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Review

Protein–Solvent Interactions in Pharmaceutical Formulations

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The stability of proteins is affected by a variety of solvent additives. Sugars, certain amino acids and salts, and polyhydric alcohols stabilize proteins in solution and during freeze-thawing. Urea and guanidine hydrochloride destabilize proteins under either condition. These effects can be explained from the preferential interactions of the cosolvents with the proteins; i.e., the protein stabilizers are preferentially excluded from the proteins, while the destabilizers bind to them. There is a class of compounds, such as polyethylene glycol and 2-methyl-2,4-pentanediol, that destabilize proteins at high temperature but stabilize them during freeze-thawing. Such effects can be accounted for by their preferential exclusion from the native proteins determined at room temperature and from their hydrophobic character, which depends on temperature. During freeze-drying, only a few sugars appear to be effective in protecting proteins from inactivation, as most other stabilizers cannot exert their action on proteins without water. The stabilization is due to hydrogen bonding between the sugars and the dried proteins, the sugars acting as water substitute. Understanding the mechanism of the effects of solvent additives on the protein stability should aid in the development of a suitable formulation for protein.

KEY WORDS: protein–solvent interaction; protein stability; freeze-thawing; freeze-drying; hydrophobic interaction.

INTRODUCTION

Proteins are marginally stable under physiological conditions, the free energy of denaturation being 5 to 20 kcal/mol (1–4). This is because stabilizing free energy, arising from interresidue interactions in a globular structure, is largely canceled by a loss of entropy that arises from the protein's compactness. A variety of compounds has been shown to affect the stability of proteins in solution. Sugars, polyols, and certain amino acids and salts are known to be protein stabilizers (5–12). On the other hand, hydrophobic organic compounds, chaotropic salts, urea, and guanidine hydrochloride are known to be protein destabilizers (6,7,13–15). The above protein stabilizers are also effective in the frozen state as well as in solution. Certain organic compounds such as dimethyl sulfoxide, 2-methyl-2,4-pentanediol, and polyethylene glycol are also effective as protein stabilizers in the frozen state, but not in solution (in particular at higher temperatures), whereas urea and guanidine hydrochloride are destabilizers in the frozen state as well.

Although a broad range of compounds can serve as protein stabilizers in solution and frozen state, Carpenter and Crowe (16) have recently shown that only carbohydrates can protect a labile enzyme (i.e, phosphofructokinase) from inactivation by freeze-drying. This suggests that a fundamental difference exists in stabilizing mechanism between the dried state and the solution or frozen state, as has recently been discussed by Crowe *et al.* (17).

We summarize the solvent additives that have been used to enhance the stability of proteins in solution and during freeze-thawing and freeze-drying and then describe the current knowledge of the mechanism of their actions. Finally, we attempt to show how one can use this knowledge to achieve suitable formulations for protein pharmaceuticals.

Cosolvent Effects in Solution

Examples of cosolvent-induced stabilization of proteins in solution may be arbitrarily divided into two groups, i.e., stabilization against reversible denaturation induced by different types of perturbation and stabilization against time-dependent irreversible denaturation or slow reversible denaturation. In their classical work, von Hippel and Wong (12) showed that potassium phosphate and ammonium sulfate increase the transition temperature of the globular protein, ribonuclease A, and hence stabilize it from a reversible denaturation, NaCl and KCl have no effect, and $CaCl_2$ and KSCN are destabilizers. Similar effects of the salts were observed on the stability of a nonglobular protein, collagen (11), and on the thermal stability of myosin (18). In the latter study, the bromide salts were the strongest destabilizers; sodium citrate and the other stabilizing salts increased the transition temperature. Salt-induced stabilization and destabilization can be found with many other proteins (19–21). β-Lactoglobulin can be stabilized against urea-induced unfolding by salts (at 0.2 *M*), the stabilization following the order of chloride < tartrate < sulfate < phosphate < citrate

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(19). Antithrombin III, which undergoes heat-induced denaturation followed by the formation of aggregates, is strongly stabilized by phosphate and sulfate and destabilized by iodide and thiocyanate. It is noteworthy that ethylenediaminetetraacetic acid and citrate are extremely strong stabilizers.

Effects of polyhydric alcohols on the thermal stability of globular proteins were extensively studied by Gerlsma (6,7). Glycerol, erythritol, and sorbitol increase the transition temperature of the protein regardless of pH, the increase being greater at higher cosolvent concentrations. Stabilizing effects of polyhydric alcohols have been demonstrated with collagen (23,24), chymotrypsinogen (7), lysozyme (22) and other proteins (25). Ethylene glycol decreases the transition temperature of ovalbumin, while the effect on ribonuclease A is more complex, being a protein stabilizer at pH 2.3 but a destabilizer at pH 5.5. This suggests that the stabilizing effect of ethylene glycol is variable; it induces concentration-dependent denaturation of β-lactoglobulin (14) and decreases the thermal stability of lysozyme (22), while it increases the transition temperature of collagen (23).

Sugars are also effective protein stabilizers as measured by their ability to increase the transition temperature of collagen (23). Sucrose and maltose are most effective, ribose and deoxyribose least effective, and glucose and mannose are intermediate. The transition temperature of ovalbumin increases in the presence of various sugars, including arabinose, glucose, galactose, and mannose. A polysaccharide, dextran 10, but not Ficoll, also stabilizes the protein (15). Lee and Timasheff (26) found that sucrose increases the transition temperatures of chymotrypsinogen, α-chymotrypsin, and ribonuclease. These results demonstrate that the effects of sugars are general and independent of the proteins used.

Sugars also have been shown to protect proteins from time-dependent denaturation. The effect of varying concentrations of sucrose on the colchicine binding ability of tubulin has been studied (27). Tubulin can bind colchicine only when it is in the native state. Sucrose (1 *M*) can give complete protection from inactivation, while at lower sucrose concentrations there is a gradual loss of the ability of tubulin to bind colchicine. Glucose also can stabilize tubulin against time-dependent denaturation (28).

Certain amino acids and amine compounds also serve as protein-stabilizing cosolvents. The transition temperatures of lysozyme were determined in the absence and presence of various amino acids (e.g., L-proline, L-serine, α-alanine, and β-alanine) and related compounds (e.g., trimethylamine *N*-oxide, taurine, γ-aminobutyric acid, sarcosine, and betaine) (8). The transition temperature was increased by the inclusion of these additives. Monosodium glutamate, lysine hydrochloride, glycine, and betaine at 1 *M* also have been shown to increase the thermal stability of bovine serum albumin and lysozyme (9,10).

These compounds, at moderate concentrations, also protect proteins from time-dependent denaturation and inactivation and offset the deleterious effects of urea on protein stability (29–31). Monosodium glutamate (28) and ε-aminocaproic acid (32), respectively, can protect tubulin and reduce degradation of allergen component proteins in aqueous pollen extracts.

All of the compounds described above, except ethylene glycol, also enhance self-association of proteins, protein–protein interactions, and interactions of proteins with other ligands and decrease protein solubility. Sucrose, glycerol, and monosodium glutamate enhance microtubule assembly (56,59–61), and structure-stabilizing salts enhance actin polymeration (62), β-lactoglobulin dimerization (19), and hemocyanin self-association (63). Polyethylene glycol is widely used as a protein precipitant and to enhance protein–protein interactions, yet it is not a protein stabilizer; it decreases the transition temperature of some monomeric proteins (64,65). Polyethylene glycol 8000 (PEG 8000) enhances the association between glycolytic enzymes and between glycolytic enzymes and F-actin (66), among other proteins (67,68).

Cosolvent Effects During Freeze-Thawing

The bulk of the research on protein cryopreservation has been on enzymes, where stabilization is reflected in the maintenance of catalytic activity upon thawing. Protection against freeze-thawing can be afforded by the compounds described above and even other proteins (e.g., bovine serum albumin) (33–35). Usually cosolvent concentrations exceeding 0.2 *M* are needed for cryopreservation.

Loomis *et al.* (36,37) have demonstrated that end products of anaerobic metabolism, which are thought to protect certain organisms against freeze-induced damage in nature, can also stabilize labile enzymes such as lactate dehydrogenase and phosphofructokinase. These compounds include strombine, alanopine, octopine, lactate, succinate, and propionate.

Carpenter and colleagues (38–41) have shown that combinations of certain divalent cations (e.g., Zn^{2+}) and organic cosolvents (e.g., sugars, polyhydric alcohols, amino acids, and related compounds) can provide synergistic protection for labile enzymes and antibodies. For example, full activity of phosphofructokinase is recovered when this sensitive enzyme is frozen in the presence of 0.6 m*M* zinc sulfate and 5 m*M* trehalose. With either additive alone, no enzyme activity is measurable after freeze-thawing. This phenomenon could have practical advantages in protein formulation since reduced amounts of stabilizers could be used.

Cosolvent Effects During Freeze- and Air-Drying

Many cryoprotectants are unable to stabilize labile enzymes during drying and rehydration (17,35). Carpenter and colleagues found that only carbohydrates stabilize phosphofructokinase during either freeze-drying or air-drying. The greatest degree of protection is seen with the disaccharides, trehalose, sucrose, maltose, and lactose (16,42–44). Monosaccharides (e.g., glucose and galactose) and *myo*-inositol are much less effective.

Synergistic stabilization of phosphofructokinase can be realized during freeze-drying and air-drying with combinations of divalent zinc and carbohydrates (42,43). However, even in the presence of zinc, cryoprotectants such as amino acids and glycerol do not preserve the enzyme in the dried state.

MECHANISM OF STABILIZATION OF PROTEINS BY COSOLVENTS

Since a broad spectrum of cosolvents can stabilize pro-

teins under widely different conditions, it is unlikely that the stabilization of proteins by the cosolvents in solution and during freeze-thawing stems from a specific binding effect. The preferential interaction of proteins with solvent components can explain, probably without exception, the effects of cosolvents on the stability of proteins both in solution and during freeze-thawing (33,45).

Here we describe the preferential interaction of proteins with solvent components and the thermodynamic consequences. The protein–ligand interaction is described by the bindings of water (designated with the subscripts w) and ligand (s) to a protein (p), according to Eq. (1); at constant temperature, pressure, and chemical equilibrium;

$$(\partial g_s/\partial g_p)_{T,\mu_w,\mu_s} = g_w A_s - g_s A_w \quad (1)$$

where g_i is the concentration of component i (s, p, or w) in grams per gram of water in the system, T is Kelvin temperature, μ_i is the chemical potential of component i, and A_s and A_w are the bindings of ligand and water expressed as gram per gram of protein. At high ligand concentrations, the second term, i.e., the binding of water, contributes significantly to the observed value. Under such conditions, the preferential binding, $(\partial g_s/\partial g_p)$ (subscripts omitted), becomes different from the total ligand binding, A_s. The parameter $(\partial g_s/\partial g_p)$ can be either positive or negative. When the parameter is negative, water is in excess in the protein domain over its concentration in the bulk, i.e., the protein is preferentially hydrated. The preferential hydration parameter $(\partial g_w/\partial g_p)$ can be calculated from

$$(\partial g_w/\partial g_p) = -(1/g_s)(\partial g_s/\partial g_p) \quad (2)$$

The preferential interaction parameter expressed in grams can be converted to that in moles by Eq. (3):

$$(\partial m_s/\partial m_p) = (M_p/M_s)(\partial g_s/\partial g_p) \quad (3)$$

where M_i is the molecular weight, and m_i is the molal concentration of component i.

Preferential interaction parameters have been determined for salts (46–54), amino acids and related compounds (8,52), polyhydric alcohols (55–57), and sugars (26,58) with various proteins. Typical results for salts, amino acids, and sugars are shown in Tables I and II. These compounds have large negative values of $(\partial g_s/\partial g_p)$. The structure stabilizing compounds are excluded from the protein surface. The structure destabilizing compounds, such as $MgCl_2$ and KSCN, have been shown to be bound to the proteins (49,50,53).

Table III lists the preferential interaction values of several polyhydric alcohols with bovine serum albumin; the protein is preferentially hydrated and these cosolvents are excluded from the protein. The preferential exclusion of glycerol was also observed with tubulin and other proteins (56,57). Ethylene glycol shows a small preferential exclusion from bovine serum albumin, yet binds to β-lactoglobulin (14), indicating that the preferential interaction of ethylene glycol may depend on the kind of protein. This observation is consistent with the fact that ethylene glycol stabilizes some proteins while destabilizing others.

Preferential interaction simply reflects the perturbation of the chemical potential of the protein by the ligand (69,70). Equation (4) shows that a negative value of $(\partial m_s/\partial m_p)$

$$(\partial m_s/\partial m_p) = -(\partial \mu_p/\partial m_s)/(\partial \mu_s/\partial m_s) \quad (4)$$

means that $(\partial \mu_p/\partial m_s)$ is positive; namely, the addition of ligand (stabilizer) increases the chemical potential of the protein and, thus, the free energy of the system. This is thermodynamically unfavorable. If, in the course of the denaturation reaction, the chemical nature of the interactions between the protein and the stabilizer does not change, this situation should become even more thermodynamically unfavorable for the unfolded state of the protein due to the increase in the surface area of contact between protein and solvent. Therefore, the reaction is pushed toward the native state, resulting in stabilization of the native structure.

For a wide variety of compounds, exclusion is determined by the effect of the additive on the surface tension of water. Cosolvents perturb the cohesive force of water and, hence, its surface tension. This results in either an excess or a deficiency of the cosolvent in the protein surface layer (71). Those compounds which increase the surface tension of water should be excluded from the protein surface (49–53).

Although exclusion predominates for the structure stabilizing compounds, nevertheless these can bind to proteins through hydrophobic interaction, hydrogen bonding, or electrostatic interactions. The net interaction observed between proteins and stabilizing compounds is the balance between binding to the protein and exclusion. If the binding of a cosolvent increases more than the exclusion does upon denaturation, the result should be protein destabilization, while

Table I. Preferential Interactions of Cosolvents with Proteins

Solvent	Protein	$(\partial g_s/\partial g_p)$	$(\partial m_s/\partial m_p)$	$(\partial g_w/\partial g_p)$
1 *M* sucrose	Tubulin	−0.106	−38.0	0.243
1 *M* sucrose	Ribonuclease A	−0.190	−7.6	0.437
2 *M* glucose	BSA[a]	−0.099	−37.4	0.212
3.4 *M* glycerol	BSA	−0.113	−83.4	0.212
2 *M* glycine	BSA	−0.069	−62.2	0.416
2 *M* betaine	BSA	−0.125	−72.5	0.428
2 *M* Na glutamate	BSA	−0.171	−68.8	0.417
1 *M* Na glutamate	BSA	−0.088	−35.5	0.477
1 *M* Na glutamate	Tubulin	−0.058	−37.3	0.393
1 *M* NaCl	BSA	−0.0145	−16.8	0.243
1 *M* Na_2SO_4	BSA	−0.074	−35.4	0.524
1 *M* NaOAc	BSA	−0.027	−22.4	0.312
1 *M* $MgSO_4$	BSA	−0.047	−26.5	0.388

[a] Bovine serum albumin.

Table II. Interaction Parameters of Lysozyme with Amino Acids and Related Compounds at pH 6.0

Solvent	$(\partial g_s/\partial g_p)$ (g/g)	$(\partial g_w/\partial g_p)$ (g/g)
1 *M* L-proline	−0.0406 ± 0.0093	0.322 ± 0.074
1 *M* L-serine	−0.0497 ± 0.0030	0.444 ± 0.027
0.667 *M* taurine	−0.0331 ± 0.0051	0.377 ± 0.058
1 *M* γ-aminobutyric acid	−0.0699 ± 0.0082	0.629 ± 0.074
1 *M* sarcosine	−0.0461 ± 0.0038	0.485 ± 0.040

Table III. Preferential Interaction Parameters of Bovine Serum Albumin with Solvent Components in Water–Polyhydric Alcohol Systems at 25°C

Alcohol (%)	$(\partial g_s/\partial g_p)$ g/g	$(\partial g_w/\partial g_p)$ g/g	$(\partial m_w/\partial m_p)$ mol/mol
	Water–ethylene glycol		
0			
20 (v/v)	−0.041 ± 0.020	0.148	560
40	−0.097 ± 0.027	0.130	490
60	−0.222 ± 0.041	0.137	520
	Water–glycerol		
10 (v/v)	−0.020 ± 0.010	0.143	540
20	−0.052 ± 0.023	0.165	620
30	−0.101 ± 0.020	0.187	710
40	−0.154 ± 0.024	0.185	700
	Water–xylitol		
20 (w/v)	−0.030 ± 0.021	0.129	490
30	−0.055 ± 0.011	0.146	550
	Water–mannitol		
10 (w/v)	−0.022 ± 0.006	0.205	770
15	−0.034 ± 0.007	0.204	770
	Water–sorbitol		
5 (w/v)	−0.009 ± 0.009	0.174	660
10	−0.022 ± 0.009	0.205	770
15	−0.033 ± 0.010	0.198	750
20	−0.054 ± 0.011	0.229	870
30	−0.092 ± 0.016	0.245	930
40	−0.128 ± 0.018	0.234	880
	Water–inositol		
5 (w/v)	−0.021 ± 0.003	0.407	1,540
10	−0.041 ± 0.005	0.387	1,460

the opposite should be true for the structure stabilizers. For strong protein denaturants, binding always predominates.

The effect of stabilizers on a reaction involving protein–protein contacts, such as protein self-assembly or precipitation, may be viewed in the same way as described for the denaturation reaction. Exclusion of the stabilizer per monomeric protein unit is decreased upon formation of protein–protein contacts; namely, the associated form of the precipitate is less unfavorable thermodynamically in the presence of the stabilizer.

The mechanism of protein stabilization described above should apply both in solution and during freeze-thawing. However, polyethylene glycol and 2-methyl-2,4-pentanediol (MPD) are unique in that they are preferentially excluded from the protein, but they denature or destabilize proteins in solution. Nevertheless, they can stabilize proteins during freeze-thawing.

The postulated mechanism by which these compounds exert such a unique effect (34,54) is through exclusion from the native protein due to steric exclusion and/or repulsion from protein charges and binding to the denatured protein due to hydrophobic interaction. Therefore, the cosolvent exclusion will increase upon denaturation, but the cosolvent binding will do so even more strongly, and the net result is a decrease in the preferential exclusion upon denaturation.

How do these cosolvents stabilize protein during freeze-thawing? At subzero temperatures, the hydrophobic character of these compounds will become weak, and hence hydrophobic interaction between the proteins and these compounds, which is the driving force of destabilization, will no longer contribute to the preferential interaction. Consequently, preferential exclusion of these compounds will dominate for the native and denatured states of the protein, and hence the net result is stabilization. The same argument should apply to the proteins undergoing self-association, since these compounds stabilize monomeric proteins and enhance their associations at subzero temperatures.

We now turn to the mechanism of cosolvent effect on proteins during freeze-drying. As noted above, only carbohydrates are effective at protecting phosphofructokinase during either freeze-drying or air-drying (42,43). The observation that many effective cryoprotectants fail to protect dried phosphofructokinase indicates that the mechanism of cosolvent-induced protein stabilization in the dried state is fundamentally different from that for proteins in aqueous or frozen systems (16,17,44). In addition, the thermodynamic arguments that are needed to explain protein stabilization by preferentially excluded cosolvents are not applicable when water is removed from the system.

It has been suggested (44) that certain carbohydrates protect proteins by binding to the dried protein, thus serving as a "water substitute," when the hydration shell of the protein is removed. Fourier transform infrared spectroscopy (16) indicated not only that hydrogen bonding occurs between proteins and stabilizing carbohydrates but also that solute binding is requisite for labile proteins to be preserved during drying. For example, the presence of the protein (bovine serum albumin or lysozyme) leads to a pronounced decrease in absorbance in the fingerprint region of the infrared spectrum for trehalose, major shifts in band position, and a loss of band splitting. The protein-induced spectral changes can be titrated by freeze-drying the sugar with increasing amounts of either protein (18).

The significance of the influence of proteins on the vibrational spectrum of dried trehalose can best be appreciated when compared to the effects of water on the spectrum of the hydrated sugar. Typical spectra for trehalose dried in the presence of either lysozyme or bovine serum albumin are remarkably similar to that for hydrated trehalose, while all are very different from a spectrum of crystalline trehalose. Thus, it appears that proteins serve the same role for dried trehalose as does water for the hydrated sugar, i.e., they form hydrogen bonds with the polar groups in the sugar.

It is implicit in the conclusion that proteins serve as water substitutes for dried carbohydrates that the converse must also be true. To test this suggestion, the influence of trehalose on the infrared spectrum of lysozyme was investigated (16). When lysozyme is dried without the sugar, there is an increase in the frequency of the amide I band from 1652.1 cm^{-1}, seen for the fully hydrated protein, to 1659 cm^{-1}. The amide II band is broadened and shifts from about 1543 to almost 1530 cm^{-1} in the dried protein. In addition, the band assigned to the carboxylate in the hydrated protein (72) at 1583 cm^{-1} is not detectable with the dried protein.

When lysozyme is freeze-dried in the presence of trehalose (5 g sugar/g protein), the amide I band of the dried protein is shifted back to 1658.1 cm^{-1}. A band corresponding to that for carboxylate at 1583 cm^{-1} also appears. The most dramatic effect of the sugar is to shift the amide II band back to 1542 cm^{-1}, almost the identical position noted for the hydrated protein. In addition, the band shape is essentially the same as that seen for the hydrated protein.

The position of the amide II band was used as a means to characterize the effect of varying the amount of sugar on the vibrational spectrum of dried lysozyme. Figure 1A shows there is an increase in the amide II frequency as the initial sugar concentration is increased up to 100 mg/ml. With 100–200 mg/ml trehalose, the amide II band is centered at about 1542 cm^{-1}. However, when the sugar concentration is greater than 200 mg/ml, there is a progressive decrease in the frequency of the amide II band, such that with 400 mg/ml trehalose, amide II is shifted back to 1531 cm^{-1}. A concentration of 400 mg/ml is very near the solubility of trehalose at room temperature. In the lysozyme samples freeze-dried with this level of sugar, the bands in the trehalose fingerprint region show a high degree of splitting, a characteristic feature of dried crystalline trehalose (cf. 16). Crystallization of trehalose during sublimation could decrease the availability of the sugar for forming hydrogen bonds with the protein (16).

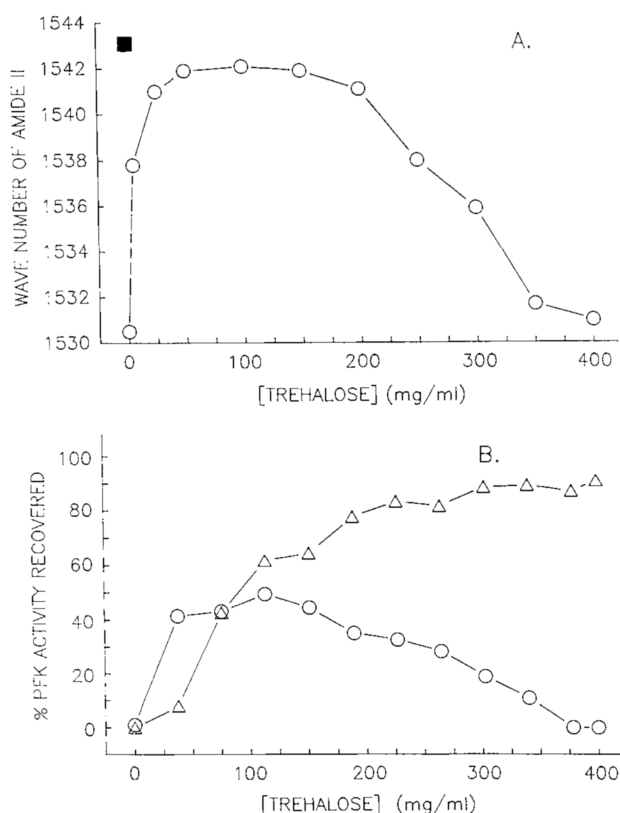

Fig. 1. (A) Effects of trehalose on the wavenumber for the amide II band of dried lyzoyme. Lysozyme (20 mg/ml) was freeze-dried in the presence of the indicated amounts of trehalose. The filled square represents the wavenumber for the amide II band of hydrated lysozyme. (B) Comparison of the percentage of phosphofructokinase activity recovered after freeze-thawing (open triangles) or freeze-drying and rehydration (open circles) in the presence of trehalose. [Reprinted from Ref. 16. Copyright (1989), American Chemical Society.]

Significantly, the greatest degree of protection of phosphofructinase during freeze drying (16) is noted with intermediate amounts of sugar (Fig. 1B). With trehalose concentrations greater than 150 mg/ml, there is a decrease in activity recovered. These results indicate that whenever trehalose is at a concentration that does not influence the frequency of the amide II band for dried lysozyme, this sugar concentration is also ineffective at preserving dried, labile proteins. That is, hydrogen bonding of the sugar to the protein appears to be mandatory for the sugar to preserve dried proteins.

During freeze-thawing, however, the presence of high concentrations of trehalose leads to increased recovery of activity (Fig. 1B), as expected, since cryopreservation is due to the preferential exclusion of the stabilizing cosolvent from the surface of the protein.

In summary, sugars such as trehalose can serve to satisfy partially the hydrogen-bonding requirements of the polar groups on dried proteins and, thus, serve as water substitutes. The carbohydrate may prevent the formation of intra- and interprotein hydrogen bonding in the dried state, which could induce unfolding and/or aggregation of protein molecules upon rehydration. It is clear that this is distinctly different from the preferential exclusion mechanism that is operative in the aqueous and frozen state. Therefore, it is important, for discussions regarding stabilization of biomolecules, that freeze-thawing and freeze-drying, although both dependent on a freezing step, be viewed as distinctly different stress vectors (cf. Refs. 16, 17, 35).

PRACTICAL CONSIDERATIONS

It is clear that certain salts, sugars, and amino acids and related compounds can protect proteins from denaturation or inactivation in solution and during freeze-thawing. Carbohydrates can also protect proteins from damages that occur during drying. Since these compounds stabilize proteins at high and low temperatures, it is not absolutely necessary to keep the protein solution at low temperatures. However, those compounds which destabilize the proteins at high temperatures (e.g., PEG and MPD) but stabilize them at low temperature must be used with caution.

All the compounds described above enhance protein–protein and protein–ligand interactions. Therefore, they might enhance the binding of proteins to glass vials or lead to a decrease in the protein solubility. Polyethylene glycol is often used to prevent proteins from binding to glass during sample storage and column chromatography. The denaturation action of polyethylene glycol at high temperatures is extremely weak (37,48,49), but its precipitating action is extremely strong. The reason why this compound does not enhance the protein binding to the glass is probably due to the binding affinity of polyethylene glycol itself to the glass surface. Therefore, although polyethylene glycol is not a protein stabilizer in solution (in particular at high temperature), it may be used to reduce surface adsorption of proteins and stabilize proteins from freeze-thawing, provided that ap-

propriate concentration and molecular weight of polyethylene glycol are chosen to avoid protein precipitation.

Finally, it should be noted that the interaction between carbohydrates and proteins, and thereby the stabilization of the proteins by the carbohydrates in the dried state, depends on the initial concentration of the carbohydrates. Excess carbohydrates may eliminate the interaction and hence the stabilization effect on the proteins, suggesting that proper initial concentration of carbohydrates is important for optimal freeze-drying of the proteins.

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