

Factors affecting short-term and long-term stabilities of proteins[☆]

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Abstract

Proteins are marginally stable and, hence, are readily denatured by various stresses encountered in solution, or in the frozen or dried states. Various additives are known to minimize damage and enhance the stability of proteins. This review discusses the current knowledge of the mechanisms by which these additives stabilize proteins against acute stresses, and also the various factors to be considered for long-term storage of proteins in solution. © 2001 Elsevier Science B.V. All rights reserved.

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Abbreviations: PEG, polyethylene glycol; MPD, 2-methyl-2,4-pentanediol; FTIR, Fourier transform infrared; PFK, phosphofructokinase; G-CSF, granulocyte-colony stimulating factor; FGF, fibroblast growth factor.

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1. Introduction

The isolation and purification of a protein is often detrimental to its structure and biological activity, particularly in the case of intracellular proteins that are protected by the cellular milieu. A number of factors can cause damage to isolated proteins, including concentrations of macromolecules and low-

molecular-mass compounds, pH, ionic strength, redox potential, and temperature. As a consequence, proteins may be covalently or non-covalently modified, resulting in conformational changes and/or aggregation. It has been customary to use a so-called protein stabilizer to maintain the native conformation of proteins during isolation and storage [1–6].

Most proteins are only marginally stable at neutral pH and room temperature and hence are readily denatured by increasing (occasionally decreasing) the temperature and pressure, by pH changes, addition of urea, guanidine HCl and chaotropic salts, as well as freeze–thawing and freeze-drying procedures [7–10]. It has been shown that many additives, such as sugars, certain amino acids, some amines, and glycerol, stabilize proteins against these stresses [11–29]. Still, in many cases the use of these additives is insufficient to stabilize proteins completely.

Freeze–thawing and freeze-drying are the most commonly used methods for the long-term storage of proteins, although many proteins are not stable against these stresses [30–34]. Recently, Carpenter and Crowe [30] examined a number of compounds for their effects on the retention of enzyme activity upon freeze–thawing and freeze-drying. Those compounds that are known to stabilize proteins in solution protected proteins from freeze–thaw damage and those compounds that are known to denature or destabilize proteins enhanced damage due to freeze–thawing. Thus, it was proposed that the mechanism for the co-solute stabilization of proteins during freeze–thawing is the same as that described for stabilization in the solution state [30]. On the other hand, those researchers found that only saccharides protected proteins from freeze-drying damage [32,33,35,36]. Crowe et al. [37] concluded from these results that stresses arising from freeze-drying are fundamentally different from those due to freeze–thawing.

In vitro stabilization of proteins by co-solutes appears to parallel the means for stabilizing macromolecules that is utilized by a number of organisms that are adapted to extreme conditions, such as high osmolality, freezing and dehydration. Those organisms living under conditions of high osmolality, or that are resistant to freezing, accumulate a variety of compounds, so-called osmolytes, to raise the osmotic pressure of the cytoplasm or serve as cryoprotect-

ants, respectively. These compounds are apparently non-perturbing to the biological functions of the macromolecules and are also protein stabilizers [38–44]. On the other hand, the compounds that are used by organisms that can survive at low water activity are limited to disaccharides [45–48]. Similarly, for in vitro solutions, proteins and other biological components are protected from dehydration only by these sugars [35–37,49,50].

The mechanism of protection and stabilization of proteins by glycerol and sugars in solution and during freeze–thawing, despite their extensive use, was poorly understood until research by Timasheff and colleagues on the preferential interaction of proteins with solvent components. The experimental and theoretical analysis of the observed co-solute-induced stabilization and destabilization of proteins in solution has shown that the protein–solvent interaction determines the effect of the co-solutes on the stability of the proteins, rather than direct interaction of solutes with proteins (reviewed in [49–51]). Carpenter and Crowe [35–38] demonstrated that the same mechanism can be extended to protein stabilization in the frozen state, but not to the dried state. Clearly, thermodynamic arguments for protein stabilization in solution are not applicable when water is removed from the system.

The mechanism of protein stabilization by saccharides against freeze-drying is also poorly understood. Recently, Carpenter and Crowe [36,90] have shown that direct interaction between sugars and proteins through hydrogen bonding is essential for the observed stabilization. This was further extended by the collaborative work of Prestrelski, Arakawa, and Carpenter, which demonstrated that protein dehydration often induces conformational changes. However, the native conformation is maintained even in the dried state if the protein is dehydrated in the presence of stabilizing sugars, that is, recovery of activity upon rehydration is dependent on preventing drying-induced conformational changes.

In this review, we will describe the current knowledge of the mechanism of protein stabilization by various co-solutes in solution and during freeze–thawing and freeze-drying. Although the short-term stabilization of proteins can be achieved by these co-solutes, it is becoming more evident that methods for long-term stabilization of proteins in solution and

in the dried state must be developed. Many proteins can be produced in large quantities using recombinant DNA techniques. Solution formulation of these proteins requires that other (even more important) factors need to be considered for prolonged storage. We will also review these factors.

2. Factors affecting solution-state stability

Detailed discussion of the mechanism of protein stabilization in solution induced by co-solutes will be presented later. Briefly, it has been observed experimentally that there is a deficiency in the stabilizing co-solute in the immediate vicinity of the protein (relative to the bulk solution), and that the protein is preferentially hydrated. That is, these co-solutes are preferentially excluded from contact with the surface of the protein. Thus, as Timasheff and his colleagues explained, the presence of these co-solutes in a protein solution creates a thermodynamically unfavorable situation, since the chemical potentials of both the protein and the additive are increased. In other words, an entropically unfavorable state occurs when co-solute molecules are preferentially excluded from contact with the protein. Consequently, the native structure of monomers and the polymerized form of oligomeric proteins are stabilized because denaturation or dissociation would lead to a greater contact surface between the protein and the solvent and, therefore, augment this thermodynamically unfavorable effect.

The effects of co-solutes can be examined more quantitatively. It has been observed that stabilization of proteins by co-solutes is concentration-dependent, and that the stabilization becomes apparent only at relatively high (e.g. >0.3 M) co-solute concentrations. At such high concentrations, interaction does not imply stoichiometric complexation, but it accounts for the averaged amounts of the components present in the solvent volume permanently or transiently perturbed by the protein, reflecting both strong and weak as well as attractive and repulsive interactions [52–55]. In addition, interaction includes the amount of water binding (hydration) to the protein, since, for a three-component system, the interaction parameter (ξ_3) is expressed at constant pressure and temperature by Eq. (1) [52]:

$$\xi_3 = \left(\frac{\partial g_3}{\partial g_2} \right) = A_3 - g_3 A_1 \quad (1)$$

where the solution components are expressed by the subscripts 1, 2 and 3 (component 1 = H₂O, component 2 = protein, and component 3 = co-solute), g_i is the concentration of component i in grams per gram of H₂O, and A_i is the amount of component i binding (interacting) to the protein in grams per gram. (For simplicity, the subscripts that express constant temperature and pressure are omitted.)

The parameter ξ_3 can be determined experimentally by equilibrium dialysis. In Eq. (1), it is evident that, at high co-solute concentration, protein hydration makes a significant contribution to the experimentally determined interaction value.

More importantly, the preferential interaction (not the actual binding amount, A_3) is the interaction that determines the effect of the co-solute on the stability of the protein. Let us assume the following equilibrium denaturation:



where N and D are the native and denatured forms of a protein, respectively, and the equilibrium is expressed by a constant $K (= [D]/[N])$. The free energy of each state (G^N and G^D) is related to K by:

$$-RT \ln K = G^D - G^N = \Delta G \quad (2)$$

If we express with the subscript w the parameters for a protein solution in the absence of co-solute and with no subscript for the presence of co-solute, the difference is given by Eq. (3):

$$\begin{aligned} -RT \ln \frac{k}{k_w} &= (G^D - G_w^D) - (G^N - G_w^N) \\ &= \Delta \mu_2^D - \Delta \mu_2^N \end{aligned} \quad (3)$$

where the $\Delta \mu_2^D$ and $\Delta \mu_2^N$ are the free energies of transfer of the denatured and native protein, respectively, from water to solution containing co-solute. The transfer free energy is related to the preferential interaction parameter, since:

$$\left(\frac{M_2}{M_3} \right) \left(\frac{\partial g_3}{\partial g_2} \right) = \left(\frac{\partial m_3}{\partial m_2} \right) \quad (4)$$

and

$$\left(\frac{\partial \mu_2}{\partial m_3}\right) = -\left(\frac{\partial m_3}{\partial m_2}\right)\left(\frac{\partial \mu_3}{\partial m_3}\right)$$

where M_i is the molecular weight of component i , μ_i is the chemical potential, and m_i is the molality [56]. That is, a negative value of preferential interaction parameter, $(\partial m_3/\partial m_2)$, means that the chemical potential of the protein is increased. Integration of $(\partial \mu_2/\partial m_3)$ with regard to m_3 :

$$\Delta \mu_2 = \int \left(\frac{\partial \mu_2}{\partial m_3}\right) dm_3 \quad (5)$$

gives the transfer free energy of the protein from water to an aqueous co-solute solution. Thus, if one can determine the preferential interactions for the native and denatured states as a function of m_3 , then $\Delta \mu_2$ for both states, i.e., $\Delta \mu_2^N$ and $\Delta \mu_2^D$, can be obtained.

The preferential interaction parameter (ξ_3) can be negative since, at high concentrations of co-solute, the hydration term in Eq. (1) becomes significant. In such a case, the co-solute is preferentially excluded from the protein. If the co-solute is excluded to a greater degree from the denatured protein,

$$\left(\frac{\partial m_3}{\partial m_2}\right)^D < \left(\frac{\partial m_3}{\partial m_2}\right)^N < 0,$$

then the co-solute will stabilize the proteins, since

$$\left(\frac{\partial \mu_2}{\partial m_3}\right)^D > \left(\frac{\partial \mu_2}{\partial m_3}\right)^N \quad \text{and} \quad \Delta \mu_2^D > \Delta \mu_2^N$$

Although this mechanism applies to the reversible denaturation of proteins, the same co-solutes can stabilize proteins against irreversible denaturation and deactivation. The irreversible process may be best described as $N \rightleftharpoons D \rightarrow D'$, where D' represents the product of an irreversible reaction, such as aggregation or chemical modification. Therefore, the stabilizing co-solutes are expected to slow this process down by shifting the equilibrium toward the native state, as has been observed [4,25,57,58].

Preferential interaction measurements have been carried out for a variety of co-solutes and several proteins [23,59–69]. These measurements were made on which ever state the protein assumes under the experimental conditions. When the co-solutes are stabilizers, the experimental values refer to the native

state. When the co-solutes are strong denaturants and are at high-enough concentrations, the experimental values refer to the denatured state. It is not possible to generate two states of a protein under identical solution conditions without irreversibly denaturing the protein, and hence no experimental values are available for both states under identical conditions.

Numerous experimental results of preferential interaction measurements are now available for a variety of protein stabilizers, including sugars (sucrose, lactose, and glucose), amino acids (glycine, alanine, and proline), amines (betaine and trimethylamine *N*-oxide), polyols (mannitol and sorbitol), and certain salts (ammonium, sodium, and magnesium sulfate). Although these compounds differ widely in chemical properties, one thing they share in common is preferential exclusion from the surface of native proteins. They have negative values of ξ_3 and $(\partial m_3/\partial m_2)$ and hence, positive values of $(\partial \mu_2/\partial m_3)$.

Taking glucose as an example [23], the values of $(\partial m_3/\partial m_2)$ were determined at various concentrations of glucose and plotted against m_3 (Fig. 1). Assuming a linear dependence, $(\partial m_3/\partial m_2)$ was graphically integrated with m_3 and the transfer free energy thus calculated is plotted in Fig. 2. This corresponds to the free energy required to transfer the protein from water to a solution containing glucose. The values are positive and, hence, the transfer is thermodynamically unfavorable. As described in Eq. (3), the effect of glucose on the stability of protein is determined by the difference in transfer free energy between the native and denatured states. So far, no data are available for the denatured state. The assumption has been made that the preferential exclusion will be greater for the denatured state. With this assumption, ξ_3 [and $(\partial m_3/\partial m_2)$] is more negative for the denatured state and hence $(\partial \mu_2/\partial m_3)$ is more positive. Consequently, the transfer free energy of the denatured protein from water to co-solute is more positive, and thus the transfer is more unfavorable than that of the native state. This should result in protein stabilization.

The free energy diagram for the above case is depicted in Fig. 3. The free energy difference between the native and denatured states is increased by the addition of glucose, since a larger transfer free energy of the denatured state makes the free energy of denaturation even greater.

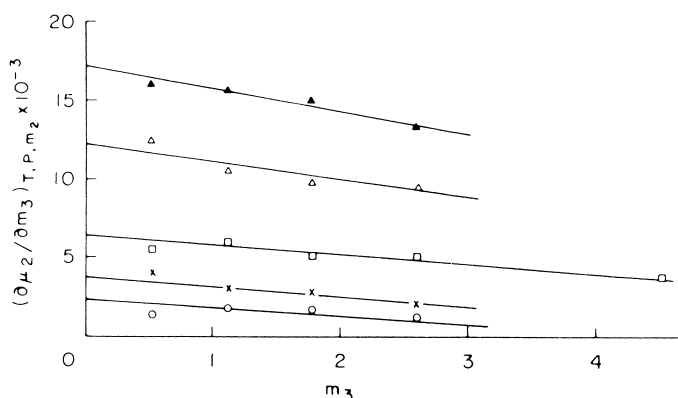


Fig. 1. Plot of the preferential interaction parameter $(\partial\mu_2/\partial m_3)$ vs. concentration of glucose (m_3). Symbols are ribonuclease (○), bovine serum albumin at pH 6.0 (△) and at pH 3.0 (▲), chymotrypsinogen (□), and lysozyme (X). Reproduced with permission from Arakawa and Timasheff [23]. Copyright 1982, American Chemical Society.

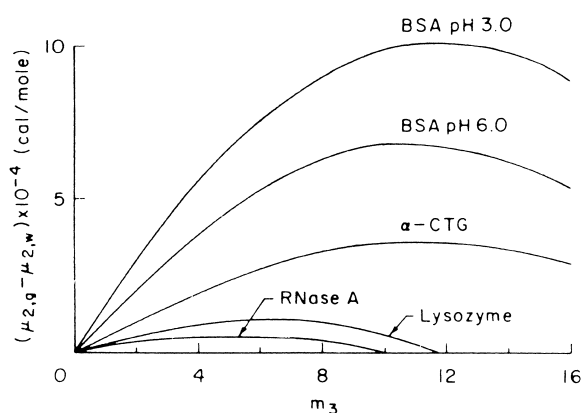


Fig. 2. Transfer free energy $(\Delta\mu_2^N)$ of various proteins from water to glucose solution. Reproduced with permission from Arakawa and Timasheff [23]. Copyright 1982, American Chemical Society.

Preferential interaction measurements have also been performed on those compounds that are known to destabilize or denature proteins [70–73]. These compounds show either a much weaker preferential exclusion or a preferential binding, implying that they have a greater tendency to bind to the proteins than to be excluded from the surface. Preferential binding decreases the chemical potential. Here again, data are unavailable for the preferential interactions of both the native and denatured states under identical conditions. However, it is expected that the binding will be greater for the denatured state with a larger solvent-exposed surface. This leads to a more

positive value of ξ_3 for the denatured state and hence a larger decrease in chemical potential:

$$\left(\frac{\partial\mu_2}{\partial m_3}\right)^D < \left(\frac{\partial\mu_2}{\partial m_3}\right)^N < 0.$$

Thus, the transfer of the denatured state from water to a solution containing such co-solutes is more favorable than that of the native state, and hence they should destabilize proteins. Examples of the substances belonging to this class are MgCl_2 , guanidine hydrochloride, KSCN, and urea.

This case is also depicted in Fig. 3. In the presence of destabilizers, the free energy of denaturation becomes smaller and hence the stability of the protein is decreased. In denaturing solvent systems, the much more favorable transfer free energy for the denatured state reverses the free energy states of the native and denatured states. Thus, in this case the denatured state has a lower free energy than the native state and hence the denatured protein is more stable (Fig. 3).

A strong correlation between solution stabilization and preferential interaction has been observed for many compounds. Those compounds that are strongly excluded from the protein surface stabilize proteins against various stresses imposed on the proteins in solution. There are, however, some exceptions to this rule, e.g. PEG (polyethylene glycol) and MPD (2-methyl 2,4-pentanediol) [74–76]. Their exclusion from the protein surface is quite large, and even

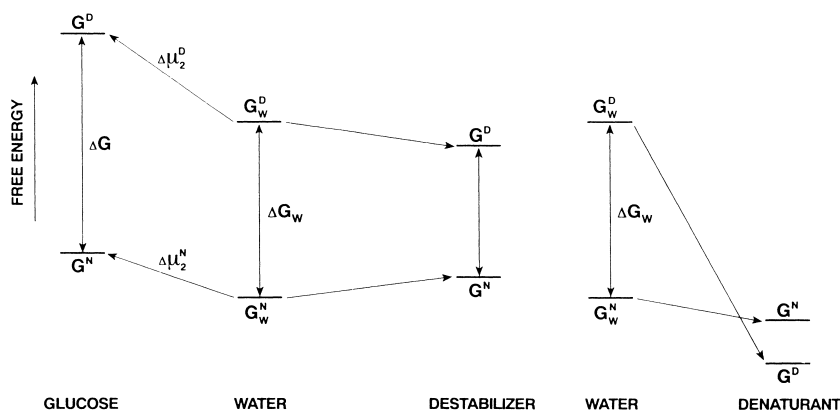


Fig. 3. Free energy diagram of a protein in different solvent systems.

exceeds the level of exclusion achieved by the above well-known protein stabilizers. However, these compounds are weak protein destabilizers. They do not denature proteins at room temperature, but they do decrease the melting temperature of the proteins [62,73,77,78].

Why does their behavior not follow their preferential interactions with proteins? It must be recalled here that the effects of co-solutes are determined by the difference in preferential interaction between the native and denatured states. The preferential interaction values for MPD and PEG are determined at 20°C, i.e. for the native state. We have no information on their interactions with the denatured states under identical conditions. One may ask how we can analyze those data of protein stabilizers despite the fact that no data on the denatured state are available. One major difference between the protein stabilizing and destabilizing excluded compounds is in their chemical nature. The first class of compounds is very polar and has almost no hydrophobic groups in the molecules, whereas the second class has hydrophobic character. This implies that co-solutes such as PEG and MPD, may have some affinity for the denatured state of the protein, which has many more exposed hydrophobic groups. A possible interaction of MPD and protein is depicted in Fig. 4, where the exclusion occurs due to repulsion between MPD molecules and charged groups. In the native state (left side), MPD is strongly excluded from the surface of the native, compact protein carrying a large number of charges, but shows little binding, while in the denatured state,

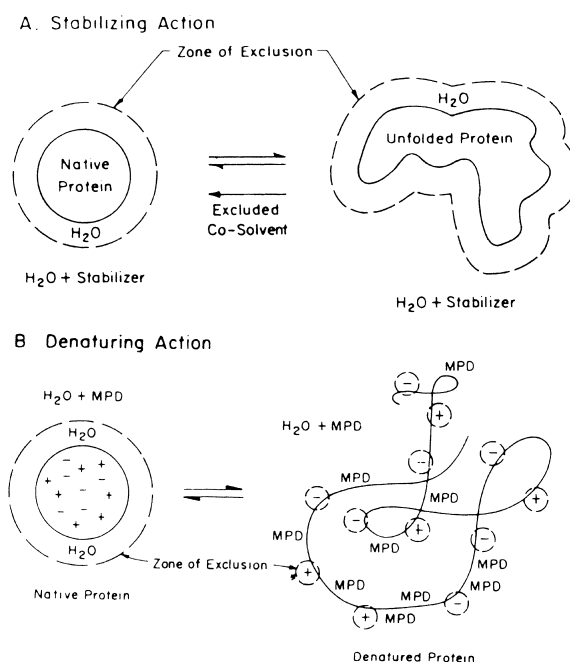


Fig. 4. Schematic illustration of preferential interactions of a protein with a protein stabilizing co-solute (A) and MPD (B). Reproduced with permission from Arakawa et al. [73]. Copyright 1990, American Chemical Society.

MPD is less excluded due to decreased charge density and shows enhanced interaction with the solvent-exposed hydrophobic surface. This should have a consequence that ξ_3 is greater for the denatured state and, thus:

$$\left(\frac{\partial \mu_2}{\partial m_3}\right)^D < \left(\frac{\partial \mu_2}{\partial m_3}\right)^N$$

Therefore, the transfer of the denatured state to a MPD-containing solution becomes more favorable (or less unfavorable) than that of the native state, leading to destabilization of the native state. For comparison, a case of the stabilizing co-solute is depicted in Fig. 4A. It is interesting to point out here that MPD and PEG are excellent cryoprotectants for proteins, comparable to other protein stabilizers [30]. Thus, the effects of MPD and PEG on protein stability are temperature-dependent [79]. This will be explained further in the following section, when the mechanism of protein stabilization by co-solutes against freeze–thawing stress is described.

3. Factors affecting freeze–thaw stability

There are several points in the processing, storage and shipping of proteins that freezing and freeze–thawing stresses may arise, either by design or accidentally. Some proteins are frozen for long-term storage and then thawed before use. Occasionally, lyophilized proteins are rehydrated, divided into aliquots and stored frozen. Frozen storage may also serve as an interim measure for holding batches of protein, prior to the preparation of a large lot by freeze-drying. However, even in liquid formulations, there is a need to stabilize proteins against freeze–thawing, because of the risks of accidental freezing during shipping and handling. Finally, the freezing stress arising during freeze-drying must be taken into consideration when designing formulation strategies for lyophilization. This stress-specific stabilization of freeze-dried proteins will be considered in detail in the next section of this review.

The most critical stresses to which a protein is exposed during freezing are low temperature and the formation of ice. Cold denaturation has been clearly documented for several proteins and, by itself is sufficient to account for the inactivation of many proteins that is noted during freeze–thawing [80–82]. Also, since the protein is excluded from the ice crystals during freezing, protein molecules are subjected to the chemical and physical changes that occur in the non-ice phase. As ice is formed, the

concentration of all solutes increases dramatically. If the solutes present in the non-ice fraction are destabilizing [83], then this concentrating effect can lead to protein denaturation. For the most part, the qualitative influence of solutes on protein stability during freeze–thawing (e.g. Refs. [83,84]) mimics that noted in non-frozen aqueous solution (certain exceptions will be discussed later). Finally, it should be noted that a solution containing buffer components such as sodium phosphate can undergo a dramatic decrease in pH at the eutectic point [85,86].

Even when such individual stresses are not damaging to the protein, a combination of factors arising during freeze–thawing, including low temperature and high destabilizing salt concentration, can be very destructive [30,80]. Also, the duration of exposure of a protein to perturbing conditions can influence how much damage occurs (e.g. [87]). This is reflected in the finding that loss of enzyme activity during freezing and thawing often is inversely correlated with the cooling and warming rates [85,88,89].

The stability of a protein against these perturbations can be increased in a protein-specific fashion by several means. In general, any factor that alters protein stability in non-frozen aqueous solution will tend to have the same qualitative effect during freeze–thawing. That is, if solution conditions such as pH and protein concentration are chosen that give maximum stability in solution, then the protein will have maximum inherent stability (defined as the ability of a protein to withstand a given stress independent of any solutes or co-factors) during freeze–thawing. For example, with many proteins, it has been found that increasing the protein concentration will increase the stability of the protein in solution and also during freeze–thawing (reviewed in [30]). Similarly, even though pH may change during freeze–thawing, an initial pH that is optimum for solution stability will tend to give the highest recovery of native protein after freeze–thawing [80,87]. Finally, specific substrates, co-factors and/or allosteric modifiers all can influence protein stability during freeze–thawing [85].

Even when all of these factors are optimized, many proteins are still denatured to a high degree during freeze–thawing. That is, their inherent stability is not sufficient to withstand the stresses involved. In these cases, cryoprotectants can be employed. A

Table 1
Cryoprotectants for proteins

Sugars:	Sucrose, lactose, glucose, trehalose, maltose
Polyols:	Inositol, ethylene glycol, glycerol, sorbitol, xylitol, mannitol, 2-methyl-2,4-pentanediol
Amino acids:	Sodium glutamate, proline, α -alanine, β -alanine, glycine, lysine-HCl, 4-hydroxyproline
Polymers:	Polyethylene glycol, dextran, polyvinylpyrrolidone
Inorganic salts:	Sodium sulfate, ammonium sulfate, potassium phosphate, magnesium sulfate, sodium fluoride
Organic salts:	Sodium acetate, sodium polyethylene, sodium caprylate, propionate, lactate, succinate
Miscellaneous:	Trimethylamine <i>N</i> -oxide, sarcosine, betaine, γ -aminobutyric acid, octopine, alanopine, strombine, dimethylsulfoxide, ethanol

wide variety of compounds (see Table 1) have been used to cryopreserve proteins (reviewed in [30,51]). The more common and obvious choices are sugars, polyols, synthetic polymers and certain amino acids. Recently, it has been found that imino acids, lactate, succinate and propionate also protect frozen proteins [91].

Perhaps not as obvious as cryoprotectants are stabilizing, inorganic salts. These are the same ions that are known as ‘salting-out’ compounds and protein stabilizers in non-frozen aqueous solution. Thus, freezing-induced concentration of salts is not necessarily detrimental to protein stability. For example, it has been found that if lactate dehydrogenase is freeze-thawed in 10 mM potassium phosphate buffer (with no other solutes), less than 20% of the initial activity is recovered [30]. However, if initial concentrations of potassium phosphate exceeding 1 M are used, greater than 60% activity is recovered. Clearly, freezing-induced concentration of salt is not responsible for the inactivation noted in the former case, since the salt itself serves as a cryoprotectant.

A wide variety of chemically diverse compounds can provide cryoprotection to proteins. Although several mechanisms have been proposed for this effect (see [30]), the only one that seems to be universally applicable to all presently known cryoprotectants is the preferential exclusion mechanism described in Section 2 of this review for the stabilization of proteins in non-frozen aqueous solutions. In fact, the one unifying characteristic of all of the cryoprotectants listed in Table 1 is that they (or chemically similar compounds) have all been shown to be preferentially excluded from the surface of proteins (reviewed in [30]). Conversely, those com-

pounds that are known to bind preferentially to proteins, such as urea and guanidine-HCl, foster additional damage during freeze-thawing [30,79]. Thus, the mechanism demonstrated by Timasheff and his colleagues for the stabilization of proteins in non-frozen, aqueous systems apparently applies equally well to protein cryopreservation [30]. That is, sub-zero temperatures, freezing-induced concentration of solutes, alterations in solution pH, and other destabilizing conditions that may arise during freeze-thawing can be viewed simply as other types of perturbations induced in solution. Preferentially excluded solutes serve to prevent these stresses from denaturing the protein during freezing and thawing. This conclusion is straightforward if one considers that the interactions of both destabilizing and stabilizing solutes with proteins during freeze-thawing actually occur in the non-ice, aqueous phase. Also, the basic thermodynamic principles governing protein stability in the frozen state should not be different from those operative in non-frozen, aqueous systems.

However, as noted above, there is one group of compounds for which their influence on protein stability in aqueous solution does not correlate with their effect during freeze-thawing. Compounds such as PEG and MPD have been shown to be excluded from the surface of proteins at 20°C and are excellent cryoprotectants (reviewed in [79]). In fact, PEG is the most effective cryoprotectant for proteins tested to date [30]. However, these same compounds destabilize proteins at higher temperatures, by lowering the thermal denaturation temperature. This apparent paradox can be resolved by considering the temperature dependence of hydrophobic interactions between proteins and co-solutes. At relatively low

temperatures, hydrophobic interactions are weaker and, for the reasons described in Section 2, the co-solutes are preferentially excluded. In contrast, at relatively high temperatures ($>25^{\circ}\text{C}$), the hydrophobic character of these compounds predominates and leads to preferential binding. This phenomenon has been considered in detail in a recent review by Arakawa et al. [79].

In summary, although a number of factors can be considered to destabilize proteins during the freeze–thaw process, the addition of a variety of compounds that are known to stabilize the proteins in solution can protect them from freeze–thaw stress. The mechanism of the co-solute-induced stabilization is probably preferential exclusion of the co-solutes from the protein surface in the frozen state. Although freeze-drying also involves the freezing process, as described below, much more specialized stabilizers are required to eliminate denaturation due to stresses arising during freeze-drying.

4. Factors affecting freeze-dry stability

Freeze-drying, or lyophilization is often used in the preparation of protein products that are not sufficiently stable for long-term storage as aqueous solutions. The lyophilization process consists of freezing the protein solution and subsequent drying in vacuo. The drying process takes place in two phases: primary drying, which removes frozen water through sublimation, and secondary drying which removes non-frozen ‘bound’ water. Freeze-drying often presents stability problems due to the conformational instability of many proteins when subjected to freezing and subsequent dehydration stresses [37]. The protein must be stabilized against both of these fundamentally different stresses. To maximize stability, parameters of the lyophilization process, such as shelf temperatures, times, and residual moisture must be optimized [92]. However, in many cases optimization of these parameters is insufficient. To further enhance the stability of freeze-dried proteins, excipients often are added to the protein solution. In this section, the mechanism of protein stabilization by additives will be reviewed.

Numerous reports have detailed empirical results obtained for freeze-drying various proteins using

different additives to enhance stability during freeze-drying and subsequent storage [93–100]. The mechanism of co-solute stabilization during freeze-drying has also been the topic of many reports and is clearly more complex than that of cryoprotection [37]. For example, phosphofructokinase (PFK) is a tetrameric enzyme that dissociates into its monomeric components upon freezing and/or drying, a process that irreversibly denatures it. Protection can be conferred against either of these two stresses by the addition of stabilizing co-solutes [30–32,35,36]. However, the requirements for protection against dehydration are much more specific [32,36,37]. Many effective cryoprotectants have no stabilizing effect during dehydration. The only solutes that do provide stabilization are disaccharides. This mechanism is not yet understood completely but it cannot involve preferential exclusion of co-solutes. The failure of many cryoprotectants to adequately protect dried PFK indicates that the mechanism of solute-induced stabilization during dehydration is fundamentally different from that for proteins in aqueous or frozen systems [36]. In addition, the thermodynamic arguments necessary to explain protein stabilization by preferential exclusion of co-solutes are not applicable when the solvent is removed from the system.

In previous reports on the stabilization of dried proteins, Carpenter and Crowe [35] suggested that certain carbohydrates might protect proteins because these co-solutes hydrogen-bond to the dried protein, thus serving as a water substitute, when the hydration shell of the protein is removed. To explore this hypothesis, Carpenter and Crowe used Fourier transform infrared spectroscopy (FTIR) to characterize the interactions between dried proteins and carbohydrates. It was found not only that hydrogen bonding occurs between proteins and stabilizing carbohydrates but also that such bonding is a requisite for labile proteins to be preserved during drying [36]. The results describing trehalose and protein interactions in the dried state illustrate these points. Fig. 5 compares the ‘fingerprint’ region of the infrared spectrum for trehalose alone with that for trehalose dried with either lysozyme or bovine serum albumin. The presence of the protein leads to a pronounced decrease in absorbance in the entire region, major shifts in band position, and a loss of band splitting. These protein-induced spectral changes can be ti-

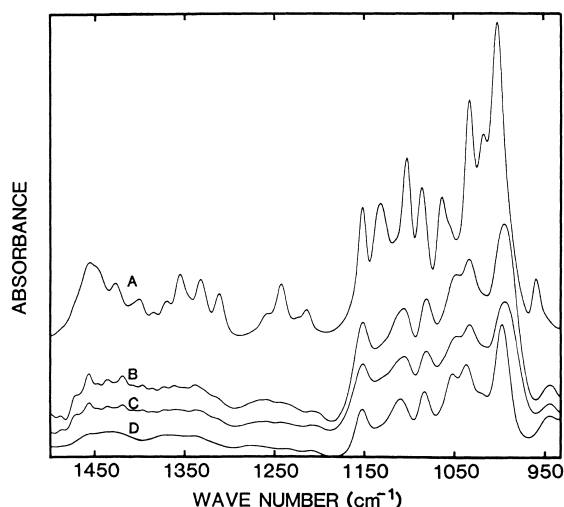


Fig. 5. Infrared spectra in the fingerprint region for trehalose freeze-dried alone (spectrum A), in the presence of 0.3 g of lysozyme/g of trehalose (spectrum B), and in the presence of 0.1 g of BSA/g of trehalose (spectrum C) and for hydrated trehalose (spectrum D). The spectra for the dried samples have been corrected for differences in the amounts of trehalose present in the samples and can be compared quantitatively as well as qualitatively. The spectrum for the hydrated sugar has been normalized, relative to the band at approximately 1000 cm^{-1} (which was previously normalized to the corresponding band in the bovine serum albumin/sugar spectrum). Reproduced with permission from Carpenter and Crowe [36]. Copyright 1989, American Chemical Society.

trated by freeze-drying the sugar with increasing amounts of protein [36]. 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 (Fig. 5). Typical spectra for trehalose dried in the presence of either lysozyme or bovine serum albumin are remarkably similar to those for hydrated trehalose, but very different from a spectrum of crystalline trehalose. Thus, proteins apparently 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.

Implicit in the conclusion that proteins serve as water substitutes for dried carbohydrates, by satisfying the hydrogen-bonding requirements of polar groups, is that the converse must also be true. To test this hypothesis, the influence of trehalose on the infrared spectrum of lysozyme was investigated.

When lysozyme is dried without the sugar, the frequency of the amide I band increases from 1652 cm^{-1} , seen for the fully hydrated protein, to 1659 cm^{-1} (Fig. 6). The amide II band is broadened and shifts from about 1543 cm^{-1} to almost 1530 cm^{-1} in the dried protein. In contrast, 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 cm^{-1} (Fig. 6). 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. It is further observed in this study that when high initial concentrations of trehalose are used, the protein spectrum is shifted back to the form noted for protein dried without the sugar. The spectrum of trehalose under these conditions is very similar to that of crystalline trehalose. Crystallization of trehalose during sublimation diminishes the availability of the sugar for forming hydrogen bonds with the protein. At similarly high concentrations, the ability of trehalose to protect the activity of PFK is abolished. Thus, Carpenter and Crowe concluded that whenever

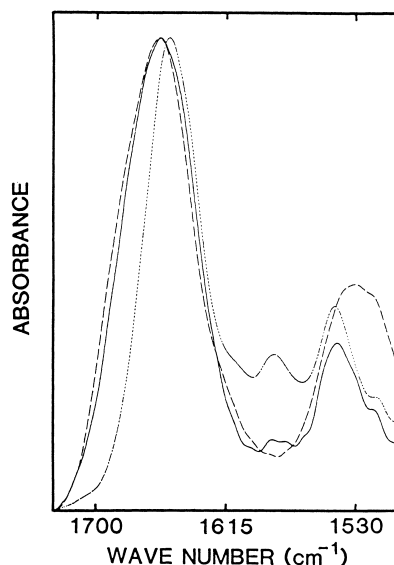


Fig. 6. Amide band region for hydrated lysozyme (dotted line), lysozyme freeze-dried alone (dashed line), and lysozyme (20 mg/ml) freeze-dried in the presence of 100 mg/ml trehalose (solid line). All spectra have been normalized. Reproduced with permission from Carpenter and Crowe [36]. Copyright 1989, American Chemical Society.

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, they proposed that hydrogen-bonding of the sugar to the protein is mandatory for the sugar to preserve dried proteins.

A report by Tanaka et al. [101] employed a different approach to examine the mechanism of freeze-drying protection by saccharides. These investigators examined the protection of catalase activity during freeze-drying by titrating various concentrations of the enzyme with different saccharides. The results indicated that the parameter that directly correlated with the protection of enzyme activity was the weight ratio of the excipient to the protein and not the bulk concentration of excipient. This result argues that a direct interaction between the excipient molecules and the protein is the basis for lyoprotection. Clearly, if direct interaction were not a requisite factor and the mechanism involved glass formation or water ordering, the bulk concentration of the additive would correlate with protection. This was not observed. These results further support the contention that direct interaction of stabilizers with protein is necessary for stabilization. In another study, Lippert and Galinski [102] found that the stabilization of freeze-dried PFK and lactate dehydrogenase (LDH) by a class of compatible solutes known as ectoines depended on the presence of hydroxyl groups on the molecule, suggesting that hydrogen-bonding is needed for stabilization during dehydration.

Other investigators have proposed that the mechanism of protein lyoprotection by various excipients is related to glass formation in the dried state [103]. That is, immobilization of the protein and additives in a solid, amorphous (i.e. glassy) state results in protection of the protein from chemical and conformational degradation because diffusion rates, and therefore reaction rates, are slow. While this is a plausible hypothesis, several lines of evidence argue against it. Firstly, in a study of the stability of freeze-dried recombinant human growth hormone, Pikal et al. [98] observed that formulations containing dextran, which remains completely amorphous and forms a glass, were destabilizing to the protein structure and resulted in a high degree of

aggregate formation after reconstitution. Secondly, in the previously noted study by Tanaka et al. [101], the authors examined the ability of various saccharide monomers and oligomers to protect catalase during freeze-drying. It was observed that the protective ability decreased as the length of the chain increased. In contrast, as the length of the saccharide chains increased, they became better glass-formers (i.e. they underwent glass transitions at higher temperatures [103]). If glass formation were the mechanism of protection, one would expect increased stability with the longer saccharide chains. However, exactly the opposite is observed. Furthermore, these authors also examined the ability of dextran, again an amorphous, glass-forming excipient, to preserve the activity of catalase after freeze-drying. It was observed that dextran was not as effective in stabilizing catalase as the lower-molecular-mass saccharides and further, that the effectiveness of dextran decreased as the molecular mass increased. This was attributed to the inability of the large polymer to interact effectively with the surface polar groups of the enzyme. Thus, while formation of an amorphous glassy state is clearly a necessary condition for protein stabilization during dehydration (in that it allows hydrogen-bonding), these two independent studies strongly indicate that glass formation is not a sufficient condition. Clearly, other interactions, such as those described above, are necessary.

During drying, the major stress that must be overcome is the removal of the protein's hydration shell, which, for at least some labile proteins, can result in irreversible loss of biological activity. The results reviewed here demonstrate that sugars such as trehalose can serve to satisfy, at least to a limited degree, the hydrogen-bonding requirements of the polar groups on dried proteins, and thus serve as water substitutes for dried proteins. However, the mechanism by which this interaction results in the preservation of enzyme activity is not completely understood. Recently, Prestrelski et al. [105] examined the conformation of lyophilized proteins using resolution-enhanced FTIR spectroscopy. These studies provide additional insight into the mechanism of protein destabilization during dehydration and solute-induced stabilization. Comparison of infrared spectra of proteins in the dried state to the native, hydrated protein indicates that moderate to rather

large conformational changes (depending on the protein studied) are induced during dehydration (Fig. 7). In several cases, denaturation and aggregation were observed during rehydration, indicating that the changes observed in dried proteins are sometimes irreversible. In contrast, when these same proteins were lyophilized under the same conditions but in the presence of stabilizing co-solutes (i.e. those that are effective at preserving the activity of labile enzymes during freeze-drying and rehydration), the native or aqueous structure was preserved in the dried state, as shown in Fig. 8. Comparison of these spectra with the aqueous spectra in Fig. 7 shows that, in the presence of stabilizing additives, the structure of the dehydrated proteins are essentially identical to those in the aqueous state. Furthermore, upon rehydration, the native state was also retained when the proteins were lyophilized in the presence of stabilizers. Based on these results, it is hypothesized that

preservation of the native structure is necessary to preserve biological activity upon reconstitution, and that the effect that stabilizing co-solutes have is through maintenance of the native structure in the dehydrated state.

Prestrelski et al. [105] also examined the conformation of a model polypeptide, poly-L-lysine, upon lyophilization. They found that, as with proteins, lyophilization induced a conformational transition. Poly-L-lysine is transformed from a random conformation in solution to a β -sheet in the dried state. The transition was attributed to loss of hydrogen bonding to water, because a random coil in solution has its hydrogen bonds satisfied by water molecules. Upon dehydration, these water molecules are removed and the peptide hydrogen bonds with each other. Saccharides were observed to inhibit the observed conformational transition, presumably by serving as a water substitute. These studies support

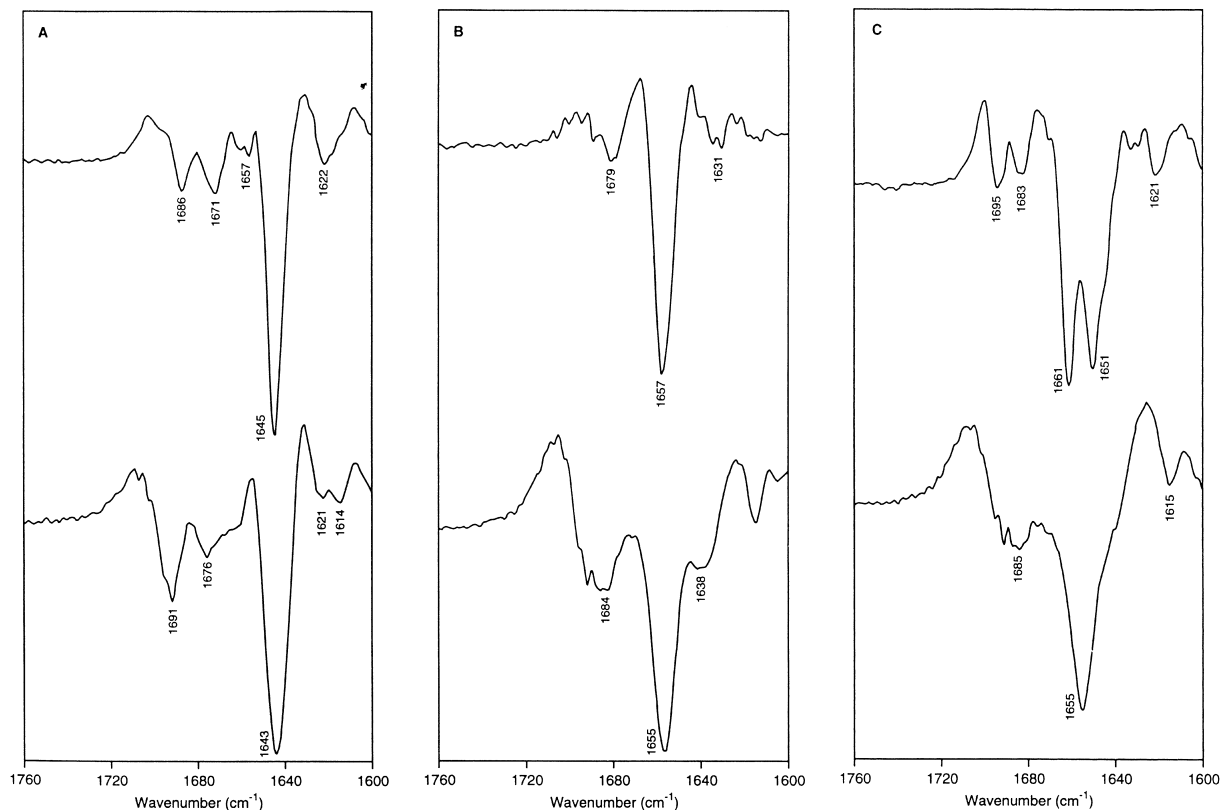


Fig. 7. Second derivative infrared spectra of (A) basic fibroblast growth factor, (B) γ -interferon and (C) α -lactalbumin in the aqueous state (upper curves) and dehydrated state (lower curves). Reproduced from Prestrelski et al. [105].

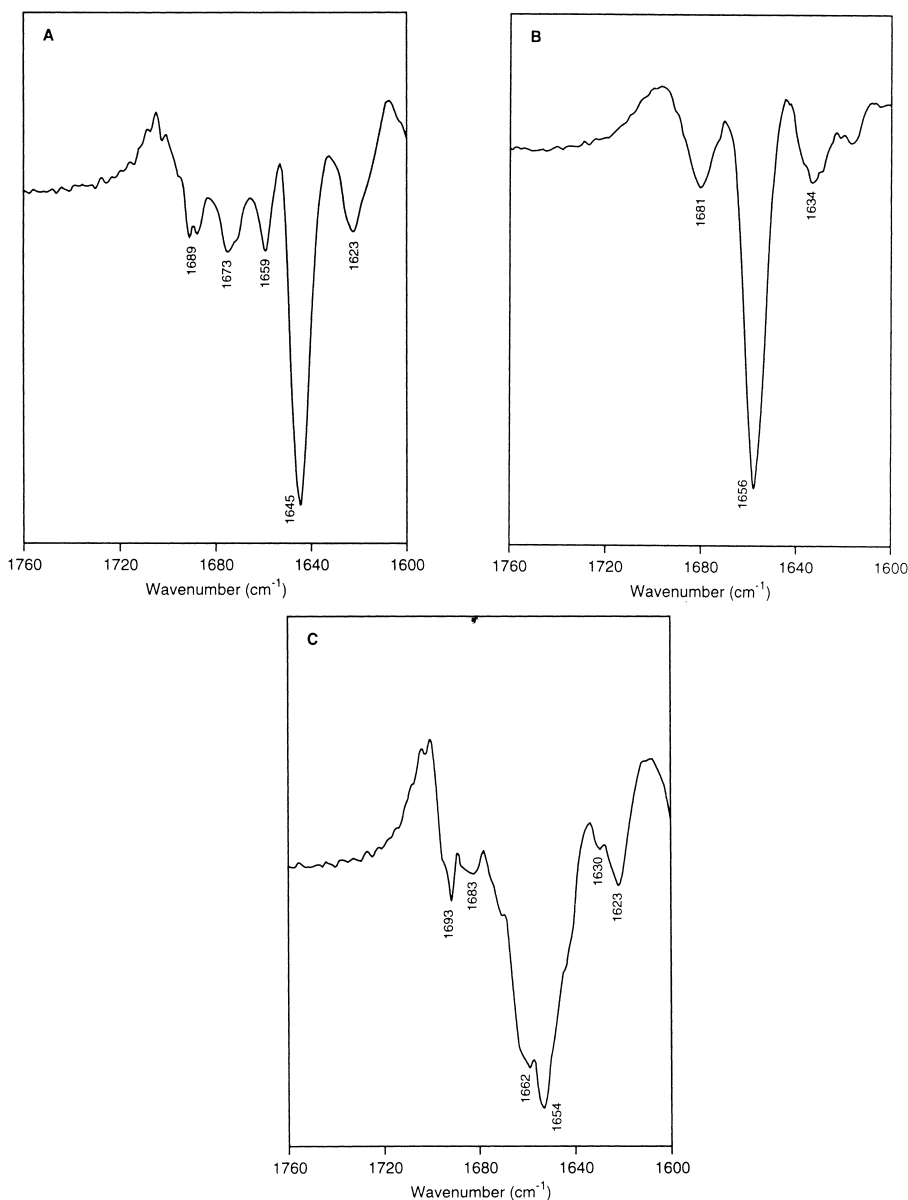


Fig. 8. Second derivative infrared spectra in the amide I region for (A) basic fibroblast growth factor, (B) γ -interferon and (C) α -lactalbumin. Lyophilized in the presence of 200 mg/ml sucrose. Reproduced from Prestrelski et al. [105].

the argument that conformational changes (i.e. unfolding) are responsible for loss of biological activity during freeze-drying and that saccharides protect proteins by preserving the native structure, presumably by hydrogen bonding [104].

An apparent dilemma arises from the argument that certain additives stabilize dried proteins through

hydrogen-bonding to proteins in place of water. If only this interaction was needed for protein preservation during the entire process of freeze-drying, then mono- and disaccharides should provide similar protection. This is not the case. When PFK is freeze-dried in the presence of 0.2–0.4 M trehalose, over 60% of the initial activity is recovered after rehydra-

tion [32]. In contrast, when similar amounts of glucose are used, the recovery is less than 5%. PFK and other labile proteins are sensitive to both the freezing and the subsequent dehydration stresses encountered during lyophilization [30–32]. Glucose does not protect freeze-dried PFK because it provides minimal stabilization during freezing, whereas trehalose is effective at stabilizing the enzyme during both freezing and dehydration [31,32]. Thus, a given additive could be highly effective at stabilizing a protein against dehydration stress. However, if this same compound does not provide cryoprotection to the protein, preservation during freeze-drying will not be realized. Since cryopreservation derives from the preferential exclusion of the additive from the protein's surface, increasing the concentration of the additive will increase protein-stabilization during freezing [30,49]. However, sugars (at high initial concentrations) can crystallize during freeze-drying and, hence, not be available to hydrogen bond to, and protect, the dried protein [36].

To resolve this apparent dilemma, the freezing and drying stresses to the protein, arising during the lyophilization process, must be treated separately. Many proteins are sensitive to both the freezing and dehydration stresses arising during lyophilization. Recently, Carpenter et al. [107] developed a two-component excipient system for stress-specific stabilization of proteins during lyophilization, in which one excipient provides cryoprotection and the second provides protection during dehydration [106,107]. In this stabilization scheme, PEG was used as a cryoprotectant and various carbohydrates were used as lyoprotectants. As shown in Fig. 9A, PEG is a very effective cryoprotectant but provides little or no stabilization during dehydration. However, when small amounts of trehalose, as well as other saccharides and polyhydric alcohols are titrated in, the two components provide excellent stabilization of proteins. In contrast, trehalose (or other saccharides and polyhydric alcohols) alone provide no stabilization at these concentrations (Fig. 9B). Thus, under conditions where cryoprotection is provided by another co-solute, glucose and trehalose are equally effective in stabilizing the dried enzymes.

In a complementary structural study of stress-specific stabilization using infrared spectroscopy, Prestrelski et al. [108] found that the recovery of

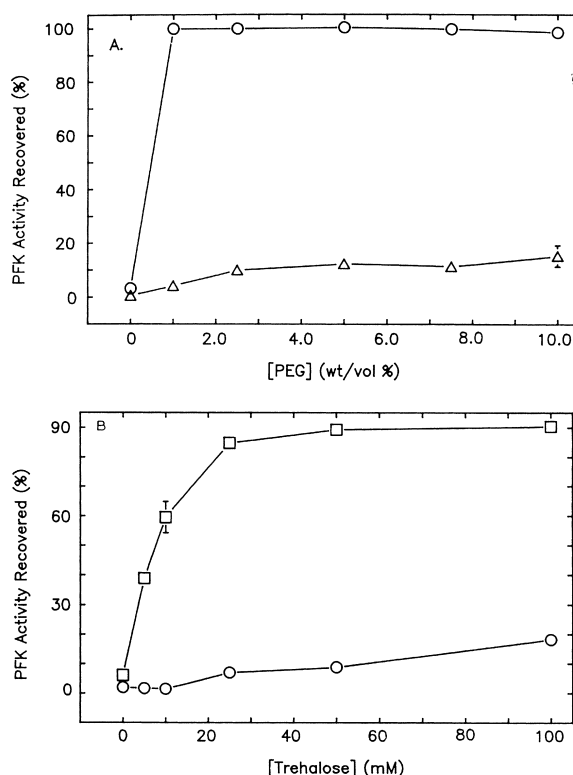


Fig. 9. (A) Effect of polyethylene glycol on phosphofructokinase stability during freeze-thawing (○) and freeze-drying (△). (B) Comparison of the influence of trehalose alone (○) and trehalose with 1% (w/v) polyethylene glycol (□) on the stability of freeze-dried phosphofructokinase.

activity after rehydration correlated directly with the ability of the excipient systems to preserve the native structure of labile enzymes in the dried state. When a combination of PEG and carbohydrates is lyophilized with the proteins, the native structure is observed in the dried state. However, when these excipients are used alone, or no excipients are used, an unfolded structure is observed in the dried state, that is, preservation of the native structure during dehydration is required for recovery of activity after rehydration.

In summary, the mechanism of solute-induced stabilization of proteins during freeze-drying involves direct interaction of solute molecules with the protein through hydrogen-bonding. The solute molecules serve as a water substitute. The effect of the water substitution is to preserve the native structure

of the protein during dehydration. An amorphous state of solute molecules appears important to allow maximal hydrogen bonding between the solute molecules and proteins. Finally, for certain labile proteins, a cryoprotectant also must be used to protect the protein during the freezing step.

The studies of protein stabilization during freeze-drying described above have emphasized stabilization during the lyophilization process itself. However, it has become evident that long-term stability of dried protein preparations is also an important factor. Again, much of the literature reports to date provide empirical results with few mechanistic data [94–100]. Izutsu et al. [96] have examined the effects of additives on the long-term stability of β -galactosidase at elevated temperatures. Many additives, including saccharides, amino acids and sugar alcohols, were able to stabilize the protein during the lyophilization process, but only disaccharides were able to impart long-term stability at 70°C. Results of differential scanning calorimetry studies of rehydrated enzymes indicate that, in the absence of disaccharides, the enzymes unfolded during storage. Conversely, disaccharides were able to preserve the folded structure of the enzyme. Thus, it appears that maintenance of the proper folded structure during long-term storage is necessary for stabilization. More studies are necessary as only the one report by Izutsu et al. has addressed this question to any degree. The recent advances in IR spectroscopic examination of dried protein conformation should provide valuable information on the conformational state at elevated temperatures.

5. Factors affecting long-term shelf stability

As noted in the previous sections, the process of freeze–thawing or freeze-drying can be detrimental to protein stability, although if the protein is stable to these procedures, then long-term storage in a frozen or freeze-dried state is often possible. However, the process of achieving these states can be more detrimental to the protein than the final states themselves.

Storing proteins in a liquid state can have a number of advantages. The process of thawing or

rehydration is not necessary and, particularly with therapeutic proteins, less manipulation is required for a liquid formulation than for a frozen or freeze-dried form. Some of what follows is equally applicable to freeze-dried, frozen, or liquid formulations, but is perhaps most relevant to the latter storage conditions.

With the emergence of recombinant proteins of therapeutic importance, the need to establish conditions for long-term storage of proteins is particularly important. In order to do so, certain events that may compromise storage stability should be understood. For example, some of the side chains of amino-acid residues that make up the polypeptide backbone of proteins are, relatively speaking, more reactive than others. Cysteiny l residues in proteins can occur as free sulfhydryl groups, or in disulfide linkages with other cysteiny l residues. During long-term storage, free sulfhydryl groups may be oxidized to the disulfide form. If a number of Cys residues are present in a protein, then several different disulfide linkages are possible. In addition to intrachain disulfide bonds, interchain disulfide bonds may form between different molecules of the protein, leading to disulfide-linked dimers, trimers, and higher-molecular-mass aggregates. In order to minimize the reactivity of Cys residues in proteins, an oxidation step is often included in the manufacturing process to convert these residues to the less active disulfide form. Non-productive disulfide linkages and higher-molecular-mass aggregates can then be removed in subsequent purification steps.

For proteins that contain an even number of Cys residues, all of the thiol groups can often be oxidized so that no free thiol remains. Some naturally occurring proteins of therapeutic interest, however, contain an odd number of Cys residues and hence, even after an oxidation step has taken place, a free cysteiny l residue remains in the final protein product. If this thiol group is not essential for activity, it can be mutated to a less reactive amino-acyl residue through the use of molecular biological and recombinant protein techniques. As a consequence, the protein analog will have an even number of Cys residues that are stabilized by disulfide linkages. An example of such a mutation is interleukin-2, in which the non-essential Cys residue at position-125 has been changed to alanine [109] or to serine [110]. The remaining Cys residues at positions 58 and 105 form

a disulfide linkage that stabilizes the tertiary structure of the protein.

Other proteins may not require mutation of a free Cys residue if it is buried within the tertiary structure of the protein and is rendered less reactive due to the localized environment. Granulocyte-colony stimulating factor (G-CSF) falls into this category, as it contains five Cys residues, four of which are involved in two disulfide bonds. The remaining free Cys residue is buried within the protein [111] and, under most conditions, is unavailable to react with other Cys residues or solvent components.

Unique examples of proteins having both types of Cys residues within a single molecule are acidic and basic fibroblast growth factors (FGFs). Based on X-ray crystal structure, basic FGF and acidic FGF have two and one solvent-exposed Cys residues, respectively. Both of the growth factors have two Cys residues that are buried, i.e. inaccessible to the solvent [112]. Mutation of the two solvent-exposed Cys residues of basic FGF reduces the propensity for formation of interchain disulfide bonds [113].

Occasionally, protein disulfide linkages cannot be formed due to lack of a nearby Cys residue in the tertiary structure of the protein. The approach then is to couple this residue oxidatively with low-molecular-mass, naturally occurring compounds, such as glutathione or cysteine. In this way, again, the disulfide linkage stabilizes the thiol residue for long-term storage.

Methionyl residues can also compromise the long-term storage of proteins. This amino acid contains a sulfur moiety in a thio-ether linkage which, over time, can become oxidized to the sulfoxide. How serious this reaction is to storage properties is in part dependent upon the local environment of the side chain. Also, this oxidation step may be minimized by decreasing the oxygen tension of the protein solution.

High on the list of reactions that can compromise the long-term stability of proteins are those of the peptide bond itself. Three reactions are significant in this regard. First is hydrolysis of the peptide bond, to form two, smaller, polypeptide chains. Second, N (nitrogen) to O (oxygen) migration can lead to a different structure at the affected position. The third involves α -carboxy to β -carboxy migration of the peptide linkage. These reactions are sequence-sensi-

tive and are most prevalent where Asp or Asn residues are one of the components of the peptide bond.

Other amino-acyl side chains that can undergo modification over a period of time include amides (deamidation of Asn and Gln), aromatic moieties (oxidation of Trp and Tyr), amino groups (acylation of the α -amino group of protein and the ϵ -amino group of lysine), and carboxyl (esterification of Glu, Asp, or C-terminal residue). Except for deamidation, which is not unlike hydrolysis of a peptide bond, these latter reactions are perhaps more specific to individual proteins and the microenvironment in which these side chains reside.

In addition to the individual amino-acyl residues, instability over time can arise from alteration of the secondary, tertiary and quaternary structure of the protein. Many forces, including electrostatic, hydrophobic and solvent-associated forces, are involved in maintaining the protein structure. Conceptually, it can be envisioned that micro regions of the protein breathe and momentarily expose certain surfaces to the environment. Thus, over time, the protein may acquire a different structure or a mixture of different structures. Moreover, hydrophobic surfaces of two proteins can associate to form dimers, which weaken the overall structure of the protein. This in turn leads to further exposure of hydrophobic areas and, consequently, increased aggregation. Conditions for long-term storage must favor the desired structure and diminish the possibility for non-productive, protein–protein interactions.

5.1. Excipient properties

When proteins are to be used in a clinical setting as a human therapeutic, certain criteria restrict the type of excipients that may be used and the conditions under which the protein is subjected to long-term storage. Excipient components must be safe, i.e. non-toxic and non-infective. The solution should be isotonic with respect to the route and mode of administration. Tonicity modifiers include salts such as sodium chloride, carbohydrates such as glucose, or mannitol. The pH should be in an appropriate range. The choice and amount of buffer used must take into account the desired pH and whether or not the buffer can be metabolized upon administration.

5.2. Stability-monitoring techniques

To assess factors that influence the long-term storage of proteins, methods must be available to recognize these changes. A bioassay is often insufficient, since it is not only labor-intensive but also relatively insensitive and variable. Optimally, stability-indicating techniques should be able to measure changes of only a few percent. Assays should be easy to perform and allow for the screening of a large number of samples. Many techniques now in use characterize the electrophoretic and chromatographic properties of the protein. Rather than measuring changes or the disappearance of the desired protein, these techniques often detect the appearance of new species in a region of the electrophoretogram or chromatogram that showed nothing when the parent protein was subjected to such a procedure. For example, sodium dodecylsulfate–polyacrylamide gel electrophoresis detects the appearance of polypeptides arising from hydrolysis of peptide bonds and of interchain-disulfide-linked dimers, trimers, and higher molecular-mass forms. Isoelectric focusing can detect the appearance of alternate forms, based on changes in charge, such as those brought about by deamidation. Size-exclusion chromatography can detect the appearance of aggregates. Changes in hydrophobicity of the protein can be measured by reversed phase-high performance liquid chromatography. Often during the development of a product, the most useful stability-indicating techniques become readily apparent for a particular protein as its electrophoretic and chromatographic properties are characterized.

5.3. Protein stability

Identifying suitable conditions for long-term stability must be performed on a protein-by-protein basis. Although considerable information exists in the literature on stabilizing proteins, this information is relatively sparse when it is restricted to those conditions that are suitable for use as a parenteral human therapeutic. Many times, such information is proprietary during development of the protein product. However, patents and published clinical studies usually give a description of protein-storage conditions. Once a protein is approved for use, the

ingredients in the formulation buffer become publicly available through the package insert or in compendia such as the *Physicians' Desk Reference*. One can surmise that excipient conditions listed in these materials are suitable for long-term storage of the particular protein being considered. Other conditions may be just as suitable. This is readily apparent for a protein where one company supplies it as a lyophilized powder while another company may supply it in liquid form.

Stability protocols should be developed concomitantly with protein–drug development. During this time, information becomes available regarding the characteristics of the protein, e.g. amino-acid composition and sequence, hydrophobicity, and techniques that identify instability. This allows for optimal manufacturing procedures at the pre-formulation and formulation stages.

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