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Mechanism of the stabilization of ribonuclease A by sorbitol: Preferential hydration is greater for the denatured than for the native protein

GUIFU XIE AND SERGE N. TIMASHEFF

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

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Abstract

The effect of interactions of sorbitol with ribonuclease A (RNase A) and the resulting stabilization of structure was examined in parallel thermal unfolding and preferential binding studies with the application of multicomponent thermodynamic theory. The protein was stabilized by sorbitol both at pH 2.0 and pH 5.5 as the transition temperature, T_m, was increased. The enthalpy of the thermal denaturation had a small dependence on sorbitol concentration, which was reflected in the values of the standard free energy change of denaturation, $\delta\Delta G^\circ = \Delta G^\circ(\text{sorbitol}) - \Delta G^\circ(\text{water})$. Measurements of preferential interactions at 48 °C at pH 5.5, where protein is native, and pH 2.0, where it is denatured, showed that sorbitol is preferentially excluded from the denatured protein up to 40%, but becomes preferentially bound to native protein above 20% sorbitol. The chemical potential change on transferring the denatured RNase A from water to sorbitol solution is larger than that for the native protein, $\Delta\mu_2^D > \Delta\mu_2^N$, which is consistent with the effect of sorbitol on the free energy change of denaturation. The conformity of these results to the thermodynamic expression of the effect of a co-solvent on denaturation, $\Delta G_W^\circ + \Delta\mu_2^D = \Delta G_S^\circ + \Delta\mu_2^D$, indicates that the stabilization of the protein by sorbitol can be fully accounted for by weak thermodynamic interactions at the protein surface that involve water $\rightleftharpoons$ co-solvent exchange at thermodynamically non-neutral sites. The protein structure stabilizing action of sorbitol is driven by stronger exclusion from the unfolded protein than from the native structure.

Keywords: preferential hydration; protein stabilization; sorbitol; transfer free energy

Polyhydric compounds are among the most prevalent molecules used by nature to protect organisms against the stresses of high osmotic pressure (osmolytes) and freezing (cryoprotectant) (Yancey et al., 1982; Santoro et al., 1992; Carpenter et al., 1993). The same compounds have been found to be effective stabilizers of the native conformation of globular proteins and biological assemblies when added at high concentration (≥1 M) (Jarabak et al., 1962; Gerlsma, 1968, 1970; Bradbury & Jakoby, 1972; Gerlsma & Stuur, 1972, 1974; Myers & Jakoby, 1973, 1975; Timasheff et al., 1976; Gekko & Morikawa, 1981b; Gekko & Timasheff, 1981a, 1981b; Lee & Timasheff, 1981; Na & Timasheff, 1981; Arakawa & Timasheff, 1982; Timasheff, 1993). Studies aimed at understanding the mechanism by which these compounds stabilize globular proteins have uncovered the common feature that, at 20 °C, all of these solvent additives (known as co-solvents) are preferentially excluded from the surface of the native protein, i.e., in their presence proteins are preferentially hydrated (Timasheff & Arakawa, 1988). This is true of sugars (sucrose, glucose, lactose, trehalose) (Lee & Timasheff, 1981; Arakawa & Timasheff 1982; Lin & Timasheff, 1996), glycerol (Gekko & Timasheff, 1981a; Na & Timasheff, 1981), and polyhydric alcohols (ethylene glycol, xylitol, sorbitol, mannitol, and inositol) (Gekko & Morikawa, 1981a). For the vast majority of these co-solvents, the preferential hydration was found to induce protein stabilization. Yet this was not universally true. For example, aqueous 2-methyl-2,4-pentanediol induced the denaturation of ribonuclease A (RNase A) (Arakawa et al., 1990a, 1990b) even though the native protein was very strongly preferentially hydrated (Pittz & Timasheff, 1978). A detailed analysis of preferential hydration in various solvent systems (Arakawa et al., 1990b) has led to the classification of co-solvents into two categories: (1) those in which the preferential hydration is independent of solution conditions (pH, co-solvent concentration); and (2) those in which it varies with conditions. The observation was that co-solvents that belong to the first class stabilize the structure of globular proteins, whereas the others do not always do so. Therefore, the fact that a protein is preferentially hydrated in the native state in a given solvent system is not a sufficient criterion of structure stabilizing action.

Thermodynamically, whether a co-solvent acts as a stabilizer of the native structure or not relative to water (dilute buffer) as solvent is determined by the transfer free energies from water to the solvent system, $\Delta\mu_2^D$ and $\Delta\mu_2^N$, of the protein in the denatured and

Reprint requests to: Serge N. Timasheff, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254.

native states, where the transfer free energy is defined as the difference between the chemical potentials of the protein in the solvent system and in water, $\Delta\mu_2 = \mu_{2,S} - \mu_{2,W}$. In the absence of other interactions, at any temperature the effect of a co-solvent in the stabilization of a protein, expressed as the difference between the standard free energy changes of denaturation in the presence (ΔG_S°) and absence (ΔG_W°) of co-solvent, $\delta\Delta G^\circ = \Delta G_S^\circ - \Delta G_W^\circ$, is equal to the change in transfer free energy during the course of denaturation, $\delta\Delta\mu_2$ (Timasheff, 1992, 1993), i.e.,

$$\delta\Delta G^\circ = \Delta G_S^\circ - \Delta G_W^\circ = \Delta\mu_2^D - \Delta\mu_2^N = \delta\Delta\mu_2. \quad (1)$$

This equality has never been tested, because it requires measurements of solvent interactions with the native and denatured protein under a rigid set of criteria. In order to test this equality for stabilizing co-solvents, parallel measurements have been undertaken of the effects of these additives on the free energy of protein denaturation and on their preferential interactions with proteins in the native and denatured states. To this end, preferential binding of sorbitol to native and denatured RNase A was measured at identical temperature and the results are presented in this paper.

Results

Thermal stabilization

Typical thermal transition curves (heating) of RNase A at various sorbitol concentrations at pH 2.0 and 5.5 are shown in Figure 1A and C. The midpoints of the transition (T_m) are listed in Table 1. It is evident that the presence of sorbitol raises the thermal transition temperature of RNase A at both pH values. The reversibility of the process was tested by a cooling cycle. The transition curves obtained on cooling, shown in Figure 1B and D, are mirror images of the heating curves, and the midpoints, listed in Table 1, are essentially identical to those measured on heating. This clear indication of the reversibility of the denaturation permits application of equilibrium thermodynamics. At pH 2.0, sorbitol manifested an apparently greater stabilization of the protein than at pH 5.5 when T_m was used as the criterion. Thus, in 40% sorbitol, the transition temperature was raised by about 13 °C at pH 2.0 and by 8 °C at pH 5.5.

A better quantitative description of stabilization by co-solvents is found in the thermodynamic parameters. These were obtained from the transition curves by setting at each solvent composition and temperature the equilibrium constant for the $N \rightleftharpoons D$ transition equal to $K = \alpha/(1 - \alpha)$, where α is the degree of conversion from the initial to the final state in the two-state process. In determining α, the relative amounts of the protein in its native and unfolded states were estimated from the UV absorbance, following the procedure of Biltonen and Lumry (1969), and Lee and Timasheff (1981). The data were fitted to a truncated form of the integrated van't Hoff equation (Glasstone, 1947; Lee & Timasheff, 1981; Kiefhaber et al., 1990):

$$\ln K = a + b(1/T) + c \ln T$$
$$\Delta H^\circ = R(cT - b) \quad (2)$$
$$\Delta C_p = Rc.$$

The standard enthalpy change of unfolding, ΔH°, was found to have a small dependence on sorbitol concentration both at pH 2.0 and pH 5.5, as shown in Table 1. Its lack of dependence on temperature over the transition range in which the $\ln K$ points were taken (e.g., at pH 2.0, transition occurred between 27 °C and 51 °C

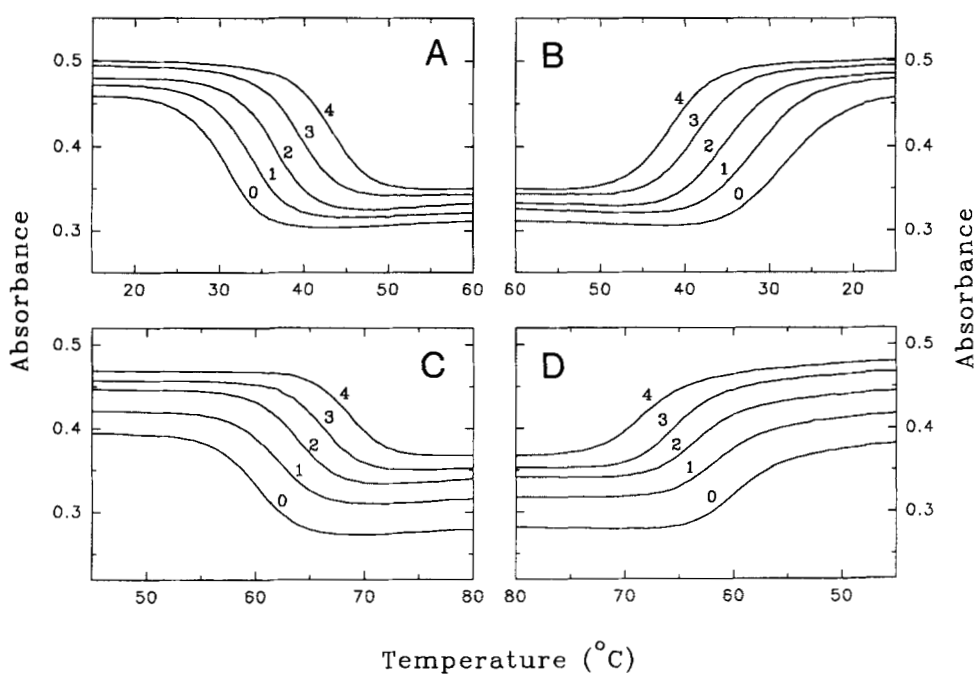

Fig. 1. Thermal transitions of RNase A. **A:** In a pH 2.0, 0.04 M glycine buffer: curves 0, 1, 2, 3, and 4 are for 0%, 10%, 20%, 30%, and 40% sorbitol solutions, respectively. **B:** Same as A. **C:** In a pH 5.5, 0.04 M sodium acetate buffer: curves 0, 1, 2, 3, and 4 are for 0, 10%, 20%, 30%, and 40% sorbitol solutions, respectively. **D:** Same as C.

Table 1. *Thermodynamic parameters of thermal denaturation of RNase A at pH 2.0 and pH 5.5 in sorbitol–water systems*

Sorbitol (w/v)	T_m (°C) Heating (unfolding)	T_m (°C) Cooling (refolding)	$\Delta H°$ at T_m (kcal/mol)	$\Delta G°$ (20 °C) (kcal/mol)	δ ($\Delta G°$) (48 °C) (kcal/mol)
pH 2.0					
0	30.1 ± 0.3	29.1 ± 0.4	72.83 ± 1	2.60 ± 0.3	0 ± 0.3[a]
10%	33.1	32.2	69.90	2.89	0.64
20%	36.3	35.5	73.60	3.72	1.20
30%	39.6 ± 0.3	39.0 ± 0.4	75.49 ± 5	4.61 ± 0.3	2.00 ± 0.4
40%	43.1	42.5	78.46	5.54	2.75
pH 5.5					
0	60.4 ± 0.2	59.8 ± 0.4	108.36 ± 6	13.60 ± 0.5	0 ± 0.5[a]
10%	62.0	61.6	112.53	14.10	0.63
20%	64.0	63.5	117.67	15.29	1.45
30%	66.6 ± 0.4	65.6 ± 0.5	124.91 ± 7	16.88 ± 0.3	2.52 ± 0.5
40%	68.6	68.2	129.60	18.28	3.62

[a]Values of $\Delta G°$ in water at 48 °C were −4.15 at pH 2.0 and 4.15 at pH 5.5.

in 30% sorbitol and between 17 °C and 42 °C in dilute buffer) permitted us to evaluate in a consistent manner $\Delta G°$ values at various temperatures between 20 °C and 48 °C. Because stabilization is expressed only by $\delta(\Delta G°) \equiv \Delta G_S^\circ - \Delta G_W^\circ$, errors introduced into the individual $\Delta G°$ values at a given temperature by the neglect of ΔC_p can be expected to cancel, because they would be in the same direction and of similar magnitude in dilute buffer and in the sorbitol solutions. The values of $\Delta G°$ at 20 °C obtained in this manner and of $\delta\Delta G°$ at 48 °C are listed in Table 1. As shown in the table, both the $\Delta G°$ and $\Delta H°$ values are higher at pH 5.5 than at pH 2.0. Most of this difference occurs for denaturation in water (dilute buffer). These values are similar to those reported previously for this protein in dilute aqueous buffer (Richards & Wyckoff, 1971; Pace, 1975).

The quantitative effect of sorbitol on RNase A stability was obtained by calculating the increment in the standard free energy change of denaturation, $\delta(\Delta G°)$ (see Equation 1), brought about by the addition of any given amount of sorbitol to the system. The values of $\delta(\Delta G°)$ at 48 °C are listed in the last column of Table 1.

Preferential interaction parameters

The thermodynamic stabilization of RNase by sorbitol, which is expressed quantitatively by its increase of the standard free energy of unfolding, $\delta\Delta G°$, reflects the changes in the interactions of the protein with the components of the solvent system during unfolding. In the absence of specific interactions at sites, this manifests itself through $\delta\Delta\mu_2$, i.e., the change in the transfer free energy, as expressed in Equation 1. The latter can be obtained directly by integration of the preferential interaction parameter, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, in the native ($N$) and denatured ($D$) states of the protein:

$$\delta\Delta\mu_2 = \int_0^{m_3} (\partial\mu_2/\partial m_3)^D_{T,P,m_2}\, dm_3 - \int_0^{m_3} (\partial\mu_2/\partial m_3)^N_{T,P,m_2}\, dm_3. \quad (3)$$

This operation requires that the preferential binding parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ be measured as a function of co-solvent concentration for the protein in both the native and denatured states (see Equation 16). Therefore, the preferential binding (dialysis equilibrium) of sorbitol to RNase A was measured at the set of conditions necessary to define $\delta\Delta\mu_2$ ($N \rightarrow D$). The auxiliary parameters characteristic of the sorbitol solutions were measured as described in Materials and methods. Their values are listed in Table 2.

Proper measurement of $\delta\Delta\mu_2$ requires that the preferential interactions be determined for both the native and the denatured states of the protein at identical temperatures, and, therefore, by necessity at different pH values. The appropriate conditions were established from the thermal transition results of Table 1 and Figure 1. The temperature chosen was 48 °C, because, at that temperature, RNase A is native at pH 5.5, but denatured at pH 2.0 at all the sorbitol concentrations used. This, in turn, necessitated the demonstration that the preferential interactions of sorbitol with native RNase A are identical at pH 2.0 and 5.5. To this end, dialysis

Table 2. *Characteristic parameters of sorbitol solutions*

Sorbitol (w/v)	g_3 (g/g)	m_3 (mol/1,000 g H_2O)	ρ_0 (g/mL)	$\bar{v}_3$ (mL/g)	γ_3[a]	$(\partial \ln \gamma_3/\partial m_3)$
10%	0.1070	0.587	1.0348 ± 0.002	0.651	1.008	0.004
20%	0.2301	1.263	1.0691 ± 0.002	0.654	1.023	0.021
30%	0.3736	2.050	1.1031 ± 0.002	0.656	1.047	0.030
40%	0.5431	2.990	1.1365 ± 0.003	0.659	1.093	0.044

[a]Data of Bonner (1982).

Table 3. *Partial specific volumes and preferential interaction parameters of native RNase A with sorbitol solutions at 20 °C, pH 5.5 and pH 2.0*

Sorbitol (w/v)	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]
pH 5.5							
10%	0.700 ± 0.001	0.720 ± 0.001	−0.0634 ± 0.003	+0.593 ± 0.030	−4.76 ± 0.2	+450 ± 22	+4,734 ± 237
20%	0.701 ± 0.001	0.729 ± 0.001	−0.0995 ± 0.004	+0.432 ± 0.015	−7.47 ± 0.3	+328 ± 12	+3,536 ± 126
30%	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
40%	0.703 ± 0.001	0.742 ± 0.003	−0.1766 ± 0.009	+0.325 ± 0.016	−13.26 ± 0.7	+247 ± 12	+2,923 ± 150
pH 2.0							
10%	0.698 ± 0.001	0.717 ± 0.001	−0.0602 ± 0.003	+0.563 ± 0.030	−4.52 ± 0.2	+428 ± 22	+4,496 ± 236
20%	0.699 ± 0.001	0.725 ± 0.001	−0.0924 ± 0.004	+0.402 ± 0.015	−6.94 ± 0.3	+306 ± 12	+3,285 ± 126
30%	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
40%	0.701 ± 0.002	0.739 ± 0.002	−0.1720 ± 0.009	+0.317 ± 0.016	−12.91 ± 0.7	+241 ± 12	+2,845 ± 150

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

[b] The values of $(\partial\mu_3/\partial m_3)_{T,P,m_2}$ at 20 °C: 994.6, 473.4, 301.6, and 220.4 cal-(mol of sorbitol)$^{-2}$ in 1,000 g H_2O for 10%, 20%, 30%, and 40% sorbitol solutions, respectively.

equilibrium experiments were performed on the native protein at pH 2.0 and pH 5.5, 20 °C as a function of sorbitol concentration. The results presented in Table 3 show an essential identity of interactions at the two pH values. This permitted identification of these parameters as those of the preferential interactions of sorbitol with native protein at room temperature (20 °C) for both pH 2.0 and 5.5. As a further control, measurements were done at pH 2.0 at the temperature of completion of the unfolding at each sorbitol concentration as determined by Figure 1. These conditions were: 10% sorbitol, 42 °C; 20% sorbitol, 45 °C; 30% sorbitol, 48 °C; 40% sorbitol, 51 °C. The partial specific volumes and preferential interaction parameters of RNase A with sorbitol at high temperature are presented in Table 4. The results of all these measurements show that the values of the preferential binding parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, are negative for the denatured protein at 48 °C, pH 2.0, and for the native protein at 20 °C, both at pH 2.0 and 5.5. This means that sorbitol is preferentially excluded from the protein domain at all these conditions. For the native protein at 48 °C, however, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ became positive at sorbitol concentrations above 20% (w/v), i.e., the co-solvent was preferentially bound to the

Table 4. *Partial specific volumes and preferential interaction parameters of native RNase A (pH 5.5, 48 °C) and denatured RNase A (pH 2.0 48 °C and 42–51 °C) with sorbitol*

Sorbitol (w/v)	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]
pH 5.5, 48 °C							
10%	0.699 ± 0.001	0.706 ± 0.004	−0.0222 ± 0.008	+0.207 ± 0.074	−1.67 ± 0.6	+157 ± 56	+1,820 ± 650
20%	0.698 ± 0.002	0.700 ± 0.005	−0.0071 ± 0.012	+0.031 ± 0.054	−0.53 ± 0.9	+24 ± 42	+275 ± 481
30%	0.699 ± 0.004	0.692 ± 0.006	+0.0279 ± 0.020	−0.075 ± 0.053	+2.09 ± 1.5	−57 ± 40	−691 ± 493
40%	0.698 ± 0.005	0.668 ± 0.009	+0.1195 ± 0.028	−0.220 ± 0.051	+8.97 ± 2.1	−167 ± 39	−2,166 ± 505
pH 2.0, 48 °C							
10%	0.700 ± 0.001	0.708 ± 0.006	−0.0254 ± 0.011	+0.237 ± 0.103	−1.91 ± 0.8	+180 ± 78	+2,081 ± 910
20%	0.698 ± 0.003	0.711 ± 0.008	−0.0462 ± 0.020	+0.201 ± 0.085	−3.47 ± 1.5	+153 ± 64	+1,800 ± 761
30%	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
40%	0.697 ± 0.005	0.713 ± 0.007	−0.0637 ± 0.024	+0.117 ± 0.044	−4.78 ± 1.8	+89 ± 33	+1,154 ± 432
pH 2.0, 42–51 °C							
10%	0.700 ± 0.001	0.710 ± 0.002	−0.0317 ± 0.005	+0.296 ± 0.044	−2.38 ± 0.4	+225 ± 33	+2,545 ± 381
20%	0.698 ± 0.002	0.713 ± 0.001	−0.0533 ± 0.005	+0.232 ± 0.023	−4.00 ± 0.4	+176 ± 17	+2,055 ± 205
30%	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
40%	0.696 ± 0.002	0.711 ± 0.005	−0.0597 ± 0.014	+0.110 ± 0.025	−4.48 ± 1.0	+84 ± 19	+1,092 ± 254

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

[b] Values of $(\partial\mu_3/\partial m_3)_{T,P,m_2}$ at 48 °C: 1,089.3, 518.6, 330.4, and 241.5 cal-(mol of sorbitol)$^{-2}$ in 1,000 g H_2O for 10%, 20%, 30%, and 40% sorbitol solutions, respectively; and 1,069.3 for 10% sorbitol at 42 °C, 513.8 for 20% sorbitol at 45 °C, 330.4 for 30% sorbitol at 48 °C and 243.8 cal-(mol sorbitol)$^{-2}$ for 40% sorbitol at 51 °C.

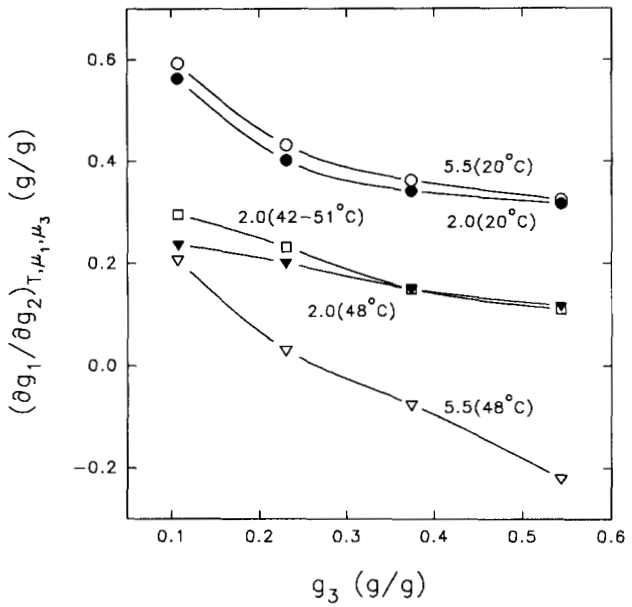

Fig. 2. Sorbitol concentration dependence of the preferential hydration for RNase A. (○) pH 5.5, 20 °C; (●) pH 2.0, 20 °C; (▽) pH 5.5, 48 °C; (▼) pH 2.0, 48 °C; (□) pH 2.0, 42–51 °C.

protein above 20% sorbitol. Thus, in 40% sorbitol, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ is +0.120 g/g at 48 °C, whereas at 20 °C it is −0.177 g/g. For the denatured protein, on the other hand, this parameter remained negative up to the highest value of co-solvent concentration used, −0.064 g/g in 40% sorbitol, 48 °C.

In Figure 2, the results of the dialysis equilibrium measurements are compared in terms of the preferential hydration parameter, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$. This brings out clearly the observations that: (1) at 20 °C, the protein is equally preferentially hydrated at pH 2.0 and 5.5; (2) when the temperature was raised to 48 °C, the preferential hydration of the native protein (pH 5.5) decreased sharply (by ca. 0.40 g water per g protein) to the point of assuming negative values above 20% (w/v) sorbitol; (3) denatured protein (pH 2.0) at 48 °C, on the other hand, maintained positive values of preferential hydration, albeit lower ones than those of native protein at 20 °C, over the entire concentration range of sorbitol. At all conditions, the preferential hydration decreased with sorbitol concentration, although this dependence was quite shallow for the denatured protein. What is the meaning of these results? In molecular terms, this means that, despite the preferential hydration, some sorbitol molecules interact with (bind very weakly[1] to) RNase A, i.e., that, as a time average, they occupy loci on the protein surface in a nonthermodynamically indifferent mode (Schellman, 1987a, 1987b, 1990, 1993; Timasheff, 1992, 1993). This binding is most extensive to the native protein at the elevated temperature (48 °C).

[1] In this context, binding does not mean complex formation at specific sites. Rather, it means the existence of weak attractive interactions between sorbitol molecules and loci on the protein molecules that favor sorbitol over water in the $P{\cdot}H_2O + S \rightleftharpoons P{\cdot}S + H_2O$ exchange reaction (Schellman, 1987a, 1990, 1993). The exchange equilibrium constants are of the order of 0.02 m^{-1} and the corresponding free energies of interaction per site are $<|0.05|$ kcal-mol^{-1}.

Binding of sorbitol

The extent of co-solvent binding[1] can be estimated if the extent of hydration is known. Preferential (dialysis equilibrium) binding is a thermodynamic quantity that describes the net global balance of protein surface site occupancy by water and co-solvent (Schellman, 1987a, 1987b, 1990, 1993; Timasheff, 1963, 1992, 1993). It is related to the actual numbers of the water and co-solvent molecules in contact with the protein surface as a time average by (Timasheff & Inoue, 1968; Inoue & Timasheff, 1972; Kupke, 1973; Reisler et al., 1977):

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = A_3 - g_3 A_1$$

$$\bar{\nu}_3 = (\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = B_3 - \frac{m_3}{m_1} B_1, \qquad (4)$$

where A_i is the total amount of component i that occupies loci on component 2, expressed in grams of component i per gram of component 2, and B_i is the same parameter in units of moles of i per mole of protein. Neither A_1 nor A_3 are thermodynamic quantities per se, because they are unresolvable parts of the exchange equilibria (Schellman, 1990; Timasheff, 1992). In molecular terms, therefore, any change in preferential binding reflects changes in the numbers of molecules of water and co-solvent in contact with loci on the protein surface. Making the plausible assumption as a first approximation that, in an aqueous solution of sorbitol, the protein retains the same amount of hydration as in pure water, the values of A_3 were calculated by Equation 4 using for A_1 the value of 0.355 grams water per gram protein, which had been determined from hydration data at a water activity of 0.92 and at 25 °C (Bull & Breese, 1968). Values of A_3 estimated in this manner actually correspond to the maximal amount of sorbitol present in the immediate domain of the protein. The results of the calculation are presented as a function of the sorbitol concentration in Figure 3. It is evident that, at all the conditions, the amount of sorbitol present within the solvation layer of the protein increases with increasing co-solvent concentration, which is consistent with the Law of Mass Action. This amount is very small for the native protein at 20 °C,

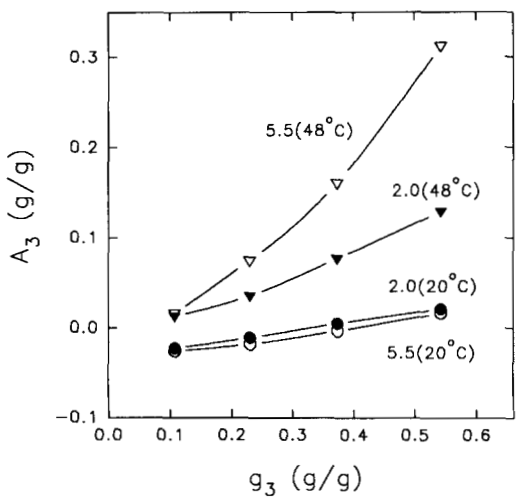

Fig. 3. Dependence of the total interaction, A_3, of sorbitol with RNase A on sorbitol concentration. (○) pH 5.5, 20 °C; (●) pH 2.0, 20 °C; (▽) pH 5.5, 48 °C; (▼) pH 2.0, 48 °C.

in fact not far from zero. At high temperature (48 °C), the amount of sorbitol in excess of bulk solvent composition increases in the solvation layer of the native protein and reaches a value of 0.3 gram per gram of protein at 40% sorbitol, which is 23.4 mol of sorbitol per mole of RNase A.[2] For the denatured protein, the similar increase in sorbitol binding is approximately half that for the native protein. As a consequence, the denatured protein is still preferentially hydrated at 48 °C, whereas the native protein preferentially binds the polyol. This is the molecular description of the source of the stabilization. What is it in thermodynamic terms?

Free energy of interaction

In thermodynamic terms, when the reference state is a solvent of a given composition, stabilization is determined by the difference of the preferential interaction parameters, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, of the protein in the native and denatured forms. When the reference state is water, it is determined by the difference between the transfer free energies, $\Delta\mu_2$, of the protein in the two forms as expressed in Equation 1. To calculate $\Delta\mu_2$, the values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ determined under the various conditions were plotted as a function of m_3, fitted to quadratic functions in sorbitol concentration and integrated according to Equation 3. The corresponding data fitting equations obtained were:

Native protein (pH 2.0, 20 °C):

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = 5.73 - 2.48m_3 + 0.51m_3^2 \text{ (r.c: } -0.97)$$

$$\Delta\mu_2^N = 5.73m_3 - 1.24m_3^2 + 0.17m_3^3. \quad (5)$$

Native protein (pH 5.5, 20 °C):

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = 5.95 - 2.42m_3 + 0.47m_3^2 \text{ (r.c: } -0.97)$$

$$\Delta\mu_2^N = 5.95m_3 - 1.21m_3^2 + 0.16m_3^3. \quad (6)$$

Native protein (pH 5.5, 48 °C):

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = 3.00 - 2.23m_3 + 0.17m_3^2 \text{ (r.c: } -0.97)$$

$$\Delta\mu_2^N = 3.00m_3 - 1.12m_3^2 + 0.06m_3^3. \quad (7)$$

Denatured protein (pH 2.0, 48 °C):

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = 2.45 - 0.64m_3 + 0.06m_3^2 \text{ (r.c: } -0.97)$$

$$\Delta\mu_2^D = 2.45m_3 - 0.32m_3^2 + 0.02m_3^3. \quad (8)$$

For the denatured state at the temperature that corresponds to the end of the transition at the different sorbitol concentrations (the data from Table 4), the similar plots were

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = 3.21 - 1.16m_3 + 0.15m_3^2 \text{ (r.c: } -0.97)$$

$$\Delta\mu_2^D = 3.21m_3 - 0.58m_3^2 + 0.05m_3^3. \quad (9)$$

[2]The degree of protein hydration is expected to depend on temperature. Although there are no data for 48 °C similar to those at 25 °C (Bull & Breeze, 1968), decrease of the hydration by a factor of two from the value at 25 °C reduces the 48 °C values of A_3, but does not alter the qualitative conclusion. For bovine serum albumin, A_1 increases with temperature (Gekko & Morikawa, 1981a).

The resulting values of the transfer free energy of the protein from water to the sorbitol solutions ($\Delta\mu_2 = \mu_S - \mu_W$) are plotted as a function of m_3 in Figure 4. All the values of $\Delta\mu_2$ are positive, which indicates that the interaction of RNase A, in both the native and denatured states, with the aqueous sorbitol system is thermodynamically unfavorable when water is taken as the reference state, at least up to the highest concentration of the polyol used. These interactions are increasingly unfavorable for the native protein at 20 °C, both at pH 5.5 and at 2.0 (curves 1 and 2 in Fig. 4). The same is true of the denatured protein at 48 °C (curve 3) (curve 4, obtained at varying temperatures, is, in fact, very similar to that measured at 48 °C). In contrast, the behavior of the native protein at 48 °C, pH 5.5 (curve 5), is bell shaped. The interaction attains a maximally unfavorable state at ca. 1.5 molal sorbitol and then decreases again, tending toward $\Delta\mu_2 = 0$ at some concentration above 3.5 m. This reflects the increasing site occupancy by sorbitol at pH 5.5, 48 °C. The essential identity of the $\Delta\mu_2$ dependence on sorbitol concentration at 20 °C, pH 2.0 and 5.5, indicates essential identity of the state of the protein surface at the two pH values. A test of this identity in the denatured state was performed by translating the data obtained at 20 °C (pH 2.0) to 48 °C and comparing it with the pH 5.5 measurements at that temperature. This was based on the observation that, for native RNase A (pH 5.5), $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ decreased by essentially a constant value when the temperature was changed from 20 °C to 48 °C. Taking this difference at 10, 20, and 30% sorbitol (40% sorbitol was not used due to the large uncertainty in the 48 °C value), an average value of the difference was obtained as 3.3 kcal/(mol protein-mol sorbitol). The resulting values calculated for native protein at pH 2.0, 48 °C were fitted by the quadratic function and its integrated form:

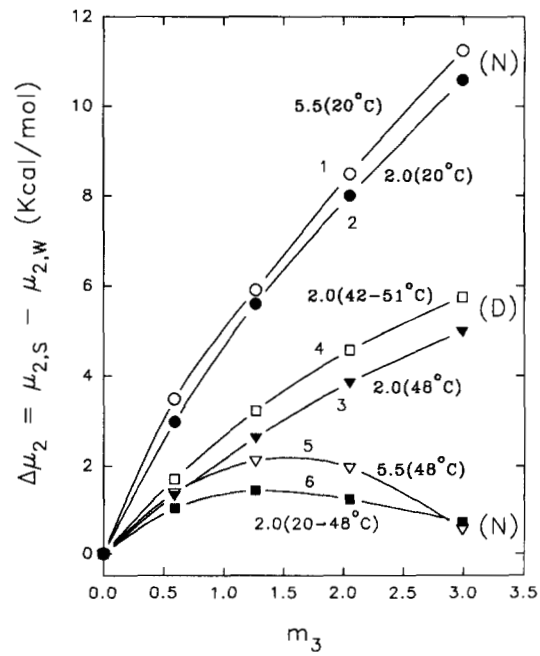

Fig. 4. Chemical potential change of RNase A on transferring it from water to an aqueous solution of sorbitol. Curve 1 (○) pH 5.5, 20 °C; curve 2 (●) pH 2.0, 20 °C; curve 3 (▼) pH 2.0, 48 °C; curve 4 (□) pH 2.0, 42–51 °C; curve 5 (▽) pH 5.5, 48 °C; and curve 6 (■) pH 2.0, calculated at 48 °C by translating 20 °C data (see text).

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = 2.43 - 2.48m_3 + 0.15m_3^2 \text{ (r.c: } -0.97)$$

$$\Delta\mu_2 = 2.43m_3 - 1.24m_3^2 + 0.17m_3^3. \quad (10)$$

The resulting curve (curve 6 of Fig. 4) was similar in character to that measured at pH 5.5, 48 °C.

Discussion

RNase stabilization by sorbitol

The results of the present study show that the stabilization of RNase A by sorbitol is determined by the change in preferential interactions during the protein unfolding reaction. At 20 °C, the protein is identically preferentially hydrated at pH 2.0 and 5.5. At higher temperature (48 °C), the preferential hydration decreases. Unfolded RNase A remains preferentially hydrated up to 40% (w/v) sorbitol (the highest concentration used). For the native protein, however, at 48 °C, preferential hydration changes to preferential binding of sorbitol above 20% (w/v). These observations are consistent with earlier studies, which have shown that, in polyhydric alcohol–water mixtures (glycerol, ethylene glycol, xylitol, mannitol, sorbitol, and inositol) (Gekko & Morikawa, 1981a, 1981b; Gekko & Timasheff, 1981a; Na & Timasheff, 1981) and sugars (sucrose, lactose, and glucose) (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982), all the proteins examined were preferentially hydrated in the native state at 20 °C. No such measurements have been done with denatured proteins up to now, except for one point with polyethylene glycol 1000 (Lee & Lee, 1987). When judged by the effect on T_m, the stabilization of RNase A by sorbitol appears to be greater at pH 2.0 than at pH 5.5. When stabilization is assessed by the change in the standard free energy of unfolding, $\delta\Delta G°$, its values are somewhat greater at pH 5.5 than at pH 2.0. Thus, the effect appears to be opposite of that on T_m. This divergence stems from the difference between the enthalpies of denaturation at the two pH values, which must reflect the difference in the state of ionization of the protein. The isoelectric point of RNase A being 9.6, at pH 2.0 all the acidic groups are protonated, which renders the protein highly positively charged. Hydrodynamically, RNase A is slightly expanded at pH 2.0 (Tanford & Hauenstein, 1956; Richards & Wyckoff, 1971). This evidently does not affect the preferential hydration of the protein measured at 20 °C because the values at pH 2.0 and 5.5 are identical. This result also means that a change in the ionization of carboxyl groups has no effect on the preferential exclusion of sorbitol from the protein surface. The identity of the preferential interactions at the two pH values for the native protein at 20 °C led to the assumption that this identity would hold at 48 °C as well, and permitted the direct comparison at 48 °C of native protein at pH 5.5 with denatured protein at pH 2.0.

Stabilization is caused by general nonspecific interactions

The effect of a co-solvent on protein stability can be examined with respect to two reference states: (1) solvent of a given composition, or (2) water. When the reference state is solvent of a given composition, the stabilizing effectiveness of the co-solvent is expressed through the mutual perturbation of the chemical potentials of the protein and the solvent system, $(\partial\mu_3/\partial m_2)_{T,P,m_3} = (\partial\mu_2/\partial m_3)_{T,P,m_2}$. The positive values of the preferential interaction parameter, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, listed in column 8 of Tables 3 and 4 for the native protein at 20 °C and the denatured protein at 48 °C, indicate an unfavorable thermodynamic interaction between protein and sorbitol at all solvent compositions. For the native protein at 48 °C, however, the same is true only up to 20% sorbitol, above which further addition of sorbitol reverses the effect and any infinitesimal increase in solvent composition renders the thermodynamic interaction favorable. Quantitatively, the effect on the unfolding reaction at any given solvent composition with reference to that solvent composition is expressed by the linkage equation (Wyman, 1964):

$$-\left(\frac{d\Delta G°}{d\mu_3}\right) = \left(\frac{d\ln K}{d\ln a_3}\right) = \left(\left(\frac{\partial\mu_2}{\partial m_3}\right)^N_{T,P,m_2} - \left(\frac{\partial\mu_2}{\partial m_3}\right)^D_{T,P,m_2}\right)\Bigg/\left(\frac{\partial\mu_3}{\partial m_3}\right)_{T,P,m_2}, \quad (11)$$

where a_3 is the activity of the co-solvent, $a_3 = m_3\gamma_3$.

Comparison of the values of the preferential interaction parameter for the native and denatured protein at 48 °C (Tables 3, 4) shows that, at all sorbitol concentrations, the value for the denatured protein is more positive than that for the native protein. Therefore, addition of the co-solvent lowers the equilibrium constant and, hence, displaces the $N \rightleftharpoons D$ equilibrium toward the native state. For example, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ was 1.8 and 1.4 kcal/(mol protein-mol sorbitol) for denatured protein in 20% or 30% sorbitol (pH 2.0, 48 °C), but only 0.3 and −0.7 kcal/(mol protein-mol sorbitol) for the native protein (pH 5.5, 48 °C) (Table 4, last column). In other words, at both co-solvent concentrations, contact of sorbitol with the denatured state is thermodynamically more unfavorable than with the native state. Application of Equation 11 results in a stabilizing power of −2.9 kcal/(mol protein-mol sorbitol in 1,000 g water) for 20% sorbitol and −6.3 kcal/(mol protein-mol sorbitol in 1,000 g water) for 30% sorbitol with reference to the given solvent composition.

The more positive gradient of protein chemical potential with the addition of sorbitol for the denatured protein than that for the native one means, by Equation 3, that at any solvent composition the free energy of interaction of the protein with the solvent system, relative to water as reference state, must be more positive for the denatured state than for the native state. If the conformations of the native and the unfolded states of RNase A are not affected by addition of sorbitol and the total effect of the co-solvent is expressed through the transfer free energy, then $\Delta G°_W + \Delta\mu_2^D = \Delta\mu_2^N + \Delta G°_S$ (see Equation 1), and the thermodynamic box must close. Now, if $\Delta\mu_2^D > \Delta\mu_2^N$, then $\Delta G°_S > \Delta G°_W$, and, in the presence of sorbitol, more free energy must be expended to unfold the protein. Let us test the conformity of the present data to this situation by calculating the proper thermodynamic parameters. The thermal denaturation data at pH 2.0, gave $\Delta G°_W = -4.15$ and $\Delta G°_S = -2.15$ kcal/mol for RNase A in water and in the 30% sorbitol solution at 48 °C, respectively. On the other hand, integration of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ gave for 30% sorbitol and 48 °C, $\Delta\mu_2^D = 3.85$ kcal/mol (pH 2.0) and $\Delta\mu_2^N = 1.96$ kcal/mol (pH 5.5) (calculated with Equations 8 and 7, respectively). These measured values give for RNase A in the 30% sorbitol solution:

$$\Delta G°_W + \Delta\mu_2^D = -4.15 + 3.85 = -0.30 \text{ kcal/mol},$$

$$\Delta G°_S + \Delta\mu_2^N = -2.15 + 1.96 = -0.19 \text{ kcal/mol},$$

or, rearranging,

$$\Delta\mu_2^D - \Delta\mu_2^N = 3.85 - 1.96 = 1.89 \text{ kcal/mol},$$

$$\Delta G_S^\circ - \Delta G_W^\circ = -2.15 - (-4.15) = 2.00 \text{ kcal/mol}.$$

These results, plotted in the form of a thermodynamic box in Figure 5, show that it closes within 0.11 kcal/mol. Therefore, the change in transfer free energy on denaturation ($\Delta\mu_2^D - \Delta\mu_2^N$) can account fully for the stabilization of RNase A structure by addition of sorbitol ($\Delta G_S^\circ - \Delta G_W^\circ$). This permits us to conclude that the effect of sorbitol on the unfolding of RNase A is of a strictly nonspecific thermodynamic nature. There is no evidence of any specific conformational effect or other reactions induced in the protein, as all the stabilizing free energy can be accounted for by the weak interactions with the water–sorbitol system expressed through preferential binding (exclusion).

If the same calculation is redone using the data obtained at pH 5.5, the appropriate parameters are $\Delta G_W^\circ = 4.15$ and $\Delta G_S^\circ = 6.67$ kcal/mol for RNase A in water and the 30% sorbitol solution at 48 °C, respectively. Then

$$\Delta G_W^\circ + \Delta\mu_2^D = 4.15 + 3.85 = 8.00 \text{ kcal/mol},$$

$$\Delta G_S^\circ + \Delta\mu_2^N = 6.67 + 1.96 = 8.63 \text{ kcal/mol},$$

or, rearranging,

$$\Delta\mu_2^D + \Delta\mu_2^N = 3.85 - 1.96 = 1.89 \text{ kcal/mol},$$

$$\Delta\mu_S^\circ + \Delta\mu_W^\circ = 6.67 - 4.15 = 2.52 \text{ kcal/mol}.$$

The thermodynamic box closes again within 0.63 kcal/mol, which is within experimental error in view of the integrations needed to obtain the $\Delta\mu_2$ values. In general, comparison of the differences between the transfer free energies of the denatured and native proteins with the $\delta\Delta G^\circ$ values shows a general overlap of the two sets of parameters, seeing the spread in their values (Table 1; Fig. 4).

Why is sorbitol preferentially excluded?

As a conclusion, it seems desirable to ask the question: What are the sources of the preferential exclusion of sorbitol from the protein surface and why does it change as it does on denaturation? This can be considered in light of Equation 4, i.e., the decomposition of the preferential interaction (thermodynamic binding) into contacts at protein surface loci of water and sorbitol molecules, respectively. Ribonuclease has a high ratio of polar amino acid residues to nonpolar ones (1.73) and a very low hydrophobicity (Bigelow, 1967). With such a high polarity, RNase A has a very hydrophilic surface. It has been shown that glycerol has an affinity for polar regions on a protein surface (Gekko & Timasheff, 1981a). Similarly, sorbitol molecules, being hydrophilic co-solvents, should have an affinity for the polar residues on the protein surface. Because these interactions with sorbitol would be weaker than with water molecules, the exchange reaction (Schellman, 1987a, 1990) would favor preferential hydration. The remaining fraction of the protein surface is nonpolar and is occupied by atoms that have no ability to form hydrogen bonds (Bull & Breese, 1968). Sorbitol molecules should be excluded preferentially from these loci because sorbitol, just as glycerol, is a solvophobic agent (Gekko & Morikawa, 1981a). Furthermore, the steric exclusion principle (Kauzmann, as quoted in Schachman & Lauffer, 1949) should make the distance of closest approach of a co-solvent with a large molecular volume greater than that of water. As a result, the volume fraction occupied by sorbitol at the surface of the macromolecule should be less than that in the bulk solvent. Thermodynamically, this manifests itself as preferential hydration. Therefore, the preferential hydration of proteins in sorbitol–water mixtures may be regarded as the result of the delicate balance between various weak interactions.

On denaturation, the surface of the protein increases. This increase is accompanied by the exposure of nonpolar residues buried in the interior of a globular protein and by the breaking of peptide–peptide hydrogen bonds. The resultant increase in the nonpolar residues exposed to solvent and hydration of the peptide bonds would be translated into enhanced preferential hydration of the denatured protein relative to the native protein, which would account for the observed change in preferential interactions on unfolding. These concepts were probed further by an examination of the temperature dependence of the preferential interactions of RNase A with co-solvents. These studies are described in the companion paper (Xie & Timasheff, 1997).

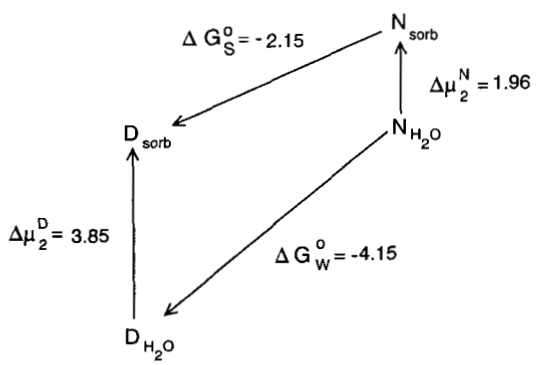

Fig. 5. Thermodynamic box of the stabilization of RNase A by 30% sorbitol at 48 °C (the values are in kcal/mol).

Materials and methods

Materials

D-sorbitol (lot 28F-0870), glycine (reagent grade), and RNase A (type IIA from bovine pancreas) were purchased from Sigma. The protein was further purified on a Sulfoethyl–Sephadex c-25 column in 0.1 M phosphate buffer, pH 6.47, following essentially the procedure of Crestfield et al. (1962, 1963). The fractionated protein was collected at about 60 effluent mL from a 0.9 × 60-cm column. It was then subjected to exhaustive dialysis against deionized water at 4 °C, and finally lyophilized. SDS electrophoresis indicated that the purified RNase A consisted of only one band, whereas unpurified RNase A and RNase A that had only been dialyzed against water and then lyophilized showed two bands. Hence, the purified RNase A was homogeneous, whereas the starting material was not, and dialysis against water was not sufficient to purify the protein. The molecular porous membrane tubing (23 × 100, M.W. cutoff: 6,000–8,000) was purchased from Spec-

trum. Fisher standardized 1 N HCl solution was used for the pH adjustment of solutions.

All solutions contained 0.04 M glycine at pH 2.0, or 0.04 M sodium acetate at pH 5.5. The protein concentrations were determined by UV absorbance on a Perkin-Elmer UV/Vis lambda 3B spectrophotometer. The extinction coefficients were obtained by gravimetric measurements. The extinction coefficients for RNase A [dL/(g·cm)] at 277 nm in various concentrations of sorbitol were found to be 7.03, 7.10, 7.20, 7.27, and 7.34 for 0, 10%, 20%, 30%, and 40% sorbitol at pH 2.0; and 7.12, 7.18, 7.23, 7.27, and 7.34 for 0, 10%, 20%, 30%, and 40% sorbitol at pH 5.5.

Thermodynamics of thermal denaturation

The change in absorbance of the protein with change of temperature was followed on a Gilford Response II UV/Vis spectrophotometer. In all experiments, 1 mg/mL RNase was used. The solutions were degassed for 3–5 min under reduced pressure to prevent bubble formation during heating. The wavelength used was 287 nm. The rate of heating was ~0.35 °C per min. Because the interaction measurements required the protein solutions to be kept at high temperature, e.g., 48 °C, for 20 h, control experiments were conducted. In these, the protein solutions in the presence of trehalose were kept at the high temperature for 20 h both at pH 2.0 and 5.5. They were then cooled for 2 h and subjected to the thermal transition measurements. The transition curves obtained were found to be fully reversible and identical with those of untreated protein, showing that no irreversible changes had taken place. Furthermore, turbidity measurements at 350 nm showed that heating for 20 h had no effect.

Preferential interactions

The preferential interactions of the solvent components with the protein were calculated from the apparent partial specific volumes of the protein measured with a Precision DMA-02 density meter (Anton Paar, Gratz). Density measurements are measurements of co-solvent concentration. At any given protein concentration, the solution density was measured with and without dialysis against the solvent system. Dialysis adjusts the cosolvent concentration inside the bag as a consequence of its interaction with the protein, because at chemical equilibrium the chemical potential of sorbitol must be identical inside and outside the bag (isopotential conditions). The difference between the two values of the apparent partial specific volume measured with (isopotential) and without (isomolal) dialysis gives the extent of binding (or exclusion) of the co-solvent to (from) the protein. Therefore, at each protein concentration, parallel experiments were performed. In one, the protein was dialyzed against the solvent system at the chosen temperature for a given length of time (20 h). In the other, it was subjected to exactly the same regimen (duration of exposure to the given temperature, e.g., 20 h at 48 °C). This resulted in apparent partial specific volumes at conditions at which, respectively, the chemical potential of sorbitol or its molality were equal in the solvent and the protein solution. All density measurements were made at 20 °C and the general procedures used were similar to those described previously (Lee & Timasheff, 1974, 1981; Lee et al., 1979; Arakawa at al., 1990a). Solutions that contained sorbitol at the various concentrations were filtered through a sintered-glass filter. In constant molality experiments, six samples (6–20 mg) of RNase were dried in small glass test tubes over phosphorus pentoxide at 40 °C for 2 days in a vacuum oven. After being cooled to room temperature, about 1 mL of sorbitol solution was added and the tubes were sealed immediately and tightly with Parafilm and left to stand overnight at the designated experimental temperatures. If the samples were to be kept in a heated water bath, the tubes had to be sealed with five layers of Parafilm and two layers of Saran Wrap to prevent the Parafilm from breaking at high temperature. The samples were then equilibrated at 20 °C for 3–4 h before the density measurements. In constant chemical potential experiments, i.e., dialysis equilibrium with the co-solvent, each of the seven samples of protein at concentrations between 8 and 25 mg/mL was dissolved in about 1.1 mL of sorbitol solution and transferred into a dialysis bag that had been cleaned by boiling in deionized water and then rinsed in running deionized water. These samples were dialyzed at the given temperature for about 20 h against 800–1,000 mL of sorbitol solution. The samples were then transferred into small tubes, sealed tightly to avoid evaporation, and kept for 3–4 h at 20 °C prior to densimetry. After the density measurement, each protein solution was diluted gravimetrically with solvent to a final optical density of about 0.7 (~1 mg/mL) and the concentration was determined spectrophotometrically. Repeated measurements of individual apparent partial specific volume values gave good reproducibility. In a control experiment, RNase A solutions in 30% sorbitol both at pH 2.0 and 5.5 were dialyzed for 20 h at 48 °C. The temperature was then lowered to 20 °C and dialysis was continued for 10 h. The resulting values of the apparent partial specific volumes were found to be close to identical with those measured after dialysis at 20 °C, without heating. This showed that the system was fully reversible.

Calculation of the preferential interaction parameters

The apparent partial specific volume of a protein, ϕ, is related to density measurements by (Kielley & Harrington, 1960):

$$\phi = (1/\rho_0)[1 - (\rho - \rho_0)/c], \tag{12}$$

where ρ and ρ_0 are the densities of the protein solution and reference solvent, respectively, and c is the concentration of protein in grams/mL. The apparent partial specific volumes, ϕ_2 and ϕ_2', were 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. Setting component 1 = water, component 2 = protein, component 3 = additive (here sorbitol), in accordance with the notation of Scatchard (1946) and Stockmayer (1950), the preferential binding, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, is calculated from (Casassa & Eisenberg, 1964; Cohen & Eisenberg, 1968):

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = \rho_0(\phi_2^\circ - \phi_2'^\circ)/(1 - \rho_0\bar{v}_3), \tag{13}$$

where g_i is the concentration of component i in grams per gram of water, T is the thermodynamic (Kelvin) temperature, μ_i is the chemical potential of component i, and $\bar{v}_3$ is the partial specific volume of component 3. The measured values of ρ_0 and $\bar{v}_3$ are listed in Table 2. The superscript 0 indicates extrapolation to zero protein concentration. The preferential binding parameter is a measure of the excess of component 3 in the immediate domain of the protein over its concentration in the bulk solvent. Within a negligible approximation, it is equal to the binding measured by dialysis equilibrium and related techniques (Stigter, 1960). A positive value

of this parameter indicates an excess of component 3; a negative value means a deficiency of component 3, i.e., an excess of water, component 1, in the domain of the protein. The corresponding preferential hydration parameter, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, is (Timasheff & Kronman, 1959; Inoue & Timasheff, 1972; Reisler et al., 1977):

$$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3} = -\frac{1}{g_3}\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}. \quad (14)$$

Expressed in units of moles of ligand bound per mole of protein, which is $\bar{\nu}_3$ in Scatchard (1949) notation, the preferential binding is:

$$\bar{\nu}_3 \equiv (\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (M_2/M_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}, \quad (15)$$

where m_i is the molality of component i and M_i is its molecular weight. Thermodynamically, the measured preferential binding parameter (dialysis equilibrium binding) is a measure of the readjustment of solvent component concentrations in the domain of the protein due to the mutual perturbations of the chemical potentials of components 2 and 3 (Kirkwood & Goldberg, 1950; Casassa & Eisenberg, 1961, 1964):

$$\begin{aligned}(\partial\mu_2/\partial m_3)_{T,P,m_2} &= (\partial\mu_3/\partial m_2)_{T,P,m_3} \\ &= -(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_3/\partial m_3)_{T,P,m_2} \\ &= -nRT[1/m_3 + (\partial \ln \gamma_3/\partial m_3)_{T,p,m_2}] \\ &\quad \times (\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}, \end{aligned} \quad (16)$$

where R is the universal gas constant, n is the number of particles into which co-solvent dissociates (it is 1 for sorbitol), and γ_i is the activity coefficient of component i. The values of the pertinent thermodynamic parameters of sorbitol solutions are listed in Table 2. Detailed descriptions of these measurements and calculations are given in Lee et al. (1979) and Timasheff and Arakawa (1988).

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