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Thermal stability of α-Lactalbumin in the presence of various sugars as osmolytes

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ABSTRACT

Thermal denaturation of α-Lactalbumin in the absence and presence of various concentrations of sucrose, sorbitol, glucose and galactose as sugar osmolytes were measured by monitoring changes in the absorptions that carried out in a Lambd 35 UV-Vis double beam spectrophotometer at pH 6.0. These measurements gave values of T_m (midpoint of denaturation), ΔH_m (enthalpy change at T_m), and ΔCp (constant-pressure heat capacity change) under a given solvent condition. Using these values of ΔH_m, T_m and ΔC_p , ΔG_D° (Gibbs energy change), was determined at a given concentration of each sugar. It has been observed that each sugar stabilizes the α-Lactalbumin in terms of T_m and ΔG_D°. The temperature that corresponds to maximum protein stability, T_S, is increased in the presence of these osmolytes. The same trend was also observed for T_H, the temperature corresponding to zero enthalpy change of denaturation.

Keywords: Protein stability; Sugar osmolytes; Thermal denaturation; α-Lactalbumin

INTRODUCTION

α-Lactalbumin (αLA) is a 123-residue mixed α + β protein with 4 disulfide bonds which in the native state binds Ca^{2+}. It is readily available from milk, where it acts as a regulatory subunit of the dimeric enzyme lactose synthase. Its function is to allow galactosyltransferase to use glucose as a substrate in the synthesis of lactose from glucose and galactose. αLA has been studied for decades as a model for protein stability and folding due to its conformational versatility [1–7]. It remains folded between pH 4.2 and 9.5; in the pH-range 4.2–3.0 and above pH 9.5 it forms the so-called A-state, which is prone to aggregation and interaction with [8]as well as fusion of [9] phospholipids vesicles. The molten globule state formed below pH 3 also binds to both zwitterionic [10] and anionic [11] lipids, forming a stable complex that survives transition to neutral pH.

Recently, Svanborg and co-workers have reported a novel property of human αLA, namely an ability to induce apoptosis in a wide array of cancer cells [12–14]. The phenomenon is known as HAMLET (Human α-Lactalbumin Made Lethal to Tumour cells) and requires αLA to assume a specific conformational state in which the ca $^{2+}$ co-factor is removed and oleic acid is bound [15].

Considerable time in nearly all fields of biochemical sciences is devoted to improving protein stability, which is the result of a balance between the intramolecular interactions of protein functional groups and their interaction with solvent environment [16-18]. Naturally occurring osmolytes are co-solvents that are used to protect organisms from denaturation by harsh environmental stresses. These molecules stabilise proteins, not by interacting with them directly but by altering the solvent properties of the surrounding water and hence the protein–solvent interactions [18]. Their effect seems to be general for all proteins. They have no inhibitory or enhancing effects on biological activity under physiological conditions hence are called compatible osmolyte [17,19]. Stabilizing

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osmolytes are chemically diverse and include such chemical classes as polyols, certain amino acids and their derivatives, and methylamine compounds [19]. There are various mechanisms that have been used to explain the observation on the effect of osmolytes on the protein denaturation equilibrium; native (N) state ↔ denatured (D) state [20-23]. The most widely used mechanism is due to Timasheff [22]. According to this mechanism osmolytes stabiliseNstate because they are preferentially excluded from the protein surface, for the preferential exclusion increases the chemical potential of the protein proportionately to solvent exposed surface area. Thus, by Le Chatelier's principle, osmolytes favour the more compact state, i.e., the N state over the structurally expanded state, i.e., D state. Hence according to this mechanism ΔG_D, the Gibbs free energy change associated with the denaturation process, N state ↔ D state, should increase in the presence of osmolytes, for $\Delta G_D = -RT\ln([D]/[N])$, where square bracket represents concentration. The most recent mechanism of stabilisation of proteins by osmolytes is due to Bolen and co-workers [23]. According to this mechanism osmolytes stabilise N state because of their overwhelming unfavourable interaction with the peptide backbone. Thus, this "osmophobic effect" favours the N state over the D state of proteins. Hence, according to this mechanism ΔG_D should increase in the presence of osmolytes.

Previous studies showed that osmolytes such as sugar and polyols effect on denaturation and have found that sugar and polyols have a stabilizing effect, increasing thermal denaturation temperature of globular proteins [24-34]. The main conclusion of these studies is that all osmolytes act independently on the protein, i.e., none of the osmolytes alters the efficacy of the other in forcing the protein to fold or unfold.

In the present work, the roles of sucrose, sorbitol, glucose and galactose as sugar osmolytes on the thermodynamic stability of α-Lactalbumin during heat stress have been extensively studied at various sugar concentrations.

EXPERIMENTAL SECTION

Chemicals

Commercially lyophilized bovine α-Lactalbumin (αLA) was purchased from Sigma Chemical Co. D-sorbitol, D-glucose, D-galactose and D-sucrose were also obtained from Sigma. All salts used for buffer preparation were analytical grade and dissolved in douhle distilled water. The 50 mM phosphate huffer pH 6.0 was used as buffers. All of the solutions were used freshly after preparation. Protein stock solutions were filtered using 0.45 μm milipore filter paper. The concentration of α-Lactalbumin was determined experimentally using a value of 29210 $M^{-1}.cm^{-1}$ for the molar absorption coefficient (ε) at 280 nm and pH 6.0.

Thermal Denaturation of αLA

Thermal denaturation studies were carried out in a Lambd 35 UV-Vis double beam spectrophotometer with a heating rate of 1°C/min. The requirement for equilibrium conditions was achieved by this scan rate. Each sample was heated from 20 to 95 °C. The change in absorbance of αLA at a fixed concentration of each osmolyte with increasing temperature was followed at 295 nm. The basic observation was a heat-induced transition curve. i.e. a plot of an optical property against temperature. To obtain values of T_m (the midpoint of the transition curve) and ΔHm (the enthalpy change upon denaturation at T_m), a nonlinear least-squares analysis was used to fit all the data points of the transition curve according to this relation [35]:

$$y(T) = \frac{y_N(T) + y_D(T)\exp[-\frac{\Delta H_m}{R}(\frac{1}{T} - \frac{1}{T_m})]}{1 + \exp[-\frac{\Delta H_m}{R}(\frac{1}{T} - \frac{1}{T_m})]} \quad (1)$$

Where y(T) is the optical property at temperature T(k), $y_N(T)$ and $y_D(T)$ are the optical properties of the native and denatured protein molecules at T, respectively, and R is the gas constant. In the analysis of the transition curve, it was assumed that a parabolic function describes the dependence of the optical properties of the native and denatured protein molecules (i.e., $y_N(T) = a_N + b_N T + c_N T^2$ and $y_D(T) = a_D + b_D T + c_D T^2$, where a_N, b_N, c_N, a_D, b_D, and c_D are temperature-independent coefficients) [36, 37]. A plot of ΔH_m versus T_m gave the value of ΔC_p, the temperature-independent heat capacity change at constant pressure. $\Delta G_D(T)$, the value of ΔG_D at any temperature T was estimated using Gibbs–Helmholtz equation with values of T_m, ΔH_m and ΔC_p,

$$\Delta G = \Delta H_m(1 - \frac{T}{T_m}) - \Delta C_p[(T_m - T) + T\ln\frac{T}{T_m}] \quad (2)$$

RESULTS

All denaturation curves were measured at least three times. Fig. 1 shows the representative denaturation curves of αLA in the presence and absence of sucrose, sorbitol, glucose and galactose. To convert the reversible heat-induced optical transition data into thermodynamic parameters, the following assumptions were made. First, the transition between N and D states follows a two-state mechanism. Second, the temperature dependencies of Y_N and Y_D are parabolic. Third, osmolytes have no effect on the conformational ΔC_P of αLA. Making use of the first two assumptions, the thermal transition curves were analysed according to eqn (1), and the analysis yielded values of T_m and ΔH_m with their uncertainties. Data fitting was done using Sigma Plot 10 software [38].

The denaturation results in 30% and 40% w/v galactose could not be analysed to estimate fitting parameters due to high dispersion of the experimental points. It seems the assumptions that have been made for analysing the transition curve failed under these conditions.The values of T_m, ΔT_m, ΔH_m and $\Delta\Delta H_m$ (the difference between ΔH_m in he preceence and absence of osmolytes) for αLA in the presence of different concentrations of sucrose, sorbitol, glucose and galactose are collected in Table 1.

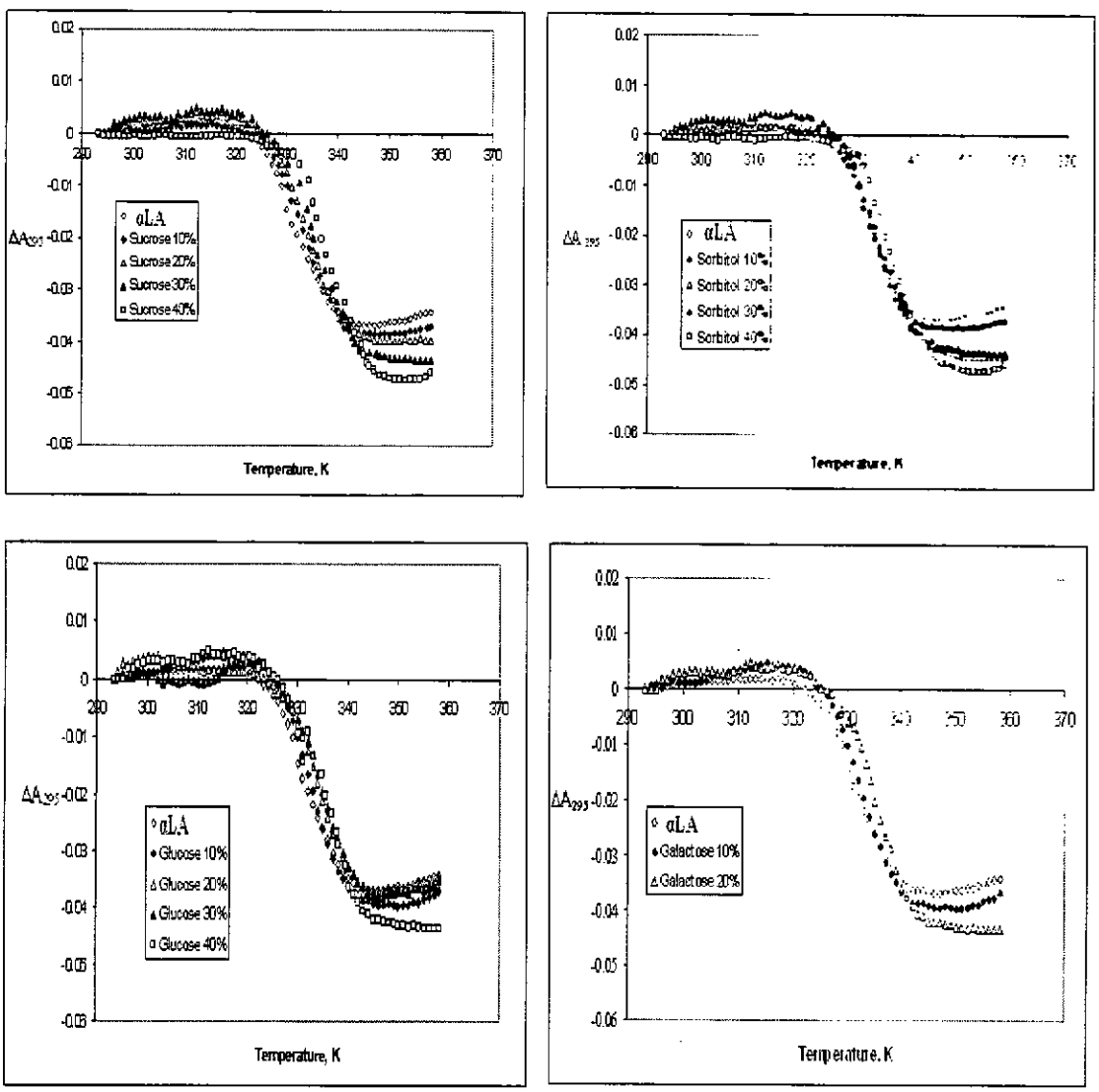

Fig. 1. Thermal denaturation curves of αLA in the absence and presence of various sugar osmplytes.

Making use of the third assumption (independence of ΔC_P from osmolyte concentration), we plotted ΔH_m as a function of T_m at each fixed concentration of an osmolyte. The value of 5.71 $kJ.mol^{-1}.K^{-1}$ obtained for ΔC_P of αLA. Thermal stability curve, i.e., the variation of $\Delta G_D(T)$ versus T, was constructed for αLA in the presence of various concentrations of osmolytes and shown in Fig. 2. Tables 1. present the values of ΔG_D° (Gibbs free energy change at 25 °C) at different concentrations of sucrose, sorbitol, glucose and galactose for αLA. This table also shows $\%\Delta\Delta G_D^\circ$, the percent change in ΔG_D° of the protein due the presence of sugars(s); $\%\Delta\Delta G_D^\circ$ =100 [ΔG_D° (in the presence of sugar(s)) - ΔG_D° (in the absence of sugar)] /ΔG_D° (in the absence of sugar).

The value of T_S (the temperature in which the protein has maximum stability) was obtained exactly from Fig. 2 (temperature of the maximum point in plot of ΔG° against T). The values of T_S and ΔH_S were used in eqn (3) to estimate T_H, the temperature at which the enthalpy changes of denaturation equals zero [39].

$$T_H = T_S - (\Delta H_S / \Delta C_p) \quad (3)$$

Since ΔH at T_S (ΔH_S) is equal to ΔG at T_S (ΔG_S; the maximum of ΔG) according to $\Delta G = \Delta H - T\Delta S$ with $\Delta S = 0$ at Ts, eqn (3) may be simplified to

$$T_H = T_S - (\Delta G_S / \Delta C_p) \quad (4)$$

Another important thermodynamic parameter that can be determined from thermal stability profiles is T_G', the temperature at which the Gibbs energy change of denaturation is zero but the entropy change of denaturation is negative. TG′ characterizes the cold denaturation of a protein and can be derived from continuing the left side of the thermal stability curve. This parameter was estimated from the following equation [40]:

$$T_G' = \frac{T_m^2}{3T_m - 2T_H} \quad (5)$$

The estimated values of T_m and T_H were used to determine T_G' with the help of eqns (5). The values of T_G', $\Delta T_G'$ (the difference between T_G' in he precence and absence of osmolytes), T_H, ΔT_H(the difference between T_H in he precence and absence of osmolytes), T_S and ΔT_S thus obtained at various concentrations of osmolytes are given in Tables 2. for αLA.

Table 1. Stability parameters of αLA in the presence of various concentrations of sugar osmolytes at pH 6.0

Osmolytes	M	T_m/K	ΔT_m/K	ΔH_m(kJ.mol^{-1})	$\Delta\Delta H_m$(kJ.mol^{-1})	ΔG_D°(kJ.mol^{-1})	$\%\Delta\Delta G_D^\circ$
Control	00	328.6± 1.2	0.0	254.2 ± 2.3	0.00	15.24± 0.6	0.00
Sucrose	10	330.9± 1.3	2.3	266.5± 2.4	12.3	16.80± 0.6	10.24
	20	331.6± 1.5	3.0	271.2± 2.4	17.0	17.38± 0.5	14.04
	30	333.1± 1.4	4.5	279.0± 2.6	24.8	18.42± 0.4	20.87
	40	334.2± 1.3	5.6	286.7± 2.5	32.5	19.40± 0.5	27.30
Sorbitol	10	330.8± 1.3	2.2	266.4± 2.4	12.2	16.77± 0.5	10.04
	20	331.5± 1.5	2.9	270.9± 2.4	16.7	17.33± 0.5	13.71
	30	333.0± 1.4	4.4	278.7± 2.6	24.5	18.36± 0.6	20.47
	40	334.1± 1.3	5.5	286.5± 2.5	32.3	19.36± 0.5	27.03
Glucose	10	329.9± 1.3	1.3	260.7± 2.3	6.5	16.07± 0.7	5.45
	20	330.6± 1.5	2.0	264.6± 2.6	10.4	16.57± 0.5	8.72
	30	331.9± 1.4	3.3	272.5± 2.5	18.3	17.56± 0.4	15.22
	40	332.8± 1.3	4.2	277.6± 1.3	23.4	18.23± 0.4	19.62
Galactose	10	329.5± 1.3	0.9	260.4± 2.3	6.2	15.98± 0.7	4.86
	20	330.4± 1.2	1.8	264.3± 2.4	10.1	16.50± 0.5	8.27

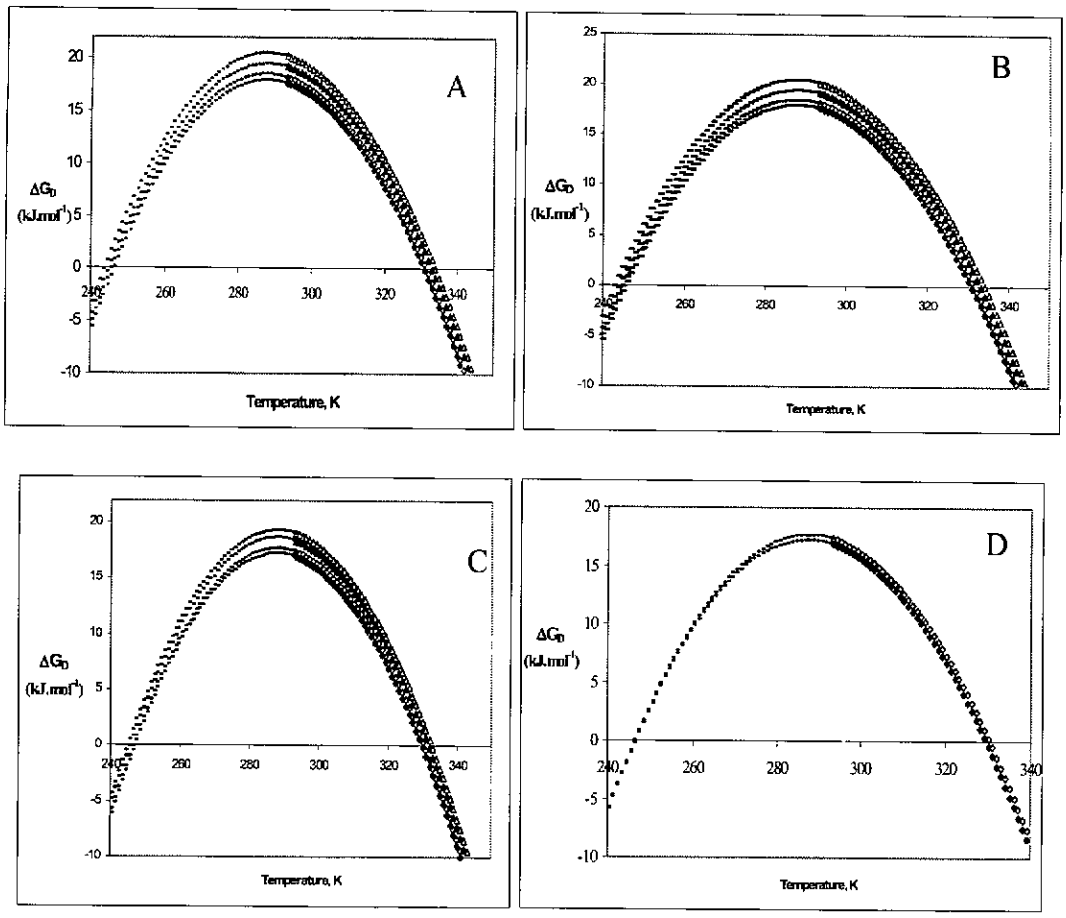

Fig. 2. Thermal stability curves for β-lactoglobulin B in the presence of Sucrose (A), Sorbitol (B), Glucose(C) and Galactose (D). The points joined by continuous lines correspond to the experimental measurements and the dashed lines have been calculated using eqn (2).

Table 2. The values of T_G', $\Delta T_G'$, T_H, ΔT_H, T_S and ΔT_S associated with thermal denaturation of αLA in the absence and presence of various concentrations of sugar osmolytes

Osmolytes	%w/v	T_G'/K	$\Delta T_G'$/K	T_H/K	ΔT_H/K	T_S/K	ΔT_S/K
Control	00	258.28 ± 1.2	0.00	284.27± 1.2	0.00	286.9±0.8	0.00
Sucrose	10	258.43 ± 1.3	0.15	284.41± 1.2	0.14	287.8±0.7	0.9
	20	258.51 ± 1.2	0.23	284.90± 1.3	0.63	288.2±0.8	1/3
	30	258.80 ± 1.5	0.52	284.91± 1.2	0.64	288.3±0.9	1/4
	40	258.77 ± 1.4	0.49	284.76± 1.4	0.49	288.5±0.8	1/6
Sorbitol	10	258.42 ± 1.7	0.22	284.43± 1.2	0.16	287.7±0.7	0.8
	20	258.55 ± 1.6	0.27	284.91± 1.5	0.64	288.2±0.9	1/3
	30	258.97 ± 1.3	0.69	284.73± 1.2	0.46	288.3±0.8	1/4
	40	258.80 ± 1.2	0.52	284.78± 1.4	0.51	288.4±0.8	1/5
Glucose	10	258.35± 1.2	0.07	284.33± 1.2	0.06	287.6±0.8	0.7
	20	258.46± 1.3	0.18	284.45± 1.5	0.18	288.0±0.7	1/1
	30	258.41± 1.2	0.13	284.88± 1.2	0.61	288.2±0.6	1/3
	40	258.80± 1.4	0.52	284.56± 1.3	0.29	288.3±0.8	1/4
Galactose	10	258.35± 1.2	0.07	284.32± 1.3	0.05	287.5±0.9	0.6
	20	258.42± 1.3	0.14	284.47± 1.2	0.20	287.9±0.8	1/0

DISCUSSION

All thermodynamic quantities, (given in Tables. 1) were obtained from the analysis of heat denaturation curves of αLA in the presence and absence of different sugars (e.g., see Figs. 1). This analysis according to eq. (1) assumes that the transition between the native and denatured states is a two-state process. It can be seen in Fig. 1 that y_D has a stronger dependency on temperature and osmolyte concentration than y_N, suggesting that osmolytes are more effective on the denatured state of αLA. In other words, osmolytes affect the denatured state of the protein more than its native state, leading to a change in protein stability.

It should be noted that such measurements were not possible in the presence of fructose individually or in a mixture containing other monosaccharides. This is due to the fact that fructose has a very strong absorption in the near-UV region [41].

It has been observed that a disaccharide has more stabilizing effect than the individual monosaccharide, and that, the order of stahilization is: sucrose, sorbitol > glucose, galactose. It is noteworthy that cytochrome-c was also shown to follow the same ranking, i.e., the order of stabilization wa tetra- > tri- > di- > mono-saccharide [42].

Moreover, our curves in Fig. 1 show that at the conditions of this study, the transition can be assumed as a change between two states and an intermediate state is not clear in this case. Calculated denaturation temperatures show that T_m for αLA in buffer are 328.6 K at pH 6.0. Recently, Singh [43] showed the T_m value of the αLA solution without co solvent was 327.6 K at pH 6.0 and shifted to 331.8 with the presence of 1.0 M glycine betaine.

It is seen in Fig. 1 (also see Tables 1) that T_m of αLA at pH 6.0 increases linearly with an increase in the concentration of individual sugar. The ΔH_m values of many proteins remain unchanged in the presence of various osmolytes [44-49]. We have also observed that the ΔH_m of αLA in the presence of different sugars shows insignificant dependence on type and concentration of the sugar. This and earlier observations suggest that sugar osmolytes have no significant affinity on the protein.

We have determined $\Delta C_p = \left(\frac{\partial \Delta H_m}{\partial T_m}\right)_p$ from the linear plot of ΔH_m and T_m values at pH 6.0. The value of ΔCp in the presence of different concentrations of sugars is 5.71 kJ $mol^{-1}K^{-1}$ for αLA. A DSC (differential scanning calorimetry) study of αLA was reported that in aqueous solutions ΔC_P=6.5 kJ $mol^{-1}K^{-1}$ [50].

The effect of sugars on protein stability have been explained in terms of preferential binding and preferential exclusion of these cosolutes [18, 22, 51], which is supported by recent observations on the transfer-free energy of protein groups from the solvent water to the co-solvent aqueous solutions [52]. Both Timasheff's and Bolen's group have argued that the source of stabilization of protein by sugars is the shifting of denaturation equilibrium towards the N state [52, 53]. Thus, what effects co-solvents will have on the denaturation equilibrium, N state ↔ D state under the native condition will be known only by measuring ΔG_D°. It is seen from Tables 1 that the effect of sugars on ΔG_D° of protein increases with increasing sugar concentrations at pH 6.0. It is seen that the $\%\Delta\Delta G_D^\circ$ increases with the molar concentration of the additive.

Although, there is no universal molecular theory that can explain the mechanism by which these stabilizing osmolytes interact with proteins to affect their stability. However, a new mechanism based on the observation of transfer-free energy of the protein backbone from water to aqueous osmolyte solution predicts that the interaction between the protein backbone and osmolytes polar group is more favourable than the corresponding interaction with non-polar groups [54,55]. Thus stabilizing/destabilizing osmolytes will be preferentially excluded/accumulated around protein backbone. This prediction is consistent with the thermodynamics of preferential interaction of stabilizing and destabilizing osmolytes [53, 56 and 57]. This new molecular mechanism for osmolyte-induced protein stability also predicts that osmolytes having the same fraction of the polar contact surface area will have the same effect on the protein denaturation equilibrium [58].

T_G' increases with rising sugars concentration. It means that the cold resistance of αLA decrease with rising sugars concentration. Changes in T_H show an increase at all concentrations of sugar osmolytes. Following Baldwin's suggestion that a protein has the least solubility at T_H [58], it

seems that sugar osmolytes increases the solubility of αLA at all concentrations. The temperature at which αLA have the most stability, T_S, follows the same trend as T_H. It can be asserted that T_S is related to the rate of ΔG_D changes with temperature. Therefore, the more T_S increases, the more the rate of change of ΔG_D increases with temperature. On the other hand, the sensitivity of the thermodynamic stability of the protein increases with temperature. Of course, one should be careful in this interpretation, because this comment holds true as long as T_m is constant.

CONCLUSION

Overall, protein stability should depend upon a fine balance between favorable and unfavorable interactions of the native and the denatured protein states with the cosolvent molecules . In summary, sucrose, sorbitol, glucose and galactose as sugar osmolytes stabilize αLA by shifting the denaturation equilibrium toward the native state at pH 6.0. It has been observed that a disaccharide has more stabilizing effect than the individual monosaccharide.

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