Thermal Stability of Proteins in Aqueous Polyol Solutions: Role of the Surface Tension of Water in the Stabilizing Effect of Polyols

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Polyols and sugars are known to increase the thermal stability of proteins in aqueous solutions and lead to their preferential hydration. While the mechanism of action of sugars has been proposed to be essentially due to the increase in the surface tension of water in their presence, polyols including glycerol have been reported to lower the surface tension of water and act via the solvophobic effect. Surface tension measurements of polyols in water carried out by us, on the contrary, indicate a substantial increase in the surface free energy of water. These increments are comparable to those found in the case of sugars. To investigate the role of the surface tension of the solvent medium on the thermal stability of proteins, we have carried out a comprehensive study on the effects of a series of polyols with varying numbers of hydroxyl groups and stereochemistry, such as mannitol, inositol, sorbitol, xylitol, and adonitol, on the thermal stability of five proteins varying in their physicochemical characteristics, viz., ribonuclease A, α-chymotrypsinogen, lysozyme, cytochrome *c*, and trypsin inhibitor. The studies have been carried out at pH 2.5, 4.0, and 7.0 to elucidate the contribution of the surface charges toward the polyol-mediated thermal stability of proteins. A very good correlation between an increase in the surface tension of water in the presence of a polyol and an increase in the themal stability of proteins has been observed. Correlation of the thermal stability of proteins with physicochemical properties such as net charge and net hydrophobic surface exposed on unfolding indicates that none of these properties is a dominant factor in governing protein thermal stability. The results indicate that polyol-induced water-mediated effects such as an increase in the surface tension of water play a major role in the stabilization of proteins and that the preferential hydration of proteins observed in their presence is a consequence of the increase in the surface free energy of water.

Introduction

In the last several years there has been a considerable effort toward improving the thermal stability of proteins. The majority of the work done has focused on the site-directed mutagenesis of the amino acid residues to enhance their thermal stability.[1] In addition to the role of amino acids in imparting structural stability to the protein, the solvent environment has been known to play a significant role for several decades. Among the cosolvents employed for this purpose are sugars,[2−6] some salts,[7,8] polyols,[9,10] and amino acids.[11] While sugars and polyols have always been found to enhance the thermal stability of proteins by several degrees depending on their concentration, some of the amino acids have even been found to destabilize the protein conformation[12] and the effect of salts depends much on the pH of the medium and their chemical nature.

Some sugars and polyols have been found to increase the thermal denaturation temperature of proteins by as much as 15 °C at 2−3 M concentration.[2] Uedaira and Uedaira[13] have correlated the stabilizing effect of sugars with the number and positions of hydroxyl groups. The sugar effect seems to be nonspecific, independent of the nature of the protein, and mediated through water around the proteins. Extensive hydrogen bonding of water in the hydration shell around proteins in the presence of sugars has been considered to be a major driving force toward their stabilizing effect.[9] Proteins have also been found to be preferentially hydrated in the presence of sugars.[5,6,14]

Polyols have also been shown to impart preferential hydration to bovine serum albumin (BSA) and ribonuclease A (RNase A).[3,15] Sugars are known to increase the surface tension of water, thereby increasing the energy requirement to create a cavity in such a medium upon protein denaturation.[5] An increase in the surface tension of water has therefore been considered as the major factor in the stabilization of proteins by sugars. The observed preferential hydration of proteins in sugars has also been considered as resulting from the surface tension effect.[16] However, the surface tension of water has not been thought to play any major role in protein stabilization by polyols, as glycerol decreases the surface tension of water while increasing protein stability[17] and other polyols have been reported to decrease the surface tension of the solvent.[18] Preferential hydration, a decrease in the hydrogen-bond rupturing capacity of the medium,[9,19] and solvophobic effects[20] have been proposed to be the operative forces for protein stabilization in the presence of polyols. It is proposed that these forces are strong enough to offset the effect of decreased surface tension in their presence.[18] On the contrary, we have observed a substantial increase in the surface tension of water at room temperature in the presence of all the polyols studied by us, viz., mannitol, inositol, sorbitol, xylitol, and adonitol. This paper reports the correlation of the increase in the surface tension of water in the presence of polyols with their effect on an increase in the thermal stability of five different proteins, viz., RNase

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A, α-chymotrypsinogen (α-CTgen), lysozyme, cytochrome *c* (cyt c), and trypsin inhibitor (trp-inh), varying in their physicochemical properties. The studies have been carried out at pH 2.5, 4.0, and 7.0 to elucidate the contribution of surface charges toward the polyol-mediated thermal stability of proteins. The selection of five different proteins has also enabled us to find any possible correlation between the net hydrophobicity of the proteins and their thermal stability in polyols.

Experimental Section

Materials. RNase A, lysozyme (hen egg white, HEW), α-CTgen, cyt c, and trp-inh (HEW) were all from Sigma Chemical Co. They were dialyzed against distilled deionized water and lyophilized. The lyophilized samples were dried over P_2O_5. The polyols sorbitol, mannitol, adonitol, and xylitol were also procured from Sigma while myoinositol was obtained from Himedia labs, India. Glycine, sodium acetate, and disodium and monosodium hydrogen phosphate were from E. Merck, India. Glass doubly distilled water was used to make the polyol solutions. The pH of the polyol solutions was adjusted on a Consort or Radiometer PHM84 research pH meter by adding HCl or NaOH solutions. Also, 20 mM glycine HCl buffer at pH 2.5, 40 mM acetate buffer at pH 4.0, and 20 mM phosphate or MOPS (Sigma Chemical Co.) buffer at pH 7.0 were used from their stock solutions. The pH 7.0 solutions were made in the presence of 1.5 M guanidium chloride (GdmCl) (Amresco, OH).

Thermal Denaturation Experiments. Thermal denaturation experiments were carried out using a Cecil 599 or Shimadzu 160A model UV/vis spectrophotometer to which a linear temperature programmer CE-247 (Cecil) was attached. The concentrations of the protein solutions were ~0.5 mg/mL except for cyt c, where a 0.1 mg/mL protein concentration was used. The protein solutions were loaded in a 0.5 mL masked and Teflon-stoppered quartz cuvette (Hellma). A temperature scan rate of 1 °C/min was used in all the experiments. The wavelengths for monitoring the conformational changes 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. These wavelengths were selected on the basis of the protein difference spectra. The reversibility of the thermal transitions recorded for the proteins was checked by reheating the protein solutions. In all the cases except that for α-CTgen at pH 4.0, the thermal transitions were reversible and hence amenable to thermodynamic analysis.

Surface Tension Measurements. Surface tension measurements of polyol−water mixtures were carried out by the drop weight method.[14] The rate of flow through the capillary was controlled to 5−6 drops per minute. The temperature of the stalagmometer was maintained at 25 ± 0.1 °C by circulating water through the glass jacket around it from a circulator bath (Haake F3-CH), which was stopped during the collection of drops to avoid any vibrations. In each experiment the weight of 55 drops was measured on a Scientech Precision Scientific weighing balance immediately after the collection of the drops.

Analysis of Data. The evaluation of thermodynamic parameters obtained from spectroscopic techniques is based on the equilibrium constant K for N ↔ D conversion for a two-state reversible transition, where N represents the native state and D the denatured state. The equilibrium constant was deduced from the equation

$$K = [\text{unfolded}]/[\text{native}]$$

or

$$K = (A_N - A_O)/(A_O - A_D) \quad (1)$$

where A_N is the absorbance of the pure native state in the transition zone after extrapolation from the pretransition region, A_D is the corresponding absorbance of the pure denatured state, and A_O is the observed absorbance at any temperature in the transition zone. However, the use of K to deduce ΔH, the enthalpy of denaturation, and ΔS, the entropy of denaturation, using the absorbance vs temperature curves obtained is fraught with inaccuracies arising especially due to the variable slopes and sometimes nonlinear behavior of the pre- and post-transition regions. This also leads to inaccurate estimation of the thermodynamic parameters outside of the narrow transition zone due to large extrapolation errors. To avoid this we have used all the experimental data points obtained and fitted them in the equations in the following manner. Equation 1 can be rewritten as

$$A_O = (A_N + KA_D)/(1 + K) \quad (2)$$

and

$$K = \exp(-\Delta G°/RT) \quad (3)$$

where $\Delta G°$ is the standard free energy change, R is the gas constant, and T is the temperature in degrees Kelvin. Substituting the value of K in eq 2 we get

$$A_O = [(A_N + A_D \exp(-\Delta G°/RT))/(1 + \exp(-\Delta G°/RT)] \quad (4)$$

A form of the Gibbs−Helmholtz equation can be written as

$$\Delta G°(T) = \Delta H_m(1 - T/T_m) - \Delta C_p(T_m - T + T \ln(T/T_m)) \quad (5)$$

where ΔH_m is the enthalpy of denaturation calculated at the T_m, ΔC_p is the heat capacity change for denaturation, T_m refers to the temperature at the midpoint of the transition, and T refers to any temperature where $\Delta G°(T)$ is calculated. Substituting the value of $\Delta G°(T)$ obtained from eq 5 in eq 4 we get

$$A_O = [A_N + A_D \exp(-1/R(\Delta H_m(1/T - 1/T_m) - \Delta C_p (T_m/T - 1 + \ln(T/T_m)))]/[1 + \exp(-1/R(\Delta H_m(1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + \ln(T/T_m)))] \quad (6)$$

Since A_N and A_D have been found to be linear functions of temperature, they can be written as

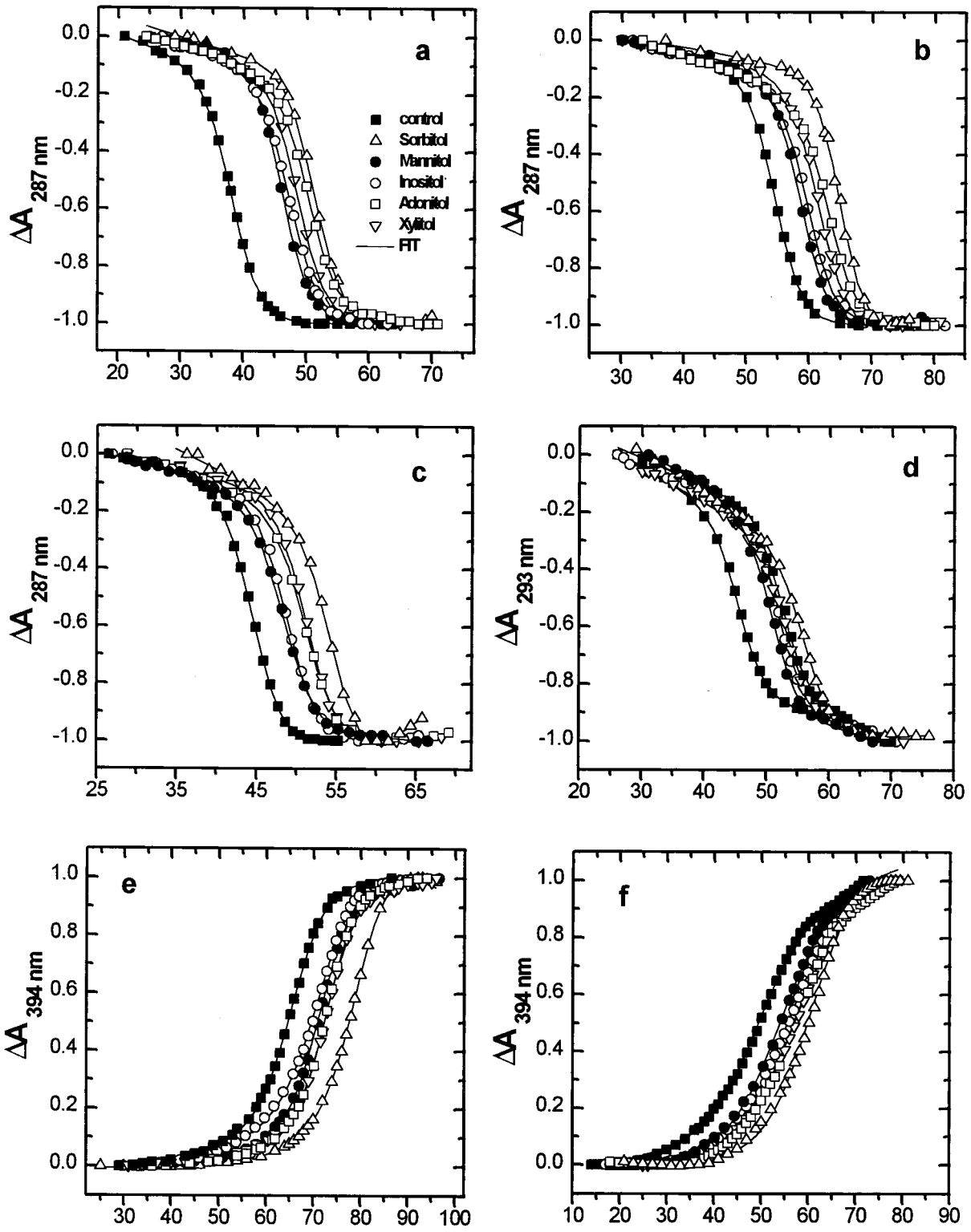
$$A_N = a_N + m_N T \quad (7)$$

$$A_D = a_D + m_D T \quad (8)$$

where a_N and a_D are the intercepts of A_N and A_D vs temperature plots and m_N and m_D are the corresponding slopes. Substituting A_N and A_D as obtained from eqs 7 and 8 in eq 6 we get

$$A_O = [a_N + m_N T + (a_D + m_D T) \exp(-1/R(\Delta H_m(1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + \ln(T/T_m)))]/[1 + \exp(-1/R(\Delta H_m (1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + \ln(T/T_m)))] \quad (9)$$

Equation 9 was used to fit the thermal denaturation data by using a nonlinear least-squares fit and successive iterations using the Marquardt−Levenberg routine as available in the Origin software (Microcal Inc., Northampton, MA). A minimum of 50 iterations or more was performed until the fractional change in the χ^2 value was within the tolerance limit which was set to



0.0005. Usually a value on the order of $10^{-5}-10^{-4}$ of the fractional χ^2 change was obtained in the final iterations. Parameters a_N, a_D, m_N, m_D, T_m, and ΔH_m were floated freely so as to fit them simultaneously to eq 9. The ΔC_p values available in the literature for individual proteins were used as constants.

Results

Figure 1 presents a set of thermal unfolding curves for RNase-A, α-CTgen, lysozyme, cyt c, and trp-inh in the presence of 1.0 M mannitol, 2.0 M sorbitol, 0.75 M myoinositol, 2.0 M adonitol, and 2.0 M xylitol. The pH values selected were 2.5, 4.0, and 7.0. In the case of cyt c, studies at pH 2.5 were not possible as it is partially denatured at this pH.[21] Trp-inh studies were not possible at pH 2.5 and 4.0 due to protein aggregation upon heating at these pH values. For all the proteins the thermal denaturation experiments carried out at pH 7.0 included 1.5 M GdmCl in the solutions. This was necessary in order to avoid aggregation of the proteins at higher temperatures and to bring the thermal transition temperature of stable proteins within the limit of experimental study. Addition of GdmCl in this way has no effect on ΔT_m values as determined earlier for lysozyme

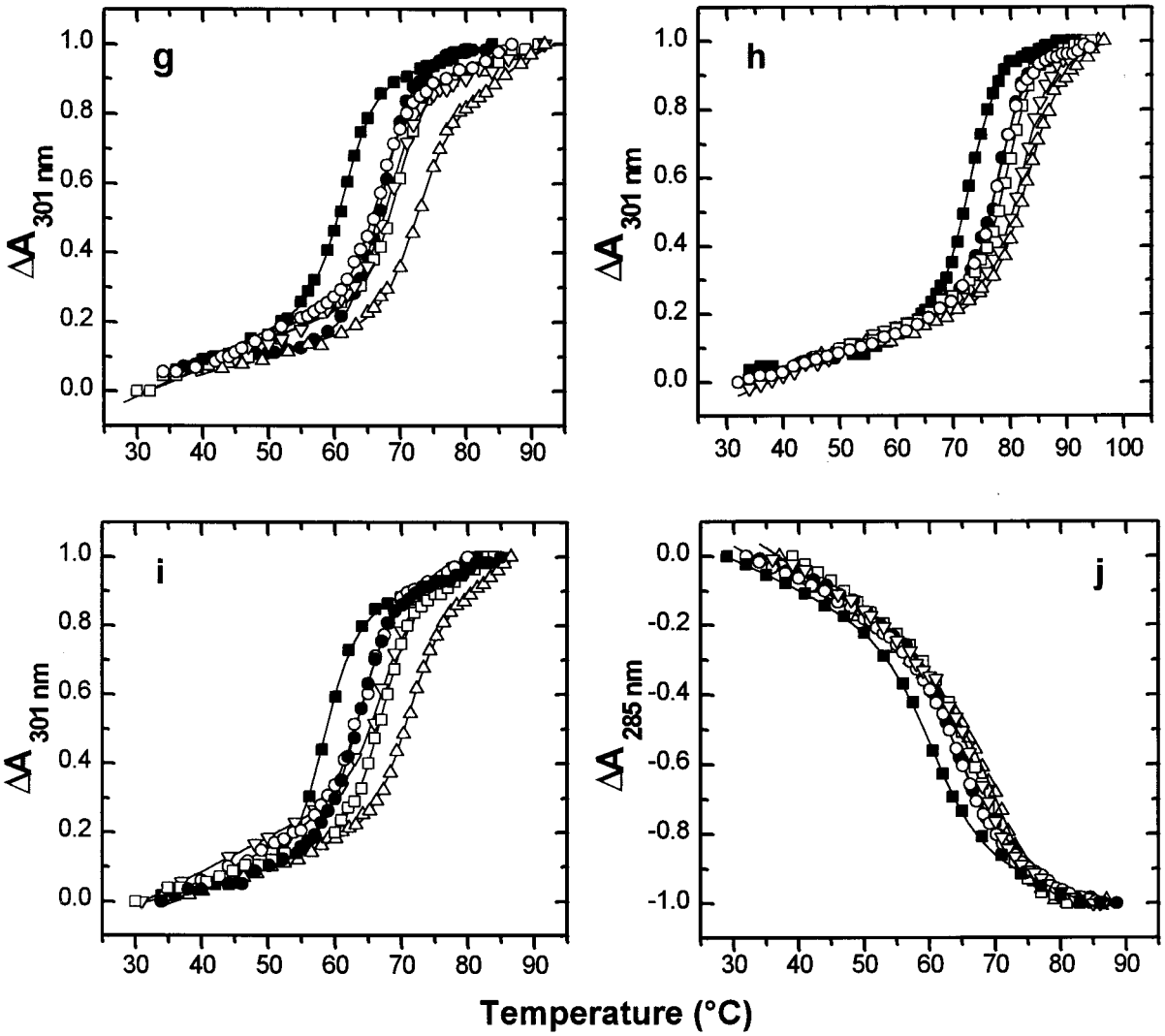


Figure 1. Thermal denaturation profiles of proteins in the presence of polyols. RNase A at pH 2.5 (a), 4.0 (b), 7.0 (c); α-CTgen at pH 2.5 (d); cyt c at pH 4.0 (e), 7.0 (f); lysozyme at pH 2.5 (g), 4.0 (h), 7.0 (i), and trp-inh at pH 7.0 (j). All the experiments at pH 7.0 were carried out in the presence of 1.5 M GdmCl. The symbols for polyols presented in panel a are used throughout this paper.

stability in the presence of osmolytes.[22] The concentrations of the polyols selected were such as to have maximal effect on the thermal stability of the proteins, keeping in view their solubility in solution as well as their tendency to precipitate proteins at higher temperatures.

The data presented in Figure 1 have been used to evaluate T_m, ΔH_m, ΔS_m, and ΔT_m. Instead of calculating the free energy gain at 25 °C we have calculated the free energy change, $\Delta G°$, of proteins in the presence of polyols at the T_m of the control, wherein $\Delta G°(T_m)$ is zero. Hence the free energy change for proteins in the presence of polyols will be the net free energy of stabilization, $\Delta\Delta G°$, evaluated at the T_m of the control. These data have been summarized in Table 1. The values presented are the averages of at least three scans. The T_m values were found to be within ±0.5 °C, whereas the uncertainties in ΔH_m, ΔS_m and $\Delta\Delta G°$ were within ±7%. The thermodynamic parameters for α-CTgen at pH 4.0 have not been calculated due to irreversible denaturation under these conditions. T_m and ΔT_m values are, however, presented for the sake of comparison.

It can be seen from Figure 1 and Table 1 that the polyols studied increase the thermal stability of all the proteins. On a molar basis, it was found that inositol was the best stabilizer with the highest value of ΔT_m, followed by the epimers mannitol and sorbitol, whereas xylitol and adonitol, which are also epimers, had the least stabilizing effect. ΔT_m values per molar concentration of polyols were calculated by assuming a linear dependence of T_m with the concentration of polyols as observed earlier by Gekko and Morikawa.[4] It has been found that as the number of hydroxyl groups increases, their stabilizing effect for the protein also increases.[4] Our results are consistent with the observation for a wide variety of proteins studied (Table 1). Although the increase in ΔH_m values in the presence of different polyols is within the experimental and fitting errors, an overall gradual increasing trend is implied. ΔS_m values also show a corresponding increase. The increase in ΔH_m values with the increase in T_m values has often been observed for several proteins studied using differential scanning calorimetry.[23−25] There is also a very good correlation of the increase in $\Delta\Delta G°$ with the increase in ΔT_m for RNase A, lysozyme, and cyt c (Table 1).

Effect of pH. Figure 2a represents the plots of ΔT_m per mole of additive (ΔT_m/mol) versus the net charge on RNase A. Since the data for mannitol and inositol are at lower concentration due to their limited solubility compared to other polyols, the data need to be normalized to a fixed concentration for all these additives. The net charges on RNase A[26] and lysozyme[27] were calculated from their pH titration curves. The data show that thermal stability increases with the net charge on RNase A,

TABLE 1: Thermodynamic Parameters for Several Proteins in Aqueous Polyol Solutions at Various pH Values

cosolvent	RNase A					α-CTgen					lysozyme					cyt c				
	T_m (°C)	ΔT_m (°C)	ΔH_m (kcal/mol)	ΔS_m (cal/mol/K)	$\Delta\Delta G°$ (kcal/mol)	T_m (°C)	ΔT_m (°C)	ΔH_m (kcal/mol)	ΔS_m (cal/mol/K)	$\Delta\Delta G°$ (kcal/mol)	T_m (°C)	ΔT_m (°C)	ΔH_m (kcal/mol)	ΔS_m (cal/mol/K)	$\Delta\Delta G°$ (kcal/mol)	T_m (°C)	ΔT_m (°C)	ΔH_m (kcal/mol)	ΔS_m (cal/mol/K)	$\Delta\Delta G°$ (kcal/mol)
pH 2.5																				
control	38.3		81.1	260	0.00	44.9		96.3	302	0.00	61.1		98.0	293	0.00					
mannitol (1 M)	46.6	8.3	87.5	274	2.12	50.5	5.6	94.0	290	1.47	67.1	7.0	100.8	296	1.70					
inositol (0.75 M)	47.0	8.7	88.6	277	2.25	52.3	7.4	100.2	308	2.04	66.8	6.4	98.0	288	1.49					
xylitol (2.0 M)	48.5	10.2	88.8	276	2.60	52.6	7.7	101.9	313	2.10	68.7	8.0	103.7	303	2.18					
adonitol (2.0 M)	50.2	11.9	89.0	275	2.98	52.5	7.6	99.5	305	1.97	69.1	9.1	101.1	295	2.21					
sorbitol (2.0 M)	51.5	13.2	91.6	282	3.38	54.7	9.8	98.2	300	2.45	71.0	9.9	103.1	300	2.78					
pH 4.0																				
control	54.2		94.0	287	0.00	59.0					73.1		98.5	284	0.00	64.5		61.5	182	0.00
mannitol (1 M)	59.0	4.8	97.2	293	1.40	63.0	4.0				77.9	4.8	100.8	287	1.32	69.8	5.4	66.5	190	0.94
inositol (0.75 M)	59.8	5.6	98.1	295	1.64	62.0	3.0				77.7	4.6	101.0	288	1.32	70.9	6.5	54.2	158	0.90
xylitol (2.0 M)	61.4	7.2	98.8	296	2.11	64.5	5.5				80.1	7.0	101.0	286	1.89	72.5	8.1	62.1	180	1.30
adonitol (2.0 M)	63.3	9.1	103.3	307	2.66	64.0	5.0				79.6	6.5	102.0	289	1.78	72.6	8.2	67.4	195	1.42
sorbitol (2.0 M)	66.0	11.8	105.3	310	3.50	65.3	6.7				82.5	9.4	104.9	295	2.57	73.8	9.3	64.8	187	1.52
pH 7.0[a]						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
mannitol (1 M)	50.2	3.9	97.6	302	1.26	64.8	5.8	71.3	211	1.08	63.5	5.0	96.6	287	1.37	52.7	4.7	38.4	118	0.50
inositol (0.75 M)	50.7	4.5	98.5	304	1.44	64.5	5.5	71.2	211	1.06	64.5	6.0	96.1	285	1.63	54.0	6.0	40.2	123	0.65
xylitol (2.0 M)	52.9	6.5	97.7	300	2.14	67.3	8.3	71.2	209	1.53	66.5	8.0	100.9	297	2.25	56.8	8.8	40.0	121	0.88
adonitol (2.0 M)	53.4	6.8	97.9	300	2.15	67.3	8.3	71.8	211	1.54	67.0	8.5	102.0	300	2.39	56.5	8.5	39.9	121	0.75
sorbitol (2.0 M)	56.1	10.3	99.5	302	2.50	71.8	12.8	73.0	212	2.22	70.1	11.6	105.5	307	3.29	61.1	13.1	45.5	136	1.37

[a] Solutions contain 1.5 M GdmCl.

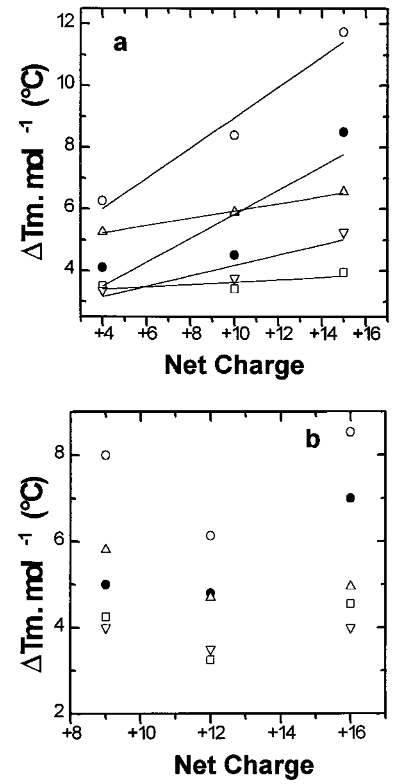


Figure 2. Effect of net charge on RNase A (a) and lysozyme (b) on the thermal stability in the presence of polyols. See Figure 1a for description of symbols.

TABLE 2: Partial Molar Heat Capacities, Volumes, and Surface Tension Values of Aqueous Polyol Solutions

polyols	$\bar{C}_p°$ (kJ mol^{-1})	$\bar{V}°$ (cm^3 mol^{-1})	σ/M (dyn cm^{-1} mol^{-1})
xylitol	346.4,[a] 326[b]	102.14[a]	72.9 ± 0.20
adonitol	375.6,[a] 354[b]	103.11[a]	72.8 ± 0.21
inositol	369[b]		74.22 ± 0.26
mannitol	455.4,[a] 461[b]	119.71[a]	73.12 ± 0.07
sorbitol	423.1,[a] 424[b]	119.16[a]	73.15 ± 0.19

[a] Reference 55. [b] Reference 41.

implying that at lower pH values the polyols are more effective in their stabilizing action. Similar trends have been observed for α-CTgen at pH 2.5 and 4.0 and for cyt c at pH 4.0 and 7.0; however, in the case of lysozyme, variation of ΔT_m with the net charge is not linear (Figure 2b). The anomaly in the variation of ΔT_m with the net charge on proteins could be due to the presence of 1.5 M GdmCl in the solution at pH 7.0 conditions, even though the contribution of ca. 15 °C to ΔT_m in the presence of GdmCl has been taken into account while plotting the results. The plots for all the proteins have not been drawn due to the availability of ΔT_m values at only two pH values for α-CTgen and cyt c and at only one pH value in the case of trp-inh.

Surface Tension Measurement and Protein Stability. Surface tension (σ) values of aqueous solutions of polyols measured by the drop weight method have been shown in Table 2. Also included are the $\bar{C}_p°$ and $\bar{V}°$ values of aqueous polyol solutions taken from the literature. Figure 3 shows the variation of ΔT_m for various proteins as a function of the surface tension of aqueous polyol solutions at pH 7.0. Although thermal stability (ΔT_m) does not increase linearly with the surface tension of the medium, a positive contribution of surface tension is obvious. Similar trends have been observed for the proteins studied at pH 2.5 and 4.0 also. It implies, therefore, that the

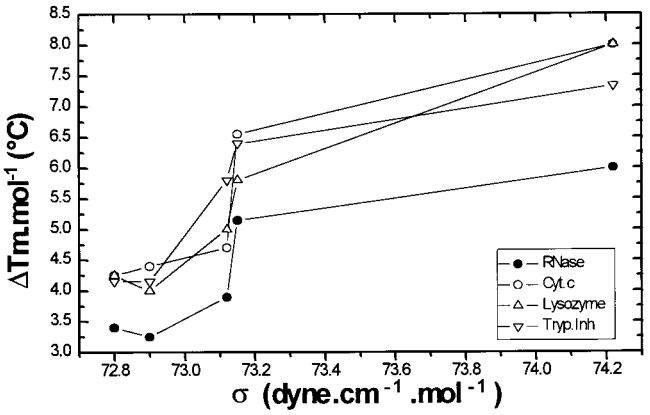


Figure 3. Effect of the surface tension of aqueous polyol solutions on the thermal stability of various proteins at pH 7.0.

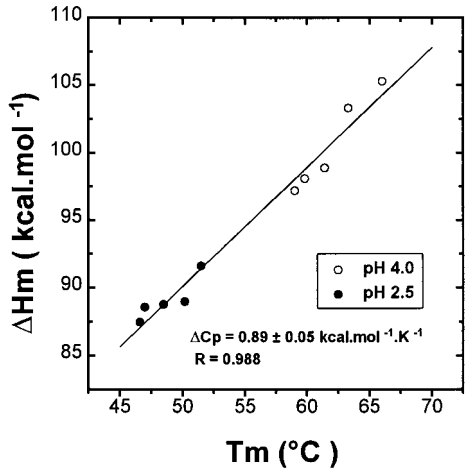


Figure 4. Enthalpy of denaturation, ΔH_m, as a function of transition temperature, T_m. Data at pH 2.5 and 4.0 have been used to evaluate the value of heat capacity. Data at pH 7.0 had a larger scatter in values and hence could not be used.

natures of both the cosolvent polyol and the protein govern protein−cosolvent interactions and hence the thermal stability.

Excess Heat Capacity of Proteins in the Presence of Polyols. The data in Table 1 clearly show that there is only a marginal increase in the enthalpy of denaturation, ΔH_m, in the presence of polyols. The change in ΔH_m as a function of transition temperature for RNase A has been shown in Figure 4. Enthalpy of denaturation increases linearly with the T_m, suggesting a positive increase in the heat capacity of RNase A upon denaturation (ΔC_p). A ΔC_p value of 0.89 ± 0.05 kcal mol^{-1} K^{-1} has been obtained in the presence of polyols. Pace and Laurents[28] have reported a value of 2.2 ± 0.3 kcal mol^{-1} K^{-1} evaluated by varying the T_m of RNase A by adding urea. A calorimetric value of 1.4 kcal mol^{-1} K^{-1} has been reported by Privalov et al.[29] Recently Liu and Sturtevant[25] have very rigorously investigated the effect of various cosolvents on the calorimetric heat capacity value of RNase A and lysozyme. They have reported a ΔC_p value of 1.74 ± 0.07 kcal mol^{-1} K^{-1} for RNase A in buffer, which differs from their earlier reported value of 2.06 kcal mol^{-1} K^{-1}.[30] Liu and Sturtevant[25] have pointed out that the origin of such a variation could be in the method and conditions employed in the evaluation of the heat capacity of proteins. In the presence of polyols we have observed a decrease in the ΔC_p values for all the proteins studied.

Role of Physicochemical Properties of Proteins. As evident from Figure 1 and Table 1, the nature of the protein plays a significant role for the stabilizing effect in the presence of polyols. Is there a physicochemical property associated with the protein which could be governing polyol−protein interactions mediated via the solvent water? To answer this question we examined in detail the correlation of properties of proteins such as net charge and net buried hydrophobic surface area, ΔASA_{NP}-ΔASA_P, with the thermal stability of proteins, where ΔASA_{NP} is the buried nonpolar accessible surface area (ASA) which gets exposed upon denaturation of the native state and ΔASA_P is the buried polar ASA getting exposed upon unfolding of the protein. ΔASA_{NP} and ΔASA_P for RNase A, lysozyme, α-CTgen, and cyt c were taken from the literature.[31] ΔASA for trp-inh was not available and hence was calculated from the equation given,[31] taking the number of total amino acid residues present in it to be 18632 and assuming that about 30% of the buried ΔASA is contributed by the polar groups, especially buried peptide bonds.

Plots of ΔT_m/M additive versus these properties at several pH values using the data for the proteins studied by us did not give any direct correlation or uniform dependence except at pH 7.0, wherein an increase in ΔT_m was observed as a function of increasing net buried hydrophobic surface area (Figures 5a and 5b). Interaction of ΔASA_{NP} with a hydrophilic solvent should drive the protein toward the native state. On the other hand, favorable interactions of the solvent with the polar groups which get exposed upon denaturation should destabilize the native conformation. The balance between these two opposing interactions should decide the role of particular additives in providing the stability to proteins. In addition, the contribution due to the peptide backbone and its interaction with the solvent medium upon protein denaturation should also be taken into consideration.[33]

Figures 5c and 5d show the effect of the net charges on the protein surface irrespective of the type of protein at a given pH. At pH 7.0, ΔT_m increases with an increase in the net charge, whereas at pH 4.0 the relationship is reversed. This clearly indicates that although net charge is important, contributions from other interactions play a compensatory role in modulating the protein−cosolvent interactions. Different properties of the proteins seem to vary in a nonparallel way, leading to a varied net effect on the protein stability.

Discussion

The data presented in Figure 1 and Table 1 clearly indicate that the polyols used in the study stabilize proteins to varying extents. The polyols selected were such as to enable the comparison of the effects of sorbitol and mannitol, having six carbons in their chains, with xylitol and adonitol, having five carbons. Also, inositol, which is a cyclic polyol with six carbon atoms, could be compared with the analogous acyclic polyols sorbitol and mannitol.

In a multicomponent system containing protein, water, and polyol, the interactions that could govern the stabilizing action of a polyol include those between polyol and water, between polyol and protein, and between water and protein. Polyols are known to induce preferential hydration for bovine serum albumin[3] and are therefore excluded from the immediate vicinity of the protein. Recent studies on RNase A in the presence of sorbitol have shown that under denaturing conditions (high temperature, low pH) the protein undergoes greater preferential hydration than in the native state (high temperature, high pH).[15] It has been proposed that unfavorable interaction with sorbitol of the denatured state of RNase A compared with that of the native state is the driving force for the stabilization of the protein

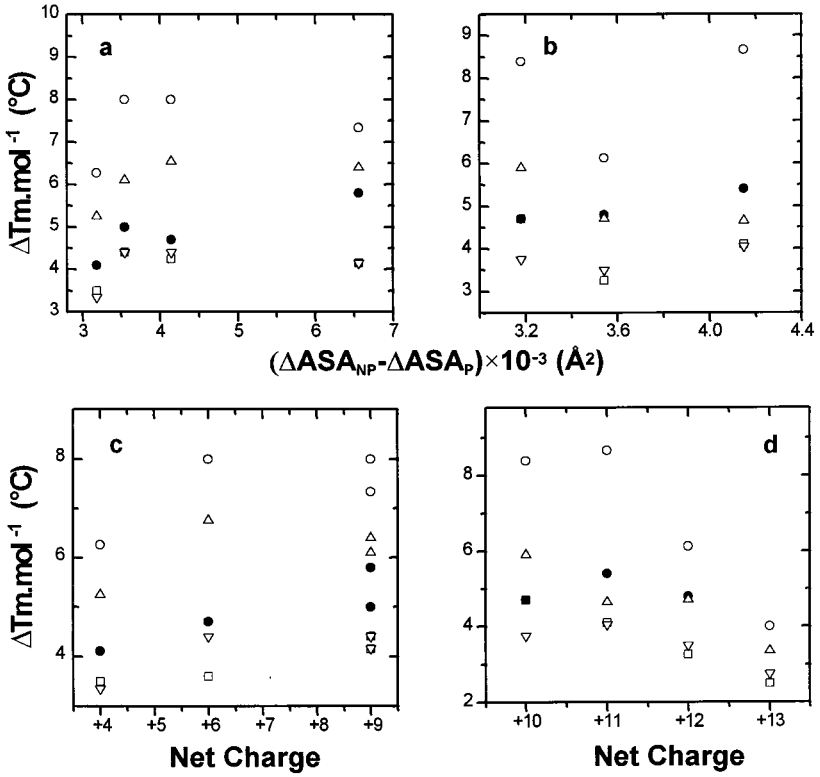


Figure 5. Dependence of the thermal stability of proteins on their physicochemical characteristics. Contribution of net hydrophobicity at pH 7.0 (a) and pH 4.0 (b) and net charge at pH 7.0 (c) and pH 4.0 (d). ΔASA_{NP}-ΔASA_P (Å^2) values for various proteins are RNase A, 3181; lysozyme, 3542; cyt c, 4148; trp-inh, 6556. Net charge on trp-inh is −9 at pH 7.0, but the data have been shown against +9 net charge. Symbols used are the same as in Figure 1a.

in sorbitol. It therefore appears that water-mediated effects in the presence of polyols may play a crucial role in the stabilization of proteins.

Role of Surface Tension. Recently, the surface tension of water and its change by the addition of cosolvents has been thought to play a major role in the stabilization of proteins in aqueous solutions.[14,34] Using rigorous thermodynamic analysis, the effect of sugars such as sucrose,[5] glucose and lactose,[6] and trehalose[14] on the surface tension of water has been found to be the main force behind the stabilization of proteins and their preferential hydration. However, polyols including glycerol have been reported to reduce the surface tension of water,[18] and their stabilizing action has been considered to be essentially solvophobic in nature.[16] On the contrary, the polyols studied by us lead to a considerable increase in the surface tension of water (Table 2). This increase is more or less identical to the increase by sugars. It was found that on a molar basis inositol leads to the largest increase in the surface tension of water, followed by sorbitol and mannitol, which gave nearly equal values. Xylitol and adonitol increased the surface tension of water to similar extents but to lesser magnitudes than sorbitol and mannitol. These data correlate well with our observation of the increase in the thermal stabilities (ΔT_m) of proteins in their presence (Figure 3).

Compounds which increase the surface tension of water are known to be excluded from the protein surface, leading to their preferential hydration. This should lead to an excess of water molecules at the protein−solvent interface in accordance with the Gibbs absorption isotherm[5,35]

$$(\partial m_3/\partial m_2)^{\sigma}_{T,\mu 1,\mu 3} = -Aa_3/RT(\partial\sigma/\partial a_3)_T \equiv -A/RT(\partial\sigma/\partial m_3)_T \quad (10)$$

where the subscripts 1, 2, and 3 represent water, protein, and the cosolvent, respectively, a is the activity of the cosolvent, m is its molality, T is the temperature, μ is the chemical potential, A is the molar surface area of the protein, R is the gas constant, and σ is the surface tension of the medium; σ in superscript indicates that the interaction parameter so obtained is calculated from the surface tension measurements rather than directly by dialysis equilibrium. Any compound leading to a positive increase in σ with increasing concentration of cosolvent should give a negative preferential interaction parameter, $(\partial m_3/\partial m_2)$, and hence lead to preferential exclusion of the cosolvent molecules from the protein surface.[20] The preferential hydration induced by the polyols in the case of BSA[3] has been found to be in the order inositol > sorbitol ≈ mannitol > xylitol. Interestingly, it correlates very well with the order of the degree of increase in the surface tension of water in the presence of these polyols as observed by us (Table 2). We have observed a good correlation of the surface tension increments by polyols with the increase in thermal stability of several globular proteins, varying in their physicochemical properties at pH 7.0 (Figure 3) and at pH 2.5 and 4.0. These observations suggest the dominant role of surface tension of the medium in providing stability to the globular proteins. We, therefore, consider that the observed preferential hydration of proteins is essentially due to the increase in the surface tension of water in the presence of polyols rather than due only to the unfavorable protein−cosolvent interactions, as has been proposed earlier,[16,18] and that the increase in the protein thermal stability is due to the increase in the surface tension of water and not due to solvophobic effect alone. This is supported by the observation of Sinanoglu and Abdulnur,[36,37] Breslow and Guo,[38] and Timasheff and co-workers,[14,34] who have proposed that the free energy of cavity formation in water to accommodate the exposed groups of proteins upon denaturation is proportional to the increase in

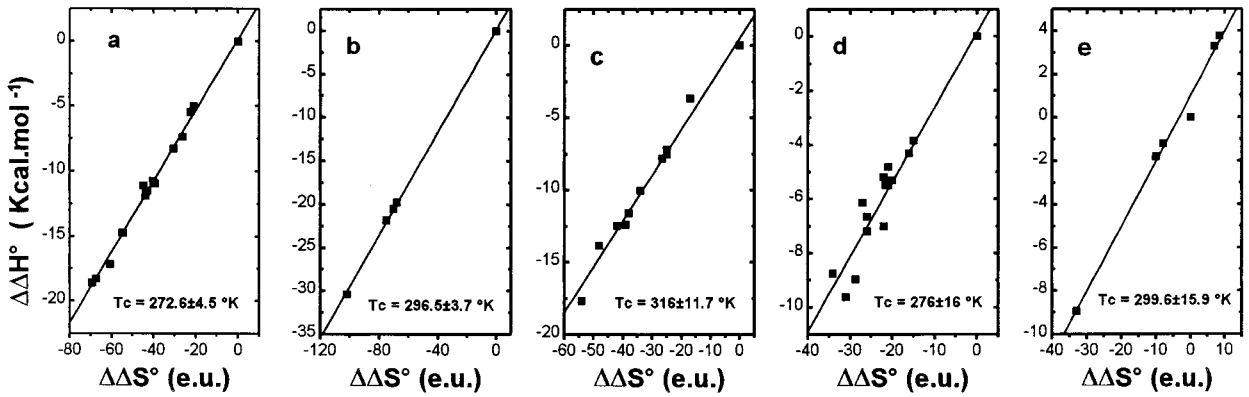


Figure 6. Enthalpy−entropy compensation curves for RNase A (a), α-CTgen (b), Cyt c (c), lysozyme (d), and trp-inh (e).

surface tension of water by various solvent additives. However, this may not be the case for all cosolvents which lead to the stabilization of proteins against denaturation, as observed in the case of amino acid salts.[14] Urea and GdmCl have been found to increase the surface free energy of water[38] and at the same time are preferentially bound to proteins at high concentrations.[39,40]

To further analyze the role of polyols in water structuring we looked at the other thermodynamic properties such as partial molar heat capacity, $\bar{C}_p°$, and volume, $\bar{V}°$, for polyol−water mixtures. It has been observed that the partial molar heat capacities of polyols in water are highly positive and related to their relative degree of fitting to water structure. A large heat capacity of water in the presence of polyols and sugars has been attributed to stronger or more extensive hydrogen bonding between solute hydroxyl groups and water molecules, as has been suggested from various spectroscopic and thermodynamic studies.[41−43] An increase in $\bar{V}°$ also indicates better structuring of water molecules in the presence of polyols. Gerlsma and Stuur[44,45] have suggested extensive hydrogen bonding between polyhydroxy compounds and water explained in terms of a specific hydration model.[46] The values of $\bar{C}_p°$ and $\bar{V}°$ for the aqueous polyol solutions studied are presented in Table 2. It can be seen that sorbitol and mannitol have the largest $\bar{C}_p°$ and $\bar{V}°$ values compared with those of adonitol and xylitol. However, inositol has a lower value of $\bar{C}_p°$ than sorbitol and mannitol, which cannot be explained considering that inositol fits better with the water structure. The distance between the hydroxyl oxygen atoms on the same side of the inositol ring has been found to correspond to the second nearest neighbor oxygen distance in water.[47] The same has been observed for D-glucose, which also has a lower $\bar{C}_p°$ and $\bar{V}°$ value than other acyclic analogues in solutions but has a greater surface tension effect on water and hence a larger stabilizing effect.[5] The lower values of $\bar{C}_p°$ and $\bar{V}°$ for inositol or glucose can be ascribed to their cyclic natures. From thermodynamic studies on model compounds, it has also been shown that cyclization leads to a lowering of $\bar{C}_p°$ and $\bar{V}°$ values.[48] However, it remains to be explained how inositol, having a lower $\bar{C}_p°$ value than sorbitol and mannitol, increases the surface tension of water to a larger extent. With the exception of inositol, there seems to be a parallel trend between the increase in the surface tension of water on one hand and the increase in $\bar{C}_p°$ and $\bar{V}°$ on the other and the ability of polyols to increase the T_m of proteins.

Thermodynamics of Protein Stabilization by Polyols. From Figure 4, it is apparent that the ΔC_p of denaturation of RNase evaluated in the presence of polyols is lower compared with the values reported in the literature in their absence.[25] The positive heat capacity of denaturation is known to depend on the extent of ΔASA upon protein denaturation[31,50,51] and has its origin in the unfavorable interactions of the solvent with the hydrophobic groups undergoing exposure.[24] The solvation of these groups contributes significantly toward a positive ΔC_p of denaturation. In the presence of polyols, it appears therefore that either the tendency of the nonpolar groups to be solvated by water is considerably reduced or their exposure to water is reduced.[49] The higher values of ΔC_p obtained by Liu & Sturtevant[25] for RNase and lysozyme in sucrose could be due to the aggregation of proteins at the high protein concentrations used in calorimetry.

A good correlation of ΔT_m with the net hydrophobicity of proteins was observed at pH 7.0 (Figure 5a), whereas at pH 4.0 (Figure 5b) and pH 2.5 no appreciable correlation was observed. This could possibly be due to changes in the hydrophobicity of different proteins as the charges disappear or appear on their surface. At lower pH, groups such as COO^- get protonated, leading to an increase in the hydrophobicity.[52] Different proteins have different compositions and distributions of charged groups, making the situation very complex. This is likely to result in a varied correlation of properties such as net hydrophobicity with the thermal stability at different pH values as observed by us.

From the thermal denaturation curves in the presence of polyols, the $\Delta H°$ and $\Delta S°$ values evaluated at a temperature equal to the T_m in the control experiment (without polyol) revealed that the stabilizing effect of polyols for the proteins is entropically controlled, i.e., the $\Delta S°$ term is decreasing to a larger extent in the presence of polyols relative to control, which compensates for the decrease in $\Delta H°$ (Figure 6) to give a small increase in $\Delta G°$, tabulated as $\Delta\Delta G°$ in Table 1. Similar observations have been made earlier in the literature.[4] The values of compensation temperature T_c, evaluated from the enthalpy−entropy compensation plots, range from 272 to 316 K and indicate the role of water in imparting thermal stability to proteins.[53,54]

On the basis of the studies of five different proteins, it can be concluded that the natures of both the protein and the polyol are important in governing the thermal stability of proteins. An increase in the surface tension of the medium in the presence of polyols appears to be the dominant factor leading to the thermal stability of proteins, and the preferential hydration of proteins in the presence of polyols observed earlier is due to the ability of the polyol to increase the surface tension of water. The fact that several physicochemical properties of proteins did not show any dominant contribution toward thermal stability in the presence of polyols indicates that a delicate balance of the interactions between the surface hydrophobic, hydrophilic, and charged residues with water, and the ability of polyols to

alter the physicochemical properties of water, especially the interfacial tension at the protein−water interface, plays a very significant role in the stabilizing action of polyols.

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