Effect of Additives on Protein Aggregation

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Abstract: This paper overviews solution additives that affect protein stability and aggregation during refolding, heating, and freezing processes. Solution additives are mainly grouped into two classes, i.e., protein denaturants and stabilizers. The former includes guanidine, urea, strong ionic detergents, and certain chaotropic salts; the latter includes certain amino acids, sugars, polyhydric alcohols, osmolytes, and kosmotropic salts. However, there are solution additives that are not unambiguously placed into these two classes, including arginine, certain divalent cation salts (e.g., $MgCl_2$) and certain polyhydric alcohols (e.g., ethylene glycol). Certain non-ionic or non-detergent surfactants, ionic liquids, amino acid derivatives, polyamines, and certain amphiphilic polymers may belong to this class. They have marginal effects on protein structure and stability, but are able to disrupt protein interactions. Information on additives that do not catalyze chemical reactions nor affect protein functions helps us to design protein solutions for increased stability or reduced aggregation.

Keywords: Protein aggregation, refolding, aggregation suppressor, low-molecular-weight additive, freeze-drying, freeze-thawing, preferential interaction, preferential exclusion.

1. INTRODUCTION

Proteins are most soluble in the native folded state in aqueous solution. When the structure deviates from the native state, proteins tend to aggregate. Such deviation can occur when proteins are subjected to environmental stresses, as the native structure is marginally stable [1-5]. Temperature increase or decrease (e.g., freezing), mechanical stresses such as shear strain, surface adsorption or foaming, pH shift and high protein concentration, all can cause structural changes [6, 7]. Such marginal structure stability also causes problems for attaining high yield in protein refolding. During refolding, the proteins are not promptly converted to the native structure and instead assume a partially folded state for a prolonged period [8, 9]. Thus, it is apparent that either protein structure must be stabilized to minimize such deviation or the structures that are not in the native state must be kept soluble. There are numerous solution additives that have been proved useful for this goal. They are mainly grouped into two classes, protein stabilizer and denaturants. However, there are some unique compounds, which do not belong in either of these classes. They affect little protein structure and stability, but appear capable of suppressing protein-protein interactions or disrupt such interactions. This chapter reviews these solution additives.

2. SOLUTION ADDITIVES

Solution additives have been developed to control protein folding, stability and aggregation. Some additives stabilize native fold, while the others destabilize or denature the protein structure. Others affect little protein structure and stability, but effectively suppress aggregation. This section describes additives that are widely used to prevent protein misfolding and aggregation.

Denaturant

Urea and guanidine (Gdn) are the most commonly used protein denaturants. High concentration of denaturants unfolds almost all proteins. Since Anfinsen's demonstration of spontaneous *in vitro* folding of ribonuclease A [10], the denaturing property of Gdn and urea has been a valuable tool for thermodynamic and kinetic analyses of protein folding and more importantly for recombinant protein production. These denaturants exert their effects by complete disruption of both intra- and inter-molecular interactions, leading to denaturation (unfolding) and solubilization. Orsini and Goldberg [11] described the first report on aggregation suppression by protein denaturants during refolding process [11]. The proteins that are difficult to refold have been successfully refolded in the presence of non-denaturing concentrations of denaturant [12, 13]. The positive effect of Gdn to suppress aggregation during refolding is caused by solubilization of hydrophobic moieties that are exposed to the solvent in the folding intermediates or misfolded species [13]. Although the details of interaction between protein and denaturants are not fully understood, extensive studies have been conducted. Molecular dynamics simulation has shown that electrostatic interaction between Gdn and both the charged residues and the peptide backbone is the dominant force, by which proteins are destabilized in Gdn solution [14]. Thermodynamic measurements suggested that Gdn interacts with the aromatic side chains, the peptide backbone and the negatively charged side chains, resulting in stabilization of the colloidal dispersion state of aggregation-prone species or folding intermediates and hence prevention of aggregation [15-19]. The interaction between Gdn and aromatic groups may be due to the planar stacking with cation-π interactions [19, 20].

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1389-2010/09 $55.00+.00

Detergent

Sodium dodecyl sulfate (SDS) denatures protein structure by binding to hydrophobic regions of the polypeptide. SDS is an anionic detergent that imparts a negative charge to all proteins. SDS strongly binds to the polypeptide at a ratio of approximately 1.4 g SDS per 1.0 g protein, as elegantly demonstrated by Takagi's group [21]. They proposed that SDS micelle binds to the polypeptide chain as a necklace-like shape (Fig. **1**), leading to an elongated structure with varying amounts of secondary structures depending on proteins [22-24]. Accordingly, SDS above critical micelle concentration (CMC) can fully denature the protein structure. When SDS is used for solubilization and unfolding, refolding is done by lowering SDS concentration below CMC. However, SDS monomer can bind to the protein so tightly that dilution or dialysis does not completely dissociate SDS molecules. Hydrophobic cyclodextrin (CD) or cycloamylose is used to absorb SDS molecules (see Fig. **1**). A combination of solubilizing detergent and hydrophobic cyclic sugars that strip off the detergent is called an "artificial chaperone". A large number of artificial chaperone-assisted refolding has been examined: carbonic anhydrase B [25-28], lysozyme [26, 28, 29], α-amylase [30], and citrate synthase [26, 28, 31]. The artificial chaperones prevent protein aggregation by forming protein-detergent complexes, in which the detergent shields the hydrophobic regions of the unfolded polypeptide, and the detergent is stripped off from the protein by the addition of the stripping agent (e.g., CD). However, the method is not versatile due to the complicated process and toxicity of the detergent.

Ionic Liquid and Non-Detergent Surfactants

Recently, ionic liquids, i.e., organic salts with melting point at room temperature have been reported as a refolding additive. Ethylammonium nitrate (EAN) at concentrations up to 0.5 M effectively suppressed aggregation during oxidative refolding of hen egg white lysozyme [32]. Ionic liquid-rich solvents were used for a reversible thermal unfolding/refolding reaction and a long term storage stabilization against aggregation and hydrolysis of lysozyme [33].

Non-detergent surfactant is different from strong detergents, such as SDS, which bind to protein too tightly to be removed from the protein surface, although strong detergents are excellent protein solubilizing agents as described above. Non-detergent sulfobetaines (NDSBs) are more favorable for aggregation suppression than the detergents. NDSBs have a short hydrophobic group and a hydrophilic sulfobetaine head group, which is a zwitterion over a wide pH range. The hydrophobic group in NDSBs is, however, too short to form micelles even at concentrations as high as 1 M; thus NDSBs do not behave like detergents. It is the first report that some NDSBs increase the refolding yield of *Escherichia coli* β-D-galactosidase [34]. NDSBs prevent protein aggregation by interacting with early folding intermediates [35]. Since these earlier studies, NDSBs have been used in the refolding of several proteins, such as *E. coli* tryptophan synthase, bovine serum albumin, a monoclonal antibody [36]. Rudolph *et al.* reported that *N*'-alkyl- and *N*'-(ω-hydroxyalkyl)-*N*-methylimidazolium chlorides improved the refolding of lysozyme and anti-oxazolone single-chain antibody fragment [37]. NDSBs have been used for refolding of chemically or thermally denatured proteins, because they can be easily removed by dialysis. They are useful additives for oxidative refolding and aggregation suppression due to their weak binding to the proteins.

Arginine

Arginine (Arg) as an aggregation suppressor during refolding was first reported in a patent application by Rudolph and Fischer [38]. Since then, Arg has been used for refolding of a variety of proteins, including human tissue type plasminogen activator (t-PA) [41], Fab antibody fragments [39-42], single-chain immunotoxins [41, 43], interleukin-6 receptor [44], interleukin-21 [45], human matrix metalloproteinase-7 [46], casein kinase II [47], and human neurotrophins [48, 49]. Stepwise reduction of denaturant concentration in combination with the addition of Arg is a most conventional method for protein refolding [40]. Arg is also a versatile additive for protein formulation and affinity column chromatography [50-53]. The molecular mechanism of Arg as a solution additive has been reported; Arg does not accelerate the refolding kinetics, but increases the solubility of aggregation-prone molecules [12, 54]; also chapter 4.2 and 4.3 in this issue. Although Arg contains a guanidino group, it does not destabilize the native structure of the proteins [14, 54-57]. Arg also suppresses heat-induced aggregation of proteins, including interleukine-6 and monoclonal antibody [58]. Among amino acids, Arg is the most effective suppressor for heat-induced aggregation of several proteins [56].

Amino Acid Derivative

Arginine ethylester (ArgEE) is a highly effective additive for preventing heat-induced aggregation of lysozyme [59]. Fig. (**2**) shows the effect of 100 mM ArgEE on heat-induced aggregation of lysozyme. It is evident that a much larger fraction of protein remains soluble after heating at 98 °C in

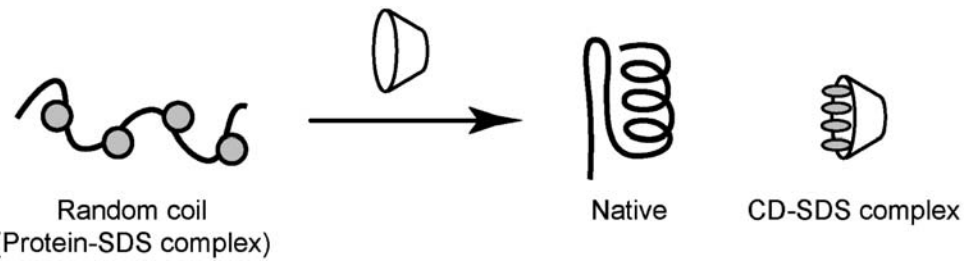

Fig. (1). Schematic presentation of protein refolding from protein-SDS complex using cyclodextrin (CD) as detergent-stripping agent.

the presence of ArgEE (open circle). It appears that ArgEE is stronger than Arg in aggregation suppression. Furthermore, amino acid alkylesters prevent heat-induced aggregation of lysozyme as effectively as ArgEE [60]. Although amino acid alkylesters are promising candidates for preventing heat-induced aggregation of lysozyme, these additives tend to be hydrolyzed to alcohols and amino acids at high temperature or alkaline pH. On the other hand, amidated amino acids are as effective as amino acid alkylesters for preventing heat-induced aggregation [61]. Argininamide appears to be the most prominent additive for oxidative refolding of lysozyme among the low molecular weight additives tested so far [62]. These amino acid derivatives have been used for crystallization of lysozyme to decrease the tendency to form amorphous aggregates [63, 64]. As these amino acid derivatives are moderately toxic and expensive for use in commercial bioprocesses, the more useful analogues should be explored.

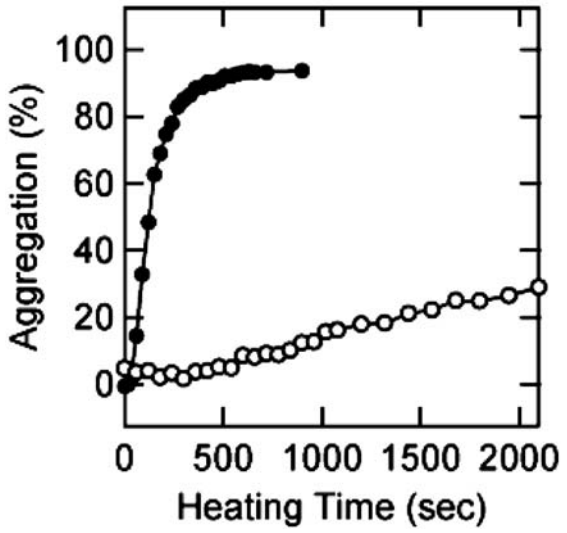

Fig. (2). Heat-induced aggregation of lysozyme in the presence of 100 mM additives. The samples containing 1.0 mg/ml lysozyme in the absence (closed circles) or presence (open circles) of ArgEE were heated at 98 ºC at various periods. The curves shown by the solid line were fitted to a single exponential equation.

Other Amino Acids

Proline (Pro) has been reported as an aggregation suppressor during refolding of bovine carbonic anhydrase [65], hen egg-white lysozyme [66], arginine kinase [67], creatine kinase [68], and aminoacylase [69]. Pro has an extremely high aqueous solubility (>7 M) at room temperature and is the most soluble of all the amino acids [70]. The unusual properties of Pro may be due to the inter-molecular self-association due to hydrophobic stacking in aqueous solution [71]. Such hydrophobic nature of proline shields hydrophobic region of the proteins, through which this amino acid effectively suppresses protein aggregation [72]. Histidine has also been shown to suppress heat-induced aggregation of interferon-tau [73], while imidazole enhanced refolding of chemically-denatured green fluorescent protein and suppressed its aggregation during heat-treatment [74]. β-Alanine suppressed heat-induced inactivation of lactate dehydrogenase [75]. Amino acids are favored for biotechnological applications because of their low cost and safety.

Polyamine

Putrescine, spermidine, and spermine are naturally occurring polyamines, which are present in almost all organisms [76]. Polyamines, specifically spermine and spermidine, slightly destabilize the native structure of hen-egg white lysozyme, but greatly increase the solubility of aggregation-prone compounds [77]. The addition of small amounts of polyamines (typically 0.1 M) prevents lysozyme from heat-induced aggregation more effectively than does Arg [77]. The essential structure of polyamines for their function as aggregation suppressor is due to the presence of multiple amines [78, 79]. Such an ability of amines to suppress aggregation can be seen even in ammonium salts, as ammonium sulfate and chloride prevent heat-induced aggregation of lysozyme more effectively than their corresponding sodium salts (see Fig. **3**) [79]. However, it has been reported that spermidine promotes aggregation during refolding of lysozyme [80] and amyloid formation of α-synuclein [81]. This implies that spermidine suppresses aggregation via hydrophobic interaction, but promotes formation of hydrogen bonds, which are responsible in part for lysozyme and α-synuclein aggregation.

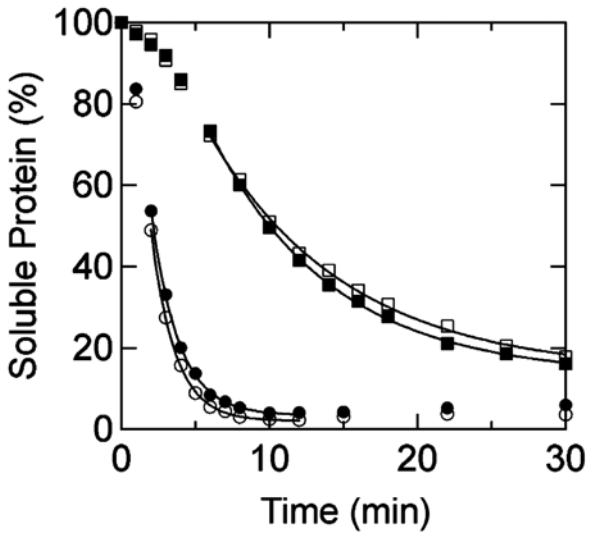

Fig. (3). Heat-induced aggregation of lysozyme in the presence of various salts. The samples containing 1.0 mg/ml lysozyme, 50 mM Na-phosphate (pH 7.4) and 600 mM salt were heated at 98 ºC for indicated periods. Closed circles, NaCl; open circles, Na_2SO_4; closed squares, NH_4Cl; open squares, $(NH_4)_2SO_4$. The continuous lines show the theoretical curves fitted to the data with single exponential equations.

Amphiphilic Polymer

Polyethylene glycol (PEG) is one of the most versatile water-soluble polymers for refolding of recombinant proteins [82] and stabilization of proteins by chemical modification (i.e., PEGylation) [83, 84]. PEG specifically binds to the first refolding intermediate of bovine carbonic anhydrase B to perturb the self-association of the aggregation-prone inter-

mediate [85, 86]. PEG has been successfully used for refolding of interferon [87] and xylanase [88]. Although PEG does not act as a denaturant, it slightly decreases the thermal stability of proteins [89]. Analysis of solvent-protein interactions indicates that PEG interacts favorably with the hydrophobic side chains exposed upon unfolding at elevated temperatures [89].

Polyvinylpyrrolidone (PVP), a similar amphiphilic polymer, has been applied for pharmaceutical use due to its low toxicity [90, 91]. Refolding of bovine carbonic anhydrase B in the presence of PVP showed that this polymer binds to the early molten-globule like refolding intermediate, leading to the protection of the exposed hydrophobic surface of the aggregation-prone intermediate and thereby an increase in the refolding rate [92]. The favorable refolding additives result from the highly polar amide group on PVP monomer, which confers to this polymer the hydrophilic properties and the hydrophobic nonpolar backbone and ring structures. Such properties of PVP can protect proteins from thermal aggregation of protein [93-96].

Osmolyte

Yancey *et al.* [97] have developed a concept of osmolyte, i.e., naturally organic osmotic solutes, found in bacteria, plants, and animals that can survive in salty environments. Although many types of osmolytes, such as carbohydrates, amine compounds, and amino acids, have been reported, trimethylamine N-oxide (TMAO) has the most interesting properties in terms of biotechnological applications. TMAO is a solute present at fairly high concentrations in the urea-rich cells, e.g., elasmobranchs and coelacanth, to offset denaturation action of urea [98, 99]. The ability of TMAO to fold protein is due to its solvophobic effect on the peptide backbone; i.e., TMAO disfavors contact with the backbone, forcing it to be shielded from solvent [100]. Osmolyte stabilizes the protein structure; it enhances the stability by preferential hydration mechanism as well as solvophobic effect [101]. Folding kinetics and thermodynamic analyses have been performed in the presence of TMAO with or without urea for lysozyme [101], FKBP12 [102], ribonuclease A [103], ribonuclease T1 [98,99], glycogen phosphorylase b [104], lactate dehydrogenase [105], chymotrypsin inhibitor 2 [106], and prion protein [107, 108]. Although osmolyte is thought to be compatible with cellular function and activity, some chemical activities have been reported, e.g., antioxidant property for polyols and taurine, redox balance for glycerol, detoxifying sulfide for hypotaurine, and stabilization of membranes under freezing temperatures for trehalose [109].

Salts

At low concentrations, salts can stabilize proteins through nonspecific electrostatic interactions, dependent only on the ionic strength of the medium [110]. At high concentrations, however, salts exert specific effects on proteins depending on the type and concentration of the salts, resulting in either the stabilization or destabilization of proteins, or even denaturation [111, 112]. Salts usually increase interfacial tension between the protein surface and bulk solvent, leading to an alteration of the protein solubility [113, 114]. Although the stabilizing effect of salt on protein structure is closely correlated with the salting-out effect described by Hofmeister, known as a Hofmeister series [115], there is controversy regarding the molecular nature of the origin of salt effects [116]. Ammonium sulfate is widely used as a precipitant for protein purification and for crystallization, as this salt can prevent amorphous aggregation. Ammonium salts have recently been reported to prevent heat-induced aggregation of lysozyme independent of the surface tension increment of the solution [79]. As shown in Fig. (**4**), the surface tension correlates with the aggregation rate for sodium or potassium salts, but not for ammonium salts, which showed little change in aggregation rate with increasing surface tension. As discussed above, polyamines effectively suppressed heat-induced aggregation; thus the amino group may play a key role in preventing heat-induced aggregation. It is worth noting that salts increase the solubility of aggregation-prone protein in the presence of organic solvent and amphiphilic polymer [117, 118].

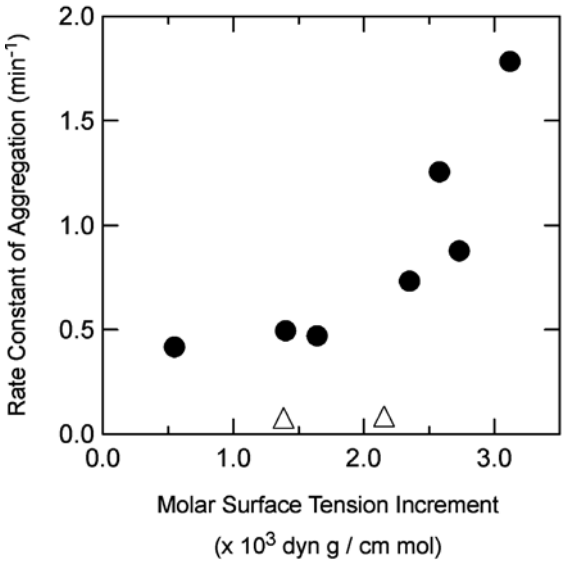

Fig. (4). Relationship between rate constant of aggregation and molar surface tension increment. Closed circles, sodium or potassium salts; open triangles, ammonium salts (NH_4Cl and $(NH_4)_2SO_4$).

3. BACKGROUND AND APPLICATION

This section introduces a theory of protein-solute interaction and briefly summarizes solution stresses that affect protein refolding, stability and aggregation during heating, freeze-drying, and freeze-thawing.

Theoretical Aspects of Solution Additives

We have summarized above a variety of solvent additives that affect protein stability and aggregation. They share a common property that all require high concentration in the range of 100 mM to 2 M, meaning that their affinity is weak. Any ligands can affect reversible reaction by binding to the protein [119-122]. Specific ligands, such as substrate and inhibitors, bind to the native, active structure with high affinity, resulting in protein stabilization. Solvent additives that require high concentration bind to proteins with low affinity.

For example, urea and Gdn binding occur in molar range, resulting in protein unfolding [123, 124]. A major difference between specific ligands and solvent additives is the mechanism via their effects on water molecules. Both specific ligands and solvent additives can interact with water. However, they must interact with a large number of water molecules to alter the free energy of protein molecules. This can only be achieved with sufficient number of solvent additives. For example, Mg ion can bind to proteins at high affinity and affect protein reactions [125-128]. Mg salts also have effects on protein stability and solubility at high concentration in the molar range [111, 112, 129]. A proposed mechanism of the latter effect is water binding of the salt [130]. Suppose each Mg ion binds 10 water molecules. Thus, 1 mM Mg can bind 10 mM water molecules, which account only for 0.018 % of total water (55.5 M). Namely, a large fraction of water molecules is free from the influence of salt ions. When Mg salts are present at 1 M, then they trap 10 M water molecules, which account for 18 %. It is evident that 1 mM Mg salt cannot affect protein reaction through its effect on water and a sufficient additive concentration is required based on this mechanism. This is consistent with the known fact that the additive effect increases with the concentration through this mechanism, i.e., via effects on water molecules.

How do the solvent additives then affect protein stability and aggregation via its effect on water? One such mechanism is water binding or cohesive force on water by the additives, which leads to increased surface tension as described in the previous section. Another mechanism is excluded volume, which leads to increased hydration of proteins [131]. Both mechanisms lead to deficiency of the additives at the protein surface. Regardless of the mechanism, such deficiency can be determined by dialysis equilibrium and is defined as preferential exclusion of the additives. Conversely, the excess amount of the additives at the protein surface is defined as preferential binding. These data have been compiled primarily by Timasheff's group. Preferential exclusion of additives is thermodynamically unfavorable, leading to increased free energy of the protein, which is reduced when protein self-associate (i.e., aggregation is enhanced as schematically depicted in Fig. **5**) or protein remains folded (i.e., enhanced protein stability also shown in Fig. **5**). On the other hand, preferentially bound additives can suppress aggregation.

Heating and Refolding Stresses

Aggregation can be triggered by exposing proteins to elevated temperatures in aqueous solution. High temperature treatment is indispensable for certain pharmaceutical products for virus inactivation [132, 133]. Upon heating, fluctuation of native structure ensemble increases. Kinetic analysis of the thermal aggregation of protein has been classically described by the Lumry-Eyring model [134],

$$U <=> A \qquad \text{Scheme 1}$$

$$A + A_m -> A_{m+1} \qquad \text{Scheme 2}$$

where U is the thermally unfolded state, A is a heat-denatured state and A_m and A_{m+1} are aggregates of heat-denatured state with the aggregate size of A_m and A_{m+1}. This is also depicted in Fig. (**5**). The reaction mechanism indicates that the rate of aggregation depends on both thermodynamic unfolding reaction and kinetics of protein-protein interaction and that the reaction order for the rate-limiting step determines the apparent order of the aggregation reaction. It is thus clear that there are two mechanisms for reducing aggregation of the proteins. The first is related to Scheme 1, in which the thermodynamic stability of the native state is increased as conferred by protein stabilizers (see Fig. **5**). The second is related to Scheme 2, in which intermolecular interactions are reduced, as conferred by aggregation suppressor (see Fig. **5**) [1]. It is interesting to note that a number of proteins showed to follow the first-order aggregation kinetics in the absence [135, 136] and presence of additives [59, 77].

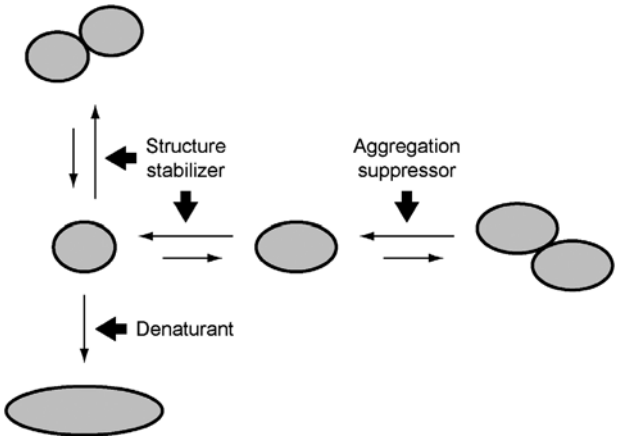

Fig. (5). Schematic presentation of the effects of additives on protein folding and aggregation.

On the other hand, correct refolding from a denaturant-induced unfolded state competes with higher order reaction of the intermolecular aggregation [137-139]. In fact, refolding-induced aggregates have a different structure from the heat-induced aggregates analyzed by FT-IR using lysozyme as a model (Fig. **6**) [80]. The refolding reaction is governed by two competing reactions, i.e., an intramolecular reaction (folding) and an intermolecular reaction (aggregation);

$$U -> N \qquad \text{Scheme 3}$$

$$U + U -> A_2 \qquad \text{Scheme 4}$$

where U, N, and A_2 represent the unfolded state, the native state, and the aggregated state, respectively [140]. This simple model suggests that a fine balance between refolding (Scheme 3) and aggregation (Scheme 4) determines the refolding yield. Folding and aggregation resemble each other as a condensation reaction, both being cooperative [140]. Folding is, however, a mono-molecular reaction, while aggregation is a multi-molecular reaction. During refolding reaction, hydrophobic interaction drives the unfolded protein to sequester their hydrophobic patches from contact with water when the denaturant concentration is reduced [141]. The competition between inter-molecular and intra-molecular interactions is responsible for the decreased yield of biologically active protein, in particular at high protein concentration.

Additives that prevent aggregation are conventionally used to assist refolding. Such additives include Gdn, urea,

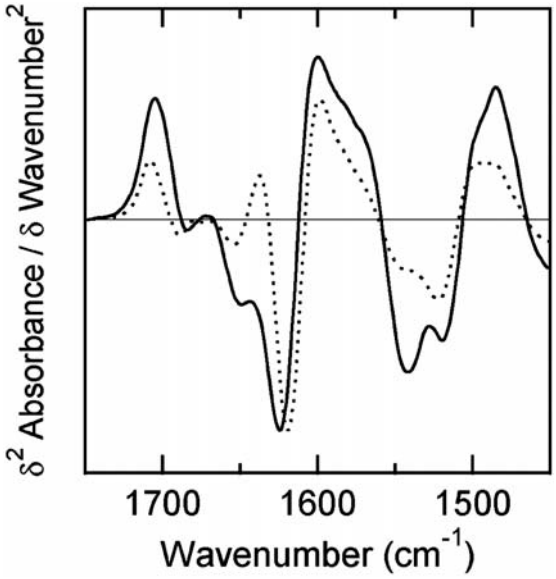

Fig. (6). FT-IR second-derivative spectra in amide I and amide II regions of aggregated lysozyme. Solid line, spectrum of heat-induced aggregates; dotted line, spectrum of refolding-induced aggregates.

Arg, and amphiphilic polymers. Although all these additives appear to bind to the proteins, their precise binding mechanism is not clear (reviewed in chapter 4.3). Nevertheless, they are effective in suppressing aggregation during protein refolding. Although these additives can reduce refolding rate by destabilizing the native state, their aggregation suppressive effect usually overwhelms the slow kinetics, resulting in higher refolding yield. Arg appears to be the most useful additive for mitigation of both refolding and heating stresses of protein in solution. The guanidino group on Arg may interact with the aromatic ring of unfolded polypeptide that favors suppression of aggregation [19, 142]. However, the mechanism of Arg effects on protein refolding remains controversial [19, 20, 54, 57, 62]. Detailed understanding on the arginine binding would lead to its broader and more systematic applications as a refolding additive. Conversely, although protein structure stabilizing additives may accelerate refolding kinetics, they can enhance aggregation, leading to a reduced refolding yield.

Freeze-Drying and Freeze-Thawing

Protein in solution is susceptible to degradation, such as aggregation, hydrolysis, deamination, oxidation, and Asp-Gly isomerization [6, 7]. Thus, lyophilization is widely used to enhance long term storage stability [143]. Although removal of water by freeze-drying or lyophilization therefore minimizes damages on proteins, the processes of freezing and drying themselves can lead to irreversible damages on labile proteins [144, 145]. Different stresses arise from freeze-thawing and freeze-drying of proteins [146, 147]. The most critical damages on proteins during freeze-thawing are the formation of ice crystal. This appears to enhance protein unfolding and hence the protein stabilizers, such as polyols and amino acids, are effective against freezing damages [148, 149]. The addition of trehalose, sucrose, and Pro increased the recovery of the enzymatic activity of phosphofructokinase from frozen state [150, 151]. PEG does not stabilize proteins from drying, but is one of the best cryoprotectants during freeze-thawing [152, 153]. The most critical damage during drying of frozen protein solution as well as air-drying is the removal of water from the protein surface, resulting in irreversible denaturation of protein [146, 147]. The dried protein, if not appropriately done, can result in aggregation or loss of biological function upon rehydration [153-156]. Although several protectants have been developed to maintain the protein function during freezing, only disaccharides protect protein from drying damages [97, 146, 147, 151, 156]. Several amino acids have been used as additives, such as glutamate and lysine, for freeze denaturation of lactate dehydrogenase [157], and lyophilization of tissue-type plasminogen activator stored at high temperature in solution state [158]. The interaction of Arg and multivalent counter ions increases the hydrogen bonding network for the frozen and freeze-dried states of proteins, which reduces the mobility of molecules and hence stabilizes the proteins [159].

ACKNOWLEDGMENT

This work was partly supported by Grant-in-Aid for Young Scientist (No. 18750140) from the MEXT of Japan and Tsukuba Industrial Liaison and Cooperative Research Center.

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Received: June 25, 2008 **Revised: August 08, 2008** **Accepted: August 08, 2009**