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Stabilization of yeast hexokinase A by polyol osmolytes: Correlation with the physicochemical properties of aqueous solutions

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Abstract

Osmolytes of the polyol series are known to accumulate in biological systems under stress and stabilize the structures of a wide variety of proteins. While increased surface tension of aqueous solutions has been considered an important factor in protein stabilization effect, glycerol is an exception, lowering the surface tension of water. To clarify this anomalous effect, the effect of a series of polyols on the thermal stability of a highly thermolabile two domain protein yeast hexokinase A has been investigated by differential scanning calorimetry and by monitoring loss in the biological activity of the enzyme as a function of time. A larger increase in the T_m of domain 1 compared with that of domain 2, varying linearly with the number of hydroxyl groups in polyols, has been observed, sorbitol being the best stabilizer against both thermal as well as urea denaturation. Polyols help retain the activity of the enzyme considerably and a good correlation of the increase in T_m (ΔT_m) and the retention of activity with the increase in the surface tension of polyol solutions, except glycerol, which breaks this trend, has been observed. However, the ΔT_m values show a linear correlation with apparent molal heat capacity and volume of aqueous polyol solutions including glycerol. These results suggest that while bulk solution properties contribute significantly to protein stabilization, interfacial properties are not always a good indicator of the stabilizing effect. A subtle balance of various weak binding and exclusion effects of the osmolytes mediated by water further regulates the stabilizing effect. Understanding these aspects is critical in the rational design of stable protein formulations.

Keywords: Calorimetry; Thermal stability; Polyol; Yeast hexokinase A; Surface tension

1. Introduction

Understanding the intricate balance of various factors responsible for the stability of proteins and enzymes in solution is not only an academic challenge but also has enormous implications for the pharmaceutical and the biotechnology industry [1,2]. How are the forces that govern the stability of proteins disrupted or strengthened by solvent conditions is a question, the plausible answers for which are still being sought [3–7]. A variety of compounds have been shown to affect the stability and folding of proteins in aqueous solutions [8,9]. Among the solutes employed for this purpose are sugars [10–14], polyols [15–23], salts [24–27], and amino acids [28–34]. A few general schemes for the mechanisms of action of these osmolytes have been proposed [8,35–37]. Sugars and polyols, except glycerol, lead to the increase in the thermal stability of proteins and the increase in the surface tension of solvent water in their presence is considered as a contributory force for their preferential hydration effect and hence protein stability [11,14,21]. In the case of salts and amino acids which have also been found to lead to the increase in the surface tension of water, the net stabilizing effect is governed by a fine balance between the increase in the energy required for cavity formation in such a medium on protein denaturation, and their ability to bind proteins to varying extents in some cases depending on the

Abbreviations: DSC, differential scanning calorimetry; CD, circular dichroism; T_m, transition temperature; ΔT_{m1}, change in T_m of domain 1; ΔT_{m2}, change in T_m of domain 2; ΔH_{cal}, calorimetric enthalpy; Tris, Tris (hydroxymethyl) amino-methane; NADP, β-nicotinamide adenine dinucleotide phosphate; ATP, adenosine 5′-triphosphate; ΔC_p, heat capacity of denaturation; MRE, mean residue ellipticity; $\phi_c°$, apparent molal heat capacity; $\phi_v°$, apparent molal volume.

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pH of the medium [13,26,38]. However, the surface tension mechanism does not seem to be applicable in some cases, wherein an increase in the surface tension of aqueous solutions by the addition of solutes has been observed while there is a decrease in the stability of proteins in their presence and vice versa [37].

Bolen and coworkers [36,37] have proposed osmophobic mechanism of protein stabilization by a variety of compounds, based on the free energy of transfer studies of amino acids from water to aqueous osmolyte solutions and postulate that unfavorable free energy of interaction of the osmolytes with the peptide backbone is a key factor in protein stabilization by these compounds. However, extensive pH dependence studies on the thermal stability of a number of proteins in organic osmolytes by us have suggested that the stabilizing effect should also depend on the nature of the amino side chains in proteins and that the structure of aqueous solution plays a central role in the stabilization process [14,21]. In order to resolve the anomalies observed in the surface tension hypothesis, there is a need to look for correlations of the stabilization effect with several other physicochemical properties of aqueous solutions as well.

While considerable amount of work has been carried out on the thermal stability of monomeric single domain proteins in the presence of a variety of cosolvent conditions using calorimetry or spectroscopy, little work has been carried out either on multimeric or multidomain systems. Here, we report the results of the thermal stability of the two domain protein yeast hexokinase A in the presence of a series of polyols using differential scanning calorimetry (DSC) and activity measurements. Hexokinase A is ideally suited for understanding the structure–stability–activity relationship in the presence of osmolytes as, in addition to revealing domain transitions during unfolding in DSC, it is also highly thermolabile, losing its activity rapidly even at room temperature.

Yeast hexokinase A (S-form) exists as a monomer with a molecular weight of 50,000 Da and the polypeptide chain is folded into two distinct lobes [39–41]. Within each lobe the structure and the pattern of folding is very similar to that of native hexokinase B [41,42]. Crystallographic studies show that when glucose molecule binds to the cleft, the small lobe of the molecule rotates relative to the large lobe essentially as a rigid body, enclosing it, and brings the atoms of the small lobe into contact with the large lobe [39,41]. The Differential Scanning Calorimetric (DSC) profile of hexokinase B at pH 8.5 shows two partially overlapped peaks which have been related to the presence of two structural domains in the native conformation of the enzyme that possess different thermal stabilities and are denatured more or less independently [43,44]. The binding of D-glucose enhances the interactions between the two lobes, and gives rise to DSC profiles resembling those of a single domain protein.

DSC has been used successfully to analyze the energetics of multidomain and multimeric protein thermal transitions [45,46]. It allows the visualization of the individual domains as they undergo thermal unfolding [47–50] and also gives direct estimates of sensitive thermodynamic parameters like the transition temperature, T_m, the change in enthalpy, ΔH and the change in heat capacity, ΔC_p, etc. Our previous work on the pH dependence of domain transitions of hexokinase A investigated by DSC suggested the role of acidic amino acids in the cleft region in the domain stability and merger and enabled the correlation of the structural and stability aspects of the enzyme as a function of pH [51]. In the present paper, DSC studies of hexokinase A in the presence of the polyol series glycerol, erythritol, xylitol and sorbitol, increasing in the carbon chain length and the number of –OH groups, have been undertaken to elucidate the osmolyte effect on the individual domain stability of the protein. In addition to investigating the correlation of the surface tension effect of aqueous polyol solutions and the increase in the transition temperatures T_{m1} (for domain 1) and T_{m2} (for domain 2) of hexokinase A, we have also determined the correlation with two important thermodynamic properties, the apparent molal heat capacity, ϕ_c° and volume, ϕ_v° known to reflect structure and interactions in solutions of cosolvent additives [52], to clarify the anomalous effect of glycerol. The paper also reports the results of circular dichroism (CD) spectroscopic studies of the effect of sorbitol on the urea-induced denaturation of hexokinase A and the correlation between the increase in the T_m of the enzyme in the presence of polyols and the retention of its biological activity upon incubation at 278.15 and 298.15 K to unravel the stability–activity relationship of the enzyme.

2. Materials and methods

2.1. Materials

Yeast hexokinase used in these experiments was the A isozyme obtained from Sigma Chemical Co. St. Louis, USA (type C-301). The enzyme is prepared by the method of Kaji et al. [53] and has been proteolytically cleaved by an endogenous yeast protease, resulting in the removal of 11 amino acid residues from the amino terminus [54]. This preparation exists as a monomer at alkaline pH [55,56].

Tris (hydroxymethyl) amino-methane (Tris), sorbitol, xylitol, erythritol, glucose, β-nicotinamide adenine dinucleotide phosphate (NADP), adenosine 5′-triphosphate (ATP), sodium hydroxide and urea were procured from Sigma Chemical Co., yeast glucose-6-phosphate dehydrogenase (G-6-PDH) was from Boehringer Mannheim, Germany, and glycerol, sodium dihydrogen phosphate, disodium hydrogen phosphate were from Merck, India. The chemicals used were of the highest purity grade available and were used without any further purification. Glass double distilled water was used to make polyol solutions and buffers. The pH of the solutions was adjusted on Control Dynamics pH meter model APX 175, by adding sodium hydroxide or hydrochloric acid solutions. The pH standards used for calibrating the pH meter were from Sigma Chemical Co.

2.2. Preparation of solutions

Yeast hexokinase A obtained from Sigma as an ammonium sulfate precipitate was used without further purification except

that it was desalted before use. The protein solution was mixed with 20 mM Tris–HCl buffer pH 8.5 and then loaded in Centricon concentrators (Amicon Inc., USA) to desalt it completely by centrifugation at 5000×*g*. The protein concentration was calculated based on the specific absorptivity of 0.885 cm^2 mg^{-1} [39] using Hitachi U-2000 UV–visible spectrophotometer. The protein was equilibrated with the solutions of choice, e.g., 20 mM Tris–HCl buffer, pH 8.5 containing sorbitol, xylitol, erythritol or glycerol (0.5 to 1.5 M) by passing the solution through Centricon concentrators of 30,000 Da cut off in a high speed centrifuge at 277.15 K. On comparing the DSC results of samples prepared by dialysis using conventional dialysis membranes with those using Centricon set-up, no differences were found in the DSC profiles of the proteins as well as in the evaluated thermodynamic parameters like the transition temperature, T_m, and the enthalpy of denaturation, ΔH as described by us earlier [57]. There were no evaporational losses reported for concentrated solutions by other workers using conventional dialysis [58].

The concentration of the protein used for the DSC was in the range of 0.9 mg ml^{-1} to 1.8 mg ml^{-1}. All solutions before loading were filtered through 0.22 μm membrane filter (Sartorius, Germany). Protein solutions were centrifuged for 10 min at 10,000×*g* in a Sorvall RC 5C centrifuge to remove any suspended impurities. Solutions were degassed and the protein concentration was determined before loading in the calorimeter cells.

2.3. Differential scanning calorimetry

The instrument used for the calorimetric studies was MC-2D from Microcal Inc., Northampton, USA which was calibrated with the standards provided by the company. A scan rate of 1 K min^{-1} was used and the data were acquired through an in-built data translation board DT 2801 in a 486DX personal computer using 'ORIGIN' software. A minimum of three repeats was done for an experiment and the mean values of the various parameters were reported. The raw data was processed for buffer baseline correction and concentration normalization followed by fitting the data to a non-two-state model as provided in the Origin software. DSC transitions of hexokinase were found to be independent of scan rate as observed earlier [43,44].

2.4. Circular dichroism studies

Circular dichroism spectra at 222 nm were recorded on a Jasco J-700 spectropolarimeter. The instrument was calibrated with [+]-10-camphor sulfonic acid. In general, an average of 5–10 scans was used and the data presented as mean residue ellipticity [MRE] expressed in deg cm^2 $dmol^{-1}$. The studies were carried out in increasing concentrations of urea at 298.15 K in the presence as well as the absence of 1.5 M sorbitol. The data points reported are the mean of at least three separate experiments.

2.5. Activity assay

Hexokinase activity was assayed by the glucose-6-phosphate dehydrogenase coupled method [59]. The formation of NADPH was followed by the absorbance change at 340 nm. The assays were carried out at 298.15 K and pH 7.8 after incubating the enzyme at pH 8.5 for various time intervals at 278.15 K and 298.15 K in the buffer and 1 M polyol solutions. All the studies were carried out in triplicates using the Hitachi spectrophotometer.

Table 1
Thermodynamic parameters of hexokinase A denaturation evaluated by differential scanning calorimetry in the absence and presence of polyols at pH 8.5

Cosolvent	T_m (K)		ΔT_m (K)		ΔH (kJ mol^{-1})	
	T_{m1}	T_{m2}	ΔT_{m1}	ΔT_{m2}	ΔH_1	ΔH_2
Buffer (pH 8.5)	308.9±0.4	320.5±0.2	–	–	188±17	427±33
Glycerol						
0.5 M	309.7±0.2	320.5±0.3	0.8±0.4	0.0±0.4	335±13	385±4
1.0 M	310.9±0.3	321.1±0.2	2.0±0.5	0.6±0.3	351±21	368±21
1.5 M	312.8±0.4	321.5±0.4	3.9±0.6	1.0±0.4	268±46	377±54
Erythritol						
0.5 M	310.9±0.1	321.0±0.3	2.0±0.4	0.5±0.4	272±17	318±17
1.0 M	312.8±0.1	322.8±0.1	3.9±0.4	2.3±0.2	322±59	330±38
1.5 M	316.1±0.7	324.8±0.6	7.2±0.8	4.3±0.6	402±17	343±67
Xylitol						
0.5 M	312.2±0.3	321.1±0.1	3.3±0.5	0.6±0.2	305±29	544±79
1.0 M	315.2±0.2	323.9±0.1	6.3±0.4	3.4±0.2	289±33	464±96
1.5 M	318.6±0.1	325.4±0.2	9.7±0.4	4.9±0.3	334±25	435±29
Sorbitol						
0.5 M	313.9±0.1	323.3±0.1	5.0±0.4	2.8±0.2	318±4	393±41
1.0 M	318.5±0.1	325.1±0.1	9.6±0.4	4.6±0.2	314±17	381±25
1.5 M	322.3±0.4	327.3±0.1	13.4±0.6	6.8±0.2	339±29	397±67
Sorbitol (1.5 M)						
+1 M Urea	319.8±0.1	324.8±0.1	10.9±0.4	4.3±0.2	477±4	151±4
+2 M Urea	317.2±0.1	–	8.3±0.4		510±4	–
Urea						
0.5 M	305.4±0.2	317.8±0.1	−3.5±0.4	−2.7±0.2	226±4	251±4

A Haake model F3-CH water bath was used for the incubation of the protein at different temperatures for various time intervals.

2.6. Surface tension measurements

Surface tension measurements of polyol–water solutions were carried out by the drop weight method as described elsewhere [21,38].

3. Results

3.1. Differential scanning calorimetry

Table 1 presents data on the effect of glycerol, erythritol, xylitol, and sorbitol on the thermal denaturation of hexokinase A at pH 8.5 monitored by DSC. The thermal denaturation transition curve for the enzyme in the buffer, used as a control, has been provided in Fig. 1. Fig. 1 also includes data for the effect of sorbitol on the thermal denaturation of hexokinase A, used as a representative polyol. The control data consist of two transitions that have been assigned to the two domains in the enzyme, the small (domain 1) and the large (domain 2) domain, with a T_{m1} of 308.9 K and T_{m2} of 320.5 K, respectively [51] (Table 1). Using different buffer systems like MOPS, Hepes, Tris, and phosphate at 20 mM concentrations, it has been observed earlier by us that the nature of the buffer components do not affect the stability parameters of the enzyme like the transition temperatures and the calorimetric enthalpies of denaturation of the two domains monitored by DSC [51]. The large heat of ionization of Tris buffer, therefore, does not appear to affect the thermodynamic parameters for hexokinase denaturation.

Addition of glycerol leads to the differential stabilization of the two domains, with domain 1 being stabilized to a larger extent than domain 2 as the glycerol concentration is increased from 0.5 M to 1.5 M. As a result of larger shift in T_{m1} towards higher temperatures relative to T_{m2}, the overlap between

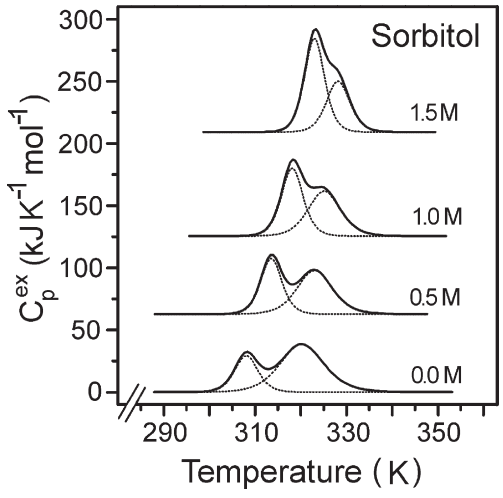

Fig. 1. Effect of sorbitol on the thermal denaturation of hexokinase A, pH 8.5 monitored by DSC. The traces have been presented after concentration normalization and baseline subtraction. The dotted lines indicate the deconvolution of the two domains.

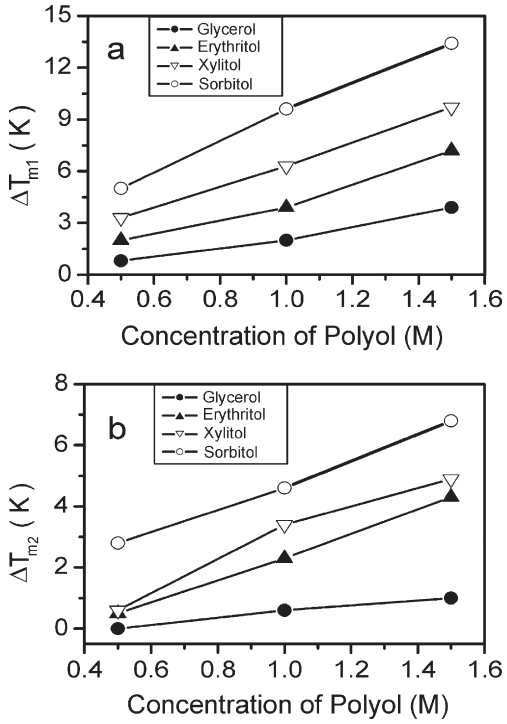

Fig. 2. Dependence of the transition temperatures of the two domains of hexokinase A on the concentrations of polyols; panel a: domain 1, panel b: domain 2. The symbols used for the various polyols are shown in the inset.

domains 1 and 2 increases. The calorimetric enthalpy of denaturation of domain 1 has also been found to increase compared to control on the addition of glycerol whereas it does not change much in the case of domain 2. In the case of erythritol, a similar trend has been observed but the shift in T_{m1} is much larger than in T_{m2} (Table 1) as compared with glycerol. In the presence of xylitol and sorbitol also, the shift in T_{m1} is much larger than that of T_{m2} bringing the two peaks closer to each other and increasing the overall overlap of the two transitions as a result of which the total transition zone for the protein denaturation decreases considerably. The ΔH values, however, do not show any appreciable change as a function of increasing concentration of polyols (Table 1).

Fig. 2a and b shows the plots of ΔT_{m1} and ΔT_{m2} for hexokinase A versus increasing concentration of polyols, respectively. Substantial increase in the magnitudes of the transition temperatures as a function of polyol concentration can be seen in these plots with more or less linear dependence. The differential stabilization of the two domains is also quite evident from these figures. It has been observed that sorbitol is the best stabilizer followed by xylitol, erythritol, and glycerol as indicated by both ΔT_{m1} and ΔT_{m2} values (Table 1). This correlates well with their effect on the thermal stability of several single domain proteins, showing a single cooperative transition, studied by us earlier [21].

3.2. Circular dichroism spectroscopy

CD data of hexokinase at 222 nm monitored by the change in ellipticity as a function of increasing urea concentration carried

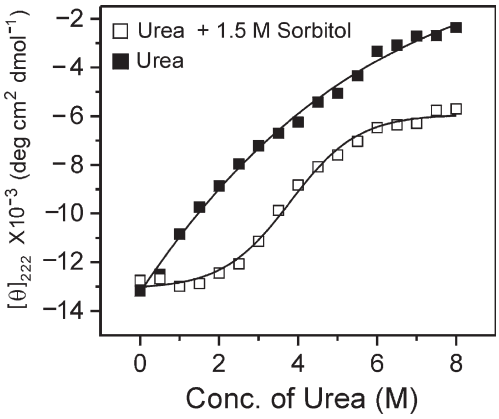

Fig. 3. Effect of sorbitol on the mean residue ellipticity of urea-induced denaturation of hexokinase A, pH 8.5 monitored by CD spectroscopy at 222 nm and 298.15 K.

out at 298.15 K, pH 8.5 in the presence as well as the absence of 1.5 M sorbitol are presented in Fig. 3. Sorbitol was chosen for the study as the representative polyol as it was found to be the best stabilizer in the polyol series used, increasing ΔT_{m1} by 13.4 K at 1.5 M concentration (Table 1). Denaturation monitored at 222 nm in increasing urea concentration by measuring the change in the helical content of the protein showed non-cooperative denaturation of the protein in the buffer, i.e., no clear transition was seen. However, in the presence of 1.5 M sorbitol, the protein denaturation curve was sigmoidal in shape showing cooperative unfolding of hexokinase (Fig. 3). To explore the reason for the cooperative denaturation behaviour of hexokinase A in the presence of 1.5 M sorbitol, DSC experiments were carried out in the presence of 0.5 M urea and a mixture of urea and sorbitol (Fig. 4). Sorbitol was able to compensate for the destabilizing effect of urea. In the presence of a solution of 1.5 M sorbitol containing 1 M urea, there was an increase in T_{m1} by 10.9 K while the increase was 8.3 K in the presence of 2 M urea with relatively small increase in T_{m2} (4.3 K in 1 M urea) compared

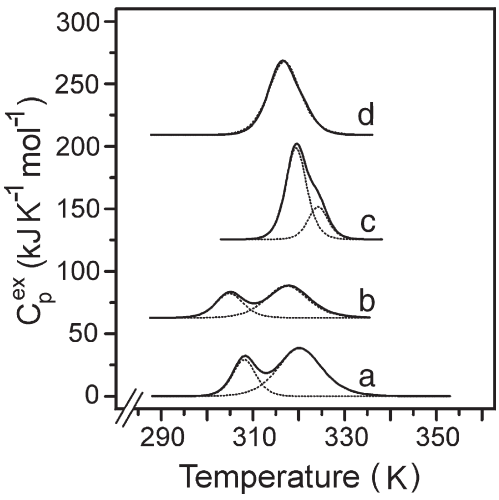

Fig. 4. Effect of urea and a mixture of urea and sorbitol on the calorimetric transitions of hexokinase A, pH 8.5. (a) Buffer control; (b) 0.5 M urea; (c) 1 M urea + 1.5 M sorbitol; (d) 2 M urea + 1.5 M sorbitol.

with buffer control devoid of urea and sorbitol (Table 1). In the presence of 0.5 M urea alone, the T_{m1} and T_{m2} decreased by 3.5 and 2.7 K, respectively. In the presence of 1 M urea no significant transition was observed (data not shown).

3.3. Activity measurements

Hexokinase activity measurements were carried out at pH 7.8 and 298.15 K after incubating the enzyme at 278.15 and 298.15 K for different time intervals in the presence of 1 M polyols at pH 8.5 (Fig. 5a, b). Upon incubation at 278.15 K, it was found that sorbitol was the best stabilizer of activity followed by xylitol, glycerol, and erythritol. Sorbitol retained the activity of hexokinase up to 80% of the native control taken as 100% even after more than 100 h of incubation at 278.15 K compared with the retention of less than 10% activity in the absence of polyols (Fig. 5a). When hexokinase was incubated at 298.15 K, the control protein lost 70% of activity in the first 2 h and had negligible activity in 24 h (Fig. 5b). In the presence of sorbitol, the activity was significantly higher than the control in the first 2 h and then showed a gradual decrease over a period of time. Compared with the control buffer, sorbitol was able to retain up to ~75% activity upon incubation for 47 h and was also found to be the best stabilizer of hexokinase activity followed by xylitol, glycerol, and erythritol (Fig. 5b). These trends were nearly identical at 278.15 and 298.15 K. Fig. 6 shows the

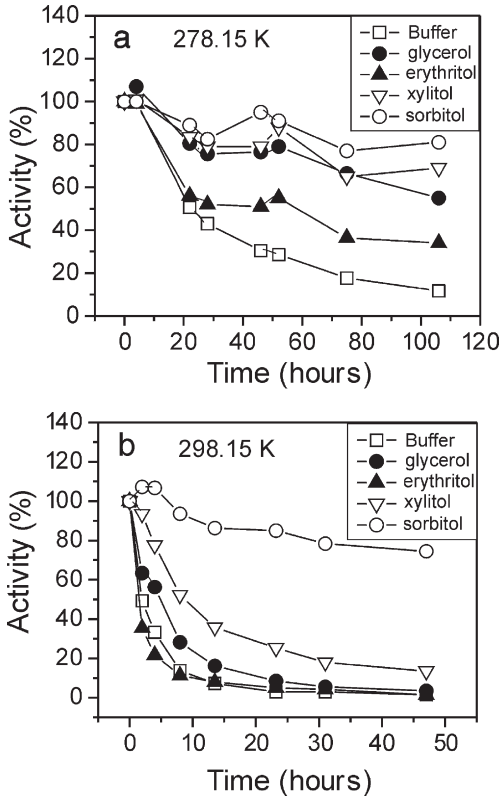

Fig. 5. Retention of the activity of hexokinase A after incubating the enzyme at 278.15 K (a) and 298.15 K (b) in the presence of 1 M concentration of polyols at pH 8.5 for varying time periods. Symbols for the polyols used are shown in the inset. The maximum errors in the activity measurements were ±5%.

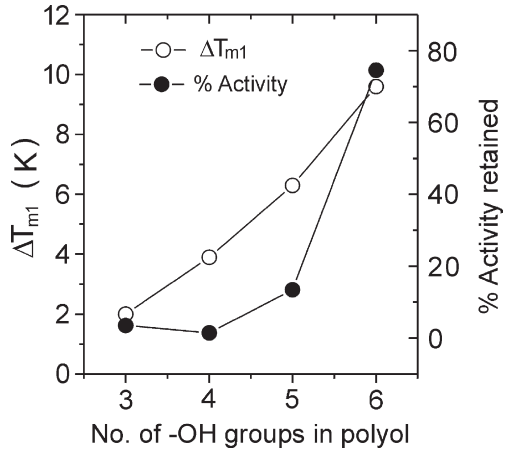

Fig. 6. Correlation of the increase in the thermal transition temperatures of domain 1 of hexokinase A, ΔT_{m1} and that of the % activity of the enzyme retained after incubation in 1 M polyols, pH 8.5 at 298.15 K for 47 h, with the number of hydroxyl groups in the polyols.

correlation of the activity of hexokinase retained after 47 h of incubation at 298.15 K and that of ΔT_{m1} in 1 M polyols relative to buffer with the number of –OH groups in the polyols. The greater the thermal stabilization of the protein in a polyol observed by DSC measurements, the greater was the activity retained in its presence and the larger the carbon chain length of the polyol or the number of hydroxyl groups the better was the functional and thermal stabilization provided. Glycerol, however, was an exception and showed marginally higher retention in the activity of hexokinase at 298.15 K and considerable retention at 278.15 K compared to erythritol (Fig. 5a, b), although it leads to a lesser increase in the T_{m1} value compared with erythritol.

3.4. Hexokinase thermal stability and physicochemical properties of polyol solutions

Fig. 7 presents plots of the variations in the thermal stability of the two domains of hexokinase, ΔT_{m1} and ΔT_{m2}, with the

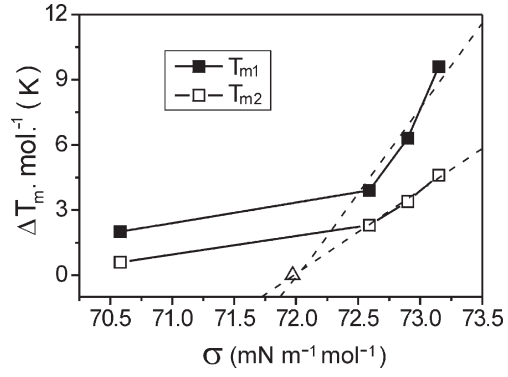

Fig. 7. Variation of the increase in the thermal transition temperatures of the two domains of hexokinase A, ΔT_{m1} and ΔT_{m2} in the presence of 1 M polyols, pH 8.5 with the surface tension of 1 M aqueous polyol solutions at 298.15 K. Surface tension of water at 298.15 K has been taken as 71.97 mN m^{-1} and shown as '△' in the plot. The dotted line is the linear regression of the values for water and other polyols, except glycerol.

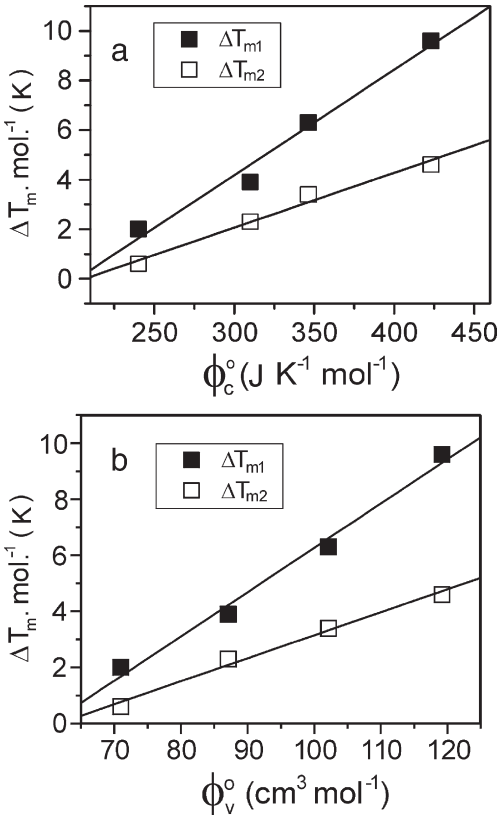

Fig. 8. Variation of the increase in the thermal transition temperatures of the two domains of hexokinase A, ΔT_{m1} and ΔT_{m2} in the presence of 1 M polyols, pH 8.5 with the apparent molal heat capacity, $\phi_c°$ (a) and apparent molal volume, $\phi_v°$ (b) of aqueous polyol solutions.

magnitudes of the surface tension values of aqueous polyol solutions. The surface tension values obtained for 1 M erythritol and glycerol solutions in water at 298.15 K were 72.59±0.05 and 70.58±0.08 mN m^{-1} mol^{-1}, respectively. Surface tension values of 1 M solutions of sorbitol and xylitol were taken from our previous work [21], and were 73.15±0.19 and 72.9±0.2 mN m^{-1} mol^{-1}, respectively. Low concentrations of the buffer ions used in the study have been found not to affect the surface tension of water significantly. It is clearly seen that erythritol, xylitol, and sorbitol fit to a straight line while glycerol shows a break owing to its tendency to lower the surface tension of water. However, when the ΔT_m values were plotted against other two physicochemical properties of polyol solutions, viz., apparent molal heat capacity and volume, $\phi_c°$ and $\phi_v°$, respectively taken from the literature [52], a linear correlation was observed (Fig. 8a, b).

4. Discussion

4.1. Differential domain stabilization by polyols

The larger extent of stabilization of domain 1 compared with domain 2 by polyols can be attributed to the differences in the structural stabilities of the two domains in the buffer at pH 8.5 [51] (Fig. 1, Table 1). It has been observed earlier by us that polyols stabilize different proteins to different extents,

depending on the physicochemical properties of the proteins [21]. Also, polyol effect is known to be highly pH dependent, e.g., the lower the T_m of RNase A in the buffer, the greater was the increase in the transition temperature in the presence of polyols [20,21]. Polyols have been shown to stabilize the acid unfolded state of cytochrome *c* by strengthening hydrophobic interactions by overcoming electrostatic repulsions among the charged residues at low pH [60]. In another study, using fluorescence anisotropy, it has been shown that sorbitol can reduce the internal motions in the nucleocapsid protein of rhabdovirus and favor a more compact state of the protein [61]. Thus, polyols known to strengthen hydrophobic interactions in protein interiors may probably be able to strengthen the structure in the loosely organized domain 1 of hexokinase with a lower T_m to a larger extent than that in domain 2 and lead to a greater increase in the T_m of domain 1. The use of higher concentrations of polyols required to have appreciable effect on the T_m values of hexokinase indicates that the effect of polyols is essentially water mediated and does not depend on direct specific or nonspecific interactions with the protein. Polyols have been earlier known to increase the thermal stability of a wide variety of proteins and a direct correlation of the number of hydroxyl groups in a polyol with the increase in T_m of proteins in their presence has been well established [21,62]. Similar correlation has been observed for the two domain protein hexokinase with the trends in the increase of T_{m1} and T_{m2} being identical to those observed in the literature. It must be pointed out that although the molecular weights of the polyols increase with the number of –CHOH groups in them, comparing the results on a molar basis (using 1 M polyol data) fixes the number of polyol molecules while varying the number of –OH groups.

The larger increase in ΔH_1 compared to negligible change in ΔH_2 in the presence of polyols compared with the buffer control could also be explained based on the above observations. However, it has been observed that with increasing concentration of polyols, the ΔH values do not show any appreciable change and the changes, whatsoever observed, are within the experimental uncertainty (Table 1). Similar trends have been observed for the stabilization of RNase A [30] and myoglobin [63] in the presence of increasing concentrations of glycine based osmolytes, and RNase A and lysozyme in polyols recently [64]. However, at low pH, the enthalpy values show a slight increase with polyol concentration. Non-compatible amino acid osmolytes, on the other hand, have been found to lower the T_m of several proteins as well as lead to a decrease in the ΔH values as their concentration increases [65].

4.2. Mechanism of polyol action

Polyols including glycerol have been found to lead to the preferential hydration of proteins [15,17]. It has been shown that sorbitol leads to the preferential hydration of RNase A and that the larger preferential hydration of the denatured state compared to the native state is responsible for the stabilizing effect of sorbitol [20]. Extensive studies carried out in our laboratory earlier on the effect of a variety of polyols (excluding glycerol) on the thermal stability of several single domain proteins have demonstrated that the stabilizing effect of polyols has a linear correlation with the increase in the surface tension of water in their presence [21], leading to the preferential hydration of proteins as observed by other workers. However, in the present study, which includes the polyols glycerol, erythritol, xylitol, and sorbitol, it has been observed that while glycerol lowers the surface tension of water considerably, yet it increases the T_m of hexokinase, though to smaller extent compared to the other polyols which lead to the increase in the surface tension of water. Surface tension effect, therefore, cannot be considered as a dominant factor leading to the stabilizing effect of proteins by polyols. This has been pointed out earlier by us [21] and by other workers [13,37]. It has also been suggested that there may be problems in extrapolating the surface tension values of the polyol solutions measured at 298.15 K to the temperatures of transition of the protein which varies with the type of the polyol used. Also, surface tension of aqueous solutions involves the air–water interface which is not the same as the protein–water interface. Further, protein surface is not inert to water and consists of a mosaic of charges and other predominantly hydrophilic groups which are likely to either interact favorably with the organic osmolytes added or repel them based on the energetics of interactions and the preference of either water or the solute to interact favorably with protein surface groups. Nonetheless, a linear correlation obtained between the ΔT_m values for hexokinase and the surface tension values of water in the presence of erythritol, xylitol, and sorbitol (Fig. 7) indicates that with the exception of glycerol, increase in the surface tension of the medium, reflecting structure in aqueous polyol solutions for the other three polyols, is a contributing factor for the increase in the thermal stability similar to what has been reported for sugars [11,12].

4.3. Anomalous effect of glycerol

Glycerol is known to lead to the preferential hydration of several proteins and increase their transition temperatures [15–18]. The stabilizing effect of glycerol has been attributed to 'solvophobic effect' which considers more favorable interactions of water molecules with the surface protein residues compared to glycerol [17,18,35] even though it reduces the surface tension of water. However, in a previous study we have observed that glycerol leads to a lowering in the T_m of insulin; the greater the concentration of glycerol, the larger was the lowering of the T_m [19]. This affect has been attributed to the possible binding of glycerol to some of the partially exposed nonpolar side chains of the less compact insulin as the temperature increases. Recently, it has been reported that while trehalose prevented the thermal inactivation of rabbit muscle phosphofructokinase, addition of glycerol resulted in 2–4 fold less recovery of activity of the enzyme with respect to control [66]. Glycerol is therefore expected to exert a dual effect, one depending on the nature of the protein and the other based on the physicochemical properties of its aqueous solutions including surface tension. Interestingly, the plots for

the ΔT_m values against two other physicochemical properties of solutions, apparent molal heat capacity and volume of polyols including glycerol at infinite dilution, ϕ_c° and ϕ_v°, respectively, lead to a linear correlation (Fig. 8). ϕ_c° and ϕ_v° have been known to be very sensitive to structure and interactions in solution. Addition of polyols to water has been observed to lead to substantial increase in these parameters suggesting an increase in the network of hydrogen bonded interactions between polyols and water molecules in aqueous solutions, which has been suggested to be responsible for increase in the stability of proteins [52]. While polyols are expected to be preferentially excluded from the protein surface, the enhanced hydrogen bonded network of water molecules in the vicinity of protein surface is expected to result in stronger protein–water hydrophilic interactions resulting in the increased T_m values as observed here. The linear dependence of ΔT_m with ϕ_c° and ϕ_v° (Fig. 8a, b) suggests that water structure making ability of polyols varies linearly with the number of –OH groups in polyols. Parallel studies carried out in our laboratory on the simple monomeric protein RNase A at pH 2.5 in the presence of 1 M polyols including glycerol using UV-thermal denaturation (data not shown) also indicate a linear correlation of ΔT_m with respect to ϕ_c° and ϕ_v° but a break in the surface tension plot with respect to glycerol. This suggests that the anomalous surface tension effect of glycerol could be applicable to several other proteins as well.

Recent studies of the effect of sugars on the hydrogen bonded network of water by Raman scattering and molecular dynamics simulations suggest that they are highly hydrated structures, with trehalose being superior compared with other disaccharides [67]. Polyols, which are expected to exert a similar effect as sugars, have also been observed to have large dynamic hydration numbers increasing with the number of hydroxyl groups in them [68]. The anomaly in the surface tension effect of glycerol, however, implies that caution should be exercised while analyzing the mechanistic aspects of the effect of solutes which affect protein stability essentially by modulating the solvent properties around it. Bulk solution properties of solutes like ϕ_c° and ϕ_v° indicative of structure and interactions in solution may be different from their interfacial property, the surface tension. This has been clearly demonstrated here by the anomalous behaviour of glycerol with respect to its affect on hexokinase thermal stability. Studies on the thermal stability of RNase A in the presence of several amino acids have revealed that even though amino acids lead to an increase in the surface tension of water, arginine hydrochloride results in a lowering of the transition temperature [13]. Similar discrepancies have been observed in the case of effect of the osmolyte trimethylamine *N*-oxide (TMAO) and urea on protein stability, with the former reducing surface tension but enhancing protein stability while the opposite is true for urea [37,38]. Recently, it has been observed that TMAO could even destabilize proteins at acidic pH conditions [69]. The effect of the osmolytes on protein stability should, thus, be governed by a fine balance between their ability to increase/decrease the surface tension of water and their ability to either induce preferential hydration for proteins or exert direct binding to the protein surface residues. In those situations where glycerol is strongly repelled by the protein surface, the surface tension effect is reversed by the preferential exclusion (or preferential hydration) effect leading to stabilization, while in some other situations where preferential binding is facilitated, structural destabilization is expected. The anomalous effect of glycerol observed here can be explained in light of the above discussion.

4.4. Structure–stability–activity relationship

Fig. 3 shows a gradual increase in the hexokinase ellipticity at 222 nm in the presence of increasing urea concentration and a non-cooperative unfolding transition. DSC profile in the presence of buffer, pH 8.5 (Fig. 1) shows that the onset of denaturation (unfolding) for the protein starts around 298.15 K and the transition is spread over a large temperature interval, showing non-cooperative unfolding. In the presence of 0.5 M urea there is a considerable decrease in the T_m and the enthalpy of denaturation values suggesting breakdown of the non-covalent forces to a considerable extent (Fig. 4). Both the peaks shift to the lower temperature side leading to further lowering of the temperature of the onset of denaturation. This could be the reason that no clear transition is seen in the urea denaturation CD curve monitored at 298.15 K (Fig. 3). Interestingly, the plot of ellipticity versus concentrations of urea for hexokinase is sigmoidal in shape in the presence of 1.5 M sorbitol indicative of cooperative unfolding of hexokinase in the presence of sorbitol, which is a strong protein structure stabilizer. DSC experiments showed that in the presence of 1.5 M sorbitol and 1 M urea the shift in T_{m1} was much higher (10.9 K) compared to that for T_{m2} (4.3 K), and at 2 M urea both the peaks merged leading to a shift in T_m by 8.3 K. This resulted in an increase in the overlap of domain 1 and 2 transitions, shifting the temperature of onset of denaturation to a temperature much higher than 298.15 K. As a result, the overall transition zone showed a decrease indicating higher cooperativity of unfolding of hexokinase A with only one visible peak (Fig. 4, trace d). The more negative magnitude of the mean residue ellipticity at 222 nm in the presence of 1.5 M sorbitol at all the concentrations of urea compared with urea alone also indicates that sorbitol is stabilizing the helical structure of the protein against urea denaturation as well.

Sorbitol stabilizes the activity of hexokinase when incubated at 278.15 K to the maximal extent. There is a good correlation of the increase in T_m by the polyols and their ability to retain the activity at 278.15 K except for glycerol which stabilizes hexokinase activity to a better extent than expected from the stability data (Fig. 5a). When hexokinase A is incubated at 298.15 K for different time periods, the stabilization provided by sorbitol was also observed to be the highest followed by xylitol, while glycerol and erythritol provided very little protection of activity (Fig. 5b). The data in Fig. 6, which presents the correlation of the increase in T_{m1} in 1 M polyol and that of the % hexokinase activity retained after incubation at 298.15 K for 47 h with the number of hydroxyl groups in

polyols, indicate that there is a good correlation of the thermal stability (T_m) and activity retained with the number of –OH groups in the polyol, except that there is a linear increase in the former and an exponential increase in the latter. It can, thus, be concluded that shift in the T_m values or the onset of denaturation has to be much higher in the presence of polyols relative to control for retaining the enzyme activity to significant extents as a function of time.

The DSC curves of hexokinase in the presence of these polyols show that the onset of transition in the presence of sorbitol is at a much higher temperature compared to other polyols. The onset of temperature of unfolding is very near to 298.15 K in the case of glycerol and erythritol which may be leading to the slow denaturation of the protein at 298.15 K and hence rapid loss of its activity. Hexokinase incubated at 298.15 K in the presence of 1 M sorbitol retains activity for exceptionally longer periods of time. Even after incubation for 47 h nearly 75% activity is retained. This is likely due to the shift in its T_{m1} to 318.5 K and the temperature onset of denaturation to a much higher temperature. The protein, thus, does not seem to be undergoing slow unfolding process at 298.15 K as happens in the case of other polyols especially erythritol and glycerol. These results also indicate that the stability of domain 1 is very important for the retention of hexokinase activity. This conclusion correlates very well with the crystallographic data for the protein showing that the active site for glucose binding is present on the smaller lobe of the protein, i.e., domain 1 [41].

In conclusion, calorimetric data reveal differential stabilization of the two domains in the presence of polyols owing to differences in their stabilities based on the extent of intra-domain interactions. The thermal stability of the two domains increases linearly with the increase in the number of hydroxyl groups in a polyol, similar to the observations in several single-domain proteins, and correlates well with the retention of biological activity of the enzyme after incubation at 298.15 K. Sorbitol which is the best stabilizer among the polyol series also protects the enzyme against urea denaturation. The results presented here in conjunction with those obtained for several other proteins in the literature suggest that surface tension of aqueous osmolyte solutions is not a good indicator of their stabilizing effect. While bulk solution physicochemical properties of polyols reflecting structure of aqueous solutions are important contributors toward the stabilizing effect of proteins, in addition to the peptide group contribution, much would also depend on the nature of the surface groups in proteins, which may either repel the solute molecules or have favorable interactions with them. A balance of all these forces would contribute toward the overall stabilizing or destabilizing effect of proteins by these osmolytes.

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