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Stability of proteins in the presence of polyols estimated from their guanidinium chloride-induced transition curves at different pH values and 25 °C

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Abstract

We have recently concluded from the heat-induced denaturation studies that polyols do not affect ΔG°_{D} (the Gibbs free energy change (ΔG_D) at 25 °C) of ribonuclease-A and lysozyme at physiological pH and temperature, and their stabilizing effect increases with decrease in pH. Since the estimation of ΔG°_{D} of proteins from heat-induced denaturation curves requires a large extrapolation, the reliability of this procedure for the estimation of ΔG°_{D} is always questionable, and so are conclusions drawn from such studies. This led us to measure ΔG°_{D} of ribonuclease-A and lysozyme using a more accurate method, i.e., from their isothermal (25 °C) guanidinium chloride (GdmCl)-induced denaturations. We show that our earlier conclusions drawn from heat-induced denaturation studies are correct. Since the extent of unfolding of heat- and GdmCl-induced denatured states of these proteins is not identical, the extent of stabilization of the proteins by polyols against heat and GdmCl denaturations may also differ. We report that in spite of the differences in the structural nature of the heat- and GdmCl-denatured states of each protein, the extent of stabilization by a polyol is same. We also report that the functional dependence of ΔG_D of proteins in the presence of polyols on denaturant concentration is linear through the full denaturant concentration range. Furthermore, polyols do not affect the secondary and tertiary structures of the native and GdmCl-denatured states.

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

1. Introduction

Osmolytes are small organic solutes accumulated at high concentrations by cells/tissues in response to osmotic stress [1,2]. Osmolytes increase stability of folded proteins and provide protection against denaturing stresses [3,4]. In addition to the ability of osmolytes to stabilize proteins, their effects on the functional activity of proteins are equally important, especially at high concentrations [5]. Those having no inhibitory or enhancing effect on the functional activity of enzymes are called compatible osmolytes [1,2]. The mechanism of osmolyte compatibility and osmolyte-induced stability has, therefore, attracted considerable attention in recent years [5]. We have been interested for some time in understanding the thermodynamic basis of stabilization of proteins by naturally occurring osmolytes [6–10]. The main conclusions of our studies are that compatible osmolytes do not perturb the protein denaturation equilibrium, N (native) state ↔ D (denatured) state near physiological pH (6.0–7.0) and temperature (25 °C), and they stabilize proteins at lower pH values by shifting the denaturation equilibrium toward the left. These conclusions were reached from the measurements of heat-induced denaturation curves in the presence and absence of compatible osmolytes and their analyses for ΔG°_{D}, the Gibbs energy change at 25 °C, associated with this denaturation equilibrium. It is obvious that the determination of ΔG°_{D} from the heat-induced denaturation curves

Abbreviations: ΔG_D, Gibbs free energy change; ΔG°_{D}, Gibbs free energy change in absence of denaturant at 25 °C; RNase-A, ribonuclease-A; C_m, midpoint of chemical denaturation; CD, circular dichroism.

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requires a long extrapolation of the data measured in the temperature range far away from 25 °C, and the extrapolation range increases in the presence of osmolytes. The ΔG_D° value thus obtained has a large error, which increases with an increase in T_m (the midpoint of thermal denaturation) caused by the presence of compatible osmolytes. For instance, if values of T_m (midpoint of denaturation), ΔH_m (enthalpy change at T_m) and ΔC_p (constant pressure–heat capacity change) are 86.5 °C, 564.0 ± 40 kJ mol^{-1} and 6.77 ± 0.54 kJ mol^{-1} K^{-1} [9], respectively, the ΔG_D° is estimated with an error of 17% (ΔG_D° = 58.62 ± 9.86 kJ mol^{-1}) and this error increases with the rise in T_m due to the presence of osmolyte. These considerations cast doubt on our earlier conclusions regarding the effect of polyols on ΔG_D° of proteins [9]. We thought it would be worthwhile to measure ΔG_D° of lysozyme and ribonuclease-A (RNase-A) using a more accurate method, i.e., from the guanidinium chloride (GdmCl)-induced denaturation at 25 °C and under the solvent conditions (polyols and pH values) used in thermal denaturation studies of these proteins [9].

Various conformational techniques have shown that heat-induced denatured state retains residual structures that can be cooperatively removed by the addition of GdmCl [11–13]. On the other hand, GdmCl gives a denatured state devoid of almost all noncovalent interactions present in the native state [14]. Evidence has been presented to show that osmolytes stabilize proteins due to its overwhelming unfavorable interaction with the protein peptide backbone [15]. Since the denatured state induced by GdmCl is more extended than that induced by heat [14], it is expected that the exclusion of osmolytes from the GdmCl-denatured state will be more than that from the heat-denatured state. In this communication we report results of measurements of ΔG_D° from the GdmCl-induced denaturation curves of RNase-A and lysozyme in the presence of different concentrations of various polyols and at different pH values used in thermal denaturation studies of these proteins [9]. These measurements led us to conclude (i) that our earlier conclusions regarding the effect of polyols on ΔG_D° from the heat-induced denaturation of proteins [9] are correct, (ii) that, interestingly, polyols have the same stabilizing effect on both the GdmCl- and heat-denatured states, and (iii) that the ΔG_D versus GdmCl concentration plot of proteins in the presence of osmolytes is linear throughout the denaturant concentration range.

2. Materials and methods

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

RNase-A and lysozyme solutions were dialyzed extensively against 0.1 M KCl at pH 7 in cold (~4 °C). Protein stock solutions were filtered using 0.45 m millipore filter paper. Both proteins gave a single band during polyacrylamide gel electrophoresis. Concentration of the protein solution was determined experimentally using ε, the molar absorption coefficient (M^{-1} cm^{-1}) values of 9800 at 277.5 nm for RNase-A [16] and 39,000 at 280 nm for lysozyme [17]. The concentrations of urea and GdmCl in buffer solutions were determined refractometrically using tabulated values of the solution refractive index [18]. 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). Since pH of the protein solution may change on the addition of cosolvents, pH of each solution was also measured after the denaturation experiments. 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 observed pH of protein solutions, i.e., only apparent pH is reported here.

GdmCl-induced denaturation of lysozyme and RNase-A in the presence and absence of polyols was followed by measuring changes in $[\theta]_{222}$ as a function of GdmCl concentration at 25 °C. It should be noted that each solution was kept for adequate time to allow equilibration for denaturation by GdmCl. Assuming a two-state model of denaturation, optical transition data were converted into ΔG_D°, the Gibbs energy change using the relation,

$$\Delta G_D = -RT\ln\{(y - y_N)/(y_D - y)\}, \quad (1)$$

where y is the observed optical property and y_N and y_D are, respectively, the properties of the native and denatured protein molecules under the same experimental condition in which y has been determined. ΔG_D ($-5.4 \le \Delta G_D$ (kJ mol^{-1}) ≤ 5.4) [14] was plotted against [d], the molar concentration of the denaturant, and a linear least-squares analysis was used to fit the (ΔG_D, [d]) data to the relation,

$$\Delta G_D = \Delta G_D^\circ - m_d[d], \quad (2)$$

where ΔG_D° is the value of ΔG_D at 0 M denaturant, and m_d gives the linear dependence of ΔG_D on [d].

Urea-induced denaturation of proteins was also measured. The denaturation curves were analyzed for ΔG_D° and m_d using Eqs. (1) and (2). GdmCl-induced transition curves of proteins were also obtained at several fixed urea and polyols concentrations, and ΔG_D values were determined using Eq. (1). ΔG_D values in the presence of GdmCl–urea mixture at a given polyol concentration, temperature and pH were assumed to be the sum of three mutually independent free energy changes [19],

$$\Delta G_D = \Delta G_D^\circ - m_g[\text{GdmCl}] - m_u[\text{urea}], \quad (3)$$

where ΔG_D° is the value of ΔG_D at zero molar concentrations of both GdmCl and urea, and m_g[GdmCl] and m_u[urea] are GdmCl and urea contributions to ΔG_D, respectively. It follows from this equation that the values of ΔG_D measured in the presence of the denaturant mixture can be corrected for the effect of urea on the GdmCl-induced equilibrium between N and D states. The corrected free energy change (ΔG_D^{cor}) is thus given by the relation,

$$\Delta G_D^{cor} = \Delta G_D + m_u[\text{urea}] = \Delta G_D^\circ - m_g[\text{GdmCl}]. \quad (4)$$

3. Results

All denaturation curves of lysozyme and RNase-A were measured three times. All GdmCl-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 osmolytes does not affect the structure of both

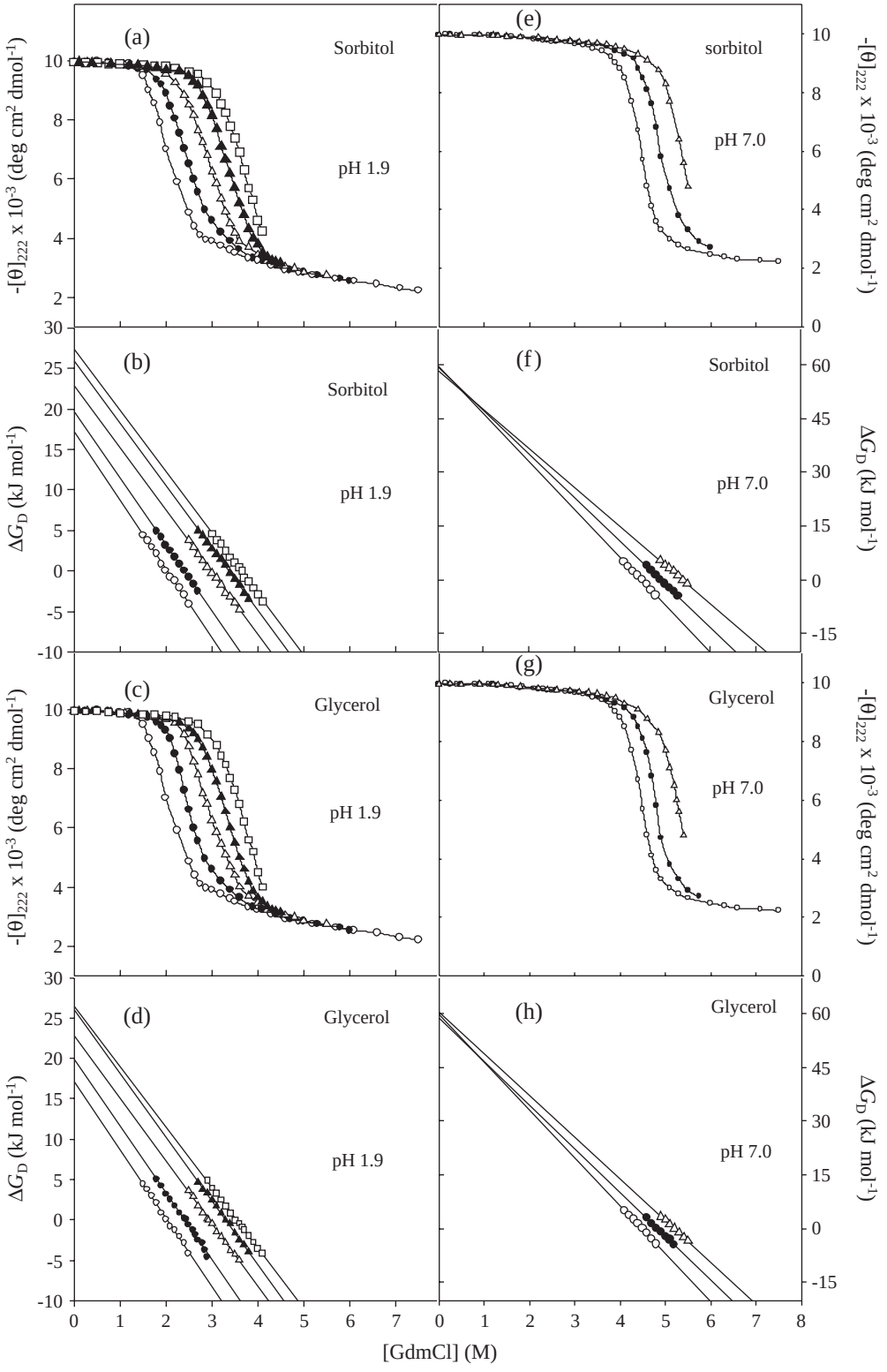

Fig. 1. Representative GdmCl-induced unfolding profiles and ΔG_D versus [GdmCl] plots of lysozyme in the presence of different concentrations of polyols at 25 °C. Unfolding curves (panels a, c, e and g) and ΔG_D versus [GdmCl] plots (panels b, d, f and h) in the presence of 0% (>), 10% (⋆), 20% (△), 30% (▲) and 40% (□) (w/v) sorbitol; and 0% (>), 10% (⋆), 20% (△), 30% (▲) and 40% (□) (v/v) glycerol at the indicated pH values.

the native and denatured states, all optical transition data were converted into thermodynamic parameters using appropriate relations.

GdmCl-induced denaturation curves of lysozyme and RNase-A in the absence and presence of 10%, 20%, 30% and 40% (w/v) sorbitol; 10%, 20%, 30% and 40% (v/v)

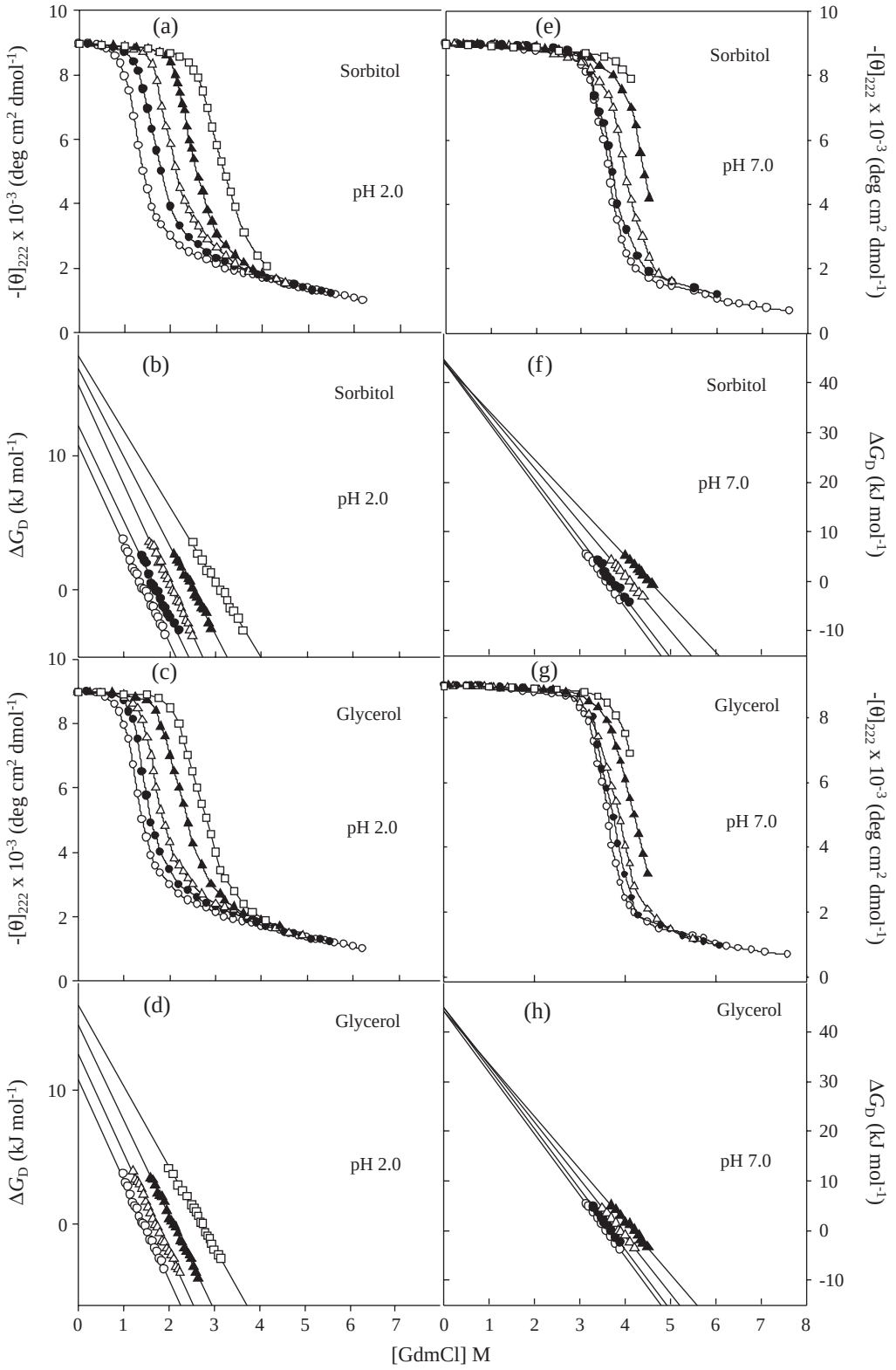

Fig. 2. Representative GdmCl-induced unfolding profiles and ΔG_D versus [GdmCl] plots for RNase-A in the presence of different concentrations of polyols at 25 °C. Unfolding curves (a, c, e and g) and ΔG_D versus [GdmCl] plots (panels b, d f and h) in the presence of 0% (>), 10% (⋆), 20% (△), 30% (▲) and 40% (□) (w/v) sorbitol; and 0% (>), 10% (⋆), 20% (△), 30% (▲) and 40% (□) (v/v) glycerol at the indicated pH values. In order to maintain clarity, the ΔG_D versus [GdmCl] plot in the presence of 10% glycerol at pH 2.0 is not shown.

Table 1
Parameters characterizing the GdmCl unfolding of lysozyme and RNase-A at different pH values and 25 °C [a,b,c]

	C_m	ΔG°_D	C_m	ΔG°_D	C_m	ΔG°_D	C_m	ΔG°_D	C_m	ΔG°_D
Lysozyme										
Concentration	pH 1.9		pH 2.4		pH 4.8		pH 6.0		pH 7.0[d]	
%, w/v[e]				Sorbitol						
0	2.0	17.14	2.6	27.08	3.9	49.82	4.3	56.01	4.5	59.43
	(2.0)	(17.10)							(4.6)	(58.23)
10	2.4	19.64	2.9	30.68	4.4	51.12	4.8	57.89	4.9	59.06
	(2.4)	(19.58)							(4.9)	(58.49)
20	2.9	22.57	3.3	35.53	4.7	52.25	5.3	57.22	5.4	58.94
30	3.4	25.99	3.7	39.08	5.2	53.21	–	–	–	–
40	3.7	27.17	3.9	40.17	–	–	–	–	–	–
%, v/v[f]				Glycerol						
10	2.5	19.85	2.8	28.97	4.3	50.95	4.7	58.26	4.8	58.52
	(2.5)	(19.75)							(4.7)	(59.15)
20	2.8	22.86	3.1	32.52	4.5	51.45	5.1	57.15	5.2	60.19
30	3.4	25.99	3.5	38.70	4.8	51.95	–	–	–	–
40	3.5	26.33	3.7	38.03	–	–	–	–	–	–
				Xylitol						
M										
0.25	2.2	17.97	2.9	28.84	4.1	50.79	4.4	56.64	4.7	59.31
0.50	2.5	19.23	3.0	30.51	4.4	51.41	4.6	57.48	5.1	59.80
	(2.5)	(19.45)							(5.0)	(60.04)
0.75	2.8	20.57	3.3	33.02	4.6	51.83	4.8	57.21	5.2	59.56
1.00	3.0	22.73	3.4	36.07	–	–	–	–	–	–
				Adonitol						
0.25	2.2	17.56	2.7	28.42	4.0	51.00	4.5	56.85	4.8	59.66
0.50	2.4	19.80	2.9	30.10	4.3	51.62	4.9	57.27	5.0	59.69
	(2.4)	(19.84)							(4.9)	(58.32)
0.75	2.9	20.92	3.4	33.52	4.5	52.04	5.0	57.35	5.2	60.26
1.00	3.2	23.20	3.5	35.53	–	–	–	–	–	–
				Mannitol						
0.25	2.1	17.76	2.8	28.84	4.1	50.75	4.5	56.43	4.8	59.68
0.50	2.4	19.65	2.9	30.51	4.4	51.33	5.8	57.06	4.9	59.36
	(2.4)	(19.59)							(4.9)	(57.55)
0.75	2.8	20.64	3.2	33.86	4.6	51.75	4.9	57.18	5.1	59.10
1.00	3.1	22.93	3.4	36.37	–	–	–	–	–	–
RNase-A										
	pH 2.0		pH 3.5		pH 5.5		pH 6.0		pH 7.0[d]	
%, w/v[e]				Sorbitol						
0	1.4	10.74	2.2	19.95	2.9	37.07	3.2	41.17	3.6	44.31
	(1.4)	(10.63)							(3.5)	(43.97)
10	1.7	12.20	2.5	22.18	3.3	38.28	3.5	41.75	3.7	44.60
	(1.7)	(12.48)							(3.7)	(42.27)
20	2.1	15.29	2.7	25.58	3.8	38.91	3.9	42.68	4.1	44.80
30	2.5	16.46	3.1	27.12	4.2	39.96	4.3	43.59	4.5	44.18
40	3.1	17.47	3.5	30.43	–	–	–	–	–	–
%, v/v[f]				Glycerol						
10	1.6	11.62	2.4	21.54	3.1	36.82	3.4	41.38	3.7	43.89
	(1.6)	(10.77)							(3.8)	(41.56)
20	1.8	12.66	2.7	25.70	3.6	37.53	3.7	42.17	3.8	44.60
30	2.3	14.79	2.8	26.95	3.9	37.74	4.0	42.46	4.2	44.14
40	2.8	16.05	3.2	27.78	4.3	38.87	–	–	–	–
				Xylitol						
M										
0.25	1.6	12.04	2.0	20.23	3.0	37.87	3.3	41.80	3.6	43.85
0.50	1.8	13.88	2.1	25.50	3.2	38.21	3.5	42.09	3.9	44.18
	(1.8)	(13.78)							(3.9)	(46.75)
0.75	2.0	14.59	2.6	28.29	3.4	38.46	3.6	42.68	4.0	44.34
1.00	2.2	15.17	2.7	29.18	3.6	38.58	3.8	42.72	4.2	44.60
				Adonitol						
0.25	1.7	12.46	2.0	20.57	3.0	36.78	3.2	41.72	3.7	43.51
0.50	1.8	14.30	2.3	24.87	3.3	38.12	3.6	42.34	3.8	44.64
	(1.8)	(14.54)							(3.9)	(43.08)
0.75	1.9	14.92	2.5	27.29	3.6	38.67	3.7	42.64	4.0	44.48
1.00	2.2	15.47	2.6	28.68	3.7	39.67	3.7	42.76	4.3	44.94
				Mannitol						
0.25	1.6	12.21	2.0	21.34	3.0	37.87	3.3	41.42	3.7	43.93
0.50	1.7	12.87	2.2	25.12	3.3	38.21	3.5	42.22	3.8	44.06
	(1.7)	(12.79)							(3.8)	(42.03)
0.75	1.9	15.01	2.5	26.44	3.6	38.54	3.6	42.68	3.9	44.48
1.00	2.2	15.80	2.6	27.89	3.7	39.21	3.8	42.76	4.1	44.39

[a]Dimensions of C_m and ΔG°_D are M and kJ mol^{-1}, respectively. [b]Values of thermodynamic parameters given in parentheses are obtained from unfolding measurements using $\Delta\varepsilon_{300}$ and $\Delta\varepsilon_{287}$ as probes for lysozyme and RNase-A, respectively. [c]From triplicate measurements values of maximum errors from the mean are 2% and 10% in C_m and ΔG°_D, respectively. [d]Data at pH 7.0 are taken from [10]. [e]10%, 20%, 30%, and 40% (w/v) of sorbitol are equivalent to 0.55, 1.10, 1.65 and 2.20 M, respectively. [f]10%, 20%, 30% and 40% (v/v) of glycerol are equivalent to 1.37, 2.74, 4.10 and 5.48 M, respectively.

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 the denaturant concentration at pH values in the range 7.0–1.9 and 25 °C. Figs. 1 and 2 (panels a, c, e, g) show, respectively, the representative GdmCl-induced denaturation curves of lysozyme and RNase-A in the presence and absence of different concentrations of sorbitol and glycerol at two extreme pH values. It has been observed that for each protein the dependencies of y_N and y_D on [GdmCl] are independent of the polyol type and its concentration. These dependencies for lysozyme and RNase-A at all pH values are given by Eqs. (5) and (6) and Eqs. (7) and (8), respectively.

$$y_N, \text{deg cm}^2\,\text{dmol}^{-1} = 168(\pm 25)\,[\text{GdmCl}] - 10062(\pm 48) \quad (5)$$

$$y_D, \text{deg cm}^2\,\text{dmol}^{-1} = 262(\pm 32)\,[\text{GdmCl}] - 4160(\pm 194) \quad (6)$$

$$y_N, \text{deg cm}^2\ \text{dmol}^{-1} = 101(\pm 4)\ [\text{GdmCl}] - 8962(\pm 2) \quad (7)$$

$$y_D, \text{deg cm}^2\ \text{dmol}^{-1} = 352(\pm 3)\ [\text{GdmCl}] - 3187(\pm 15) \quad (8)$$

These observations were used to estimate values of ΔG_D in the range $-5.4 \le \Delta G_D$ (kJ mol^{-1}) ≤ 5.4 associated with each GdmCl-induced denaturation curve using Eq. (2), which are plotted against [GdmCl] in panels b, d, f, h of Figs. 1 and 2. It should be noted that either denaturation curves could not be induced or they could be induced partially in the presence of some concentrations of polyols because of the experimental constraints. However, the partially observed transition curves were analysed using the same dependence of y_D on [GdmCl] given above. Each plot of ΔG_D versus [GdmCl] was analysed for ΔG°_D and m_d using Eq. (2). Table 1 shows the values of ΔG°_D and C_m, the midpoint of denaturation ($=\Delta G^\circ_D/m_d$) in the presence and absence of different concentrations of polyols.

We have also measured GdmCl-induced denaturation curves of lysozyme and RNase-A at two extreme pH values in the

absence and presence of 10% each of sorbitol and glycerol and 0.5 M each of xylitol, adonitol and mannitol by following changes in $\Delta\varepsilon$, the difference molar absorption coefficient ($\Delta\varepsilon_{300}$ for lysozyme and $\Delta\varepsilon_{287}$ for RNase-A) as a function of the denaturant concentration at 25 °C (transition curves not shown). Each transition curve, which was measured three times, was analyzed for ΔG°_{D} and C_m using the procedure described above (results shown in parentheses, Table 1).

In order to investigate whether the linear extrapolation is true in the presence of polyols in the full concentration of [GdmCl], we have carried out GdmCl-induced denaturation of both lysozyme and RNase-A at fixed concentrations of urea (1.00, 2.75 and 3.30 M) in the presence of 20% sorbitol, 20% glycerol and 0.50 M xylitol; we did not measure transition curves in the presence of epimers of sorbitol (i.e., mannitol) and xylitol (i.e., adonitol). The pH chosen were 1.9 and 3.0 for lysozyme and RNase-A, respectively. The denaturation profiles are shown in Figs. 3 and 4 (left panels). It was observed that y_N does not depend on the type and concentration of the denaturants while y_D was found to depend slightly on the denaturant type and concentration. The observed dependencies were used to analyze the denaturation curves, and values of ΔG_D were calculated as a function of [GdmCl] at each urea concentration (see insets in Figs. 3 and 4). The values of ΔG_D were corrected for the effect of urea on the GdmCl-induced equilibrium between N and D states using Eq. (3). All corrected ΔG_D (ΔG_D^{cor}) values, are mapped onto curve 1 in Figs. 3 and 4 (right panels).

The far- and near-UV CD spectra of the native lysozyme and RNase-A in the absence and presence of all polyols have been measured at different pH values and 25 °C. As reported earlier [9,10], there is no significant change in the secondary

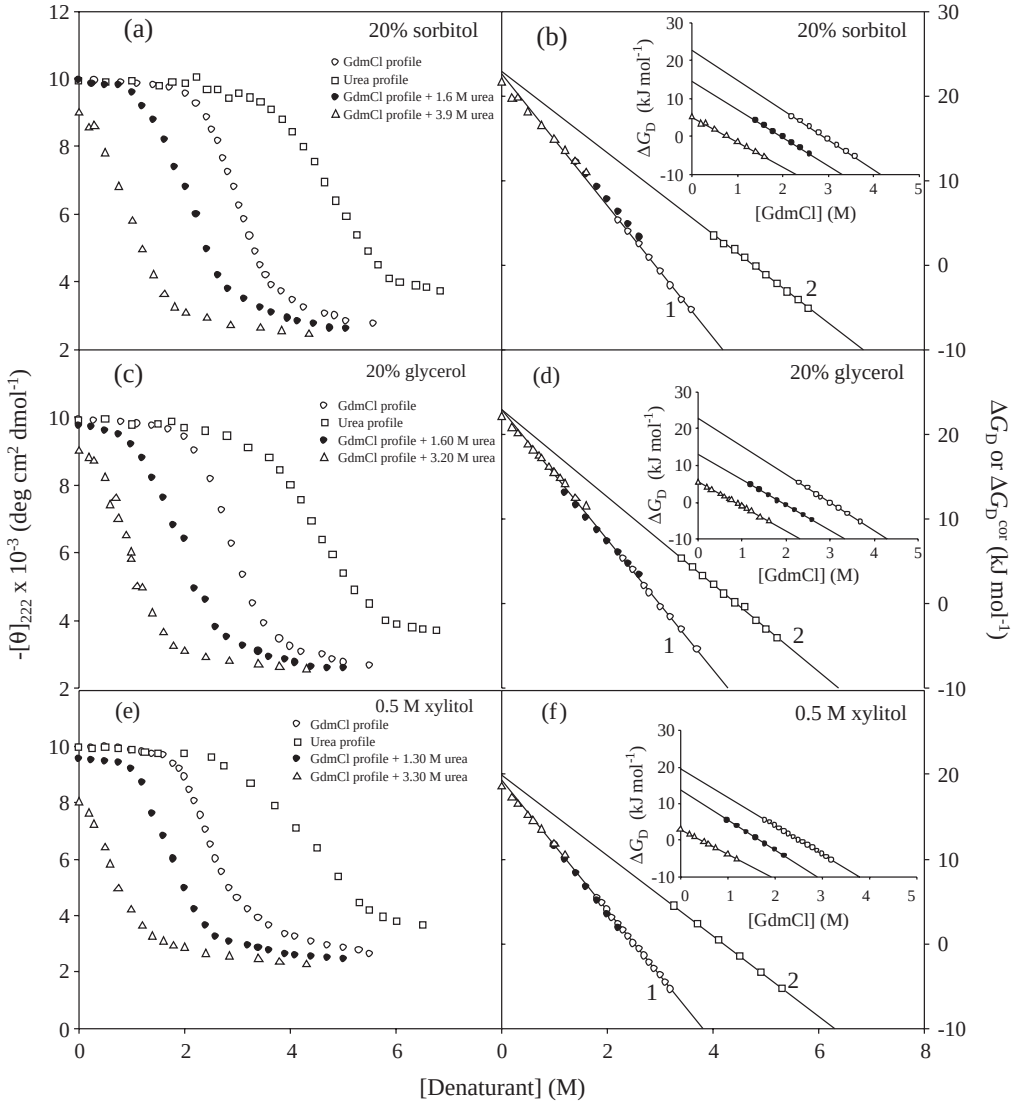

Fig. 3. Denaturation of lysozyme under different solvent conditions at pH 1.9 and 25 °C. Panels a, c and e: urea profile, urea denaturation curve in the presence of polyols at the indicated concentrations; GdmCl profile, GdmCl denaturation curve in the presence of polyols at the indicated concentrations; and GdmCl profile+x M urea (where x is [u]), GdmCl denaturation curve in the presence of polyol and urea at the indicated concentrations. Panels b, d and f: curves 1 and 2 drawn using Eq. (2) with ΔG°_{D} and m_d values given for urea and GdmCl in Table 3, respectively. Insets in panels b, d and f show the ΔG°_{D} versus [GdmCl] plots in the presence of different concentrations of urea at the indicated polyol concentration. Each symbol in panels b, d and f has the same meaning as in a, c and e, respectively.

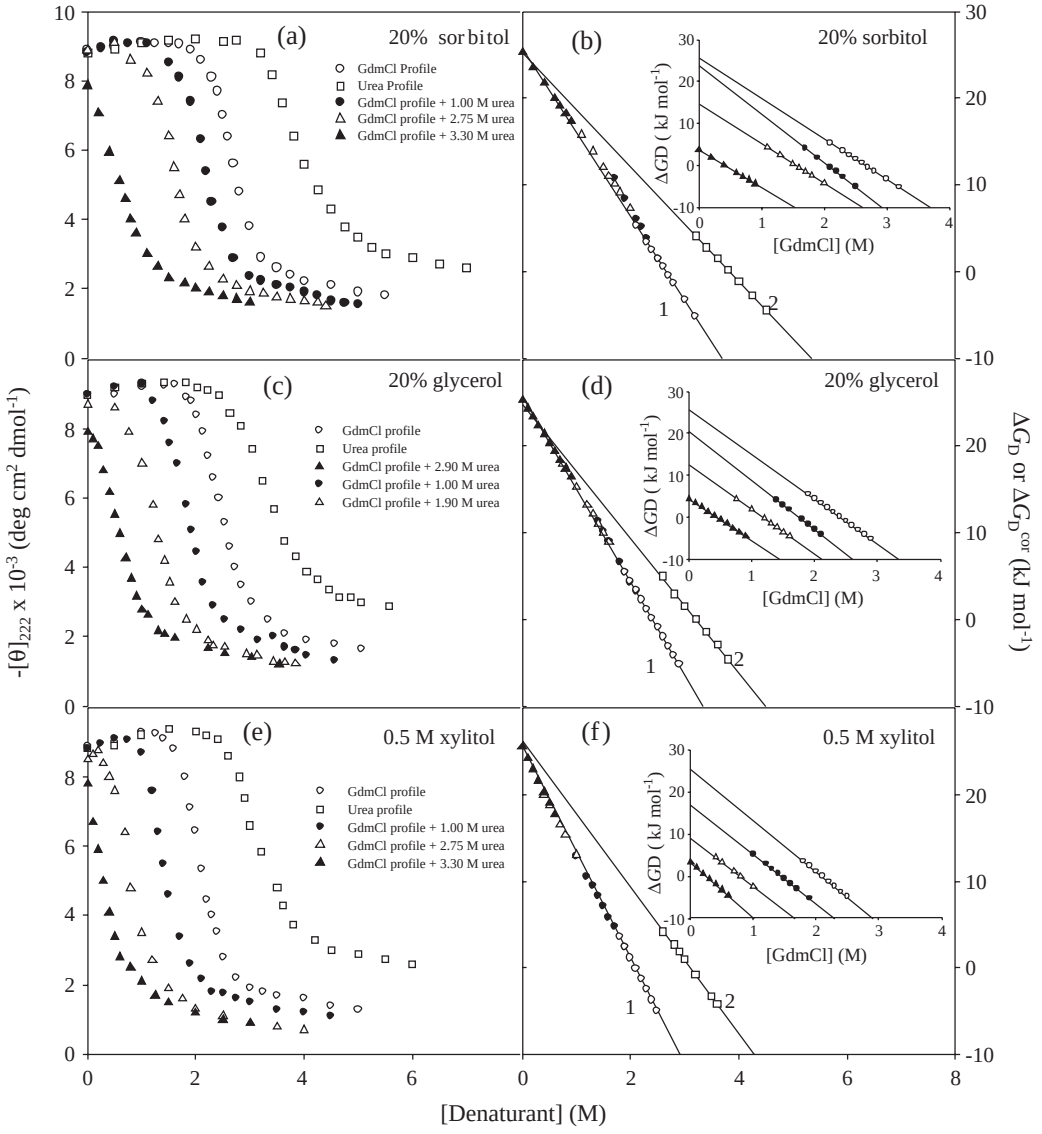

Fig. 4. Denaturation of RNase-A under different solvent conditions at pH 3.5 and 25 °C. Panels a, c and e: urea profile, urea denaturation curve in the presence of polyols at the indicated concentrations; GdmCl profile, GdmCl denaturation curve in the presence of polyols at the indicated concentrations; and GdmCl profile+x M urea (where x is [u]), GdmCl denaturation curve in the presence of polyol and urea at the indicated concentrations. Panels b, d and f: curves 1 and 2 drawn using Eq. (2) with ΔG°_{D} and m_d values given for urea and GdmCl in Table 3, respectively. Insets in panels b, d and f show the ΔG°_{D} versus [GdmCl] plots in the presence of different concentrations of urea at the indicated polyol concentration. Each symbol in panels b, d and f has the same meaning as in a, c and e, respectively.

and tertiary structures of the native protein on the addition of polyols (curves grouped as 1 in Fig. 5). These figures also show the far- and near-UV CD spectra of the GdmCl-denatured lysozyme and RNase-A in the presence of polyols at different pH values and 25 °C (curves grouped as 2). It should, however, be noted that the far- and near-UV CD spectra of both proteins in the presence of polyols at pH 7.0 are in excellent agreement with those reported earlier [10].

4. Discussion

Our earlier study of the determination of ΔG°_{D} of RNase-A and lysozyme from the heat-induced denaturation curves led us to conclude that polyols do not stabilize proteins at physiological pH, and their stabilizing effect increases with decreasing pH [9]. It was also concluded that proteins in the presence of polyols are stabilized in terms of T_m at all pH values [9]. As mentioned above, determination of ΔG°_{D} from the thermal denaturation studies involves a long extrapolation, which introduces large error in ΔG°_{D}, and this error increases with an increase in T_m. In order to confirm our earlier observations from the thermal denaturation studies [9], we carried out the isothermal (25 °C) GdmCl-induced denaturation of lysozyme and RNase-A 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 these transition curves according to Eq. (2), three assumptions were made. First, it has been assumed that the GdmCl-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

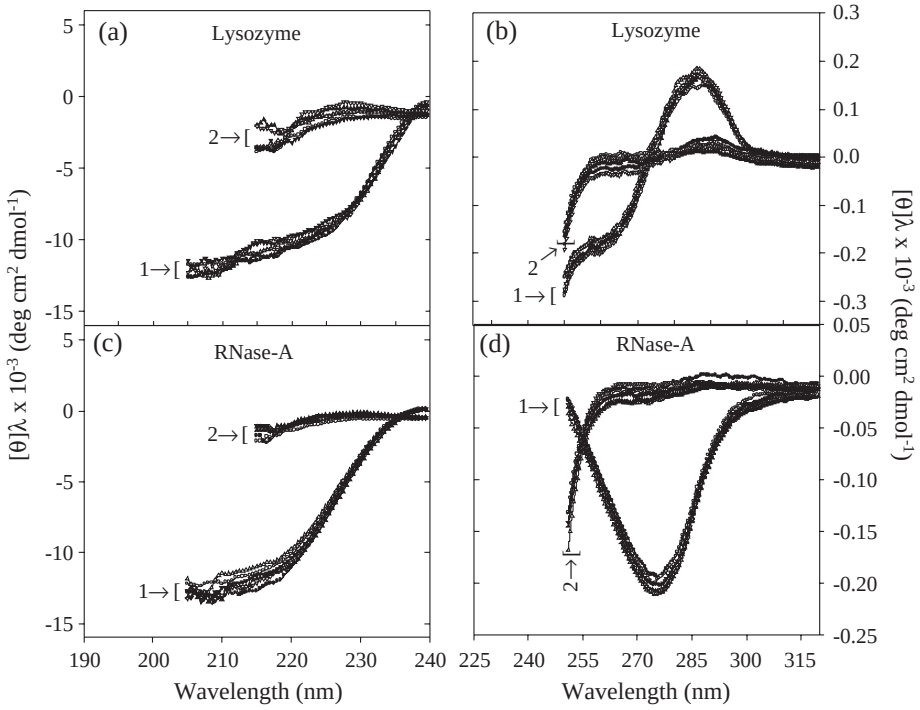

Fig. 5. Effect of polyols on the secondary and tertiary structures of the native and GdmCl-denatured states of lysozyme (pH 7.0–1.9) and RNase-A (pH 7.0–2.0) at 25 °C. The far-UV (a) and near-UV (b) CD spectra of the native state (1) and 6.0 M GdmCl-denatured state (2) of lysozyme in the absence (>) and presence of 10% each of sorbitol (⋆) and glycerol (△); and 0.50 M each of mannitol (▲), adonitol (▽) and xylitol (▼). The far-UV (c) and near-UV (d) CD spectra of the native and 6.0 M GdmCl-denatured state of RNase-A. Curve numbers and symbols have the same meaning as in (a).

proteins in the absence of osmolytes [20,21]. In order to check whether a two-state assumption is also valid in the presence of polyols, GdmCl-induced denaturation curves at extreme pH values were also measured by observing changes in the difference absorption coefficients $\Delta\varepsilon_{300}$ and $\Delta\varepsilon_{287}$ of lysozyme and RNase-A, respectively, in the presence of 10% each of sorbitol and glycerol and 0.5 M each of mannitol, adonitol and xylitol (transition curves not shown). These transition curves were analysed for ΔG°_{D} and C_m values. We compared ΔG°_{D} and C_m values obtained from the absorption measurements with those obtained from CD measurements. It has been observed that both measurements gave, within experimental errors, identical values of thermodynamic parameters (see Table 1). Thus, the assumption that GdmCl-induced 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 GdmCl-induced transition curves of RNase-A and lysozyme is that the structural characteristics of the two end states of the 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. 5). It is seen in Fig. 5 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 showing that the native structure of a protein is not perturbed in the presence of osmolytes [22]. It is interesting to note that osmolytes have been shown to have no effect on the dimensions of the native folded molecules either [4,23,24]. Thus, measurements of optical properties (this study), dimensional properties [4,23,24] and X-ray data [22] 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 GdmCl-induced denatured proteins in the presence and absence of all the five polyols at the lowest pH were measured. It has been observed that polyols do not affect the secondary and tertiary structures of the GdmCl-induced denatured state of RNase-A and lysozyme (see Fig. 5). 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.

The third assumption used in the analysis of the GdmCl-induced denaturation curves of lysozyme and RNase-A is that the dependence of ΔG_D on [GdmCl] is linear under all experimental conditions. In fact, we have shown earlier that ΔG_D of both proteins in the absence of polyols varies linearly with [GdmCl] and [urea] throughout the denaturant concentration range [19,25]. In order to determine the exact functional relationship between ΔG_D and [GdmCl] in the presence of polyols in the full denaturant concentration range, we have measured urea-induced denaturation (□) and GdmCl-induced denaturations in the presence of different concentrations of urea

Table 2
Thermodynamic parameters of GdmCl-induced transition of lysozyme (pH 1.9) and RNase-A (pH 3.0) in the presence of polyol–urea mixtures at 25 °C

Polyol concentration	[u]	$\Delta G_D^{\circ,[u]}$	$m_g^{[u]}$	$C_m^{[u]}$
	M	(kJ mol^{-1})	(kJ mol^{-1} M^{-1})	(M)
Lysozyme				
20% (w/v) sorbitol	1.60	14.63±0.79	7.44±0.42	2.0
	3.90	5.02±0.38	6.56±0.33	0.8
20% (v/v) glycerol	1.60	12.96±0.75	6.90±0.38	1.9
	3.20	5.48±0.54	6.69±0.33	0.8
0.50 M xylitol	1.40	13.59±0.96	8.15±0.54	1.7
	3.30	2.88±0.25	6.73±0.33	0.4
RNase-A				
20% (w/v) sorbitol	1.00	23.62±1.59	11.45±0.71	2.1
	2.75	14.67±0.79	9.45±0.92	1.6
	3.30	3.66±0.54	8.86±0.59	0.4
20% (v/v) glycerol	1.00	20.31±1.21	11.62±0.63	1.8
	1.90	12.41±0.71	10.62±1.04	1.2
	2.90	4.39±1.09	9.95±0.79	0.4
0.50 M xylitol	0.75	17.01±1.23	11.75±0.71	1.5
	1.90	9.20±1.00	11.54±0.38	0.8
	2.60	3.30±0.25	13.08±1.25	0.3

Superscript [u] represents the urea concentration at which parameters were determined.

(>, ⋆, Δ) at 25 °C and pH 1.9 for lysozyme and pH 3.0 for RNase-A (see Figs. 3 and 4). From these measurements, values of ΔG_D were estimated using Eq. (1). Plots of ΔG_D versus [urea] (curve 1) and ΔG_D versus [GdmCl] (curve 2) are shown in Figs. 3 and 4. Insets in these figures show the [GdmCl]-dependence of ΔG_D in the presence of urea, where it is seen that each ΔG_D versus [GdmCl] plot at each fixed [urea] is linear, and a least-squares analysis according to Eq. (2) of these plots yielded values of thermodynamic parameters, which are given in Table 2. Each ΔG_D value of a protein in the urea–polyol mixture was corrected for the effect of urea on the GdmCl-induced denaturation using Eq. (3) with the appropriate value of m_u [u] given in Table 3. Values of ΔG_D^{cor}, thus obtained are mapped on the ΔG_D versus [GdmCl] plot (see curve 1 in Figs. 3 and 4). It is seen in these figures that all ΔG_D^{cor} values fall on the same line (curve 1) drawn using (ΔG_D, [GdmCl]) data with the parameters given in Table 3. Thus, our assumption that functional dependence of ΔG_D on [GdmCl] is linear for both proteins in the presence of polyols is valid. Support to this linear dependence of ΔG_D on [GdmCl] comes also from observations reported earlier that there is neither direct interaction between polyols and protein [26,27] nor any interaction between denaturants and polyols [28].

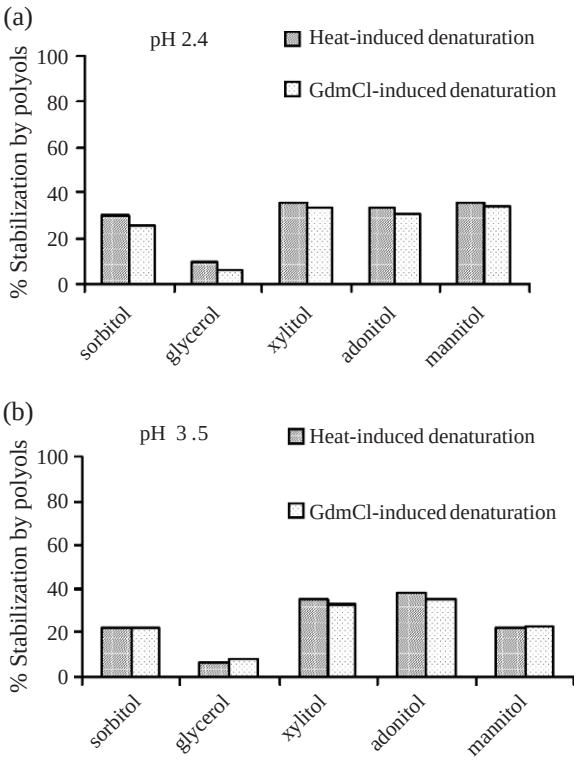

Fig. 6. Representative bardiagrams for the extent of stabilization of proteins by polyols. Bardiagrams of % stabilization of lysozyme (a) and RNase-A (b) by 1 M of each polyol. For the estimation of % stabilization see Ref. [9].

Values of ΔG_D° and C_m obtained from the analysis of the GdmCl-induced transition curves of lysozyme and RNase-A in the presence of different concentrations of polyols are given in Table 1. These values in the absence of the polyol are in excellent agreements with those reported earlier [20,21]. It is seen in Table 1 that ΔG_D° of lysozyme and RNase-A in the presence of all polyols is unperturbed at pH 7.0, and it increases with decreasing pH. Thus our earlier conclusions from the measurements of heat-induced denaturation curves of lysozyme and RNase-A in the presence of polyols are correct. That is ΔG_D° of protein is not significantly perturbed by polyol osmolytes near neutral pH, and ΔG_D° values of proteins are

Table 3
Parameters characterizing the GdmCl and urea unfolding of lysozyme (pH 1.9) and RNase-A (pH 3.0) in the presence of different polyols at 25 °C

Polyol concentration	GdmHCl			Urea		
	ΔG_D° (kJ mol^{-1})	m (kJ mol^{-1} M^{-1})	C_m (M)	ΔG_D° (kJ mol^{-1})	m (kJ mol^{-1} M^{-1})	C_m (M)
Lysozyme						
20% (w/v) sorbitol	22.57±1.59	7.78±0.46	2.9	23.70±1.84	4.97±0.17	4.8
20% (v/v) glycerol	22.86±1.59	8.16±0.29	2.8	22.99±1.63	5.18±0.21	4.4
0.50 M xylitol	19.23±1.38	7.69±0.33	2.5	19.90±1.38	4.72±0.17	4.2
RNase-A						
20% (w/v) sorbitol	25.58±1.25	9.66±0.04	2.7	25.16±1.84	6.56±0.75	3.8
20% (v/v) glycerol	25.70±0.67	10.66±0.46	2.4	23.16±0.38	7.23±0.84	3.2
0.50 M xylitol	25.50±1.00	12.21±0.04	2.1	26.63±1.51	8.53±1.00	3.1

increase with a decrease in pH [9]. It is noteworthy that Cioni et al. [29] have examined the influence of polyols on the stability of wild type metal free azurin and its various mutants. They observed that the effect of polyols on the protein stability is more for the destabilised mutants than that on the wild type. This observation is in agreement with our conclusion that stabilizing effect of polyols increases with extent of destabilization of the protein by a decrease in pH (see Table 1, [9]).

Furthermore, we have also estimated % stabilization of lysozyme and RNase-A by polyols at a given pH using the procedure described earlier [9]. Fig. 6 shows the representative bar diagrams for the extent of stabilization of proteins by 1 M each of polyol against GdmCl-induced and heat-induced denaturations. It is seen in this figure that the extent of stabilization of proteins by polyols against both modes of denaturation is almost the same. It has been reported that when a protein is present in the polyol solution, two types of interactions are observed [24], namely the osmophobic effect (i.e., the unfavourable interaction between peptide units and polyol) and osmophilic effect (i.e., the favourable interaction between side chains and polyol). Both of these effects will be larger in GdmCl-denatured protein than that in the heat-denatured protein, for GdmCl-denatured state has all the protein groups (peptide units and side chains) exposed to solvent, whereas the heat-denatured state retains residual structure (i.e., some peptide units and side chains are exposed to solvent) [13]. In the light of these findings [14,24], the observed effect of polyols on GdmCl and heat denaturations shown in Fig. 6 seems to be an unexpected observation. One most possible explanation for this unexpected observation is that in the case of GdmCl-denatured state the osmophobic effect is reduced by the osmophilic effect to an extent that the observed stabilizing effect of polyols on GdmCl denaturation becomes almost the same as that on the heat denaturation.

In summary, polyols do not affect the equilibrium between N and D states near physiological pH and room temperature, but they stabilize proteins by shifting the denaturation equilibrium toward the native state at lower pH values.

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