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Effect of polyol osmolytes on ΔG_D, the Gibbs energy of stabilisation of proteins at different pH values

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Abstract

Thermal denaturation curves of lysozyme and ribonuclease-A were determined by measuring their far-UV circular dichroism (CD) spectra in the presence of different concentrations of five polyols (sorbitol, glycerol, mannitol, xylitol and adonitol) at various pH values in the range 7.0–1.9. The denaturation curve at each polyol concentration and pH was analysed to obtain values of T_m (midpoint of denaturation) and ΔH_m (enthalpy change at T_m), and these ΔH_m and T_m values obtained at different pH values were used to obtain ΔC_p (constant-pressure heat capacity change) at each polyol concentration. Using values of ΔH_m, T_m and ΔC_p in the Gibbs–Helmholtz equation, $\Delta G_D°$ (Gibbs energy change at 25 °C) was determined at a given pH and polyol concentration. Main conclusions of this study are that polyols have no significant effect on $\Delta G_D°$ at pH 7.0, and they stabilise proteins in terms of $\Delta G_D°$ against heat denaturation at lower pH values. Other conclusions of this study are: (i) T_m at each pH increases with increasing polyol concentration, (ii) ΔH_m remains, within experimental error, unperturbed in the presence of polyols, and (iii) ΔC_p depends on polyol concentration. Furthermore, measurements of the far- and near-UV CD spectra suggested that secondary and tertiary structures of both proteins in their native and denatured states are not perturbed on the addition of polyols.

Keywords: Protein stability; Thermal denaturation; Protein structure; Polyol osmolyte; Ribonuclease-A; Lysozyme

1. Introduction

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 [1–3]. 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 [3]. 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 [2,4]. Stabilizing osmolytes are chemically diverse and include such chemical classes as polyols, certain amino acids and their derivatives, and methylamine compounds [4]. Among these chemical classes polyhydric alcohols (polyols) are among the most prevalent molecules used by nature to protect organisms against the stresses of high osmotic pressure and freezing [2,5]. Polyols belong to the class of compatible osmolytes [6–9]. They have also been found to be effective stabilisers of proteins and biological assemblies when added at high concentrations [3,10–14].

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)

Abbreviations: ΔG_D, Gibbs free energy change; ΔG_D°, Gibbs free energy change at 25 °C; RNase-A, ribonuclease-A; ΔC_p, constant-pressure heat capacity change; T_m, midpoint of thermal denaturation; ΔH_m, enthalpy change at T_m; CD, circular dichroism.

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state [15–18]. The most widely used mechanism is due to Timasheff [17]. According to this mechanism osmolytes stabilise N state 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 [18]. 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.

Recently, we studied the effect of trimethylamine N-oxide on the thermal denaturations of several proteins at different pH values [19]. The main conclusion of this study is that this osmolyte increases $\Delta G_D°$ (value of ΔG_D at 25 °C) of proteins at pH values above its pK_a (pK_a=4.66±0.10 [20,21]), and it decreases $\Delta G_D°$ of proteins at pH values below its pK_a. Since polyol osmolytes are nonionizable molecules, it is expected that, as predicted by all mechanisms of stabilisation of proteins [15–18], they should increase $\Delta G_D°$ of proteins at all pH values. In order to see whether this is indeed true, we have measured thermal denaturations of lysozyme and RNase-A in the absence and presence of five polyols (sorbitol, glycerol, xylitol, adonitol and mannitol) at different pH values in the range 7.0–1.9. We report for the first time that $\Delta G_D°$ of each protein is, within experimental errors, unchanged in the presence of all five polyols at pH 7.0, and it increases with decreasing pH. We also report that polyols have no effect on the secondary and tertiary structures of the two end states of the equilibrium N state↔D state.

2. Materials and methods

Commercial lyophilised preparations of RNase-A (type III-A) and hen egg white lysozyme were purchased from Sigma Chemical Co. D-Sorbitol, D-glycerol, D-mannitol, D-adonitol and D-xylitol were also obtained from Sigma Chemical Co. Guanidinium chloride (GdmCl) was the ultra pure sample from Schwarz/Mann. These and other chemicals, which were of analytical grade, were used without further purification.

RNase-A and lysozyme solutions were dialysed extensively against 0.1 M KCl at pH 7.0. Protein stock solutions were filtered using 0.45 μm millipore filter paper. Both proteins gave single band during polyacrylamide gel electrophoresis. Concentration of the protein stock solution was determined experimentally using ϵ, the molar absorption coefficient (M^{-1} cm^{-1}) values of 9800 at 277.5 nm for RNase-A [22] and 39,000 at 280 nm for lysozyme [23]. All solutions for optical measurements were prepared in the desired degassed buffer containing 0.1 M KCl. For various pH ranges, the buffers used were 0.05 M glycine hydrochloride buffer (pH range 1.9–3.5) and 0.05 M cacodylic acid buffer (pH range 5.0–7.0). It may be noted that a known amount of the unbuffered protein stock solution was used to prepare solutions at different pH values. Since pH of the protein solution may change on heating or on the addition of GdmCl, pH of each solution was also measured after the denaturation experiment. It was observed that the change in pH was not significant. It should, however, be noted that no corrections were made for the possible effect of co-solvents on the pH of protein solutions.

2.1. Thermal denaturation measurements

Thermal denaturation studies were carried out in a Jasco J-715 spectropolarimeter equipped with a peltier type temperature controller (PTC-348 WI) with a heating rate of 1 °C/min. This scan rate was found to provide adequate time for equilibration. Change in CD at 222 nm of the protein solution (concentration range 0.3–0.5 mg/ml) was measured in the temperature range 20 to 85 °C. About 650 data points of each transition curve were collected. After denaturation, the sample was immediately cooled down to measure reversibility of the reaction at different temperatures. It was observed that data from the renaturation experiments fell on the denaturation curve. All solution blanks showed negligible change in ellipticity with temperature and were, therefore, neglected during the data analysis. The raw CD data were converted into $[\theta]_\lambda$, the mean residue ellipticity (deg cm^2 $dmol^{-1}$) at a given wavelength λ using the relation,

$$[\theta]_\lambda = \theta_\lambda M_o / 10lc \quad (1)$$

where θ_λ is the observed ellipticity (millidegrees) at wavelength λ, M_o is the mean residue weight of the protein, c is the protein concentration (mg/cm^3), and l is the pathlength (cm). Each heat-induced transition curve was analysed for T_m (midpoint of denaturation) and ΔH_m (enthalpy change at T_m) using a non-linear least-squares analysis according to the relation,

$$y(T) = \frac{y_N(T) + y_D(T)\exp[-\Delta H_m/R(1/T - 1/T_m)]}{1 + \exp[-\Delta H_m/R(1/T - 1/T_m)]} \quad (2)$$

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 K, 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., $y_N(T)=a_N+b_NT+c_NT^2$ 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) [24,25]. A plot of ΔH_m versus T_m gave the value of ΔC_p, the temperature-independent heat capacity change at constant pressure. Using values of T_m, ΔH_m and ΔC_p, the value of ΔG_D at any temperature T, $\Delta G_D(T)$ was estimated with the help of the Gibbs–Helmholtz equation,

$$\Delta G_D(T) = \Delta H_m \left(\frac{T_m - T}{T_m} \right) - \Delta C_p \left[(T_m - T) + T \ln \left(\frac{T}{T_m} \right) \right] \quad (3)$$

3. Results

All denaturation curves of lysozyme and RNase-A were measured three times. All heat-induced denaturations of proteins in the presence and absence of various osmolytes were reversible at all pH values. Assuming that (i) the transition between N and D states follows a two-state mechanism, and (ii) the presence of the osmolyte does not affect the structure of both the native and denatured states, all optical transition data were converted into thermodynamic parameters using appropriate relations.

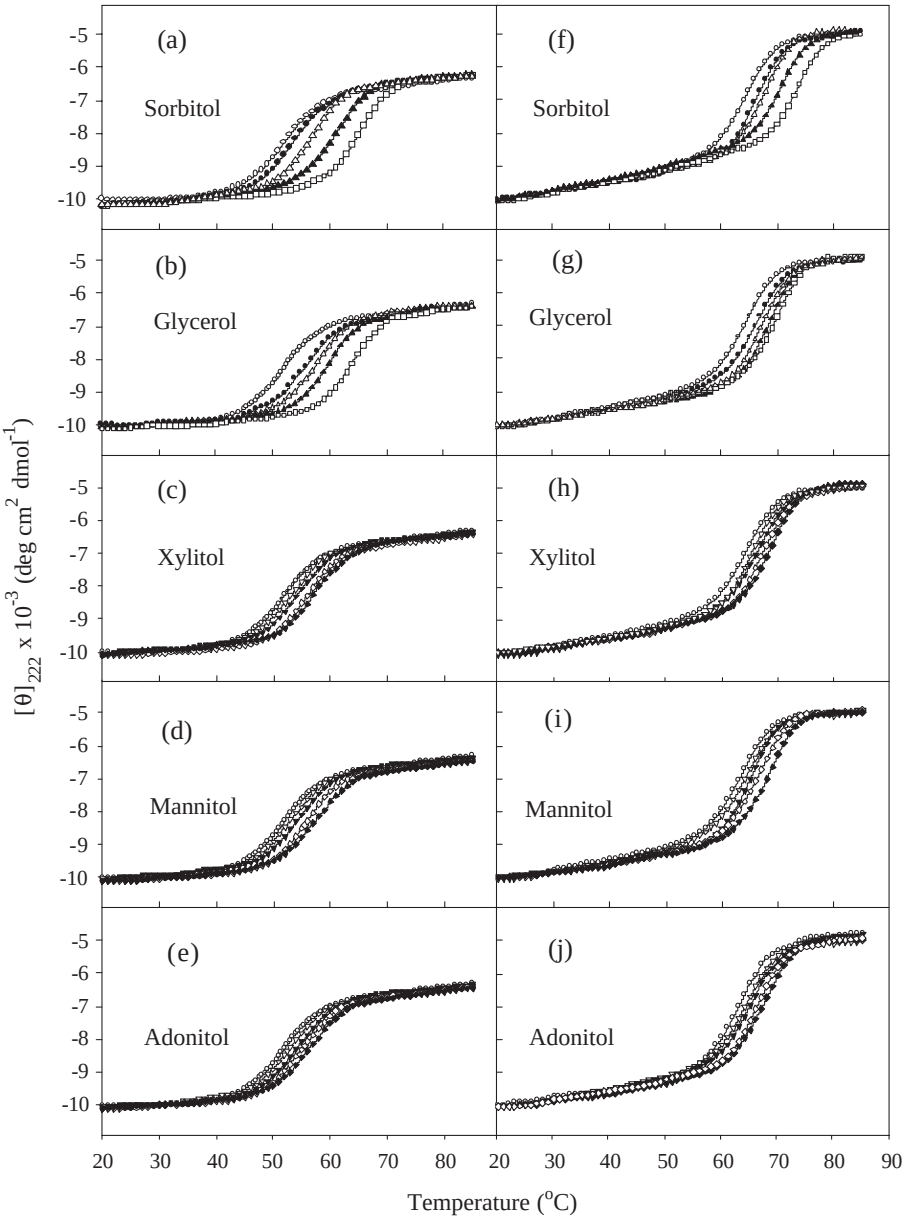

Fig. 1. Representative thermal denaturation profiles of lysozyme in the presence of different concentrations of polyols at pH 1.9 and 7.0. Denaturation curves in the absence (>) and presence of 10 (⋆), 20 (△), 30 (▲) and 40 (□) % sorbitol (w/v); 10 (⋆), 20 (△), 30 (▲) and 40 (□) % glycerol (v/v); and 0.25 (▽), 0.50 (▼), 0.75 (◇) and 1.00 (♦) M mannitol, adonitol and xylitol. Panels (a)–(e) and panels (f)–(j) show results at pH 1.9 and 7.0, respectively. In order to maintain clarity some data points are not shown. Temperature dependencies of y_D are: $y_D = 12885\ (\pm 1031) - 127\ (\pm 7)T + 0.2\ (\pm 0.0147)T^2$ at pH 1.9; $y_D = 222300\ (\pm 17765) - 1279\ (\pm 120)T + 1.7920\ (\pm 0.1085)T^2$ at pH 2.4; $y_D = 123625\ (\pm 11126) - 851\ (\pm 68)T + 1.3680\ (\pm 0.1231)T^2$ at pH 4.8; $y_D = 52169\ (\pm 3651) - 315\ (\pm 25)T + 0.4329(\pm 0.281)T^2$ at pH 6.0; and $y_D = 237383\ (\pm 22538) - 1294\ (\pm 90)T + 1.7250\ (\pm 0.1897)T^2$ at pH 7.0.

Table 1
Stability parameters of lysozyme and RNase-A in the presence of polyols[a,b]

Concentration	pH 1.9				pH 2.4				Lysozyme[c] pH 4.8				pH 6.0				pH 7.0				ΔC_p
	T_m	ΔH_m	ΔG_D°	$\%\Delta\Delta G_D^\circ$	T_m	ΔH_m	ΔG_D°	$\%\Delta\Delta G_D^\circ$	T_m	ΔH_m	ΔG_D°	$\%\Delta\Delta G_D^\circ$	T_m	ΔH_m	ΔG_D°	$\%\Delta\Delta G_D^\circ$	T_m	ΔH_m	ΔG_D°	$\%\Delta\Delta G_D^\circ$	
Sorbitol																					
0%, w/v	52.0	326	19.2±1.6	0	62.0	385	28.0±3.0	0	79.0	493	46.4±5.0	0	84.1	548	55.5±5.5	0	86.5	564	58.5±7.8	0	6.77±0.54
10%, w/v	54.5	334	21.8±2.3	13	65.4	393	31.7±2.5	13	82.1	481	48.0±4.5	4	87.0	539	58.6±5.8	6	88.2	535	58.2±6.3	1	6.02±0.38
20%, w/v	57.5	347	24.9±1.8	30	70.8	405	36.2±4.6	30	85.7	489	54.4±4.1	13	88.8	527	59.5±5.9	7	90.0	518	58.3±5.2	0	5.56±0.38
30%, w/v	60.5	364	28.4±2.4	48	73.1	418	39.7±4.1	42	87.7	493	55.4±3.4	20	92.6	535	61.1±4.6	10	91.8	506	58.5±8.6	0	5.23±0.33
40%, w/v	65.9	368	31.4±3.3	63	79.4	418	42.3±5.1	51	90.5	489	56.5±3.3	22	95.0	510	61.2±5.3	10	94.0	506	60.2±6.3	3	5.02±0.29
Glycerol																					
10%, v/v	55.8	339	22.1±2.6	15	64.4	393	30.5±3.0	9	81.1	489	47.1±5.1	2	86.4	543	56.9±6.1	3	87.5	543	57.1±8.4	3	6.44±0.46
20%, v/v	57.4	355	25.5±3.1	33	65.6	405	33.6±2.5	19	82.9	497	52.2±3.9	13	87.6	535	59.4±5.1	7	88.0	523	58.1±8.6	0	5.77±0.38
30%, v/v	59.0	360	28.1±1.7	46	71.5	410	38.9±3.8	39	84.5	485	54.8±3.6	18	90.0	510	60.6±6.3	9	88.9	506	59.3±8.9	1	4.97±0.29
40%, v/v	62.5	376	31.3±4.0	63	72.5	410	39.9±4.6	42	85.4	493	56.8±3.2	26	91.0	506	60.5±5.2	9	90.5	502	59.3±6.8	1	4.89±0.33
Xylitol																					
0.25 M	52.9	343	21.6±1.8	13	63.5	401	31.9±2.1	13	80.0	497	49.6±3.3	7	85.0	543	58.6±5.8	6	87.0	548	59.8±7.4	2	6.10±0.29
0.50 M	54.2	347	24.0±1.9	24	63.7	406	33.8±2.3	21	81.3	489	52.0±3.3	12	86.0	527	59.4±6.7	7	88.0	523	59.3±7.1	1	5.43±0.29
0.75 M	55.5	360	26.3±2.9	37	65.6	401	35.9±1.9	28	82.7	485	54.9±3.2	18	86.5	510	60.2±4.9	9	89.1	502	59.8±3.9	2	4.81±0.38
1.00 M	57.0	372	28.9±2.6	50	66.6	410	38.5±3.5	37	83.1	489	57.3±4.4	23	87.5	506	61.9±5.6	12	90.0	489	59.8±6.1	2	4.47±0.33
Adonitol																					
0.25 M	52.5	334	20.9±1.3	9	63.6	389	30.4±2.7	9	80.7	485	48.2±5.5	4	84.5	539	57.8±4.6	4	87.2	539	58.2±8.4	0	6.10±0.33
0.50 M	53.8	351	23.6±2.7	24	64.2	397	33.1±3.2	18	81.3	489	51.5±4.3	11	85.5	531	59.8±5.6	8	88.0	535	61.0±7.3	4	5.52±0.33
0.75 M	54.9	355	25.3±2.2	30	65.0	401	34.7±3.0	23	81.9	481	52.7±4.4	14	86.3	523	60.5±4.4	9	88.5	514	59.8±7.1	2	5.14±0.21
1.00 M	56.4	364	27.5±3.3	43	66.0	410	37.6±2.4	34	82.5	477	54.3±3.7	17	87.0	514	61.9±5.5	12	89.3	501	58.0±5.7	0	5.10±0.33
Mannitol																					
0.25 M	52.7	330	20.5±1.9	7	64.6	389	30.5±3.5	9	81.1	493	48.8±5.3	5	85.2	535	56.4±5.8	2	87.5	548	59.0±9.6	0	6.23±0.42
0.50 M	54.2	343	23.7±1.6	18	65.8	385	31.2±2.4	11	82.8	502	52.1±5.1	12	87.0	531	57.9±6.2	4	88.5	535	58.9±5.9	0	5.89±0.33
0.75 M	56.9	355	25.3±2.4	32	69.6	401	35.1±2.4	25	84.3	493	52.4±3.4	13	88.2	535	60.3±4.4	9	89.3	527	59.1±6.8	0	5.64±0.25
1.00 M	58.1	360	27.5±2.6	38	70.1	406	36.4±4.2	30	85.0	489	52.9±4.4	14	89.0	531	61.1±5.5	10	90.0	518	59.1±7.3	0	5.43±0.29

RNase-A

	PH 2.0				pH 3.5				pH 5.5				pH 6.0				pH 7.0				
Sorbitol																					
0%, w/v	36.0	339	11.0±0.9	0	49.7	401	25.8±1.5	0	61.0	447	38.0±2.34	0	62.4	485	42.6±2.9	0	63.0	489	44.0±5.3	0	5.06±0.42
10%, w/v	39.3	339	13.8±0.9	25	54.4	422	30.9±2.0	20	65.0	468	42.8±2.01	13	66.7	480	45.5±3.6	6	66.0	476	44.4±3.6	0	5.10±0.38
20%, w/v	41.8	313	14.3±1.0	30	57.0	414	31.5±2.3	22	68.3	456	42.5±2.51	12	69.1	468	45.8±4.1	7	68.4	464	43.6±5.8	0	5.31±0.29
30%, w/v	45.1	334	17.5±0.5	59	60.5	418	34.1±1.8	30	70.6	476	45.7±2.59	20	71.5	481	46.7±3.1	10	69.7	468	44.2±4.4	0	5.52±0.42
40%, w/v	49.2	339	19.9±1.3	81	63.7	422	34.9±2.3	35	72.8	476	45.4±2.68	19	73.5	485	46.8±3.5	10	72.1	472	44.5±6.3	0	5.89±0.33
Glycerol																					
10%, w/v	38.5	339	13.1±0.8	19	50.9	410	27.2±1.8	6	62.7	447	38.8±1.3	2	63.6	489	44.1±3.7	3	64.0	484	43.9±4.6	0	5.18±0.33
20%, w/v	41.7	343	16.4±1.3	47	51.8	414	28.1±1.1	9	63.2	451	39.4±2.4	4	64.5	493	45.0±4.4	6	64.7	485	44.1±3.8	0	5.27±0.42
30%, w/v	43.0	339	16.3±0.9	48	53.2	423	29.4±1.2	14	64.5	456	39.8±2.4	5	65.4	493	44.8±4.2	5	65.5	489	44.3±3.2	0	5.60±0.46
40%, w/v	46.8	339	23.1±0.9	72	54.3	426	29.8±2.0	16	65.8	472	41.0±2.8	8	66.3	506	25.3±4.4	6	66.2	489	43.2±5.9	2	6.19±0.50
Xylitol																					
0.25 M	37.6	347	12.7±0.7	16	50.3	410	26.9±1.8	4	61.2	460	39.4±1.9	4	63.2	481	43.2±3.1	1	63.7	481	43.5±3.3	0	5.06±0.33
0.50 M	38.7	351	13.8±0.7	25	51.0	422	28.3±1.1	10	62.0	468	40.7±1.9	7	63.9	493	44.9±3.7	5	64.5	493	45.3±5.3	3	5.14±0.33
0.75 M	39.7	351	14.6±1.1	33	52.4	426	29.5±1.5	15	62.9	472	41.5±1.5	9	64.6	497	45.5±4.9	7	65.0	493	45.3±4.6	3	5.31±0.42
1.00 M	41.3	360	16.3±1.5	47	53.5	435	30.9±1.6	20	63.6	476	42.0±2.1	11	64.6	502	45.6±4.0	7	65.2	497	45.4±1.5	3	5.48±0.46
Adonitol																					
0.25 M	37.5	347	12.6±0.4	14	51.6	410	27.7±2.0	7	61.1	464	39.5±2.0	4	63.7	476	42.7±4.6	0	63.7	376	42.6±3.3	2	5.23±0.29
0.50 M	38.7	355	13.9±1.4	27	52.4	418	28.8±1.3	12	62.1	456	39.1±1.4	3	64.0	497	44.9±4.0	5	64.4	489	44.3±5.1	0	5.35±0.29
0.75 M	39.7	360	15.0±1.3	36	52.8	430	30.1±1.7	17	62.9	468	40.7±1.9	7	65.2	502	46.1±3.5	8	65.2	485	44.2±4.6	0	5.39±0.42
1.00 M	41.2	360	16.2±1.3	47	54.8	430	31.5±2.1	22	63.4	464	42.6±2.9	12	65.7	506	46.8±4.8	10	66.0	481	43.9±3.6	0	5.48±0.50
Mannitol																					
0.25 M	37.5	347	12.7±0.9	15	51.2	418	28.2±1.3	10	61.7	456	39.4±2.4	4	63.4	481	43.3±3.3	2	64.0	476	43.2±5.4	2	5.06±0.38
0.50 M	38.7	351	13.9±0.8	26	52.7	414	29.0±1.4	12	62.7	456	39.9±1.8	5	64.4	485	44.3±3.9	4	64.8	481	44.1±5.4	0	5.10±0.25
0.75 M	39.9	355	15.0±1.5	36	55.3	426	31.9±1.1	24	64.5	468	42.5±2.8	12	65.2	489	45.4±4.4	6	65.5	476	43.9±4.0	0	5.18±0.29
1.00 M	41.0	355	15.9±1.4	44	55.0	426	31.5±1.8	22	64.8	464	42.8±2.7	10	66.6	493	46.3±5.3	9	66.0	481	44.3±4.8	1	5.31±0.25

[a] T_m is in °C, ΔH_m and $\Delta G_D°$ are in kJ mol^{-1}, and ΔC_p is in kJ mol^{-1} K^{-1}.

[b] From triplicate measurements values of maximum errors from the mean are 1% and 9% in T_m and ΔH_m, respectively.

[c] ΔH_m and T_m values of lysozyme are corrected for the effect of 2.0 M GdmCl at pH values 4.8, 6.0 and 7.0 (see text).

3.1. Thermal denaturation of lysozyme

Heat-induced denaturation curves of lysozyme in the absence and presence of 10%, 20%, 30% and 40% (w/v) sorbitol; 10%, 20%, 30% and 40% (v/v) glycerol; and 0.25, 0.50, 0.75 and 1.00 M each of mannitol, adonitol and xylitol were measured by following changes in $[\theta]_{222}$ as a function of temperature at five pH values (1.9, 2.4, 4.8, 6.0 and 7.0). Fig. 1 shows the representative thermal denaturation curves of lysozyme in the presence and absence of different concentrations of sorbitol, glycerol, mannitol, adonitol and xylitol. The entire ($[\theta]_{222}$, T) data of each transition curve were fitted to Eq. (2) with eight free parameters (a_N, b_N, c_N, a_D, b_D, c_D, ΔH_m and T_m). It has been observed that the temperature dependence of y_N is independent of the polyol type and its concentration at all pH values (e.g., see Fig. 1). This dependence is described by the relation, y_N, deg cm^2 dmol^{-1} = −12380 (±975) − 14.95 (±6.31)T + 0.0797 (±0.0102)T^2; each temperature-independent coefficient is the average of all measurements in the presence of different polyol concentrations and pH values, and a '±' is the average of the standard deviations. However, the temperature dependence of y_D is independent of the polyol type and its concentration only at a given pH. This temperature dependence of y_D at each pH is given in the legend of Fig. 1; each temperature-independent coefficient is equal to the average of all measurements in the presence of different polyols and their concentrations at a fixed pH, and a '±' is the average of the standard deviations.

Table 1 shows values of ΔH_m and T_m at each pH and polyol concentration; each thermodynamic parameter represents the mean of the triplicate measurements. It should, however, be noted that the thermal denaturation experiments for lysozyme carried out at pH values 4.8, 6.0 and 7.0 included 2.0 M GdmCl. The reason for adding this GdmCl concentration was to bring thermal denaturation curves in the measurable temperature range at these pH values. In order to correct for the effect of this concentration of GdmCl on ΔH_m and T_m values, we also measured thermal transition curves of lysozyme at three more concentrations of GdmCl, namely, 1.5, 3.0 and 3.5 M at pH values 4.8, 6.0 and 7.0 (results not shown). Analysis of these transition curves using Eq. (2) gave values of ΔH_m and T_m at a given [GdmCl], the molar concentration of GdmCl, and pH. At a given pH plots of ΔH_m versus [GdmCl] and T_m versus [GdmCl], which were found to be linear, were used to get values of ΔH_m and T_m in the absence of GdmCl, and these are the values that are given in Table 1. At a given polyol concentration values of ΔH_m and T_m obtained at different pH values were used to construct a plot of ΔH_m versus T_m. The linear least-squares analysis of such a plot gave ΔC_p

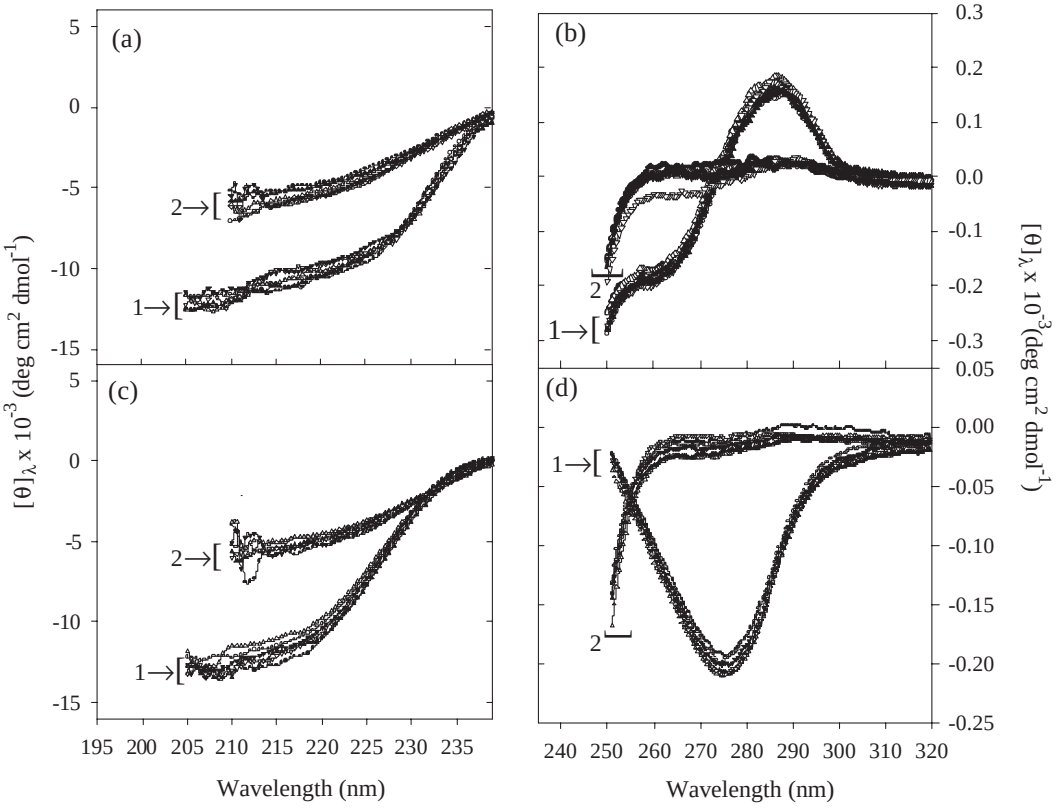

Fig. 2. Effect of polyols on the secondary and tertiary structures of the native and heat-denatured states of lysozyme and RNase-A. The far-UV (a) and near-UV (b) CD spectra of lysozyme in the native state at pH 7.0 and 25 °C (1) and heat-denatured state at pH 1.9 and 85 °C (2) in the absence (>) and presence of 40% sorbitol (⋆), 40% glycerol (Δ), 1.00 M mannitol (▲), 1.00 M adonitol (▽) and 1.00 M xylitol (▼). The far-UV (c) and near-UV (d) CD spectra of RNase-A in the native state at pH 7.0 and 25 °C (1) and heat-denatured state at pH 2.0 and 85 °C (2). Symbols have the same meaning as in (a).

(=($\delta\Delta H_m/\delta T_m)_p$). Values of ΔC_p with their standard deviations at each polyol concentration are also given in Table 1.

Values of $\Delta G_D°$ (ΔG_D value at 25 °C) of lysozyme in the presence and absence of various polyols at different pH values were determined using Eq. (3) with the corresponding values of T_m, ΔH_m and ΔC_p given in Table 1 (see Discussion). These values of $\Delta G_D°$ are shown in Table 1. This table also shows $\%\Delta\Delta G_D°$, the percent stabilisation of the protein by polyols. In order to have a feel of the procedure for estimating $\%\Delta\Delta G_D°$, we show a model calculation. $\%\Delta\Delta G_D°$ of lysozyme in the presence of 40%

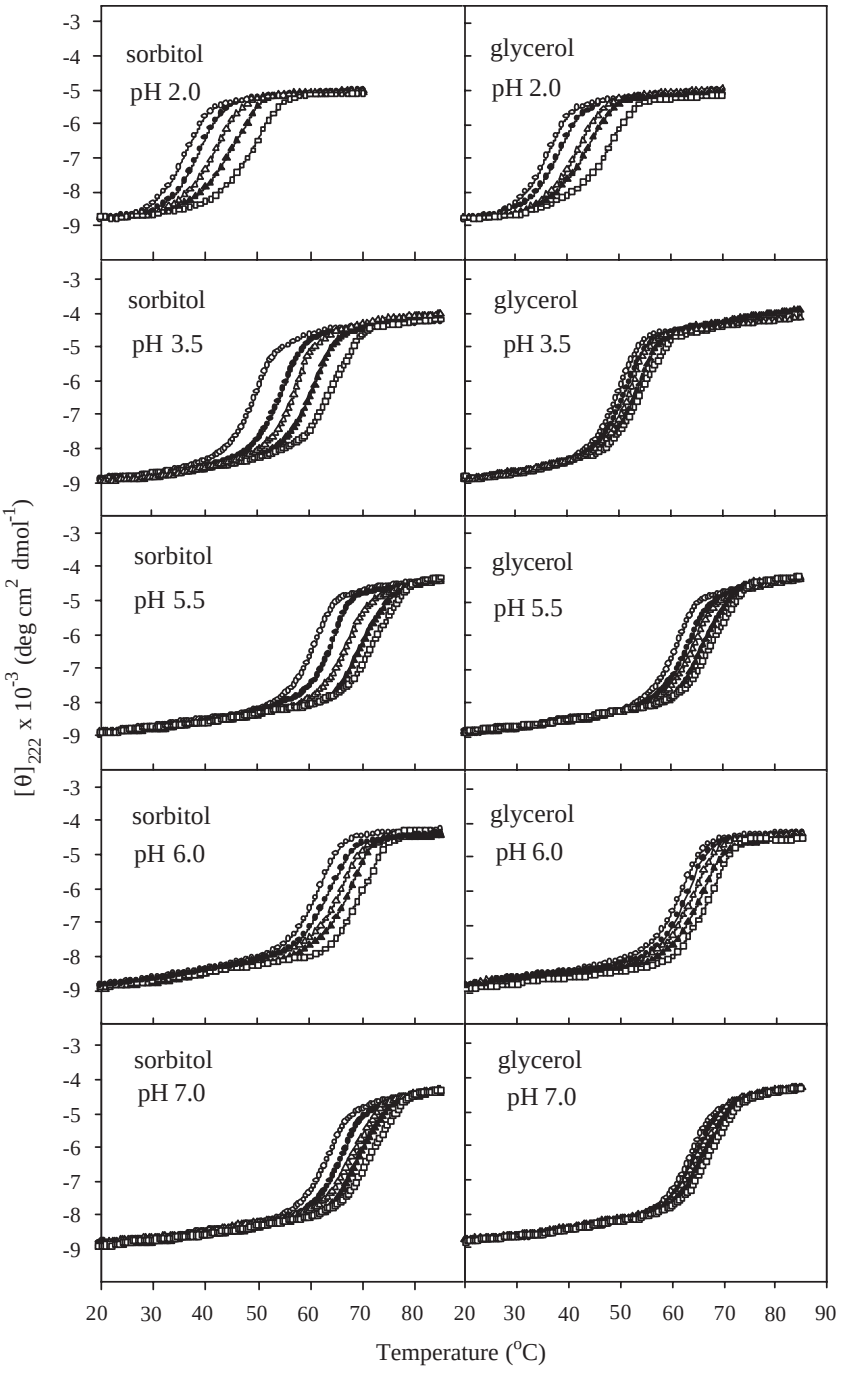

Fig. 3. Representative thermal denaturation profiles of RNase-A in the presence of different concentrations of polyols at different pH values. Denaturation curves in the absence (>) and presence of sorbitol (w/v) and glycerol (v/v) at concentrations 10 (⋆), 20 (Δ), 30 (▲) and 40 (□) % and different pH values indicated in the figure. Denaturation curves at pH 3.0 are not shown here. Temperature dependencies of y_D are: $y_D = -53640\ (\pm 4827) + 274\ (\pm 22)T - 0.3865\ (\pm 0.0347)T^2$ at pH 2.0; $y_D = -11980\ (\pm 895) + 26\ (\pm 2)T - 0.0153\ (\pm 0.0008)T^2$ at pH 3.5; $y_D = -541383\ (\pm 4622) + 274\ (\pm 30)T - 0.3750\ (\pm 0.0187)T^2$ at pH 3.5; $y_D = -73255\ (\pm 7577) + 416\ (\pm 41)T - 6243\ (\pm 0.0374)T^2$ at pH 5.5; $y_D = -108253\ (\pm 7577) + 556\ (\pm 39)T - 0.7432\ (\pm 0.0668)T^2$ at pH 6.0; and $y_D = -58649\ (\pm 5571) + 325\ (\pm 26)T - 0.4825\ (\pm 0.0241)T^2$ at pH 7.0.

sorbitol at pH 6.0 = 100 × [($\Delta G_D°$ in the presence of 40% sorbitol − $\Delta G_D°$ in the absence of polyol)/$\Delta G_D°$ in the absence of polyol)] = 100 × [(61.23 − 55.47)/55.47] = 10 (see Table 1).

3.2. Effect of polyols on N and D states of lysozyme

Fig. 2a and b show the far- and near-UV CD spectra of the native lysozyme in the absence and presence of the highest concentrations of all polyols at pH 7.0 and 25 °C (curves grouped as 1). It is seen in these figures that there is no significant change in the secondary and tertiary structures of the native protein on the addition of polyols. These figures also show the far- and near-UV CD spectra of the heat-denatured lysozyme in the absence and presence of highest concentration of all polyols at pH 1.9 and 85 °C (curves grouped as 2). These measurements suggest that there is no significant difference in the characteristic CD spectra of the heat-denatured protein in the absence and presence of polyols.

3.3. Thermal denaturation of RNase-A

Thermal transition curves of RNase-A in the absence and presence of 10%, 20%, 30% and 40% (w/v) sorbitol and 10%, 20%, 30% and 40% (v/v) glycerol and 0.25, 0.50, 0.75 and 1.00 M each of mannitol, adonitol and xylitol were measured at six pH values, namely, 2.0, 3.0, 3.5, 5.5, 6.0 and 7.0. Typical denaturation profiles of the protein are shown in Fig. 3. It has been observed that the temperature dependence of y_N is independent of polyol type and its concentration at all pH values. This dependence is described by the relation, y_N, deg cm^2 dmol^{-1} = −14930 (±1075) − 172.5 (±6.921)T + 0.321 (±0.011)T^2; each temperature-independent coefficient is equal to the average of all measurements in the presence of different polyol concentrations and pH values, and a '±' is the mean of the standard deviations. However, the temperature dependence of y_D is independent of the polyol type and its concentration only at a given pH value. This temperature dependence of y_D at each pH is given in the legend of Fig. 3; each temperature-independent coefficient is equal to the average of all measurements in the presence of different polyols and their concentrations at a fixed pH, and a '±' is the average of the standard deviations. As we did in the case of lysozyme, each transition curve of RNase-A at a fixed pH and polyol concentration was analysed for ΔH_m and T_m using Eq. (2). Table 1 shows values of ΔH_m and T_m at each pH and different polyol concentrations; each thermodynamic parameter represents the mean of all measurements at the fixed polyol concentration and pH. Value of ΔC_p at each polyol concentration was obtained from the plot of ΔH_m versus T_m using the linear least-squares analysis. These values of ΔC_p with their standard deviations are also given in Table 1. $\Delta G_D°$ values of RNase-A in the absence and presence of various osmolytes at different pH values were determined using Eq. (3) with the corresponding values of T_m, ΔH_m and ΔC_p given in Table 1 (see Discussion). These values of $\Delta G_D°$ are shown in Table 1. This table also shows $\%\Delta\Delta G_D°$ under different experimental conditions.

3.4. Effect of polyols on N and D states of RNase-A

Figs. 2c and d show the far- and near-UV CD spectra of the native RNase-A in the absence and presence of the highest concentration of each polyol used in this study at pH 7.0 and 25 °C (curves grouped as 1). It is seen in these figures that the secondary structure (Fig. 2c) and tertiary structure (Fig. 2d) of the native protein remain, within experimental errors, unchanged on the addition of polyols. These figures also show the far- and near-UV CD spectra of the heat-denatured protein in the absence and presence of the highest concentration of polyols at pH 2.0 and 85 °C (curves grouped as 2). These measurements suggest that there is no significant change in the characteristic CD spectra of the heat-denatured protein on the addition of polyols.

4. Discussion

In order to see whether the effect of a polyol on the protein stability in terms of $\Delta G_D°$ (Gibbs energy change at 25 °C) is different at different pH values, thermal denaturation curves of lysozyme and RNase-A were measured in the presence and absence of different concentrations of five polyols by following changes in $[\theta]_{222}$ at various pH values. In the analysis of the thermal transition curves according to Eq. (2), two assumptions were made. First, it has been assumed that the heat-induced denaturation of proteins in the absence and presence of polyols at different pH values follows a two-state mechanism. This is indeed true for these proteins in the absence of osmolytes [26]. Furthermore, a comparison of T_m and ΔH_m of lysozyme and RNase-A in the absence of polyols given in Table 1 with those obtained from DSC measurement [26] gave excellent agreement. This agreement led us to believe that our measurements of transition curves and their analyses for thermodynamic parameters are authentic and accurate. In order to check whether the two-state assumption is also valid in the presence of polyols, thermal denaturation curves were measured by observing changes in the difference absorption coefficients $\Delta\epsilon_{300}$ and $\Delta\epsilon_{287}$ of lysozyme and RNase-A, respectively, in the presence of 40% each of sorbitol and glycerol and 1.0 M each of mannitol, adonitol and xylitol (results not shown). We compared T_m and ΔH_m values obtained from the absorption measurements with the ones obtained from CD measurements. It has been observed that both measurements gave, within experimental errors, identical values of thermodynamic parameters. Thus, the assumption that thermal denaturation of RNase-A and lysozyme in the presence of polyol osmolytes is a two-state process seems to be valid.

Another assumption made in the analysis of the thermal transition curves of RNase-A and lysozyme is that the structural characteristics of the two end states of the thermal denaturation are not affected on the addition of polyols. In order to see whether this assumption is valid for the native proteins, the far- and near-UV CD spectra of lysozyme and RNase-A were measured in the absence and presence of 40% each of sorbitol and glycerol and 1.0 M each of mannitol, xylitol and adonitol at pH 7.0 and 25 °C (see Fig. 2). It is seen in Fig. 2 that both secondary and tertiary structures of the native proteins are, within experimental errors, identical in the absence and presence of all polyols. This observation is consistent with the recent X-ray diffraction results [27] and earlier CD data [28] showing that the native structure of a protein is not perturbed in the presence of osmolytes. It is interesting to note that osmolytes have been shown to have no effect on the dimensions of the native folded molecules either [29–31]. Thus, measurements of optical properties [28, this study], dimensional properties [29–31] and X-ray data [27] support our assumption that the native structure of a protein is the same in the presence and absence of polyols. In order to see whether the same is also true for the denatured states of RNase-A and lysozyme, i.e., polyols do not affect the structural characteristics of the denatured state of a protein, the far- and near-UV CD spectra of heat-denatured proteins in the presence and absence of all the five polyols at the lowest pH and 85 °C were measured. It has been observed that polyols do not affect the secondary and tertiary structures of the heat-denatured state of RNase-A and lysozyme (see Fig. 2). This observation is also in agreement with the earlier CD data on chymotrypsinogen [28]. Thus, our assumption that polyols do not affect the structural characteristics of the two end states of the process N state↔D state seems to be reasonable. Hence a comparison of a thermodynamic property of the protein in the presence and absence of polyols is valid.

It is seen in Table 1 that T_m of each protein increases with an increase in the concentration of each polyol at all pH values. It is also seen in this table that at a given osmolyte concentration, T_m of a protein in the presence of xylitol is, within experimental error, same as that in the presence of its epimer adonitol, and T_m in the presence of sorbitol is, within experimental error, same as in the presence of its epimer mannitol. Furthermore, it is seen in this table that the extent of stabilisation in terms of T_m of both proteins is pH-dependent, i.e., more at lower pH than at higher pH conditions. All these findings are in agreement with the observations reported earlier [11–14,32].

It is mentioned in Table 1 that the maximum standard deviation in the measurements of ΔH_m is 9%. It may be seen from the results given in this table that the change in ΔH_m in the presence of the highest concentration of each polyol is in the range 0–8% for RNase-A at all pH values, and it is in the range 0–11% for lysozyme at all pH values with the exception of glycerol and xylitol data at pH 1.9 where ΔH_m is increased by 14–15% on the addition of these polyols. Although there is a definite increasing trend of the change in ΔH_m with an increase in the polyol concentration, these results led us to conclude that ΔH_m of the protein does not depend significantly on the type and concentration of polyols. This conclusion is in agreement with earlier reports [11–13,28].

Our main aim is to determine $\Delta G_D°$ of lysozyme and RNase-A in the presence and absence of polyols at different pH values using Eq. (3). It is possible only when ΔC_p of each protein is known under these experimental conditions, for ΔH_m and T_m are known from the analysis of thermal denaturation curves (Table 1). It is seen in Table 1 that ΔC_p at a given pH cannot be estimated from the variation of ΔH_m with T_m as the polyol concentration was varied, for the change in ΔH_m in the presence of polyols is not significant. However, we have estimated ΔC_p of the protein using a procedure which involves (i) measurements of heat-induced denaturation of the protein in the presence of a fixed polyol concentration at different pH values and (ii) determination of ΔC_p $(=(\delta\Delta H_m/\Delta T_m)_p)$ from the linear plot of ΔH_m versus T_m [26]. Values of ΔC_p in the presence of different concentrations of polyols are given in Table 1. ΔC_p values of 5.06 and 6.77 kJ mol^{-1} K^{-1}, respectively, for RNase-A and lysozyme in the absence of polyols (see Table 1) are in excellent agreements with calorimetric ΔC_p values of 5.10 kJ mol^{-1} K^{-1} for RNase-A and 6.77 kJ mol^{-1} K^{-1} for lysozyme [26]. The molecular interpretation of the origin of ΔC_p of proteins is that it mainly reflects the interactions with solvent water of the polar and apolar groups, which are exposed upon protein denaturation [33]. Unfortunately, this viewpoint alone does not lead to an unambiguous interpretation of the observation that polyols increase ΔC_p of RNase-A, whereas they decrease the ΔC_p of lysozyme (see Table 1). This is so because the heat capacity changes associated with the exposure of apolar and polar parts of the protein have different sign in water, and these heat capacity changes might change in an unknown manner in the presence of osmolytes [33].

Following the procedure of Becktel and Schellman [34] we have determined $\Delta G_D°$ values of lysozyme and RNase-A in the presence of a given polyol concentration at different pH values using Eq. (3). It may be noted that the errors in the estimation of $\Delta G_D°$ given in Table 1 are due to errors in the measurements of ΔH_m and ΔC_p. A few comments are, therefore, necessary. (i) The values of $\Delta G_D°$ at a given polyol concentration was obtained using Eq. (3) with all best fitting parameters. For example, a value of 58.52 kJ mol^{-1} for $\Delta G_D°$ of lysozyme in the absence of polyol at pH 7.0 (see Table 1) is obtained using the best fitted parameters, ΔH_m (=564 kJ mol^{-1}), T_m (=86.5 °C) and ΔC_p (=6.77 kJ mol^{-1} K^{-1}). (ii) Following Becktel and Schellman [34], we have obtained upper and lower values of $\Delta G_D°$ of lysozyme using Eq. (3) with upper and lower limits of standard errors in the measurements of ΔH_m, which is ±9%, and ΔC_p, which is ±0.54 kJ mol^{-1} K^{-1}. For example,

the upper value of $\Delta G_D°$ (=66.63 kJ mol^{-1}) in the absence of polyol at pH 7.0 is obtained using ΔH_m (=564+9%)=615 kJ mol^{-1}, T_m=86.5 °C and ΔC_p (=6.77−0.54)=6.23 kJ mol^{-1} K^{-1}, and the lower value of $\Delta G_D°$ (=50.83 kJ mol^{-1}) is obtained using ΔH_m (=564−9%)=513 kJ mol^{-1}, T_m=86.5 °C and ΔC_p=(6.77+0.54)=7.31 kJ mol^{-1} K^{-1}. (iii) The error in each $\Delta G_D°$ value given in Table 1 represents deviations from the mean of the upper and the lower values of $\Delta G_D°$, which is ±7.80 kJ mol^{-1} of lysozyme in the absence of polyols (see Table 1). (iv) We did not consider the effect of the error in the determination of T_m on $\Delta G_D°$, for in our study it has insignificant effect on $\Delta G_D°$.

The polyol–water system has its effect on the denaturation equilibrium, N conformation↔D conformation through its preferential interactions with protein groups. Two types of interactions have been proposed. One is due to the repulsive forces between the polyol and the nonpolar regions located on the protein surface [32] and between the polyol and the peptide backbone [18]. This interaction is a source of stabilisation of proteins by polyols, for the preferential exclusion of the polyol from the protein domain (i.e., preferential hydration of the protein) shifts the denaturation equilibrium toward the N state [17,32]. Second is due to the penetration of the polyol into the solvation sheath of the protein [32], for the polyol is essentially hydrophilic as water is [32]. This interaction is a source of destabilisation of the protein by polyols, for this preferential binding shifts the denaturation equilibrium toward D conformation [17,32]. The observed effect of a polyol on $\Delta G_D°$ is through the delicate balance between these two opposing effects on the denaturation equilibrium. It is seen in Table 1 that $\Delta G_D°$ of lysozyme and RNase-A is unperturbed in the presence of all polyols at pH 7.0. The most probable explanation for this effect is that the two opposing effects are in perfect balance at this pH. It is also seen in Table 1 that stabilisation effect of each protein at a given polyol concentration increases with decreasing pH. This pH dependence of $\Delta G_D°$ of the protein is explained in the light of the report that different charge states of a protein affect its hydrophobicity [35]. Kuhn et al. [35] have argued that the protein hydrophobicity increases with a decrease in pH due to the protonation of COO^- groups, and hence the degree of exclusion (repulsion) of polyol from the protein domain (the stabilising effect) increases with increasing protonation of COO^- groups. Our results suggest that the stabilisation effect (repulsive forces between the polyol and the protein) not only increases with decreasing pH but it is also a dominating effect. Interestingly, it has been reported that the RNase-A and lysozyme are stabilised by trehalose and the stabilisation increases with decreasing pH [36].

The protein stabilisation in terms of $\Delta G_D°$ depends on the two factors namely, $\Delta H_D°$ (ΔH_D value at 25 °C) and $\Delta S_D°$ (ΔS_D, the denaturational entropy change at 25 °C). One can estimate enthalpy and entropy contributions to $\Delta G_D°$ in a given solvent condition using the values of T_m, ΔH_m and ΔC_p, given in Table 1, in appropriate thermodynamic relations. These are: $\Delta H_D° = \Delta H_m - \Delta C_p(T_m - 298.15)$ and $\Delta S_D° = (\Delta H_m/T_m) + \Delta C_p \ln(298.15/T_m)$. It has been observed that there is a perfect enthalpy–entropy compensation in cases of both proteins in the presence of all polyols at pH 7.0, i. e., $\Delta G_D°$ is unperturbed. However, the protein stabilisation by all polyols becomes enthalpy and entropy effects for lysozyme and RNase-A at pH values below pH 7.0, respectively. The finding that the driving force for polyol-induced stabilisation of lysozyme is an enthalpy effect is in agreement with the earlier report [37].

It is noteworthy that two other laboratories [11–13] also reported values of T_m, ΔH_m and $\Delta G_D°$ (value of ΔG_D at 25 °C) of RNase-A and lysozyme obtained from the measurements of thermal denaturation curves in the presence of all

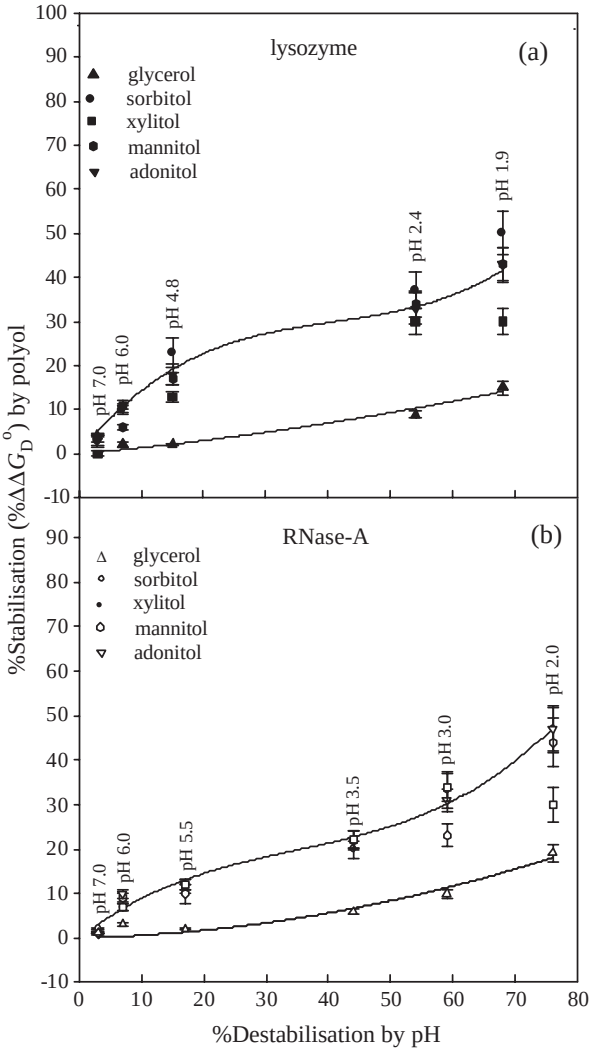

Fig. 4. Plots of %stabilisation (%$\Delta\Delta G_D°$) by polyol versus %destabilisation by pH of lysozyme (a) and RNase-A (b) in the presence of 1 M of each polyol. See text for the estimation of %polyol stabilisation (%$\Delta\Delta G_D°$). %pH destabilisation at any pH x with respect to pH 7.0 is equal to $100\times(\Delta G_D{}^{o(pH\ 7.0)} - \Delta G_D{}^{o(pH\ x)})/\Delta G_D{}^{o(pH\ 7.0)}$). A sample calculation for the estimation of %destabilisation of RNase-A in the absence of polyols on decreasing pH from 7.0 to 2.0 is given here. Thus, %destabilisation of RNase-A at pH 2.0=100×(43.97−11.03)/43.97=75. Results of RNase-A at pH 3.0 are also included.

polyols used in this study. Xie and Timasheff [11,12] reported the effect of only one osmolyte (sorbitol) at only one concentration (30%) on the thermal denaturation of RNase-A at two pH values in one report and at four pH values in another report. Kaushik and Bhat [13] reported the effect of various polyols at only one concentration on the thermal denaturation of several proteins at only three different pH values. A comparison of their results with those obtained here suggests several agreements but one disagreement. Agreements are that for a protein, (i) T_m increases with an increase in polyol concentration, (ii) the extent of stabilisation in terms of T_m by a polyol increases with a decrease in pH, and (iii) ΔH_m is not significantly perturbed in the presence of polyols. The disagreement is between $\Delta G_D°$ values observed here and those reported earlier [11–13]. Xie and Timasheff [11] have measured $\Delta G_D°$ values of RNase-A in the presence of different concentrations of sorbitol at pH 5.5 and 2.0. The apparent disagreement is due to the different procedures used to analyse denaturation curves. For example, Xie and Timasheff [11] have reported a value of 48.07 kJ mol^{-1} for $\Delta G_D°$ in the absence of polyol at pH 5.5, which has been obtained by analysing thermal denaturation curves assuming a value of zero for ΔC_p. Since ΔC_p of RNase-A is zero neither in the absence nor in the presence of sorbitol, we have corrected their $\Delta G_D°$ values using their ΔH_m (453±25 kJ mol^{-1}) and T_m (60.4±0.2 °C) values and a ΔC_p value of 5.06 kJ mol^{-1} K^{-1} [26] and found that $\Delta G_D°$ value is reduced from 48.07 to 38.20 kJ mol^{-1}, which is in excellent agreement with the value 37.99±2.34 kJ mol^{-1} observed in this study (see Table 1). It is noteworthy that Xie and Timasheff [12] have determined ΔG_D value also by measuring the chemical potentials of the native and denatured RNase-A in the presence of 30% sorbitol at pH 5.5 and 48 °C. They reported that the protein is stabilised by 10.53±2.09 kJ mol^{-1}. Using Eq. (3) with the values of ΔH_m, T_m and ΔC_p of RNase-A in 30% sorbitol at pH 5.5 given in Table 1, we obtained a value of 11.08±0.80 kJ mol^{-1} for ΔG_D at 48 °C, which is in excellent agreement with their value (ΔG_D=10.53±2.09 kJ mol^{-1}). Furthermore, Kaushik and Bhat [13] estimated ΔG_D values of both proteins in the presence of polyols at T_m of the control at each pH. They have observed that polyols stabilise proteins in terms of ΔG_D at T_m, which is supported by our observation. Since these authors have not estimated $\Delta G_D°$ (value of ΔG_D at 25 °C), our results at 25 °C could not be compared with theirs. It should, however, be noted that their ΔG_D of a protein in the presence of polyol estimated at the T_m of control will not be same as that expected at 25 °C, for ΔC_p of RNase-A and lysozyme in the absence and presence of polyol is not zero (see Table 1).

Fig. 4 summarizes the observation on the effect of polyol osmolytes on $\Delta G_D°$, the themodynamic stability of proteins at different pH values. Two important conclusions are derived from the results shown in this figure. (i) The stabilising effect of a polyol increases with an increase in the destabilisation of the native protein by lowering the pH from 7, i.e., with an increase in the protonation of the COO^- groups. (ii) At a given polyol concentration and pH the stabilising effect of glycerol on the protein is less than that of other polyols. The latter observation may be explained in the light of a finding that the stabilising effect increases with the length and number of OH groups of polyhydric alcohol [38].

In summary, we are sure of one thing that, contrary to trimethylamine N-oxide [19], polyol osmolytes can be used to stabilize less stable proteins at all pH values.

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