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The Mechanism of Cryoprotection of Proteins by Solutes

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We have tested the capacity of 28 different compounds to protect lactate dehydrogenase from damage during freeze–thawing. These solutes come from very dissimilar chemical classes including sugars, polyols, amino acids, methylamines, and lyotropic salts. All the compounds tested, except NaCl, protected the enzyme, to varying degrees, from inactivation. The only characteristic that these compounds have in common, as a group, is that they have all been shown to be preferentially excluded from contact with the surface of proteins in aqueous solution. It has been demonstrated previously (via thermodynamic arguments) that this interaction of solutes with proteins leads to the stabilization of proteins in nonfrozen, aqueous systems. Conversely, those solutes, e.g., urea and guanidine HCl, that bind to proteins destabilize proteins in solution, and we have found that they also enhanced the inactivation of lactate dehydrogenase during freeze–thawing. Based on the results of our freeze–thawing experiments and a review of the theory of protein stabilization in nonfrozen, aqueous solution we propose that the cryoprotection afforded to isolated proteins by solutes can be accounted for by the fact that these solutes are preferentially excluded from contact with the protein's surface.

Over 25 years ago, Shikama and Yamazaki provided the first quantification of denaturation of an isolated protein, catalase, during freeze–thawing (30). More significantly, in this pioneering study they demonstrated that the addition of stabilizing solutes to catalase solutions could increase, to varying degrees, the recovery of activity after thawing. This cryoprotective property was not limited to a certain distinct class of chemical compounds, rather it was found that diverse types of solutes such as sugars, polyols, other proteins, and even certain salts were effective at preserving catalase (30). In subsequent years there have been many other investigations that characterized the effectiveness of additives, usually sugars or polyols, as cryoprotectants for isolated proteins (see Table 1 for a partial summary). The results from these phenomenological studies have led to much speculation about the mechanism by which the solutes exert their protective influence (see Discussion). However, to date there has been no mechanism proposed that could satisfactorily account, at least in our minds, for the fact that cryoprotection is afforded by a broad array of chemical compounds. We believe that this situation has developed because in most investigations only a few different cryoprotectants have been tested and thus any hypothetical model for stabilization has been limited to just those few compounds. The purpose of the present study was to demonstrate, using one straightforward freeze–thawing protocol, that a large variety of chemically distinct additives could afford cryoprotection to a labile enzyme, lactate dehydrogenase (LDH). Furthermore, based on the results of these experiments and a review of the literature on protein stabilization, we wished to formulate a single mechanism to explain the basis for the cryoprotection imparted by all the compounds tested.

The idea that an all encompassing mechanism for cryoprotection of isolated proteins is not only feasible, but necessary, comes from reviewing the literature on the stabilization of soluble proteins in aqueous, nonfrozen systems. There is a striking par-

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TABLE 1
List of Solutes that Afford Cryoprotection to Isolated Enzymes and that Are Known To Be Preferentially Excluded from Contact with the Surface of Proteins

Solute	Protein(s) preserved[a]	Reference(s)
Glycerol	Catalase, LDH, GDH, TPD, α-amylase, PFK	(10, 14, 15, 30, 38)
Ethylene glycol	Catalase, LDH, α-amylase	(15, 30, 38)
Inositol	PFK	(14)
Glucose	Catalase, PFK	(14, 30)
Sucrose	Catalase, chymotrypsinogen, LDH, α-amylase, PFK	(12, 14, 15, 30, 38)
Na glutamate	LDH, MDH, ADH, PK, Glu-6-PDH, myosin	(1, 32)
Glycine	PFK	(14)
Proline	PFK	(14)
Trimethylamine *N*-oxide	PFK	(14)
Na acetate	Catalase	(30)
$(NH_4)_2SO_4$	Catalase	(30)

[a] Abbreviations: LDH, lactate dehydrogenase; MDH, malate dehydrogenase; TPD, triosephosphate dehydrogenase; ADH, alcohol dehydrogenase; PK, pyruvate kinase; Glu-6-PDH, glucose-6-phosphate dehydrogenase.

allel between the types of compounds that have been found to stabilize proteins against solution-induced perturbations, such as thermally induced unfolding and pH-induced dissociation (2, 4, 6, 8, 13, 22), and those compounds that have been chosen as cryoprotectants for isolated proteins (1, 10, 12, 14, 15, 30, 32, 38). Fortunately, through the efforts of Timasheff, Arakawa, and their colleagues, the mechanism by which these solutes exert their stabilizing influence on proteins in aqueous solution has been determined (2–9, 11, 19, 20, 23, 26, 27, 33, 34). These researchers have observed experimentally that there is a deficiency of the stabilizing solute (relative to the bulk solution) in the immediate vicinity of the protein and that the protein is preferentially hydrated. That is, these solutes are preferentially excluded from contact with the surface of the protein. Thus, as Timasheff and his colleagues explain, the presence of these solutes in a protein solution creates a thermodynamically unfavorable situation since the chemical potentials of both the protein and the additive are increased. The detailed arguments that explain how such a situation could arise are beyond the scope of this paper. However, a brief summary of the reasoning used is presented under Discussion. For our purposes it is more important to understand the consequences of the entropically unfavorable state that occurs when solute molecules are preferentially excluded from contact with the protein. That is, the native structure of monomers and the polymerized form of oligomeric proteins are stabilized because denaturation or dissociation, respectively, would lead to a greater contact surface between the protein and the solvent and, therefore, augment this thermodynamically unfavorable effect (for a detailed and intuitively satisfying review of this argument see (25)).

Preferential hydration of proteins has been shown to occur in the presence of a diversity of chemically unrelated compounds including sugars, polyols, amino acids, methylamines, and inorganic salts (2–9, 11, 19, 20, 23, 26, 27, 33, 34). We believe that it is not simply coincidental that many of these same compounds have also been found to stabilize a variety of different proteins during many different freeze–thawing regimes. A list of cryoprotectants that are also known to be preferentially excluded from contact with the surface of proteins is

given in Table 1. In the present study we have tested the ability of the compounds listed in Table 1, as well as most of the other solutes that are known to be preferentially excluded from the surface of proteins, to protect lactate dehydrogenase during freeze–thawing. Based on the data generated by these experiments and our discussion of these results, we hope we can convince the reader that the mechanism established by Timasheff and colleagues for the stabilization of proteins in nonfrozen, aqueous systems is sufficient to account for the stability afforded to isolated proteins by the wide array of known cryoprotectants.

MATERIALS AND METHODS

Materials. Rabbit muscle (M_4 isoenzyme) lactate dehydrogenase (Type V-S, Lot 65F-95251) was purchased from Sigma and used without further purification. All other compounds were of reagent grade and were used as obtained from the supplier.

Assay of LDH. LDH activity was measured at 25°C. The 2.0-ml reaction mixture contained 80 m*M* Tris/HCl buffer (pH 7.5), 100 m*M* KCl, 2 m*M* pyruvate, and 0.15 m*M* NADH. The reaction was initiated by the addition of 2–10 μl of the LDH preparation and was followed by measuring the decrease in absorbance at 340 nm.

Freeze–thawing experiments. Prior to each experiment LDH was dialyzed (4°C) for several hours against 10 m*M* potassium phosphate buffer (pH 7.5 at 23°C). In one case, 10 m*M* sodium phosphate (pH 7.5 at 23°C) was substituted as the buffer. To avoid complications due to enzyme adsorption to glass, all phases of the freeze–thawing experiments were performed in polypropylene Eppendorf test tubes. An aliquot of the stock enzyme was added to the appropriate preparation of a given solute (prepared in the same type of buffer against which the enzyme was dialyzed) to give a final LDH concentration of 25 μg/ml and the highest concentration of additive to be tested. Samples of various concentrations of the additive were obtained by diluting the above mixture with a solution containing 25 μg/ml of LDH in the buffer alone. After the catalytic activity of a given sample was measured, 75 μl was transferred into another test tube and frozen by immersion in liquid nitrogen for 30 sec. Samples were then thawed at room temperature, immediately mixed, and assayed for residual activity. The results are expressed as the percentage of prefreeze activity recovered after thawing.

RESULTS

We tested the capacity of 28 different compounds to protect LDH during freeze–thawing. These solutes come from very dissimilar chemical classes and the only characteristic that they have in common, as a group, is that they have all been shown to be preferentially excluded from contact with the surface of proteins (2–9, 11, 19, 20, 23, 26, 27, 33, 34). The major chemical classes used were sugars, polyols, amino acids, methylamines, and lyotropic salts, and for the convenience of presenting the results each class is considered separately.

The first group of compounds we tested were sugars, which have been used for decades to stabilize proteins during freeze–thawing (12, 14, 15, 30, 38). As can be seen in Fig. 1. when LDH was frozen in potassium phosphate buffer alone, only about 20% of the initial activity was recovered after thawing. In the presence of glucose, lactose, and sucrose there was generally an increase in the activity recovered as the sugar concentration was increased. With 1 *M* glucose or sucrose, greater than 60 and 80%, respectively, of the initial activity were recovered. Lactose is much less soluble and was only tested at concentrations up to 0.5 *M*, yet even with this level of sugar more than 70% of the prefreeze activity was preserved.

It has been noted previously in the cryobiology literature that changes in buffer pH during freeze–thawing can affect the stabil-

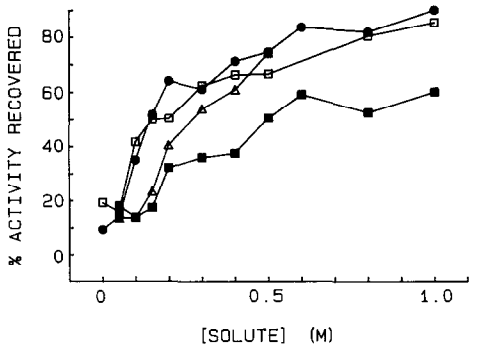

FIG. 1. Comparison of the percentage of lactate dehydrogenase activity recovered after freeze–thawing in the presence of glucose (■), lactose (△), sucrose (□) prepared in 10 m*M* potassium phosphate buffer (see Methods), and sucrose prepared in 10 m*M* sodium phosphate buffer (●).

ity of isolated enzymes (17, 31, 35, 36). To see how this phenomenon might influence the cryoprotective capacity of a sugar, we compared the stabilization provided by sucrose in sodium phosphate buffer, which is known to show a large decrease in pH at the eutectic point, to that noted in potassium phosphate buffer in which these changes are minimal (35). Without added sugar the enzyme was somewhat less stable during freeze–thawing in sodium phosphate buffer (Fig. 1). However, in the presence of sucrose the enzyme was stabilized to about the same degree in either buffer system (Fig. 1).

Another group of compounds that frequently have been used to protect soluble enzymes during freeze–thawing are the polyols (10, 14, 15, 30, 38). In Fig. 2 it can be seen that glycerol, sorbitol, mannitol, and ethylene glycol all provided about the same degree of protection to LDH, with about 70% of the prefreeze activity recovered when these solutes were present at 1 *M* concentrations. Xylitol was somewhat less effective, and inositol provided no stabilization except at the highest concentration tested (0.7 *M*).

For comparison, we have also included here (Fig. 2) results for 2-methyl-2,4-pentanediol (MPD), even though it is not actually a polyol since only two of the six carbons have hydroxyl groups. MPD was far superior to the above solutes at protecting LDH, with over 70% of the initial activity being recovered in 50 m*M* MPD and essentially no loss of activity occurring if MPD was at a concentration of $\geq$0.4 *M*. Even more impressive were the results with poly(ethylene glycol) (MW 600). There was almost no loss of activity when this solute was present at concentrations as low as 25 m*M* (Fig. 2).

Although amino acids are not used as often as the sugars or polyols as cryoprotectants, sodium glutamate and proline previously have been employed for this purpose (1, 14, 32). From the results presented in Fig. 3, it is apparent that the ability to stabilize frozen enzymes is not unique to these particular amino acids. In the pres-

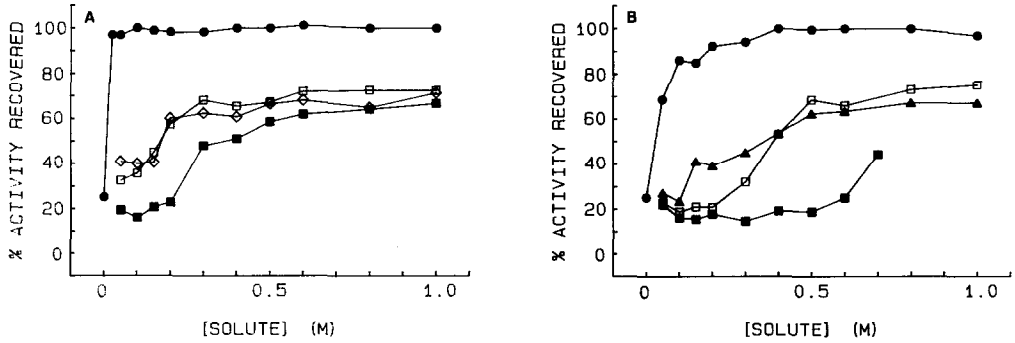

FIG. 2. Comparison of the percentage of lactate dehydrogenase activity recovered after freeze–thawing in the presence of (A) poly(ethylene glycol) (●), ethylene glycol (□), glycerol (◇), and xylitol (■); and (B) 2-methyl-2,4-pentanediol (●), sorbitol (□), mannitol (▲), and inositol (■).

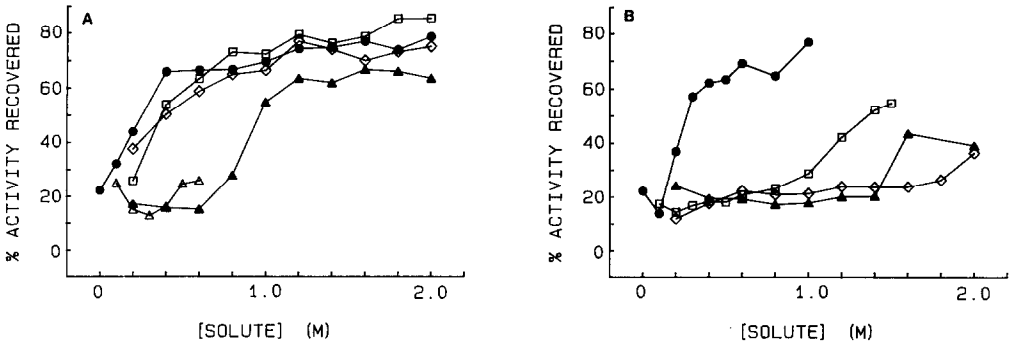

FIG. 3. Comparison of the percentage of lactate dehydrogenase activity recovered after freeze–thawing in the presence of (A) lysine HCl (□), proline (●), Na glutamate (◇), serine (▲), taurine (△); and (B) γ-aminobutyric acid (●), α-alanine (□), β-alanine (◇), and glycine (▲).

ence of >1 *M* proline, sodium glutamate, or lysine HCl almost 80% of the initial activity was recovered after thawing. At 1 *M* concentration, γ-aminobutyric acid provided a similar level of protection. Serine was somewhat less effective, with at most about 65% of the prefreeze activity remaining. With 1.5 *M* α-alanine, about 55% of initial activity remained after thawing, whereas the same level of β-alanine provided no protection, and even at a concentration of 2.0 *M* less than 40% recovery was noted with the β form. The results with glycine were very similar to those for β-alanine, except that the maximum recovery was seen with 1.6 *M* glycine. In contrast to the other amino acids, taurine provided only a minimal degree of protection, which may be attributed to its limited solubility; this amino acid could only be used at concentrations up to 0.7 *M*.

The next group of compounds we tested were the methylamines, which includes trimethylamine *N*-oxide, sarcosine, and the amino acid betaine. These molecules have only recently been recognized to have the capacity to stabilize proteins in solution (8, 22), and one of them, trimethylamine *N*-oxide, has been shown to protect labile enzymes during freeze–thawing (14). At concentrations of ≤0.5 *M*, trimethylamine *N*-oxide was the most effective at stabilizing LDH during freeze–thawing (Fig. 4). However, at higher concentrations sarcosine provided almost equivalent protection, with about 60% of the initial activity recovered. In contrast, with 1.0 *M* betaine only about 45% of the prefreeze activity remained after thawing.

The final set of stabilizing molecules tested were inorganic salts, which have received little attention as possible cryoprotectants. However, salting-out salts have long been recognized as stabilizers of proteins in solution and it is known that they exert this effect in the same manner as do organic solutes (3, 5, 6). Therefore, if our general hypothesis, that preferential exclusion of cryoprotectants from the surface of proteins leads to stabilization, is correct, then it is imperative that salting-out salts also stabilize enzymes during freeze–

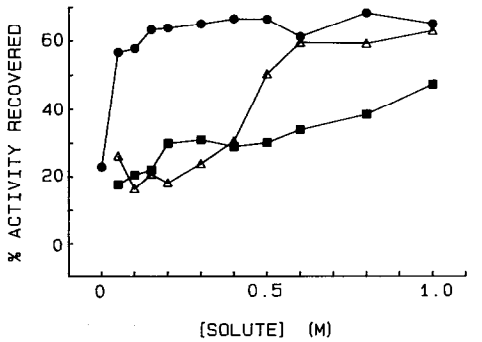

FIG. 4. Comparison of the percentage of lactate dehydrogenase activity recovered after freeze–thawing in the presence of trimethylamine *N*-oxide (●), sarcosine (△), and betaine (■).

thawing. In Fig. 5 it can be seen that this is the case. At the highest concentrations tested, freeze–thawing in the presence of $(NH_4)_2SO_4$, $MgSO_4$, Na_2SO_4, and potassium phosphate led to a recovery of 55, 62, 39, and 64%, respectively, of the initial LDH activity. With the latter three salts, at the lower concentrations tested (ca. <0.5 *M*) the enzyme was actually less stable than it was without added salt. This same sort of trend can be seen in the results with some of the organic solutes as well (e.g., glucose, lactose (Fig. 1); xylitol, sorbitol, inositol (Fig. 2); and serine, taurine, α-alanine, β-alanine, γ-aminobutyric acid (Fig. 3)); this phenomenon is considered further under Discussion.

The only salt tested that did not protect LDH during freeze–thawing was NaCl (Fig. 5). In the presence of ≤0.5 *M* concentrations of this salt the enzyme was completely inactivated. At higher concentrations there was some activity recovered, which suggests that, as noted above, the destabilizing influence of a given compound can be overcome by higher concentrations of that same compound. However, even with the addition of 3.0 *M* NaCl only 20% of the initial activity was recovered, which was less than that noted without added salts.

We have also included sodium acetate here (Fig. 5) because, although this compound is not an inorganic salt, it is known to be very effective at salting-out proteins (3). Of the salts tested, sodium acetate proved to be the best cryoprotectant with about 80% recovery of activity seen with concentrations of 1.6 *M* or greater.

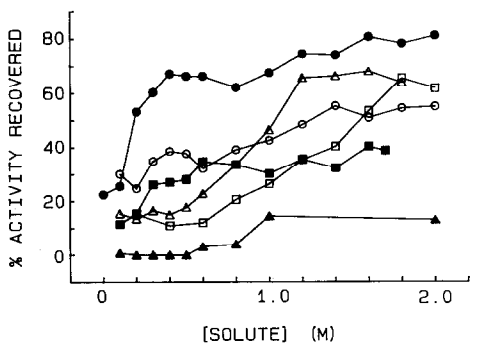

FIG. 5. Comparison of the percentage of lactate dehydrogenase activity recovered after freeze–thawing in the presence of potassium phosphate (△), Na acetate (●), $MgSO_4$ (□), $(NH_4)_2SO_4$ (○), Na_2SO_4 (■), and NaCl (▲).

Finally, it has been well established that solutes that denature proteins in solution have an interaction with the protein opposite that of stabilizing solutes. That is, compounds such as urea and guanidine HCl bind preferentially to the protein, and this binding can foster unfolding of polypeptide chains (6, 28). Therefore, one would expect that if the "rules" that apply to protein stability in solution apply during freeze–thawing, then urea and guanidine HCl should destabilize frozen and thawed enzymes. To test this hypothesis, it was necessary to start with a system in which LDH was more stable against freeze–thaw-induced inactivation. The simplest means to accomplish this was to increase the concentration of the enzyme used, an adjustment that has long been recognized to increase the stability of LDH and other enzymes (15, 21, 31, 32). We found that the recovery of initial activity could be increased to greater than 60% when the enzyme was frozen at concentrations of 175 μg/ml or greater (Fig. 6). As predicted, the addition of guanidine HCl or urea to a preparation containing 175 μg/ml of LDH greatly destabilized the enzyme (Fig. 6). With urea there was a monotonic decrease in recovered activity with increasing urea concentration, and less than 27% of the initial activity remained with 200 m*M* urea. Guanidine HCl was an even more potent destabilizer, with a sharp drop in activity seen at concentrations greater than 20 m*M* and no LDH activity measurable after the enzyme was frozen and thawed with ≥80 m*M* guanidine HCl. Prior to freezing, the presence of either urea or guanidine HCl at the highest concentrations tested led to a less than 8% reduction in the activity of the enzyme.

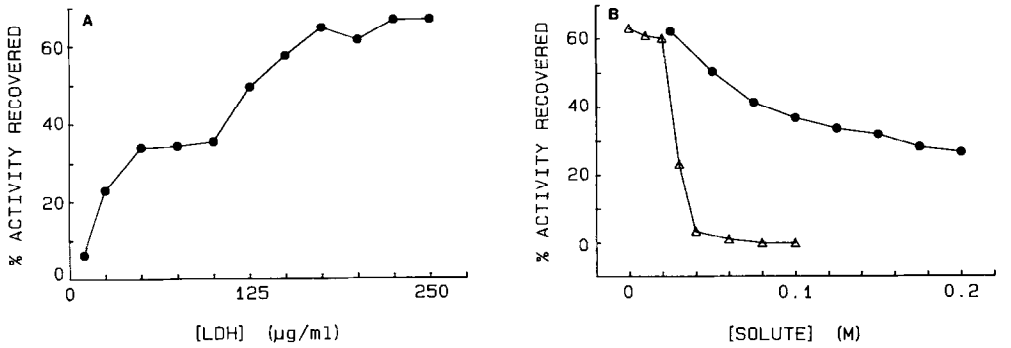

FIG. 6. (A) Effect of varying enzyme concentrations on the stability of lactate dehydrogenase during freeze–thawing. (B) Influence of urea (●) and the guanidine HCl (△) on the stability of lactate dehydrogenase (175 μg/ml) during freeze–thawing.

DISCUSSION

Several possible mechanisms for solute-induced stabilization of proteins during freeze–thawing emerge from a study of the literature: (i) binding of the solute to the protein; (ii) colligative action of the solute; (iii) prevention of potentially damaging changes in solution properties; (iv) inhibition of formation of interprotein disulfide bridges; and (v) preferential exclusion of the solute from the protein surface. We discuss the appropriateness of each of these alternatives in the following paragraphs.

Previously Proposed Mechanisms for Protein Cryoprotection

Solute binding. In this study we have demonstrated that all but one of 28 different compounds that are known to stabilize and/or salt out proteins in nonfrozen aqueous solution (2–9, 11, 19, 20, 23, 26, 27, 33, 34) are effective cryoprotectants for lactate dehydrogenase. Since this protective influence is provided by distinctly different classes of chemicals, which vary greatly in structure, relative hydrophobicity, charge, and orientation of dipoles, any mechanism to describe this protection that invokes a specific interaction of a solute with the protein is unlikely. In addition, the fact that relatively high concentrations of solutes (e.g., >0.5 *M*) are usually needed to achieve cryoprotection would argue against any specific binding being the dominant force involved in the stabilization.

Recently, a mechanism invoking nonspecific solute binding to proteins has been proposed (1, 32) to account for the cryoprotection afforded by sodium glutamate. It is suggested that ionized glutamate is associated with the surface of the protein, leading to an increased negative charge and then to increased hydration of the protein. It is then stated that these factors prevent (by some undescribed mechanism) the alteration of the protein's conformation and the aggregation of protein molecules that are induced by removal of water during freezing. One mechanism by which potent protein denaturants, such as urea and guanidine HCl, operate is by binding to the protein, and it does not seem feasible thermodynamically that similar nonspecific binding of glutamate would lead to stabilization. We deal with a related phenomenon, the association of proline with certain proteins (29), in another context below.

Colligative properties. Although the influence of the stabilizing solutes could be based on their colligative properties, this is clearly not the case since other solutes, urea and guanidine HCl, actually destabilize lactate dehydrogenase during freeze–thawing (Fig. 6).

Prevention of changes in solution prop-

erties. The suggestion here has been that cryoprotectants prevent the alteration of solution properties that occur during freeze–thawing (e.g., the change in pH at the eutectic point) and/or serve to dilute the high concentrations of salts that occur as ice is formed (17, 31). It seems unlikely that the broad array of solutes that we have found to be cryoprotectants (many of which are salts) could all be expected to have the capacity to block these putatively destructive alterations in solution properties, and the resulting protein denaturation, during freeze–thawing. Furthermore, such an hypothesis would not explain our finding that in 10 m*M* potassium phosphate buffer LDH is greatly inactivated during freeze–thawing, but at much higher concentrations of the same buffer (with no other additives) the enzyme is protected.

Disulfide bridges. Another hypothesis that has been advanced to explain the loss of enzyme activity is that during freeze–thawing individual protein molecules unfold, and as ice is formed the increased protein concentration fosters the formation of intermolecular disulfide bridges (24). This aggregation is thought to prevent the enzyme molecules from returning to their native, active conformation after thawing. It seems unlikely that this model for protein inactivation is generally correct since enzyme inactivation during freeze–thawing can occur when reducing agents are present (14). Furthermore, if aggregate formation did lead to protein inactivation, then increasing the concentration of protein should foster even greater inactivation rather than the increased stability that is usually noted (15, 21, 31, 32). However, even if this model were correct, the role of the cryoprotectant should be to prevent the protein from unfolding and not, as has been suggested (24), to intercalate between the unfolded protein molecules and block the formation of disulfide bridges. If the protein were to unfold in the presence of stabilizing solutes, then the increase in the chemical potential of the protein that is induced by the solutes should lead to an increase in the degree of protein aggregation.

Preferential Exclusion of Cryoprotectants from Contact with Proteins

Timasheff and his colleagues have already provided an explanation that accounts for the fact that proteins can be protected in nonfrozen, aqueous systems by a diverse array of compounds. They determined that stabilizing and salting-out solutes all exert their influence because they are preferentially excluded from the surface of the proteins in aqueous solution (2–9, 11, 19, 20, 23, 26, 27, 33, 34). Conversely, guanidine HCl and urea bind preferentially to proteins and induce denaturation (since more binding sites are exposed as the protein unfolds), especially in the face of additional stress on the protein such as high temperature or freeze–thawing (6, 8, 28) (Fig. 6). Since we found that all but one of the solutes that are known to be preferentially excluded from contact with proteins were effective cryoprotectants (and those that bind to proteins enhance inactivation of LDH during freeze–thawing), we conclude that the mechanism demonstrated by Timasheff for the stabilization of proteins in nonfrozen, aqueous systems applies equally well as an explanation for how solutes act as cryoprotectants for isolated proteins.

We are suggesting that subzero temperatures, the osmotic shock that occurs as ice is formed, alterations in solution pH, and other destabilizing conditions that may arise during freeze–thawing (17, 31, 35) can be viewed simply as other types of solution-induced perturbations. As Timasheff and his colleagues have proposed for such perturbations in unfrozen solutions, we suggest that the stabilizing solutes serve to prevent these forces from denaturing the protein in the frozen state. This conclusion should not be surprising since the interactions of both destabilizing and stabilizing solutes with proteins during freeze–thawing

actually occur in the aqueous phase. Furthermore, as stressed by Franks (18), the basic thermodynamic principles governing protein stability in frozen systems should not be different than those that have been defined for the unfrozen state.

Basis for preferential exclusion: Effects on surface tension of water. Unfortunately, there are other compounds that have been used to preserve isolated proteins during freeze–thawing for which there are no published reports on the nature of their interaction with proteins. It is possible to infer, however, that these cryoprotectants are also preferentially excluded from contact with proteins by examining the mechanisms by which this situation has been shown to arise with chemically related solutes. For example, we have found that the disaccharides trehalose and maltose are very effective cryoprotectants for the extremely labile enzyme phosphofructokinase (14). Timasheff and his colleagues have shown that other sugars are excluded from the surface of proteins because the sugars raise the surface tension of water, and thereby increase the cohesive forces within the water structure (2, 23). In this situation, the lower surface tension bulk water tends to hydrate the protein since disruption of the solute–water interactions to form protein–water interactions is energetically unfavorable. Thus, the high surface tension solute water with its solute is excluded from contact with the protein (cf. (25)). Since this effect on the surface tension of water is a general property of sugars (and many other solutes) (2–5, 23) it is reasonable to suggest that trehalose and maltose should also be preferentially excluded from contact with the surface of proteins and that this property accounts for their cryoprotective capacity.

Basis for preferential exclusion: Steric effects. Other effective cryoprotectants, for which the nature of their interactions with proteins has not been determined, are the polymers polyvinylpyrrolidone and dextran (10). For these solutes the basis by which they might be preferentially excluded from contact with proteins can be attributed to the mechanism described for poly(ethylene glycol). That is, these bulky solutes are sterically hindered from making contact with the protein's surface, whereas water can hydrogen bond to almost any region on the protein's surface without geometric constraint (9). Thus, the protein will be preferentially hydrated and the bulkier polymer will be displaced away from the protein.

Steric effects on protein–protein interaction. Extension of these arguments might even account for the increased stability that is noted during freeze–thawing as the concentration of the enzyme is increased (15, 21, 31, 32). This phenomenon has been demonstrated for a number of enzymes, and furthermore it has been found that the addition of even a different protein (e.g., bovine serum albumin) will also increase the stability of an enzyme during freeze–thawing (15, 21, 32). We propose that, for enzymes that do not undergo self-association into polymers, the steric hinderance of protein–protein binding effectively mimics the situation seen with other nonprotein polymers. Thus, since individual protein molecules cannot penetrate the hydration shell of neighboring protein molecules, the arguments set forth by Timasheff to explain protein stabilization by solutes that are preferentially excluded from contact with the protein surface should be applicable.

Exceptions to the preferential exclusion rule. In general, demonstration that a given compound is excluded from contact with any protein in solution should be sufficient to predict that the solute will protect other proteins during freeze–thawing. However, this is not always the case since we have found that NaCl, which is known to foster preferential hydration of some proteins, did not protect frozen LDH. Timasheff (2–9, 11, 19, 20, 23, 26, 27, 33, 34) and others (16, 37) have shown that the influence of a given

solute on the stability of a protein derives from a balance between the destabilizing effects of solute binding to a protein and the stabilization imparted when the solute is excluded from the protein. The relative contributions of either force depend on the surface properties of the protein as well as the concentration of solute present. For example, all salts will be attracted electrostatically to polar regions of a protein and will bind to those regions. Only when this binding is offset and overcome by repulsive forces (e.g., the presence of hydrophobic groups or the surface tension effects noted above) will the salt serve as a stabilizer (cf. (25)). Thus, when it is said that a solute is preferentially excluded from contact with a protein this does not necessarily mean that no solute molecules are able to penetrate the hydration shell of the protein; rather it means, that to some degree, there is a deficiency of the solute in the immediate domain of the protein relative to the solute concentration in the bulk solution.

It is now possible to offer an explanation for our finding that NaCl failed to preserve LDH during freeze–thawing even though this salt is known to be preferentially excluded from contact with (and to salt out) other proteins (3). This failure is most likely due, within the range of NaCl concentrations we tested, to an excess of salt binding to LDH relative to repulsion of the salt from the protein. The same argument can also be put forth to explain the decrease in enzyme stability we noted when LDH was frozen and thawed in the presence of a low concentration of a solute, which at higher concentrations provided cryoprotection. That is, at low solute concentration the dominant interaction is probably solute binding to the protein, but above a certain concentration the stabilizing effect of solute exclusion from the protein would be expected to supersede (cf. (6)). This points to the importance of testing a wide range of solute concentrations when trying to determine if a given solute can preserve a given enzyme during freeze–thawing.

Another apparent exception to the rule that must be explained is proline. There is one report showing that the solubility of β-lactoglobulin was increased in the presence of proline at concentrations ≥ 2 *M* (29). It was proposed that the hydrophobic pyrrolidine ring of the amphiphile proline associates with the hydrophobic portions of proteins, thus converting these groups into hydrophilic regions and thereby increasing the solubility of the proteins (29). If this were a general property of proline, then one would predict (see results for urea and guanidine HCl) that at very high concentrations this amino acid should actually destabilize LDH during freeze–thawing. We found, however, that in the presence of 3 and 4 *M* proline LDH was very stable, with 92 and 97%, respectively, of the initial activity recovered after thawing (also see Fig. 3). These results, in concert with the finding that proline is preferentially excluded from contact with lysozyme (8), suggest that the putative binding of proline to proteins, and the concomitant increase in protein solubility, is not a ubiquitous property of this amino acid. Rather, it appears that binding dominates over exclusion only for certain relatively hydrophobic proteins such as β-lactoglobulin (2, 29).

Summary and Conclusions

We conclude that the bulk of the available evidence is consistent with the preferential exclusion hypothesis and that the thermodynamic arguments advanced by Timasheff and colleagues for stabilization of proteins by solutes in aqueous solution apply equally well to stabilization in the frozen state. However, we must offer the caveat that the demonstration that a solute is preferentially excluded from contact with one protein does not necessarily assure that this solute will have the capacity to preserve another protein during freeze–

thawing. Nevertheless, we suggest that those compounds that are shown to afford cryoprotection to a given protein will be found to be preferentially excluded from contact with the surface of that protein.

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