CHAPTER 4

THERMAL STABILITY OF YEAST HEXOKINASE A IN AQUEOUS POLYOL SOLUTIONS
INTRODUCTION

Understanding the 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. What are the forces that govern the stability of proteins and how are they disrupted or strengthened by solvent conditions is a problem that is under intense scrutiny (Dill, 1990; Makhatadze & Privalov, 1995). In addition to the use of protein engineering approach to enhance the thermal stability of proteins (Pace, 1990a,b; Querol et al., 1996; Takano et al., 1997a,b, 1998), a variety of compounds have been shown to affect the stability of proteins in aqueous solutions (Arakawa et al., 1991; Timasheff, 1998). Among the cosolvents employed for this purpose are sugars (Back et al., 1979; Lee & Timasheff, 1981; Arakawa & Timasheff 1982a; Lin & Timasheff, 1996), polyols (Gerlisma, 1968; Gekko & Morikawa, 1981a,b; Gekko & Timasheff, 1981a,b; Gupta & Bhat, 1995; Xie & Timasheff, 1997a,b; Kaushik & Bhat, 1998; Radha et al., 1998), salts (Arakawa & Timasheff 1982b; Busby & Ingham, 1984; Arakawa et al., 1990a; Kaushik & Bhat, 1999), and amino acids (Arakawa & Timasheff, 1983, 1985a; Santoro et al., 1992; Gopal & Ahluwalia, 1993; Taneja & Ahmad, 1994; Sabulal & Kishore, 1995). A few general schemes, which need further strengthening in terms of elucidating the mechanisms of action of additives, have been worked out (Timasheff, 1995, 1998). Sugars and polyols except glycerol have been found to lead to the increase in the thermal stability of proteins due to the increase in the surface tension of the solvent water in their presence (Lee & Timasheff, 1981; Kaushik & Bhat, 1998). However, 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 pH of the medium (Arakawa et al., 1990a,b; Kita et al., 1994; Lin & Timasheff, 1996).

Here, we report the results of the thermal stability of the two domain protein yeast hexokinase A at pH 8.5 in the presence of a series of polyols using Differential Scanning Calorimetry (DSC). DSC studies of hexokinase A will allow us to see the effect of the polyol series chosen, viz., glycerol, erythritol, xylitol and sorbitol, increasing in the carbon chain length and the number of -OH groups, on the individual domain stability of the protein. Recently, surface tension of aqueous polyols has been found to be an important contributing
factor toward the stability of proteins in their presence. We have investigated the correlation of this parameter with the thermal stability of the domains in terms of the increase in the transition temperatures $T_{m1}$ and $T_{m2}$. Comparison has also been made with two other thermodynamic properties of solutions, viz., apparent molal heat capacity, $\phi^o_c$ and volume, $\phi^o_v$. The study also reports the results of circular dichroism spectroscopic (CD) 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 protein in the presence of polyols with the retention of its biological activity upon incubation at 5 and 25°C for several hours. An attempt has also been made to analyze the nature of the calorimetric transitions obtained in the light of the available structural data on the protein.

RESULTS

Differential Scanning Calorimetry

At pH 8.5, yeast hexokinase A unfolds into two partially overlapped peaks indicating independent unfolding of the two domains. The first transition corresponding to domain 1 has lower enthalpy compared to the second transition due to domain 2. These transitions have been ascribed to the melting of the smaller and the bigger structural domains of hexokinase A, respectively, that possess different thermal stabilities and are denatured more or less independently (Chapter 3). At pH 8.5 domain 1 unfolds with a transition temperature, $T_m$ of 35.7°C while the domain 2 unfolds with a $T_m$ of 47.3°C (Chapter 3).

Figures 1 to 4 show the effect of polyols, viz., glycerol, erythritol, xylitol, and sorbitol with increasing number of hydroxyl groups, on the domain transitions of hexokinase A monitored by DSC. The studies have been carried out as a function of concentration of each of these polyols. In the case of glycerol (Figure 1), differential stabilization of the two domains can be seen, with domain 1 being stabilized to a larger extent than domain 2. The observed peak due to domain 1 is sharper with a higher peak maximum than domain 2, and with increasing concentration of glycerol the sharpness and the height of peak 1 increases compared to that of peak 2 which decreases considerably. As a result of larger shift in $T_{m1}$ towards higher temperatures relative to $T_{m2}$, the overlap between domain 1 and 2 increases. The calorimetric enthalpy of denaturation of domain 1 has been found to increase on the addition of glycerol whereas it does not seem to change much in the case of domain 2.
TABLE I: Thermodynamic parameters of hexokinase A denaturation evaluated by differential scanning calorimetry in the absence and presence of polyols at pH 8.5.

<table>
<thead>
<tr>
<th>Cosolvent</th>
<th>$T_m$ (°C)</th>
<th>$\Delta T_m$ (°C)</th>
<th>$\Delta H_{cal}$ (kcal mol$^{-1}$)</th>
<th>$\Delta C_p$ (kcal K$^{-1}$ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{m1}$</td>
<td>$T_{m2}$</td>
<td>$\Delta T_{m1}$ $\Delta T_{m2}$</td>
<td>$\Delta H_1$ $\Delta H_2$</td>
</tr>
<tr>
<td>Buffer (pH 8.5)</td>
<td>35.7 ± 0.41</td>
<td>47.3 ± 0.21</td>
<td>----  ----</td>
<td>44.6 ± 4.2</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.5 M</td>
<td>36.5 ± 0.21</td>
<td>47.3 ± 0.3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>1.0 M</td>
<td>37.7 ± 0.29</td>
<td>47.9 ± 0.22</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>1.5 M</td>
<td>39.6 ± 0.38</td>
<td>48.3 ± 0.41</td>
<td>3.9</td>
</tr>
<tr>
<td>Erythritol</td>
<td>0.5 M</td>
<td>37.7 ± 0.14</td>
<td>47.8 ± 0.29</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>1.0 M</td>
<td>39.6 ± 0.12</td>
<td>49.6 ± 0.12</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>1.5 M</td>
<td>42.9 ± 0.68</td>
<td>51.6 ± 0.58</td>
<td>7.2</td>
</tr>
<tr>
<td>Xylitol</td>
<td>0.5 M</td>
<td>39.0 ± 0.25</td>
<td>47.9 ± 0.13</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>1.0 M</td>
<td>42.0 ± 0.15</td>
<td>50.7 ± 0.08</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>1.5 M</td>
<td>45.4 ± 0.04</td>
<td>52.2 ± 0.15</td>
<td>9.7</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0.5 M</td>
<td>40.7 ± 0.02</td>
<td>50.1 ± 0.08</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>1.0 M</td>
<td>45.3 ± 0.04</td>
<td>51.9 ± 0.14</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>1.5 M</td>
<td>49.1 ± 0.41</td>
<td>54.1 ± 0.15</td>
<td>13.4</td>
</tr>
<tr>
<td>Sorbitol (1.5 M)</td>
<td>+ 1 M Urea</td>
<td>46.6 ± 0.1</td>
<td>51.6 ± 0.12</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td>+ 2 M Urea</td>
<td>44.0 ± 0.02</td>
<td>----  ----</td>
<td>8.3</td>
</tr>
<tr>
<td>Urea</td>
<td>0.5 M</td>
<td>32.2 ± 0.25</td>
<td>44.6 ± 0.07</td>
<td>(-)3.5</td>
</tr>
</tbody>
</table>
Figure 1. Effect of different concentrations of glycerol on the calorimetric transitions of hexokinase A in 20mM Tris-HCl buffer, pH 8.5. a: the raw data after concentration normalization; b, c, and d: the analyzed curves after baseline subtraction and deconvolution of the two transitions.

In the case of erythritol (Figure 2) similar trend has been observed wherein domain 1 is stabilized to a larger extent compared to domain 2. The shift in T_{m1} is much larger than T_{m2} (Table I) as compared to that in glycerol. In xylitol and sorbitol also (Figures 3, 4) 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. The ΔH values do not show any appreciable change as a function of increasing concentration of polyols (Table I). At 0.5M concentration of polyols, ΔC_p values are similar to that observed in the buffer. However, there is a marked decrease in the ΔC_p values at higher polyol concentrations (Table I).
Figure 2. Effect of different concentrations of erythritol on the calorimetric transitions of hexokinase A in 20mM Tris-HCl buffer, pH 8.5. a: the raw data after concentration normalization; b, c, and d: the analyzed curves after baseline subtraction and deconvolution of the two transitions.

Figure 5a,b shows the plots of $\Delta T_{m1}$ and $\Delta T_{m2}$ 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 $\Delta T_{m1}$ and $\Delta T_{m2}$ values (Table I). This correlates well with their effect on the thermal stability of several proteins studied earlier by us (Kaushik & Bhat, 1998).
Figure 3. Effect of different concentrations of xylitol on the calorimetric transitions of hexokinase A in 20mM Tris-HCl buffer, pH 8.5. a: the raw data after concentration normalization; b, c, and d: the analyzed curves after baseline subtraction and deconvolution of the two transitions.

Circular Dichroism Spectroscopy

CD data of hexokinase at 222nm monitored by the change in ellipticity as a function of increasing urea concentration carried out at 25°C, pH 8.5 in the presence as well as the absence of 1.5M sorbitol are presented in Figure 6. 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_m by 13.4°C at 1.5 M concentration (Table I). Denaturation monitored at 222nm in increasing urea concentration, indicative of the change in the helical content of the protein, showed non-cooperative behaviour of the protein in the buffer, i.e., no clear transition was seen. However, in the presence of 1.5M sorbitol, the protein denaturation curve was sigmoidal in shape showing cooperative unfolding of hexokinase (Figure 6).
Figure 4. Effect of different concentrations of sorbitol on the calorimetric transitions of hexokinase A in 20mM Tris-HCl buffer, pH 8.5. a: the raw data after concentration normalization; b, c, and d: the analyzed curves after baseline subtraction and deconvolution of the two transitions.

Figure 5. Increase in the thermal stability of the two domains of hexokinase A in the presence of varying concentration of polyols, a: domain 1, b: domain 2. The symbols used for the various polyols are shown in the inset.
Figure 6. Effect of 1.5M sorbitol on the urea-induced denaturation of hexokinase A monitored by mean residue ellipticity of the enzyme at 222 nm at pH 8.5 and 25°C.

To explore the reason for the cooperative denaturation behaviour of hexokinase A in the presence of 1.5M sorbitol, DSC experiments were carried out in the presence of 0.5M urea and a mixture of urea and sorbitol (Figure 7). In the presence of a solution of 1.5M sorbitol containing 1M urea, there was an increase in Tm1 by 10.9°C while the increase was 8.3°C in the presence of 2M urea with relatively small increase in Tm2 (4.3°C in 1M urea) (Table I). In the presence of 0.5M urea alone, the Tm1 and Tm2 decreased by 3.5 and 2.7 °C, respectively.

Activity Measurements

Hexokinase activity measurements were carried out at pH 7.8 and 25 °C after incubating the enzyme at 5 and 25 °C for different time intervals in the presence of 1M polyols at pH 8.5 (Figure 8 a,b). Upon incubation at 5 °C, it was found that sorbitol is the best stabilizer of activity followed by xylitol, glycerol, and erythritol. Sorbitol retained the activity of hexokinase upto 80% of the native control taken as 100% even after more than 100 hours of incubation at 5 °C compared to the retention of less than 10% activity in the absence of polyols (Figure 8a). When hexokinase was incubated at 25 °C, the control protein lost 70% of activity in the first two hours and had negligible activity in 24 hr. (Figure 8b).

In the presence of sorbitol, the activity was significantly higher than the control in the first two hours and then showed a gradual decrease over a period of time. Compared to the control buffer, sorbitol was able to retain upto ≈75% activity upon incubation for 47 hr. and was also found to be the best stabilizer of hexokinase activity followed by xylitol, glycerol,
Figure 7. Effect of 0.5M urea and a combination of urea and sorbitol on the calorimetric transition of hexokinase A in 20 mM Tris HCl buffer, pH 8.5. a: the raw data after concentration normalization; b, c, and d: the analyzed curves after baseline subtraction and deconvolution of the two transitions.

Figure 8. % Activity of hexokinase A retained after incubating the enzyme at 5 °C (a) and 25 °C (b) in the presence of 1M concentration of various polyols and 20 mM Tris-HCl buffer, pH 8.5 for varying time periods. Symbols for the polyols used are shown in the inset. The error in the activity measurements was ± 5%.
Figure 9. Correlation of the increase in the thermal stability of domain 1 of hexokinase A, $\Delta T_{m1}$, and that of the % activity of the enzyme retained after incubation in 1M polyols, pH 8.5 at 25°C for 47 hrs., with the number of hydroxyl groups in the polyols.

Figure 10. Variation of the increase in the thermal stability of the two domains of hexokinase A, $\Delta T_{m1}$ (■) and $\Delta T_{m2}$ (□) in the presence of 1M polyols, pH 8.5 with the surface tension of 1M aqueous polyol solutions at 25 °C. Surface tension of water at 25 °C has been taken as 71.97 dyne.cm⁻¹ and plotted as $\Delta$ in the figure.

and erythritol (Figure 8b). These trends were nearly identical at 5 and 25°C. Figure 9 shows the correlation of the activity of hexokinase retained after 47 hours of incubation at 25 °C and that of $\Delta T_{m1}$ with the number of -OH groups in the polyols. The higher the thermal stabilization of the protein in a polyol, the higher is the activity retained in its presence and larger the carbon chain length of the polyol or the number of hydroxyl groups better is the functional and thermal stabilization provided. Glycerol, however, was an exception and showed marginally higher retention in the activity of hexokinase at 25 °C and considerable retention at 5°C compared to erythritol, although it leads to a lesser increase in the $T_{m1}$ value compared to erythritol.

**Hexokinase thermal stability and physico-chemical properties of polyols**

Figure 10 presents plots of the variations in the thermal stability of the two domains of hexokinase, $\Delta T_{m1}$ and $\Delta T_{m2}$, with the magnitudes of the surface tension values of aqueous polyol solutions. The surface tension values obtained for 1 M erythritol and glycerol solutions at 25 °C were 72.59 ± 0.05 and 70.58 ± 0.08 dyne.cm⁻¹mol⁻¹, respectively. Surface tension values of 1 M solutions of sorbitol and xylitol were taken from our previous work (Kaushik & Bhat, 1998), and were 73.15 ± 0.19 and 72.9 ± 0.2 dyne.cm⁻¹mol⁻¹, respectively. 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 (Figure 10). However, when the $\Delta T_m$ values are plotted against two other physico-chemical properties of polyol solutions, viz., apparent molal heat capacity and volume, $\phi_c^o$ and $\phi_v^o$, respectively taken from the literature (DiPaola & Belleau, 1977), a linear correlation is observed (Figure 11 a,b). These properties were selected as they are known to be very sensitive to structural changes in aqueous solutions related to solute-solvent interactions (Kauzmann, 1959).

![Figure 11](image)

**Figure 11.** Variation of the increase in the thermal stability of the two domains of hexokinase A, $\Delta T_{m1}$ and $\Delta T_{m2}$ in the presence of 1M polyols, pH 8.5 with the apparent molal heat capacity, $\phi_c^o$ (a) and apparent molal volume, $\phi_v^o$ (b) of aqueous polyol solutions.

**DISCUSSION**

In the presence of polyols, domain 1 is stabilized to a larger extent than domain 2 (Figures 1-4) as evident from the respective $T_m$ values, and the stabilizing effect of the polyols for both the domains increases with the increasing number of -OH groups as we go from glycerol to sorbitol (Figure 5; Table I). The plots of $\Delta T_m$ values versus concentration of polyols are linear showing that the effect of polyols is more or less additive upto 1.5M concentration. 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 (Gekko & Koga, 1983; Kaushik & Bhat, 1998). Similar correlation has been observed for the two domain protein hexokinase with the trend in the increase of $T_{m1}$ and $T_{m2}$ being identical to those observed in the literature.
Polyols including glycerol have been found to lead to the preferential hydration of proteins (Gekko & Timasheff, 1981a; Gekko & Morikawa, 1981a). Recently Xie & Timasheff (1997a) have 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. Extensive studies carried out in our laboratory on the effect of a variety of polyols on the thermal stability of several proteins have demonstrated that the stabilizing effect of polyols studied results from the increase in the surface tension of water in their presence (Kaushik & Bhat, 1998), 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, the other three polyols lead to its increase. Increased surface tension of the solution, therefore, can not be considered as a sole factor leading to the stabilizing effect. This has been pointed out earlier by us (Kaushik & Bhat, 1998) and by other workers (Lin & Timasheff, 1996). Also, there may be problems in extrapolating the surface tension values of the polyol solutions measured at 25 °C to the temperatures of transition of the protein which varies with the type of the polyol used. 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 (Figure 10), indicates that for the three polyols studied, increase in the surface tension of the medium leads to an increase in the thermal stability just as has been reported for sugars (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a), glycerol being an exception. Glycerol has been reported to lead to preferential hydration of proteins owing to "solvophobic effect" which means that the surface residues on the protein molecule have more affinity for water than glycerol (Gekko and Timasheff, 1981a,b). It has been observed by us earlier that glycerol unexpectedly lowers the T_m of insulin while other polyols studied increased the T_m (Gupta and Bhat, 1995). This observation in conjunction with the data of the effect of glycerol on the thermal stability of several other proteins available in the literature (Back et al., 1979; Gekko and Timasheff, 1981b; Gekko and Koga, 1983) suggests that glycerol effect is highly protein dependent and that the nature of glycerol-protein interactions mediated via solvent water plays an important role. The anomalous behaviour of glycerol observed here therefore, could, stem primarily from its varied interactions with the protein surface residues mediated via solvent water as compared to other polyols.
Interestingly, the plots for the $\Delta T_m$ values against two other physico-chemical properties of solution, apparent molal heat capacity and volume of polyols including glycerol at infinite dilution, $\Phi_c^o$ and $\Phi_v^o$, respectively, lead to a linear correlation (Figure 11). It, therefore, 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 $\Phi_c^o$ and $\Phi_v^o$ 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 protein thermal stability. Recently Bolen and coworkers (Liu and Bolen, 1995; Wang and Bolen, 1997) based on the free energy of transfer studies of amino acids and a model peptide from water to osmolyte solutions, have demonstrated that the peptide backbone and not the side chains play a dominant role in protein stabilization by osmolytes. Similar studies in the presence of polyols would throw further light on the role of the components by the protein in the stabilizing effect by polyols.

It has been observed that with increasing concentration of a polyol, domains 1 and 2 start overlapping. This is due to the differential stabilization of the two domains of hexokinase A with domain 1 being stabilized to a much higher extent compared to domain 2 (Table I, Figures 1-4). It has been observed earlier that a polyol stabilizes different proteins to varying extents depending on the various physicochemical properties of proteins (Kaushik & Bhat, 1998). Also, it has been seen that RNase A is stabilized to different extents in the presence of polyols as a function of pH. Lower the $T_m$ of RNase A in buffer higher is the stabilizing effect of a polyol (Xie & Timasheff, 1997a; Kaushik & Bhat, 1998). Polyols are known to increase the strength of inter- and intra-molecular hydrogen bonding in the water structure and peptide chain, respectively (Gerlsma & Stuur, 1972, 1974). They also lead to the lowering of the dielectric constant of the medium which results in the strengthening of electrostatic interactions, though marginally (Akerlof, 1932).

Recent studies carried out in our laboratory indicate a dominant role of the increase in the surface tension of the medium and its relevance in strengthening the hydrophobic interactions in the protein core, leading to increased protein thermal stability (Kaushik & Bhat, 1998), glycerol being an exception. It, therefore, appears that polyols are able to strengthen one or all of the above interactions more in the loosely structured domain 1 compared to domain 2 and lead to the differential stabilization of the two domains as seen in
the DSC profiles. The larger increase in $\Delta H_1$ compared to $\Delta H_2$ in the presence of 0.5 M polyols could also be attributed to this effect. However, it has been observed that with increasing concentration of polyols, the $\Delta H$ values do not show any appreciable change. The changes whatsoever observed are within the experimental uncertainty. Similar trends have been observed for the stabilization of RNase A in the presence of increasing concentrations of glycine based osmolytes (Santoro et al., 1992). $\Delta C_p$ of denaturation of hexokinase in buffer has been observed to be highly positive and can be considered to be due to significant exposure of the nonpolar accessible surface area upon protein denaturation, as has been observed for several proteins (Myers et al., 1995). $\Delta C_p$ values have been found to be remaining constant, within the limits of experimental uncertainty, in the presence of 0.5M polyols. However, there is a considerable decrease in the values as the polyol concentrations are increased further. This could be due to the reduction in the degree of exposure of the nonpolar groups and hence their solvation in the denatured state in the presence of high concentration of the polyols. Similar reduction in the $\Delta C_p$ values, evaluated by UV spectroscopy, has been observed by us for RNase A in the presence of a number of polyols (Kaushik & Bhat 1998) and those determined by DSC for several proteins in the presence of varying pH conditions (Makhatadze et al., 1997).

Figure 6 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 (Chapter 3: Figure 1) shows that the onset of denaturation (unfolding) for the protein starts around 25 °C 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 (Figure 7). 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 25°C (Figure 6). 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 structure stabilizer. DSC experiments showed that in the presence of 1.5M sorbitol and 1M urea the shift in $T_{m1}$ was much higher (10.9 °C) compared to that for $T_{m2}$ (4.3 °C), and at 2M urea both the peaks merged leading to a shift in $T_m$ by 8.3°C. 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 25 °C. As a result the overall transition zone showed a decrease indicating higher cooperativity of unfolding of hexokinase A with only one visible peak (Figure 7d). The more negative magnitude of the mean residue ellipticity in the presence of 1.5M sorbitol at all the concentrations of urea compared to 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 5°C to the maximal extent. There is a good correlation of the increase in Tₘ by the polyols and their ability to retain the activity at 5°C except for glycerol which seems to be stabilizing hexokinase to a better extent than expected (Figure 8a). This anomalous behaviour of glycerol again remains unexplained. When hexokinase A is incubated at 25 °C 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 (Figure 8b). The data in Figure 9, which presents the correlation of the increase in Tₘ and that of the % hexokinase activity retained after incubation at 25°C for 47 hr. with the number of hydroxyl groups in polyols, indicate that there is a good correlation of the thermal stability and activity retained with the number of -OH groups in the polyol, except that there is a gradual increase in the former and an exponential increase in the latter. It can, thus, be concluded that shift in the Tₘ 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 25 °C in the case of glycerol and erythritol which may be leading to the slow denaturation of the protein at 25 °C and hence rapid loss of its activity. Hexokinase incubated at 25 °C in the presence of 1M sorbitol retains activity for exceptionally longer periods of time. Even after incubation for 47 hr. nearly 75% activity is retained. This is likely due to the shift in its Tₘ to 45.3°C and the temperature onset of denaturation to a much higher temperature. The protein thus, does not seem to be undergoing slow unfolding process at 25 °C as it does 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 (Bennett & Steitz 1980b).

Analysis of the calorimetric data on hexokinase and the structural information of the enzyme leads to the conclusion that there is 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 and correlates well with the retention of biological activity of the enzyme after incubation at 25 °C. There is a decrease in the ΔC_p values with the increase in the concentration of the polyols which may be resulting from a decrease in the nonpolar accessible surface area upon denaturation of the protein. Sorbitol which is the best stabilizer among the polyol series also protects the enzyme against urea denaturation. Although, increase in the surface tension of the medium appears to be a significant factor in modulating protein stability by erythritol, xylitol, and sorbitol, yet glycerol lowers the surface tension of water while increasing protein stability. Caution should, thus, be exercised in extrapolating the interfacial properties of the solutes in aqueous solutions to their physico-chemical properties in the bulk.