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Pharmaceutical Research, Vol. 20, No. 9, September 2003 (© 2003)

Review

Physical Stability of Proteins in Aqueous Solution: Mechanism and Driving Forces in Nonnative Protein Aggregation

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Received February 26, 2003; accepted May 1, 2003

Irreversible protein aggregation is problematic in the biotechnology industry, where aggregation is encountered throughout the lifetime of a therapeutic protein, including during refolding, purification, sterilization, shipping, and storage processes. The purpose of the current review is to provide a fundamental understanding of the mechanisms by which proteins aggregate and by which varying solution conditions, such as temperature, pH, salt type, salt concentration, cosolutes, preservatives, and surfactants, affect this process.

KEY WORDS: formulation; pharmaceuticals; denaturation; second virial coefficient; conformational stability.

INTRODUCTION

The issue of protein stability was first explained on a fundamental level by Hsien Wu in 1931 (1), when he proposed a theory on protein denaturation after publishing 12 papers on his experimental observations on this topic (2). In 1954, Lumry and Eyring published a seminal paper (3) — "Conformation Changes of Proteins" — that laid the groundwork for what we know today about protein structure, folding, stability, and aggregation.

Protein stability is a particularly relevant issue today in the pharmaceutical field and will continue to gain more importance as the number of therapeutic protein products in development increases. Proteins provide numerous unique and critical treatments for human diseases and conditions (e.g., diabetes, cancer, hemophilia, myocardial infarction). There are already dozens of protein products on the market and hundreds more in preclinical and clinical development (4). However, if a therapeutic protein cannot be stabilized adequately, its benefits to human health will not be realized. The shelf life required for economic viability of a typical protein pharmaceutical product is 18–24 months (5). Achieving this goal is particularly difficult because proteins are only marginally stable and are highly susceptible to degradation, both chemical and physical (6–9). Chemical degradation refers to modifications involving covalent bonds, such as deamidation, oxidation, and disulfide bond shuffling. Physical degradation includes protein unfolding, undesirable adsorption to surfaces, and aggregation (6,8–10). Nonnative aggregation is particularly problematic because it is encountered routinely during refolding, purification, sterilization, shipping, and storage processes. Aggregation can occur even under solution conditions where the protein native state is highly thermodynamically favored (e.g., neutral pH and 37°C) and in the absence of stresses. This review examines the mechanisms and driving forces in nonnative protein aggregation.

Nonnative protein aggregation (hereafter referred to simply as "aggregation") describes the assembly from initially native, folded proteins of aggregates containing nonnative protein structures. Aggregation is often irreversible, and aggregates often contain high levels of nonnative, intermolecular β-sheet structures (11). Protein aggregation behaviors, such as onset, aggregation rate, and the final morphology of the aggregated state (i.e., amorphous precipitates or fibrils) have been found to depend strongly on the properties of a protein's solution environment, such as temperature, pH, salt type, salt concentration, cosolutes, preservatives, and surfactants (10,12–16) as well as the relative intrinsic thermodynamic stability of the native state (17–20).

This review first examines how different solution conditions affect protein stability. Case studies and fundamental insights into how each solution condition affects protein stability are discussed. The second part of this review discusses characteristics, mechanisms, energetics, and driving forces of nonnative protein aggregation. Recent studies on two therapeutic proteins — recombinant human interferon-γ (rhIFN-γ) and recombinant human granulocyte colony-stimulating

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ABBREVIATIONS: rhIFN-γ, recombinant human interferon-γ; rhGCSF, recombinant human granulocyte colony-stimulating factor; ΔG_{unf}, free energy of unfolding; B_{22}, osmotic second virial coefficient; GdnHCl, guanidine hydrochloride.

0724-8741/03/0900-1325/0 © 2003 Plenum Publishing Corporation

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factor (rhGCSF) — will be used to provide insight into the mechanistic issues. The present review is not aimed at providing a comprehensive literature review in the area of protein stability and aggregation. Each protein is unique both chemically and physically and therefore will exhibit unique stability behavior. The purpose of the current review is to provide a fundamental understanding of the mechanisms by which proteins aggregate and by which varying solution conditions may affect this process. This insight in turn can be used in the rational design of stable aqueous formulations of therapeutic proteins.

FACTORS INFLUENCING PROTEIN STABILITY

Temperature

Most proteins fold to a specific globular conformation that is essential for their biologic functions. The thermodynamic stability of the native protein conformation is only marginal, about 5–20 kcal/mole in free energy more stable than unfolded, biologically inactive conformations under physiologic conditions (21–25). This thermodynamic stability is much weaker than covalent or ionic bonds (~150 kcal/mole) (26) or the thermal energy of a protein (5–20 kcal/mole is less than one tenth of k_BT per residue, where k_B is Boltzmann's constant and T is the absolute temperature) (21). The small net conformational stability of protein results from a unique balance between large stabilizing and large destabilizing forces. Contributions to the free energy of folding arise from hydrophobic interactions, hydrogen bonding, van der Waals' forces, electrostatic forces (classic charge repulsion or ion pairing), and intrinsic propensities (local peptide interactions) (21). The main force opposing protein folding is the protein's conformational entropy. Both local entropy (e.g., translational, rotational, and vibrational degrees of freedom on the molecular scale) and nonlocal entropy (e.g., excluded volume and chain configurational freedom) are increased on unfolding (21). Because of the small conformational stability of the protein native state, relatively small changes of external variables (e.g., temperature, pH, salt, etc.) in the protein–solvent system can destabilize the structure of the protein, i.e., induce its unfolding.

The thermodynamic stability of the native protein conformation, characterized by the free energy of unfolding (ΔG_{unf}), typically shows a parabolic profile as a function of temperature (27–30). ΔG_{unf} therefore becomes negative at two temperatures, accounting for the unfolding of proteins at both high (e.g., 50–100°C) and low temperatures (e.g., less than 10°C) (21,27,29,30). The molecular origin of the effect of temperature on ΔG_{unf} is complex and is the subject of much ongoing research (21,22,29–31) and is not considered further here.

It has long been known that incubating protein solutions at high temperatures results in physical degradation. Although thermally induced denaturation may be reversible for some proteins, high temperatures usually lead to irreversible denaturation because of aggregation. Examples include the concomitant unfolding and aggregation of recombinant human Flt2 ligand (32), streptokinase (33), recombinant human keratinocyte growth factor (34,35), recombinant consensus interferon (36), rhIFN-γ (37), and ribonuclease A (38,39).

Typically, high temperatures perturb the native protein conformation to a sufficient degree to promote aggregation (13). Importantly, it is usually observed during heating that aggregation starts at temperatures well below the equilibrium melting temperature of the protein (11). This observation suggests that aggregates are not formed from fully unfolded molecules. Rather, as discussed in more detail below, it appears that partially unfolded protein molecules are the reactive species that form aggregates.

Temperature also strongly affects reaction kinetics because rate constants increase exponentially with temperature for activated reactions. Increasing temperature increases the thermal kinetic energy of reactants. As a result, reactant collision frequency, as well as the probability of collisions with enough energy to overcome activation energies, increases with increasing temperature (40). For diffusion-controlled reactions, increasing temperature increases the rate of diffusion of reactant species, thus increasing the rate of reaction. Protein aggregation rates are similarly increased at high temperatures.

Solution pH

pH has a strong influence on aggregation rate. Proteins are often stable against aggregation over narrow pH ranges and may aggregate rapidly in solutions with pH outside these ranges. Examples include recombinant factor VIII SQ (41), low-molecular-weight urokinase (42), relaxin (43), rhGCSF (17,20), deoxyhemoglobin (44), interlukin-1β (45), ribonuclease A (46), and insulin (47).

Solution pH determines the type (positive or negative) and total charge on the protein, thereby affecting electrostatic interactions. There are two different ways in which electrostatic interactions can affect protein stability. First, classic electrostatic effects are the nonspecific repulsions that arise from charged groups on a protein when it is highly charged, for example, at pH far removed from the isoelectric point (pI) of the protein (21). As the number of charged groups on a protein is increased by increasing the acidity or basicity of the solution, increased charge repulsion within the protein destabilizes the folded protein conformation because the charge density on the folded protein is greater than on the unfolded protein. Thus, pH-induced unfolding leads to a state of lower electrostatic free energy (21). Second, specific charge interactions, such as salt bridges (or ion pairing), can also affect protein conformational stability. In contrast to the nonspecific electrostatic effect, where increasing charges destabilize the folded state, salt bridges stabilize it (21,48,49).

In addition to their effects on protein conformation, charges on protein molecules also give rise to electrostatic interactions between protein molecules. When proteins are highly charged, repulsive interactions between proteins stabilize protein solution colloidally, making assembly processes such as aggregation energetically unfavorable (20,26,37,38). When proteins possess both positively and negatively charged groups (e.g., at pH values close to the pI), anisotropic charge distribution on the protein surface could give rise to dipoles. In such cases, protein–protein interactions could be highly attractive, making assembly processes such as aggregation energetically favorable (20,50).

Ligands and Cosolutes

The Wyman linkage function and related theories applied by Timasheff *et al.* [see, for example, Timasheff (51)] to protein conformational stability can be used to explain the effects of cosolutes, such as strong binding ligands, excipients, and salts, on protein physical stability. By the Wyman linkage function, differential binding of ligand in a two-state equilibrium will shift the equilibrium toward the state with the greater binding. Thus, for example, binding of polyanions to the native state of acidic fibroblast growth factor (52) or native recombinant keratinocyte growth factor (34) greatly shifts the equilibrium between the native and unfolded states to favor the native state. Likewise, binding of Zn^{2+} to human growth hormone increases the free energy of unfolding (53).

The Wyman linkage function can also be used to explain the effect of weakly interacting ligands (i.e., cosolutes) that affect protein conformational stability and equilibrium solubility at relatively high concentrations. It has been recognized for over a century that high concentrations ($\geq$1 M) of certain solutes (e.g., sugars, polyols, and certain salts, such as ammonium sulfate) stabilize the native state of proteins, whereas other solutes act as protein denaturants [e.g., urea and guanidine hydrochloride (GdnHCl)] (51,54,55). These observations can be explained by differences in binding of these weakly interacting solutes to native and unfolded states. Denaturants bind to the unfolded state to a higher degree than to the native state, thus favoring unfolding.

Protein stabilizers such as sucrose and glycerol are preferentially excluded from the surface of a protein molecule, and the degree of exclusion is proportional to its solvent-exposed surface area (51,56,57). These cosolutes are depleted in the domain of the protein, and as a result, water is enriched in that domain. Preferential exclusion can thus be interpreted as negative binding. During unfolding, protein surface area increases, leading to a greater degree of preferential exclusion (e.g., larger negative binding). The net effect of greater negative binding to the unfolded state is to favor the native state.

Another way to state the mechanism by which preferential exclusion stabilizes the native state is to consider that this interaction coincides with an increase in protein chemical potential. By LeChatelier's principle, the system will tend to minimize this unfavorable effect. Thus, protein states with reduced surface area that exhibit lower preferential exclusion are favored over more solvent-exposed states. As a result, the free energy of unfolding is increased in the presence of preferentially excluded solutes. In addition, assembly of monomers into native oligomers, which reduces the specific protein surface area exposed to solvent, is also favored. The same mechanism also explains the decrease in equilibrium solubility of proteins in the presence of preferentially excluded solutes, such as ammonium sulfate (58).

Ligands and cosolutes that alter protein conformational stability also influence the rate of formation of nonnative aggregates. For example, in the presence of polyanions, aggregation of acidic fibroblast growth factor (52) and native recombinant keratinocyte growth factor (34) is greatly inhibited. It has also been shown that the addition of weakly interacting preferentially excluded solutes can reduce the rate of protein aggregation. For example, sucrose has been shown to inhibit aggregation of hemoglobin (59), rhIFN-γ (60,61), keratinocyte growth factor (62), immunoglobulin light chains (15,19), and rhGCSF (17). In contrast, cosolutes (e.g., GdnHCl) that exhibit greater binding to the denatured state can accelerate aggregation (15,18,19,63). The mechanism for the effects of cosolutes on protein aggregation is discussed below.

Salt Type and Concentration

Electrolytes have complex effects on protein physical stability by modifying conformational stability, equilibrium solubility (e.g., salting-in and salting-out), and rate of formation of nonnative aggregates (38,64–67). For example, Yamasaki *et al.* found that bovine serum albumin could be stabilized against thermal unfolding by kosmotropic salts such as NaSCN and $NaClO_4$ and destabilized by chaotropic salts at high ionic strength (68). However, low concentrations of chaotropes (10–100 mM) stabilized bovine serum albumin (68). The equilibrium solubility of recombinant human tissue factor pathway inhibitor was decreased in the presence of NaCl (69). The rates of aggregation of recombinant factor VIII SQ (41) and recombinant keratinocyte growth factor (70) were decreased in the presence of NaCl. In contrast, NaCl increased the aggregation rate for rhGCSF (20).

Salts bind to proteins. Ions can interact with unpaired charged side chains on the protein surface. Binding of multivalent ions to these side chains can cross-link charged residues on the protein surface, leading to the stabilization of the protein native state (65). Because the peptide bond has a large dipole moment resulting from a partial positive charge on the amino group and partial negative charge on the carbonyl oxygen, ions can bind to peptide bonds (67), potentially destabilizing the native state. Consistent with the Wyman linkage theory described above, destabilization occurs if ions bind more strongly to nonnative than to native protein states (65).

Electrolytes modulate the strength of electrostatic interactions between the charged groups, both within the protein and between protein molecules. Thus, whereas intramolecular charge–charge interactions affect conformational stability, intermolecular electrostatic interactions affect equilibrium and rate of aggregate formation, as is described in more detail below.

At low concentrations, the predominant effect of ions in solution results from charge shielding, which reduces electrostatic interactions. However, at high concentrations of certain salts, in addition to charge-shielding effects, preferential binding of ions to the protein surface can result in a decrease in thermodynamic stability of the native conformation and an increase in equilibrium solubility (64). Other salts that are preferentially excluded from protein surface show stabilizing or salting-out effects (71).

The net effect of salt on protein stability is thus a balance of the multiple mechanisms by which salt interacts with protein molecules and by which salt affects protein–protein interactions. Because pH determines the type, total, and distribution of charges in a protein, salt-binding effects may be strongly pH dependent.

Preservatives

Antimicrobial preservatives, such as benzyl alcohol and phenol, are often needed in protein liquid formulations to ensure sterility during its shelf life. In particular, multidose

formulations of proteins require effective preservatives to prevent microbial growth after the first dose has been removed from a product vial. Preservatives are also required for certain drug delivery systems, e.g., injection pens that are used for multiple doses, minipumps that are used for continuous injection, and topical applications for wound healing. However, preservatives often induce aggregation of protein in aqueous solution. For example, preservatives (e.g., phenol, *m*-cresol, and benzyl alcohol) have been shown to induce aggregation of human growth hormone (16), recombinant interleukin-1 receptor (72), human insulin-like growth factor I (73), and rhIFN-γ (74).

The mechanism for preservative-induced protein aggregation is not well understood. However, it has been observed that addition of benzyl alcohol perturbed the tertiary structure of rhINF-γ without affecting its secondary structure, and the rate of rhINF-γ aggregation increased as the molar ratio of benzyl alcohol to protein increased (74). Also, preservatives reduced the apparent melting temperature of recombinant interleukin-1 receptor (72). These results suggest that preservatives bind to and populate unfolded protein states that are prone to aggregation. However, further research is needed to test this hypothesis and to determine rational strategies to inhibit preservative-induced protein aggregation.

Surfactants

Nonionic surfactants are often added to protein solutions to prevent aggregation and unwanted adsorption (e.g., to filter and container surfaces) during purification, filtration, transportation, freeze-drying, spray-drying, and storage. Surfactants are amphiphilic molecules that tend to orient so that the exposure of the hydrophobic portion to the aqueous solution is minimized. For example, surfactants adsorb at air/water interfaces, forming a surface layer of surfactant molecules oriented so that only their hydrophilic ends are exposed to water. Such orientation and surface adsorption can also occur at solid/water interfaces such as those found in vials, syringes, tubing, and other containers [for a review see Randolph *et al.* (10) and references therein]. Protein molecules are also surface active and adsorb at interfaces. Surface tension forces at interfaces perturb protein structure, often resulting in aggregation. Surfactants inhibit interface-induced aggregation by limiting the extent of protein adsorption (10,75).

As with other cosolutes, differential binding of surfactants to native and unfolded states of protein influences the protein's conformational stability. For some proteins, surfactants bind more strongly to the native state and increase the free energy of denaturation [e.g., human growth hormone (76)]. A more common effect is preferential binding of surfactants to the unfolded state, resulting in a decrease in the native protein state stability (10). Despite that surfactants often cause a reduction in thermodynamic stability of protein conformation, surfactants still can kinetically inhibit proteins aggregation at interfaces. In addition, surfactants have been shown to act as chemical chaperones, increasing rates of protein refolding and thus reducing aggregation (77). The readers are directed to the following reviews, and references therein, for further information: Randolph *et al.* (10), Jones *et al.* (75), and Jones (78).

MECHANISM OF PROTEIN AGGREGATION

Structural Transitions Accompanying Aggregation

Protein aggregation is accompanied by the loss of native protein structure. Such structural transitions have been well documented by Fourier transform infrared spectroscopy (FTIR) studies [for a review, see Dong *et al.* (11) and references therein] A common feature of protein aggregates — formed in response to thermal, chemical, or physical stresses, or even in the absence of any applied stress — is an increased level of nonnative intermolecular β-sheet structures (11). This structural transition occurs regardless of the initial secondary structural composition of the native protein (11) or the final morphology (amorphous or fibrillar) of the aggregates (14,15,17–19).

Characterization of the Aggregation-Competent Species

Based mostly on studies of thermally induced precipitation, research on protein aggregation first led to the proposal that protein aggregates form from the fully unfolded state [reviewed by Dong *et al.* (11)]. Subsequent research has led to the hypothesis that partially unfolded states aggregate (14,79–86). These partially unfolded states (also called molten globules or acid-denatured "A" states) generally adopt a collapsed conformation that is more compact than the unfolded state and has substantial secondary structure and little tertiary structure (79). They have large patches of contiguous surface hydrophobicity and are much more prone to aggregation than both native and completely unfolded conformations (14).

Recently, several studies have found that even under physiologic solution conditions that are not perturbing of protein tertiary structure and that thermodynamically greatly favor the native state, proteins can form aggregates and precipitate (17–19,87). The protein native conformation is flexible and does not exist as a discrete, single structure (12,63,88,89). Rather, at any instant in time, there exists an ensemble of native substates with a distribution of structural expansion and compaction. Kendrick *et al.* showed that the aggregation of rhIFN-γ proceeds through a transiently expanded conformational species within the native state ensemble (61). Compared to the most compact native species, the expanded species has a 9% increase in surface area (18,87). This conformational expansion is only about 30% of that required for the complete unfolding of rhIFN-γ (87). Furthermore, Webb *et al.* showed that the surface area increase to form the structurally expanded species that precedes rhIFN-γ aggregation is independent of GdnHCl concentration, pressure, or temperature, suggesting a common intermediate for aggregation under these various stresses (87).

Krishnan *et al.* recently showed that under physiologic conditions (neutral pH, 37°C, with no added denaturants), where the native state is greatly favored thermodynamically, rhGCSF aggregated readily (17). The surface area increase needed to form the expanded conformation leading to aggregation was only approximately 15% of that for unfolding (17).

Aggregation Models, Energetics, and Rates

In order to transform protein molecules from natively folded monomers (or higher-order native assemblies, e.g., native dimers) to structurally perturbed, higher-order aggre-

$$N \leftrightarrow TS^* \rightarrow A_I \quad (1a)$$

$$A_I + A_m \rightarrow A_{m+I} \quad (1b)$$

Scheme 1. Lumry-Eyring framework of protein aggregation.

gates, protein molecules in the native state need to undergo both structural changes and assembly processes. The aggregation pathways of many proteins have been analyzed in the well-known Lumry-Eyring framework (3,18,90,91). A representation of this framework, shown in Scheme 1, involves a reversible conformational change of a protein (Scheme 1a) followed by irreversible assembly of the nonnative species to form aggregates (Scheme 1b) (3,90,91).

In Scheme 1, N is the native protein, TS* represents the transition state preceding the formation of an aggregation intermediate A_I, and A_m and A_{m+I} are aggregates containing m and $m + I$ protein molecules, respectively.

It is generally known that the rate of a reaction is controlled by both thermodynamics and kinetics. The transition state theory used in the model depicted in Scheme 1 can be graphically represented on a reaction coordinate diagram as shown in Fig. 1. The free energies of reactant (N), transition state (TS*), and products (A_n and A_m) are shown on an arbitrary free energy y-axis. The x-axis represents the course of individual reaction events. A_m is expected to be favored thermodynamically and therefore has the lowest free energy. Each reaction proceeds through energy barriers (curved lines in Fig. 1), which represent energies of the different molecular configurations between reactants and products. The maximum energy configuration is the transition state, and the free energy difference between the transition state and reactant is called activation free energy ($\Delta G^{\ddagger}$). For a multiple-step reaction, such as protein aggregation, the step that has the highest $\Delta G^{\ddagger}$ is the rate-limiting step.

Scheme 1 describes a reversible reaction to form a transition state, followed by irreversible reactions. The reaction order for the rate-limiting step determines the apparent order of the aggregation reaction. A number of proteins have been found to follow first order aggregation kinetics (3,18), suggesting that the rate-limiting step is unimolecular (e.g., a conformational change) rather than a bimolecular reaction limited by collision frequency.

In contrast, the aggregation of rhGCSF in pH 7 phosphate buffer saline (PBS) follows a second-order reaction, suggesting that the rate-limiting step is biomolecular (17). Krishnan *et al.* proposed the mechanism in Scheme 2 for rhGCSF aggregation. Native rhGCSF (N) undergoes a bimolecular, second-order irreversible reaction ($2N \rightarrow A_2$) to form a dimeric aggregation-competent intermediate A_2. This step proceeds through the formation of a transition state N*, which is a transiently expanded conformational species within the native state ensemble (17). N* then irreversibly dimerizes to form A_2, and this step is rate limiting (17). A_2 then undergoes assembly reactions to form aggregates. N* could also react irreversibly with an existing aggregate A_n to form a larger aggregate A_{n+1}. The reaction coordinate diagram for Scheme 2 is shown in Fig. 2. Also shown in Fig. 2 is the unfolded state (U), which is thermodynamically unstable with respect to the native state by 9.5 kcal/mole (17,20).

$$2N \leftrightarrow 2N^* \rightarrow A_2 \quad (2a)$$

$$N^* + A_n \rightarrow A_{n+1} \quad (2b)$$

$$A_2 + A_m \rightarrow A_{m+2} \quad (2c)$$

Scheme 2. rhGCSF aggregation mechanism with N* as the transition state species (17).

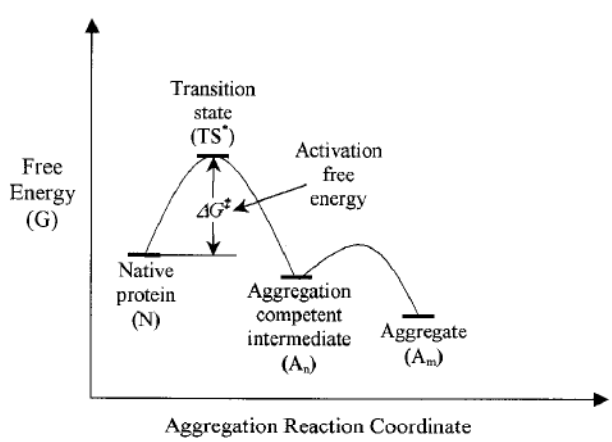

Fig. 1. Schematic reaction coordinate diagram of a protein aggregation (Scheme 1) on an arbitrary free energy y-axis. Curved lines illustrate kinetic energy barriers.

Role of Conformational Stability

It is apparent that the intrinsic conformational stability of the protein native state plays an important role in aggregation. First, aggregation is accompanied by the loss of native protein structures. Second, partially unfolded protein molecules are especially prone to aggregation. Third, the aggregation transition state of some proteins has been identified as a structurally expanded species within the protein native state ensemble (17,18). Hence, aggregation is governed by the conformational stability of the protein native state relative to that of the aggregation transition state.

Kendrick *et al.* showed that the addition of a thermodynamic stabilizer (e.g., sucrose) that increased ΔG_{unf} of

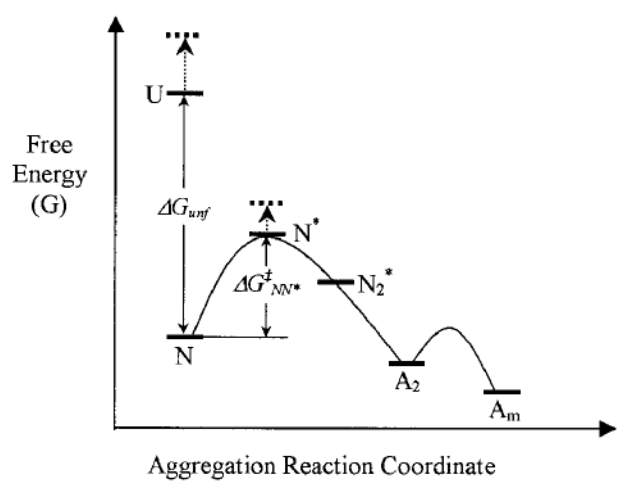

Fig. 2. Schematic reaction coordinate diagram of rhGCSF aggregation in pH 7 PBS. N* is the transitions state species, and $\Delta G^{\ddagger}_{NN^*}$ is the activation free energy of aggregation. A_2 is the dimeric aggregation intermediate. Dotted arrows illustrate, relative to protein native state (N), shifts in the free energies of unfolded state (U) and N* when sucrose is added (20).

rhIFN-γ decreased its aggregation rate (Fig. 3a). The same trend was observed for the aggregation of rhGCSF (Fig. 3b) (17). As discussed earlier, stabilizers such as sucrose increase protein thermodynamic stability because they are preferentially excluded from the surface of protein molecules (92). By the Wyman linkage theory, the addition of sucrose thus drives reactions toward the least solvent-exposed states. The aggregation-prone partially folded states and structurally expanded transition states both have greater surface area than the most compact native state and are therefore expected to be disfavored energetically by the addition of sucrose. Sucrose thus shifts the molecular population toward the most compact species within the native state ensemble. This is verified experimentally by the observation that sucrose greatly reduces the rate of hydrogen-deuterium (H-D) exchange within proteins (17–19,63). A reduction in H-D exchange indicates that the time-averaged conformation of the protein in the presence of sucrose was more compact than in the absence of this stabilizing solute (17–19,63).

The effects of sucrose on ΔG_{unf} and $\Delta G^{\ddagger}$ (which controls the rate of aggregation) are illustrated by dashed arrows in Figs. 2 and 4. The addition of sucrose increases the free energies, relative to N, of U and N* (upward dashed arrows in Figs. 2 and 4). Increasing the free energy of N* relative to N results in a larger $\Delta G^{\ddagger}$, thus shifting the equilibrium between

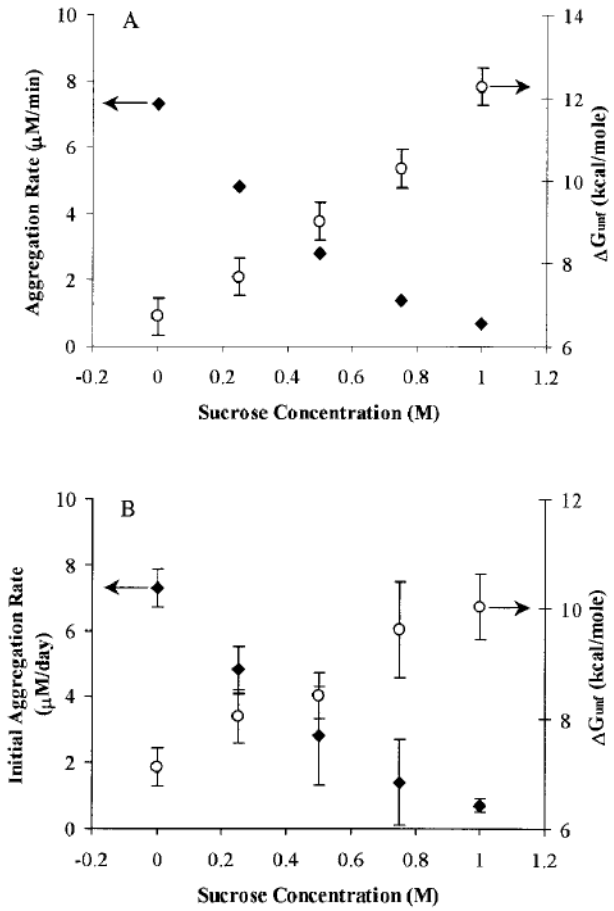

Fig. 3. The effect of conformational stability on the aggregation rates of (A) rhIFN-γ (18,87) and (B) rhGCSF (17). Increasing the free energy of unfolding (ΔG_{unf}) by the addition of sucrose decreased protein aggregation rates.

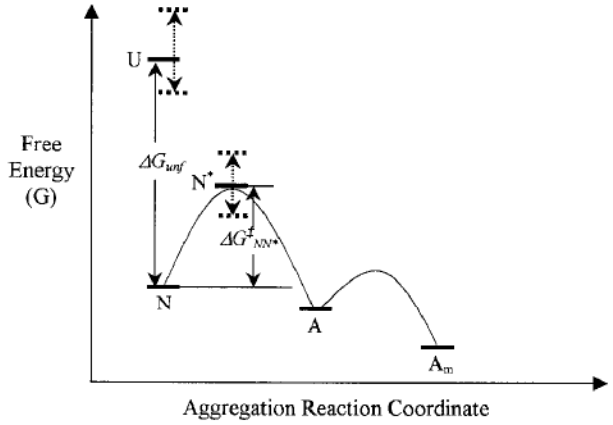

Fig. 4. Schematic reaction energy profile of rhIFN-γ aggregation. A is the monomer aggregation intermediate. Dotted upward and downward arrows illustrate, relative to the protein native state (N), shifts in the free energy of the aggregation transition state (N*) when sucrose or GdnHCl is added, respectively.

N and N* toward N. As a result, the rates of rhIFN-γ and rhGCSF aggregation decrease with the addition of sucrose.

The opposite effect, also illustrated in Fig. 4, of increasing aggregation rate by the addition of chaotropes (e.g., urea and GdnHCl) that destabilize the conformational stability of the native protein, has also been observed (18,19,61). Kim *et al.* found that urea, which decreased the ΔG_{unf} of immunoglobulin light chains (and presumably reduced $\Delta G^{\ddagger}$), enhanced their fibril formation by both reducing nucleation lag time and increasing fibril growth rate (15). Furthermore, the destabilizing effect of urea could be counteracted by the addition of preferentially excluded cosolutes sorbitol and betaine (15).

Cosolutes that increase the stability of the native conformation have been demonstrated to be effective at reducing protein aggregation. Is conformational stability the only factor controlling the rate of protein aggregation? When denatured by high temperature, ribonuclease A aggregated readily at pH 7.8 at temperatures both above and below the apparent melting temperature, where the protein was mostly unfolded and folded, respectively (38,39). In contrast, ribonuclease A did not aggregate at pH 3 even when exposed to 75°C for 24 h (38,39). The same effect of pH was observed during thermal scanning of rhGCSF. The protein aggregated rapidly at pH 7 and 6.1, but no aggregation was observed at pH 3.5 (20). We discussed earlier that even minor perturbations to the native protein structure can lead to rapid aggregation. However, even when the native conformation of ribonuclease A and rhGCSF became significantly perturbed at high temperatures, these proteins did not aggregate at acidic pH. Thus, protein conformation alone cannot explain the aggregation behaviors of ribonuclease A and rhGCSF observed at different pH during thermally induced denaturation.

Furthermore, as shown in Fig. 3b, ΔG_{unf} values of rhGCSF in pH 7 PBS correlated well with its initial aggregation rate during incubation at 37°C. However, at different solution pHs, very different aggregation behaviors were observed even when the ΔG_{unf} values were comparable (20). ΔG_{unf} values measured for rhGCSF in several different solutions, pH 3.5 HCl, pH 6.1 PBS, pH 7 PBS, were 11.3 ± 0.7, 11.4 ± 0.6, and 9.5 ± 0.5 kcal/mole, respectively (20). Aggre-

gation occurred in the pH 6.1 and 7 solutions but did not occur in the pH 3.5 solution (Fig. 5) (20). In addition, aggregation was observed in solution at pH 3.5 in the presence of 150 mM NaCl, although the change in ΔG_{unf} caused by addition of NaCl was statistically insignificant (20). Thus, rhGCSF aggregation behavior in different solutions cannot be explained by its conformational stability alone.

Role of Colloidal Stability

In addition to the structural changes that occur during aggregation, protein molecules also assemble to form higher-order aggregates. Molecular assembly processes occur as a result of attractive intermolecular interactions. Thus, an understanding of protein aggregation also requires information about the nature and magnitude of these interactions. The osmotic second virial coefficient (B_{22}) is a thermodynamic solution parameter that directly quantifies overall protein–protein interactions on the molecular level, which include hard-sphere, electrostatic, van der Waals', and all other short-range interactions. Positive B_{22} values indicate the overall dominance of repulsive forces between protein molecules, where protein–solvent interactions are favored over protein–protein interactions (93) (i.e., proteins are colloidally stable). Negative B_{22} values reflect overall attractive forces between proteins, with protein–protein interactions being favored over protein—solvent interactions (i.e., proteins are colloidally unstable). B_{22} is fundamentally linked to protein phase behavior (94–96) and solubility (97–100), and B_{22} measurements have been used to characterize and predict solution conditions for protein assembly into crystals and for salting out (93,95,98,99,101,102).

The onset of native protein crystallization or precipitation and the morphology of the solid phases formed are predominantly determined by the mechanisms of molecular approach, reorientation, and incorporation of native proteins, which are governed by the strength and range of protein colloidal interactions (103). Assembly of protein molecules into nonnative aggregates by definition involves the formation of higher-molecular-weight assemblies from initial lower-molecular-weight species. Thus, the same intermolecular interactions that govern protein crystallization and salting out are also expected to be important in the formation of nonnative protein aggregates.

Two major contributions to interactions between protein molecules in aqueous solutions are Coulombic electrostatic interactions and van der Waals' interactions (Fig. 6A). Electrostatic interactions between isocharged surfaces are always repulsive and are described by double-layer interactions whose range and strength are modulated by electrolyte concentration (26). As illustrated in Fig. 6A, when two isocharged particles such as protein molecules approach each other (e.g., starting from a particle surface separation distance marked as point ***a*** in Fig. 6A), they need to overcome an energy barrier, ΔW_1 (located at a separation distance marked as position ***b***) to come into physical contact. At distances less than ***b***, molecules experience attractive forces, resulting in coagulation. When ΔW_1 is high, particles remain kinetically stable as dispersed particles (Fig. 6B, case *i*). When ΔW_1 is small (Fig. 6B, case *ii*) or negative (Fig. 6B, case *iii*), particles become colloidally unstable, and coagulation occurs. Energetics of particle assembly processes are thus controlled by the interaction energy (or ΔW_1) between them.

Assembly processes in protein aggregation pathways discussed so far include Scheme 1b and Schemes 2b and 2c. These are the reactions that transform lower-molecular-weight proteins into higher-order aggregates. A more subtle assembly reaction is the formation of the dimeric intermediate A_2 (Scheme 2a) from N in rhGCSF aggregation. Although in pH 7 PBS, N* is the transition state for the reaction $2N \rightarrow A_2$ (Fig. 2), the reaction also must proceed through a dimeric molecular configuration, N_2*, to form A_2 (Fig. 2). A + pH 3.5, where rhGCSF is highly charged, electrostatic repulsion is stronger than at pH 7, near the isoelectric point. This causes the energy barrier for collisions between protein molecules (ΔW_1) to increase. If ΔW_1 is sufficiently high, assembly reac-

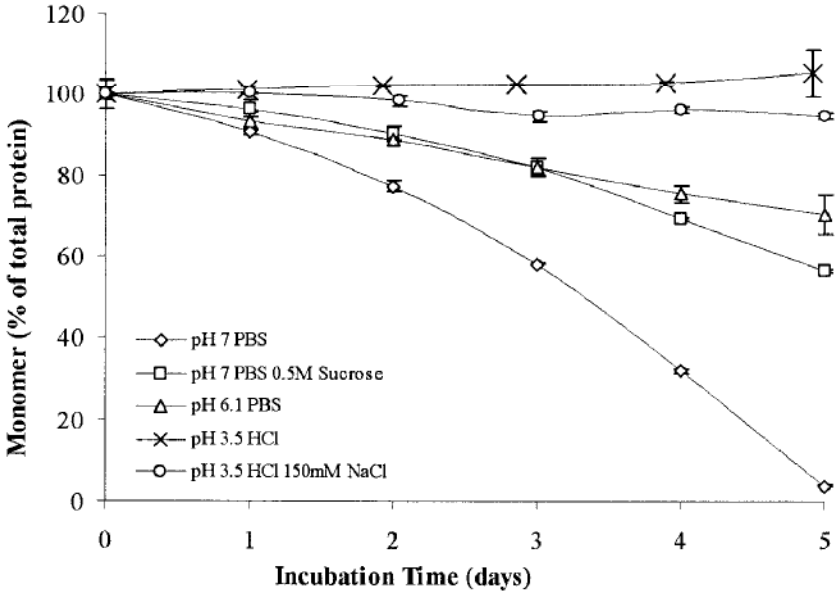

Fig. 5. Aggregation profiles of rhGCSF in different solution conditions during 5 days of incubation at 37°C (17,20).

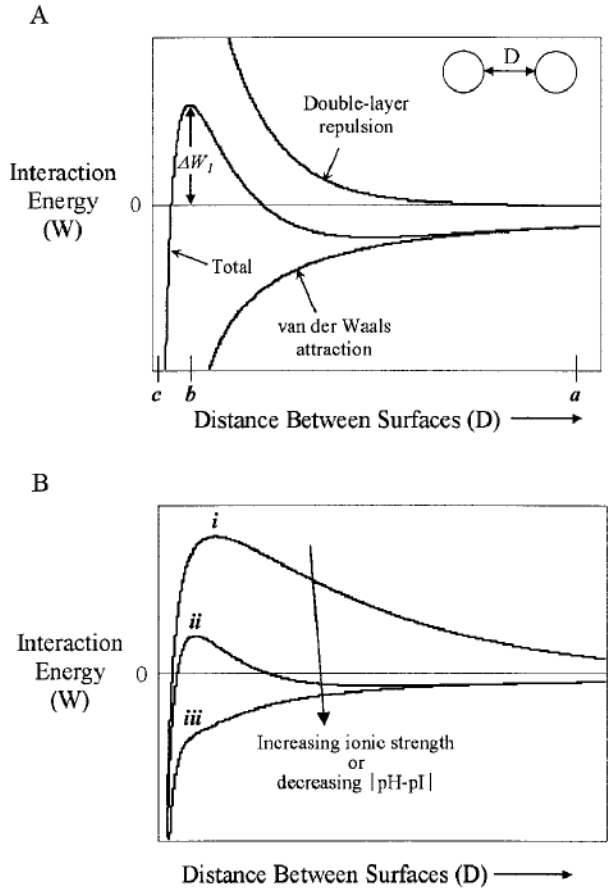

Fig. 6. Schematic interaction energy of two spherical particles interacting at constant and uniform surface potential. A, Total interaction energy is the sum of electric double-layer repulsion and van der Waals' attraction (26). ΔW_1 represents the maximum interaction energy barrier. B, Increasing salt concentration screens double-layer repulsion, resulting in a decrease of ΔW_1. Decreases in ΔW_1 could also result from a decrease in the absolute value of the difference between solution pH and the isoelectric point (|pH – pI|) of a protein.

tion becomes the rate-limiting step, and N_2* is the transition state (20) (Fig. 7).

B_{22} values reflect similar information. As shown in Fig. 8, B_{22} values of rhGCSF in pH 7 and 6.1 PBS are negative, indicating that protein–protein interactions are attractive (Fig. 6B, case *iii*) (20). As pH is lowered to 3.5, B_{22} value became large and positive, indicating that the overall interactions between rhGCSF molecules changed from attractive to highly repulsive (Fig. 6B, case *i*) such that no aggregation occurred (20). Solution pH thus changed rhGCSF colloidal stability, from being colloidally unstable at pH 7 and 6.1 to colloidally stable at pH 3.5. This dominant role of colloidal interaction also explains the observation that even when the native state became significantly unfolded during thermal unfolding at pH 3.5, aggregation still did not occur (20).

At pH 3.5, rhGCSF aggregation could be induced by the addition of 150 mM NaCl (Fig. 5) whereas ΔG_{unf} remained relatively unchanged by the addition of NaCl (20). The effect of ionic strength on B_{22} values measured at pH 3.5 is shown in Fig. 9. Salt screens repulsive electrostatic interactions, reducing ΔW_1 (or $\Delta G^{\ddagger}_{NN_2*}$) sufficiently so that aggregation occurred (Fig. 6B and Fig. 8, case *iii*). Under these conditions,

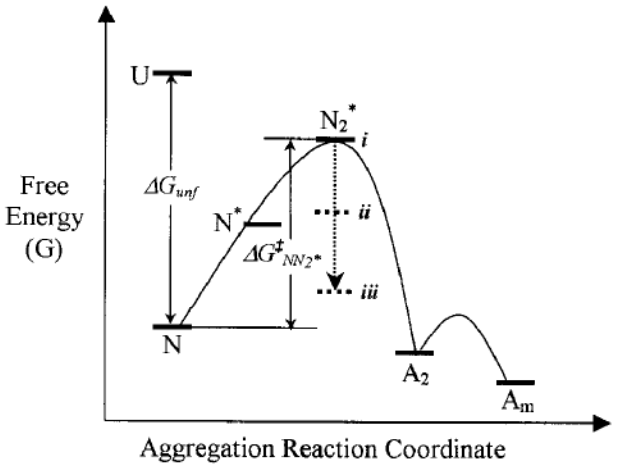

Fig. 7. Schematic reaction energy profile of rhGCSF in pH 3.5 HCl. N_2* is the transition state species, and $\Delta G^{\ddagger}_{NN_2*}$ is the activation free energy of aggregation. Dotted arrows indicate that increases in solution ionic strength (or decrease in |pH – pI|) decrease $\Delta G^{\ddagger}_{NN_2*}$. At low ionic strength (*i*), ΔW_1 is large and positive, resulting in a high $\Delta G^{\ddagger}_{NN_2*}$. Increasing ionic strength sufficiently led to a negative ΔW_1 (*iii*), lowering $\Delta G^{\ddagger}_{NN_2*}$ enough that N_2* is no longer the transition state of the aggregation reactions. At high ionic strength, N* is expected to be the transition state of aggregation (20).

assembly reaction is no longer rate limiting; the transition state is N* rather than N_2*. Thus, at pH 3.5 and high ionic strength, conformational stability of the native state again becomes the dominant factor governing rate of rhGCSF aggregation (20).

Nucleation-Dependent Aggregation

Thus far, we have discussed in detail the mechanism and driving forces that are important during the initial stages of aggregation. Following the onset of aggregation, both the number and size of aggregates increase while the native protein population is depleted. Aggregates grow and eventually precipitate out of solution and form visible particles. Aggregation can thus often be detected visually. However, the aggregation of some proteins exhibits a distinct lag phase during which the protein solution remains clear and the loss of native

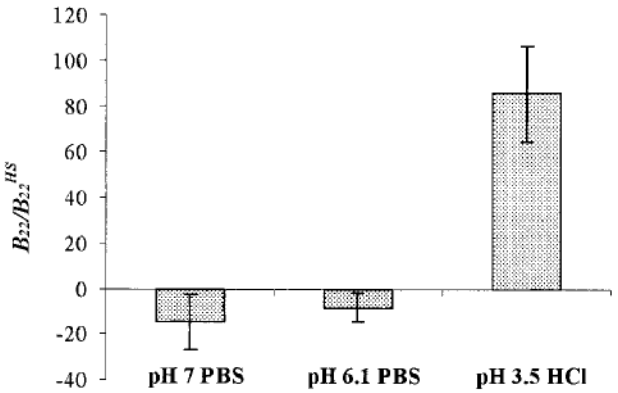

Fig. 8. Osmotic second virial coefficient, normalized to hard sphere contribution, of rhGCSF in different pH solution conditions. Protein–protein interactions changed from strongly repulsive at pH 3.5 to increasingly attractive at pH 6.1 and 7. Error bars are standard errors from the linear regression of light-scattering data (20).

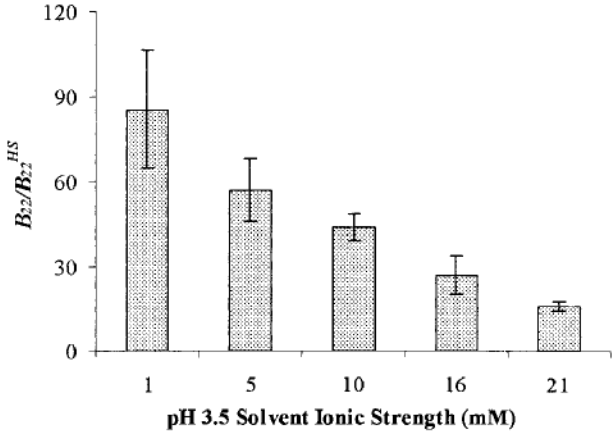

Fig. 9. Osmotic second virial coefficient, normalized to hard sphere contribution, of rhGCSF in pH 3.5 solutions with different ionic strength. Protein–protein charge repulsion becomes increasingly shielded as the ionic strength of the solution is increased. Error bars are standard errors from the linear regression of light-scattering data (20).

protein often is nearly undetectable, followed by rapid, often complete, native protein loss. For example, when α-synuclein was incubated at 37°C, insoluble aggregates were not detected during the first 15 days (17). This lag phase was followed by a rapid loss of monomeric α-synuclein, where after 30 days, essentially all native α-synuclein were lost to aggregation (104). This type of nucleation-dependent aggregation behavior has been observed for a number of other proteins, sulch as β-lactoglobulin (105), immunoglobulin light chains (15), prion protein (PrP96-111M) (106), and the SH3 domain of the α-subunit of bovine phosphatidylinositol-3′-kinase (107). Furthermore, seeding (e.g., with preformed aggregates) has been found to dramatically reduce lag time and promote aggregation (105,106,108).

Protein aggregation is inherently a nucleation and growth phenomenon where aggregates accumulate, eventually exceeding their solubility and precipitate. The existence of a lag phase in the aggregation of some proteins is caused an energy barrier to assembly (or nucleation). The energy barrier results from the free energy required to create a new solid-liquid interface and depends on aggregate size (109,110). When the size of the new phase is above a critical size where energy barrier is highest, then growth of the nucleus occurs. Nucleation-dependent aggregation behavior is a result of a rate-limiting nucleation step. Furthermore, the barrier to assembly may be orientationally specific. If there is an orientation with a lower free energy to assemble, then growth will occur preferentially in that orientation, resulting in ordered aggregate morphology. For example, fibrils are often associated with nucleation-dependent assembly (105,106,108,111).

For some protein systems undergoing nucleation-dependent assembly, the rate-limiting step is the formation of a prenucleus species (15,104). For example, with α-synuclein the prenucleus was a ditryrosine crosslinked dimer that formed due to protein oxidation (104). When the dimer concentration reached a critical level of about 1–2% of the total protein population, nucleation occurred rapidly. Seeding a sample that contained no dimers with preformed dimers rapidly induce fibril formation, whereas inhibition of dimer formation with methionine (which servers as a radical scavenger) prevented fibril formation.

Similar pathways for therapeutic proteins probably are important for their physical degradation, although there are no published examples in the literature. In these cases, soluble aggregates (which can be noncovalently or covalently crosslinked) can form, for example, during long-term storage in an aqueous formulation, without any assembly into higher order aggregates or precipitates. However, if the soluble aggregate is a prenucleus species and it reaches a critical level (e.g., 1%), it can foster rapid assembly into large aggregates. Thus, a product that appears to have acceptable physical stability for several months, could in the space of a few weeks (or even days) suddenly have unacceptable levels of aggregated protein. Similarly, if the bulk drug product already has soluble aggregates present, the long-term stability of the final formulated product may be compromised.

Thus, it is important for pharmaceutical scientists to pay close attention to the levels of soluble aggregates present in both bulk drug product, as well as the final formulation. For a given therapeutic protein product, it may become essential to have processing steps to reduce the levels of soluble aggregates, in order to obtain acceptable long-term stability in the final formulation.

CONCLUSIONS

Protein aggregation is problematic both *in vivo* and *in vitro*. Because of the low thermodynamic stability of the native protein structure, aggregation often occurs, even under solution conditions where the native protein is both conformationally stable and at concentrations well below its equilibrium solubility. Non-native aggregation of a protein involves at least two steps – conformational changes to the protein native state and assembly of protein molecules into higher order aggregates, and their energetics are controlled by conformational stability, expressed as ΔG_{unf}, and colloidal stability, reflected in the values of B_{22}, respectively. Under solution conditions where conformational stability dominates (i.e., large ΔG_{unf} and negative B_{22}), the first step is rate-limiting. Increasing ΔG_{unf} (e.g., by the addition of sucrose) is effective at decreasing aggregation. In solutions where colloidal stability is high (i.e., large and positive B_{22} values), assembly step is rate-limiting. Solution conditions (e.g., pH and ionic strength) that increases B_{22} are effective at reducing aggregation.

Protein aggregation is therefore controlled by both conformational stability and colloidal stability, and, depending on the solution conditions, either could be rate limiting. To successfully stabilize protein against aggregation, solution conditions need to be chosen not only to stabilize the protein native conformation but also to stabilize protein against attractive intermolecular forces. During development of formulations for therapeutic proteins, the latter goal is often achieved empirically during preformulation studies, where ionic strength, pH, and buffer type are optimized to minimize precipitation and other adverse events (e.g., deamidation). Manipulation of solution conditions during preformulation studies so as to maximize B_{22} may be useful in development of formulations exhibiting long-term storage stability.

ACKNOWLEDGMENTS

We gratefully acknowledge support from the National Science Foundation Grant BES-0138595 and Amgen, Inc. E.Y.C. was supported by graduate fellowships from the National Science Foundation and U.S. Department of Education's Graduate Assistantships in Areas of National Need (GAANN).

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