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Temperature dependence of the preferential interactions of ribonuclease A in aqueous co-solvent systems: Thermodynamic analysis

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Abstract

The temperature dependence of preferential solvent interactions with ribonuclease A in aqueous solutions of 30% sorbitol, 0.6 M $MgCl_2$, and 0.6 M $MgSO_4$ at low pH (1.5 and 2.0) and high pH (5.5) has been investigated. This protein was stabilized by all three co-solvents, more so at low pH than high pH (except 0.6 M $MgCl_2$ at pH 5.5). The preferential hydration of protein in all three co-solvents was high at temperatures below 30 °C and decreased with a further increase in temperature (for 0.6 M $MgCl_2$ at pH 5.5, this was not significant), indicating a greater thermodynamic instability at low temperature than at high temperature. The preferential hydration of denatured protein (low pH, high temperature) was always greater than that of native protein (high pH, high temperature). In 30% sorbitol, the interaction passed to preferential binding at 45% for native ribonuclease A and at 55 °C for the denatured protein. Availability of the temperature dependence of the variation with sorbitol concentration of the chemical potential of the protein, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, permitted calculation of the corresponding enthalpy and entropy parameters. Combination with available data on sorbitol concentration dependence of this interaction parameter gave (approximate) values of the transfer enthalpy, $\Delta\bar{H}_{2,tr}$, and transfer entropy $\Delta\bar{S}_{2,tr}$. Transfer of ribonuclease A from water into 30% sorbitol is characterized by positive values of the transfer free energy, transfer enthalpy, transfer entropy, and transfer heat capacity. On denaturation, the transfer enthalpy becomes more positive. This increment, however, is small relative to both the enthalpy of unfolding in water and to the transfer enthalpy of the native protein from water to a 30% sorbitol solution.

Keywords: preferential binding; protein–solvent interactions; protein stabilization by co-solvents; transfer enthalpy; transfer free energy

In the companion paper (Xie & Timasheff, 1997), protein (RNase A) stabilization by sorbitol in aqueous solution was examined in concerted manner by thermal denaturation and measurement of preferential interactions at conditions in which the protein (RNase A) was either native or unfolded at identical temperatures. It was established that at the limited condition of 48 °C, RNase A was in the native state at pH 5.5, but was denatured at pH 2.0. At 48 °C sorbitol was preferentially excluded from the denatured protein, but preferentially bound to the native one. The higher preferential hydration of the denatured protein than that of the native one is the thermodynamic source of the stabilization.

It is generally accepted that knowledge of the temperature dependence of the partial specific volume of proteins should provide important information on solvent–protein interactions (Kauzmann, 1959; Kupke, 1973; Xie & Timasheff, 1997). Gekko and Morikawa (1981a) measured the preferential interaction parameters of bovine serum albumin in 30% aqueous solutions of glycerol and sorbitol at several temperatures in the range of 10–35 °C, where the protein is in the native state. Xie and Timasheff (1997) measured the preferential interaction parameters of RNase A in pH 2.0 and pH 5.5 sorbitol solutions at 20 °C and 48 °C. Little work has been done, however, on the temperature dependence in three-component systems that contain protein, water, and co-solvent. In order to get more detailed thermodynamic information on the protein–solvent interactions, systematic measurements of the preferential interaction parameters of RNase A in 30% sorbitol have been conducted as a function of temperature in the range of 4–55 °C, at both pH 2.0 and pH 5.5. For comparison, the 0.6 M $MgCl_2$ and 0.6 M $MgSO_4$ systems at pH 1.5 and at pH 5.5 have also been examined as a function of temperature. The results are presented in this paper.

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Results

Thermal stabilization

The results of thermal transition experiments as a function of pH are presented in Table 1 and the T_m values are compared in

Table 1. *Thermodynamic parameters of the thermal denaturation of RNase A*

Solvent	pH	T_m (°C) (unfolding)	$\Delta H°$ at T_m (kcal/mol)	$\Delta G°$ (20 °C) (kcal/mol)	$\delta(\Delta G°)$ (20 °C) (kcal/mol)	$\delta(\Delta G°)$ at T_m (kcal/mol)
Buffer	1.5	26.5 ± 0.9	66.8 ± 5	1.6 ± 0.6		
	2.0	30.1 ± 0.3	72.8 ± 1	2.6 ± 0.3		
	2.8	41.1 ± 0.2	78.0 ± 5	5.5 ± 0.3		
	3.0	44.8	81.0	6.3		
	3.2	46.6	82.4	6.8		
	5.5	60.4 ± 0.2	108.4 ± 4	13.6 ± 0.5		
	5.8	61.4	112.3	13.9		
0.6 M $MgCl_2$	1.5	43.5 ± 0.1	71.3 ± 2	5.8 ± 0.1	4.2 ± 0.6	4.1 ± 0.6
	2.0	45.5	82.5	6.6	4.0	3.7
	3.0	52.8	89.7	9.0	2.7	2.0
	5.5	60.5 ± 0.2	108.3 ± 5	13.4 ± 0.3	0.0 ± 0.6	0.0 ± 0.6
0.6 M $MgSO_4$	1.5	51.1 ± 0.7	77.3 ± 5	7.9 ± 0.2	6.3 ± 0.6	6.0 ± 0.6
	2.0	53.1	88.1	8.9	6.3	5.6
	3.0	60.7	101.6	12.3	6.0	4.0
	5.5	67.4 ± 0.3	111.2 ± 6	16.9 ± 0.3	3.3 ± 0.6	2.3 ± 0.6
30% Sorbitol	1.5	36.4 ± 0.2	71.5 ± 4	3.8 ± 0.2	2.2 ± 0.6	2.4 ± 0.6
	2.0	39.6 ± 0.3	75.5 ± 5	4.6 ± 0.3	2.0 ± 0.4	2.2 ± 0.4
	3.0	51.6	85.1	8.4	2.1	1.7
	5.5	66.6 ± 0.4	124.9 ± 7	16.9 ± 0.3	3.3 ± 0.6	2.0 ± 0.6

Figure 1. It is clear that the stability of RNase A increases with pH between pH 1.5 and pH 5.5, whether the medium is dilute buffer, sorbitol (Xie & Timasheff, 1997), $MgCl_2$, or $MgSO_4$ (Table 1). In dilute buffer, the transition midpoints (T_m) of protein denaturation are 26.5, 30.1, and 60.4 °C for pH 1.5, 2.0, and 5.5, respectively. This trend is in good agreement with literature values (Arakawa et al., 1990a) and reflects the increasing positive charge of the protein as pH is lowered. There is a distinct difference between the stabilization patterns by sorbitol and the two salts. For 30% sorbitol, the pH dependence of T_m is almost parallel to that in water, as T_m increased by 9.5 °C at pH 2.0 and by 6.2 °C at pH 5.5. Both $MgCl_2$ and $MgSO_4$ exert a much greater stabilization at low pH, as T_m increased by 17.0 °C in 0.6 M $MgCl_2$ and by 24.6 °C in 0.6 M $MgSO_4$ at pH 1.5. On the other hand, it did not change at pH 5.5 for 0.6 M $MgCl_2$ and increased by only 7 °C in $MgSO_4$. The decrease in the stabilizing power of the two salts with an increase in pH reflects the protein ionization pattern: the attraction for the Mg^{2+} ions increases as the net positive charge on the protein decreases. An interesting feature of these results is the close to parallel variation with pH of the T_m values in 0.6 M $MgSO_4$ and $MgCl_2$. This must reflect the difference between the preferential exclusion capacities of the SO_4^{2-} and Cl^- ions that has been noted previously for their salts with Na^+, guanidinium$^+$, and Mg^{2+} ions at a single pH value (Arakawa & Timasheff, 1984b). It is evident that the attraction of Mg^{2+} ions to negatively charged sites on the protein overcomes the exclusion of Cl^- ions much better than that of SO_4^{2-} ions.

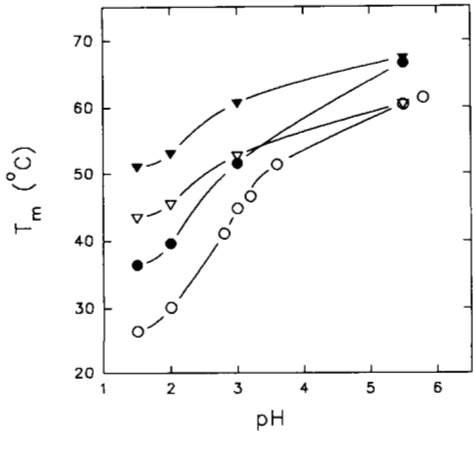

Fig. 1. pH dependence of the effect of co-solvents on the transition midpoint temperature of RNase A denaturation: (○) buffer; (●) 30% sorbitol; (△) 0.6 M $MgCl_2$; and (▲) 0.6 M $MgSo_4$.

The thermodynamic parameters were calculated from the transition curves using the method described by Biltonen and Lumry (1969) and Lee and Timasheff (1981). The standard enthalpy change of unfolding, $\Delta H°$, was calculated from the truncated form of the integrated van't Hoff equation (Glasstone, 1947; Lee & Timasheff, 1981; Kiefhaber et al., 1990; Xie & Timasheff, 1997):

$$\ln K = a + b(1/T) + c \ln T$$

$$\Delta H° = R(cT - b)$$

$$\Delta C_p = Rc. \quad (1)$$

As shown in Table 1, $\Delta H°$ was found to increase with pH in all the solvent systems. Analysis of the transition region in each case (a span of ca. 20 °C about T_m) gave close to 0 values for the fitting constant c. This did not permit us to evaluate ΔC_p. Nevertheless, $\delta\Delta G° = \Delta G_S^\circ - \Delta G_W^\circ$ values were estimated, since the errors in $\Delta G°$ for water and co-solvent should be of the same sign and close in magnitude and, therefore, tend to cancel. The standard free

energy change increments, $\delta\Delta G°$, with addition of the co-solvents, are listed in Table 1 for 20 °C and for the transition temperature, T_m. Both sets of values show again that, for the two salts, the stabilizing capacity decreases with increasing pH. It falls sharply at pH 5.5, where all the carboxyls are deprotonated and, in fact, vanishes for $MgCl_2$. The close-to-constant difference between the $\delta\Delta G°$ values of the $MgSO_4$ and $MgCl_2$ systems give a measure of the difference between the structure stabilizing capacities of the SO_4^{2-} and the Cl^- ions. In contrast, for sorbitol, the stabilizing capacity remains close to invariant with pH. This confirms the conclusion (Xie & Timasheff, 1997) that the effect of sorbitol is essentially independent of the state of charge of the protein.

Preferential interactions

The partial specific volumes and the preferential interaction parameters of RNase A in 30% sorbitol, 0.6 M $MgSO_4$, and 0.6 M $MgCl_2$ at different temperatures between 4 °C and the completion of the transition reaction at low pH (51–64 °C) are presented in Tables 2, 3, and 4. From the thermal transition data, it is known that, in 30% sorbitol, RNase A was in the native state at pH 5.5 in the temperature range of 4–55 °C (the transition occurs between 60.0 and 72.8 °C). At pH 2.0, however, RNase A denatured if the temperature was raised to 30 °C and above (the transition range is 26.8–50.8 °C). For 0.6 M $MgCl_2$, the protein was native in the range of 4–51 °C at pH 5.5, but it denatured when the temperature was raised above 32 °C at pH 1.5. For 0.6 M $MgSO_4$, the protein was native in the range 4–57 °C at pH 5.5, but at pH 1.5, it denatured when the temperature was raised above 40 °C. Therefore, at the high pH (here 5.5), the protein was in the native state at all the temperatures used in all three solvents. At low pH, however (here 1.5 or 2.0), it underwent a transition as the temperature was raised. Therefore, at low pH, the preferential binding measurements spanned from native protein at low temperatures to denatured protein at high temperature after passing through the transition zone.

Examination of Tables 2, 3, and 4 at isomolal conditions that are insensitive to interactions with solvent components and reflect only the state of compactness of the protein molecule shows that the values of ϕ_2° of the protein had only a very weak dependence on temperature in all the solvent systems used, indicating no changes in structure. At isopotential conditions, which reflect interactions with solvent (dialysis equilibrium), the apparent partial specific volume, $\phi_2'^\circ$, was found to be strongly temperature dependent. At low temperature (ca. <30 °C), the $\phi_2'^\circ$ values at the two pH values were essentially identical and displayed little dependence on temperature, which indicated that pH had no effect on the preferential interactions of native protein. At close to 30 °C, all the $\phi_2'^\circ$ values (except for 0.6 m $MgCl_2$, pH 1.5) started decreasing. This decrease set in at lower temperatures for the native protein (high pH) than for protein undergoing denaturation (low pH).

The preferential binding parameters, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, and $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, calculated from the above partial specific volumes are listed in columns 4, 5, and 6 of Tables 2, 3, and 4. It is evident that an increase in temperature was accompanied by a decrease both in preferential exclusion and in preferential hydration for all the systems (except for $MgCl_2$ at low pH). These changes reflect the variation with temperature of the chemical potential gradient of the protein with increasing co-solvent concentration, $(\partial\mu_2/\partial m_3)_{T,\mu_1,\mu_3}$. As seen in the last column of the

Table 2. *Temperature dependence of the preferential interaction parameters of RNase A with 30% sorbitol at pH 5.5 and pH 2.0*

Temperature (°C)	ϕ_2° (mL/g)	$\phi_2'^\circ$ (mL/g)	$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial m_1}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$ [a,b,c]
pH 5.5, g_3 = 0.3736, m_3 = 2.050							
4	0.701 ± 0.001	0.741 ± 0.001	−0.1597 ± 0.004	+0.427 ± 0.011	−11.99 ± 0.3	+325 ± 8	+3,520 ± 85
15	0.701 ± 0.002	0.737 ± 0.001	−0.1437 ± 0.006	+0.385 ± 0.016	−10.79 ± 0.5	+293 ± 12	+3,199 ± 133
20	0.701 ± 0.000	0.735 ± 0.001	−0.1357 ± 0.002	+0.363 ± 0.006	−10.19 ± 0.2	+276 ± 4	+3,073 ± 45
25	0.700 ± 0.001	0.733 ± 0.002	−0.1317 ± 0.006	+0.353 ± 0.016	−9.89 ± 0.5	+268 ± 12	+3,034 ± 138
33	0.700 ± 0.002	0.727 ± 0.001	−0.1078 ± 0.006	+0.289 ± 0.016	−8.09 ± 0.5	+220 ± 12	+2,548 ± 141
40	0.700 ± 0.003	0.710 ± 0.002	−0.0399 ± 0.010	+0.107 ± 0.026	−3.00 ± 0.8	+81 ± 20	+967 ± 242
48	0.699 ± 0.004	0.692 ± 0.006	+0.0279 ± 0.020	−0.075 ± 0.053	+2.09 ± 1.5	−57 ± 40	−691 ± 493
55	0.699 ± 0.004	0.673 ± 0.009	+0.1038 ± 0.026	−0.278 ± 0.070	+7.79 ± 2.0	−211 ± 53	−2,630 ± 657
pH 2.0, g_3 = 0.3736, m_3 = 2.050							
4	0.701 ± 0.000	0.740 ± 0.002	−0.1557 ± 0.004	+0.417 ± 0.011	−11.69 ± 0.3	+317 ± 8	+3,334 ± 85
15	0.701 ± 0.001	0.735 ± 0.001	−0.1357 ± 0.004	+0.363 ± 0.011	−10.19 ± 0.3	+276 ± 8	+3,021 ± 89
20	0.701 ± 0.000	0.733 ± 0.001	−0.1277 ± 0.002	+0.342 ± 0.005	−9.59 ± 0.2	+260 ± 4	+2,892 ± 45
25	0.701 ± 0.001	0.732 ± 0.001	−0.1237 ± 0.004	+0.331 ± 0.011	−9.29 ± 0.3	+252 ± 8	+2,850 ± 92
33	0.700 ± 0.002	0.731 ± 0.002	−0.1237 ± 0.008	+0.331 ± 0.021	−9.29 ± 0.6	+252 ± 16	+2,926 ± 189
40	0.700 ± 0.002	0.727 ± 0.003	−0.1078 ± 0.010	+0.289 ± 0.055	−9.09 ± 0.8	+220 ± 20	+2,929 ± 271
48	0.698 ± 0.003	0.712 ± 0.002	−0.0559 ± 0.010	+0.150 ± 0.026	−4.19 ± 0.7	+114 ± 20	+1,385 ± 247
55	0.697 ± 0.003	0.701 ± 0.006	−0.0160 ± 0.018	+0.041 ± 0.048	−1.20 ± 1.4	+33 ± 37	+405 ± 455

[a] Calories (mol of sorbitol)$^{-1}$ (mol protein)$^{-1}$ in 1,000 g H_2O.

[b] Value of $(\partial \ln \gamma_3/\partial m_3)$ was 0.030 calculated from Bonner (1982).

[c] Values of $(\partial\mu_3/\partial m_3)_{T,P,m_2}$ were 285.2, 296.5, 301.6, 306.8, 315.0, 322.2, 330.4, and 337.6 calories (mol sorbitol)$^{-2}$ in 1,000 g H_2O for 30% sorbitol at 4, 15, 20, 25, 33, 40, 48, and 55 °C, respectively.

Table 3. *Temperature dependence of the preferential interaction parameters of RNase A with 0.6 M $MgSO_4$ at pH 5.5 and pH 1.5*

Temperature (°C)	ϕ_2° (mL/g)	$\phi_2'^\circ$ (mL/g)	$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial m_1}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$ [a,b,c]
pH 5.5, g_3 = 0.0724, m_3 = 0.601							
4	0.702 ± 0.001	0.728 ± 0.001	−0.0288 ± 0.001	+0.398 ± 0.015	−3.27 ± 0.1	+302 ± 12	+3,277 ± 126
20	0.702 ± 0.001	0.728 ± 0.001	−0.0288 ± 0.001	+0.398 ± 0.015	−3.27 ± 0.1	+302 ± 12	+3,466 ± 133
30	0.700 ± 0.001	0.718 ± 0.002	−0.0199 ± 0.002	+0.275 ± 0.023	−2.26 ± 0.2	+209 ± 17	+2,476 ± 206
40	0.699 ± 0.001	0.701 ± 0.002	−0.0022 ± 0.002	+0.030 ± 0.022	−0.25 ± 0.2	+23 ± 17	+283 ± 212
50	0.698 ± 0.002	0.688 ± 0.008	+0.0111 ± 0.006	−0.153 ± 0.077	+1.26 ± 0.6	−116 ± 58	−1,472 ± 736
57	0.697 ± 0.003	0.671 ± 0.003	+0.0288 ± 0.003	−0.398 ± 0.046	+3.27 ± 0.4	−302 ± 35	−3,903 ± 450
pH 1.5, g_3 = 0.0724, m_3 = 0.601							
4	0.701 ± 0.001	0.725 ± 0.002	−0.0265 ± 0.002	+0.366 ± 0.023	−3.01 ± 0.2	+278 ± 17	+3,015 ± 188
20	0.701 ± 0.001	0.725 ± 0.002	−0.0265 ± 0.002	+0.366 ± 0.023	−3.01 ± 0.2	+278 ± 17	+3,189 ± 199
30	0.701 ± 0.002	0.723 ± 0.001	−0.0243 ± 0.002	+0.336 ± 0.023	−2.76 ± 0.2	+255 ± 17	+3,024 ± 206
40	0.701 ± 0.002	0.715 ± 0.007	−0.0155 ± 0.005	+0.214 ± 0.069	−1.76 ± 0.6	+163 ± 52	+1,992 ± 640
50	0.701 ± 0.002	0.708 ± 0.006	−0.0077 ± 0.004	+0.106 ± 0.061	−0.88 ± 0.5	+81 ± 46	+1,021 ± 587
57	0.701 ± 0.003	0.696 ± 0.004	+0.0055 ± 0.004	−0.076 ± 0.053	+0.63 ± 0.4	−58 ± 41	−745 ± 526
64	0.701 ± 0.003	0.682 ± 0.009	+0.0210 ± 0.007	−0.290 ± 0.092	+2.39 ± 0.8	−220 ± 69	−2,906 ± 919

[a]Calories (mol sorbitol)$^{-1}$ (mol protein)$^{-1}$ in 1,000 g H_2O.
[b]Value of $(\partial \ln \gamma_3/\partial m_3)$ was −0.755 calculated from Robinson and Stokes (1955).
[c]Values of $(\partial \mu_3/\partial m_3)_{T,P,m_2}$ were 1,001.1, 1,058.9, 1,095.0, 1,131.1, 1,167.2, 1,192.5, and 1,217.8 calories (mol $MgSO_4$)$^{-2}$ in 1,000 g H_2O for 0.6 M $MgSO_4$ at 4, 20, 30, 40, 50, 57, and 64 °C, respectively.

same tables, this parameter became less positive with increasing temperature for all the systems (except for 0.6 M $MgCl_2$ at pH 1.5) and, in fact, assumed negative values for 0.6 M $MgSO_4$ and for native protein in 30% sorbitol. This means that, with the given solvent concentrations as reference states, the thermodynamic interaction of the solvent systems with the protein is less unfavorable at higher temperature and, in fact, can become favorable if the temperature is raised sufficiently.

Preferential interactions of a protein with solvent components can be expressed in terms of three interrelated parameters: (1) preferential binding of co-solvent, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$; (2) preferential hydration, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, frequently referred to as the preferential exclusion of co-solvent; (3) perturbation of the chemical potential of the protein by addition of a co-solvent, $(\partial \mu_2/\partial m_3)_{T,P,m_2}$. The three are interrelated by Equations 11 and 12. In Figure 2, the interaction results of Tables 2, 3, and 4 are presented in the three

Table 4. *Temperature dependence of the preferential interaction parameters of RNase A with 0.6 M $MgCl_2$ at pH 5.5 and pH 1.5*

Temperature (°C)	ϕ_2° (mL/g)	$\phi_2'^\circ$ (mL/g)	$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial m_1}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$ [a,b,c]
pH 5.5, g_3 = 0.0578, m_3 = 0.607							
4	0.705 ± 0.001	0.725 ± 0.001	−0.0268 ± 0.001	+0.464 ± 0.023	−3.85 ± 0.2	+353 ± 18	+12,177 ± 609
20	0.705 ± 0.001	0.725 ± 0.001	−0.0268 ± 0.001	+0.464 ± 0.023	−3.85 ± 0.2	+353 ± 18	+12,880 ± 644
27.5	0.705 ± 0.002	0.725 ± 0.001	−0.0268 ± 0.002	+0.464 ± 0.035	−3.85 ± 0.3	+353 ± 26	+13,209 ± 991
35	0.703 ± 0.002	0.715 ± 0.003	−0.0161 ± 0.003	+0.279 ± 0.058	−2.31 ± 0.5	+212 ± 44	+8,123 ± 1,692
45	0.701 ± 0.002	0.709 ± 0.004	−0.0107 ± 0.004	+0.185 ± 0.069	−1.54 ± 0.6	+141 ± 53	+5,591 ± 2,097
51	0.700 ± 0.002	0.708 ± 0.004	−0.0107 ± 0.004	+0.185 ± 0.069	−1.54 ± 0.6	+141 ± 53	+5,697 ± 2,136
pH 1.5, g_3 = 0.0578, m_3 = 0.607							
4	0.709 ± 0.001	0.729 ± 0.003	−0.0268 ± 0.003	+0.464 ± 0.046	−3.85 ± 0.4	+353 ± 35	+12,177 ± 1,218
20	0.709 ± 0.001	0.729 ± 0.003	−0.0268 ± 0.003	+0.464 ± 0.046	−3.85 ± 0.4	+353 ± 35	+12,880 ± 1,288
35	0.709 ± 0.001	0.729 ± 0.003	−0.0268 ± 0.003	+0.464 ± 0.046	−3.85 ± 0.4	+353 ± 35	+13,539 ± 1,354
51	0.709 ± 0.001	0.728 ± 0.003	−0.0255 ± 0.003	+0.441 ± 0.046	−3.66 ± 0.4	+335 ± 35	+13,539 ± 1,425
58	0.709 ± 0.002	0.727 ± 0.007	−0.0241 ± 0.006	+0.417 ± 0.104	−3.46 ± 0.9	+317 ± 79	+13,076 ± 3,269

[a]Calories (mol sorbitol)$^{-1}$ (mol protein)$^{-1}$ in 1,000 g H_2O.
[b]Value of $(\partial \ln \gamma_3/\partial m_3)$ was 0.267, calculated from Robinson and Stokes (1955).
[c]Values of $(\partial \mu_3/\partial m_3)_{T,P,m_2}$ were 3,162.8, 3,345.4, 3,431.0, 3,516.6, 3,630.7, 3,699.2, and 3,779.1 calories (mol $MgCl_2$)$^{-2}$ in 1,000 g H_2O for 0.6 M $MgCl_2$ at 4, 20, 27.5, 35, 45, 51, and 58 °C, respectively.

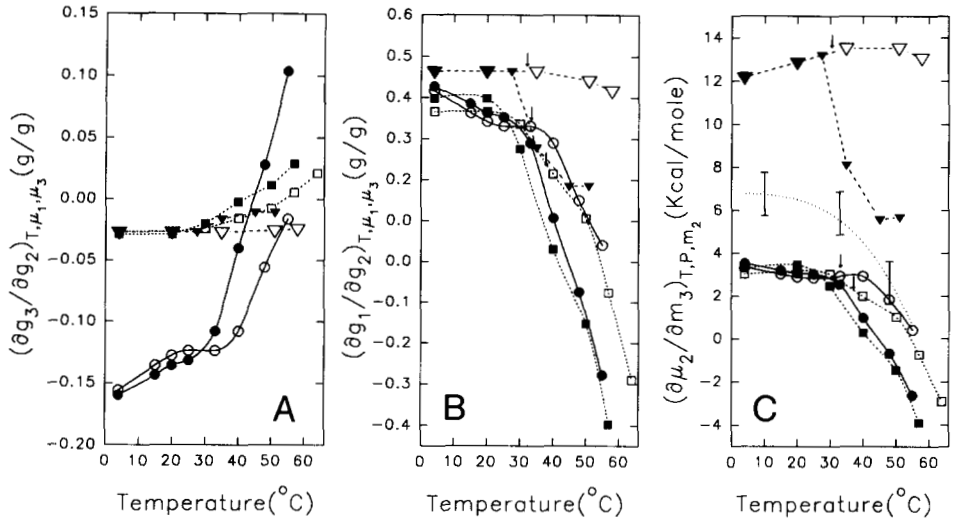

Fig. 2. Temperature dependence of the preferential interactions of co-solvents with RNase A. **A:** Preferential binding. 30% sorbitol: (○) pH 2.0, (●) pH 5.5; 0.6 M $MgCl_2$: (△) pH 1.5, (▲) pH 5.5; 0.6 M $MgSO_4$: (□) pH 1.5, (■) pH 5.5. **B:** Preferential hydration. 30% sorbitol: (○) pH 2.0, (●) pH 5.5; 0.6 M $MgCl_2$: (△) pH 1.5, (▲) pH 5.5; 0.6 M $MgSO_4$: (□) pH 1.5, (■) pH 5.5. Arrows indicate the onset of the thermal transition. **C:** Temperature dependence of the chemical potential perturbation, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$. 30% sorbitol: (○) pH 2.0, (●) pH 5.5; 0.6 M $MgCl_2$: (△) pH 1.5, (▲) pH 5.5; 0.6 M $MgSO_4$: (□) pH 1.5, (■) pH 5.5. The dotted line is the parameter calculated at 48 °C for the denatured protein in 30% sorbitol (see text). Arrows indicate the onset of the thermal transition.

forms because each emphasizes a different aspect of the interaction. Figure 2A shows preferential binding of co-solvent (whether positive or negative), i.e., the result obtained directly in dialysis equilibrium experiments. Figure 2B shows preferential hydration, i.e., the excess (or deficiency) of water in the immediate domain of the protein relative to that in the bulk solvent. Figure 2C shows the same results as the mutual thermodynamic effect of protein and co-solvent on each other. The last, in fact, is the fundamental statement of the interactions, since it indicates the direction in which addition of an infinitesimal amount of co-solvent to a system of a given solvent composition will displace the interaction, i.e., make it more favorable or more unfavorable.

Comparison of Figures 2A, B, and C brings out some similarities, as well as a striking difference between the dialysis equilibrium results (Fig. 2A) and the actual thermodynamics of the interactions (Fig. 2C). At low temperatures (< ca. 30 °C) for all three systems, each of the three parameters displayed identical values at acid and close to neutral pH. This means that the deficiency of the co-solvent, in the immediate domain of the protein, i.e., the unfavorable thermodynamic interaction, was independent of the charge state of the protein. When viewed as preferential binding (dialysis equilibrium result), the values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ remained very small at all temperatures for the two salts. For sorbitol, however, they spanned a five times broader range, from an exclusion of −0.16 g sorbitol per g protein at low temperature to +0.10 g sorbitol per g protein at 55 °C, pH 5.5. This striking difference disappears when expressed in terms of preferential hydration or perturbation of the chemical potential. From Figure 2B it is clear that, at low temperature, the preferential hydration of the protein is very similar for all three systems. Its magnitude, 0.37–0.46 g of H_2O/g of protein, is similar to the generally observed hydration values of most globular proteins (Bull & Breese, 1968; Kuntz, 1971; Kuntz & Kauzmann, 1974). This indicates that, below 30 °C, co-solvent is close to being excluded totally from the immediate domain of the protein. The thermodynamic expression of the interactions (Fig. 2C) reveals that, in fact, the chemical potential of the protein is perturbed much more strongly by $MgCl_2$ than $MgSO_4$ or sorbitol, even though dialysis equilibrium (Fig. 2A) showed identical binding of $MgCl_2$ and $MgSO_4$ below 30 °C. Comparison of the data for 30% sorbitol and 0.6 M $MgSO_4$ of Figure 2A and C reveals another striking feature. The thermodynamics of the interactions (Fig. 2C) are strikingly identical for the two systems at all temperatures and both pH values. Yet these identical perturbations of the chemical potential of the protein manifest themselves in totally different experimental observations in dialysis equilibrium (Fig. 2A). Thus, it seems clear that a full description and understanding of the degree and strength of interactions requires that the results of dialysis equilibrium experiments be examined in terms of the various aspects of the interactions and not just in terms of the usual binding parameter.

Let us now compare the interaction results at high temperature, i.e., above the onset of unfolding. For all three systems, the observations were identical. The preferential binding parameter (Fig. 2A) was always more negative at low pH than at high pH, meaning that the co-solvents are more strongly preferentially excluded from the denatured protein than from the native one. This in turn means that, thermodynamically, the interactions are more unfavorable (or less favorable) with the denatured protein than the native form: as seen in Figure 2C, the values of $(\partial\mu_2/\partial m_3)_{T,\mu_1,\mu_3}$ are always less negative (more positive) at low pH than at high pH. What do these observations mean in terms of the structural stabilization of the protein?

Thermodynamic stabilization

At any given solvent composition, the effect of increasing infinitesimally the concentration of any ligand, in this case co-solvent, on protein stability is given by the linkage relation (Wyman, 1964):

$$\left(\frac{d\Delta G^\circ}{d\mu_3}\right)_{T,P,m_2} = \left[\left(\frac{\partial\mu_2}{\partial m_3}\right)^D_{T,P,m_2} - \left(\frac{\partial\mu_2}{\partial m_3}\right)^N_{T,P,m_2}\right]\Big/\left(\frac{\partial\mu_3}{\partial m_3}\right)_{T,P,m_2}, \quad (2)$$

where $d\mu_3 = RTd\ln a_3$. The stabilizing ability of a co-solvent is defined, therefore, by the difference between the chemical potential gradients for the protein in the native and denatured states. Such a comparison can be made directly on Figure 2C. Once the zone of transition is reached, the experimental chemical potential gradient with increasing co-solvent concentration of the denaturing protein (acid pH) maintains more positive values than those of the native protein (pH 5.5). Therefore, the effect is always one of stabilization. What is the effect below the zone of transition? The results show identical interactions of the native protein at the two values of pH for all three co-solvents. The stabilizing ability below the transition temperature requires knowledge of the interactions of the denatured protein. This was estimated by extrapolating the $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ curve at low pH to the low temperature zone, with the assumption that, in the transition zone, the measured preferential binding is the weighted sum of these parameters for the native and denatured states of the protein.[1] Then, if f_N and f_D are the fractions of the native and denatured protein,

$$\left(\frac{\partial m_3}{\partial m_2}\right)^{Exper}_{T,P,\mu_3} = f_N\left(\frac{\partial m_3}{\partial m_2}\right)^N_{T,P,\mu_3} + f_D\left(\frac{\partial m_3}{\partial m_2}\right)^D_{T,P,\mu_3}$$

$$\left(\frac{\partial\mu_2}{\partial m_3}\right)^{Exper}_{T,P,m_2} = f_N\left(\frac{\partial\mu_2}{\partial m_3}\right)^N_{T,P,\mu_3} + f_D\left(\frac{\partial\mu_2}{\partial m_3}\right)^D_{T,P,\mu_3}. \quad (3)$$

This calculation was performed for the 30% sorbitol system using the points in the transition region, with the further assumption that

$$\left(\frac{\partial m_3}{\partial m_2}\right)^D_{T,P,\mu_3} - \left(\frac{\partial m_3}{\partial m_2}\right)^D_{T,P,\mu_3}$$

is independent of temperature. The resulting expected temperature dependence of $(\partial\mu_2/\partial m_3)^D_{T,P,m_2}$ for the unfolded RNase A below the measurable transition is shown on Figure 2C by the dotted line. This gives a graphic explanation of the reason why the low pH experimental curves turn down at higher temperatures than those measured at high pH.

Thermodynamic parameters

Knowledge of the temperature dependence of the preferential interactions opens the possibility of conducting for the first time a complete thermodynamic analysis of protein solvent interactions. It should be recalled that the preferential interaction parameter measured by dialysis equilibrium, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, is the variation with the concentration of the co-solvent of the partial molal free energy, $\bar{G}_2$. This, in turn, is the sum of variations with co-solvent concentration of the partial molal enthalpy, $\bar{H}_2 = (\partial H_2/\partial m_2)_{T,P,m_3}$, and the partial molal entropy, $\bar{S}_2 = (\partial S_2/\partial m_2)_{T,P,m_3}$. Hence,

$$\left(\frac{\partial\mu_2}{\partial m_3}\right)_{T,P,m_2} = \left(\frac{\partial\bar{G}_2}{\partial m_3}\right)_{T,P,m_2} = \left(\frac{\partial\bar{H}_2}{\partial m_3}\right)_{T,P,m_2} - T\left(\frac{\partial\bar{S}}{\partial m_3}\right)_{T,P,m_2}. \quad (4)$$

The variation of the partial molal enthalpy of the protein with co-solvent concentration was calculated from the temperature dependence of the variation of the partial molal free energy by applying Equation 1, in which $\ln K$ was replaced by $-(\partial\mu_2/\partial m_3)_{T,\mu_1,\mu_3}/RT$:

$$-\frac{1}{RT}\left(\frac{\partial\mu_2}{\partial m_3}\right)_{T,P,m_2} = a + b(1/T) + c\ln T$$

$$\left(\frac{\partial\bar{H}_2}{\partial m_3}\right)_{T,P,m_2} = R(cT - b)$$

$$\left(\frac{\partial\bar{C}_{p,2}}{\partial m_3}\right)_{T,P,m_2} = Rc. \quad (5)$$

The calculations and their results for 30% sorbitol at pH 5.5 (native RNase) are summarized on Figure 3. Figure 3A shows the van't Hoff plot of the preferential interaction parameter. The data for native protein in 30% sorbitol, taken from Table 2, last column, were fitted to Equation 5 and the resulting values of $(\partial\bar{H}_2/\partial m_3)_{T,\mu_1,\mu_3}$ as a function of temperature are presented in Figure 3B. These are seen to increase with temperature, from -14.8 kcal/mol^2 at 4 °C to 89.5 kcal/mol^2 at 55 °C, which gives a positive value of the co-solvent concentration variation of the partial molal heat capacity, $(\partial\bar{C}_{p,2}/\partial m_3)_{T,\mu_1,\mu_3} = 2.0$ kcal deg^{-1} (mol protein)$^{-1}$ (mol co-solvent)$^{-1}$.

Knowledge of $(\partial\bar{H}_2/\partial m_3)_{T,P,m_2}$ as a function of co-solvent concentration should make it possible to calculate the sorbitol concentration dependence of the partial molal enthalpy, $\Delta\bar{H}_{2,m_3}$, of the protein, i.e., the transfer enthalpy, since

$$\Delta\bar{H}_{2,m_3} = \bar{H}_{2,m_3} - \bar{H}_{2,w} = \int_0^{m_3}\left(\frac{\partial\bar{H}_2}{\partial m_3}\right)_{T,P,m_2} dm_3 = \Delta\bar{H}_{2,tr}, \quad (6)$$

and of the partial molal entropy, $\Delta\bar{S}_{2,m_3}$, since

$$\Delta\bar{S}_{2,m_3} = \frac{1}{T}(\Delta\bar{H}_{2,m_3} - \Delta\mu_{2,tr}) = \Delta\bar{S}_{2,tr}. \quad (7)$$

This defines fully the thermodynamics of transferring a protein from water to a solvent system of composition m_3 at any given temperature in terms of transfer free energy, $\Delta\mu_{2,tr}$, transfer enthalpy, $\Delta\bar{H}_{2,tr}$, and transfer entropy, $\Delta\bar{S}_{2,tr}$. Such a calculation requires knowledge of the temperature dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ at several co-solvent concentrations. In the absence of detailed such data, an approximate calculation was carried out as follows. The co-solvent concentration dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ for native RNase A is known at two temperatures (20 °C and 48 °C) (Xie & Timasheff, 1997). The values for 10%, 20%, and 40% sorbitol were plotted (see Fig. 3A) alongside the detailed data obtained in 30% sorbitol and curves were constructed through these points with the assumption that the form of the temperature dependence is the same at all co-solvent concentrations, as long as the protein remains native. Application of Equation 5 yielded values of $(\partial\bar{H}_2/\partial m_3)_{T,P,m_2}$ for all the sorbitol concentrations. These are shown as a function of temperature in Figure 3B and as a function of sorbitol concentration at 20 °C and 48 °C, the only two temper-

[1] This in fact is an approximation. The exact relation requires that the difference between the thermodynamic states of the protein in the native and denatured forms in water be taken into account at each temperature.

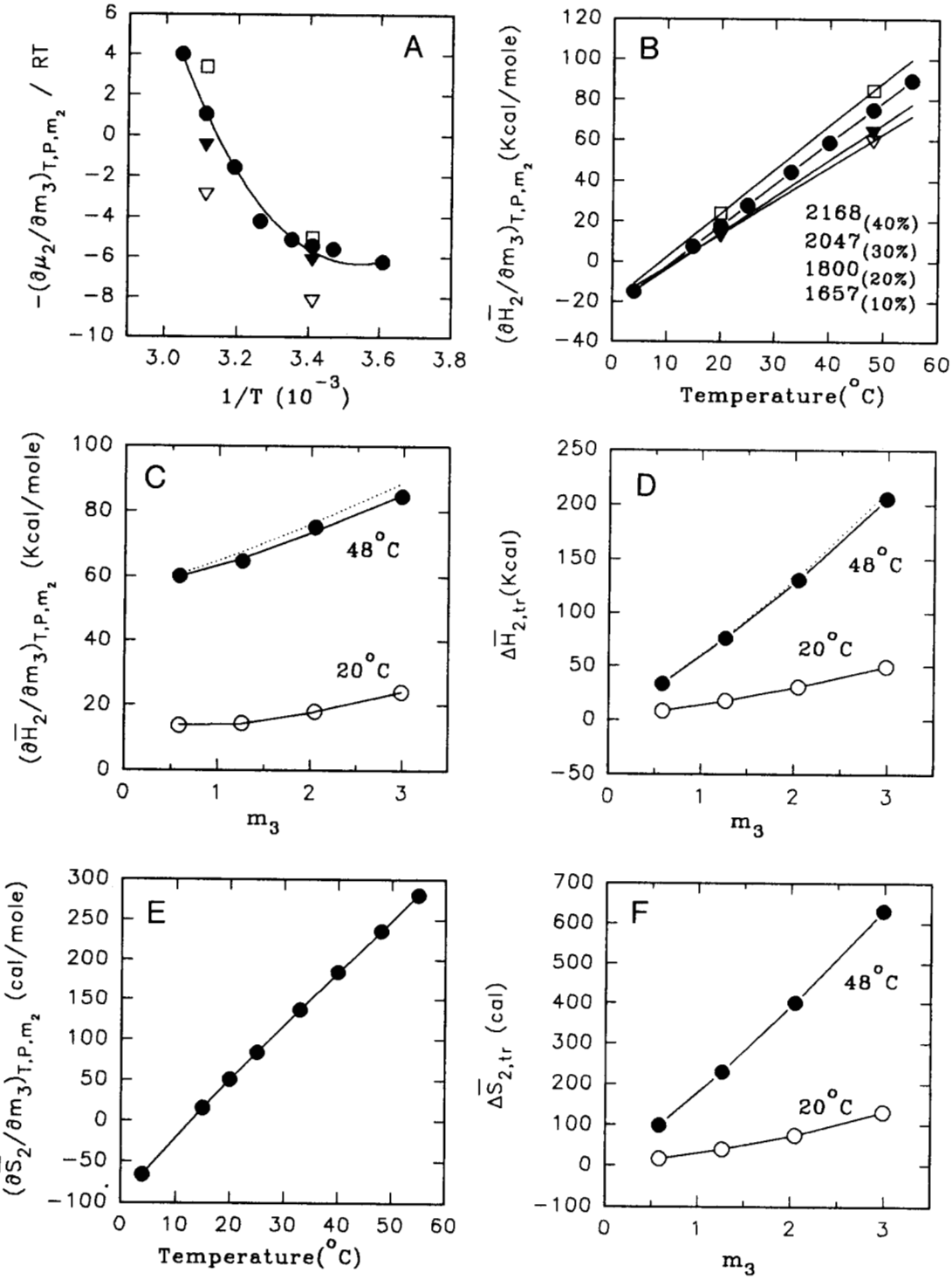

Fig. 3. Thermodynamics of the preferential interactions of aqueous sorbitol solutions with native RNase A. **A:** Van't Hoff plot of the transfer free energy variation with sorbitol concentration: (▽) 10% sorbitol; (▼) 20% sorbitol; (●) 30% sorbitol; (□) 40% sorbitol. **B:** Temperature dependence of the variation of the transfer enthalpy with sorbitol concentration at 10% (▽), 20% (▼), 30% (●), 40% (□) sorbitol. Numbers on the figure are the slopes, which represent $(\partial\bar{C}_{p,2}/\partial m_3)_{T,P,m_2}$ in cal-deg^{-1}-mol^{-1}. **C:** Dependence on sorbitol concentration of the transfer enthalpy variation with sorbitol concentration at 20 °C and 48 °C. The dotted line is the parameter calculated at 48 °C for the denatured protein. **D:** Dependence of the transfer enthalpy on sorbitol concentration at 20 °C and 48 °C. The dotted line is the parameter calculated at 48 °C for the denatured protein. **E:** Temperature dependence of the variation of the transfer entropy with sorbitol concentration at 30% sorbitol. **F:** Dependence of the transfer entropy on sorbitol concentration at 20 °C and 48 °C.

atures at which detailed data are available, in Figure 3C. Integration of the data of Figure 3C according to Equation 6 gave the values of the transfer enthalpy, $\Delta\bar{H}_{2,tr}$ at the two temperatures for the four co-solvent concentrations for which data are available. These are shown in Figure 3D. Combination of the values of Figure 3B with the experimental values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ (Xie &

Timasheff, 1997), as well as of the values of Figure 3D with experimental values of $\Delta\mu_{2,tr}$ (Xie & Timasheff, 1997) gave, by Equations 4 and 7, the temperature dependence of $(\partial\bar{S}/\partial m_3)_{T,P,m_2}$, shown in Figure 3E for 30% sorbitol, and the sorbitol concentration dependence of the transfer entropy, $\Delta\bar{S}_{2,tr}$, at 20 °C and 48 °C, shown in Figure 3F.

We emphasize that the numbers shown in Figure 3C, D, and F must be regarded as illustrative because the calculations required the assumption that the form of the temperature dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ was identical at the various sorbitol concentrations. Nevertheless, these numbers give at least a qualitative thermodynamic description of the solvent interactions. With this caution, it is evident that the transfer of RNase A from water into an aqueous sorbitol medium is accompanied by positive changes in enthalpy ($\Delta\bar{H}_{2,tr}$) and entropy ($\Delta\bar{S}_{2,tr}$). The fact that the $\Delta\bar{H}_{2,tr}$ values are more positive at 48 °C than at 20 °C indicates further that this process is characterized by a positive value of $\Delta\bar{C}_{p,2}$, as well.

In similar manner, the temperature dependences of $(\partial\bar{H}_2/\partial m_3)_{T,P,m_3}$ and $(\partial\bar{S}_2/\partial m_3)_{T,P,m_3}$ were calculated for the $MgSO_4$ and $MgCl_2$ systems from the van't Hoff plot of $-(\partial\mu_2/\partial m_3)_{T,P,m_3}/RT$ with the use of Equation 5. The results are presented in Figure 4. Both systems are characterized by increasing values with temperature of the partial molal enthalpy and partial molal entropy variations with co-solvent concentration. Above 10 °C, all the values of these parameters are positive. Furthermore, both are characterized by positive increments of $\Delta\bar{C}_{p,2}$, as the values of $(\partial\bar{C}_{p,2}/\partial m_3)_{T,P,m_2}$ are 2.2 kcal deg^{-1} (mol protein)$^{-1}$ (mol co-solvent)$^{-1}$ for $MgSO_4$ and 3.4 kcal deg^{-1} (mol protein)$^{-1}$ (mol co-solvent)$^{-1}$ for $MgCl_2$.

Discussion

Stabilization: Balance of preferential interactions

The present examination of the temperature dependence of preferential interactions has shown that for three co-solvent systems, 30% sorbitol, 0.6 M $MgSO_4$, and 0.6 M $MgCl_2$, the preferential hydration of a native globular protein, RNase A, decreases as temperature increases. This is particularly pronounced above ca. 30 °C. Denaturation is accompanied by an increase in the preferential hydration in all three co-solvents. This is reflected by the observation that, above the transition temperature, the preferential exclusion remains at higher values for the denatured protein than for the native one. In other words, the denatured protein is always more preferentially hydrated than the native one. This is the source of the stabilization. At any solvent composition, the stabilizing ability of the co-solvent is expressed by Equation 2, or with the application of Equation 12, by the difference between the preferential bindings of the co-solvent to the protein in the two end states of the equilibrium, i.e., $(\partial m_3/\partial m_2)^D_{T,P,\mu_3} - (\partial m/\partial m_2)^N_{T,P,\mu_3}$. It is evident, then, that there is no requirement that co-solvent be preferentially excluded from the native protein in order to be a structure stabilizer. While all the previous studies done at 20 °C have invariably given this result, the present measurements have shown that at high temperature a co-solvent can be preferentially bound to the native protein, yet be a stabilizer. What is required is that the preferential binding to the denatured form be smaller (Timasheff, 1992). For example, dialysis equilibrium of native RNase in 30% sorbitol at 55 °C resulted in preferential binding, $(\partial m_3/\partial m_2)^N_{T,P,\mu_3} = 7.8$ mol sorbitol per mol protein. The same experiment with the denatured protein gave $(\partial m_3/\partial m_2)^D_{T,P,\mu_3} = -1.2$ mol sorbitol per mol protein. The difference, −9.0 mol sorbitol per mol protein, indicates stabilization with an increase of the chemical potential gradient of +3.0 kcal (mol protein)$^{-1}$ (mol sorbitol)$^{-1}$ for denaturing the protein. In the case of 0.6 M $MgSO_4$ at 57 °C, both dialysis equilibrium measurements gave *binding* of the co-solvent. Yet there was stabilization of structure since $(\partial m_3/\partial m_2)^D_{T,P,\mu_3} - (\partial m_3/\partial m_2)^N_{T,P,\mu_3} = 0.63 - 3.27 = -2.64$ mol $MgSO_4$ per mol protein, with a stabilizing increase in the chemical potential gradient of +3.2 kcal (mol protein)$^{-1}$ (mol sorbitol)$^{-1}$.

Thermodynamics of stabilization

The free energy of stabilization, expressed through the difference between the transfer free energies from water to the solvent system of the protein in the denatured and native states, stems from the changes of the transfer enthalpies and transfer entropies during denaturation. At any temperature and solvent composition,

$$\delta\Delta\mu_{2,tr} = \Delta\bar{H}^D_{2,tr} - \Delta\bar{H}^N_{2,tr} - T\Delta\bar{S}^D_{2,tr} + T\Delta\bar{S}^N_{2,tr}. \quad (8)$$

The analysis of the temperature dependence of the preferential interactions for native RNase, presented in Results, has given ap-

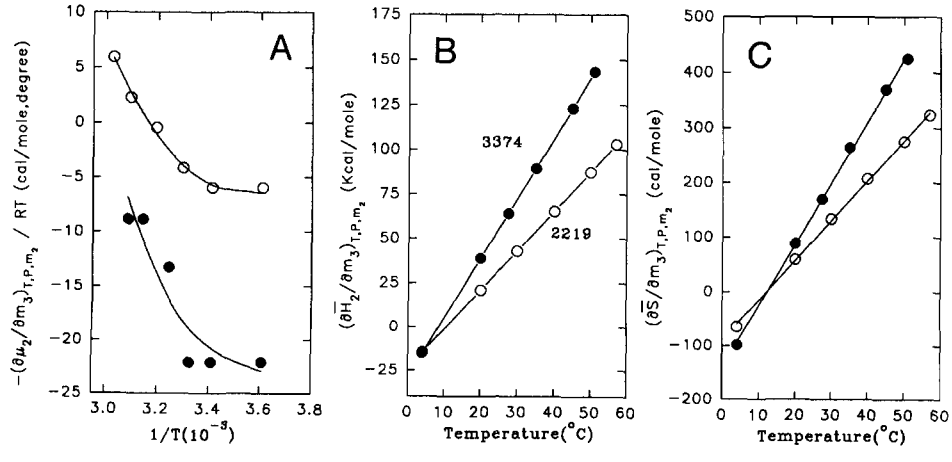

Fig. 4. Temperature dependence of the variations with cosolvent concentration of (**A**) transfer free energy, (**B**) transfer enthalpy, and (**C**) transfer entropy of RNase A in 0.6 M $MgCl_2$ (●) and 0.6 M $MgSO_4$ (○) solutions. Numbers next to the curves are the slopes.

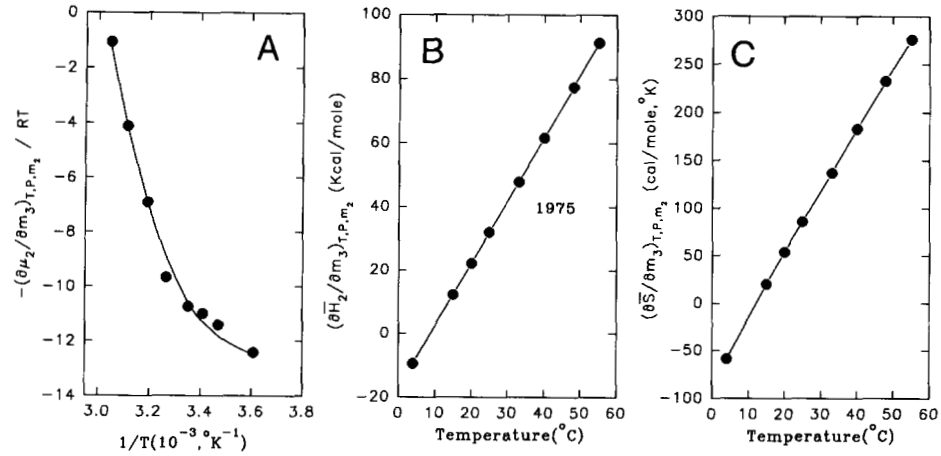

Fig. 5. Temperature dependence of the variations with sorbitol concentration of the calculated (**A**) transfer free energy, (**B**) transfer enthalpy, and (**C**) transfer entropy of denatured RNaseA in 30% aqueous sorbitol solution.

proximate values of these parameters for one end state of the $N \rightleftharpoons D$ equilibrium in sorbitol, namely for N. While data are even more scarce for the denatured protein than for the native one, an approximate temperature variation of $(\partial\bar{H}_{2,tr}/\partial m_3)_{T,P,m_3}$ and, hence, of the corresponding entropy function can be generated from the extrapolation of the temperature dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ at pH 2.0, shown in Figure 2C. The corresponding van't Hoff plot is given in Figure 5A and the temperature dependence of the partial molal enthalpy and entropy variations with co-solvent concentrations, calculated with Equations 5 and 4, respectively, are given in Figure 5B and 5C. Just as for the native protein, both parameters are positive and increase with temperature. This highly approximate calculation suggests that the thermodynamic parameters for the denatured protein will have a qualitatively similar dependence on sorbitol concentration as those obtained for the native protein.

At this point it seems of interest to examine the contribution of the changes in partial molal enthalpy and entropy upon denaturation to the measured changes in standard enthalpy and entropy of denaturation due to the presence of cosolvent. Just as with free energy, the contribution of the co-solvent to the enthalpy and entropy of denaturation is given by the difference between the corresponding transfer quantities of the denatured and native protein:

$$\delta\Delta G^\circ = \Delta G^\circ_{m_3} - \Delta G^\circ_w = \Delta\mu^D_{2,tr} - \Delta\mu^N_{2,tr} = \delta\Delta\mu^{N\to D}_{2,tr} \quad (9a)$$

$$\delta\Delta H^\circ = \Delta H^\circ_{m_3} - \Delta H^\circ_w = \Delta\bar{H}^D_{2,tr} - \Delta\bar{H}^N_{2,tr} = \delta\Delta\bar{H}^{N\to D}_{2,tr} \quad (9b)$$

$$\delta\Delta S^\circ = \Delta S^\circ_{m_3} - \Delta S^\circ_w = \Delta\bar{S}^D_{2,tr} - \Delta\bar{S}^N_{2,tr} = \delta\Delta\bar{S}^{N\to D}_{2,tr}. \quad (9c)$$

For the free energy change, the values are reported in the companion paper (Xie & Timasheff, 1997). The enthalpy contributions were estimated from the ΔH° values of denaturation at 48 °C as a function of sorbitol concentration. The calculations are summarized in Table 5. Following Equation 9b, $\delta\Delta\bar{H}^{N\to D}_{2,tr}$ was taken as the difference between the standard enthalpy of denaturation changes in the co-solvent and dilute aqueous medium, $\Delta H^\circ_{m_3} - \Delta H^\circ_w$, taken from Xie and Timasheff (1997). This was combined with the values of $\Delta\bar{H}_{2,tr}$ deduced above for the native protein and presented in Figure 3D and Table 5, column 4. The results of the calculation, $\Delta\bar{H}^D_{2,tr} = \delta\Delta\bar{H}^{N\to D}_{2,tr} + \Delta H^N_{2,tr}$, are presented in Table 5, column 5 based on the data measured at pH 5.5 and 2.0. The corresponding numbers are plotted in Figure 3D as the dotted line. The slopes of this plot as a function of sorbitol concentration are plotted as the dotted line in Figure 3C, $(\partial\bar{H}^D_2/\partial m_3)_{T,P,m_2}$, and listed in the last

Table 5. *Transfer enthalpies of native and denatured RNase A at 48 °C in aqueous sorbitol solutions*

Sorbitol (w/v)	m_3 (mol/1,000 g H_2O)	$\Delta H^\circ_{m_3} - \Delta H^\circ_w$ [a,d]	$\Delta\bar{H}^N_{2,tr}$ [b]	$\Delta\bar{H}^D_{2,tr}$ [c,d]	$\left(\frac{\Delta\bar{H}^D_{2,tr}}{\partial m_3}\right)_{T,P,m_2}$ [d,e]
10%	0.587	−0.9 (−3.0)	33.5	32.6 (30.5)	68.7 (63.9)
20%	1.263	4.2 (0.6)	76.0	80.2 (76.6)	74.4 (70.1)
30%	2.050	11.3 (2.5)	131.1	142.4 (133.6)	81.0 (77.4)
40%	2.990	15.9 (5.3)	205.8	221.7 (211.1)	89.0 (86.1)

[a] kcal-mol^{-1}, data taken from Xie and Timasheff (1997).
[b] kcal-mol protein^{-1}, values calculated by Equations 8 and 9, as described in the text.
[c] kcal-mol protein^{-1}.
[d] The numbers listed are at pH 5.5; numbers in parentheses are at pH 2.0.
[e] kcal-mol protein^{-1} mol sorbitol^{-1}.

column of Table 5. Comparison of the value for 30% sorbitol,48 °C (79 ± 2 kcal-mol protein^{-1} mol sorbitol^{-1}) with that calculated by the extrapolation of $(\partial\mu_2/\partial m_3)_{T,P,m_3}$ of the denatured protein (Fig. 5B) (78 kcal mol protein^{-1} mol sorbitol^{-1}) shows good agreement, which lends some confidence in this approximate analysis since the numbers were deduced from independent experiments.

Examination of the various measured and calculated parameters leads to two conclusions. First, the increment of the denaturation enthalpy due to the presence of the co-solvent is small relative to both the standard enthalpy change of denaturation in water and the transfer enthalpy of the native protein from water to the sorbitol medium. Second, the transfer enthalpies of the denatured protein are more positive than those of the native protein. Again the increments are not large relative to the values of the same parameter for the native protein.

Nature of the interactions

Of the three co-solvents used in this study, two are ionic and one is a polyhydric alcohol. All are known to stabilize the structure of globular proteins. Sorbitol is always a good stabilizer (Gekko & Morikawa, 1981a, 1981b; Xie & Timasheff, 1997; present results), and so is $MgSO_4$, although its stabilizing ability decreases with increasing pH (Arakawa et al., 1990a; present results). The action of $MgCl_2$ depends strongly on conditions. At low pH and low concentration (<2 M), it acts as a stabilizer (Arakawa et al., 1990a, 1990b). As pH increases, its stabilizing ability decreases and may vanish (see Results). In fact, it has been reported to be a structure destabilizer and salting-in agent (von Hippel & Schleich, 1969; Collins & Washabaugh, 1985). Under stabilizing conditions, all three co-solvents are preferentially excluded from native proteins at 20 °C. In the present study, it has been shown that at high temperature (>45 °C) sorbitol and $MgSO_4$ become preferentially bound to RNase A. Nevertheless, the balance between the interactions with the denatured and native proteins gives a net preferential exclusion increment on unfolding. What is the source of this exclusion and why is it greater for the denatured protein? Some insight can be gained from the present thermodynamic analysis.

We first take sorbitol. By analogy with glycerol, this linear polyhydric alcohol can be regarded as a solvophobic agent and to have a weak affinity for polar groups on the protein surface (Gekko & Morikawa, 1981a; Gekko & Timasheff, 1981). The interaction of this polyol with a protein surface is characterized by positive values of the transfer enthalpies, entropies, and heat capacity. This is fully consistent with a hydrophobic, or in this case, solvophobic mode of interactions. Its weak affinity for polar groups on the protein requires exchange with more strongly bound water molecules (Schellman, 1987, 1990, 1993; Timasheff, 1992, 1993, 1994). Replacement of the water hydrogen bonds by the weaker polyol ones can be expected to be accompanied by a positive enthalpy increment and an increase in entropy. Liu and Bolen (1995) have reported that osmolytes, co-solvents, that differ greatly in their chemical structure, namely sucrose and sarcosine, interact similarly with amino acid side chains and peptide groups. Both have a weak affinity for polar groups and some nonpolar residues (tryptophan and histidine in the case of sucrose). Both have lower affinity than water for peptide groups. Assuming that sorbitol displays a generally similar behavior, replacement of water molecules by the polyol at those sites where the relative affinities favor interaction with the latter would then be entropy driven, due to the liberation of water molecules. This could increase with a rise in temperature as a consequence of the accompanying positive values of $\Delta\bar{H}_{2,tr}$ and $\Delta\bar{C}_{p,2,tr}$. On denaturation, release of peptide groups and additional side chains to contact with solvent should create a large number of new interaction sites of opposite relative affinity between water and sorbitol. The prevalence of unfavorable interactions, probably of peptide groups, with the sorbitol, should lead to an increase in preferential exclusion. The small increments in the transfer free energy, enthalpy, and entropy changes during denaturation would be due to a near balance between the solvent interactions of newly exposed sites with higher and lower affinities, respectively, for sorbitol than for water in the exchange reaction (Schellman, 1993; Timasheff, 1992, 1993).

In the case of the two salts, the nature of the anion must be taken into consideration. At low pH, both are strong stabilizers. As pH increases, their stabilizing ability decreases. Both contain Mg^{2+} ions, known to have a weak affinity for negatively charged groups on proteins, which would manifest itself more strongly at the higher pH values. This attraction, however, must compete with the anions, both of which contribute to preferential exclusion from proteins (Arakawa & Timasheff, 1984a, 1984b). At identical concentrations of Mg^{2+} ions, this attraction should be identical whether the anion is Cl^- or SO_4^{2-}, hence, the parallel variation with pH of the T_m values in 0.6 M $MgSO_4$ and $MgCl_2$. The constant difference between the two curves reflects the charge-independent nonspecific contribution of the anions to the preferential interactions, as expected from the ranking of the SO_4^{2-} and Cl^- ions in the Hofmeister series (von Hippel & Schleich, 1969; Arakawa & Timasheff, 1984b).

Materials and methods

Materials

All materials and methods were the same as in the companion paper (Xie & Timasheff, 1997). $MgCl_2$ and $MgSO_4$ were purchased from Fisher. All solutions contained 0.04 M glycine at pH 1.5 and pH 2.0, and 0.04 M sodium acetate at pH 5.5. Protein concentrations were determined by UV absorbance. Extinction coefficients used in the determination of RNase A concentration were 7.27 dl/g cm at pH 2.0 and pH 5.5 for 30% sorbitol, 7.11 for 0.6 M $MgCl_2$, and 0.6 M $MgSO_4$ at pH 1.5, and 7.12 at pH 5.5 at 277 nm.

Preferential interaction parameters

Using the notation of Scatchard (1946) and Stockmayer (1950) that component 1 is water, component 2 is protein, component 3 is the additive, the preferential binding parameter, $(\partial g_3/\partial g_2)_{T\mu_1,\mu_3}$, was calculated from apparent partial specific volumes extrapolated to zero protein concentration, ϕ_2°, and $\phi_2'^\circ$, determined at conditions at which the molalities of solvent components and their chemical potentials were, in turn, kept identical in the protein solution and in the reference solvent (Cohen & Eisenberg, 1968):

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} = \left(\frac{M_3}{M_3}\right)\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3} = \rho_0(\phi_2^0 - \phi_2'^\circ)/(1 - \rho_0\bar{v}_3), \tag{10}$$

where g_i is the concentration of component i in grams per gram of water, m_i is the molality of component i and M_i is its molecular weight, T is the thermodynamic (Kelvin) temperature, $\bar{v}_3$ is the

partial specific volume of component 3, R is the universal gas constant, $\mu_i = nRT \ln M_i + nRT \ln \gamma_i + \mu_i^\circ(T,P)$ is the chemical potential of component i, γ_i is its activity coefficient, P is pressure, and n is the number of particles into which component 3 dissociates. The values of the partial specific volume of component 3, $\bar{v}_3$, were: 0.656 mL/g for 30% sorbitol, 0.210 mL/g for 0.6 M $MgCl_2$, and 0.030 mL/g for 0.6 M $MgSO_4$.

The corresponding preferential hydration parameter, $(\partial g_1/\partial g_2)_{T\mu_1,\mu_3}$ (Timasheff & Kronman, 1959; Inoue & Timasheff, 1972; Reisler et al., 1977), is:

$$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3} = -\frac{1}{g_3}\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}. \quad (11)$$

The preferential binding parameter is a direct expression of the mutual perturbations of the chemical potentials of components 2 and 3 (Casassa & Eisenberg, 1961, 1964). The corresponding chemical potential gradients are:

$$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2} = \left(\frac{\partial \mu_3}{\partial m_2}\right)_{T,P,m_3} = -\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}\left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2}$$

$$= -\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}\left[\frac{nRT}{m_3} + nRT\left(\frac{\partial \ln \gamma_3}{\partial m_3}\right)_{T,P,m_2}\right]. \quad (12)$$

Values of $(\partial \ln \gamma_3/\partial m_3)_{T,P,m_2}$ were calculated from osmotic coefficient data of Bonner (1982) and Robinson and Stokes (1955); n is 1 for sorbitol, 2 for $MgSO_4$, and 3 for $MgCl_2$.

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