to proteins at high concentrations (Kita et al., 1994). On the other hand, excipients decreasing surface tension of water may still stabilize a protein. Betaine and glycerol decrease the surface energy of water but induce protein preferential hydration (Kita et al., 1994). Four organic solvents, PEG, 2-methyl-2,4-pentanediol (MPD), DMSO, and TMAO, decrease the surface tension of water but induce preferential hydration of lysozyme and BSA (Kita et al., 1994). This has been attributed to the presence of two types of agents which are preferentially excluded: (1) those that are totally independent of the chemical nature of protein surface and excipient concentration; and (2) those that are not (Timasheff, 1993, 1998). The first-type agents achieve preferential exclusion either by increasing the surface free energy of water or by steric exclusion. The second-type agents rely on the solvophobic effect such as glycerol, PEG, 2-methyl-2,4-pentanediol (MPD), and may stabilize or destabilize a protein depending on its concentration and solvent conditions (Kita et al., 1994; Frye and Royer, 1997).

Alternative explanations for excipient-induced protein stabilization have been offered recently through studies on hydrogen exchange (HX) in proteins by NMR. Different types of protons have different rates of HX in a protein, including slow-exchanging amide protons mainly from the compact unfolded state and the fast and intermediate amide protons mainly from the native state (Wang et al., 1995). Sucrose at 1 M decreases the HX rate of slow-exchanging amide protons by decreasing the population of unfolded protein, suggesting that sucrose may favor protein folding (Wang et al., 1995). An increased rate of folding is also the primary reason for stabilization of staphylococcal nuclease by xylose (Frye and Royer, 1997).

The H-D exchange rate and solvent accessibility are considered a measurement of protein flexibility, which inversely correlates protein stability (Tang and Dill, 1998). It has been found that both the H-D exchange rate in IL-1ra and the reactivity of Cys (free thiol groups) with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NbdCl) are reduced by increasing sucrose concentrations, suggesting reduction of solvent accessibility and conformational mobility of rhIL-1ra by sucrose (Kendrick et al., 1997). The apparent hydrodynamic diameter of the protein also decreases gradually with increasing sucrose concentration, which indicates increased packing of the protein structure and decreased solvent accessibility.

#### 4.2. *Stabilization of proteins by excipients*

The most common method for stabilizing liquid protein pharmaceuticals is the use of a formulation excipient(s), which should be at least soluble and nontoxic. These excipients are also referenced as chemical additives (Li et al., 1995c), co-solutes (Arakawa et al. 1993) or co-solvents (Timasheff, 1993, 1998; Lin and Timasheff, 1996). These excipients can also retard certain chemical degradations in proteins. Their stabilizing effects are usually concentration- and protein-dependent, although high concentrations of excipients may not be necessarily more effective, and in some cases, can have negative effects. For example, sorbitol reduces heat-induced aggregation of aFGF at

55°C effectively at concentrations below 0.5 M but is less effective at higher concentrations (Tsai et al., 1993).

Protein-stabilizing excipients can be broadly divided at least into the following types: sugars and polyols, amino acids, amines, salts, polymers, and surfactants. Their stabilizing effects can be significantly different (Table 4). For example, the amounts of excipients required to reduce 50% of heat-induced aggregation of aFGF are 0.55 mM for sodium sulfate, 1.6 mM for His, 180 mM for Gly, 155 mM for trehalose, 250 mM for dextrose and 310 mM for sorbitol (Tsai et al., 1993).

#### 4.2.1. Stabilizing buffer

Usually, a protein is stable only in a very narrow pH range, such as recombinant FVIII SQ, whose stable pH range is between 6.5 and 7 in 0.1 or 1 M NaCl solution (Fatouros et al., 1997a). Accurate control of the pH of a protein formulation is the first step toward protein stabilization. Unfortunately, no general rules have been reported for buffer selection. In a few cases, phosphate buffer has been shown to be detrimental. About 38% of hEGF at 0.5 µg/ml are deamidated in Tris-HCl at pH 7.0 on incubation at 60°C for 2 days, and the amounts of deamidated hEGF increase to 83, 63, 52, 51, and 49%, respectively, in buffers of PBS, sodium phosphate, sodium borate, sodium citrate, and sodium acetate (Son and Kwon, 1995). Similarly, at 37°C, rhKGF in 100 mM phosphate buffer (pH 7.0) has a half-life of 15 days, but the half-life increases to 25 and 88 days, respectively, in 100 and 500 mM sodium citrate buffers (Chen et al., 1994a). The better stability of rhKGF in citrate buffer may be due to the favorable interaction of citrate with positively charged residues in rhKGF as hypothesized in the stabilization of pig heart mitochondrial malate dehydrogenase (*phm*-MDH) (Jensen et al., 1996). In a different study, however, increasing citrate buffer concentrations increase the formation of bFGF particles at pH 5, and the citrate buffer also causes aggregation of bFGF at pH 3.7, whereas acetate buffer at pH 3.8 does not (Wang et al., 1996b). On the other hand, phosphate has been shown to stabilize certain proteins. Increasing phosphate buffer concentration from 4.6 to

9.2 mg/ml significantly decreases the rate of aFGF aggregation (Won et al., 1998). In the presence of 0.1 or 1 mM phosphate, the GdnHCl concentration for unfolding 50% alkaline phosphatase molecules is increased from 2.46 to 2.67 and 2.72 M, respectively (Hung and Chang, 1998). Phosphate at 0.05 M can also stabilize maize leaf phosphoenolpyruvate carboxylase (*ml*-PEPC) upon incubation at 40°C, although it is not as effective as citrate at the same concentration (Jensen et al., 1996).

Both buffer species and concentration may affect physical and chemical stability of a protein. Pikal et al. (1991) examined the effect of three phosphate buffer concentrations (0.11, 0.23 and 0.45 mg sodium phosphate/mg hGH) on the stability of hGH. While maximum aggregation occurs at 0.23 mg sodium phosphate/mg hGH, chemical degradations (oxidation and deamidation) are less significant at both 0.23 and 0.45 mg sodium phosphate/mg hGH. At a lower buffer concentration of 0.11 mg sodium phosphate/mg hGH, chemical degradation is relatively high. Therefore, a balanced buffer concentration should be selected to maximize protein stability. An ideal buffer should be able to inhibit both physical and chemical instabilities simultaneously.

#### 4.2.2. Sugars and polyols

Sugars and polyols represent often-used non-specific protein stabilizers. Their stabilizing effect is demonstrated and widely interpreted as the result of preferential exclusions, weak and non-specific (Xie and Timasheff, 1997a,b; Timasheff, 1998). Among various sugars, sucrose and trehalose appear to be the most often-used stabilizers. Sucrose has been shown to effect concentration-dependent stabilization of rhDNase at pH 6.8 (Chan et al., 1996), and inhibition of IL-1ra dimer formation (Chang et al., 1996a) and GdnHCl-induced rhIFN-γ aggregation (Kendrick et al., 1998a). Similarly, trehalose is able to increase the  $T_m$  of RNase A (Lin and Timasheff, 1996), to inhibit heat-induced aggregation of aFGF (Tsai et al., 1993), and to stabilize rhDNase in a concentration-dependent manner (Chan et al., 1996).

Table 4  
Examples of protein stabilization by excipients in liquid state

Proteins	Formulation compositions	Study conditions	Remaining activity (or as stated)	References
Baker's yeast ADH	50 µg/ml in 20 mM potassium phosphate, pH 7.6	50°C for 10 min	50%	Ramos et al., 1997
	Control+500 mM KCl	50°C for 10 min	~90%	Ramos et al., 1997
	Control+500 mM trehalose	50°C for 10 min	~70%	Ramos et al., 1997
	Control+500 mM mannosylglycerate	50°C for 10 min	~90%	Ramos et al., 1997
hEGF	0.5 µg/ml in 50 mM sodium phosphate, pH 8.0	60°C for 1 day	49%	Son and Kwon, 1995
	Control+0.02% Triton X-100	60°C for 1 day	84%	Son and Kwon, 1995
	Control+0.01% Tween 20	60°C for 1 day	75%	Son and Kwon, 1995
	Control+0.05% fibronectin	60°C for 1 day	85%	Son and Kwon, 1995
	Control+6 mM ZnCl <sub>2</sub>	60°C for 1 day	76%	Son and Kwon, 1995
GDH	50 µg/ml in 20 mM potassium phosphate, pH 7.5	50°C for 10 min	0%	Ramos et al., 1997
	Control+500mM KCl	50°C for 10 min	~90%	Ramos et al., 1997
	Control+500 mM trehalose	50°C for 10 min	~2%	Ramos et al., 1997
	Control+500 mM mannosylglycerate	50°C for 10 min	~90%	Ramos et al., 1997
rIL-2	0.2 µg/ml in PBS, pH 7.0	Moderate shaking at 4°C for 96 h	~30%	Wang and Johnston, 1993a
	+10% (w/w) Pluoronic F-127	Moderate shaking at 4°C for 96 h	100%	Wang and Johnston, 1993a
rhKGF	0.5 mg/ml in 140 mM NaCl and 10 mM sodium phosphate, pH 7.0	37°C	$T_{1/2}+2$ days (loss of soluble aggregates)	Chen et al., 1994a
	0.5 mg/ml in 100 mM sodium phosphate, pH 7.0	37°C	$T_{1/2}+15$ days	Chen et al., 1994a
	0.5 mg/ml in 100 sodium citrate buffer, pH 7.0	37°C	$T_{1/2}+25$ days	Chen et al., 1994a
	0.5 mg/ml in 500 sodium citrate, pH 7.0	37°C	$T_{1/2}+88$ days	Chen et al., 1994a
	0.5 mg/ml in 10 mM sodium phosphate, 140 mM NaCl, and 0.5% (w/v) heparin, pH 7.0	37°C	$T_{1/2}+105$ days	Chen et al., 1994a
Rabbit muscle LDH	50 µg/ml in 20 mM potassium phosphate, pH 7.6	50°C for 10 min	5%	Ramos et al., 1997
	Control+500 mM KCl	50°C for 10 min	~20%	Ramos et al., 1997
	Control+500 mM trehalose	50°C for 10 min	~25%	Ramos et al., 1997
	Control+500 mM mannosylglycerate	50°C for 10 min	~90%	Ramos et al., 1997
Urease	0.33 mg/ml in phosphate buffer and 1 mM EDTA, pH 7.0	Moderate shaking at 4°C for 96 h	~40%	Wang and Johnston, 1993a
	Control+10% (w/w) Pluoronic F-127	Moderate shaking at 4°C for 96 h	100%	Wang and Johnston, 1993a
Urease	0.33 mg/ml in phosphate buffer and 1 mM EDTA, pH 7.0	50°C for 96 h	AUC = 4868 (activity-time plot)	Wang and Johnston, 1993b
	Control+1% Pluoronic F-127	50°C for 96 h	AUC = 6722	Wang and Johnston, 1993b

In all these examples, the stabilizing effect of sugars depends on their concentrations. A concentration of 0.3 M (or 5%) sugar or polyols has been suggested to be the minimum to achieve significant protein stabilization (Arakawa et al. 1993). This seems true in many cases. As high as 1 M sucrose or 10% glycerol have been commonly used to protect the activity of proteins during their isolation (Timasheff, 1993). Other examples include stabilization of thrombin by 20% dextrose or sorbitol or 50% glycerol (Boctor and Mehta, 1992) and rhG-CSF by 5% mannitol (Herman et al., 1996), inhibition of bFGF aggregation by  $\geq 30\%$  sucrose (Wang et al., 1996b), IL-1ra dimer formation by 40% (w/v) sucrose (Chang et al., 1996a), and IgG aggregation by 33% sorbitol (Ganzález et al., 1995), and significant increase in the  $T_m$  of RNase A by 0.5–1 M sucrose or sorbitol (Liu and Sturtevant, 1996; McIntosh et al., 1998) and porcine pancreatic elastase by 20% sucrose, sorbitol, lactose, or mannitol (Chang et al., 1993).

Different sugars or polyols may stabilize a protein to a similar or different degree depending on the protein. It has been found that lactose, sucrose, trehalose, or mannitol stabilizes rhDNase at pH 6.8 to a similar degree based on their effect on the  $T_m$  (Chan et al., 1996). In a different case, while 0.55 M sucrose increases the  $T_m$  of RNase from 35.5 to 44.9°C, the same concentration of sorbitol only increases the  $T_m$  to 40.5°C (McIntosh et al., 1998). Similarly, different concentrations of excipients (155 mM trehalose, 250 mM dextrose, or 310 mM sorbitol) are required to reduce 50% of heat-induced aggregation of aFGF (Tsai et al., 1993). Sucrose at 10, 300, or 600 mg/ml is more effective than sorbitol or mannitol in the stabilization of FVIII SQ (Fatouros et al., 1997b).

Sugars and polyols can also protect proteins from chemical degradations. For example, Li et al. (1996a,b) studied the effect of sugars and polyols on the metal-catalyzed oxidation of human relaxin and found that sugars and polyols, such as glucose, mannitol, glycerol, and ethylene glycol, can significantly inhibit the oxidative degradation. The inhibitory effect was shown to be due to their complexation with metal ions instead of the commonly-accepted radical-scav-

enging mechanisms. Due to the weak complexation, as high as 50 mM mannitol is needed to have a significant protective effect.

Not all proteins can be stabilized by sugars or polyols. At 37°C, the half-life for the loss of soluble rhKGF in 10 mM phosphate buffer (pH 7.0) and 140 mM NaCl is 1.8 days and replacing NaCl with 9% (w/v) sucrose or 5% sorbitol does not improve its half-life (Chen et al., 1994a). Sorbitol does not show any effect against interfacial denaturation of pGH and is marginally effective to prevent precipitation by thermal and guanidine dilution denaturation (Charman et al., 1993). Mannitol at 5 mg/ml even destabilizes IL-1R by decreasing its  $T_m$  from 48.1 to 46.7°C (Remmele et al., 1998).

In the selection of sugars for protein stabilization, reducing sugars should be avoided whenever possible, as these sugars have the potential to react with amino groups in proteins via the Maillard reaction (see Section 3.2).

#### 4.2.3. Surfactants

There are basically two types of surfactants—nonionic and ionic. These surfactants drop surface tension of protein solutions and decrease the driving force for protein adsorption and/or aggregation at hydrophobic surfaces.

Nonionic surfactants are generally preferred in protein stabilization. Low concentrations of nonionic surfactants are often sufficient to prevent or reduce protein surface adsorption and/or aggregation due to their relatively low critical micelle concentrations (CMC) (Bam et al. 1995). Complete or significant inhibition has been reported of surface adsorption loss of TGF- $\beta_1$  by Tween 80 at 0.01% (Gombotz et al., 1996), glass surface-induced rConIFN aggregation by Tween 20 or Tween 80 at 0.01% (Ip et al., 1995), shear (or shaking)-induced rhGH aggregation by Pluronic F88 or Tween 20 at 0.1 mg/ml (or at  $\geq 4:1$  Tween 20:rhGH molar ratio) (Maa and Hsu, 1997; Bam et al., 1998), shaking-induced hemoglobin aggregation by Tween 80 at 0.045% (Kerwin et al., 1998), vortex-induced hGH aggregation by Tween 80, Brij 35, or Pluronic F68 at respective concentrations of 0.1%, 0.013%, and 0.1% (Katakam et al., 1995), refolding-induced rhGH aggregation by

Tween 20, 40, and 80 at a Tween:rhGH molar ratio of 10 (Bam et al., 1996), shaking-induced loss of urease and rIL-2 activity by poloxamer 407 (Pluronic F-127) at 10% (w/w) (Wang and Johnston, 1993a), and agitation-induced rhFXIII aggregation by Tween 20 at around 0.007% (w/v) (Kreilgaard et al., 1998). Tween 20 seems to be relatively more effective in a few cases. The rank order of minimizing rhG-CSF adsorption to PVC surface is Tween 20 > Tween 80 > Pluronic F127 > Pluronic F68 (Johnston, 1996). Tween 20 is also more effective than Tween 80 or Tween 40 in the inhibition of shaking-induced aggregation of rhGH (Bam et al., 1998). Not all proteins can be stabilized by surfactants and Tween 80 at 0.1% has no effect on the aggregation of IL-1 $\beta$  at 100  $\mu$ g/ml at pHs from 3 to 7 (Gu et al., 1991).

Nonionic surfactants can also inhibit chemical degradations in proteins. hEGF degrades mainly by deamidation and loses 51% of its mitogenic activity in 50 mM sodium phosphate buffer (pH 8.0) during storage at 60°C for 1 day, and in the presence of 0.02% Triton X-100 or 0.01% Tween 20, the remaining activity is, respectively, 84 and 75% (Son and Kwon, 1995). At the same time, surfactants may be contaminated with alkyl peroxides, which can accelerate oxidation of proteins. rhCNTF has been found to dimerize by alkyl peroxides present in Tween 80 (Knepp et al., 1996). Inclusion of certain antioxidants, such as Cys, Met, or glutathione, can significantly retard the reaction. A correlation between the peroxide level in Tween 80 and the degree of oxidation in rhG-CSF has also been reported, and the peroxide-induced oxidation appears more severe than that induced by atmospheric oxygen present in the vial headspace (Herman et al., 1996). For this reason, only 0.004% Tween 80 is formulated in the liquid rhG-CSF formulation.

Ionic surfactants are usually not used in stabilizing proteins because they can bind to both polar and nonpolar groups in proteins and cause denaturation (Giancola et al., 1997). Anionic surfactants such as SDS generally bind more effectively than cationic detergents such as decyltrimethyl ammonium chloride. The binding of ionic detergents such as SDS to a protein is a function of the free detergent concentration and

may become saturated beyond the critical micelle concentration (Volkin and Klivanov, 1989). Nevertheless, these surfactants have been reported to have stabilizing effect on proteins. It has been found that SDS stabilizes BSA up to a SDS/protein molar ratio of 10 (Giancola et al., 1997) and reduces aggregation of aFGF in a concentration-dependent manner (Won et al., 1998). SDS at either 0.1 or 1% effectively prevents aggregation of heat-denatured RNase at pH 7.8, possibly by increasing repulsion of the added negative charges on the protein (Tsai et al., 1998b). Anionic detergents have also been used to increase thermal stability of proteins in non-aqueous solvents by stoichiometric formation of complexes with proteins (hydrophobic ion pairing) (Manning et al., 1995). In addition, certain ionic (including nonionic) surfactants in a limited concentration range may help protein refolding. Rhodanese at 1 mg/ml denatures in a mixture of 6 M GdnHCl and 200 mM  $\beta$ -mercaptoethanol, and the denatured enzyme at 50  $\mu$ g/ml can be reactivated to various extents in the presence of 1 mg/ml ionic (sodium cholate, cetyltrimethylammonium bromide, sodium lauryl sulfate), zwitterionic (*N*-octyl-, *N*-decyl-, *N*-dodecyl-, *N*-tetradecyl- and *N*-hexadecyl-*N,N*-dimethyl-3-ammonio-1-propane-sulfonate), and nonionic surfactants (Tween 20, Triton X-100, dodecyl  $\beta$ -D-maltoside) (Tandon and Horowitz, 1987). The activity recovery of the denatured enzyme is bell-shaped as a function of cetyltrimethylammonium bromide concentration in the range of 0–5 mg/ml, indicating different effects at different concentrations.

Nonionic surfactants also bind weakly to proteins. The binding stoichiometry between Tween 20 and rhGH has been determined to be 2.5–3.5 (Tween 20/rhGH) by electron paramagnetic resonance (EPR) (Bam et al., 1995). A recent study shows that complete blocking of shaking-induced rhGH aggregation can be achieved only at a Tween 20:rhGH molar ratio of  $\geq 4:1$  (Bam et al., 1998). This molar ratio is close to the binding stoichiometry, suggesting that the inhibition of rhGH aggregation is probably due to Tween 20 binding to and blocking the aggregation-prone hydrophobic sites on the protein surface. Since the Tween 20 concentration required for complete

protection of the protein is much higher than the reported CMC of Tween 20 (59  $\mu\text{M}$ ), CMC does not appear to play a critical role. Therefore, the net effect of surfactants may depend on their concentrations relative to that of the protein (Katakam et al., 1995). In a different case, maximum protection of rhFXIII from agitation-induced aggregation is found at Tween 20 concentrations close to its CMC, independent of initial protein concentration (Kreilgaard et al., 1998). Therefore, additional mechanisms of protein protection by Tween 20 exist, which seem to arise from its competition with proteins for adsorption on denaturation interfaces.

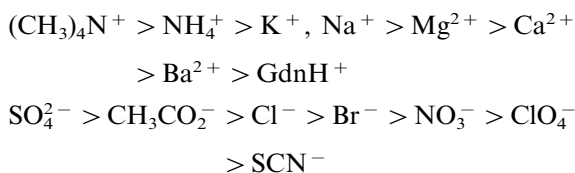
Surfactant–protein interactions are hydrophobic in nature because proteins with more hydrophobicity bind more surfactants (Bam et al., 1995). Therefore, it has been suggested that surfactants should be avoided when formulating hydrophobic proteins (Dix et al., 1995). Since hydrophobic interaction increases with increasing temperature, a stronger hydrophobic protein–surfactant interaction is expected at high temperatures. This may explain why nonionic surfactants destabilize certain proteins, especially at higher temperatures or toward thermal denaturation. For example, poloxamer 407 at 1% significantly increases the stability of urease at 0.33 mg/ml in phosphate buffer (pH 7.0) at 37 or 50°C but decreases its stability at 75°C (Wang and Johnston, 1993b). This surfactant at 0.5% also decreases the stability of IL-2 at about 0.2  $\mu\text{g}/\text{ml}$  in phosphate buffer (pH 7.0) at 50°C (Wang and Johnston, 1993b). rhCNTF, a hydrophobic and physically unstable protein, is destabilized by Tween 80 when subjected to thermal incubation (Dix et al., 1995). Either Pluronic F68 at 0.1 mg/ml or Tween 80 at 1 mg/ml destabilizes IL-1R by decreasing its  $T_m$  (Remmele et al., 1998). Decreased  $T_m$  is also observed for rhGH at 3 mg/ml in the presence of Tween 80, Tween 40, or Tween 20 at a surfactant:rhGH molar ratio of 10:1 (Bam et al., 1998).

Poloxamers 407 and 188 (Pluronic F68) have been shown to inhibit thermally-induced aggregation/precipitation of recombinant hGH or methionyl pGH (Charman et al., 1995; Katakam and Banga, 1997). In comparison with Tweens, poloxamers may increase viscosity of a protein solution, restraining the motion of protein backbone to achieve thermal stabilization (Wang and Johnston, 1993a).

#### 4.2.4. Salts

As discussed in Section 3.3, salts may stabilize, destabilize, or have no effect on protein stability depending on type and concentration of salt, nature of the ionic interactions, and the charged residues in a protein (Kohn et al., 1997). The net effect is a balance among non-specific (Debye-Hückel) electrostatic shielding, specific ion binding to a protein, and its effect on solvent properties.

The effect of salt at high concentrations correlates with the Hoffmeister lyotropic series both for anions and cations (Volkin and Klibanov, 1989; Timasheff, 1993, 1998):



in which anions and cations to the left of the series are the most stabilizing. By increasing ionic strength of a solution, these salts enhance hydrophobic interactions and reduce solubility of hydrophobic groups in proteins. In addition, they enhance accumulation of water molecules around proteins, leading to preferential hydration. The combination of these two effects makes proteins more compact and stable. Ions on the right are destabilizing by binding extensively to the charged groups or the dipoles (peptide bonds) of proteins.

NaCl, the often-used salt, has been found to play a critical role in stabilizing certain proteins. It increases both the denaturation temperature and enthalpy of BSA due to increased compactness of the protein structure resulting from an increase in ionic strength and possible reduction in long-range electrostatic repulsion between the net charges (Giancola et al., 1997). Recombinant factor VIII SQ (rFVIII SQ) at 125 IU/ml is not stable at low salt concentrations and precipitates at <0.1 M NaCl at pH 7 (Fatouros et al., 1997a). A higher amount of rFVIII SQ needs higher NaCl concentrations to prevent precipitation. Increasing NaCl concentration (up to 1 M) also linearly increases the  $T_m$  of RNase T1 from 59 to 63°C (Mayr and Schmid, 1993). Recently, NaCl has been found to be the most effective in improving



the thermal stability of IL-1R among all the excipients tested, including ascorbic acid, sugars (mannitol, lactose, sucrose, glucose), polyols (PEGs, glycerol, ethanol), polymers (PVP 10k, dextran 40), salts ( $\text{CaCl}_2$ ), amino acids (Gly, Lys, Cys, Ala, Arg) and surfactants (Pluoronic F68, Tween 80) (Remmele et al., 1998).

Another similar salt, KCl, is also a powerful protein stabilizer. It has been shown to protect the activity of yeast ADH and bovine liver glutamate dehydrogenase (GDH) against thermal inactivation more effectively than trehalose, a commonly-used nonspecific protein stabilizer (Ramos et al., 1997).

The separate contribution of anions and cations in salts to the stability of a protein can be significantly different or even in opposite direction (Timasheff, 1998). For example, GdnHCl at 3 M destabilizes RNase at pH 7.0 by decreasing its  $T_m$  from 60 to 25°C, but  $(\text{GdnH})_2\text{SO}_4$  at 3 M stabilizes the protein by increasing its  $T_m$  by 10°C due to the dominant contribution of stabilizing  $\text{SO}_4^{2-}$  (Timasheff, 1993). GdnHCl at low concentrations (up to 0.3 M) stabilizes RNase T1 against thermal and urea-induced unfolding, probably due to the significant contribution of the stabilizing effect of  $\text{Cl}^-$  (Mayr and Schmid, 1993). This also seems to be the case in the stabilization of *ml*-PEPC (Jensen et al., 1996),

#### 4.2.5. Polyethylene glycols (PEGs)

PEGs are often used as protein cryoprotectants and precipitating/crystallizing agents in aqueous media at high concentrations. They are hydrophobic in nature and may interact with hydrophobic side chains in proteins, promoting unfolding, especially at high temperatures (Farruggia et al., 1997). Nevertheless, PEGs of different molecular weights have been demonstrated to stabilize certain proteins. For examples, PEG 300 at 0.5 or 2% inhibits the aggregation of rhKGF in 10 mM potassium phosphate buffer (pH 7.0) containing 5% mannitol at 45°C (Zhang et al., 1995). PEG 200, 400, 600, and 1000 stabilize lysozyme and BSA, even though they decrease the surface tension of water (Kita et al., 1994). PEG 4000 (up to 15%) inhibits the thermal aggregation of LMW-UK in a concentration-dependent manner (Vrkljan et al., 1994).

The stabilization mechanism of PEGs in solution has not been well established. Stabilization seems dependent on the protein and the size of PEGs. Liquid IL-1R is stabilized by PEGs, and the stabilization effect is  $\text{PEG 300} > \text{1000} > \text{3350}$ , possibly because PEG 300 is the least hydrophobic (Remmele et al., 1998). On the contrary, PEG 1000 and 4000 destabilize human albumin by decreasing its thermal transition temperature, while high-molecular-weight PEG 8000 and 10 000 stabilize the native state of the protein (negative preferential interaction with the protein) (Farruggia et al., 1997). Similarly, PEG 8000 at 1% significantly reduces the nebulization-induced aggregation and glass surface adsorption of rConIFN (Ip et al., 1995). Protein stabilization by high-molecular-weight PEGs is probably due to their steric hindrance of protein-protein interactions.

#### 4.2.6. Polymers

Various kinds of polymers have been shown to stabilize proteins. Stabilization is generally due to one or more of these properties of polymers: surface activity, preferential exclusion, steric hindrance of protein-protein interactions, and increased viscosity limiting protein structural movement.

Serum albumin has been used often for inhibition of protein surface adsorption and general stabilization. However, its potential contamination with blood-borne pathogens limits its future applications. Among other polymers examined for protein stabilization, hydroxypropyl- $\beta$ -cyclodextrin (HP- $\beta$ -CD) appears to be one of the most valuable, partly because it is a good solubilizing agent and parenterally safe. HP- $\beta$ -CD has been shown to prevent the thermal and interfacial denaturation and precipitation of pGH (Charman et al., 1993), to inhibit the aggregation of rhKGF (Zhang et al., 1995), and to stabilize IL-2 and bovine insulin (Brewster et al., 1991). Other polymers include dextran (51 and 18 kD) in increasing the  $T_m$  of thrombin (Boctor and Mehta, 1992), dextran (38 and 82 kD) in increasing the  $T_m$  of porcine pancreatic elastase (Chang, et al., 1993), PVP 10 k in increasing the  $T_m$  of IL-1R (Remmele et al., 1998), fibronectin (0.05%) in improving the

mitogenic activity of hEGF in 50 mM sodium phosphate (pH 8.0) at 60°C (Son and Kwon, 1995), heparin (0.5%) in the inhibition of rhKGF aggregation in 10 mM phosphate buffer (pH 7.0) at 37°C (Chen et al., 1994a), 16-kD heparin in the concentration-dependent inhibition ( $IC_{50} = 2 \mu\text{g/ml}$ ) of the heat-induced aggregation of aFGF in PBS at pH 7.2 (Volkin et al., 1993), gelatin type A and B (up to 1%) and hydroxyethyl (heta) starch (up to 20%) in the concentration-dependent inhibition of the thermal aggregation of LMW-UK (Vrkljan et al., 1994; Manning et al., 1995). A variety of polymers has been found effective in inhibiting the aggregation of rhKGF in 10 mM phosphate buffer (pH 7.0) containing 5% mannitol, including heparin, dextran sulfate, polyphosphoric acid, poly-L-glutamic acid, poly-L-lysine, etc (Zhang et al., 1995).

The effect of polymers on protein stability is strongly protein-dependent, and in certain cases destabilization may occur. While  $\alpha$ -CD and  $\gamma$ -CD do not affect the stability of hen-egg lysozyme (HEL), some of its derivatives including M- $\beta$ -CD, HE- $\beta$ -CD, HP- $\beta$ -CD, and HB- $\beta$ -CD lower the protein's  $T_m$  (Brunchu et al., 1996). Type A gelatin at 0.1% destabilizes LMW-UK but increases its stability at higher concentrations (Vrkljan et al., 1994). PVP at 2, 5, or 10% increases the heat-induced aggregation of LMW-UK (Vrkljan et al., 1994). The failure of PVP to protect the protein could be attributed to its relatively hydrophobic nature as temperature increases. The increased hydrophobicity may promote preferential binding to proteins and facilitate protein unfolding because the denatured state has more hydrophobic binding sites than the native state.

Proteins can be stabilized by polymers through multiple electrostatic interactions. A number of sulfated polysaccharides, sulfated polymers, and poly-amino acids have been shown to stabilize aFGF against aggregation, including low-MW heparin (5 kD), sulodexide, dextran sulfate, fucoidan, pentosan polysulfate, polyvinyl sulfate, keratan sulfate, poly-Asp, and poly-Glu (Volkin et al., 1993; Won et al., 1998). Increasing the sulfation level of both heparin and sulfated  $\beta$ -cyclodextrin results in dramatic enhancement of their ability against thermal inactivation of aFGF.

It is the sulfate ions that bind to specific sites in aFGF (in the vicinity of Lys<sup>118</sup>/Lys<sup>112</sup>/Arg<sup>22</sup> region) to achieve stabilization. Poly-Arg, a positively charged polymer, destabilizes aFGF. Detailed discussion can be found elsewhere on the structure, interaction, and specificity of aFGF polyanion binding sites (Volkin and Middaugh, 1996). By similar stabilization mechanisms, the aggregation of rhKGF, another member of the FGF family, is significantly inhibited by polyanions, including poly(acrylic acid), poly(methacrylic acid), heparin, dextran sulfate (8 and 500 kD), and pentosan polysulfate (Chen et al., 1994b). In a recent study, as many as 13 model proteins have been stabilized to a different degree against thermal inactivation by forming multiple electrostatic complexes with selected soluble polyelectrolytes (such as DEAE-dextran) and polyhydroxyl compounds (such as ethylene glycol) (Gibson, 1996). However, the protection against aggregation of heat-denatured RNase by dextran sulfate at 0.1 or 1% is apparently due to the increased repulsion of the added negative charges on the protein (Tsai et al., 1998b).

Polymers may inhibit chemical degradations in proteins. For example, dextran can inhibit the metal-catalyzed oxidation of human relaxin (Li et al., 1996a,b). All the polyanions that protect aFGF against heat-induced aggregation also protect the protein at least partially against copper-catalyzed oxidation (Volkin et al., 1993). Among the polymers, heparin is the most effective. The protection could be due to both the dominant interaction of polymers with the protein and chelation of divalent metal cations.

Polymers used for protein stabilization should be stable enough against chemical and/or enzymatic degradations so that they can keep their stabilizing effect during storage. Unfortunately, proteins may be contaminated with trace amounts of enzymes, which may degrade a polymer stabilizer and result in loss of formulation quality or even protein stability. For example, hydroxyethylcellulose (HEC) has been formulated with recombinant bFGF. The viscosity of the formulation decreases with time due to the presence of trace amounts of a cellulase-like activity that originates from the *E. coli* lysate in the production of the protein (Shahrokh et al., 1995).

#### 4.2.7. Metal ions

Some metal ions (such as calcium, magnesium, and zinc) can be used as protein stabilizers as they bind to a protein and make the overall protein structure more rigid, compact, and stable (Kristjánsson and Kinsella, 1991). This is one of the mechanisms responsible for the high stability of thermophilic proteins (Mozhaev and Martinek, 1984). In addition to stabilization, metal ions may enhance the activity of a protein such as RNase H (Goedken and Marqusee, 1998). Among these metal ions, calcium is a well-known stabilizer for many enzymes at both high and low temperatures (Daniel et al., 1996).

Different metal ions may require different concentrations to achieve protein stabilization, and their specificity in protein stabilization varies. The effect of some divalent metal ions on rFVIII SQ has been investigated, including  $\text{Ca}^{+2}$ ,  $\text{Cu}^{+2}$ ,  $\text{Mn}^{+2}$ ,  $\text{Mg}^{+2}$ ,  $\text{Zn}^{+2}$ ,  $\text{Sr}^{+2}$ , and  $\text{Fe}^{+2}$  at 1 and 10 mM (Fatouros et al., 1997a). At 1 mM, only  $\text{Ca}^{+2}$  and  $\text{Sr}^{+2}$  increase the stability relative to the control (0.1 mM  $\text{Ca}^{+2}$ ) and  $\text{Fe}^{+2}$  sharply destabilizes rFVIII SQ. At 10 mM,  $\text{Ca}^{+2}$  and  $\text{Sr}^{+2}$  further increase the stability of rFVIII SQ up to about 50 mM.  $\text{Cu}^{+2}$ ,  $\text{Zn}^{+2}$  and  $\text{Fe}^{+2}$  all sharply reduce the stability at 10 mM.  $\text{Mn}^{+2}$  and  $\text{Mg}^{+2}$  do not have any significant effect at both concentrations. In another study, the  $T_m$  of rhD-Nase increases with increasing  $\text{Ca}^{2+}$  ( $\text{CaCl}_2$ ) concentrations and reaches a maximum at around 100 mM  $\text{CaCl}_2$  (Chan et al., 1996). However,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Zn}^{2+}$  destabilize the protein. The stabilizing effect of  $\text{Ca}^{2+}$  probably results from its binding to the protein and preventing the breaking of a disulfide bridge. Other examples of protein stabilization by metal ions include the inhibition of insulin fibrillation by calcium or zinc ions (Brange et al., 1997), the stabilization of 19 kD catalytic fragment of human fibroblast collagenase by calcium (Lowry et al., 1992), the stabilization of RNase H by  $\text{Mn}^{2+}$  (Goedken and Marqusee, 1998), the increase in  $T_m$  of porcine pancreatic elastase by 10 mM  $\text{CaCl}_2$  (Chang, et al., 1993), the stability dependence of bovine pancreatic DNase on  $\text{Ca}^{2+}$  (Shire, 1996), and the increased hEGF mitogenic activity in the presence of 6 mM  $\text{ZnCl}_2$  in 50 mM sodium phosphate

buffer (pH 8.0) during storage at 60°C (Son and Kwon, 1995). Calcium or zinc stabilizes insulin probably by neutralizing negative charges in the center of insulin hexamer and enhancing native hydrophobic interaction. In a different case,  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ , and  $\text{KSCN}$  at concentrations below 0.5 M destabilize albumin and lysozyme by preferential binding to these proteins (Picó, 1996).

The effect of metal ions on protein stability can be significantly influenced by the negative counter ions. Picó (1996) studied the effect of sodium halides at 0.3 M on the stability of human serum albumin in 20 mM sodium phosphate (pH 7.4) and found that the negative ions increase the melting temperature and enthalpy of unfolding of albumin in the following order:  $\text{I}^- > \text{Br}^- > \text{Cl}^- > \text{F}^-$ .

#### 4.2.8. Amino acids

Certain amino acids, either alone or in combination with other excipients, stabilize proteins most likely by preferential exclusion (Jensen et al., 1996). The aggregation of rhKGF in 10 mM potassium phosphate buffer (pH 7.0) containing 5% mannitol at 45°C can be inhibited to various degrees in the presence of the following amino acids: His, Gly, sodium aspartate, glutamate, and lysine hydrochloride (Zhang et al., 1995). Similarly, the stability of *ml*-PEPC can be significantly increased in the presence of 0.05 M sodium glycinate, sodium glutamate, sodium aspartate, or lysine hydrochloride upon incubation at 40°C (Jensen et al., 1996). Replacing 40 mM NaCl with 0.4% (w/v) L-Asp and 0.7% (w/v) L-Glu significantly increases the half-life of rhKGF from 1.8 to 2.6 and 2.4 days, respectively, for the loss of soluble protein at 37°C (Chen et al., 1994a). Gly at 1 M increases the  $T_m$  of RNase A from 65 to 68°C at pH 6.0 (Liu and Sturtevant, 1996) and at 2 M increases the  $\Delta G_{f \rightarrow u}$  of cytochrome c by 17.3 kJ/mol (Foord and Leatherbarrow, 1998). Use of amino acids with propylene glycol improves the structural stability of rhCNTF (Dix et al., 1995). L-Lys and L-Arg increase the stability of IL-1R by increasing its  $T_m$ , although Gly and L-Ala decrease it (Remmele et al., 1998).

Certain chemical degradations in proteins can be reduced in the presence of certain amino acids.

Met is an effective antioxidant. His at 2.5 mM can completely inhibit the oxidation-induced inactivation of papain by the ascorbate/Cu(II)/O<sub>2</sub> system, possibly through the formation of Cu-His complex (Kanazawa et al., 1994).

#### 4.2.9. Miscellaneous compounds/approaches

The identification of certain cellular accumulants in thermophilic organisms upon temperature or salinity increase has led to the choice of some of these compounds as protein stabilizers. For example, potassium salt of cyclic diphosphoglycerate can stabilize the thermolabile GADPH at 90°C (Hensel and König, 1988). Another compound is 2-*O*- $\beta$ -mannosylglycerate in the stabilization of several mesophilic proteins, including rabbit muscle LDH, baker's yeast ADH, and bovine liver GDH, which is more effective than trehalose (Ramos et al., 1997). Specifically, this compound at 500 mM keeps 90% of LDH activity while trehalose (or KCl) at the same concentration keeps about 30% activity after incubation of the protein at 50  $\mu$ g/ml in phosphate buffer at 50°C for 10 min.

A few other compounds have been reported to increase the thermal stability of proteins. These include dipalmitoylphosphatidylglycerol (DPPG, an anionic lipid) for RNase and cytochrome c (Lo and Rahman, 1998), 2-bromo-2-chloro-1,1,1-trifluoroethane for serum albumin (Tanner et al., 1999), and some anionic phosphate species such as ATP, inorganic phosphates, and phosphorylated inositols for aFGF (Volkin et al., 1993). Perfluorodecalin, a non-aqueous solvent, has recently been used to stabilize plasma-derived factor IX and recombinant human  $\alpha$ -IFN in the absence of moisture (<0.001%) (Knepp et al., 1998). Its stabilization mechanism is briefly discussed in Section 3.3.

Another potential way of stabilizing proteins is the use of so-called molecular chaperones. Folding of newly synthesized polypeptides in cells requires the assistance of molecular chaperone proteins because of the tendency of folding intermediates to aggregate at relatively high protein concentrations (Hendrick and Hartl, 1995; Lewis and Cowan, 1996; Ruddon and Bedows, 1997). For example, the GroEL minichaperone in bacte-

ria has been found to facilitate protein folding by preventing protein aggregation and correcting protein misfolding (Golbik et al. 1998).  $\alpha$ -Crytallin exhibits chaperone-like properties and inhibits the aggregation of denatured-reduced lysozyme (Raman et al., 1997). Recombinant human protein disulfide isomerase (rhPDI) enhances folding of C125A rhIL-2 by accelerating thiol-disulfide interchange (Du et al., 1998). Therefore, inclusion of a molecular chaperone in a protein formulation is foreseeable but seems practical only if the chaperone can be easily obtained and is safe and stable.

Reduction of dissolved oxygen in protein solutions should decrease oxygen-initiated oxidation. It has been found that replacing the air in container headspace with nitrogen reduces the loss of rFVIII SQ activity from 69 to 19% during storage at 7°C (Fatouros et al., 1997a). To prevent potential oxidation of proteins, oxygen dissolved in protein solutions can be simply and effectively reduced by a few low-temperature cycles of normal-low-normal pressure changes (Fransson et al., 1996).

Computer graphics can be used to facilitate design of more effective protein stabilizers based on proteins' steric structures. Such a molecule, benzene-1,4-disulfonic acid, has been designed to fill the center space of insulin hexamer and appears to stabilize the hexamer (Manallack et al., 1985).

#### 4.3. Stabilization of proteins by structural modification

Although proteins are very delicate molecules, they can be made more stable by careful modification of their amino acids either chemically or genetically. Again, the key issue is preservation of protein activity during and after the modification.

##### 4.3.1. Modification of amino acid and site-directed mutagenesis

Site-directed mutagenesis or chemical modification can be used to modify protein stability by mutating or blocking specific amino acids. Structural characteristics of labile proteins may be changed toward those of thermophilic proteins (see Section 2.3).

After analyzing the effect of amino acid replacement on protein thermal stability, Querol et al. (1996) made the following recommendations for amino acid replacement: (1) maintaining or enhancing secondary structure propensity; (2) replacing preferentially solvent accessible residues; (3) replacing residues by introducing a negative charge at N-cap or a positive charge at the C-cap of the helix (same for  $\beta$ -stands); (4) substituting with residues of enhancing hydrogen bonding or van der Waals contacts; (5) favoring the formation of an additional disulfide bridge, metal binding site, or glycosylation site; and (6) substituting with Pro in loops.

Even with these clear-cut guidelines, it has not been always successful to increase protein stability simply by amino acid substitution because of the complexity of protein structure and the limited number of amino acids responsible for the extra protein stability (Daniel et al., 1996). For example, the higher thermostability of the novel bacillar ADH is apparently due to the replacement of only three amino acids (Glu<sup>11</sup> → Lys, Lys<sup>14</sup> → Gln, and Pro<sup>242</sup> → Ala) (Cannio et al., 1994). It is no surprise that amino acid substitution has been done often on a trial-and-error basis. Even after well-defined amino acid exchange(s), it is often difficult to predict the protein stability (Jaenicke, 1990, 1996; Lee and Vasmatzis, 1997). Recently, using tables of amino acid substitution and propensity, Topham et al. (1997) were able to correlate the predicted stability change after amino acid substitution with experimentally determined free energy difference ( $\Delta\Delta G$ ) or difference in melting temperature between a mutant and the wild-type protein. A correlation coefficient of 0.80 is obtained for 83 staphylococcal nuclease and 68 barnase mutants. Also, mutations at the buried sites are more reliably predicted than those at the exposed or partially-buried positions, and better predictions are obtained with residues not engaged in hydrogen bonding interactions with neighboring residues than those that are.

Protein activity may change significantly after amino acid substitution. Both successful and unsuccessful examples exist in this regard. aFGF has three Cys residues (Cys-16, -83, -117), and all three single Ser mutants are as active as the wild

type in the presence of heparin. In the absence of heparin, the triple mutant is the most active and stable (Volkin and Middaugh, 1996). However, substitution of Glu<sup>58</sup> with Ala in the wild-type ribonuclease T1 not only destabilizes the protein by 0.8 kcal/mol ( $\Delta\Delta G = -0.8$ ) but also removes the majority of the hydrolytic activity (Shirley et al., 1989). A more recent study on the stability of T4 lysozyme mutants shows that those mutants with increased protein stability have either abolished or reduced protein activity (Shoichet et al., 1995).

There are other concerns in modifying protein structure for increased stability. One of them is the immunogenicity of a protein, which may change significantly. Therefore, mutant proteins have to go through extensive toxicity studies if they are pharmaceutical candidates. Replacement of any hydrophilic amino acids with hydrophobic ones may change the solubility of a protein, presenting formulation challenges. Thus, any chemical or genetic modification of proteins should be carefully considered.

#### 4.3.2. Glycosylation

Glycosylation can affect protein activity, antigenicity, solubility, proteolytic resistance, and stability (Liu, 1992). Different glycoforms frequently have different physical and chemical properties. Therefore, proteins can be properly glycosylated to increase their stability. Several stabilization mechanisms of protein glycosylation have been proposed. These include formation of hydrogen bonds with the polypeptide backbone or surface hydrophilic amino acids, and steric interaction with adjacent peptide residues (Baek and Vijayalakshmi, 1997; Runkel et al., 1998). Again, the key issue is preservation of protein activity after glycosylation.

There are many successful cases of protein stabilization by glycosylation. Glycosylation of RNase A dramatically increases its stability with its activity essentially unchanged (Baek and Vijayalakshmi, 1997). The native RNase A at 0.3 mg/ml in 10 mM phosphate buffer (pH 8.0) loses more than 90% of its original activity after incubation at 90°C for 15 min, while the glycosylated protein maintains about 75% of its activity under

the same condition. Glycosylated  $\alpha_1$ -antitrypsin has been found to be more resistant to urea-induced unfolding and thermal denaturation (an aggregation process) than non-glycosylated form (Kwon and Yu, 1997).

Insulin may form fibrils in solution, probably due to the presence of insulin monomer and its conformational change at a hydrophobic interface. To examine the effect of glycosylation on insulin fibrillation, several *p*-succinamidophenylglucopyranoside-insulin conjugate (SAPG-insulin) have been synthesized, including monosubstituted, disubstituted, and trisubstituted insulins. It has been found that the tendency to form insulin dimers/hexamers decreases and the physical stability increases as the number of SAPG moieties increases (Baudyš et al., 1995). This is seemingly due to increased hydrophilicity of the monomer surface and/or steric hindrance by SAPG moieties, preventing fibril formation. These derivatives also significantly increase insulin stability against shaking-induced fibrillation. While native insulin starts to form insoluble aggregates (fibrils) in about 0.5 day upon shaking at 37°C, it takes 18.8 days for (SAPG)<sub>3</sub>-insulin to form any aggregates. At the same time, the bioactivity for most of these derivatives is close to the native insulin ( $\pm 20\%$ ) except the Gly<sup>A1</sup>-Lys<sup>B29</sup>-substituted derivative, which shows about 65% activity of the native insulin.

#### 4.3.3. Formation of disulfide bonds

Naturally-occurring disulfide bonds generally increase thermodynamic stability of proteins (Jaenicke, 1991; Darby and Creighton, 1997; Johnson et al., 1997). This seems to result from a reduction in the configurational entropy ( $\Delta S_{\text{conf}}$ ) of the unfolded state.

Crosslinking proteins (formation of external 'braces') with bi(poly)-functional agents such as glutaraldehyde and imidoesters can prevent/reduce unfolding of tertiary structure and dissociation of oligomeric proteins (Mozhaev and Martinek, 1984; Kristjánsson and Kinsella, 1991). This is true especially when a disulfide bond (or other crosslink) is created connecting two groups that are sequentially far apart but spatially close, since this creates physical constraint to unfolding (reducing

entropy of the unfolded state) (Lee and Vasmatzis, 1997). For example, Matsumura et al. (1989) found that single or multiple disulfide-bonded mutants of T4 lysozyme significantly increase the  $T_m$  of the wild type. Similarly, Johnson et al. (1997) demonstrated that introduction of a single or multiple disulfide bonds increases the thermodynamic stability of barnase.

On the other hand, the added disulfide bonds may have potential stability problems particularly at a higher pH and temperature due to possible  $\beta$ -elimination-induced disulfide scrambling (see Section 3.2).  $\beta$ -Elimination of disulfide bonds has been observed for more than a dozen unrelated proteins at 100°C in the pH range of 4–8 (Volkin and Klivanov, 1987).

#### 4.3.4. Pegylation

Conjugation of PEGs to proteins is referred to as 'PEGylation' (Katre, 1993). PEGs are mostly attached to Lys amino groups in proteins. Generally, PEG-modified proteins exhibit increased stability, improved solubility, decreased immunogenicity, increased circulation half lives, and low toxicity (Li et al., 1995b). For example, conjugation of PEG<sub>20000</sub> to insulin (insulin-Gly<sub>1</sub>-PEG<sub>20000</sub> or insulin-Lys<sub>29</sub>-PEG<sub>20000</sub>) increases the protein's enzymatic stability in blood (Caliceti and Veronese, 1998). Conjugation of recombinant human megakaryocyte growth and development factor (rhMGDF) with PEG increases the storage stability of the protein in phosphate buffer at pH 7 (Guerra et al., 1998).

Pegylation may or may not change protein activity. The activity of recombinant consensus interferon (IFN-Con<sub>1</sub>) does not change after pegylation (Jensen-Pippo et al., 1995). However, the *in vitro* bioactivity of interleukin-6 (IL-6) decreases linearly with increasing degree of PEG-modification, while the thrombopoietic activity of PEG-modified IL-6, in which 54% of the Lys amino groups are coupled with PEG, is more than 10 times higher (Tsutsumi et al., 1995).

PEGs are generally considered safe and have very low toxicity. However, adverse reactions such as seizure and anaphylactic shock have been seen in humans after topical or oral administration of PEG-containing formulations (Katre, 1993).

#### 4.4. Formulation of liquid protein pharmaceuticals

There are two major dosage forms for protein pharmaceuticals: liquid and freeze-dried solid. Generally, it is the protein stability that dictates selection of the final dosage form. Liquid formulation has advantages of simple processing, less manipulation, and easy application, whereas freeze-dried solid protein formulation is generally more stable, although lyophilization often causes significant protein instability. There are cases when proteins in a liquid formulation are as stable as or more stable than those in a solid state. For example, oxidation of Met<sup>59</sup> in hIGF-I is a major modification pathway, and its rate in solution is roughly the same as that in a freeze-dried formulation in air-filled vials at either 25 or 30°C (Fransson et al., 1996). Liquid mannitol formulation of IFN- $\gamma$  is more stable at 5°C than a freeze-dried formulation of the same composition during storage (Perlman and Nguyen, 1992).

The science of protein formulation had been primarily empirical in the past (Middaugh, 1990). Although significant progress has been made in recent years, there is still no single pathway to follow in formulating proteins due to their structural diversities and complexities. There are several stages that require careful consideration and extensive experimentation in formulating a stable protein product. First, the route of drug administration should be addressed based on proposed indications. The remaining critical stages in protein formulation include: (1) purification, in which a protein is satisfactorily purified such that no harmful contaminants (such as proteases) exist and the process yields reproducibly stable active drug; (2) preformulation, in which physicochemical properties (such as protein *pI* and solubility), excipient compatibility, aggregation, and/or decomposition pathways are performed; and (3) final formulation, in which several key issues should be addressed, including choice of buffer, pH, stabilizers, and other excipients, stability-indicating assays, decomposition pathways, and optimal storage conditions.

##### 4.4.1. Route of drug administration

Bolus intravenous injection generally requires

an aqueous protein solution. This solution, usually not exceeding 10 ml, can be either a liquid formulation or reconstituted freeze-dried formulation. The tonicity of the solution is not a great concern since the volume is small. Other types of solutions, such as suspensions, emulsions, and cosolvent or oleagenous systems, may be used for intramuscular (up to 3 ml) or subcutaneous (up to 2 ml) injection. The buffer and pH in these systems should be carefully adjusted to alleviate irritation and provide maximum protein solubility and stability.

##### 4.4.2. Preformulation

To develop a protein formulation successfully, the basic properties of a protein should be understood thoroughly. These include protein purity, *pI*, and solubility at different pHs. Other critical issues can then be addressed, including the effect of different buffer systems, pH, tonicity modifiers, stabilizers, freeze/thaw cycles, and protein adsorption. Before conducting any preformulation studies, the purity of a protein should be adequate; usually a purity level of 95% or better is recommended (Berry, 1996).

##### 4.4.3. Composition of a liquid protein formulation

Multiple excipients are often required in a liquid protein formulation, although a single-excipient protein formulation may be sufficient, such as tPA, which is formulated in 0.5 M arginine-phosphate buffer (pH 7.3) (Overcashier et al., 1997). Both the type and level of excipients can significantly affect protein stability. These excipients include buffering agents, stabilizers, tonicity modifiers, and antimicrobial agents. Solubilizers should be used if a protein has limited solubility, such as rIFN- $\beta$ -1b, which has a solubility of <0.05 mg/ml at neutral pH and is solubilized by SDS or Tween 80 (Lin et al., 1996c). To ensure adequate solubility of a protein, the formulation pH should be at least 0.5 unit below or above its *pI* (Cleland et al., 1993). When multiple excipients are used, they should not interact with one another, and, more importantly, should not adversely affect protein stability. Thus, compatibility studies should be conducted.

Almost without any exception, an optimum pH (or a range) is required for maximum protein stability, and this pH may shift depending on the presence of other excipients. The often-used buffering agents are phosphate for pHs between 6 and 8 and citrate for pHs below 6. If these two agents adversely affect protein stability, other buffering agents may be used such as acetate for rhG-CSF at pH 4.0 (Herman et al., 1996), Tris-HCl for hEGF at pH 7.0 (Son and Kwon, 1995), and succinate for IFN- $\gamma$  at pH 5.0 (Lam et al., 1996).

In close relation to the selection of formulation excipients, a compatible container should be used. Type I borosilicate glass is usually the material of choice for containers due to its strong chemical resistance and low level of leachables. Closures should be carefully chosen based on their compatibility with protein formulation, resistance to formulation pH, excipients and sterilization, moisture/vapor transfer property, and resealability.

#### 4.4.4. Rapid screening of protein formulations

Development of a liquid protein formulation often requires screening of many excipients and their combinations at different pHs, even under rigorous computer-aided formulation design. Therefore, use of an efficient method for screening these excipients is recommended to expedite excipient selection without undertaking time- and labor-extensive stability studies. A few screening methods are briefly described here, which should be used with caution as they may or may not lead to the identification of ideal formulations. These methods include: (1) comparison of protein unfolding temperature ( $T_m$ ) in different formulations; (2) comparison of half denaturation concentration,  $C_{\text{half}}$ , in the presence of a denaturant; and (3) comparison of IR spectra of different protein formulations with a reference spectrum.

Although  $T_m$  does not have a defined relationship with the free energy of protein unfolding (an indicator of protein stability), it is widely accepted that any increase in  $T_m$  should lead to an increase in protein stability (Dill et al., 1989). Therefore, a change in  $T_m$  may indicate whether a particular

excipient has any stabilizing or destabilizing effect. For example, an increase in the amount of IL-1R aggregates correlates roughly with a gradual decrease in  $T_m$  in the presence of one of the three preservatives: phenol, m-cresol, and benzyl alcohol (Remmele et al., 1998). On the other hand, a protein stabilizer may not always increase  $T_m$ . EDTA and two reducing agents, dithiothreitol and  $\beta$ -mercaptoethanol, can protect aFGF from thermally-induced aggregation, but at low concentrations these agents do not affect the protein's  $T_m$  (Tsai et al., 1993). The  $T_m$  of a protein in the presence of two or more excipients can be roughly estimated without experimentation by calculating the algebraic sum of  $T_m$ s obtained in the presence of individual excipient.  $T_m$  estimation by this method seems rather reliable whether the excipients have the same or opposite effects (Allison et al., 1996).

$C_{\text{half}}$  is the concentration of a denaturant at which 50% of protein molecules are unfolded or denatured. It can be estimated from a protein denaturation curve as discussed in Section 3.4. The effect of a formulation excipient on protein stability is reflected in the change in  $C_{\text{half}}$ . The higher the  $C_{\text{half}}$ , the more stable the protein in the formulation. It should be noted that the time needed to unfold a protein by a denaturant may be very slow. Complete unfolding of the wide type Rop in 4 M GdnHCl takes 48 h (Munson et al., 1996).

IR has been used extensively to probe protein structural changes in liquid, frozen, and dried states (Dong et al. 1995b). The effect of excipients on protein stability can be examined by comparing the IR spectrum of a sample in the sensitive amide I region with that of a reference. Comparison can be based on the degree of spectral correlation ( $r$ ) (Prestrelski et al., 1993a,b), or the extent of spectral area overlap (Heimburg and Marsh, 1993; Allison et al., 1996; Kendrick et al., 1996), although these parameters may not necessarily reflect changes in protein activity. Using the correlation coefficient ( $r$ ), Prestrelski et al. (1995) successfully selected the formulation pH and stabilizers in designing a freeze-dried IL-2 formulation. Nevertheless, overlap comparison of area-normalized second-derivative or deconvol-



luted spectra is seemingly more reliable and objective. To overcome the interference of water absorption, protein IR spectra have to be determined either in D<sub>2</sub>O or at higher protein concentrations (> 10 mg/ml) as discussed in Section 3.4.

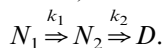
#### 4.4.5. Stability studies

To accelerate formulation development, stability studies are often conducted under accelerated (stressed) conditions. These stressed conditions include high temperature, high humidity, intensive lighting, extreme pHs, increased air/water interfaces by vortexing or shaking, and repeated freeze/thaw cycles. These studies are usually short but very helpful in screening protein formulations. Parameters used in monitoring stability studies include protein activity, rate of protein degradation, product formation and aggregation/precipitation, solution color, pH and viscosity, sterility, and pyrogenicity.

Accelerated stability studies are often conducted at high temperatures. The key issue is whether and how well the data from accelerated stability studies can be extrapolated to those under real-time conditions. Extrapolation of stability data from high to lower temperature is usually limited to a temperature range over which the same degradation pathway is operative. Very often, protein stability results obtained at high temperatures do not reflect or predict what happens under real-time conditions. This is due to the complexity of multiple protein degradation pathways at different temperatures. For example, temperature-dependent degradation mechanisms have been reported for interleukin 1 $\beta$  (IL-1 $\beta$ ) (Gu et al., 1991), and hGH (Pikal et al., 1991). Nonlinear Arrhenius relationships were observed for relaxin between 5 and 30°C (Nguyen and Shire, 1996) and for *phm*-MDH between 4 to 65°C in the presence of a variety of excipients, such as salts, salts of amino acids, and polyols (Jensen et al., 1997). The temperature nonlinearity may suggest at least two major degradation pathways in proteins.

Nevertheless, if the multiple degradation processes in proteins can be described separately, or the rate-limiting degradation step does not change with temperature, prediction of protein stability

based on accelerated stability studies is very optimistic. For example, the following thermal denaturation model with two different reaction rates representing two isoenzymes of  $\beta$ -galactosidase has been used to describe the biphasic irreversible denaturation process, and linear Arrhenius plots are obtained for both  $k_1$  and  $k_2$  (Yoshioka et al., 1994b):



Linear Arrhenius relationships have also been reported for a number of proteins in certain temperature ranges. Examples include the inactivation of *ml*-PEPC in the presence of 0.8 M sodium glutamate between 7.6 and 48°C (Jensen et al., 1997), thermal inactivation of recombinant hepatitis B surface antigen (HBsAg) between 60 and 90°C (Volkin et al., 1996), and second-order oxidation of hIGF-I between 7 and 37°C (Fransson et al., 1996).

## 5. Conclusions

Native proteins are properly folded, which involves many forces, including hydrophobic interactions, electrostatic interactions (charge repulsion and ion pairing), hydrogen bonding, intrinsic propensities, and van der Waals forces. Among these forces, hydrophobic interactions seem to be the dominant. Proteins are generally not very stable, as stabilization energy of the native state is mostly between 5 and 20 kcal/mol, which is equivalent to that of a few hydrogen bonds. Most mesophilic proteins such as those from human beings can be unfolded/denatured easily at temperatures between 50 to 80°C.

A common phenomenon of protein instability is formation of protein aggregates, which can be soluble or insoluble, chemical or physical, and reversible or irreversible. There are many factors affecting protein stability. These include at least temperature, pH, ionic strength, metal ions, surface adsorption, shearing, shaking, additives, solvents, protein concentration, purity, morphism, pressure, and freeze-thawing/drying. Chemical transformations that lead to protein instability include at least deamidation, oxidation, hydroly-

sis, isomerization, succinimidation, disulfide bond formation and breakage, non-disulfide crosslinking, and deglycosylation. Today, many analytical techniques are available to monitor protein instability. Even with the sophistication of present instrumentation, no single technique can satisfactorily provide sufficient information about a protein. Thus, a combination of analytical methods needs to be used to characterize a protein and to monitor protein instability.

To develop a liquid protein pharmaceutical, the basic properties of a protein need to be examined first. These include protein purity,  $pI$ , and solubility and stability at different pHs and in different buffer systems. With these data, protein formulation issues can then be addressed. These include the indication, route of drug administration, selection of proper formulation buffer and excipients, and finally, stability studies. To facilitate rapid development of a protein formulation, a formulation screening method may be used with caution, such as the determination of  $T_m$ ,  $C_{half}$ , or degree of IR spectral similarities. Alternatively, accelerated stability studies may be conducted under high-temperature conditions, but stability results obtained at high temperatures may not reflect or predict what happens under real-time conditions. For proteins, real-time stability studies are necessary for selection of the final formulation.

In the development of a protein formulation, the most challenging task is the stabilization of a protein to achieve an acceptable shelf life. Due to complication of structural modifications, proteins are commonly stabilized by excipients. The often-used protein stabilizers include sugars, polyols, surfactants, salts, PEGs, polymers, metal ions, and amino acids. Unfortunately, there is no single pathway to follow in selection of a suitable stabilizer(s), partly due to the lack of a clear and definitive understanding of protein-cosolute interactions and proteins' multiple inactivation mechanisms. Depending on the protein, these traditional protein stabilizers may increase protein stability only to a limited level. Therefore, new types of protein stabilizers need to be further explored. One obvious direction is to identify additional cellular components in hyperthermophilic organisms or cellular accumulants under various

stressed conditions, which may have the potential to stabilize proteins.

In summary, the most formidable challenge in formulating a liquid protein pharmaceutical is to preserve the biological activity of the protein for an acceptable shelf life. Unfortunately, there is no single pathway to follow in formulating such a product. Usually, proteins have to be evaluated on a case-by-case basis. Much more effort is still needed to understand the basic behavior of proteins, their instability factors and mechanisms, and their stabilization mechanisms in a broader and clearer perspective. This further understanding will definitely depend on further advancement of sophisticated analytical instrumentation. Therefore, research activities directed toward a general solution to protein instability will continue for at least a few decades (Richards, 1997).

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