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Review

Protein aggregation and its inhibition in biopharmaceutics

Wei Wang*

Biotechnology Division, Bayer HealthCare, 800 Dwight Way, Berkeley, CA 94701, USA

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Abstract

Protein aggregation is arguably the most common and troubling manifestation of protein instability, encountered in almost all stages of protein drug development. Protein aggregation, along with other physical and/or chemical instabilities of proteins, remains to be one of the major road barriers hindering rapid commercialization of potential protein drug candidates. Although a variety of methods have been used/designed to prevent/inhibit protein aggregation, the end results are often unsatisfactory for many proteins. The limited success is partly due to our lack of a clear understanding of the protein aggregation process. This article intends to discuss protein aggregation and its related mechanisms, methods characterizing protein aggregation, factors affecting protein aggregation, and possible venues in aggregation prevention/inhibition in various stages of protein drug development.
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Keywords: Protein aggregation; Aggregation mechanism; Protein refolding; Protein formulation; Protein stabilization

Abbreviations: Aβ, amyloid β peptide; BSA, bovine serum albumin; CAB, carbonic anhydrase B; CD, circular dichroism; rConIFN, recombinant consensus α-interferon; CspA, cold shock protein A; rhDNase, recombinant human deoxyribonuclease; DSC, differential scanning calorimetry; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; rFVIII, recombinant factor VIII; rFIX, recombinant factor IX; rFXIII, recombinant factor XIII; rhGCSF, recombinant human granulocyte colony stimulating factor; GDH, glutamate dehydrogenase; pGH, porcine growth hormone; rhGH, recombinant human growth hormone; GdnHCl, quanidine hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; rHA, recombinant human albumin; HP-β-CD, hydroxypropyl-β-cyclodextrin; HSA, human serum albumin; IFN-β, interferon-β; IFN-γ, interferon-γ; IgG, immunoglobulin G; IL-1β, interleukin-1β; IL-2, interleukin-2; rhIL-1ra, recombinant human interleukin-1 receptor antagonist; IR, infrared spectroscopy; rhKGF, recombinant human keratinocyte growth factor; LDH, lactate dehydrogenase; LMW-UK, low molecular weight urokinase; Mab, monoclonal antibody; rhMGDF, recombinant human megakaryocyte growth and development factor; NMR, nuclear magnetic resonance spectroscopy; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PEG, polyethylene glycol; PVA, polyvinyl alcohol; RH, relative humidity; RP-HPLC, reversed phase HPLC; RNase A, ribonuclease A; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; SDS, sodium dodecyl sulfate; SEC-HPLC, size exclusion HPLC

* Present address: Pfizer Inc., 700 Chesterfield Parkway West, Chesterfield, MO 63017, USA. Tel.: +1 636 247 2111; fax: +1 636 247 5030.
E-mail address: wei.2.wang@pfizer.com.

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1. Introduction

The past two decades saw an explosive growth in biopharmaceutics, fueled by the advancement of sensitive and high-throughput analytical methodologies. Yet, a rapid commercialization of protein drug candidates has not been fully realized due to the presence of many road barriers. One undisputable

barrier is the physical and chemical instabilities of proteins (Wang, 1999, 2000). Among these physical and chemical deterioration pathways, protein aggregation is arguably the most common and troubling manifestation of protein instability, almost in all phases of protein drug development. Presence of any insoluble aggregates in a protein pharmaceutical is generally unacceptable for product release.

Protein aggregation is also well represented in human etiology. More than 20 different diseases are caused at least partially by abnormal protein aggregation (Stefani and Dobson, 2003), which may result from mutations and physical or chemical changes of cellular environment (Koo et al., 1999). Among these are Alzheimer's disease, Parkinson disease, prion diseases (bovine spongiform encephalopathy and Creutzfeldt-Jacob diseases), Huntington's disease, Down's syndrome, cataract, and sickle cell disease. Some of these diseases are characterized by formation of protein fibrils, such as sickling cell disease (intracellular fibrillation of hemoglobin) and Alzheimer disease (extracellular fibrillation of amyloid β peptide (Aβ) (Koo et al., 1999). It is the protein aggregates (such as fibrils) that usually cause cytotoxicities (Stefani and Dobson, 2003).

Although significant progress has been made, our current understanding of protein aggregation is still incomplete (Gupta et al., 1998; Carpenter et al., 1999; Chi et al., 2003). Its prevention or even moderate inhibition has been mostly experimental. Therefore, achieving a better understanding of protein aggregation is critical not only in various biopharmaceutical processes but also in finding a solution to those devastating diseases. This review article intends to discuss protein aggregation and its related mechanisms, to analyze factors affecting protein aggregation, and more importantly, to summarize possible venues in aggregation prevention/inhibition. It is not the intention of this paper to be exhaustive in literature review but to stimulate more intensive and vigorous investigation on protein aggregation.

2. Protein aggregation and its influencing factors

Protein molecules may aggregate simply by physical association with one another without any changes in primary structure (physical aggregation) or by formation of a new covalent bond(s) (chemical aggregation). Formation of such a bond(s) can either directly crosslink proteins (aggregation), or indirectly alter the aggregation tendency of the original protein (Finke et al., 2000). Both mechanisms can occur simultaneously to a protein and may lead to formation of either soluble or insoluble aggregates, depending on the protein, environmental condition, and stage of the aggregation process. For example, insulin can undergo both physical aggregation process, leading to formation of either soluble hexamers or insoluble fibrils and chemical aggregation process, leading to formation of either soluble dimmers via cyclic anhydride intermediate or insoluble disulfide-bonded aggregates (Sluzky et al., 1991, 1992; Costantino et al., 1994a, 1994b; Darrington and Anderson, 1995).

Protein aggregation has been observed frequently in several key biopharmaceutical processes, including fermentation (Georgiou and Valax, 1999; Finke et al., 2000), refolding (van den Berg et al., 1999a, 1999b; Smith and Hall, 2001; Nguyen and Hall, 2002), shearing/shaking (Katakam et al., 1995), freeze-thawing (Kreilgaard et al., 1998a, 1998b), drying (Andya et al., 1999), reconstitution (Zhang et al., 1995a, 1995b), and storage (Vemuri et al., 1993; Costantino et al., 1994a, 1994b). The following section will discuss aggregation mechanisms and its influencing factors.

2.1. Mechanisms of physical aggregation

2.1.1. Folding/unfolding intermediates and protein aggregation

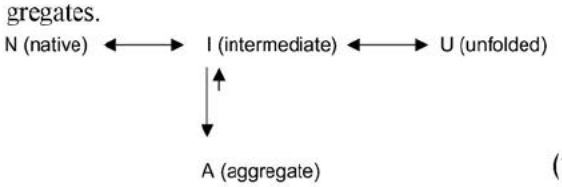
A traditional view of protein aggregation is the association of the unfolded state(s) of proteins. This view is supported by model predictions and experimental data as well (De Young et al., 1993; Stigter and Dill, 1993). However, there is overwhelming evidence that protein folding/unfolding intermediates are precursors in protein aggregation (Fields et al., 1992; Fink, 1998), even though the intermediates are usually not stable and poorly populated (Murphy et al., 1992). In contrast, completely folded or unfolded proteins do not aggregate easily as the hydrophobic side chains are either mostly buried out of contact with water, or randomly scattered (Uversky et al., 1999). It is the patches of contiguous hydrophobic groups in the folding/unfolding intermediates that initiate the aggregation process. Aggregation of many proteins has been shown to be initi-

ated by intermediates, such as scrapie amyloid (prion) protein (PrP27–30) (Safar et al., 1994), carbonic anhydrase B (CAB) (Cleland and Wang, 1992; Hammarstrom et al., 1999), recombinant human growth hormone (rhGH) (Bam et al., 1996), insulin (Brange et al., 1997), human lysozyme variants (Booth et al., 1997), P22 tailspike polypeptide (Speed et al., 1997; Schuler et al., 1999), and phage P22 wild-type coat protein and its mutants (A108V, G232D, F353L) (Teschke, 1999). Computer simulation studies also demonstrate origination of the aggregation process primarily from interactions of partially folded intermediates (Gupta et al., 1998; Istrail et al., 1999). Thermal treatment can easily generate protein-unfolding intermediates, which can rapidly aggregate, such as ovalbumin (Kato and Takagi, 1988), and a mutant of Cro repressor (Fabian et al., 1999). Another factor contributing to the rapid aggregation of intermediates is their high rate of diffusion relative to the folded state (Damodaran and Song, 1988). The high diffusion rate can significantly increase the chance of association of the intermediates.

The intermediates can be formed either from the folded or unfolded state (Wetzel, 1996). In many cases, more than one intermediate may exist, such as CAB (Wetlaufer and Xie, 1995), staphylococcal nuclease (Uversky et al., 1999), a mutant of Cro repressor (Fabian et al., 1999), and immunoglobulin G (IgG) (Vermeer and Norde, 2000). In addition, the number and structure of folding intermediates may be different from those of unfolding intermediates (Kim and Yu, 1996). Since the folding intermediates can be formed locally or globally, these intermediates may have significant amount of secondary structures, and even tertiary structures (Safar et al., 1994; Kendrick et al., 1998a, 1998b; Uversky et al., 1999).

2.1.2. Nucleation and growth of protein aggregates

Based on the above analysis, the aggregation process can be described as scheme (1), where proteins form reversible unfolding intermediates, which then form reversible unfolded proteins or irreversible/reversible aggregates.



(1)

The intermediate state (I) is equivalent to the aggregation-competent state (A) or transition state (TS^*) as proposed recently by other investigators (Krishnamurthy and Manning, 2002; Chi et al., 2003). The process from N to A can be considered as the nucleation step, which is usually rate limiting; in another word, the aggregation process is nucleation dependent. Nucleation has been demonstrated or suggested to be the initial step for fibrillation of a sequence of *E. coli*. OsmB protein (Jarrett and Lansbury, 1993), aggregation of K97I interleukin 1β during refolding (Finke et al., 2000), and aggregation of β-amyloid peptide (Lomakin et al., 1997; Szabo et al., 1999).

Further growth of protein aggregates after nucleation can be divided into two types: monomer–cluster aggregation (addition of a monomer to a growing multimer) and cluster–cluster aggregation (addition of a multimer to another multimer) (Speed et al., 1997). These two processes can be described as schemes (2) and (3) (Patro and Przybycien, 1994).

$$nI + A_m \rightarrow (n-1)I + A_{m+1} \quad (2)$$

$$A_m + A_n \rightarrow A_{m+n} \quad (3)$$

where A_m and A_n are aggregates composed of m and n monomers. These two processes can occur at the same time to the same protein. Examples of the first type of aggregation include staphylococcal nuclease (Uversky et al., 1999), and amyloid formation (Tomski and Murphy, 1992; Lomakin et al., 1997). Cluster–cluster polymerization was responsible for the aggregation of P22 tailspike polypeptide (a trimer) chains during in vitro refolding (Speed et al., 1997). The initial protein aggregates are soluble but gradually become insoluble as they exceed certain size and solubility limits (Fink, 1998; Uversky et al., 1999). Such examples include aggregation of CAB during refolding (Cleland and Wang, 1990) and aggregation of human serum albumin (HSA) during storage at 40, 55 or 70 °C (Oliva et al., 1999).

2.1.3. Reversibility and specificity of physical aggregation

The reversibility of protein aggregation is usually dependent on the stage of the aggregation process. The initial formation of soluble aggregates (nucleation) may be reversible but the later formation of insoluble aggregates is usually irreversible, unless precipitation is artificially induced such as during salt-

ing out. The two different stages correspond, respectively, to thermodynamically unfavorable and favorable processes (also see next section). Thermally induced protein aggregation is often irreversible, such as thrombin (Boctor and Mehta, 1992), recombinant pGH (Charman et al., 1993), recombinant human megakaryocyte growth and development factor (rhMGDF) (Narhi et al., 1999).

Physical aggregation is generally believed as a result of strong but non-specific protein–protein interactions (Durbin and Feher, 1996). For example, bovine serum albumin (BSA) aggregates easily because of the formation of incorrect intermolecular salt bridges (Giancola et al., 1997). Yet, recent evidence suggests that aggregation may occur by specific interaction of certain conformations of protein intermediates rather than by non-specific interactions (Speed et al., 1997; Fink, 1998). The fibril growth of a sequence of *E. coli*. OsmB protein was specific, as each peptide could be nucleated by fibrils of the same peptide but not by fibrils of closely related sequences (Jarrett and Lansbury, 1993). In mapping an aggregation nucleation site in an intermediate of human CAB, it was found that the aggregation of CAB is highly specific with intermolecular interactions in the vicinity of position 206 at the edge of β-strand 7 (Hammarstrom et al., 1999). Specificity was also implicated in the in vitro/in vivo immunoglobulin light chain amyloidosis (AL) (Helms and Wetzel, 1996) and fibrillation of insulin (Nielsen et al., 2001a, 2001b).

2.1.4. *Thermodynamics and kinetics of protein aggregation*

The thermodynamic change associated with the aggregation process for folded proteins has been carefully modeled. In a Monte Carlo simulation study on protein aggregation in a two-dimensional space, Patro and Przybycien (1994) analyzed the detailed free energy change associated with the aggregation process, assuming two types of protein surfaces—hydrophobic and hydrophilic, and no electrostatic interactions. They demonstrated that protein aggregation initially led to an increase in the overall free energy of the system mainly due to the loss of certain number of monomer conformational and translational states (loss of entropy). This initial aggregation process is analogous to the nucleation step, where it is thermodynamically unfavorable and rate limiting. Therefore, it is predicated that proteins with low native energies have a higher energetic barrier for aggregation and are less likely to aggregate (Istrail et al., 1999). After the initial step, further aggregation results in a decrease in the overall free energy of the system and the aggregation process is thermodynamically favored (Patro and Przybycien, 1994). This is because mutual attraction of hydrophobic residues/patches (aggregation) minimizes the area of unfavorable protein–solvent interface (interaction).

Kinetically, the initial aggregation process can be linear or, in many cases, non-linear. The non-linear process can be exponential or parabolic (Ferrone, 1999). Exponential growth is often associated with a significant lag time, a period usually attributable to nucleation. Rapid protein aggregation eventually slows down and the aggregation signal reaches a plateau as the soluble protein monomers gradually deplete (Won et al., 1998; Zlateva et al., 1999). These different aggregation behaviors have been well documented, including linear polymerization of isolated P22 tailspike β-helix domain (Bhx) (Schuler et al., 1999) and non-linear exponential polymerization with an initial lag phase for the aggregation of acid-denatured cold shock protein A (CspA) in solution (Alexandrescu and Rathgeb-Szabo, 1999). A lag time was also seen in the aggregation of acidic fibroblast growth factor (aFGF) at 0.1 mg/ml in 4.6 mg/ml phosphate buffer (pH 7.4) at 50 °C (Won et al., 1998) and apomyoglobin in aqueous urea solutions (De Young et al., 1993).

Physical protein aggregation arising from protein–protein interactions generally exhibits an apparent reaction order of 2 (Fink, 1998; De Bernardez Clark et al., 1999). Several aggregation processes clearly start with the formation of a dimer such as synthetic β-amyloid peptide, β1–40 (Tomski and Murphy, 1992), CAB (Cleland and Wang, 1990), recombinant human granulocyte colony stimulating factor (rhGCSF) (Krishnan et al., 2002) and HSA (Oliva et al., 1999). Dimerization can lead to further formation of multimers and precipitates/fibrils. Protein aggregation with a higher reaction order >2 was also reported (Hevehan and De Bernardez-Clark, 1997). Sometimes, protein aggregation may follow apparent first-order kinetics. This would be the case if the initial formation of unfolding intermediates is rate limiting. Both heat and denaturant-induced aggregations of recombinant human interferon-γ (IFN-γ) apparently follow first-order kinetics (Kendrick et al., 1998a, 1998b). At 60 °C, the aggregation/precipitation of interleukin 1β (IL-1β)

follows apparent first-order up to 30% drug remaining (Gu et al., 1991). If the percentage of precipitates/aggregates increases with increasing protein concentrations, multimolecular aggregation/precipitation processes is usually involved.

2.1.5. Computer-assisted probing of protein aggregation

Protein aggregation mechanisms may be conveniently probed by computer simulations (Patro and Przybycien, 1994; Istrail et al., 1999). 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). In another study, the colloid aggregation process has been simulated for a cluster–cluster aggregation (CCA) process. The heat-induced aggregation of BSA at pH 7.0 seems to fit a reaction-limited CCA model while at pH 5.1, it fits a diffusion-limited CCA model (Hagiwara et al., 1996).

2.2. Chemically-induced protein aggregation

There are many chemical reactions that can directly crosslink protein chains or change the hydrophobicity of a protein, indirectly changing its aggregation behavior. Chemical reactions of many amino acids in proteins require certain local molecular flexibility and thus, the rate of reaction may be higher in denatured states than in native states, such as disulfide exchange in β-lactoglobulin-A (Apenten, 1998). Native protein conformation, therefore, needs to be preserved for preventing or inhibiting potential chemical degradations.

2.2.1. Disulfide bond formation/exchange

Disulfide bond formation/exchange is probably the most common pathway of chemically induced protein aggregation. Free Cys residues in proteins can be oxidized easily to form disulfide bond linkages or initiate thio-disulfide exchanges, resulting in protein polymerization/aggregation, such as bFGF (Shahrokh et al., 1994a, 1994b; Wang et al., 1996), and β-galactosidase (Yoshioka et al., 1993). The thio-disulfide exchange starts from formation of an ionized thiol group, the thiolate anion. The rate of thiol–disulfide exchange depends on the extent of ionization of the nucleophilic thiol, and therefore generally increases as the solution pH increases until the pK of the nucleophilic thiol group is exceeded.

Even without a free cysteine, disulfide-bonded proteins can still undergo aggregation through disulfide exchanges via β-elimination. This is the case for lyophilized insulin during storage (Costantino et al., 1994a, 1994b). β-elimination was also observed for interleukin-1 receptor antagonist (IL-1ra) in aqueous solution (Chang et al., 1996a, 1996b, 1996c). Therefore, the apparent aggregation process can be multimolecular, including thiol–disulfide interchange in BSA and thiol-catalyzed disulfide exchange in insulin (Costantino et al., 1994a, 1994b), or unimolecular, including those initiated by β-elimination and intrachain disulfide scrambling or formations such as IL-1ra in aqueous solution (Chang et al., 1996a, 1996b, 1996c).

Although disulfide formation is a frequent cause of protein precipitation in solid state, it is not always the case in liquid state. BSA showed a tendency to form insoluble aggregates by disulfide linkages in lyophilized state but soluble aggregates were formed in solution (Jordan et al., 1994). Freeze-dried β-galactosidase formed insoluble precipitates via disulfide bonding during storage, while non-covalent interaction caused formation of soluble aggregates in solution (Yoshioka et al., 1993).

2.2.2. Non-disulfide crosslinking pathways

Proteins may form covalent dimers or polymers by non-disulfide crosslinking pathways. Insulin has been shown to form transamidated dimers and polymers during storage mainly through reactions involving the Asn^{A-21} and Phe^{B-1} residues (Brange et al., 1992). Recent evidence suggests formation of two different insulin dimers (Asp^{A21}-Phe^{B1} and Asp^{A21}-Gly^{A1}), which arise from the initial formation of a cyclic anhydride intermediate at the C-terminal Asn both in aqueous and lyophilized formulations (Strickley and Anderson, 1997).

Other non-disulfide crosslinking pathways include dityrosine formation (Malencik and Anderson, 2003) and formaldehyde-mediated crosslinking (Schwendeman et al., 1995). The latter crosslinking pathway apparently caused significant aggregation of lyophilized tetanus and diphtheria toxoids during storage.

2.2.3. Oxidation

Complicated oxidation reactions modify the primary sequence of a protein and may indirectly cause protein aggregation. Oxidation of human relaxin at His^{A12} by ascorbate/Cu(II)/O_2 led to formation of both 2-oxo-His derivative, which is soluble and other (unidentified) His^{A12} derivative(s), which can easily aggregate/precipitate (Khossravi et al., 2000). The aggregation/precipitation of the oxidized product(s) was also pH dependent.

2.2.4. Maillard reaction

Sugars are often used as protein stabilizers both in liquid and solid states. Reducing sugars have the tendency to react with amino groups in proteins forming carbohydrate adducts in multiple steps, especially at high temperatures. This extremely complex browning pathway is known as the Maillard reaction (Ledl et al., 1989). Although the reaction intermediates may be soluble, the end-stage products are often dark brown aggregates, likely as a result of altered protein stability and solubility (Liang and Rossi, 1990; Zarina et al., 2000).

2.3. Factors affecting protein aggregation

Many factors can affect the aggregation behavior of proteins. These factors are either structural (internal factors) or environmental (external factors).

2.3.1. Primary structure

It is generally believed that the amino acid sequence ultimately determines the propensity of protein aggregation, which is strongly influenced by environmental conditions (Fink, 1998). Protein aggregability is considered to be a meaningful and consistent property of protein sequences (Istrail et al., 1999). Mutation of one amino acid in a peptide or protein can dramatically increase the aggregation propensity (Soto et al., 1995; Teschke, 1999). For example, two human lysozyme variants (I56T and D67H), unlike the wild-type protein, easily aggregate upon heating (Booth et al., 1997).

Generally, the more hydrophobic a protein is, the more likely it forms aggregates (Calamai et al., 2003). In modeling physical protein aggregation, Fields et al. (1992) demonstrated that changing the non-polar composition by only a few percent could drastically change the aggregation behavior of a protein and increasing the content of non-polar groups would require a higher concentration of a denaturant for disaggregation. Using a mean-field model in the study of aggregation behavior of apomyoglobin, Stigter and Dill (1993) demonstrated that a small increase in the fraction of non-polar monomers in the protein chain from 0.37 to 0.38 led to a substantial decrease in solubility. Further studies demonstrate that the aggregation behavior is actually more correlated with the surface content of hydrophobic groups (Patro and Przybycien, 1994).

On the other hand, protein aggregation is also strongly influenced by the relative amount and location of hydrophilic residues in proteins. In a Monte Carlo simulation study, Istrail et al. (1999) demonstrated the aggregability of a protein is related to the local concentration of hydrophobic and hydrophilic residues in contiguous blocks of the sequence and, in general, fewer blocks of either type (hydrophobic or hydrophilic) lead to increased aggregability. Indeed, the sequential location of hydrophilic residues is critical in controlling the formation of fibrils in wild-type Aβ peptide (Fraser et al., 1994) and insulin (Nielsen et al., 2001a, 2001b).

2.3.2. Secondary structure

Secondary structures in peptides or proteins may play a role in controlling aggregation (Fink, 1998) as well as stability (Querol et al., 1996) and toxicity (Sato et al., 1995). Generally, protein/peptide aggregation involves mostly β-sheets while α-helix structures seem to be less likely to form aggregates. This may be due to a stronger dipole moment of α-helices than that of β-sheets (Querol et al., 1996).

A few studies on peptide aggregation support the above contentions. The aggregation of amyloid peptides upon dilution was shown to have two steps: random/helix-to-β-sheet transitions and aggregation of β-sheets (Szabo et al., 1999). In the study of amyloid β peptide aggregation, a single mutation of V18A induced a significant increase in α-helical content and dramatically reduced aggregation of Aβ1–40 at 4.0 mg/ml by 80%; on the other hand, substitution of Q22E decreased the propensity of α-helical formation at the N-terminal domain and increased amyloid formation (Soto et al., 1995). Incorporating proline, another amino acid of low β-sheet propensity, in Aβ1–40 (or Aβ1–42) also reduced the aggregation tendency (Soto et al., 1996). In the study of the association behavior of water-soluble de novo β-sheet peptides(22–32 aa),

Table 1
External factors affecting protein aggregation

Factors	Protein examples	Protein formulations	Study conditions	Results	References
Temperature	IL-1β	0.1 mg/ml in acetate, pH 5.0	Incubation at 39–60 °C	Increased aggregation	Gu et al. (1991)
	Bovine/porcine insulin	30 mg/ml, pH 2.5	Incubation at 35–75 °C	Increased fibrillation	Brange et al. (1997)
pH	IL-1β	0.1 mg/ml	Incubation at pH 2–9 at 30 °C	Increased aggregation below or above pH 6	Gu et al. (1991)
Ionic strength	RNase A	5 mg/ml in 100 mM citrate, pH 3.0	Incubation at 75 °C	Aggregation: 500 mM NaCl > 250 mM NaCl > 0 mM NaCl	Tsai et al. (1998a, 1998b, 1998c)
Shaking	hGH	0.5 mg/ml in phosphate, pH 7.4	Vortexting for 1 min	Precipitated by 67%	Katakam et al. (1995)
Shearing	hGH	2 mg/ml in 5 mM phosphate, pH 7.4	Exposed to a shear of 10^5	Aggregated by 30%	Maa and Hsu (1997)
Hydrophobic surface	Bovine insulin	0.6 mg/ml in PBS, pH 7.4	Agitation at 37 °C with different beads	Aggregation: teflon > siliconized glass > polypropylene > glass	Sluzky et al. (1992)
Protein concentration	Bovine insulin	7.5 to 50 mg/ml, pH 2.5	Incubation at 21 °C	Aggregation: 50 > 30 > 15 > 7.5 mg/ml	Brange et al. (1997)
Organic solvent	β-Lactoglobulin	50 mg/ml at pH 7–9	Addition of ethanol at 25 °C	Aggregation/gelation within few hours at 1:1 (v/v) ratio	Renard et al. (1999)
Metal ions	Deoxyribonuclease I	~18 mg/ml in 150 mM NaCl	Incubation at 40 °C for 30 days	Complete aggregation but no visible aggregation in the presence of 1 mM $CaCl_2$	Chen et al. (1999)
Protein source	Bovine insulin	15 mg/ml, pH 2.1	Incubation at 21 °C	Lot A > lot B	Brange et al. (1997)
Pressure	H^+-ATPase	0.05 mg/ml in 3 mM $MgCl_2$, 5% glycerol, and 50 mM Tris–HCl, pH 7.0	Increasing pressure from 0 to 2.3 kbar	Increased aggregation with increasing pressure	Tsai et al. (1998a, 1998b, 1998c)
Freeze-thawing	Hemoglobin	50 mg/ml in PBS, pH 7.4	Freeze-thawing 0, 3 and 5 times	Aggregation: 5 > 3 > 0 times	Kerwin et al. (1998)
Freeze-drying	Bovine IgG	0.5 to 8 mg/ml in 0.22 M NaCl and 10 mM phosphate, pH 7.1	Freeze-drying	Increased formation of insoluble aggregates with decreasing protein concentration	Sarciaux et al. (1999)
Spray-drying or Spray-freeze drying	rhMAb	10 mg/ml	Spray-drying	Aggregated by 5.6%	(Andya et al., 1999)
	BSA	20 mg/ml	Spray-freeze drying	Particle size-dependent aggregation	Costantino et al. (2000)
Reconstitution	KGF	0.4–0.65 mg/ml in 10 mM phosphate, pH 7.0	Reconstitution with water or 0.5% heparin	Aggregation: water > heparin	Zhang et al. (1995a, 1995b)

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Janek et al. (1999) demonstrated that the degree of association correlated with the stabilization of the β-sheet structure. Amyloid formation of several Aβ analogues directly correlated with the content of β-sheet conformation (Soto and Castano, 1996).

2.3.3. External factors

Protein aggregation may be induced by a variety of external/environmental factors. These factors or conditions may destabilize the folded state, stabilize the unfolded state, or favor formation of intermediates. These factors and their effect on the aggregation of representative proteins are listed in Table 1.

Among these factors, temperature is probably the most common and critical in affecting protein aggregation. Proteins unfold above certain temperatures and thermally induced protein unfolding is often followed by immediate aggregation due to exposure of the hydrophobic residues. Increasing temperature increases the rate of protein aggregation by increasing both frequencies of molecular collision and hydrophobic interactions (Speed et al., 1997). Aggregation lag time observed at low temperature may be reduced or eliminated at high temperatures such as human IFN-γ aggregation at 1 μM (Zlateva et al., 1999). High temperatures also accelerate chemical aggregations, such as increased formation of covalent insulin oligomers and polymers at temperatures >25 °C (Brange et al., 1992). Changing temperature may also change the relative composition of secondary structures and alter the aggregation behavior (Vermeer and Norde, 2000).

The second critical factor is the solution pH. The solution pH can strongly affect the rate of protein aggregation by changing the type and distribution of charges in proteins (Schein, 1990; Kohn et al., 1997). The solution pH was found to affect aggregation of many proteins, including basic fibroblast growth factor (bFGF) (Wang et al., 1996), deoxy hemoglobin (Kerwin et al., 1998), IL-1β (Gu et al., 1991), rhIL-2 (Advant et al., 1995), human relaxin (Li et al., 1995), bovine pancreas ribonuclease A (RNase A) (Townsend et al., 1990; Tsai et al., 1998a, 1998b, 1998c), and bovine insulin (Brange et al., 1997). The pH of a protein solution designed for lyophilization can also affect the aggregation of the dried product during storage, such as lyophilized IL-1ra (Chang et al., 1996a, 1996b, 1996c).

Protein concentration is another important factor in protein aggregation. The mean-field lattice model predicts that proteins will aggregate/precipitate at sufficiently high concentrations (Fields et al., 1992). Increasing protein concentration generally increases protein aggregation, such as aFGF (Won et al., 1998), insulin (Brange et al., 1992, 1997), β-lactoglobulin (Roefs and De Kruif, 1994), IL-1β (Gu et al., 1991), low molecular weight urokinase (LMW-UK) (Vrkljan et al., 1994), and apomyoglobin (De Young et al., 1993). Alexandrescu and Rathgeb-Szabo (1999) demonstrated that increasing protein concentration reduced the lag time (to the power 2.1 of the protein concentration). A sharp increase in the aggregation of K97I interleukin-1β was observed during refolding when its concentration exceeded a critical value (Finke et al., 2000). This is because proteins may need a critical concentration to form the initial nucleus for initiation of the aggregation process (Lomakin et al., 1997). Exceptions do exist. Increasing human IFN-γ concentration from 1 to 4 μM increased the time of reaching maximum aggregation. The slower aggregation of this protein at a higher concentration is probably due to an increase in the concentration of dimers, which is less competent for aggregation (Zlateva et al., 1999).

Other factors may also influence protein aggregation, including freeze-thawing, freeze-drying, spray-drying, reconstitution, etc. (Table 1). It should be mentioned that concentrated protein solutions are often more resistant against freezing-induced protein aggregation such as labile lactate dehydrogenase (LDH) (Carpenter et al., 1990, 1997). The increased stability at high protein concentrations during freezing is likely due to one or more of the following factors—limited area of ice-water interface for interfacial denaturation, protein's self stabilization, and conversion of monomers to active and more stable dimers or multimers (Mozhaev and Martinek, 1984; Allison et al., 1996).

3. Characterization of protein aggregation

Protein aggregates usually exhibit either reduced or in many cases, no biological activity (Cleland and Wang, 1990; Townsend et al., 1990; Runkel et al., 1998; Nesta et al., 2000). However, protein aggregates often have stronger immunogenicity or toxicity, such as insulin (Brange et al., 1997) and rhIL-2 (Curatolo et al., 1997). To minimize protein aggregation, protein ag-

gregates need to be properly analyzed to identify the cause(s) of aggregation. The following section will discuss general characteristics of protein aggregates and analytical techniques in characterizing protein aggregates.

3.1. Morphology of protein aggregates

Protein aggregates come in different shapes and sizes, even for aggregates of a single protein (Schuler et al., 1999; Khurana et al., 2001). In most cases, protein aggregates are amorphous. For many small proteins or peptides, fibrils are a common form of aggregates such as insulin (Brange et al., 1997), β-amyloid peptide and its fragments (Tomski and Murphy, 1992; Shen et al., 1993), human calcitonin (Cholewinski et al., 1996), a synthetic peptide (Ac-KLKLKLELELELG-NH_2) in saline (Lazo and Downing, 1997), human lysozyme variants (I56T and D67H) (Booth et al., 1997). Certain proteins may form gels, such as heated β-lactoglobulin (Matsuura and Manning, 1993; Tobitani and Ross-Murphy, 1997) or BSA (Tobitani and Ross-Murphy, 1997), and denatured CspA at pH 2.0 (Alexandrescu and Rathgeb-Szabo, 1999). The CspA gels were made of ribbon-like aggregates, similar to protein amyloid fibrils and the polymers are long, unbranched, with an average diameter of about 12 nm.

The size of protein aggregates varies greatly from soluble submicron range to visible precipitates. Heating a 0.1% (w/w) BSA solution at 95 °C for 7 min generated aggregates with an average hydrodynamic radius of 68 nm (Hagiwara et al., 1996). Incubation of Aβ1–40 (2 mg/ml) formed amyloid-like, 8–10 nm unbranched fibrils (Soto et al., 1996). The fibrils of a synthetic peptide (Ac-KLKLKLELELELG-NH_2) formed in saline had a uniform diameter of 2 nm (Lazo and Downing, 1997). The aggregates of two human lysozyme variants (I56T and D67H) are rigid fibers of variable lengths and 8–10 nm in diameter (Booth et al., 1997), while insulin fibrils are 3–15 nm in diameter with lengths of up to several microns (Brange et al., 1997; Nielsen et al., 2001a, 2001b).

Many factors may influence the morphology of protein aggregates. Major ones include the primary sequence of the protein (Helms and Wetzel, 1996), extent and distribution of the hydrophobic surface (Patro and Przybycien, 1994), sample condition (Khurana et al., 2001), and sample preparation method and history (Shen et al., 1993). A single mutation in a protein or peptide can dramatically change the morphology of resultant aggregates (Fraser et al., 1994; Helms and Wetzel, 1996). Other factors affecting aggregate morphology include temperature, pH, protein concentration, and solution composition (Fraser et al., 1994; Roefs and De Kruif, 1994; Soto et al., 1996; Nielsen et al., 2001a, 2001b).

3.2. Structure and dissolution of protein aggregates

Protein aggregation usually leads to loss of tertiary structure except under certain treatment conditions such as salting-out procedures (Fink, 1998). However, protein aggregates usually contain significant amounts of secondary structures. In fact, the β-sheet content in proteins often increases upon aggregation (Chen et al., 1999). If a protein has both α-helix and β-sheet structures, the aggregation-induced increase in β-sheet content is often accompanied by a drop in α-helix structure, such as insulin (Nielsen et al., 2001a, 2001b), human lysozyme variants (Booth et al., 1997), and ovalbumin (Kato and Takagi, 1988). Therefore, protein aggregates are often composed of dominant β-sheet structures. Typical examples include insulin fibrils consisting of extended β-chains lying perpendicular to the fibril axis (Brange et al., 1997) and amyloid fibrils consisting of cross-β conformation (Tomski and Murphy, 1992).

Physical protein aggregates could be dissolved by adjustment of certain solvent properties, such as pH, temperature, and polarity. For example, recombinant factor XIII (rFXIII) is a dimer between pH 3.9 to 10.4 but dissociates into monomers upon lowering the pH to 3.6 without loss of any compact structure or enzymatic activity (Kurochkin et al., 1995). Heating the solution of fibrillated human calcitonin in 50% AcOH/water could reconvert the fibrils to soluble monomers (Cholewinski et al., 1996).

Strong protein denaturants often dissolve protein aggregates (mainly physical). Apomyoglobin precipitates formed in 0.5–4 M urea can be dissolved in 8 M urea (De Young et al., 1993). The insoluble aggregates of hGH can be completely dissolved in 2% sodium dodecyl sulfate (SDS) (Katakam et al., 1995). However, non-covalent insulin fibrils could not be dissolved in several solvent systems including 7 M urea from pH 2 to 8, 5 M GdnHCl, 50% acetonitrile or other detergent

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solutions due to strong hydrophobic interactions in insulin fibrils (Brange et al., 1997). The ease of dissolution also depends on the density of protein aggregates (Patro and Przybycien, 1994).

3.3. Analytical techniques in monitoring protein aggregation

There are many techniques for monitoring protein aggregation, or characterizing protein aggregates (Nguyen et al., 2003). These analytical techniques and their major applications, and protein examples are summarized in Table 2. Among these methods, the most convenient way of detecting formation of insoluble protein aggregates is, albeit subjective, visual inspection. Under a microscope, the size and shape of insoluble protein aggregates can easily be determined (Schuler et al., 1999). For smaller aggregates or higher resolution, an electron microscope may be used (Alexandrescu and Rathgeb-Szabo, 1999).

A traditional and simple instrumental method in monitoring protein aggregation is the turbidimetric method by measuring the optical density of the sample based on light scattering in the near UV or visible region, where proteins have negligible absorption. A variety of wavelengths have been used, including 300 nm for urokinase (Vrkljan et al., 1994), 350 nm for aFGF (Tsai et al., 1993; Volkin et al., 1993) or insulin (Kwon et al., 2001), 340–360 nm for met-hGH and tissue plasminogen activator (tPA) (Hsu et al., 1995), 450 nm for pGH (Charman et al., 1993), and 500 nm for IL-1ra (Chang et al., 1996a, 1996b, 1996c). A requirement of

Table 2
Analytical techniques in characterizing protein aggregation

Categories	Individual techniques	Applications	Protein examples	References
Calorimetry	DSC	Thermal protein unfolding/aggregation	FVIII SQ	Fatouros et al. (1997)
Centrifugation	Analytical centrifugation	Size and shape estimation	Insulin	Richards et al. (1998)
Chromatography	SEC-HPLC	Size estimation and quantitation	Factor IX	Bush et al. (1998)
			hGH	Pikal et al. (1991)
	RP-HPLC	Isoforms of aggregates	bFGF	Shahrokh et al. (1994a, 1994b)
Electrophoresis	SDS-PAGE	Size estimation and mechanistic probing	aFGF	Won et al. (1998)
	Native PAGE	Aggregation process and mechanistic probing	IL-2	Curatolo et al. (1997)
Light scattering	Static light scattering	Size and shape estimation	β-Amyloid peptide, β1–40 Insulin	Tomski and Murphy (1992) Sluzky et al. (1991)
	Dynamic light scattering	Size distribution of soluble aggregates	Deoxy hemoglobin	Kerwin et al. (1999)
	Light scattering/obscuration	Size estimation and relative distribution	Deoxy hemoglobin	Kerwin et al. (1999)
Rheology	Dynamic shear rheometry	Gelation characterization	BSA	Tobitani and Ross-Murphy (1997)
Spectroscopy	CD	Aggregation process	Interferon-γ	Kendrick et al. (1998a, 1998b)
	Fluorescence	Aggregation process	α_1-Antitrypsin	James and Bottomley (1998)
	IR	Aggregation process	β-Lactoglobulin, etc.	Dong et al. (1995)
	NMR	Aggregation process	CspA	Alexandrescu and Rathgeb-Szabo (1999)
	UV–vis	Soluble and insoluble aggregates	aFGF	Tsai et al. (1993)
Microscopy	Light microscopy	Shape and size determination	Immunoglobulin mutants	Helms and Wetzel (1996)
	Electron microscopy	Shape and size determination	β-Amyloid peptide	Soto and Castano (1996)
	Atomic force microscopy	Shape and size determination	β-Amyloid peptide	(Parbhu et al., 2002)

using this method is uniform distribution of all the aggregates. Although this method can be more sensitive than HPLC in the early detection of aggregate formation (Wang et al., 1996) and a reasonable linearity can be established in a certain concentration range (Chang et al., 1996a, 1996b, 1996c), results from this method can be very variable. As high as 30% variation was observed in the determination of LMW-UK aggregation (Vrkljan et al., 1994). Therefore, this method is generally used for comparison of relative degree of protein aggregation and not for accurate quantitation, unless the protein aggregates are completely dissolved in a denaturant solution before UV measurement.

In comparison with the UV method, static light scattering can estimate the size and shape of the aggregates, while dynamic light scattering can estimate both the size and relative distribution of protein aggregates (Lomakin et al., 1999). Examples of monitoring protein aggregation by light scattering include β-amyloid peptide (Tomski and Murphy, 1992; Shen et al., 1993), P22 tailspike polypeptide (Speed et al., 1997), and interleukin-1β (Finke et al., 2000). Dynamic light scattering has been used in monitoring aggregation of carbonic anhydrase (Cleland and Wang, 1990; Hammarstrom et al., 1999) and β-amyloid peptide (Tomski and Murphy, 1992; Lomakin et al., 1997). Like the UV method, static light scattering is usually used to estimate the relative degree of protein aggregation, while dynamic light scattering is limited to monitoring formation of soluble aggregates. Using light scattering principle, protein aggregation can be monitored by a fluorimeter (Chrunyk and Wetzel, 1993; Rozema and Gellman, 1996a, 1996b). Since protein folding/unfolding is often associated with a significant change in fluorescence, care must be taken not to confuse any signal changes with those resulting from protein aggregation.

Polyacrylamide gel electrophoresis (PAGE) is another simple and traditional method in the determination of protein aggregation. Disulfide-bonded aggregates can be easily probed under reduced and non-reduced conditions. In most cases, PAGE was run under denaturing conditions in the presence of SDS, which may dissociate physical aggregates. In this case, physical aggregates may not be detectable. To overcome this limitation, native PAGE or 2D native PAGE may be needed (Speed et al., 1997; Arakawa and Kita, 2000). On the other hand, any change in the charged state of a protein or its aggregates may influence the migration pattern and complicate interpretation of the results.

Generally, the above-mentioned methods are used for qualitative analysis, although quantitation by UV and PAGE is possible with additional experimental steps. In contrast, size exclusion HPLC (SEC-HPLC) is a commonly used technique for quantitative assessment of aggregate size and content. Similarly, this method has several limitations. First, since an aggregated protein sample is usually filtered through a 0.2 μm Acrodisc filter to remove any particulates before injection, SEC-HPLC method is limited for the determination of soluble aggregates. Second, 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). Third, if a protein has carbohydrates or interacts with the column, the elution profile of the protein may change, leading to an 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 and 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 detect protein 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 of bFGF dimers based on hydrophobicity differences (Shahrokh et al., 1994a, 1994b). As with SEC-HPLC, RP-HPLC method is limited for the determination of soluble aggregates. A potential issue associated with any chromatographic analysis is that a protein may experience unfolding or refolding in the mobile phase, changing the retention time and complicate data interpretation (Shahrokh et al., 1994a, 1994b). Therefore, prevention of unfolding/refolding by addition of certain modifiers in the mobile phase may be needed in these cases.

Recently, infrared (IR) spectroscopy has been extensively used in protein denaturation/aggregation studies. A common feature of thermally induced (or lyophilization-induced) protein aggregation is the formation of an intermolecular antiparallel β-sheet structure, which is characterized by a low-frequency band around 1620 cm^{-1} and an associated high-frequency but weaker band around 1685 cm^{-1} (Dong et al., 1995).

For example, an aggregation band has been found at 1619 cm^{-1} for human FXIII (Dong et al., 1997) and 1620 cm^{-1} for recombinant human IFN-γ (Kendrick et al., 1998a, 1998b). This aggregation-induced structural transition occurs regardless of the initial composition of the secondary structures in native proteins. Therefore, these bands can be used to monitor and quantify aggregation in both aqueous solution and dried solid (Dong et al., 1997; Szabo et al., 1999). In addition, IR can be used to determine the reversibility of protein aggregation based on the reversibility of the aggregate band such as human FXIII (Dong et al., 1997).

There are other assays suitable for determining the formation of certain protein aggregates. For example, the fibrils of β-amyloid peptide possessing cross-β-pleated sheet structure can bind to Congo red dye and cause spectral changes of the dye (Shen et al., 1993). A red shift was observed in using such a dye for monitoring the aggregation of acid-induced CspA (Alexandrescu and Rathgeb-Szabo, 1999). Another labeling compound, thioflavin T, can also be used as a fluorescence probe in monitoring protein aggregation (Shen et al., 1993; Wall and Solomon, 1999; Nielsen et al., 2001a, 2001b).

4. Inhibition of protein aggregation

Protein aggregation can be inhibited either by modifying proteins' structure (internally) or by changing proteins' environmental properties (externally). Modification can be made by site-directed mutagenesis or by chemical reactions (Knappik and Plhckthun, 1995; Lundblad and Bradshaw, 1997). The key issue in structural modification for stabilization is to preserve the protein activity, as the modified protein can easily lose activity such as aFGF (Volkin and Middaugh, 1996). Nevertheless, many structurally modified proteins have been shown to have reduced tendency to aggregate, including single amino acid mutants of human macrophage inflammatory protein (hMIP)-1α (Czaplewski et al., 1999), thymidylate synthase (Prasanna et al., 1999) and amyloid β peptide (Soto et al., 1995), and monosubstituted, disubstituted, and trisubstituted insulins with *p*-succinamidophenylglucopyranoside (SAPG) (Baudys et al., 1995). Conjugation of methoxypoly ethylene glycol (mPEG) to insulin substantially increased its resistance to shaking-induced fibrillation (Hinds et al., 2000).

Changing proteins' environmental properties often lead to inhibition of protein aggregation. A common method is to add excipients/additives in the protein preparation. A variety of excipients/additives are available for use, such as sugars and polyols, amino acids, amines, salts, polymers, and surfactants. These additives stabilize proteins by preferential interactions, a widely accepted concept of protein stabilization (Arakawa et al., 1991; Timasheff, 1998). Other mechanisms are also operable, such as increased rate of protein folding (Wang et al., 1995; Frye and Royer, 1997), reduction of solvent accessibility and conformational mobility (Kendrick et al., 1997), and increase in solvent viscosities (Jacob and Schmid, 1999).

The following section will discuss possible ways of adjusting proteins' environmental properties for inhibition of aggregation in various processes. Typical examples are summarized in Table 3.

4.1. Refolding of proteins

A vast body of literature addresses protein aggregation during refolding (Clark, 1998; De Bernardez Clark et al., 1999). Due to our lack of understanding of the competition between on-pathway folding and off-pathway aggregation, inhibition of protein aggregation during refolding has been a trial and error process (Hevehan and De Bernardez-Clark, 1997; Clark, 1998). Added to the difficulty is the presence of multiple folding pathways and its rapidity (Yon, 1996; Popov, 1999). Many factors have been found to affect protein aggregation during refolding, including temperature, type and concentration of the denaturant and protein, pH, ionic strength, presence of refolding catalysts, thiol–disulfide agents, and stabilizing agents.

4.1.1. Temperature

Protein folding can be considered as, to a certain degree, a diffusional process (Jacob and Schmid, 1999). Therefore, thermal motion is required during folding to cross an energy barrier—the folding transition state. Increasing the folding temperature should increase the rate of protein folding but in an apparent non-Arrhenius manner as the temperature also affects the protein-stabilizing forces (Scalley and Baker, 1997; Kuhlman et al., 1998). Since increasing temperature increases both

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Table 3
Inhibition of protein aggregation by excipients

Processes (protein examples)	Protein formulations	Study conditions	Results	References
Refolding				
rhGH	0.667 mg/ml in 4.5 M GdnHCl	Refolding by dilution	$A_{350} \cong 0.6$	Bam et al. (1996)
	+ Tween 20 (10:1 = molar Tween:rhGH)		36% reduction	
	+ Tween 40 (same ratio)		65% reduction	
	+ Tween 80 (same ratio)		55% reduction	
Lysozyme	1 mg/ml in 1 mM DTT, 5 mM GSSG, 1 mM EDTA, 0.5 M GdnHCl, 50 mM Tris–HCl, pH 8 at 25 °C	Oxidative refolding	50% folding yield	Hevehan and De Bernardez-Clark (1997)
	+0.75 M L-arginine		96% folding yield	
Thermal treatment				
aFGF	0.1 mg/ml in PBS, pH 7.2	Incubation at 55 °C	A_{350} reaches >1 instantly	Tsai et al. (1993)
	+0.033 mg/ml heparin		A_{350} reaches 0.1 in ~20 min	
	+0.033 mg/ml heparin and 2 M sucrose		No significant change in A_{350} in 30 min	
aFGF	0.1 mg/ml in 0.033 mg/ml heparin in PBS, pH 7.2	Incubation at 55 °C	Observed aggregation	Tsai et al. (1993)
	+ Sufficient sodium sulfate, trehalose, dextrose, sorbitol, glycine, histidine, or EDTA		At least 50% reduction in aggregation rate	
aFGF	0.1 mg/ml in 4.6 mg/ml phosphate buffer, pH 7.4	Incubation at 50 °C	Aggregation $d4$ (min) = 100	Won et al. (1998)
	0.1 mg/ml in 9.2 mg/ml phosphate buffer, pH 7.4		Aggregation $d4$ (min) $\cong$ 10	
rhDNase	~18 mg/ml in 150 mM NaCl, pH 6.5	Incubation at 40 °C for 30 days	Almost complete aggregation	Chen et al. (1999)
	+ 1 mM $CaCl_2$		Virtually no aggregation	
IgG	50 mg/ml in 100 mM phosphate, pH 6.8	Heating at 60 °C for 1.5 h	32.2% aggregation	Gonzalez et al. (1995)
	+33% (w/v) sorbitol		6.5% aggregation	
pGH	0.5 mg/ml in 50 mM ammonium bicarbonate, pH 8.5	Incubation at 63 °C for 1 h	~60% aggregated	Charman et al. (1993)
	+ 1% Tween 20		~40% aggregated	
rhMGDF	In 10 mM phosphate or citrate buffer (pH 4–7)	Thermal unfolding	Precipitated	Narhi et al. (1999)
	In 10 mM imidazol, hisidine, or Tris from (pH 6–8)		No visible precipitates	
RNase A	5 mg/ml in 100 mM Tris–HCl, pH 7.8	Incubation at 75 °C for 24 h	Aggregation detectable	Tsai et al. (1998a, 1998b, 1998c)
	+0.1% dextran sulfate		Aggregation not detectable	
	+0.1% SDS		Aggregation not detectable	
Urokinase	38.5 kU/ml	Incubation at 60 °C for 30 min	A_{325} = 100% (relative value)	Manning et al. (1995)
	+ 1% gelatin type B or A		$A_{325} \cong 25\%$	

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Shaking/shearing				
Bovine insulin	0.6 mg/ml in PBS, pH 7.4	Agitation at 37 °C at 100 rpm	Aggregated in 2 days	Sluzky et al. (1992)
	+ 10 mM *n*-octyl-β-D-glucopyranoside or 10 mM *n*-dodecyl-β-D-maltoside		No significant change for 40 days	
Deoxy hemoglobin	80 mg/ml in PBS, pH 7.2	Shaking at 225 rpm for 1 h	Particles (≥ 2 μm) $\cong 10^6$ ml^{-1}	Kerwin et al. (1999)
	+ 0.045% Tween 80	Shaking at 225 rpm for 1 h	Particles (≥ 2 μm) $< 10^4$ ml^{-1}	
FXIII	1 mg/ml in 0.1 mM EDTA and 10 mM Tris, pH 8	Shaking at 400 rpm at 23 °C for 24 h	Complete aggregation	Kreilgaard et al. (1998a, 1998b)
	+ 6 μM Tween 20		Complete aggregation	
	+ 60 or 120 μM Tween 20		No significant change	
rhGH	0.5 mg/ml in 20 mM phosphate, pH 7.4	Vortexing for 1 min	67% aggregated	Katakam et al. (1995)
	+ 0.1% Tween 80, 0.1% Pluronic F68, or 0.013% Brij 35		Aggregation not detectable	
pGH	0.5 mg/ml in 50 mM ammonium bicarbonate, pH 8.5	Vortexing for 1 min	~75% aggregated	Charman et al. (1993)
	+ 1% Tween 20		Aggretation not detectable	
	+ 2% (w/v) HPCD		~15% aggregated	
	+ 10% (w/v) HPCD		~10% aggregated	
Freezing/drying				
GDH	0.1 mg/ml in 10 mM citrate, pH 6.5	Quench freezing and thawing	$A_{500} > 0.05$	Chang et al. (1996a, 1996b, 1996c)
	+ 0.01% Tween 80		$A_{500} < 0.01$	
Hemoglobin	50 mg/ml in PBS, pH 7.4	Freeze-thawing for 5 times (−20 °C)	Particles (≥ 2 μm) $\cong 1.4 \times 10^4$ ml^{-1}	Kerwin et al. (1998)
	+ 0.0125% Tween 80		Particles (≥ 2 μm) $\cong 2 \times 10^3$ ml^{-1}	
	+ 0.1 M sucrose		Particles (≥ 2 μm) $\cong 7 \times 10^3$ ml^{-1}	
Bovine IgG	1 mg/ml in 0.22 M NaCl and 10 mM phosphate, pH 7.1	Freeze-drying	$A_{350} = 0.54$	Sarciaux et al. (1999)
	+ 0.02% (w/w) Tween 80		$A_{350} = 0.18$	
Bovine insulin	1 mg/ml in phosphate, pH 7.4	Freeze-drying	Light scattering >1300	Katakam and Banga (1995)
	+ 1 mg/ml dextrose		Light scattering <300	
LDH	0.1 mg/ml in 10 mM citrate, pH 6.5	Queech freezing and thawing	$A_{500} = 0.11$	Chang et al. (1996a, 1996b, 1996c)
	+ 0.01% Tween 80		$A_{500} < 0.01$	

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Table 3 (*Continued*)

Processes (protein examples)	Protein formulations	Study conditions	Results	References
rhMAb	10 mg/ml	Spray-drying	Aggregated by ~5.6%	Andya et al. (1999)
	+ Trehalose at 100 mol:1 mol protein		Aggregated by <1%	
	+ Lactose at the same ratio		Aggregated by ~1%	
	+ Mannitol at the same ratio		Aggregated by ~2.6%	
Trypsinogen	20 mg/ml in 1 mM HCl, pH 3.1	Spray-drying	Dimerization >8%	Tzannis and Prestrelski (1999)
	+ 1% sucrose		Dimerization <2%	
	+ 2% sucrose		Dimerization <0.5%	
Reconstitution				
IFN-γ	0.4 mg/ml in PBS, pH 7.0	Reconstitution with water	A_{350} (mg) = 0.525	Webb et al. (2002a, 2002b)
		Reconstitution with 0.03% Tween 20	A_{350} (mg) = 0.0.175	
KGF	0.4–0.65 mg/ml in 10 mM phosphate, pH 7.0	Reconstitution with water	~10% aggregated	Zhang et al. (1995a, 1995b)
		Reconstitution with 0.5% heparin	<2% aggregated	
Emulsification				
γ-Chymotrypsin	40 mg in 1 ml methylene chloride containing 360 mg of PLGA	Emulsified with 10% PVA (o/w type)	34% aggregated	Castellanos et al. (2003)
		Emulsified with 10% PVA (o/w type)	12% aggregated	
Liquid storage				
α_1-Anitrypsin	In 0.1 M NaCl and 20 mM phosphate, pH 7.5	Incubation at 4 °C	Aggregation at ~1.5% per week	Vemuri et al. (1993)
	+ 0.9 M NaCl		~1% per week	
rhGCSF	1.5 mg/ml in PBS, pH 7	Incubation at 37 °C for 5 days	96% aggregated	Krishnan et al. (2002)
	+ 0.5 M sucrose		<50% aggregated	
	+ 1 M sucrose		7% aggregated	
Insulin ($Lys^{B28}Pro^{B29}$)	3.5 mg/ml, 7 mM phosphate (pH 7.2), 16 mg/ml glycerol, 50 mM NaCl, 17.5 μg/ml zinc ion	Self-association at room temperature	Irregular association	Richards et al. (1998)
	+ 3.2 mg/ml *m*-cresol		Regular association	
rhKGF	0.5 mg/ml in PBS, pH 7	Incubation at 37 °C for 3 days	>60% aggregated	Chen et al. (1994)
	+ 0.05 mg/ml poly(acrylic acid) or poly(methacrylic acid)		<10 aggregated	
Solid storage				
Factor IX	0.5 mg/ml rFIX in 1% sucrose, 255 mM glycine, 0.005% Tween 80, and 10 mM phosphate, pH 7.0 Replacing phosphate with histidine	Incubation (lyophilized) at 30 °C for 3 months	>2.5% aggregation	Bush et al. (1998)
			<0.2% aggregation	
Factor XIII	2 mg/ml in 0.1 mM EDTA and 10 mM Tris, pH 8	Incubation (lyophilized) at 40 °C for 1 month	Aggregated (soluble) by ~60%	Kreilgaard et al. (1998a, 1998b)
	+ 3.5% dextran		Aggregated by ~50%	
	+ 100 mM mannitol, trehalose, or sucrose		Aggregated by ~40%, <10% or <5%	

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hGH	2 mg/ml in phosphate buffer, pH 7.4	Incubation (lyophilized) at 25 °C for 2 months	~6% aggregated	Pikal et al. (1991)
	+2 mg/ml glycine		~3.5% aggregated	
	+2 mg/ml glycine and 10 mg/ml mannitol		~2.5% aggregated	
rhIL-1ra	10 mg/ml in 2% glycine and 10 mM citrate, pH 6.5	Incubation (lyophilized) at 50 °C	Turbidity A_{500} (week) = 0.075	Chang et al. (1996a, 1996b, 1996c)
	+1% maltose, trehalose, sucrose, or sorbitol		A_{500} (week) = 0.003, 0.005, 0.008, or 0.037	
	+1% sucrose and 4% mannitol, 4% alanine, or 2% glycine		A_{500} (week) = 0.006, 0.021, or 0.008	
rHA	1 mg/ml, pH 7.3	Incubation (lyophilized) at 37 °C and 96% RH for a day	Aggregated by 81%	Costantino et al. (1995a, 1995b)
	+Dextran at 1:6, 1:1, or 3:1 (g dextran:g protein)		Aggregated by 63%, 12%, or 6%	
rHA	1 mg/ml, pH 7.3	Incubation (lyophilized) at 37 °C and 96% RH for a day	Aggregated by ~80%	Costantino et al. (1995a, 1995b)
	+Sorbitol at a ratio of 1:50, 1:6, 1:1 (g sorbitol:g rHA)		Aggregated by ~80%, ~40%, or <5%	
	+D-glucaric acid, D-glucamine, NaCl, or lactic acid at a ratio of 1:6		Aggregated by ~20%, <5%, <5%, or <5%	
Tetanus toxoid	1 mg/ml, pH 7.3	Incubation (lyophilized) at 37 °C and 86% RH for 9 days	Aggregated by 88%	Costantino et al. (1996)
	+NaCl or sorbitol at a ratio of 1:5 (1 g:5 g protein)		Aggregated by 58% or 21%	
rhMAb	10 mg/ml	Incubation (spray-dried) at 30 °C	Aggregation rate constant = 0.0022 per day	Costantino et al. (1998)
	+Mannitol at a ratio of 1:9 or 1:4 (g mannitol:g protein)		Aggregation rate constant = 0.0013 or 0.0009 per day	
TNF	0.25 mg/ml in 1% mannitol and 10 mM citrate, pH 6.5	Incubation (lyophilized) at 37 °C for 1 month	Dimerization by 1.5%	Hora et al. (1992a, 1992b)
	+2% trehalose		Dimerization by 0.2%	
	+2% dextran, 2% PEG-6000, or 0.5% sucrose		Dimerization not detectable	

hydrophobic interactions and chances of intermolecular intermediate interactions, an increase in protein aggregation is usually associated with a higher rate of folding at a higher temperature. A folding temperature of higher than 35 °C often increases protein aggregation significantly relative to protein folding, such as protein CheY (Klein and Dhurjati, 1995), β-lactamase (Georgiou et al., 1994), and phage P22 coat protein mutants (Teschke, 1999).

In reality, the optimal refolding temperature is protein dependent. For examples, at pH 8.5, the relative renaturation recovery for carbonic anhydrase was 37 °C (32%) > 25 °C (30%) > 4 °C (19%) > 50 °C (0%) (Karuppiah and Sharma, 1995). The refolding yield (about 60%) for bovine CAB at 150 min at 20 °C was greater than at either 4 or 36 °C (Xie and Wetlaufer, 1996). Among the four temperatures (10, 15, 20 and 25 °C) studied in the refolding of P22 tailspike polypeptide, refolding at 20 °C produced the highest yield of native tailspike trimer (Speed et al., 1997). Decreasing the temperature from 20 to 10 or 0 °C resulted in an increase in the renaturation yield of a fusion protein at high salt concentrations (Stempfer et al., 1996). For an unknown protein, a refolding temperature of 15 °C could be a safe bet (De Bernardez Clark et al., 1999).

4.1.2. Protein concentration

Increasing protein concentration during refolding usually increases protein aggregation due to increased chances of intermolecular interactions (Lomakin et al., 1997; Finke et al., 2000). Simulation of in vitro protein refolding and aggregation indicates that aggregation dominates over refolding at high protein concentrations and the size of protein aggregates increases with increasing protein concentrations (Gupta et al., 1998). The refolding yield of several proteins dropped gradually with increasing protein concentrations, including β-lactamase (Georgiou et al., 1994), recombinant pGH (Cardamone et al., 1995), α_1-antitrypsin (Kim and Yu, 1996), lysozyme (Rariy and Klibanov, 1997; Yasuda et al., 1998), immunoglobulin G (Maeda et al., 1996a, 1996b), rhDNase (Cleland et al., 1992), and CAB (Wetlaufer and Xie, 1995).

Therefore, proteins are refolded usually at relatively low concentrations to avoid or reduce protein aggregation. Optimal refolding concentrations have been found to be <1 mg/ml (Cleland and Wang, 1990), at 5 μM for reduced lysozyme (van den Berg et al., 1999a, 1999b) and <18 μM for K97I interleukin-1β (Finke et al., 2000). On the other hand, refolding of proteins at very low concentrations is not efficient and loss or denaturation of proteins from surface adsorption may become relatively significant (van den Berg et al., 1999a, 1999b). Thus, refolding of unknown proteins is recommended in a narrow concentration range of 10–50 μg/ml (Hevehan and De Bernardez-Clark, 1997; Clark, 1998; De Bernardez Clark et al., 1999).

4.1.3. Denaturant concentration

In close relation to protein concentration, the denaturant concentration strongly affects both the rate and extent of on-pathway folding and off-pathway aggregation during refolding (Hevehan and De Bernardez-Clark, 1997). This is because denaturants at different concentrations affect protein solubility to different degrees. Low concentrations of denaturants may decrease the solubility of a protein as the denaturants convert native to denatured and aggregation-prone species, while high denaturant concentrations should solubilize the protein as it weakens the protein–protein attractions in water (Fields et al., 1992; Stigter and Dill, 1993). Because of denaturants' effect on solubility, protein aggregation is usually favored at low denaturant concentrations. This is clearly the case in the refolding of CAB (Cleland and Wang, 1990) and K97I interleukin-1β (Finke et al., 2000). It seems that protein aggregation under such conditions results from the favorable formation of misfolded intermediates (Gupta et al., 1998).

Since denaturants also have a strong effect on the rate of protein refolding, several studies showed V-shaped relationships between the rate of protein folding ($\ln k$) and the denaturant concentration, including refolding of the N-terminal domain of the protein L9 (ribosomal protein from *Bacillus stearothermophilus*, residues 1–56) (Kuhlman et al., 1998) and protein L (Y43W) (Scalley and Baker, 1997), and modeling the kinetic folding profile of two hypothetical proteins (Clarke and Waltho, 1997). Therefore, a desirable concentration of a denaturant during refolding depends on the nature of the denaturant, the protein and its concentration, and other refolding conditions. Optimal refolding concentrations of denaturants are found to be 3.5 M urea for rpGH (Cardamone et al., 1995), 1.5 M GdnHCl for lysozyme (0.02 mg/ml) (Yasuda et al., 1998), and higher or lower than 4.5 M GdnHCl for rhGH (Bam et al., 1996).

4.1.4. Use of additives

The well-known function of molecular chaperones in bacteria led to the use of such molecular systems (such as DnaK-DnaJ-GrpE and GroEL-GroES) to inhibit protein aggregation during protein refolding (King, 1997; Golbik et al., 1998). They suppress protein aggregation by alternate binding and releasing of the folding intermediates (Thomas et al., 1997). However, two major limitations seem to be associated with a routine use of these folding modulators—production cost and the extra purification burden. Therefore, other additives have been explored to achieve the same goal during refolding.

The term "artificial chaperone" has been used to describe such non-protein additives, which have chaperone-like effect. Such additives include detergents and cyclodextrins. Detergents were found to suppress formation of protein aggregates (rather than dissolve aggregates) during protein refolding (Wetlaufer and Xie, 1995). Similarly, cyclodextrins were shown to prevent formation of protein aggregates during renaturation of carbonic anhydrase (Karuppiah and Sharma, 1995). To increase the efficiency, a combination of such additives was used for the refolding of lysozyme (Rozema and Gellman, 1996a, 1996b) and CAB (Rozema and Gellman, 1996a, 1996b).

Many other additives have been used to inhibit protein aggregation during refolding. Examples include surfactants for CAB (Wetlaufer and Xie, 1995), recombinant α- and β-chains of the class II major histocompatibility molecules (MHC-II) (Stockel et al., 1997) and rhGH (Bam et al., 1996), L-arginine or glycerol for hen egg-white lysozyme (Hevehan and De Bernardez-Clark, 1997; Rariy and Klibanov, 1997), sucrose for β-lactamase (Georgiou et al., 1994), PEG for rhDNase and rhtPA (Cleland et al., 1992), organic solvents (acetone, acetamide, or dimethyl sulfoxide) for lysozyme (Yasuda et al., 1998), and Na_2SO_4 for CAB (Rozema and Gellman, 1996a, 1996b). Several additives increased the folding yield of reduced lysozyme, including sarcosine, glycerol, ammonium sulfate, *N*-acetyl glucosamine (NAG) or glucose (Maeda et al., 1996a, 1996b). Possible mechanisms of the above additives during refolding include stabilization of the native state, destabilization of incorrectly folded molecules or intermediates, solubilization of folded molecules or folding intermediates (Clark, 1998; Arakawa and Tsumoto, 2003; Tsumoto et al., 2003).

In the oxidative refolding of reduced proteins, a proper redox pair is often used to promote disulfide bond formation or catalyze the SH–SS interchange. A common pair is the reduced and oxidized glutathione (GSH/GSSG) (De Bernardez Clark et al., 1999). Such a redox pair (2-mercaptoethanol and oxidized glutathione) was shown to facilitate the refolding of reduced immunoglobulin G by catalyzing the SH–SS interchange reaction at pH 8.0 and 4 °C (Maeda et al., 1996a, 1996b). An optimal ratio may exist, depending on the redox pair and the protein concentration (Rozema and Gellman, 1996a, 1996b). In the oxidative renaturation of hen egg white lysozyme (HEWL) at 1 mg/ml, the optimum ratio of GSH/GSSG was between 0.8 and 3, and for dithiothreitol (DTT)/GSSG, the ratio was between 0.3 and 0.6 (Hevehan and De Bernardez-Clark, 1997).

4.1.5. Miscellaneous techniques

To prevent protein aggregation during refolding, a variety of novel techniques or methods have been tried. These include refolding of proteins on a size-exclusion column during elution (Batas et al., 1997), use of fusion protein construct (with a polyionic tag) (Stempfer et al., 1996), use of a temperature leap (Xie and Wetlaufer, 1996), and use of high hydrostatic pressure (Zhang et al., 1995a, 1995b; Frye and Royer, 1997; Gorovits and Horowitz, 1998).

4.2. Processing of proteins

Many routine processing steps can lead to protein aggregation to various degrees, such as heat-treatment, filtration, shaking/shearing, atomization, freezing, drying, and reconstitution (Table 3).

4.2.1. Heat treatment

One of the viral inactivation methods in protein purification is heat treatment, which is usually performed at 60 °C. Unfortunately, many proteins aggregate at this temperature and thus, need proper protection. To protect a protein, the first step is to choose a stable solution pH, as proteins are usually stable in a narrow pH range (Vrkljan et al., 1994; Fatouros et al., 1997). Although many buffering agents are available for pH adjustment, the aggregation behavior of proteins can be significantly different in different buffer systems (Narhi

et al., 1999) and at different buffer concentrations (Won et al., 1998).

Another two critical parameters in controlling protein aggregation during thermal treatment are protein concentration and ionic strength. Generally, increasing protein concentration increases the rate of protein aggregation due to the increased chances of intermolecular interactions (Narhi et al., 1999). The effect of ionic strength is very much protein dependent (Wang, 1999). The salt type can also make a significant difference in protein aggregation (Kita and Arakawa, 2002). In addition, a stabilizer(s) is often added into a protein preparation to inhibit protein aggregation under thermal treatment (Table 3). Their stabilizing effect is generally considered as the result of preferential exclusions, weak and non-specific. These stabilizers include sugars, polyols, polymers, surfactants, and amino acids (Tsai et al., 1993; Gonzalez et al., 1995; Zhang et al., 1995a, 1995b).

Polymers may inhibit protein aggregation through one or more of these properties: surface activity, preferential exclusion, steric hindrance of protein–protein interactions, and increased viscosity limiting protein structural movement. PEGs of different molecular weights were found to inhibit aggregation of recombinant human keratinocyte growth factor (rhKGF) at 45 °C (Zhang et al., 1995a, 1995b), and to inhibit thermally induced aggregation of LMW-UK (Vrkljan et al., 1994). Both gelatin (up to 1%) and hydroxyethyl (heta) starch (up to 20%) can inhibit the thermal aggregation of LMW-UK (Vrkljan et al., 1994; Manning et al., 1995). A variety of polymers were found to inhibit heat-induced aggregation of rhKGF, including HP-β-CD, dextran sulfate, pentosan polysulfate, polyphosphoric acid, poly-L-glutamic acid, poly-L-lysine, etc. (Chen et al., 1994; Zhang et al., 1995a, 1995b). The charged polymers may protect proteins from heat-induced aggregation through multiple electrostatic interactions such as EGF (Volkin et al., 1993; Won et al., 1998) or via alteration of charge distribution on the surface of a protein such as RNase (Tsai et al., 1998a, 1998b, 1998c).

The effect of surfactants on protein aggregation has not been consistent during thermal treatment, as their effects are multifaceted (Chi et al., 2003). Inhibition of thermally induced protein aggregation was observed for BSA by Tween 80 (Arakawa and Kita, 2000), and recombinant hGH or methionyl pGH by poloxamers 407 and 188 (Pluronic, F68) (Katakam and Banga, 1997). However, Tween 80 at 0.1% showed no effect on the aggregation of IL-1β at 100 μg/ml at 60 °C (Gu et al., 1991) and POE(10)L [$CH_3(CH_2)_{11}(OCH_2CH_2)_{10}OH$)] did not inhibit aggregation of CAB upon heating (Rozema and Gellman, 1996a, 1996b). In fact, increasing concentrations of non-ionic surfactants (Tween 20, Tween 80, and Triton X-100) accelerated the aggregation of aFGF at 0.1 mg/ml in phosphate buffer (pH 7.4) at 50 °C (Won et al., 1998). Ionic surfactants are usually not used because they can bind to both polar and non-polar groups in proteins and cause denaturation. However, they can be used to inhibit protein aggregation if the denaturation binding is reversible (Rozema and Gellman, 1996a, 1996b). SDS was found to reduce aggregation of aFGF in a concentration-dependent manner at 50 °C (Won et al., 1998), and to prevent aggregation of heat-denatured RNase (Tsai et al., 1998a, 1998b, 1998c) or CAB (Rozema and Gellman, 1996a, 1996b).

4.2.2. *Shaking and shearing*

Shaking creates air/water interface. The hydrophobic property of air relative to water induces protein alignment at the interface, maximizing exposure of the hydrophobic residues to the air and initiating aggregation (Volkin and Klibanov, 1989). Shearing also exposes the hydrophobic areas of proteins, causing aggregation. Many proteins easily aggregate during shaking or shearing, such as rFXIII (Kreilgaard et al., 1998a, 1998b), hGH (Katakam et al., 1995; Katakam and Banga, 1997; Bam et al., 1998), and insulin (Brange et al., 1997; Kwon et al., 2001).

To protect proteins from shaking/shearing-induced aggregation, surfactants are most commonly used. They inhibit protein aggregation by accumulating, competitively with proteins, at hydrophobic surfaces/interfaces and/or by binding directly to proteins (Bam et al., 1995, 1998; Webb et al., 2002a, 2002b). Certain surfactants such as poloxamers may also increase the viscosity of a protein solution, restraining the motion of protein backbone to inhibit protein aggregation (Wang and Johnston, 1993). In addition to those listed in Table 3, other successful examples include inhibition of shear/shaking-induced rhGH aggregation by Pluronic F88 or Tween 20 (Maa and Hsu, 1997; Bam et al., 1998), PEG-GCSF aggregation by Tween

20 (Treuheit et al., 2002) and insulin aggregation by glyceryl monooleate (Sadhale and Shah, 1999).

4.2.3. Freezing

Freezing of a protein solution may cause protein aggregation due to one or more of the following freezing-induced stresses: low temperature, solute concentration, formation of ice-water interfaces, and potential pH changes or phase separation (Wang, 2000). Understanding the cause(s) of protein aggregation is the first step in aggregation prevention/inhibition during freezing. For proteins that denature during freezing due to their interaction with the ice-water interface, a slower rate of freezing should inhibit aggregation of these proteins as a smaller ice-water interface will be generated (Chang et al., 1996a, 1996b, 1996c). On the other hand, a lower freezing rate may facilitate crystallization of other solution components, promoting potential crystallization-induced protein aggregation (Eckhardt et al., 1991). A frequent cause of protein aggregation during freezing is the buffer-induced pH change during the freezing process. In this case, selection of a suitable buffer would prevent/inhibit protein aggregation.

Many additives can be used to inhibit freezing-induced protein aggregation. They may inhibit protein aggregation by preferential interaction and/or other mechanisms such as increased solution viscosity, steric hindrance of protein–protein interactions (Farruggia et al., 1997) or suppression of pH changes during freezing (Anchordoquy and Carpenter, 1996). Among these additives, surfactants seem to be the most commonly used (see examples in Table 3). Other examples include use of Tween 80 to inhibit the aggregation of FIX during freezing and thawing (Bush et al., 1998) and Tween 20 in reducing the formation of insoluble aggregates during freeze-thawing of rFXIII at 1, 5, or 10 mg/ml (Kreilgaard et al., 1998a, 1998b).

4.2.4. Drying

Generally, the amount of water covering the surface of a protein in a fully hydrated state is around 0.3 g/g protein (Rupley and Careri, 1991; Kuhlman et al., 1997), while the water content of a dried protein product is usually less than 10%. Therefore, the drying process removes part of the hydration layer, which may disrupt the native state of a protein and cause protein aggregation. To protect the protein from dehydration-induced aggregation, excipients, capable of forming hydrogen bonds with the protein, are added to replace water so that the hydrogen bonding requirements of polar groups on the protein surface can be satisfied (Crowe et al., 1993). These excipients serve as water substitutes (Carpenter and Crowe, 1989; Carpenter et al., 1990). The formation of hydrogen bonding has been demonstrated by IR spectroscopy between carbohydrates and many freeze-dried proteins (Carpenter and Crowe, 1989; Crowe et al., 1993; Prestrelski et al., 1993). These excipients are preferably amorphous (Lueckel et al., 1998). In fact, the formation of an amorphous glass during dehydration has been considered as a prerequisite for protein stabilization (Franks, 1994; Fox, 1995).

The commonly used stabilizing agents during drying are sugars. Both trehalose and sucrose inhibit aggregation of many proteins during lyophilization such as IL-6 (Lueckel et al., 1998) and human factor XIII (Kreilgaard et al., 1998a, 1998b). Some polymers are also effective, as they can form a glass with a high transition temperature. Examples include hydroxyethyl cellulose at 1% in the complete inhibition of aFGF aggregation (Volkin and Middaugh, 1996) and PEG in the inhibition of human FXIII aggregation during lyophilization (Kreilgaard et al., 1998a, 1998b). Probably due to polymer-associated properties, many proteins protect themselves from aggregation during drying (Sarciaux et al., 1998).

Other techniques were sometimes effective in inhibition of protein aggregation during drying, such as annealing before drying (Webb et al., 2003).

4.2.5. Miscellaneous processes

Many other processes may induce protein aggregation, such as preparation of protein delivery systems using an organic solvent (Kwon et al., 2001; Castellanos et al., 2003). Proper use of a stabilizing agent(s) may inhibit or prevent protein aggregation under these processing conditions. Hydroxypropyl-β-cyclodextrin was shown to protect both ovalbumin and lysozyme against methylene chloride/water interface-induced denaturation and aggregation (Sah, 1999). Sucrose and α-crytallin have been shown to inhibit, respectively, denaturant-induced aggregation of rhIFN-γ (Kendrick et al., 1998a, 1998b) and lysozyme (Raman et al., 1997). Small liposomes were shown to inhibit jet nebulization-induced IFN-γ aggregation (Kanaoka et al., 1999). Tween 20 significantly inhibited

nebulization-induced aggregation of rhIFN-γ (Webb et al., 2002a, 2002b) or recombinant consensus α-interferon (rConIFN) (Ip et al., 1995) and atomization-induced rhIFN-γ aggregation (Webb et al., 2002a, 2002b). L-Tyrosine was effective in preventing aggregation of caeruloplasmin and haemoglobin during irradiation (Assemand et al., 2003).

4.3. Storing of proteins

Proteins need short- and/or long-term storage for various purposes. A 18-month shelf life is usually the minimum requirement for protein pharmaceutical products. Although storage at an extremely low temperature is generally a safe bet to protect a protein from aggregation, it is not always practical. In addition, low-temperature itself may be the cause of aggregation for certain proteins. Ideally, a liquid or solid protein product needs to be stored under room conditions and this generally requires extensive optimization of the protein's immediate environment.

As discussed in the protection of proteins against thermally induced aggregation (Section 4.2), the first step in optimizing the protein's environment for both liquid and solid protein pharmaceuticals is to select a good buffering agent at a proper concentration to control the formulation pH. This should be done by testing several types of buffering agents at different concentrations and different pH's. Many examples demonstrated that the tendency of protein aggregation can be significantly different in different buffer systems and at different concentrations (Pikal et al., 1991; Eberlein et al., 1994; Wang et al., 1996).

The next step in protecting liquid proteins from aggregation during storage is to select a suitable protein stabilizer(s). Theoretically, all the additives, used in inhibiting protein aggregation during refolding or under different processing conditions, are potential stabilizing excipients during storing liquid proteins. Many of these excipients and their protein examples are listed in Table 3. Among these excipients, sugars are the commonly used (Vemuri et al., 1993; Chang et al., 1996a, 1996b, 1996c; Wang et al., 1996; Soenderkaer et al., 2004). NaCl, the often-used salt, may play a critical role in the inhibition of aggregation of certain proteins, such as recombinant factor VIII SQ (rFVIII SQ) (Fatouros et al., 1997) and α_1-antitrypsin (Vemuri et al., 1993). Many multiple-charged compounds have been shown recently to inhibit aggregation of several proteins (Maclean et al., 2002). Other excipients include PEGs and zinc chloride in the inhibition of α_1-antitrypsin aggregation during storage at 4 °C (Vemuri et al., 1993) and maltosyl-β-cyclodextrin in the suppression of the insulin aggregation (Tokihiro et al., 1997). It should be noted that surfactants, widely used to prevent protein aggregation, could actually promote aggregation of certain proteins during storage (Treuheit et al., 2002).

As discussed in Section 4.2, proteins in solid states are stabilized mainly by amorphous excipients because of their ability to form a glass and to replace water for the required formation of hydrogen bondings with proteins. In addition, several other mechanisms were proposed in protecting solid proteins from aggregation, including physical dilution and separation of protein molecules (Liu et al., 1990; Costantino et al., 1995a, 1995b; Chang et al., 1996a, 1996b, 1996c; Strickley and Anderson, 1997), excipient–water interactions (Costantino et al., 1995a, 1995b), alteration of the glass transition temperature, and inhibition of crystallization of stabilizing excipients. Among these excipients, sugars are again the most commonly used for stabilizing proteins against aggregation in solid states. In addition to those listed in Table 3, other stabilized proteins by sugars include IL-6 (Lueckel et al., 1998), IL-11 (Garzon-Rodriguez et al., 2004), insulin (Strickley and Anderson, 1997), *Humicola lanuginosa* lipase (Kreilgaard et al., 1999), and monoclonal antibodies (Andya et al., 1999, 2003; Sane et al., 2004). Some polymers are also effective in the inhibition of protein aggregation during storage. These include hydroxylethyl starch for IL-11 (Garzon-Rodriguez et al., 2004), carboxymethyl-cellulose, dextran, DEAE-dextran, or PEG for lyophilized BSA (Liu et al., 1990), and HP-β-CD for several solid proteins during storage, including insulin (Katakam and Banga, 1995), IL-2 (Hora et al., 1992a, 1992b), and tumor necrosis factor (TNF) (Hora et al., 1992a, 1992b). Other stabilizing excipients include salts in the inhibition of aggregation of lyophilized BSA (Liu et al., 1990) and recombinant human albumin (rHA) (Costantino et al., 1995a, 1995b), carboxylic acids for lyophilized rHA (Costantino et al., 1995a, 1995b), amino acids for lyophilized IL-2 (Hora et al., 1992a, 1992b), and metal ions for rhDNase (Chen et al., 1999).

A critical factor in controlling the rate of protein aggregation in solid states is the moisture content of the formulation (Breen et al., 2001). Generally, increasing the moisture content increases the rate of protein aggregation during storage, such as insulin (Costantino et al., 1994a, 1994b) and bovine pancreas RNase (Townsend et al., 1990). In many cases, however, the effect of moisture on protein aggregation is a complex function. For example, maximum aggregation (78%) of lyophilized tetanus toxoid occurred at a water content of about 36% during storage at 37 °C for 10 days, and less aggregation was observed at water contents either below or above that level (Schwendeman et al., 1995). Aggregation of both (lyophilized) rHA and BSA during storage at 37 °C had a bell-shaped relationship as a function of water content with maximum aggregation at about 32% and 28% moisture, respectively (Liu et al., 1990; Costantino et al., 1995a, 1995b). Similar bell-shaped aggregation dependence on water content was also observed for ovalbumin (chicken egg albumin), glucose oxidase, bovine β-lactoglobulin (Liu et al., 1990), and insulin (Katakam and Banga, 1995; Separovic et al., 1998). In addition, the moisture content of a solid protein formulation could dictate the aggregation pathway. The mechanism of aggregation of lyophilized tetanus toxoid (150 kDa) was different under different relative humidities during storage (Schwendeman et al., 1996).

It should be noted that the pH of a protein solution for preparation of a solid formulation may affect the aggregation of proteins in solid states during storage. This is to say that the solid-state "acidity/basicity" may still affect protein aggregation, both physically and chemically. Therefore, the pH of a protein solution before drying may have to be carefully determined to prevent or inhibit protein aggregation during storage of the dried product. Such examples were reported in the storage of lyophilized proteins such as RNase (Townsend et al., 1990), antibody-vinca conjugate (Roy et al., 1992), insulin (Costantino et al., 1994a, 1994b) or rHA (Costantino et al., 1995a, 1995b).

5. Summary

A common phenomenon of protein instability is the formation of aggregates, which can be soluble or insoluble, non-covalent or covalent, and reversible or irreversible. Although protein sequence determines the behavior of protein aggregation, many external factors play a critical role in controlling/affecting protein aggregation, including temperature, pH, ionic strength, surface adsorption, shearing, shaking, presence of metal ions, organic solvents and additives, protein concentration, purity and morphism, pressure, freezing and drying. Chemical transformations can lead to direct or indirect protein aggregation, such as disulfide bond formation/exchange, non-disulfide crosslinking, transamidation, and oxidation. Although many analytical techniques are available in monitoring protein aggregation, their differences in quantatation, accuracy, sensitivity, and ease of operation require careful selection of some of these methods in monitoring all aspects of protein aggregation.

Protein aggregation occurs readily in almost all the biopharmaceutical processes, especially during fermentation, refolding, purification, formulation, and storage. Recognition of different causes of protein aggregation in these processes is the basis for selection of different techniques/methods for aggregation inhibition. Although protein aggregation can be inhibited effectively by modifying the primary sequence of a protein, this method is seldom used mainly because of the unpredictable consequences of structural modifications. Therefore, inhibition of protein aggregation is commonly achieved by changing the immediate environment of the protein. One particularly useful and simple method is the use of a compatible excipient(s). The often-used protein stabilizing excipients include sugars, polyols, surfactants, salts, PEGs, polymers, metal ions, and amino acids.

Although there are a variety of methods and a large pool of excipients for use to inhibit protein aggregation, a satisfactory level of aggregation inhibition has not been achieved for many proteins. Trial-and-error has been the main stay in the prevention or inhibition of protein aggregation in most biopharmaceutical processes. This is partly due to our lack of a clear understanding of the protein aggregation process. Therefore, intensive and rigorous investigation of mechanisms of protein aggregation is urgently needed for an ultimate solution of protein aggregation in biopharmaceutics.

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References

Advant, S.J., Braswell, E.H., et al., 1995. The effect of pH and temperature on the self-association of recombinant human interleukin-2 as studied by equilibrium sedimentation. Pharm. Res. 12, 637–641.

Alexandrescu, A.T., Rathgeb-Szabo, K., 1999. An NMR investigation of solution aggregation reactions preceding the misassembly of acid-denatured cold shock protein A into fibrils. J. Mol. Biol. 291, 1191–1206.

Allison, S.D., Dong, A., et al., 1996. Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying, and rehydration. Biophys. J. 71, 2022–2032.

Anchordoquy, T.J., Carpenter, J.F., 1996. Polymers protect lactate dehydrogenase during freeze-drying by inhibiting dissociation in the frozen state. Arch. Biochem. Biophys. 332, 231–238.

Andya, J.D., Hsu, C.C., et al., 2003. Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulations. AAPS Pharm. Sci. 5, E10.

Andya, J.D., Maa, Y.F., et al., 1999. The effect of formulation excipients on protein stability and aerosol performance of spray-dried powders of a recombinant humanized anti-IgE monoclonal antibody. Pharm. Res. 16, 350–358.

Apenten, R.K., 1998. Protein stability function relations: beta-lactoglobulin-A sulphydryl group reactivity and its relationship to protein unfolding stability. Int. J. Biol. Macromol. 23, 19–25.

Arakawa, T., Kita, Y., 2000. Protection of bovine serum albumin from aggregation by Tween 80. J. Pharm. Sci. 89, 646–651.

Arakawa, T., Kita, Y., et al., 1991. Protein–solvent interactions in pharmaceutical formulations. Pharm. Res. 8, 285–291.

Arakawa, T., Tsumoto, K., 2003. The effects of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation. Biochem. Biophys. Res. Commun. 304, 148–152.

Assemand, E., Lacroix, M., et al., 2003. L-Tyrosine prevents aggregation of therapeutic proteins by gamma-irradiation. Biotechnol. Appl. Biochem. 38, 151–156.

Bam, N.B., Cleland, J.L., et al., 1996. Molten globule intermediate of recombinant human growth hormone: stabilization with surfactants. Biotechnol. Prog. 12, 801–809.

Bam, N.B., Cleland, J.L., et al., 1998. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J. Pharm. Sci. 87, 1554–1559.

Bam, N.B., Randolph, T.W., et al., 1995. Stability of protein formulations: investigation of surfactant effects by a novel EPR spectroscopic technique. Pharm. Res. 12, 2–11.

Batas, B., Jones, H.R., et al., 1997. Studies of the hydrodynamic volume changes that occur during refolding of lysozyme using size-exclusion chromatography. J. Chromatogr. A 766, 109–119.

Baudys, M., Uchio, T., et al., 1995. Physical stabilization of insulin by glycosylation. J. Pharm. Sci. 84, 28–33.

Boctor, A.M., Mehta, S.C., 1992. Enhancement of the stability of thrombin by polyols: microcalorimetric studies. J. Pharm. Pharmacol. 44, 600–603.

Booth, D.R., Sunde, M., et al., 1997. Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis. Nature 385, 787–793.

Brange, J., Andersen, L., et al., 1997. Toward understanding insulin fibrillation. J. Pharm. Sci. 86, 517–525.

Brange, J., Havelund, S., et al., 1992. Chemical stability of insulin. 2. Formation of higher molecular weight transformation products during storage of pharmaceutical preparations. Pharm. Res. 9, 727–734.

Breen, E.D., Curley, J.G., et al., 2001. Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. Pharm. Res. 18, 1345–1353.

Bush, L., Webb, C., et al., 1998. The formulation of recombinant factor IX: stability, robustness, and convenience. Semin. Hematol. 35, 18–21.

Calamai, M., Taddei, N., et al., 2003. Relative influence of hydrophobicity and net charge in the aggregation of two homologous proteins. Biochemistry 42, 15078–15083.

Cardamone, M., Puri, N.K., et al., 1995. Comparing the refolding and reoxidation of recombinant porcine growth hormone from a urea denatured state and from *Escherichia coli* inclusion bodies. Biochemistry 34, 5773–5794.

Carpenter, J.F., Crowe, J.H., 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochemistry 28, 3916–3922.

Carpenter, J.F., Crowe, J.H., et al., 1990. Comparison of solute-induced protein stabilization in aqueous solution and in frozen and dried state. J. Dairy Sci. 73, 3527–3636.

Carpenter, J.F., Kendrick, B.S., et al., 1999. Inhibition of stress-induced aggregation of protein therapeutics. Methods Enzymol. 309, 236–255.

Carpenter, J.F., Pikal, M.J., et al., 1997. Rational design of stable lyophilized protein formulations: some practical advice. Pharm. Res. 14, 969–975.

Castellanos, I.J., Crespo, R., et al., 2003. Poly(ethylene glycol) as stabilizer and emulsifying agent: a novel stabilization approach preventing aggregation and inactivation of proteins upon encapsulation in bioerodible polyester microspheres. J. Control Rel. 88, 135–145.

Chang, B.S., Beauvais, R.M., et al., 1996a. Physical factors affecting the storage stability of freeze-dried interleukin-1 receptor antagonist: glass transition and protein conformation. Arch. Biochem. Biophys. 331, 249–258.

Chang, B.S., Kendrick, B.S., et al., 1996b. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J. Pharm. Sci. 85, 1325–1330.

Chang, B.S., Reeder, G., et al., 1996c. Development of a stable freeze-dried formulation of recombinant human interleukin-1 receptor antagonist. Pharm. Res. 13, 243–249.

Charman, S.A., Mason, K.L., et al., 1993. Techniques for assessing the effects of pharmaceutical excipients on the aggregation of porcine growth hormone. Pharm. Res. 10, 954–962.

Regeneron Pharmaceuticals, Inc. Exhibit 2053 Page 24
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Chen, B., Costantino, H.R., et al., 1999. Influence of calcium ions on the structure and stability of recombinant human deoxyribonuclease I in the aqueous and lyophilized states. J. Pharm. Sci. 88, 477–482.

Chen, B.L., Arakawa, T., et al., 1994. Aggregation pathway of recombinant human keratinocyte growth factor and its stabilization. Pharm. Res. 11, 1581–1587.

Chi, E.Y., Krishnan, S., et al., 2003. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharm. Res. 20, 1325–1336.

Cholewinski, M., Luckel, B., et al., 1996. Degradation pathways, analytical characterization and formulation strategies of a peptide and a protein. Calcitonine and human growth hormone in comparison. Pharm. Acta Helv. 71, 405–419.

Chrunyk, B.A., Wetzel, R., 1993. Breakdown in the relationship between thermal and thermodynamic stability in an interleukin-1 beta point mutant modified in a surface loop. Protein Eng. 6, 733–738.

Clark, E.D.B., 1998. Refolding of recombinant proteins. Curr. Opin. Biotechnol. 9, 157–163.

Clarke, A.R., Waltho, J.P., 1997. Protein folding and intermediates. Curr. Opin. Biotechnol. 8, 400–410.

Cleland, J.L., Builder, S.E., et al., 1992. Polyethylene glycol enhanced protein refolding. Biotechnology (NY) 10, 1013–1019.

Cleland, J.L., Wang, D.I., 1990. Refolding and aggregation of bovine carbonic anhydrase B: quasi-elastic light scattering analysis. Biochemistry 29, 11072–11078.

Cleland, J.L., Wang, D.I., 1992. Transient association of the first intermediate during the refolding of bovine carbonic anhydrase B. Biotechnol. Prog. 8, 97–103.

Costantino, H.R., Andya, J.D., et al., 1998. Effect of mannitol crystallization on the stability and aerosol performance of a spray-dried pharmaceutical protein, recombinant humanized anti-IgE monoclonal antibody. J. Pharm. Sci. 87, 1406–1411.

Costantino, H.R., Firouzabadian, L., et al., 2000. Protein spray-freeze drying. Effect of atomization conditions on particle size and stability. Pharm. Res. 17, 1374–1383.

Costantino, H.R., Griebenow, K., et al., 1995a. Fourier-transform infrared spectroscopic investigation of protein stability in the lyophilized form. Biochim. Biophys. Acta 1253, 69–74.

Costantino, H.R., Langer, R., et al., 1994a. Moisture-induced aggregation of lyophilized insulin. Pharm. Res. 11, 21–29.

Costantino, H.R., Langer, R., et al., 1994b. Solid-phase aggregation of proteins under pharmaceutically relevant conditions. J. Pharm. Sci. 83, 1662–1669.

Costantino, H.R., Langer, R., et al., 1995b. Aggregation of a lyophilized pharmaceutical protein, recombinant human albumin: effect of moisture and stabilization by excipients. Biotechnology (NY) 13, 493–496.

Costantino, H.R., Schwendeman, S.P., et al., 1996. The secondary structure and aggregation of lyophilized tetanus toxoid. J. Pharm. Sci. 85, 1290–1293.

Crowe, J.H., Crowe, L.M., et al., 1993. Preserving dry biomaterials: the water replacement hypothesis, part 2. Biopharm 6, 40–43.

Curatolo, L., Valsasina, B., et al., 1997. Recombinant human IL-2 is cytotoxic to oligodendrocytes after in vitro self-aggregation. Cytokine 9, 734–739.

Czaplewski, L.G., McKeating, J., et al., 1999. Identification of amino acid residues critical for aggregation of human CC chemokines macrophage inflammatory protein (MIP)-1alpha, MIP-1beta, and RANTES. Characterization of active disaggregated chemokine variants. J. Biol. Chem. 274, 16077–16084.

Damodaran, S., Song, K.B., 1988. Kinetics of adsorption of proteins at interfaces: role of protein conformation in diffusional adsorption. Biochim. Biophys. Acta 954, 253–264.

Darrington, R.T., Anderson, B.D., 1995. Evidence for a common intermediate in insulin deamidation and covalent dimer formation: effects of pH and aniline trapping in dilute acidic solutions. J. Pharm. Sci. 84, 275–282.

De Bernardez Clark, E., Schwarz, E., et al., 1999. Inhibition of aggregation side reactions during in vitro protein folding. Methods Enzymol. 309, 217–236.

De Young, L.R., Dill, K.A., et al., 1993. Aggregation and denaturation of apomyoglobin in aqueous urea solutions. Biochemistry 32, 3877–3886.

Dong, A., Kendrick, B., et al., 1997. Spectroscopic study of secondary structure and thermal denaturation of recombinant human factor XIII in aqueous solution. Arch. Biochem. Biophys. 347, 213–220.

Dong, A., Prestrelski, S.J., et al., 1995. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. J. Pharm. Sci. 84, 415–424.

Durbin, S.D., Feher, G., 1996. Protein crystallization. Annu. Rev. Phys. Chem. 47, 171–204.

Eberlein, G.A., Stratton, P.R., et al., 1994. Stability of rhbFGF as determined by UV spectroscopic measurements of turbidity. PDA J. Pharm. Sci. Technol. 48, 224–230.

Eckhardt, B.M., Oeswein, J.Q., et al., 1991. Effect of freezing on aggregation of human growth hormone. Pharm. Res. 8, 1360–1364.

Fabian, H., Falber, K., et al., 1999. Secondary structure and oligomerization behavior of equilibrium unfolding intermediates of the lambda cro repressor. Biochemistry 38, 5633–5642.

Farruggia, B., Garcia, G., et al., 1997. Destabilization of human serum albumin by polyethylene glycols studied by thermodynamical equilibrium and kinetic approaches. Int. J. Biol. Macromol. 20, 43–51.

Fatouros, A., Osterberg, T., et al., 1997. Recombinant factor VIII SQ—influence of oxygen, metal ions, pH and ionic strength on its stability in aqueous solution. Int. J. Pharm. 155, 121–131.

Ferrone, F., 1999. Analysis of protein aggregation kinetics. Methods Enzymol. 309, 256–274.

Fields, G., Alonso, D., et al., 1992. Theory for the aggregation of proteins and copolymers. J. Phys. Chem. 96, 3974–3981.

Fink, A.L., 1998. Protein aggregation: folding aggregates, inclusion bodies and amyloid. Fold Des. 3, R9–R23.

Finke, J.M., Roy, M., et al., 2000. Aggregation events occur prior to stable intermediate formation during refolding of interleukin 1beta. Biochemistry 39, 575–583.

Fox, K.C., 1995. Biopreservation. Putting proteins under glass. Science 267, 1922–1923.

Franks, F., 1994. Long-term stabilization of biologicals. Biotechnology (NY) 12, 253–256.

Fraser, P.E., McLachlan, D.R., et al., 1994. Conformation and fibrillogenesis of Alzheimer A beta peptides with selected substitution of charged residues. J. Mol. Biol. 244, 64–73.

Frye, K.J., Royer, C.A., 1997. The kinetic basis for the stabilization of staphylococcal nuclease by xylose. Protein Sci. 6, 789–793.

Garzon-Rodriguez, W., Koval, R.L., et al., 2004. Optimizing storage stability of lyophilized recombinant human interleukin-11 with disaccharide/hydroxyethyl starch mixtures. J. Pharm. Sci. 93, 684–696.

Georgiou, G., Valax, P., 1999. Isolating inclusion bodies from bacteria. Methods Enzymol. 309, 48–58.

Georgiou, G., Valax, P., et al., 1994. Folding and aggregation of TEM beta-lactamase: analogies with the formation of inclusion bodies in *Escherichia coli*. Protein Sci. 3, 1953–1960.

Giancola, C., De Sena, C., et al., 1997. DSC studies on bovine serum albumin denaturation. Effects of ionic strength and SDS concentration. Int. J. Biol. Macromol. 20, 193–204.

Golbik, R., Zahn, R., et al., 1998. Thermodynamic stability and folding of GroEL minichaperones. J. Mol. Biol. 276, 505–515.

Gonzalez, M., Murature, D.A., et al., 1995. Thermal stability of human immunoglobulins with sorbitol. A critical evaluation. Vox. Sang. 68, 1–4.

Gorovits, B.M., Horowitz, P.M., 1998. High hydrostatic pressure can reverse aggregation of protein folding intermediates and facilitate acquisition of native structure. Biochemistry 37, 6132–6135.

Gu, L.C., Erdos, E.A., et al., 1991. Stability of interleukin 1 beta (IL-1 beta) in aqueous solution: analytical methods, kinetics, products, and solution formulation implications. Pharm. Res. 8, 485–490.

Gupta, P., Hall, C.K., et al., 1998. Effect of denaturant and protein concentrations upon protein refolding and aggregation: a simple lattice model. Protein Sci. 7, 2642–2652.

Hagiwara, T., Kumagai, H., et al., 1996. Fractal analysis of aggregates formed by heating dilute BSA solutions using light scattering methods. Biosci. Biotechnol. Biochem. 60, 1757–1763.

Hammarstrom, P., Persson, M., et al., 1999. Structural mapping of an aggregation nucleation site in a molten globule intermediate. J. Biol. Chem. 274, 32897–32903.

Helms, L.R., Wetzel, R., 1996. Specificity of abnormal assembly in immunoglobulin light chain deposition disease and amyloidosis. J. Mol. Biol. 257, 77–86.

Hevehan, D., De Bernardez-Clark, E., 1997. Oxidative renaturation of lysozyme at high concentrations. Biotechnol. Bioeng. 54, 221–230.

Hinds, K., Koh, J.J., et al., 2000. Synthesis and characterization of poly(ethylene glycol)–insulin conjugates. Bioconjug. Chem. 11, 195–201.

Hora, M.S., Rana, R.K., et al., 1992a. Lyophilized formulations of recombinant tumor necrosis factor. Pharm. Res. 9, 33–36.

Hora, M.S., Rana, R.K., et al., 1992b. Development of a lyophilized formulation of interleukin-2. Dev. Biol. Stand. 74, 295–303.

Hsu, C.C., Nguyen, H.M., et al., 1995. Surface denaturation at solid-void interface—a possible pathway by which opalescent particulates form during the storage of lyophilized tissue-type plasminogen activator at high temperatures. Pharm. Res. 12, 69–77.

Ip, A.Y., Arakawa, T., et al., 1995. Stability of recombinant consensus interferon to air-jet and ultrasonic nebulization. J. Pharm. Sci. 84, 1210–1214.

Istrail, S., Schwartz, R., et al., 1999. Lattice simulations of aggregation funnels for protein folding. J. Comput. Biol. 6, 143–162.

Jacob, M., Schmid, F.X., 1999. Protein folding as a diffusional process. Biochemistry 38, 13773–13779.

James, E.L., Bottomley, S.P., 1998. The mechanism of alpha 1-antitrypsin polymerization probed by fluorescence spectroscopy. Arch. Biochem. Biophys. 356, 296–300.

Janek, K., Behlke, J., et al., 1999. Water-soluble beta-sheet models which self-assemble into fibrillar structures. Biochemistry 38, 8246–8252.

Jarrett, J.T., Lansbury Jr., P.T., 1993. Amyoid fibril formation requires a chemically discriminating nucleation event: studies of an amyloidogenic sequence from the bacterial protein OsmB. Biochemistry 31, 12345–12352.

Jordan, G.M., Yoshioka, S., et al., 1994. The aggregation of bovine serum albumin in solution and in the solid state. J. Pharm. Pharmacol. 46, 182–185.

Kanaoka, E., Nagata, S., et al., 1999. Stabilization of aerosolized IFN-gamma by liposomes. Int. J. Pharm. 188, 165–172.

Karuppiah, N., Sharma, A., 1995. Cyclodextrins as protein folding aids. Biochem. Biophys. Res. Commun. 211, 60–66.

Katakam, M., Banga, A.K., 1995. Aggregation of insulin and its prevention by carbohydrate excipients. PDA J. Pharm. Sci. Technol. 49, 160–165.

Katakam, M., Banga, A.K., 1997. Use of poloxamer polymers to stabilize recombinant human growth hormone against various processing stresses. Pharm. Dev. Technol. 2, 143–149.

Katakam, M., Bell, L.N., et al., 1995. Effect of surfactants on the physical stability of recombinant human growth hormone. J. Pharm. Sci. 84, 713–716.

Kato, A., Takagi, T., 1988. Formation of intermolecular b-sheet structure during heat denaturation of ovalbumin. J. Agric. Food Chem. 36, 1156–1159.

Kendrick, B.S., Carpenter, J.F., et al., 1998a. A transient expansion of the native state precedes aggregation of recombinant human interferon-gamma. Proc. Natl. Acad. Sci. U.S.A. 95, 14142–14146.

Kendrick, B.S., Chang, B.S., et al., 1997. Preferential exclusion of sucrose from recombinant interleukin-1 receptor antagonist: role in restricted conformational mobility and compaction of native state. Proc. Natl. Acad. Sci. U.S.A. 94, 11917–11922.

Kendrick, B.S., Cleland, J.L., et al., 1998b. Aggregation of recombinant human interferon gamma: kinetics and structural transitions. J. Pharm. Sci. 87, 1069–1076.

Kerwin, B.A., Akers, M.J., et al., 1999. Acute and long-term stability studies of deoxy hemoglobin and characterization of ascorbate-induced modifications. J. Pharm. Sci. 88, 79–88.

Kerwin, B.A., Heller, M.C., et al., 1998. Effects of Tween 80 and sucrose on acute short-term stability and long-term storage at −20 °C of a recombinant hemoglobin. J. Pharm. Sci. 87, 1062–1068.

Khossravi, M., Shire, S.J., et al., 2000. Evidence for the involvement of histidine A(12) in the aggregation and precipitation of human relaxin induced by metal-catalyzed oxidation. Biochemistry 39, 5876–5885.

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Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc. IPR2023-00462

Khurana, R., Gillespie, J.R., et al., 2001. Partially folded intermediates as critical precursors of light chain amyloid fibrils and amorphous aggregates. Biochemistry 40, 3525–3535.

Kim, D., Yu, M.H., 1996. Folding pathway of human alpha 1-antitrypsin: characterization of an intermediate that is active but prone to aggregation. Biochem. Biophys. Res. Commun. 226, 378–384.

King, J., 1997. Refolding with a piece of the ring. Nat. Biotechnol. 15, 514–515.

Kita, Y., Arakawa, T., 2002. Salts and glycine increase reversibility and decrease aggregation during thermal unfolding of ribonuclease-A. Biosci. Biotechnol. Biochem. 66, 880–882.

Klein, J., Dhurjati, P., 1995. Protein aggregation kinetics in an *Escherichia coli* strain overexpressing a *Salmonella typhimurium* CheY mutant gene. Appl. Environ. Microbiol. 61, 1220–1225.

Knappik, A., Plhckthun, A., 1995. Engineered turns of a recombinant antibody improve its in vivo folding. Protein Eng. 8, 81–89.

Kohn, W.D., Kay, C.M., et al., 1997. Salt effects on protein stability: two-stranded alpha-helical coiled-coils containing inter- or intrahelical ion pairs. J. Mol. Biol. 267, 1039–1052.

Koo, E.H., Lansbury Jr., P.T., et al., 1999. Amyloid diseases: abnormal protein aggregation in neurodegeneration. Proc. Natl. Acad. Sci. U.S.A. 96, 9989–9990.

Kreilgaard, L., Frokjaer, S., et al., 1998a. Effects of additives on the stability of recombinant human factor XIII during freeze-drying and storage in the dried solid. Arch. Biochem. Biophys. 360, 121–134.

Kreilgaard, L., Frokjaer, S., et al., 1999. Effects of additives on the stability of Humicola lanuginosa lipase during freeze-drying and storage in the dried solid. J. Pharm. Sci. 88, 281–290.

Kreilgaard, L., Jones, L.S., et al., 1998b. Effect of Tween 20 on freeze-thawing-and agitation-induced aggregation of recombinant human factor XIII. J. Pharm. Sci. 87, 1597–1603.

Krishnamurthy, R., Manning, M.C., 2002. The stability factor: importance in formulation development. Curr. Pharm. Biotechnol. 3, 361–371.

Krishnan, S., Chi, E.Y., et al., 2002. Aggregation of granulocyte colony stimulating factor under physiological conditions: characterization and thermodynamic inhibition. Biochemistry 41, 6422–6431.

Kuhlman, B., Luisi, D.L., et al., 1998. Global analysis of the effects of temperature and denaturant on the folding and unfolding kinetics of the N-terminal domain of the protein L9. J. Mol. Biol. 284, 1661–1670.

Kuhlman, B., Yang, H.Y., et al., 1997. An exceptionally stable helix from the ribosomal protein L9: implications for protein folding and stability. J. Mol. Biol. 270, 640–647.

Kurochkin, I.V., Procyk, R., et al., 1995. Domain structure, stability and domain–domain interactions in recombinant factor XIII. J. Mol. Biol. 248, 414–430.

Kwon, Y.M., Baudys, M., et al., 2001. In situ study of insulin aggregation induced by water-organic solvent interface. Pharm. Res. 18, 1754–1759.

Lazo, N.D., Downing, D.T., 1997. Beta-helical fibrils from a model peptide. Biochem. Biophys. Res. Commun. 235, 675–679.

Ledl, F., Beck, J., et al., 1989. Chemical pathways of the Maillard reaction. Prog. Clin. Biol. Res. 304, 23–42.

Li, S., Nguyen, T.H., et al., 1995. Aggregation and precipitation of human relaxin induced by metal-catalyzed oxidation. Biochemistry 34, 5762–5772.

Liang, J.N., Rossi, M.T., 1990. In vitro non-enzymatic glycation and formation of browning products in the bovine lens alpha-crystallin. Exp. Eye Res. 50, 367–371.

Liu, W.R., Langer, R., et al., 1990. Moisture-induced aggregation of lyophilized proteins in the solid state. Biotechnol. Bioeng. 37, 177–184.

Lomakin, A., Benedek, G.B., et al., 1999. Monitoring protein assembly using quasielastic light scattering spectroscopy. Methods Enzymol. 309, 429–459.

Lomakin, A., Teplow, D.B., et al., 1997. Kinetic theory of fibrillogenesis of amyloid beta-protein. Proc. Natl. Acad. Sci. U.S.A. 94, 7942–7947.

Lueckel, B., Helk, B., et al., 1998. Effects of formulation and process variables on the aggregation of freeze-dried interleukin-6 (IL-6) after lyophilization and on storage. Pharm. Dev. Technol. 3, 337–346.

Lundblad, R.L., Bradshaw, R.A., 1997. Applications of site-specific chemical modification in the manufacture of biopharmaceuticals. Biotechnol. Appl. Biochem. 26, 143–151.

Maa, Y.-F., Hsu, C.C., 1997. Protein denaturation by combined effect of shear and air–liquid interface. Biotechnol. Bioeng. 54.

Maclean, D.S., Qian, Q., et al., 2002. Stabilization of proteins by low molecular weight multi-ions. J. Pharm. Sci. 91, 2220–2229.

Maeda, Y., Ueda, T., et al., 1996a. Effective renaturation of denatured and reduced immunoglobulin G in vitro without assistance of chaperone. Protein Eng. 9, 95–100.

Maeda, Y., Yamada, H., et al., 1996b. Effect of additives on the renaturation of reduced lysozyme in the presence of 4 m urea. Protein Eng. 9, 461–465.

Malencik, D.A., Anderson, S.R., 2003. Dityrosine as a product of oxidative stress and fluorescent probe. Amino Acids 25, 233–247.

Manning, M.C., Matsuura, J.E., et al., 1995. Approaches for increasing the solution stability of proteins. Biotechnol. Bioeng. 48, 506–512.

Matsuura, J., Manning, M.C., 1993. Conformation of human calcitonin gene-related peptide (8–37) in aqueous solution as determined by circular dichroism spectroscopy. J. Pharm. Biomed. Anal. 11, 89–93.

Mozhaev, V.V., Martinek, K., 1984. Structure–stability relationships in proteins: new approaches to stabilizing enzymes. Enzyme Microb. Technol. 6, 50–59.

Murphy, K.P., Bhakuni, V., et al., 1992. Molecular basis of cooperativity in protein folding. III. Structural identification of folding units and folding intermediates. J. Mol. Biol. 227, 293–306.

Narhi, L.O., Philo, J.S., et al., 1999. Reversibility of heat-induced denaturation of the recombinant human megakaryocyte growth and development factor. Pharm. Res. 16, 799–807.

Nesta, D.P., Elliott, J.S., et al., 2000. Supercritical fluid precipitation of recombinant human immunoglobulin from aqueous solutions. Biotechnol. Bioeng. 67, 457–464.

Nguyen, H.D., Hall, C.K., 2002. Effect of rate of chemical or thermal renaturation on refolding and aggregation of a simple lattice protein. Biotechnol. Bioeng. 80, 823–834.

Nguyen, L.T., Wiencek, J.M., et al., 2003. Characterization methods for the physical stability of biopharmaceuticals. PDA J. Pharm. Sci. Technol. 57, 429–445.

Nielsen, L., Frokjaer, S., et al., 2001a. Probing the mechanism of insulin fibril formation with insulin mutants. Biochemistry 40, 8397–8409.

Nielsen, L., Frokjaer, S., et al., 2001b. Studies of the structure of insulin fibrils by Fourier transform infrared (FTIR) spectroscopy and electron microscopy. J. Pharm. Sci. 90, 29–37.

Oliva, A., Santoveza, A., et al., 1999. Stability study of human serum albumin pharmaceutical preparations. J. Pharm. Pharmacol. 51, 385–392.

Parbhu, A., Lin, H., et al., 2002. Imaging real-time aggregation of amyloid beta protein (1–42) by atomic force microscopy. Peptides 23, 1265–1270.

Patro, S.Y., Przybycien, T.M., 1994. Simulations of kinetically irreversible protein aggregate structure. Biophys. J. 66, 1274–1289.

Perlman, R., Nguyen, T., 1992. Pharmaceutics of protein drugs. J. Pharm. Pharmcol. 44, 178–185.

Pikal, M.J., Dellerman, K.M., et al., 1991. The effects of formulation variables on the stability of freeze-dried human growth hormone. Pharm. Res. 8, 427–436.

Popov, E.M., 1999. Protein folding as a nonlinear nonequilibrium thermodynamic process. Biochem. Mol. Biol. Int. 47, 443–453.

Prasanna, V., Gopal, B., et al., 1999. Effect of amino acid substitutions at the subunit interface on the stability and aggregation properties of a dimeric protein: role of Arg 178 and Arg 218 at the Dimer interface of thymidylate synthase. Proteins 34, 356–368.

Prestrelski, S.J., Tedeschi, N., et al., 1993. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophys. J. 65, 661–671.

Querol, E., Perez-Pons, J.A., et al., 1996. Analysis of protein conformational characteristics related to thermostability. Protein Eng. 9, 265–271.

Raman, B., Ramakrishna, T., et al., 1997. Effect of the chaperone-like alpha-crystallin on the refolding of lysozyme and ribonuclease A. FEBS Lett. 416, 369–372.

Rariy, R.V., Klibanov, A.M., 1997. Correct protein folding in glycerol. Proc. Natl. Acad. Sci. U.S.A. 94, 13520–13523.

Renard, D., Lefebvre, J., et al., 1999. Structural investigation of beta-lactoglobulin gelation in ethanol/water solutions. Int. J. Biol. Macromol. 26, 35–44.

Richards, J.P., Stickelmeyer, M.P., et al., 1998. Self-association properties of monomeric insulin analogs under formulation conditions. Pharm. Res. 15, 1434–1441.

Roefs, S.P., De Kruif, K.G., 1994. A model for the denaturation and aggregation of beta-lactoglobulin. Eur. J. Biochem. 226, 883–889.

Roy, M.L., Pikal, M.J., et al., 1992. The effects of formulation and moisture on the stability of a freeze-dried monoclonal antibody-vinca conjugate: a test of the WLF glass transition theory. Dev. Biol. Stand. 74, 323–339, discussion 340.

Rozema, D., Gellman, S.H., 1996a. Artificial chaperone-assisted refolding of carbonic anhydrase B. J. Biol. Chem. 271, 3478–3487.

Rozema, D., Gellman, S.H., 1996b. Artificial chaperone-assisted refolding of denatured-reduced lysozyme: modulation of the competition between renaturation and aggregation. Biochemistry 35, 15760–15771.

Runkel, L., Meier, W., et al., 1998. Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-beta (IFN-beta). Pharm. Res. 15, 641–649.

Rupley, J.A., Careri, G., 1991. Protein hydration and function. Adv. Protein Chem. 41, 37–172.

Sadhale, Y., Shah, J.C., 1999. Stabilization of insulin against agitation-induced aggregation by the GMO cubic phase gel. Int. J. Pharm. 191, 51–64.

Safar, J., Roller, P.P., et al., 1994. Scrapie amyloid (prion) protein has the conformational characteristics of an aggregated molten globule folding intermediate. Biochemistry 33, 8375–8383.

Sah, H., 1999. Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation. J. Control Rel. 58, 143–151.

Sane, S.U., Wong, R., et al., 2004. Raman spectroscopic characterization of drying-induced structural changes in a therapeutic antibody: correlating structural changes with long-term stability. J. Pharm. Sci. 93, 1005–1018.

Sarciaux, J.M., Mansour, S., et al., 1999. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. J. Pharm. Sci. 88, 1354–1361.

Sarciaux, J.-M., Mansour, S., et al., 1998. Effect of species, processing conditions and phosphate buffer composition on IgG aggregation during lyophilization. Pharm. Sci. (Suppl.) 1, S-545.

Sato, K., Wakamiya, A., et al., 1995. Correlation among secondary structure, amyloid precursor protein accumulation, and neurotoxicity of amyloid b(25–35) peptide as analyzed by single alanine substitution. J. Biochem. (Tokyo) 118, 1108–1111.

Scalley, M.L., Baker, D., 1997. Protein folding kinetics exhibit an Arrhenius temperature dependence when corrected for the temperature dependence of protein stability. Proc. Natl. Acad. Sci. U.S.A. 94, 10636–10640.

Schein, C.H., 1990. Solubility as a function of protein structure and solvent components. Biotechnology 8, 308–317.

Schuler, B., Rachel, R., et al., 1999. Formation of fibrous aggregates from a non-native intermediate: the isolated P22 tailspike beta-helix domain. J. Biol. Chem. 274, 18589–18596.

Schwendeman, S.P., Costantino, H.R., et al., 1995. Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation. Proc. Natl. Acad. Sci. U.S.A. 92, 11234–11238.

Schwendeman, S.P., Costantino, H.R., et al., 1996. Strategies for stabilising tetanus toxoid towards the development of a single-dose tetanus vaccine. Dev. Biol. Stand. 87, 293–306.

Separovic, F., Lam, Y.H., et al., 1998. A solid-state NMR study of protein hydration and stability. Pharm. Res. 15, 1816–1821.

Shahrokh, Z., Eberlein, G., et al., 1994a. Major degradation products of basic fibroblast growth factor: detection of succinimide and iso-aspartate in place of aspartate. Pharm. Res. 11, 936–944.

Shahrokh, Z., Stratton, P.R., et al., 1994b. Approaches to analysis of aggregates and demonstrating mass balance in pharmaceutical protein (basic fibroblast growth factor) formulations. J. Pharm. Sci. 83, 1645–1650.

Shen, C.-L., Scott, G.L., et al., 1993. Light scattering analysis of fibril growth from amino-terminal fragment b(1–28) of beta-amyloid peptide. Biophys. J. 65, 2383–2395.

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Sluzky, V., Klibanov, A.M., et al., 1992. Mechanism of insulin aggregation and stabilization in agitated aqueous solutions. Biotechnol. Bioeng. 40, 895–903.

Sluzky, V., Tamada, J.A., et al., 1991. Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. Proc. Natl. Acad. Sci. U.S.A. 88, 9377–9381.

Smith, A.V., Hall, C.K., 2001. Protein refolding versus aggregation: computer simulations on an intermediate-resolution protein model. J. Mol. Biol. 312, 187–202.

Soenderkaer, S., Carpenter, J.F., et al., 2004. Effects of sucrose on rFVIIa aggregation and methionine oxidation. Eur. J. Pharm. Sci. 21, 597–606.

Soto, C., Castano, E.M., 1996. The conformation of Alzheimer's beta peptide determines the rate of amyloid formation and is resistance to proteolysis. Biochem. J. 314, 701–707.

Soto, C., Castano, E.M., et al., 1995. The alpha-helical to beta-strand transition in the amino-terminal fragment of the amyloid beta-peptide modulates amyloid formation. J. Biol. Chem. 270, 3063–3067.

Soto, C., Kindy, M.S., et al., 1996. Inhibition of Alzheimer's amyloidosis by peptides that prevent beta-sheet conformation. Biochem. Biophys. Res. Commun. 226, 672–680.

Speed, M.A., King, J., et al., 1997. Polymerization mechanism of polypeptide chain aggregation. Biotechnol. Bioeng. 54, 333–343.

Stefani, M., Dobson, C.M., 2003. Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. J. Mol. Med. 81, 678–699.

Stempfer, G., Holl-Neugebauer, B., et al., 1996. Improved refolding of an immobilized fusion protein. Nat. Biotechnol. 14, 329–334.

Stigter, D., Dill, K.D., 1993. Theory for protein solubilities. Fluid Phase Equilib. 82, 237–249.

Stockel, J., Doring, K., et al., 1997. Pathway of detergent-mediated and peptide ligand-mediated refolding of heterodimeric class II major histocompatibility complex (MHC) molecules. Eur. J. Biochem. 248, 684–691.

Strickley, R.G., Anderson, B.D., 1997. Solid-state stability of human insulin. II. Effect of water on reactive intermediate partitioning in lyophiles from pH 2–5 solutions: stabilization against covalent dimer formation. J. Pharm. Sci. 86, 645–653.

Szabo, Z., Klement, E., et al., 1999. An FT-IR study of the beta-amyloid conformation: standardization of aggregation grade. Biochem. Biophys. Res. Commun. 265, 297–300.

Teschke, C.M., 1999. Aggregation and assembly of phage P22 temperature-sensitive coat protein mutants in vitro mimic the in vivo phenotype. Biochemistry 38, 2873–2881.

Thomas, J.G., Ayling, A., et al., 1997. Molecular chaperones, folding catalysts, and the recovery of active recombinant proteins from *E. coli*. To fold or to refold. Appl. Biochem. Biotechnol. 66, 197–238.

Timasheff, S.N., 1998. Control of protein stability and reactions by weakly interacting cosolvents: the simplicity of the complicated. Adv. Protein Chem. 51, 355–432.

Tobitani, A., Ross-Murphy, S.B., 1997. Heat-induced gelation of globular proteins. 2. Effect of environmental factors on single-component and mixed-protein gels. Macromolecules 30, 4855–4862.

Tokihiro, K., Irie, T., et al., 1997. Varying effects of cyclodextrin derivatives on aggregation and thermal behavior of insulin in aqueous solution. Chem. Pharm. Bull. (Tokyo) 45, 525–531.

Tomski, S.J., Murphy, R.M., 1992. Kinetics of aggregation of synthetic b-amyloid peptide. Arch. Biochem. Biophys. 294, 630–638.

Townsend, M.W., Byron, P.R., et al., 1990. The effects of formulation additives on the degradation of freeze-dried ribonuclease A. Pharm. Res. 7, 1086–1091.

Treuheit, M.J., Kosky, A.A., et al., 2002. Inverse relationship of protein concentration and aggregation. Pharm. Res. 19, 511–516.

Tsai, A.M., van Zanten, J.H., et al., 1998a. I. Study of protein aggregation due to heat denaturation: a structural approach using circular dichroism spectroscopy, nuclear magnetic resonance, and static light scattering. Biotechnol. Bioeng. 59, 273–280.

Tsai, A.M., van Zanten, J.H., et al., 1998b. II. Electrostatic effect in the aggregation of heat-denatured RNase A and implications for protein additive design. Biotechnol. Bioeng. 59, 281–285.

Tsai, P.K., Volkin, D.B., et al., 1993. Formulation design of acidic fibroblast growth factor. Pharm. Res. 10, 649–659.

Tsai, Y.R., Yang, S.J., et al., 1998c. High-pressure effects on vacuolar H^+-ATPase from etiolated mung bean seedlings. J. Protein Chem. 17, 161–172.

Tsumoto, K., Umetsu, M., et al., 2003. Solubilization of active green fluorescent protein from insoluble particles by guanidine and arginine. Biochem. Biophys. Res. Commun. 312, 1383–1386.

Tzannis, S.T., Prestrelski, S.J., 1999. Moisture effects on protein–excipient interactions in spray-dried powders. Nature of destabilizing effects of sucrose. J. Pharm. Sci. 88, 360–370.

Uversky, V.N., Karnoup, A.S., et al., 1999. Association of partially-folded intermediates of staphylococcal nuclease induces structure and stability. Protein Sci. 8, 161–173.

van den Berg, B., Chung, E.W., et al., 1999a. The oxidative refolding of hen lysozyme and its catalysis by protein disulfide isomerase. EMBO J. 18, 4794–4803.

van den Berg, B., Ellis, R.J., et al., 1999b. Effects of macromolecular crowding on protein folding and aggregation. EMBO J. 18, 6927–6933.

Vemuri, S., Yu, C.T., et al., 1993. Formulation and stability of recombinant alpha 1-antitrypsin. Pharm. Biotechnol. 5, 263–286.

Vermeer, A.W., Norde, W., 2000. The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. Biophys. J. 78, 394–404.

Volkin, D.B., Klibanov, A.M., 1989. Minimizing protein inactivation. In: Creighton, T.E. (Ed.), Protein Function A Practical Approach. Information Press, Oxford, UK, pp. 1–24.

Volkin, D.B., Middaugh, C.R., 1996. The characterization, stabilization, and formulation of acidic fibroblast growth factor. Pharm. Biotechnol. 9, 181–217.

Volkin, D.B., Tsai, P.K., et al., 1993. Physical stabilization of acidic fibroblast growth factor by polyanions. Arch. Biochem. Biophys. 300, 30–41.

Vrkljan, M., Foster, T.M., et al., 1994. Thermal stability of low molecular weight urokinase during heat treatment. II. Effect of polymeric additives. Pharm. Res. 11, 1004–1008.

Wall, J., Solomon, A., 1999. Flow cytometric characterization of amyloid fibrils. Methods Enzymol. 309, 460–466.

Wang, A., Robertson, A.D., et al., 1995. Effects of a naturally occurring compatible osmolyte on the internal dynamics of ribonuclease A. Biochemistry 34, 15096–150104.

Wang, P.L., Johnston, T.P., 1993. Enhanced stability of two model proteins in an agitated solution environment using poloxamer 407. J. Parenter. Sci. Technol. 47, 183–189.

Wang, W., 1999. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int. J. Pharm. 185, 129–188.

Wang, W., 2000. Lyophilization and development of solid protein pharmaceuticals. Int. J. Pharm. 203, 1–60.

Wang, Y.J., Shahrokh, Z., et al., 1996. Characterization, stability, and formulations of basic fibroblast growth factor. Pharm. Biotechnol. 9, 141–180.

Webb, S.D., Cleland, J.L., et al., 2002a. A new mechanism for decreasing aggregation of recombinant human interferon-gamma by a surfactant: slowed dissolution of lyophilized formulations in a solution containing 0.03% polysorbate 20. J. Pharm. Sci. 91, 543–558.

Webb, S.D., Cleland, J.L., et al., 2003. Effects of annealing lyophilized and spray-lyophilized formulations of recombinant human interferon-gamma. J. Pharm. Sci. 92, 715–729.

Webb, S.D., Golledge, S.L., et al., 2002b. Surface adsorption of recombinant human interferon-gamma in lyophilized and spray-lyophilized formulations. J. Pharm. Sci. 91, 1474–1487.

Wen, J., Arakawa, T., et al., 1996. Size-exclusion chromatography with on-line light-scattering, absorbance, and refractive index detectors for studying proteins and their interactions. Anal. Biochem. 240, 155–166.

Wetlaufer, D.B., Xie, Y., 1995. Control of aggregation in protein refolding: a variety of surfactants promote renaturation of carbonic anhydrase II. Protein Sci. 4, 1535–1543.

Wetzel, R., 1996. For protein misassembly, it's the "I" decade. Cell 86, 699–702.

Won, C.M., Molnar, T.E., et al., 1998. Stabilizers against heat-induced aggregation of RPR, 114849, an acidic fibroblast growth factor (AFGF). Int. J. Pharm. 167, 25–36.

Xie, Y., Wetlaufer, D.B., 1996. Control of aggregation in protein refolding: the temperature-leap tactic. Protein Sci. 5, 517–523.

Yasuda, M., Murakami, Y., et al., 1998. Effect of additives on refolding of a denatured protein. Biotechnol. Prog. 14, 601–616.

Yon, J.M., 1996. The specificity of protein aggregation [new; comment]. Nat. Biotechnol. 14, 1231.

Yoshioka, S., Aso, Y., et al., 1993. Aggregates formed during storage of beta-galactosidase in solution and in the freeze-dried state. Pharm. Res. 10, 687–691.

Zarina, S., Zhao, H.R., et al., 2000. Advanced glycation end products in human senile and diabetic cataractous lenses. Mol. Cell Biochem. 210, 29–34.

Zhang, J., Peng, X., et al., 1995a. NMR study of the cold, heat, and pressure unfolding of ribonuclease A. Biochemistry 34, 8631–8641.

Zhang, M.Z., Wen, J., et al., 1995b. A new strategy for enhancing the stability of lyophilized protein: the effect of the reconstitution medium on keratinocyte growth factor. Pharm. Res. 12, 1447–1452.

Zlateva, T., Boteva, R., et al., 1999. Factors affecting the dissociation and aggregation of human interferon gamma. Int. J. Biol. Macromol. 26, 357–362.

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