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Increased Thermal Stability of Proteins in the Presence of Naturally Occurring Osmolytes†

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ABSTRACT: Organisms and cellular systems which have adapted to stresses such as high temperature, desiccation, and urea-concentrating environments have responded by concentrating particular organic solutes known as osmolytes. These osmolytes are believed to confer protection to enzyme and other macromolecular systems against such denaturing stresses. Differential scanning calorimetric (DSC) experiments were performed on ribonuclease A and hen egg white lysozyme in the presence of varying concentrations of the osmolytes glycine, sarcosine, *N*,*N*-dimethylglycine, and betaine. Solutions containing up to several molar concentrations of these solutes were found to result in considerable increases in the thermal unfolding transition temperature (T_m) for these proteins. DSC scans of ribonuclease A in the presence of up to 8.2 M sarcosine resulted in reversible two-state unfolding transitions with T_m increases of up to 22 °C and unfolding enthalpy changes which were independent of T_m. On the basis of the thermodynamic parameters observed, 8.2 M sarcosine results in a stabilization free energy increase of 7.2 kcal/mol for ribonuclease A at 65 °C. This translates into more than a 45 000-fold increase in stability of the native form of ribonuclease A over that in the absence of sarcosine at this temperature. Catalytic activity measurements in the presence of 4 M sarcosine give k_{cat} and K_m values that are largely unchanged from those in the absence of sarcosine. DSC of lysozyme unfolding in the presence of these osmolytes also results in T_m increases of up to 23 °C; however, significant irreversibly occurs with this protein. Naturally occurring glycine-based osmolytes appear to provide a general method of stabilizing proteins against thermal unfolding even well beyond the physiological concentration range for osmolyte, and the degree of stabilization can be extraordinary.

Considerable time in nearly all fields of biochemical sciences is devoted to stabilizing proteins and developing empirical formulations and protocols for retaining the activity of purified preparations of peptides and proteins. While protein stabilization is a vexing issue in research, it is no less of a problem in living systems (Yancey et al., 1982). Organisms have constantly had to address the problem, and have done so with far more constraints and fewer options than are faced in a laboratory setting. In particular, organisms which are subjected to environmental stresses such as heat, dehydration, or extreme solution conditions such as high salt or urea must maintain biological activity in the face of rather severe denaturing stresses (Somero & Clark, 1985; Somero, 1986;

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Yancey et al., 1982). In such organisms, it appears that a major means of providing protection against the denaturing stresses is to produce tens to hundreds of millimolar intracellular concentrations of certain low molecular weight compounds, known collectively as osmolytes (Yancey et al., 1982). The presence of such osmolytes has been shown in vitro to provide stability to enzymes without substantive alteration in their catalytic action (Borowitzka & Brown, 1974; Bowlus & Somero, 1979; Pollard & Wyn Jones, 1979; Yancey, 1985). While such studies are limited in number, they suggest that these naturally occurring compounds are able to confer stabilization to proteins in vivo without a deleterious effect on biological activity.

Chemically, the naturally occurring osmolytes appear to fall into three major classes of compounds which protect protein systems: certain polyols, amino acids, and methylamines (Yancey et al., 1982). In terms of function, however, they have been classified either as compatible solutes, which have little effect on protein function, or as counteracting solutes, which offset the structural and functional effects which deleterious solutes in the cell have on proteins (Borowitzka & Brown, 1974; Yancey et al., 1982). In the animal kingdom, counteracting osmolytes (such as certain methylamines) have evolved in cellular systems which concentrate urea (Bagnasco et al., 1986; Garcia-Perez & Burg, 1990; Somero, 1986; Yancey et al., 1982). Cartilaginous fish maintain rather high levels of urea in their cells, and the expected detrimental effects of urea appear to be offset by the presence of counteracting osmolytes. This same basic mechanism for protection against urea also appears to occur in kidney (Bagnasco et al., 1986; Garcia-Perez & Burg, 1990; Yancey & Burg, 1989, 1990).

The ability of naturally occurring osmolytes to protect collections of proteins without disturbing metabolic function would appear to provide an important and general selective advantage for organisms to adapt to environmental stresses (Somero, 1986). Such selective advantage would greatly reduce the necessity to genetically modify the organism's protein and enzyme systems in response to the environmental stress. Studies of protection against activity loss associated with naturally occurring osmolytes have generally focused on characterization of enzyme activity and stability in the presence of up to 1 M protecting osmolyte when the system is subjected to heat stress or denaturant stress such as the presence of urea (Yancey, 1985; Yancey et al., 1982). Such studies appear to support the premise that osmolytes stabilize proteins against heat and denaturant stresses and suggest that a more quantitative and detailed investigation would be useful in establishing the scope and character of the phenomena.

In this report, we look at the effects of particular methylamines in stabilizing ribonuclease A (RNase A)[1] and hen egg white lysozyme (HEW lysozyme) against thermal unfolding. For purpose of comparison, we wanted to study the effects of osmolytes which are chemically similar to one another, and this led us to investigate glycine-based osmolytes. This family includes glycine, a naturally occurring compatible osmolyte, along with sarcosine (*N*-methylglycine) and betaine (*N*,*N*,*N*-trimethylglycine), two naturally occurring counteracting osmolytes (Bowlus & Somero, 1979; Yancey et al., 1982). In order to include all members of the methylglycine series, the effects of nonnaturally occurring osmolyte, *N*,*N*-dimethylglycine, were also studied. Our objective was to provide an assessment of thermal stabilization effects in quantitative thermodynamic terms over an extended concentration range of osmolytes. In addition, we wanted to look at the related question of whether naturally occurring osmolytes might also promote refolding and reactivation of thermally unfolded protein.

Materials and Methods

Bovine pancreas ribonuclease A and chicken egg white lysozyme were purchased from Sigma Chemical Co. and used without further purification. Osmolytes and suppliers used in this study include the following: betaine and *N*,*N*-dimethylglycine from Sigma Chemical Co.; glycine from Matheson Coleman & Bell; sarcosine from Fluka. Except for sarcosine, which was recrystallized from water, the osmolytes were used as purchased without further purification.

Differential scanning calorimetry (DSC) measurements were made using a MicroCal MC-2 differential scanning calorimeter with software supplied by MicroCal and Dr. Ernesto Freire. The DSC experiments were carried out at pH 5.8 or 6.0 with protein concentrations of around 1 mg/mL in degassed 20 mM citrate buffer containing 55 mM NaCl. All such experiments were conducted under a nitrogen pressure of 30 psi at a scan rate of 1 °C/min. The raw data, in the form of heat flow (millicalories per minute), were converted to excess heat capacity (C_p, in kilocalories per mole per degrees kelvin) and fitted to a single-transition model allowing both the calorimetric and van't Hoff enthalpy changes to float. In nearly all cases, the two-state model was also found to fit the data satisfactorily. Molar absorptivities of 9800 L/(mol·cm) at 278 nm and 37 700 L/(mol·cm) at 280 nm were used for RNase A and HEW lysozyme, respectively (Sela & Anfinsen, 1957; Sophianopoulos et al., 1962).

Kinetic studies of RNase A catalyzed cyclic cytidine monophosphate hydrolysis were performed at a wavelength of 292 nm. Two types of experiments were performed: one with the enzyme first being incubated with osmolyte but assayed in substrate solutions which did not contain osmolyte and a second type of experiment in which the enzyme was both incubated and assayed in the stated concentration of osmolyte. In assays performed in the presence of osmolytes, extinction coefficient differences ($\Delta\epsilon_{292}$) between substrate and product were determined as a function of sarcosine concentration and used in evaluation of k_{cat} and K_m at the sarcosine concentrations reported. The enzyme was exposed to sarcosine prior to its addition to kinetic assays containing the same concentration of sarcosine.

Results

The midpoint temperature (T_m) of a thermal unfolding transition for a protein is one of the most commonly used indexes of protein stability. Figure 1 provides evidence that increasing concentrations of sarcosine, a glycine-based osmolyte, increase the T_m values for RNase A by rather large amounts. From a thermodynamic point of view, such data become considerably more useful if the unfolding process exhibits characteristics of reversibility and two-state behavior. All of the scans shown in Figure 1 conform very well to the two-state model ($\Delta H_{van't\ Hoff} = \Delta H_{cal}$) as evident from the close correspondence of the two-state model to experimental data. Reversibility of RNase A unfolding, as defined by the reproducibility of the endotherm obtained on rescan, was found to be 90% or better with sarcosine as a perturbant.

Measurements of the kinetic characteristics of RNase A before and after a DSC scan in 5.5 M sarcosine provide a more stringent test of reversibility. Using cyclic cytidine mono-

[1] Abbreviations: RNase A, ribonuclease A; HEW lysozyme, hen egg white lysozyme; ΔH_{cal}, calorimetric enthalpy change; $\Delta H_{van't\ Hoff}$, van't Hoff enthalpy change; T_m, midpoint temperature of a thermal unfolding transition; DSC, differential scanning calorimetry; ΔC_p, apparent heat capacity change.

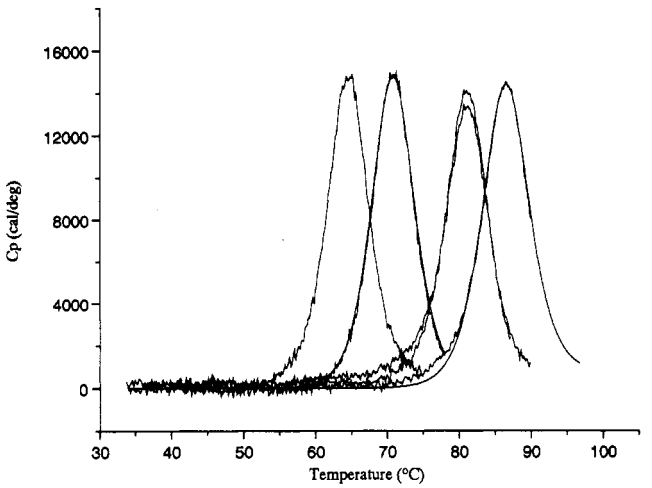

FIGURE 1: Representative DSC scans of RNase A in the presence of different concentrations of sarcosine. Endotherms from lower to higher T_m represent sarcosine concentrations of 0.0, 2.0, 5.5, and 8.2 M. The data for 5.5 M sarcosine show the first scan and the rescan of the same sample in order to illustrate the reversibility of the transition. Superimposed on the data for 2 and 8.2 M sarcosine are the fitted curves using the two-state model for protein unfolding. Small variations in the area observed in this figure are due to small changes in pre- and postdenaturation base lines from sample to sample. When these base line effects are taken into account, ΔH values for thermal unfolding as a function of T_m are essentially constant (see Figure 3A).

phosphate as substrate, k_{cat} and K_m values at 25 °C were evaluated by nonlinear least-squares fitting to the Michaelis–Menten equation, on the sample before heating and after twice heating to 94 °C at a scan rate of 1 °C/min. These kinetic assays were performed by adding the enzyme which was already in the presence of osmolyte to substrate solutions which did not contain osmolyte. Within the lower and upper nonlinear (67%) confidence limits indicated, there was no statistical difference in k_{cat} and K_m before [k_{cat} = 201 (179, 224) min^{-1}, K_m = 1.12 (0.92, 1.39) mM] and after heating [k_{cat} = 158 (131, 201) min^{-1}, K_m = 1.38 (1.03, 1.96) mM], though it appears that k_{cat} may be a bit lower after the two scans.

The observed increase in T_m of RNase with sarcosine concentration occurs to a greater or lesser degree with all of the glycine-based osmolytes (Bowlus & Somero, 1979). Panels A and B of Figure 2 show T_ms for RNase A and HEW lysozyme, respectively, as a function of concentration for each of the glycine-based osmolytes. The maximum osmolyte concentrations shown in Figure 2A represent the solubility limits of the particular osmolytes at 25 °C while in Figure 2B solubility limits for dimethylglycine and sarcosine would require reaching temperatures out of the range capable of our DSC instrument. The instrument limitation is manifested in the base line characteristics of our DSC instrument such that above about 95 °C the base line causes distortion in the shape of the endotherms. Each of the methylated glycine osmolytes in Figure 2A,B exhibits a similar trend of approaching an optimum temperature for stabilization. With some osmolytes, upon reaching a maximum T_m further addition of osmolyte results in a decrease in T_m. Such behavior suggests a tendency to destabilize the protein at very high osmolyte concentration. While the T_m versus osmolyte concentration appears to be complex, it is clear from the results with sarcosine that T_m increases of more than 20 °C can be achieved with this naturally occurring osmolyte.

The areas under the endotherms in Figure 1 appear to be constant and independent of sarcosine concentration. Figure 3A illustrates this more clearly with a plot of the RNase

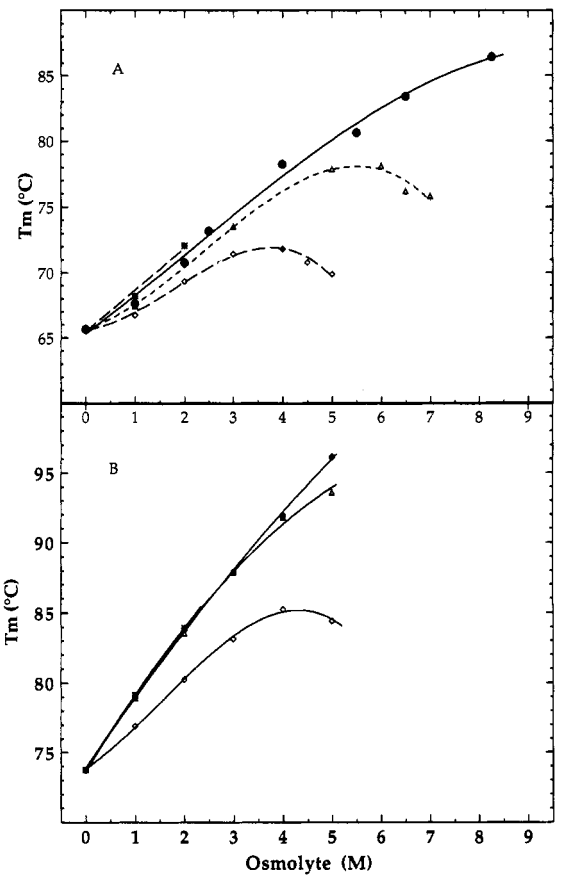

FIGURE 2: Midpoint thermal unfolding temperatures (T_m) for RNase A (A) and HEW lysozyme (B) as a function of osmolyte concentration. Osmolytes include (*) glycine, (●) sarcosine, (△) dimethylglycine, and (◇) betaine. The solid lines are empirical polynomials fitted to provide a smooth connection of the data points. For HEW lysozyme, the data for glycine, sarcosine, and dimethylglycine overlap considerably up through 2–3 M osmolyte.

unfolding enthalpy change (ΔH_{cal}) with respect to the T_m achieved in the presence of various sarcosine concentrations. This type of plot is commonly used for the purpose of evaluating the heat capacity change (ΔC_p) for protein unfolding. An apparent ΔC_p can be evaluated from the slope of such a plot, and it is evident from the data presented that the apparent ΔC_p will be approximately zero. This is to be compared with a ΔC_p of around 2.0 kcal/mol for RNase A unfolding in the absence of osmolyte (Brandts & Hunt, 1967; Tsong et al., 1970).

In contrast with RNase A, HEW lysozyme does exhibit apparent ΔC_p effects as a result of exposure to glycine-based osmolytes. Figure 3B shows ΔH_{cal} with corresponding T_ms for HEW lysozyme endotherms under conditions of different concentrations of glycine-based osmolytes. Low concentrations of osmolytes cause a positive change in ΔC_p in the range of 1.24 kcal/(mol·deg), which is a little lower than the 1.57 kcal/(mol·deg) reported for HEW lysozyme in the absence of osmolyte (Pfeil & Privalov, 1976). In the case of sarcosine, however, the apparent ΔC_p even appears to have a temperature dependence.

None of the glycine-based osmolytes cause much of a change in ΔH_{cal}. Figure 4 illustrates that ΔH_{cal} is relatively independent of osmolyte concentration and, with few exceptions, ΔH_{cal} values for all of the glycine-based osmolytes fall within the range of 115 ± 6 kcal/mol. This relative independence of ΔH_{cal} on osmolyte concentration also translates into the fact that regardless of which glycine-based osmolyte is used, ΔH_{cal} varies little with T_m (data not shown explicitly but can be

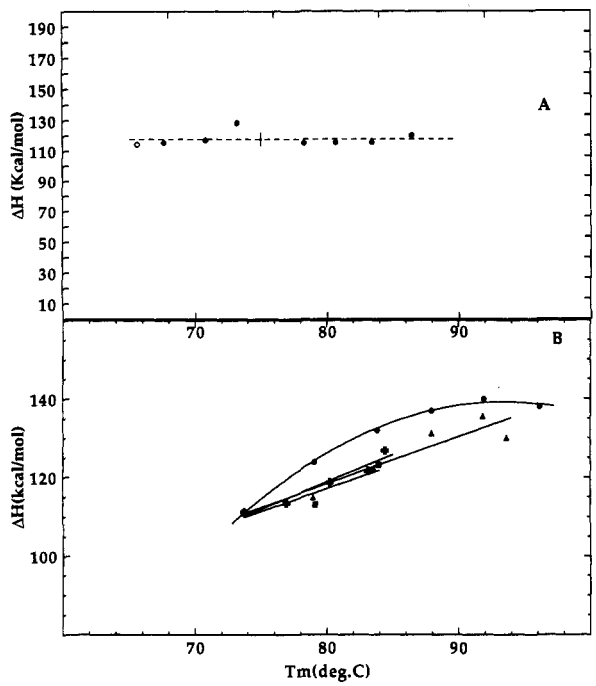

FIGURE 3: Calorimetric change in enthalpy (ΔH_{cal}) for RNase A (A) and lysozyme (B) as a function of the T_m observed at different concentrations of sarcosine. For RNase A (A), the open circle represents the observed ΔH_{cal} in buffer in the absence of osmolyte while data of increasing T_m are represented by sarcosine concentrations of 1.0, 2.0, 2.5, 4, 5.5, 6.5, and 8.2 M. The dashed line with a slope of zero ($\Delta C_p = 0$) represents the mean value of ΔH_{cal} (118 ± 4.3 kcal/mol) for these data, and the vertical error bar on the dashed line gives the standard deviation of the mean. For lysozyme (B), except in the case of sarcosine in which a polynomial was used to describe the data, the straight lines represent linear fits of the ΔH_{cal} vs T_m dependencies for glycine, dimethylglycine, and betaine.

extracted from Figures 2 and 4). Thus, the apparent ΔC_p is about zero for RNase A unfolding in the presence of any of the glycine-based osmolytes.

Naturally occurring osmolytes are presumed to have modest effects on both the structure and function of proteins (Yancey, 1985; Yancey et al., 1982). In order to investigate the extent to which osmolytes affect the catalytic activity of RNase A, we determined kinetic parameters for cCMP hydrolysis by RNase A in the presence of varying concentrations of sarcosine. In 2, 4, and 6 M sarcosine, k_{cat} was found to be 115%, 50%, and 25%, respectively, of the k_{cat} value in the absence of sarcosine. The K_m in 4 M sarcosine was found to be unchanged from what it is in the absence of sarcosine, but in 6 M sarcosine, it was 4-fold larger. These results show that even at very high concentrations, sarcosine produces very modest effects on the catalytic activity of RNase A.

In comparing the thermal unfolding characteristics of RNase A and HEW lysozyme in the presence of glycine-based osmolytes, it is worthwhile mentioning one important difference. The reversibility for thermal unfolding of HEW lysozyme is not nearly as good as that observed for RNase A. Rescans of thermally unfolded HEW lysozyme appear to be highly dependent upon the time the protein remains in the thermally unfolded state. However, even in rescans which minimize the time of exposure of unfolded HEW lysozyme at high temperature, the endotherm area recovered on a rescan is typically no greater than 50–60% in the presence of high concentrations of osmolyte.

Discussion

In the case of RNase A, we find from consecutive DSC scans (see Figure 1) that thermal unfoldings in the presence of a glycine-based osmolyte are very well behaved, with reversible two-state unfolding behavior maintained even in very high concentrations of osmolyte. The observation that k_{cat} and K_m are statistically unchanged for RNase A before and after thermal unfolding in the presence of 5.5 M sarcosine confirms high fidelity of refolding, also evident in the DSC rescans. The characteristics of reversibility and two-state unfolding are important because (1) they signal that the nature of the unfolding and refolding process in the presence of the glycine-based osmolytes is apparently unchanged from what occurs in the absence of osmolyte and (2) they permit analyses of unfolding data in the quantitative language of thermodynamics. In addition to the reversible two-state characteristics evident in Figure 1, it is important to note the large increases in T_m brought about by the osmolytes in general and sarcosine in particular. In terms of the present standards in this field, the 20-plus °C increase in T_m brought about by 8.2 M sarcosine suggests considerable stabilization for RNase A afforded by this osmolyte.

Even more remarkable than the large ΔT_m is the fact that the unfolding calorimetric ΔH for RNase A unfolding is constant, despite the high concentrations of sarcosine and the significant T_m increases brought about by this osmolyte. Since ΔC_p is equal to the derivative $d\Delta H/dT$, it is clear from the slope of Figure 3A that the apparent ΔC_p for RNase A thermal unfolding is zero in the presence of sarcosine. The Gibbs–Helmholtz equation (eq 1) shown here provides a means for

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln (T/T_m)] \quad (1)$$

determining the increase in stabilization free energy caused by the presence of this osmolyte. In the absence of sarcosine, and at the melting temperature of RNase A (T = 338 K), the unfolding free energy change is zero. In the presence of 8.2 M sarcosine, the T_m (360 K) and ΔH_{cal} (118 kcal/mol) can be combined with the apparent ΔC_p [0 kcal/(mol·deg)] to calculate the stability of the protein at 338 K. The calculation shows that at 338 K the unfolding free energy change for RNase A goes from zero in the absence of sarcosine to 7.2 kcal/mol in the presence of 8.2 M concentration of this osmolyte. This represents a 45 000-fold increase in the relative stabilization of the native form of the protein at this temperature. Considering that the stability of RNase A in aqueous solution at pH 6 and 25 °C is 8.8 kcal/mol (Pace et al., 1990), the 7.2 kcal/mol increase in the stabilization free energy at 65 °C (338 K) represents a considerable increase in RNase A stability brought about by sarcosine. Though the other glycine-based osmolytes resulted in smaller T_m increases for RNase, they also produced the same general effect, with ΔH_{cal} being essentially independent of osmolyte concentration (Figure 4), with the apparent ΔC_p nearing zero under all conditions, and with stabilization free energy increasing proportionately with $\Delta T_m/T_m$.

The increase in RNase A stabilization with increasing sarcosine concentration appears to be an entropy effect. Recalling that ΔH_{cal} does not change as a function of sarcosine concentration, the 7.2 kcal/mol increase in stabilization free energy in the presence of 8.2 M sarcosine at 25 °C results in an entropy decrease of 21 entropy units. The origin of an apparent negative entropy change is not easily attributable to any single source though configurational entropy and solvation effects are likely candidates. The current explanation for stabilization of proteins by compounds such as the osmolytes is based on preferential hydration arguments (Arakawa & Timasheff, 1983; 1985; Lee & Timasheff, 1981). Such compounds preferentially hydrate proteins, increasing the chemical

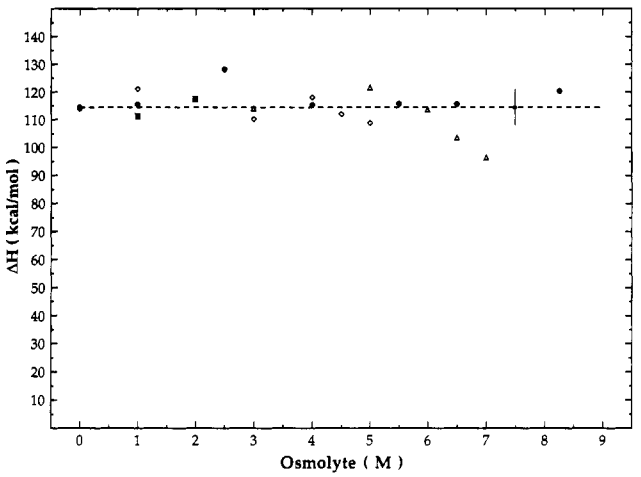

FIGURE 4: Calorimetric enthalpy change (ΔH_{cal}) for RNase A as a function of osmolyte concentration. The symbols represent the same osmolyte species as in Figure 2A,B. The dashed line and vertical error bar represent the average ΔH_{cal} value and standard deviation of all points (115 ● 6 kcal/mol), respectively.

potential of the protein with increasing osmolyte concentration. Preferential hydration opposes an increase in surface area of the protein, and since "unfolding" generally results in a surface area increase, the native ⇄ unfolded equilibrium should favor the more compact native state, resulting in an increase in T_m (Arakawa & Timasheff, 1983). Free energy changes for protein unfolding are perceived to result from the net differences between solvophobic and other forces favoring the folded state which are opposed by the gain in chain configuration entropy favoring the unfolded state. The tendency of increased osmolyte concentration to decrease protein surface area should progressively make the unfolded ensemble more compact, decreasing the configurational entropy of the unfolded state. This view provides a rationale for expecting an entropy decrease in the native ⇄ unfolded equilibrium in the presence of the glycine-based osmolytes relative to the equilibrium in the absence of osmolyte.

The question of whether decreased configurational entropy of the "unfolded" state is the source of negative entropy is complicated by consideration of solvation effects. Large positive heat capacity changes accompanying protein unfolding are often cited as evidence of hydrophobic effects associated with solvation changes in exposure of hydrophobic residues. The fact that the apparent ΔC_p for RNase A thermal unfolding goes from 2 kcal/(mol·deg) in the absence of sarcosine to 0 kcal/(mol·deg) in its presence suggests significant alterations in solvation effects normally associated with protein unfolding in the absence of osmolytes. Exactly how the solvent structure changes due to preferential hydration changes and hydrophobic exposure will contribute to the observed negative entropy change for unfolding is unclear. So little is known concerning thermodynamic behavior in concentrated solutions of osmolytes that there is a considerable risk of placing too much of a molecular interpretation on the unusual thermodynamics observed with this system.

The basic stability behavior in the presence of glycine-based osmolytes is reflected in dependencies of T_m on osmolyte and osmolyte concentration. The fact that the more highly methylated glycine osmolytes exhibit T_m optima for RNase A and HEW lysozyme (see Figure 2) as a function of osmolyte concentration suggests there are at least two effects on T_m related to the degree of methylation of the glycine-based osmolytes: (1) at low concentration of osmolyte, the greater the degree of glycine methylation, the less effective the osmolyte tends to be in increasing T_m; (2) at high osmolyte concentration, the greater the degree of glycine methylation, the greater the tendency to decrease the T_m (and protein stability) from its optimum value. On the basis of the shape of the plots of T_m vs concentration of osmolyte, it appears that in addition to dimethylglycine and betaine, sarcosine would also exhibit an optimum T_m if concentrations greater than its solubility limit could be achieved.

We may attempt to understand the T_m dependencies in terms of the degree of glycine methylation and physical characteristics of these solutes. Arakawa and Timasheff have pointed out that to understand protein stabilization by glycine and betaine both the surface area increase and the chemical nature of osmolyte and exposed protein surface need to be considered (Arakawa & Timasheff, 1983). These authors argue that betaine, with three additional methyl groups, should have a greater affinity for nonpolar substances than glycine. Such increases in nonpolar character might be expected to compete with or at least attenuate the preferential hydration effect by causing a more favorable interaction between osmolyte and exposed hydrophobic amino acid side chains, shifting the native ⇄ unfolded equilibrium more toward the unfolded species. In terms of our results on RNase A and HEW lysozyme, glycine-based osmolytes with more nonpolar character should tend to oppose the preferential hydration effect, resulting in a smaller ΔT_m than glycine osmolytes with little or no methylation. The rank order of glycine-based osmolytes with respect to ΔT_m (Figure 2) is consistent with this explanation, and this is particularly evident with T_m results involving RNase A unfolding. With respect to increasing osmolyte concentration, one might expect the more highly methyl-substituted glycine solute to increasingly favor the unfolded state, competing well enough against the surface tension effect at high concentration of osmolyte to cause an observed decline in ΔT_m from an optimal value. This, in fact, is evident in Figure 2A,B, and the rank order of osmolyte effectiveness in competing with the preferential hydration effect parallels the degree of methylation of the osmolyte. The two opposing forces are, however, of differing magnitudes with different proteins. It should be noted that the T_m increase on a molar (sarcosine) basis is greater for lysozyme than it is for RNase A. Thus, the relative position of the T_m optimum for any given protein will depend upon the balance of the two effects, one involving protein stabilization due to preferential hydration by osmolyte and the other involving destabilization effects due to a modestly favorable interaction of exposed hydrophobic side chains with apolar parts of the osmolyte.

In contrast with RNase A, DSC endotherms for unfolding of HEW lysozyme are not recovered to a high degree on rescanning the sample. In cases involving high osmolyte concentration, varying degrees of turbidity can be seen in the HEW lysozyme samples after the first DSC scan, suggesting insolubility of at least some of the protein. The shape of the endotherm is also different in the second scan, indicating that refolded HEW lysozyme is not in the same conformational state as native protein. Regardless of which of the glycine-based osmolytes were used, however, the first DSC scan was well behaved in terms of the shape of the endotherm. That is, if we ignore for the moment that the system has irreversible features and simply fit the endotherm of the first DSC scan as a single transition allowing ΔH_{cal} and $\Delta H_{van't\ Hoff}$ to float, it is found that the ratio $\Delta H_{cal}/\Delta H_{van't\ Hoff}$ is always quite near unity, regardless of osmolyte concentration. Since the unfolding has a high degree of irreversibility, we have not attempted to calculate the free energy change for stabilization

brought about by these osmolytes. Instead, the ratio $\Delta H_{cal}/\Delta H_{van't\ Hoff}$ is used in this instance with no other implication than being a convenient index of the shape of the endotherm. In the absence of osmolyte, a $\Delta H_{cal}/\Delta H_{van't\ Hoff}$ ratio of unity is achieved in the reversible unfolding of HEW lysozyme, signifying two-state behavior. The fact that the same ratio of $\Delta H_{cal}/\Delta H_{van't\ Hoff}$ occurs in the absence of osmolyte and in the first DSC scan in the presence of osmolyte is taken to mean that osmolyte holds hen egg white lysozyme in much the same conformation as exists in aqueous solution and that the osmolyte enables the protein to retain those conformational features up through the temperature at which unfolding occurs.

Of practical interest is the question of whether glycine-based osmolytes would be effective in encouraging renaturation of thermally inactivated protein. The fact that a protein like RNase A will refold in a completely reversible manner in the presence of up to 8 M sarcosine certainly indicates that the basic rules for RNase A refolding are not changed as a function of the concentration of this osmolyte. However, the irreversibility of HEW lysozyme thermal unfolding in the presence of glycine-based osmolytes suggests that such solutes will not be generally useful in regenerating or reactivating proteins which are thermally unfolded in their presence. The effects which the osmolytes have on unfolded states are quite likely to be very important in bringing about stabilization, but even with single-subunit proteins like HEW lysozyme, the solutes appear to cause aggregation to occur with the unfolded state, and this would reduce their usefulness as agents for reactivating thermally unfolded proteins.

A large number of studies concerned with protein stability have used the thermal unfolding transition or melting temperature (T_m) as an index of stability, and over the past several years, there have been serious efforts to increase protein stability (and ΔT_m) by engineering disulfide bonds into proteins (Matsumura et al., 1989a,b; Pantoliano et al., 1987; Sauer et al., 1986; Villafranca et al., 1987). These efforts have met with mixed success, but ΔT_m values as high as 23 °C have been reported using such techniques (Matsumura et al., 1989b). By comparison, we have been able to achieve ΔT_m increases of the same magnitude without much difficulty using sarcosine as a solute. The efficacy of sarcosine in increasing T_m is dependent upon the particular protein, taking 8.2 M sarcosine to cause a 22 °C increase in T_m for RNase A, 5 M sarcosine to achieve the same T_m increase with HEW lysozyme, and only 4 M to give a 23 °C increase with rabbit muscle lactate dehydrogenase (Liu and Bolen, unpublished results). While admittedly the number of proteins studied in the presence of sarcosine is limited, the potential exists for readily obtaining protein stability effects which rival some of the best design efforts using protein engineering techniques.

The study of organisms which have adapted to conditions of dehydration, heat, or high osmotic activity provides a look at how nature has approached the problem of maintaining a functional viable organism under conditions which might normally be expected to denature or inactivate a large number of that organism's macromolecules. Organisms adapting to these extreme conditions have produced and concentrated particular osmolytes believed to confer protection to the organisms without greatly affecting the functions of their enzymes. Since the osmolytes would need to stabilize a great many of the enzymes in an organism adapted to environmental stress(es), it is reasonable to assume that such osmolytes would confer stability to any enzyme or protein to which it is exposed. That is, proteins which did not evolve in the presence of osmolytes should be affected by these solutes in much the same way as enzymes which did evolve in their presence. Our results with RNase A and HEW lysozyme are consistent with this idea. We find that the glycine-based osmolytes appear to present a general method of stabilizing proteins against thermal unfolding even well beyond the physiological concentration range for osmolyte and that the degree of stabilization can be extraordinary. A clear and complete understanding of just how this is accomplished remains a point of active research.

Registry No. RNase A, 9001-99-4; cCMP, 3616-08-8; lysozyme, 9001-63-2; glycine, 56-40-6; sarcosine, 107-97-1; *N,N*-dimethylglycine, 1118-68-9; betaine, 107-43-7.

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