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Why Is Trehalose an Exceptional Protein Stabilizer?

AN ANALYSIS OF THE THERMAL STABILITY OF PROTEINS IN THE PRESENCE OF THE COMPATIBLE OSMOLYTE TREHALOSE*

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Trehalose, a naturally occurring osmolyte, is known to be an exceptional stabilizer of proteins and helps retain the activity of enzymes in solution as well as in the freeze-dried state. To understand the mechanism of action of trehalose in detail, we have conducted a thorough investigation of its effect on the thermal stability in aqueous solutions of five well characterized proteins differing in their various physico-chemical properties. Among them, RNase A has been used as a model enzyme to investigate the effect of trehalose on the retention of enzymatic activity upon incubation at high temperatures. 2 M trehalose was observed to raise the transition temperature, T_m of RNase A by as much as 18 °C and Gibbs free energy by 4.8 kcal mol^{-1} at pH 2.5. There is a decrease in the heat capacity of protein denaturation (ΔC_p) in trehalose solutions for all the studied proteins. An increase in the ΔG and a decrease in the ΔC_p values for all the proteins points toward a general mechanism of stabilization due to the elevation and broadening of the stability curve (ΔG *versus* T). A direct correlation of the surface tension of trehalose solutions and the thermal stability of various proteins has been observed. Wyman linkage analysis indicates that at 1.5 M concentration 4–7 molecules of trehalose are excluded from the vicinity of protein molecules upon denaturation. We further show that an increase in the stability of proteins in the presence of trehalose depends upon the length of the polypeptide chain. The pH dependence data suggest that even though the charge status of a protein contributes significantly, trehalose can be expected to work as a universal stabilizer of protein conformation due to its exceptional effect on the structure and properties of solvent water compared with other sugars and polyols.

Sugars have been known to protect proteins against loss of activity (1, 2), chemical (3, 4), and thermal denaturation (5–9). Among several sugars, α,α-trehalose (α-D-glucopyranosyl(1→1)-α-D-glucopyranoside) has been known to be a superior stabilizer in providing protection to biological materials against dehydration and desiccation (10, 11). It is a compatible osmolyte that gets accumulated in organisms under stress conditions (12, 13). Because of this unique property, tremendous interest has been generated in understanding the molecular basis of stress management through induction of trehalose biosynthesis (13, 14).

Trehalose has also been found to be very effective in the stabilization of labile proteins during lyophilization (15, 16) and exposure to high temperatures in solution (2, 8, 9). Sugars in general protect proteins against dehydration by hydrogen bonding to the dried protein by serving as water substitute (15, 17). Several studies carried out by Timasheff and coworkers (9, 18) show that sugars and polyols stabilize the folded structure of proteins in solution as a result of greater preferential hydration of the unfolded state compared with the native state. The mechanism is fundamentally different from stabilization in the dried state and points toward the different origins of protein denaturation under different stress conditions (17). In solution, trehalose has been observed to stabilize RNase A by increasing the surface tension of the medium, which leads to the preferential hydration of the protein (8, 9). These studies have been carried out using a representative protein at a few selected conditions only. Different proteins are expected to interact with cosolvent molecules in varied ways depending on their physico-chemical properties. In general, trehalose has been observed to provide protection to different proteins to various extents and the efficacy of protection depends on the nature of the protein (2, 4). Despite the availability of such data, the exact role of proteins and their physico-chemical properties in trehalose-mediated stability is still not clear. Studies done earlier by Gekko (19, 20), using polyol osmolytes and free energy of transfer studies, suggested that unfavorable interactions of the amino acid side chains with polyols dominate the stability effect, and peptide-polyol interactions contribute negligibly to the stability mediated by polyols. However, recently, Bolen and coworkers (21–23), based on carefully conducted transfer studies of amino acids and model compounds, have shown that cumulative interactions between amino acid side chains and osmolytes (including sucrose) favor protein unfolding, whereas their overall stabilization is achieved due to unfavorable peptide-osmolyte interactions. The exact nature of interactions that govern the osmolyte-mediated stability of proteins is, therefore, not yet very clear. Overall, protein stability should depend upon a fine balance between favorable and unfavorable interactions of the native and the denatured protein states with the cosolvent molecules (24). The stabilizing effect would, thus, depend on the nature of both the proteins as well as the cosolvent molecules and generalization of the effect may not be possible.

To understand the mechanism of trehalose-mediated thermal stability of proteins in detail, we have studied its effect on the thermal stability of a set of five well characterized globular

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proteins, *viz*., ribonuclease A (RNase A),[1] lysozyme, cytochrome *c* (cyt *c*), α-chymotrypsinogen (α-CTgen), and trypsin-inhibitor (Trp-Inh) at varying cosolvent concentrations and pH values. These proteins vary in their molecular size, ranging from 12.3 kDa for cyt *c* to 25.7 kDa for α-CTgen, their hydrophobicities, and the net charges, with pI values in the range of 4.1 for Trp-Inh to 10.7 for lysozyme. The data have been analyzed in the light of the role of bulk properties of the solvent environment and the physico-chemical properties of proteins. Because trehalose in addition to imparting thermodynamic stability to proteins also helps in the retention of activity of enzymes during storage at high temperatures, we have carried out activity studies for RNase A at elevated temperatures and denaturing conditions to understand the stability-activity relationship of the enzyme in the presence of trehalose.

EXPERIMENTAL PROCEDURES

Materials—RNase A (bovine pancreatic), lysozyme (hen egg white, HEW), α-CTgen (bovine pancreatic), cyt *c* (horse heart, type IV), Trp-Inh (HEW), trehalose, and 2′,3′-cCMP (NH_4^+ salt) were all from Sigma Chemical Co., St. Louis, MO. All the proteins were dialyzed against distilled de-ionized water and lyophilized followed by drying over P_2O_5. Glycine and sodium acetate were purchased from E. Merck, India. The pH of the solutions was adjusted on a Radiometer PHM84 research pH meter by adding HCl or NaOH solutions. 20 mM glycine HCl buffer at pH 2.5, 40 mM acetate buffer at pH 4.0, and 20 mM MOPS (Sigma Chemical Co.) buffers at pH 7.0 were used from their stock solutions. The solutions at pH 7.0 were made in the presence of 1.5 M GdmCl (Amresco, Solon, OH).

Thermal Denaturation Experiments—For monitoring the unfolding of the tertiary structure, thermal denaturation experiments were carried out using a Cecil 599 UV-visible spectrophotometer to which a linear temperature programmer (CE-247, Cecil) was attached. The concentration of the protein solutions was ~0.5 mg/ml, except for cyt *c* where 0.1 mg/ml protein was used. The protein solutions were loaded in a 0.5-ml masked and Teflon-stoppered quartz cuvette (Hellma, Germany). A temperature scan rate of 1 °C/min was used in all the experiments. The wavelengths for monitoring conformational changes related to the tertiary structure were 287 nm for RNase A, 293 nm for α-CTgen, 301 nm for lysozyme, 285 nm for Trp-Inh, and 394 nm for cyt *c* based on their difference spectra. The reversibility of the thermal transitions recorded for the proteins was ascertained by reheating the protein solutions.

To investigate the unfolding of secondary structure elements, thermal denaturation was monitored by far-UV CD measurements using a Jasco J715 spectropolarimeter at selected pH conditions, one for each protein, at a scan rate of 1 °C/min. A wavelength of 222 nm was used to specifically probe the opening up of helical regions in the proteins.

Analysis of Data—The evaluation of thermodynamic parameters from the thermal denaturation curves was based on the equilibrium constant *K*, for $N \Leftrightarrow D$ conversion for a two-state reversible transition, where *N* represents the native state and *D* is the denatured state. The equilibrium constant was deduced from the equation,

$$K = [\text{unfolded}]/[\text{native}] = (A_N - A_O)/(A_O - A_D) \qquad \text{(Eq. 1)}$$

where A_N is the absorbance of the pure native state in the transition zone after extrapolation from the pre-transition region, A_D is the corresponding absorbance of the pure denatured state, and A_O is the observed absorbance at a temperature in the transition zone. We used all the experimental data points obtained in a transition reaction to fit Equation 2, as follows, and described in detail elsewhere (25, 26),

$$A_O = \frac{[A_N + A_D \cdot \exp\cdot(-1/R(\Delta H_m(1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + 1n(T/T_m))))]}{[1 + \exp(-1/R(\Delta H_m(1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + 1n(T/T_m))))]} \qquad \text{(Eq. 2)}$$

where *R* is the gas constant, T_m is the midpoint temperature of transition in kelvin, ΔH_m is the enthalpy of protein denaturation calculated at the T_m, and ΔC_p is the apparent heat capacity of protein denaturation. For nonlinear least square analysis a minimum of 50 iterations or more using Marquardt-Levenberg routine as available in the Origin™ software (Microcal Inc., Northampton, MA) were performed until the fractional change in χ^2 value was within the tolerance limit set to 5×10^{-4}. All the parameters were floated freely to deduce their values simultaneously from thermal transition curves.

RNase A Activity Assay—RNase A catalyzed hydrolysis of 2′,3′-cCMP was measured by the change in the absorbance at 286 nm (27). Two sets of experiments were conducted in the presence of 1.5 M trehalose and 20 mM Tris, pH 7.0. In set 1, RNase A was incubated at high temperatures (66 °C and 60 °C, with 1 M GdmCl) for 13 h followed by cooling to room temperature and monitoring the activity by addition of 2′,3′-cCMP from the stock. In set 2, RNase was added to the reaction buffer and allowed to equilibrate at high temperatures (63 °C, 56 °C, and 52 °C, with 1 M GdmCl) at which the activity was monitored by the addition of 2′,3′-cyclic CMP. All the reactions were carried out in a 1.0-ml Teflon-stoppered quartz cuvette. The temperature of the cuvette was maintained by using a programmable thermoelectric cuvette holder.

RESULTS

Thermal denaturation experiments were carried out for RNase A, lysozyme, cyt *c*, α-CTgen, and Trp-Inh in the absence and presence of 1–2 M trehalose at pH 2.5, 4.0, and 7.0 by absorbance measurements. The data have been presented in Fig. 1. The *insets* in Fig. 1 (*A–E*) show thermal denaturation of the helical structure of these proteins monitored at 222 nm by CD spectroscopy in the absence and presence of 1 M trehalose. The studies were not feasible at all the pH conditions for the proteins. cyt *c* is known to be partially denatured at pH 2.5 (28), and hence the thermal stability studies at this pH were not possible. α-CTgen undergoes aggregation at pH 4.0 and 7.0 at higher temperatures, thereby limiting thermodynamic analysis. In the presence of trehalose, lysozyme at pH 4.0 and 7.0 and cyt *c* at pH 7.0 undergoes partial aggregation even in the presence of 1.5 M GdmCl. The two-state transition analysis using the van't Hoff equation in these cases was carried out only to determine the approximate values of thermodynamic parameters. Santoro *et al.* (29) have observed irreversible aggregation of lysozyme in osmolyte solutions, and its inactivation at high temperatures in buffer has also been reported (30). RNase A in the presence of trehalose undergoes a completely reversible denaturation at pH 2.5 and 4.0. At high trehalose concentrations, however, RNase A solutions at pH 7.0 showed partial aggregation even in the presence of 0.5 M GdmCl and required the addition of 1–1.5 M GdmCl for reversibility in thermal denaturation. It must be pointed out that a two-state cooperative transition has been observed for all the proteins studied. Even though Trp-Inh possesses three tandem homologous domains, only a single cooperative transition has been observed. We have observed that multidomain and multimeric proteins usually undergo irreversible thermal denaturation[2] and hence cannot be studied for the effect of trehalose by either spectroscopic or calorimetric techniques.

Effect of Trehalose on the Thermodynamics of Protein Denaturation—Thermodynamic parameters for protein denaturation in trehalose solutions at different conditions, obtained from the data in Fig. 1, have been presented in Table I. ΔT_m and $\Delta\Delta G^0$ are the increments in the midpoint of transition, T_m, and Gibbs free energy of stabilization, ΔG^0, respectively. $\Delta\Delta G^0$ in the presence of trehalose has been calculated at the T_m of the control, where ΔG^0 for control is zero. For all the proteins, both ΔT_m and $\Delta\Delta G^0$ increase linearly with an increase in the trehalose concentration. ΔH_m and ΔS_m were also observed to be increasing with trehalose concentration in the case of RNase A, α-CTgen, Trp-Inh, and

[1] The abbreviations used are: RNase A, ribonuclease A; ΔASA, change in accessible surface area; ΔC_p, apparent heat capacity of denaturation; ΔH_m, enthalpy of denaturation; ΔG^0, Gibbs energy; $\Delta\Delta G^0$, free energy of stabilization; T_m, midpoint of thermal denaturation; α-CTgen, α-chymotrypsinogen; cyt *c*, cytochrome *c*; Trp-Inh, Trypsin Inhibitor; GdmCl, guanidinium chloride; CD, circular dichroism; MOPS, 4-morpholinepropanesulfonic acid.

[2] D. P. Kumar and R. Bhat, unpublished data.

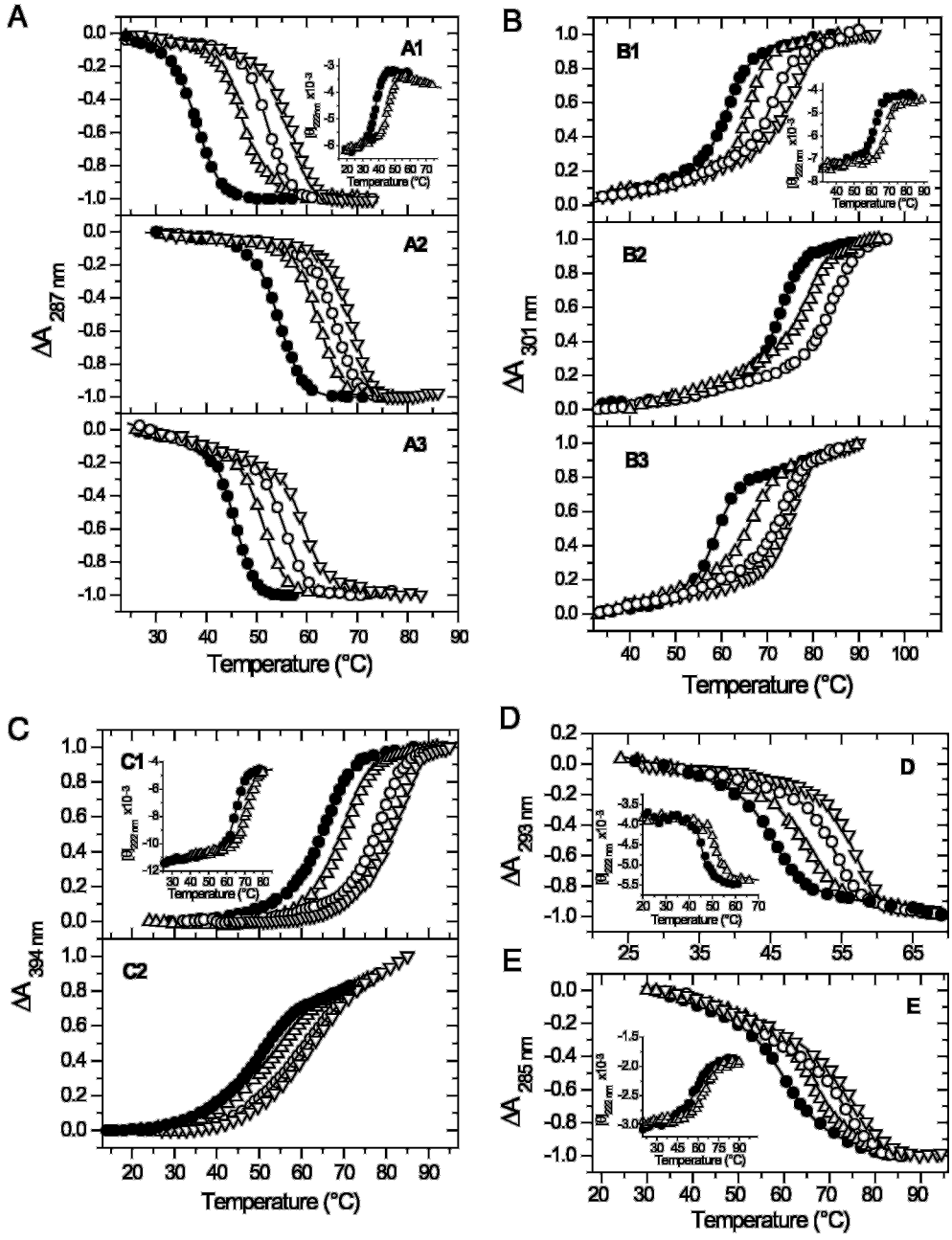


FIG. 1. **Thermal denaturation curves for different proteins at various pH values.** RNase A at pH 2.5 (*A1*), pH 4 (*A2*), pH 7 (*A3*); lysozyme at pH 2.5 (*B1*), pH 4 (*B2*), pH 7 (*B3*); cytochrome *c* at pH 4 (*C1*), pH 7 (*C2*); α-chymotrypsinogen at pH 2.5 (*D*), and trypsin inhibitor at pH 7 (*E*) in the presence of buffer (●) and 1 M (△), 1.5 M (○), and 2 M (▽) trehalose concentrations. The *solid lines* show the best fit of the denaturation data to the two-state model given by Equation 2. The normalized thermal denaturation curves were measured by the changes in the absorbance in the aromatic regions, except for cyt *c*, which was monitored in the Soret band region. The *insets* in various panels show thermal denaturation monitored by changes in the mean residue ellipticity at 222 nm for the corresponding proteins at the conditions used for main panel. Denaturation at pH 7 for the studied proteins was carried out in the presence of 1.5 M GdmCl.

cyt *c* at pH 4.0. However, these parameters show a decrease in the case of lysozyme at pH 4.0 and 7.0, and cyt *c* at pH 7.0, which could be due to partial irreversible aggregation of the proteins in the post denaturation region. Overall, barring these exceptions involving protein aggregation, trehalose was observed to gradually increase the ΔH_m and ΔS_m of protein denaturation. The experimental errors in ΔH_m were in the range of ±1–4% for RNase and cyt *c* and ±5–7% for α-CTgen, lysozyme, and Trp-Inh. The error in T_m measurements was within ±0.5 °C for all the proteins. The fitting errors were always within the experimental error limits. The CD data presented as *insets* in Fig. 1 have not been used in Table I. However, the transition temperatures and the various thermodynamic parameters obtained by CD measurements matched well with those evaluated by absorption spectroscopy.

The slopes of the curves (ΔH_m *versus* T_m) plotted in Fig. 2 represent the heat capacity of protein denaturation, ΔC_p for RNase A obtained in the presence of trehalose and are 0.87 kcal mol^{-1} K^{-1} at pH 2.5 and 4.0, and 1.1 kcal mol^{-1} K^{-1} at pH 7.0, respectively. The values are considerably lower than the spectroscopically obtained ΔC_p values of 2.07 kcal mol^{-1} K^{-1} and 2.2 kcal mol^{-1} K^{-1} evaluated by varying the T_m of RNase A using GdmCl (26) and urea (31), respectively, and calorimetric values of 1.74 kcal mol^{-1} K^{-1} in buffer and 2.16 kcal mol^{-1} K^{-1} in 1 M GdmCl reported by Liu and Sturtevant (32). Previously,

TABLE I
Thermodynamic parameters for several proteins in aqueous trehalose solutions at various pH values

Cosolvent	RNase A					α-CTgen					Lysozyme					Cytochrome *c*				
	T_m	ΔT_m	ΔH_m	ΔS_m	$\Delta\Delta G^0$	T_m	ΔT_m	ΔH_m	ΔS_m	$\Delta\Delta G^0$	T_m	ΔT_m	ΔH_m	ΔS_m	$\Delta\Delta G^0$	T_m	ΔT_m	ΔH_m	ΔS_m	$\Delta\Delta G^0$
	°C	°C	*kcal mol*$^{-1}$	*e.u.*[a]	*kcal mol*$^{-1}$	°C	°C	*kcal mol*$^{-1}$	*e.u.*	*kcal mol*$^{-1}$	°C	°C	*kcal mol*$^{-1}$	*e.u.*	*kcal mol*$^{-1}$	°C	°C	*kcal mol*$^{-1}$	*e.u.*	*kcal mol*$^{-1}$
pH 2.5																				
Control	38.3		81.0	260	0.00	44.9		96.3	303	0.00	61.1		98.0	293	0.00					
Trehalose (1.0 M)	46.6	8.3	86.0	269	2.13	50.3	5.4	98.5	304	1.59	66.5	5.4	94.5	278	1.50					
(1.5 M)	51.6	13.3	90.7	279	3.45	53.2	8.3	100.4	307	2.43	70.6	9.5	97.0	282	2.68					
(2.0 M)	56.5	18.2	95.3	289	4.48	56.4	11.5	109.7	333	3.56	74.7	13.6	101.4	292	3.96					
pH 4.0																				
Control	54.2		94.0	287	0.00						73.1		98.5	284	0.00	64.5		61.5	182	0.00
Trehalose (1.0 M)	62.4	8.2	102.9	307	2.42						79.9	6.8	95.1	269	1.83	71.2	6.7	63.1	183	1.19
(1.5 M)	66.2	12.0	104.5	308	3.49						83.7	10.6	94.0	263	2.79	78.7	14.2	67.0	190	2.56
(2.0 M)	70.5	16.3	107.1	312	4.71											81.9	17.4	68.9	194	3.15
pH 7.0[b]																				
						Trypsin inhibitor														
Control	46.0		92.0	288	0.00	59.0		56.5	170	0.00	58.5		94.9	286	0.00	48.0		38.4	120	0.00
Trehalose (1.0 M)	51.5	5.5	96.5	297	1.58	65.4	6.4	58.2	172	1.08	66.9	8.4	90.0	265	2.22	51.4	3.4	34.0	105	0.35
(1.5 M)	56.9	10.9	103.0	312	3.19	74.5	15.6	60.5	176	2.59	72.9	14.4	91.0	263	3.79	56.6	8.6	35.8	109	0.88
(2.0 M)	59.3	13.3	106.9	322	3.95						>75	>16.5	90.0	258	4.26					

[a] e.u., entropy units (cal k^{-1} mol^{-1}).
[b] Solutions at pH 7.0 also contain 1.5 M GdmCl.

FIG. 2. **Plots of enthalpy of denaturation *versus* the T_m values for RNase A in the presence of increasing concentrations of trehalose.** *A*, RNase A at pH 2.5 (○) and pH 4 (●); and *B*, RNase A at pH 7. The *solid line* is the least squares fit with slope equal to ΔC_p. At pH 7, trehalose solutions also contain 1.5 M GdmCl.

FIG. 3. **Plots of increase in the T_m values of different proteins *versus* the increase in the surface tension of aqueous trehalose solutions as a function of concentration (taken from Ref. 33).** RNase A at pH 2.5 (●) and pH 4 (○), α-CTgen at pH 2.5 (▲), lysozyme at pH 2.5 (△), and cyt *c* at pH 4 (▽).

several other osmolytes like sarcosine (29) and polyols (25) have also been observed to decrease the apparent heat capacity of denaturation of globular proteins. Neutral salts, including carboxylic acid salts (26), which increase the thermal stability of proteins, also lead to a decrease in the denaturation heat capacity of several proteins just like osmolytes. For proteins other than RNase A, *e.g.* for α-CTgen and lysozyme at pH 2.5, cyt *c* at pH 4.0, and Trp-Inh at pH 7.0, ΔH_m also increases as a function of T_m, though marginally (Table I), and results in much lower ΔC_p values than the corresponding values reported for these proteins without trehalose.

Solution Surface Tension and Protein Stability—Fig. 3 presents data showing the effect of the surface tension of trehalose solutions on the thermal stability, as monitored by T_m of various proteins studied. Surface tension of aqueous trehalose solutions has been observed to increase linearly with the concentration resulting in a slope of 1.34 dyne cm^{-1} mol^{-1} at 20 °C (33). The data presented in Fig. 3 suggest a good correlation of the effect of the increased surface tension of trehalose solutions with the increase in the T_m for all the proteins studied. Studies done by us earlier using a series of polyols (25) and carboxylic salts (26) also indicate a strong correlation of the surface tension effect with the thermal stability of proteins, suggesting an important role of water and the solvent environment in the stability of proteins.

Wyman Linkage and Interaction of Trehalose with Proteins—Trehalose stabilizes proteins by shifting the equilibrium con-

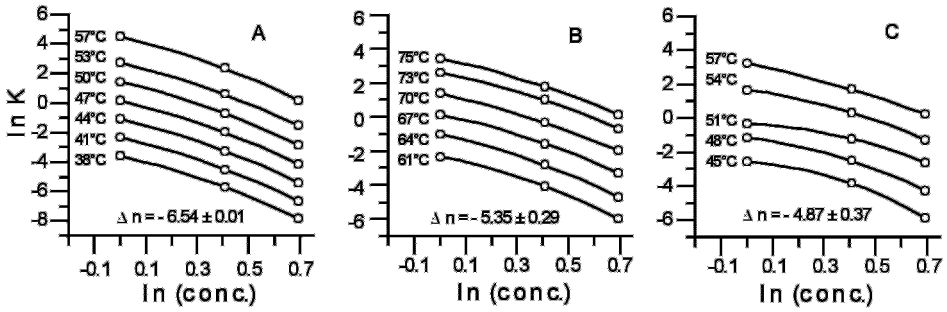


FIG. 4. **Wyman linkage plots of the effect of trehalose on the thermal denaturation of RNase A, pH 2.5 (*A*), lysozyme, pH 2.5 (*B*), and α-CTgen, pH 2.5 (*C*).** The average values of Δn at other conditions for RNase A are −5.69 (±0.14, S.D.) at pH 4.0, −5.02 ± 0.11 at pH 7.0, and for cyt *c*, −3.93 ± 0.03 at pH 4.0. The values of slopes correspond to 1.5 M trehalose. The S.D. was calculated using the Wyman slopes at various temperatures ranging from T_m in control to T_m in 2 M trehalose.

TABLE II
Relative activities of RNase A in the presence of trehalose at different temperatures

Cosolvent	Relative activities		
	Set 1[a]	Set 2[b]	
	66 °C	56 °C	63 °C
Trehalose	2.02 ± 0.1 (9.64)[c]	1.47 ± 0.08 (1.041)	2.41 ± 0.35 (2.82)
	60 °C[a]		52 °C[b]
Trehalose + 1 M GdmCl	3.03 ± 0.1 (12.3)		3.44 ± 0.05 (1.77)

[a] Activities were measured at 25 °C after 13 h of incubation at the indicated temperatures.
[b] Activities were measured at the indicated temperatures.
[c] Parentheses include the value of stabilization factor, f_{Nt}/f_{Nc}.

stant in favor of the native state. To analyze the effect of trehalose on the denaturation reaction, the Wyman linkage equation (24) given below (Equation 3) was used to determine the relative preferential interaction of trehalose with the two end states of the proteins,

$$d(\ln K)/d(\ln a) = (n_D - n_N) = \Delta n \qquad \text{(Eq. 3)}$$

where K is the equilibrium constant for the conversion reaction $N \Leftrightarrow D$, a is the cosolvent activity, and n_D and n_N are the numbers of cosolvent molecules bound to the denatured and the native protein molecules, respectively. For approximation, a second order equation was fit to a ln-ln plot of the equilibrium constant *versus* the trehalose concentration (instead of activity) for various proteins (Fig. 4). The Wyman slope of the tangent, Δn at a point on the curve provides the difference in the cosolvent molecules bound to the denatured and the native protein molecules. The Δn values obtained for various proteins at 1.5 M trehalose concentration vary from −7 to −4. The negative values indicate the preferential exclusion of trehalose from the hydration shell of the protein upon denaturation. The values of Δn extrapolated to 0.1–0.9 M trehalose agree well with those obtained by Xie and Timasheff (9) for RNase A at similar concentrations

Activity of RNase A in the Presence of Trehalose—RNase A was taken as a model enzyme to analyze the effect of trehalose on its bioactivity at high temperatures. The relative activity of RNase A in the presence of trehalose presented in Table II has been calculated by dividing the slopes of the linear zone of the corresponding activity plots by the slope of the data for control (buffer). The stabilization factor, f_{Nt}/f_{Nc} presented in Table II, wherein f_{Nt} is the fraction of the protein in the native state in the trehalose solution and f_{Nc} is the fraction of the native protein in the control buffer, was calculated from the thermal denaturation curves for RNase A in the respective solvent systems at the indicated temperatures. The data indicate a remarkable retention of activity of the enzyme in the presence of trehalose under various conditions of the experiment compared with control. Both the storage (set 1) as well as the operational (set 2) stabilities of the enzyme increased in the presence of trehalose as suggested by activity measurements under different conditions. Interestingly, greater relative retention of activity was observed in the presence of a mixture of 1.5 M trehalose and 1.5 M GdmCl compared with trehalose alone. We have obtained similar results earlier using lysozyme as a model enzyme (34).

DISCUSSION

Exceptional Stabilization by Trehalose—Among the various osmolytes selected by nature to counteract deleterious environmental effects, trehalose seems to be exceptional among the compatible osmolytes of the sugar and the polyol series, because it increases the transition temperature (ΔT_m) of proteins to a maximal extent. A comparatively high value of ΔT_m of 18.2 °C ($\Delta G^0 \sim 4.5$ kcal mol^{-1}) for RNase A at 2 M concentration of trehalose and pH 2.5 is indicative of this (Table I). This increase is much higher compared with any other sugar or polyol studied in the literature (5, 19, 25). The magnitudes of ΔT_m at other pH values for RNase A as well for several other proteins are also significantly higher. Although, several other cosolvents like carboxylic salts (26) and amino acids (29) have been observed to increase the ΔT_m of proteins to larger extents than trehalose at identical concentrations, they can have deleterious effect on the activity of some enzymes.[3] Trehalose, on the other hand, could prove to be one of the better choices as a universal stabilizer, because it is more or less inert toward the protein surface and has been observed to stabilize all the five proteins studied by us at several pH values.

Thermodynamic Basis for Protein Stabilization by Trehalose—It has been suggested that preferential interactions of cosolvents with the native and the denatured state of a protein govern their stabilization effect (24). The values of Δn, the difference in the cosolvent molecules bound to the denatured and the native proteins, that we obtained for RNase A, lysozyme, and α-CTgen clearly indicate preferential exclusion of trehalose from the vicinity of the denatured proteins (Fig. 4). The variations in the slopes for different proteins could be indicative of the subtle variations in the physico-chemical properties of proteins and hence varied protein-solvent interactions. The variations in the slopes for a given protein have also been observed to arise from changes in the pH of the trehalose solutions (Fig. 4 legend), indicating the dependence of the degree of trehalose exclusion on the charge status of the protein.

A decrease in the pH is also known to cause an increase in

[3] A. Tiwari and R. Bhat, unpublished results.

the hydrophobicity of proteins (35). This suggests that a decrease in pH should be accompanied by an increase in the degree of exclusion of trehalose due to the more hydrophobic nature of the protein. This is quite evident from the increased value of $-\Delta n$ in the case of RNase A as the pH decreases from 7.0 to 2.5 (Fig. 4). Interestingly, a decrease in the pH of the protein solutions also results in an increase in ΔT_m in the presence of trehalose. A relatively larger stability effect at low pH in the case of RNase A can be ascribed to an increased exclusion of trehalose from the vicinity of the denatured protein as compared with the native protein.

Trehalose has been observed to decrease the heat capacity of denaturation considerably for all the five proteins studied. Heat capacity is a sensitive thermodynamic parameter that can reflect upon the subtle changes in protein-solvent interactions (32, 36). Positive ΔC_p of protein unfolding is known to originate from the ordering of polar solvent around the exposed nonpolar groups in proteins, whereas the buried polar groups contribute in the opposite way (36). ΔC_p can, thus, be related to the total change in the accessible surface area (ΔASA) upon protein unfolding (37). Trehalose is expected to cause more ordering of the solvent upon protein denaturation by inducing preferential hydration and lead to a positive value of ΔC_p. However, our results are not consistent with this assumption. Because ΔC_p depends upon ΔASA or the extent of unfolding (38, 39), any residual structure present in the unfolded state could lead to a decrease in the ΔC_p value. The presence of trehalose could result in incomplete exposure of hydrophobic groups, and consequently a decrease in the value of ΔC_p. Osmolytes and other salting-out agents responsible for protein stabilization are known to induce the formation of secondary structures in proteins under denaturing conditions (40–42). Naturally occurring osmolytes like sarcosine, proline, sugars, and trimethylamine-*N*-oxide have been shown to result in the contraction of the denatured state proportional to their stabilizing power (22, 43). A relatively compact denatured state would have lesser hydrophobic surface in contact with the solvent leading to a decrease in the apparent heat capacity of protein denaturation. This is supported by our observation of a comparatively higher value of ΔC_p for RNase A at pH 7.0 in trehalose solution where 1.5 M GdmCl was also added. GdmCl is a strong chaotropic agent and unfolds the protein to a more extended conformation leading to the greater exposure of the buried hydrophobic groups.

The CD data presented as *insets* in Fig. 1 for all the five proteins studied also indicate the retention of a considerable amount of secondary structure in the presence of 1 M trehalose relative to control at the T_m for the proteins in buffer. Even at very high temperatures (70–80 °C), where the proteins are extensively denatured, there is slight retention of the secondary structure in trehalose solutions relative to that in the buffer alone. The increase in the negative ellipticity for α-CT-gen at pH 2.5 with an increase in temperature is surprising. A similar observation has been made earlier by Chalikian *et al.* (44). Nonetheless, there is an increase in the T_m of the protein in the presence of trehalose and the data match well with those obtained from UV melting curves for the protein.

A decrease in the ΔC_p value can have a marked affect on the protein stability, because it results in the flattening of the stability curve (ΔG *versus* T) leading to an increase in the free energy of stabilization over a broad range of temperatures. Interestingly, this seems to be one of the common strategies adopted by several hyperthermophilic proteins to enhance their overall stability (45–47). Also, several studies (22, 29, 43, 48) have shown clearly that natural osmolytes like sugars, sarcosine, and trimethylamine-*N*-oxide essentially reduce the entropy of the denatured state, thereby increasing the relative stability of the native state. In a similar manner, trehalose may lead to protein stabilization as the result of a decrease in the entropy of the denatured state, because the lower heat capacity of denaturation observed suggests that the denatured state of the proteins has regions that are still not fully exposed to the solvent. Therefore, it appears that decreasing the entropy of the denatured state of proteins is a common thermodynamic approach adopted to achieve higher stability and is closely related to a decrease in the heat capacity of protein denaturation.

Effect of Surface Tension and Other Physico-chemical Properties of Water—Surface tension of solvent is known to exert its affect on protein stability by increasing the energy requirement for cavity formation in the solvent to accommodate the increased surface area of proteins upon denaturation (6, 8, 24). A linear correlation of the increase in the ΔT_m of proteins with increase in the surface tension of trehalose suggests this property of water to be an important factor contributing toward protein stability.

It has been observed that, at identical concentrations, trehalose increases the surface tension of water by much larger amounts compared with other sugars and polyols. It is interesting to note that thermodynamic properties of water like partial molal heat capacity and volume, related to the structure of aqueous solutions, also show a considerable increase in the presence of trehalose (49). These values are higher in magnitude compared with those for several other mono- and disaccharides as well as polyols (49, 50). It has also been reported that trehalose has a larger hydrated volume compared with other sugars (51). The increase in the values of these parameters has been attributed to stronger and more extensive hydrogen bonding between hydroxyl groups of trehalose and water molecules. Protein denaturation in such a solution would need additional energy to accommodate its increased surface area. In addition, cosolvents increasing the surface tension of water also get depleted at the protein-solvent interface leading to the preferential hydration of proteins (24). The preferential hydration effect should lead to a loss in the entropy of solvation upon protein denaturation, rendering the unfolded state even more unstable, and resulting in a shift of the equilibrium in favor of the native state. Although the surface tension of water increases to a much larger extent for certain simple electrolytes and carboxylic acid salts (26) compared with trehalose at identical concentrations, it seems that nature has preferred trehalose over these salts as their charged nature could have inhibitory effect on the activity of enzymes.[2] These data suggest why trehalose has been selected by nature as an exceptional stabilizing agent under various stress conditions.

Role of Physico-chemical Properties of Proteins—Table I shows clearly that trehalose does not affect the stability of various proteins to the same extent. The increase in T_m of various proteins as a function of surface tension of the medium (Fig. 3) to different extents suggests that the nature of the protein also plays an important role in the trehalose-mediated thermal stability of proteins. We attempted to investigate the role of the hydrophobicity of proteins by taking into account the changes in hydrophobic interactions due to changes in nonpolar accessible surface area (ΔASAnp) and polar accessible surface area (ΔASAp) upon protein denaturation, *i.e.* $\alpha\Delta$ASAnp plus $\beta\Delta$ASAp, where α (42.5 cal/mol.Å^2) and β (-3.1 cal/mol.Å^2) are weights of each contribution (52). For simplicity, the term $\alpha\Delta$ASAnp plus $\beta\Delta$ASAp can be considered to reflect the effective hydrophobic energy driving the polypeptide chain to fold due to unfavorable interactions with the polar solvent (water). The values of ΔASAnp and ΔASAp for various proteins have

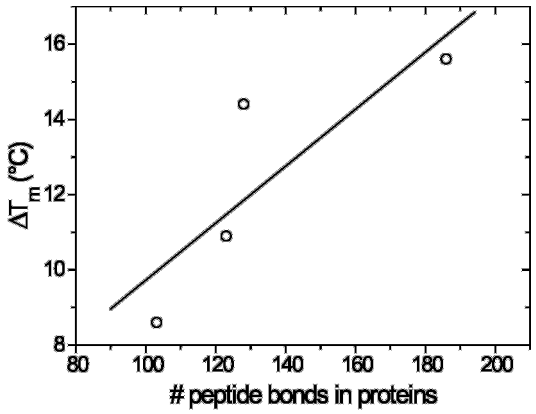


FIG. 5. **Dependence of the ΔT_m of proteins in the presence of 1.5 M trehalose on the number of peptide bonds in them at pH 7.**

been calculated from Myers *et al.* (37). A plot (not shown) between the increments in T_m (ΔT_m) due to the addition of trehalose and $\alpha\Delta$ASAnp plus $\beta\Delta$ASAp of various proteins did not show any correlation suggesting that the side chains, which determine the polarity or nonpolarity of a protein, do not predominantly affect the net stability provided by trehalose. However, these results cannot be considered conclusive due to the limited number of proteins used in this study. Also, in the presence of trehalose all the proteins may not unfold to a complete random coil, hence limiting the calculation of the true changes in the accessible surface area upon denaturation. Another reason for the lack of correlation could be that these proteins also differ from each other in other properties like net charge at a given pH (with pI values ranging from 4.1 for Trp-Inh to 10.7 for lysozyme), which should also contribute to protein-solvent interactions as suggested by the dependence of ΔT_m values in the presence of trehalose as a function of pH for a given protein.

Bolen and coworkers (21–23) have proposed that solvophobic (osmophobic) interaction between the peptide backbone and osmolytes, including sucrose, is the main force driving the protein to a more compact state. To test the validity of the above proposition, the ΔT_m values were plotted against the number of peptide bonds in proteins, and a good correlation was observed at pH 7.0 (Fig. 5). This suggests that protein backbone-cosolvent interactions are unfavorable and do contribute toward protein stability. However, the lack of such a correlation at pH 2.5 and 4.0 (plots not shown) suggests that these unfavorable interactions cannot be considered the sole factor leading to protein stabilization as has been suggested in the case of other osmolytes wherein only one pH condition was used (21–23). Because trehalose is a neutral molecule, the pH-dependent changes in protein stability mediated by trehalose should, therefore, have their origin in the nature of proteins.

Recent studies show that hydrophobic side chains favor unfolding, whereas peptide bonds favor folding of proteins in the presence of sucrose (21) and other osmolytes (22, 23). On the contrary, extensive studies on transfer of amino acids and diglycine to aqueous polyol solutions carried out by Gekko (19, 20) show the dominance of unfavorable interactions between polyols and hydrophobic side chains. The transfer free energy of peptide bond has been observed positive for small chain polyols, which becomes negligible for longer chain polyols like sorbitol (19). Based on these results it has been suggested that peptide-water interactions dominate the stabilization of chymotrypsinogen by polyols (53), whereas intensification of hydrophobic interactions dominates the polyol-induced stabilization of lysozyme (54). Recently, studies carried out by Weatherly and Pielak (55) suggest that osmolytes can interact differently with proteins and that simple models are not sufficient to understand protein-osmolyte interactions. The present study involving several proteins varying in their molecular size does indicate the contribution of unfavorable peptide-trehalose interactions in protein stability. However, in addition, the contribution of charge status of proteins based on the pH-dependent stability effect mediated by trehalose is also evident.

Activity of RNase A in the Presence of Trehalose—RNase A was taken as a model system to analyze, in general, the thermostabilization effect of trehalose especially on the bioactivity of enzymes at high temperatures. The relative activity of RNase A in the presence of trehalose (Table II) has been calculated by dividing the slope of the linear zone of the corresponding activity plots by the slopes of the data for control.

In set 1, the activity retention in the presence of trehalose is seen to depend upon its effectiveness in locking the protein molecule in its native state even under denaturing conditions as has been suggested for the action of chaperonins (56). In buffer at 66 °C, only 7% molecules of RNase A are present in the folded state. The addition of 1.5 M trehalose raises the T_m of RNase A to 67.7 °C and the population of the folded molecules to 70%. At high temperatures, trehalose is known to preferentially bind weakly to the native state of RNase A (9) and could, thus, protect against any deleterious temperature-induced kinetic reactions like aggregation and preserve the overall activity on cooling. Trehalose has also been known to suppress the aggregation of unfolded proteins *in vivo* (57) as well as heat-denatured proteins *in vitro* (41).

In the second set, the greater effectiveness of trehalose at 63 °C as compared with that at 56 °C may be explained on the basis of the extent of unfolding at the two temperatures. In the absence of any additive, RNase A has a T_m of 61.6 °C at pH 7.0 (26). There is only a marginal difference in the population of the native RNase A in the presence (f_{Nt}) and the absence (f_{Nc}) of trehalose at 56 °C, *i.e.* the stabilization factor f_{Nt}/f_{Nc} is 1.04. However, this ratio increases to 2.82 at 63 °C resulting in an increase in the relative activity retention from 1.47 at 56 °C to 2.41 at 63 °C upon addition of 1.5 M trehalose. This is essentially due to a marked thermostabilization effect of trehalose on protein conformation at the higher temperature. At 52 °C, the relative activity term, in the presence of a mixture of 1.5 M trehalose and 1 M GdmCl, was much higher than the ratio of the native states at the same temperature. This could be due to a decrease in the deleterious effect of heat on enzymes at lower temperature. The greater protective action of trehalose in the presence of GdmCl could be ascribed to the fact that GdmCl being a protein solubilizer can prevent inactivation due to aggregation of the enzyme molecules at higher temperatures (34). Solubilizing agents like GdmCl and urea have been known to increase the refolding yields of proteins (58, 59). The higher activity obtained at 25 °C after incubation of RNase A at 60 °C in the presence of 1 M GdmCl in comparison to that incubated at 66 °C without GdmCl clearly demonstrates the role of GdmCl in inhibiting the aggregation of the protein molecules during incubation. A much smaller value of relative activity compared with the corresponding stabilization factor indicates that RNase A refolds to a large extent to its native state even in the absence of trehalose.

Unlike trehalose, other mono- and disaccharides and several of the polyols have not been observed to provide thermostabilization and thermoprotection of proteins to such an extent as trehalose (1, 2, 10, 11). This is essentially due to the differences in their cosolvent molecular structure and their solution physico-chemical properties as described earlier. Most of the polyols and sugars studied so far lead to preferential hydration

of proteins at low temperatures (20–25 °C). However, studies carried out at higher temperatures (~50 °C) show considerable differences in the mode of protein-cosolvent interactions (9, 18). At low temperatures sorbitol and trehalose lead to preferential hydration of native RNase A, whereas at higher temperatures, sorbitol as well as trehalose have been observed to bind weakly to RNase A (9, 18). Trehalose in comparison to sorbitol binds to a greater extent to the native state at higher but nondenaturing temperatures. Fundamentally, both of these cosolvents stabilize the native state of RNase A. However, as far as bioactivity is concerned, the varying extent of binding of the cosolvent at elevated temperatures may have perceptible effect on the retention of activity. Strong binding of trehalose to the native state at high temperatures may provide a more compatible environment and protection from heat inactivation.

This study clearly demonstrates that trehalose-induced thermostabilization of the protein structure is also helpful in the retention of biological activity of proteins at high temperatures. It is concluded that surface tension effect dominates the stability effect of trehalose, and, although unfavorable peptide-trehalose interactions contribute to protein stability as proposed, the interactions of trehalose with various side chains of proteins also contribute to the stability effect. Even though the nature of protein molecules contributes to protein-trehalose interaction in aqueous solutions to some extent, trehalose can be expected to work as a universal protein stabilizer and could be effectively used to increase the stability of many of the industrial and therapeutic enzymes without fail.

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