Why Preferential Hydration Does Not Always Stabilize the Native Structure of Globular Proteins†,‡

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ABSTRACT: The observed preferential hydration of proteins in aqueous $MgCl_2$ solutions at low pH and low salt concentration (Arakawa et al., 1990) prompted a scrutiny of possible protein stabilization by $MgCl_2$ under the same conditions, in view of earlier observations in aqueous solutions of sugars, amino acids, and a number of salts that preferential hydration is usually accompanied by the stabilization of the native structure of globular proteins. The results of thermal transition experiments on five proteins (ribonuclease A, lysozyme, β-lactoglobulin, chymotrypsinogen, and bovine serum albumin) revealed neither significant stabilization nor destabilization of the protein structures by $MgCl_2$ both at acid conditions (except for ribonuclease A, which was stabilized, but to a much smaller extent than by $MgSO_4$) and at higher pH at which $MgCl_2$ displayed little preferential hydration. This was in contrast to the great stabilizing action of $MgSO_4$ at the same conditions. 2-Methyl-2,4-pentanediol (MPD), which gives a very large preferential hydration of native ribonuclease A at pH 5.8 [Pittz & Timasheff (1978) *Biochemistry 17*, 615-623], was found to be a strong destabilizer of that protein at the same conditions. Analysis of the preferentially hydrating solvent systems led to their classification into two categories: those in which the preferential hydration is independent of solution conditions and those in which it varies with conditions. The first always stabilize protein structure, while the second do not. In the first category the predominant interaction is that of cosolvent exclusion, determined by solvent properties, with the protein being essentially inert. In the second category interactions are determined to a major extent by the chemical nature of the protein surface. This gives rise to a fine balance between exclusion and binding of the cosolvent. The binding, being dependent on the chemical nature of the surface of the protein in contact with solvent, can be enhanced on protein unfolding due to the exposure of additional hydrophobic sites and peptide bonds, as well as to the decrease in electrostatic free energy of the protein. Therefore, the fact that a protein in the native state is preferentially hydrated in a given solvent system cannot be used as a criterion of structural stabilization. In contrast, the universally maintained correlation between preferential hydration and protein salting-out is the consequence of the immutability of the chemical nature of the protein surface on precipitation or crystallization: the patterns of preferential interaction remain the same in the two end states, protein in solution and in the solvated solid state.

In the preceding paper (Arakawa et al., 1990), it was shown that the solubility of proteins in aqueous $MgCl_2$ solutions is determined by the preferential interactions between solvent components and proteins in these systems. Under the limited conditions at which the interaction was that of preferential hydration, the effect was salting-out. From these results it might be expected that $MgCl_2$ would also stabilize proteins at the same conditions, even though it is, in general, ineffective as a protein stabilizer (von Hippel & Schleich, 1969; Collins & Washabaugh, 1985). The present study was undertaken, therefore, to examine the effect of $MgCl_2$ on protein stability at acid pH and at salt concentrations of ca. 0.5-1.5 M, conditions at which it induced preferential hydration of proteins (Arakawa et al., 1990). The results were compared with those obtained with $MgSO_4$ at acid pH, as well as those for both salts at pH 5.5, where they show a large difference in their preferential interactions with proteins (Arakawa & Timasheff, 1984a). In addition, the effect of 2-methyl-2,4-pentanediol (MPD)[1] on protein stability was examined, in view of the very strong preferential hydration of ribonuclease A in its presence (Pittz & Timasheff, 1978) and the prediction that MPD should be a protein denaturant (Pittz & Bello, 1971).

Materials and Methods

The proteins used were bovine serum albumin (BSA)[1] (lot 80F-9340), β-lactoglobulin (β-LG) (lot 106C-8070), ribonuclease A (RNase A) (lots 87C-0207 and 124F-0334), and chymotrypsinogen (CTG) (lot 66C-8125) from Sigma and lysozyme (lot 31A-993) from Worthington. $MgCl_2$ and $MgSO_4$ were of reagent grade. MPD was purified according to Bellow and Nowoswiat (1965).

$MgCl_2$ solutions were prepared by diluting a stock solution made from a fresh bottle. The pH of the salt solutions was adjusted to 5.5 in 0.04 M acetate and to 2.8, 2.0, and 1.5 in 0.04 M glycine hydrochloride buffers. The MPD solution was made in 0.01 M acetate (pH 5.8) containing 0.02 M NaCl.

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‡ This paper and the preceding one are dedicated to Professor Charles Tanford on the occasion of his retirement.
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[1] Abbreviations: BSA, bovine serum albumin; β-LG, β-lactoglobulin; RNase A, ribonuclease A; CTG, chymotrypsinogen; Gdn-HCl, guanidine hydrochloride; MPD, 2-methyl-2,4-pentanediol; PEG, poly(ethylene glycol).

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Table I: Effects of $MgCl_2$ and $MgSO_4$ on the Thermal Denaturation of Lysozyme

	transition midpoint T_m (°C)			
	pH 2.8[a]			pH 5.5
	0.6 M Gdn-HCl	0.9 M Gdn-HCl	1.2 M Gdn-HCl	1.2 M Gdn-HCl
control	56	53	51	63
0.6 M $MgCl_2$	56	53	51	63
1.2 M $MgCl_2$	(48)[b]	(46)[b]	51	63
1.5 M $MgCl_2$			52	
0.188 M $MgSO_4$			54	
0.375 M $MgSO_4$			58	
0.6 M $MgSO_4$	66		60	71
1.2 M $MgSO_4$	(64)[b]		(61)[b]	77

[a] In the absence of Gdn-HCl and no salt added, the transition occurred at 68 °C. [b] Turbidity occurred before the appearance of a transition (the number given in parentheses corresponds to the initial temperature of the absorbance increase).

The proteins were dissolved at high concentration in the appropriate dilute buffer, and an aliquot was delivered into a volumetric flask. Concentrated salt solutions, or pure MPD, and, if necessary, 6 M Gdn-HCl were then delivered to their desired final concentrations, and the flask was filled to the mark with the dilute buffer. The final protein concentrations for the transition experiments were 1 mg/mL for BSA, β-LG, and RNase A and 0.5 mg/mL for lysozyme and CTG. Protein concentrations were determined spectrophotometrically on a Cary Model 118 instrument by using absorptivity values [dL/(g·cm)] of 6.58 at 278 nm for BSA (Noelken & Timasheff, 1967), 27.4 at 281 nm for lysozyme (Roxby & Tanford, 1971), 9.6 at 278 nm for β-LG (Townend et al., 1960), 7.38 at 278 nm for RNase A (Scott & Scheraga, 1963), and 19.7 at 282 nm for CTG (Jackson & Brandts, 1970).

For the transition experiments, the protein solutions were passed through a Millipore filter (0.22-μm pore size), and helium gas was bubbled through them to avoid the formation of air bubbles on increasing the temperature. The change in absorbance with increasing temperature was followed on a Gilford Model 2600 spectrophotometer with a thermoprogrammer at a temperature scan rate of 0.25 °C/min and on a Gilford Response II UV/vis spectrophotometer at a scan rate of 1.2 °C/min. In all experiments, a control protein solution without additive was run in parallel with two sample solutions. The wavelengths used were 287 nm for BSA, β-LG, and RNase A, 293 nm for CTG, and 301 nm for lysozyme. In the cases in which part or all of the transition was masked by the appearance of turbidity, the transition was taken as occurring at, or above, the temperature at which turbidity appeared. When there was no change in absorbance up to the highest temperature measurable, the transition was regarded as completed below the initial temperature of the experiment. All protein solutions were transparent at room temperature, i.e., before heating, indicating that turbidity observed at higher temperatures reflected either the temperature dependence of the solubility of the native protein or the decreased solubility of the denatured protein, the onset of turbidity being equated with the initiation of the denaturation. A small amount of Gdn-HCl (0.6 or 1.2 M) was added when the transition temperature was higher than the measurable range in the available equipment or the small change in absorbance due to unfolding was masked by precipitation.

The preferential interaction measurements were carried out and the data analyzed as described in the preceding paper (Arakawa et al., 1990). For unfolded RNase A in 0.6 M $MgCl_2$, pH 1.5, the value of the isopotential apparent partial specific volume was obtained by dialyzing the protein for 20 h at 53 °C, removing the dialysis bag from the buffer, and cooling the protein solution to 20 °C prior to the density measurement at that temperature.

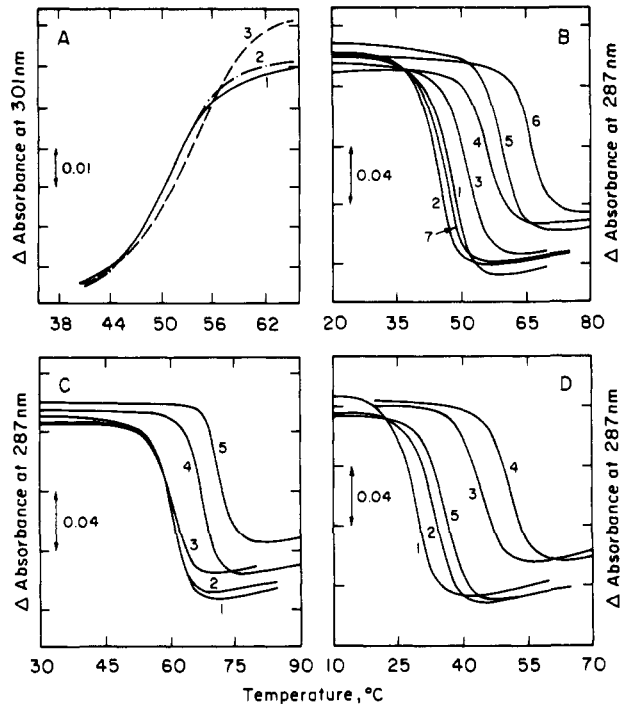

FIGURE 1: Thermal transitions of lysozyme and RNase A. (A) Lysozyme, pH 2.8: curve 1, no salt; curve 2, 0.6 M $MgCl_2$; curve 3, 1.2 M $MgCl_2$. (All the experiments with lysozyme were in the presence of 1.2 M Gdn-HCl.) (B) RNase A, pH 2.8: curve 1, no salt; curve 2, 0.1 M $MgCl_2$; curve 3, 0.6 M $MgCl_2$; curve 4, 1.2 M $MgCl_2$; curve 5, 0.6 M $MgSO_4$; curve 6, 1.2 M $MgSO_4$; curve 7, 0.3 M NaCl. (C) RNase, pH 5.5: curve 1, no salt; curve 2, 0.6 M $MgCl_2$; curve 3, 1.2 M $MgCl_2$; curve 4, 0.6 M $MgSO_4$; curve 5, 1.2 M $MgSO_4$. (D) RNase A, pH 1.5: curve 1, no salt; curve 2, 0.1 M $MgCl_2$; curve 3, 0.6 M $MgCl_2$; curve 4, 0.6 M $MgSO_4$; curve 5, 0.3 M NaCl.

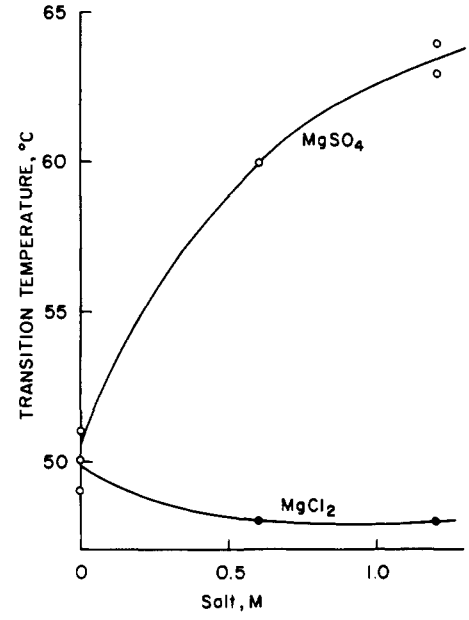

FIGURE 2: Dependence of the transition temperature of lysozyme on $MgSO_4$ and $MgCl_2$ concentration at pH 2.8. The temperatures plotted correspond to the initiation of the transition. All contained 0.6 M Gdn-HCl.

Results

Typical transition curves of lysozyme and RNase A in the presence of $MgCl_2$ and $MgSO_4$ are shown in Figure 1. A

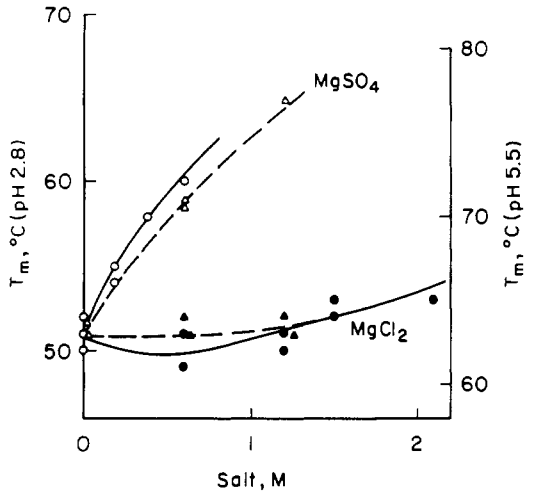

FIGURE 3: Dependence of the temperatures of the midpoint of the transition of lysozyme on salt concentration. Triangles, pH 5.5; circles, pH 2.8. All contained 1.2 M Gdn-HCl.

Table II: Effects of $MgCl_2$ and $MgSO_4$ on the Thermal Denaturation of RNase A

	transition midpoint T_m (°C)				$\Delta G_x^\circ - \Delta G_w^\circ$ (kcal/mol)[a]			
solvent/pH	5.5	2.8	2.0	1.5	5.5	2.8	2.0	1.5
control	61	48	35	29	0	0	0	0
0.1 M $MgCl_2$		44		34		−0.9		1.1
0.6 M $MgCl_2$	61	50	46	44	0	0.6	2.3	2.9
1.2 M $MgCl_2$	61	55			0	2.0		
0.6 M $MgSO_4$	67	60	54	51	2.2	2.7	3.3	5.0
1.2 M $MgSO_4$	72	66			3.3	3.8		
0.3 M NaCl		46		37		−0.4		1.6

[a] Measured at T_m of control.

comparison of the effects of $MgCl_2$ and $MgSO_4$ on the transitions of lysozyme is given in Figures 2 and 3 and Table I.[2] It is evident that $MgCl_2$ had little effect on the transition temperature at both pH 2.8 and pH 5.5, whereas $MgSO_4$ increased greatly the stability of the protein, with 1 M salt raising the transition temperature by ca. 10 °C. The transition temperature at pH 5.5 was higher by 12 °C than that at pH 2.8, as expected from the general electrostatic effect (Tanford, 1968, 1970). The near superposition of the dependencies on salt concentration of the protein stability in the two salts at pH 2.8 and 5.5, after shifting the zero salt result to the same point, indicates that the effects of $MgCl_2$ and $MgSO_4$ on the stability of lysozyme are not electrostatic in nature, since they are little dependent on solvent pH and, therefore, on protein charge. For $MgCl_2$, this result is contrary to what might have been expected from the strong pH dependence of the preferential interactions (Arakawa & Timasheff, 1984a; Arakawa et al., 1990), whereas for $MgSO_4$ the expected parallelism is realized.

The results of a similar study made with RNase A are summarized in Table II. Transition curves, shown in Figure 1, indicate that at pH 2.8 both salts stabilized the protein,

[2] In the case of lysozyme, precipitation in 0.6 and 1.2 M $MgCl_2$ or $MgSO_4$, pH 2.8, did not permit transition measurements without the addition of Gdn-HCl (0.6 or 1.2 M). The rationale of the addition of Gdn-HCl was that its effect should be additive, i.e., its weakening of protein stability should be independent of the concentration of the magnesium salt (von Hippel & Schleich, 1969). This was verified in experiments that showed identical displacements (−9 °C/M Gdn-HCl) of the transition temperature of lysozyme on the addition of the same amount of Gdn-HCl to solutions containing no salt, 0.6 M $MgSO_4$, and 0.6 M $MgCl_2$.

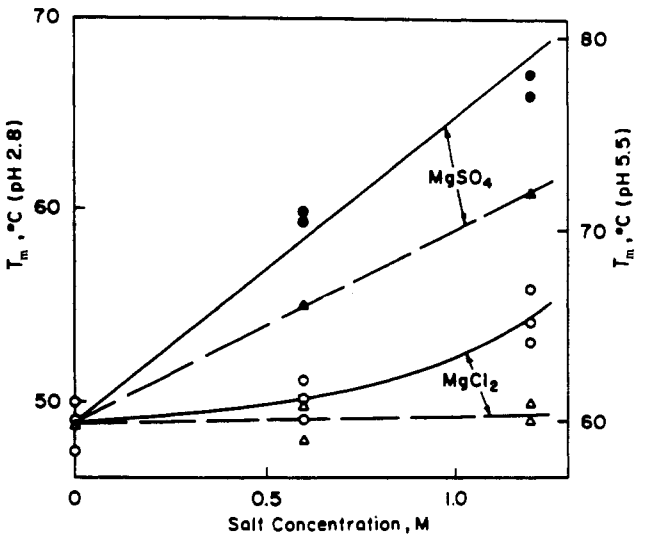

FIGURE 4: Dependence of the temperatures of the midpoint of the transition of RNase A on salt concentration. Triangles, pH 5.5; circles, pH 2.8. No Gdn-HCl added.

$MgSO_4$ being the much stronger stabilizer. The effects of the two salts on the midtransition temperature (T_m) at pH 2.8 and 5.5 are shown in Figure 4, with the results at pH 5.5 shifted down by 11 °C. Just as for lysozyme, $MgSO_4$ was a strong stabilizer in this case, stronger at pH 2.8 than at pH 5.5. To the contrary, $MgCl_2$ at pH 5.5 did not stabilize RNase A. At the lower pH values 2.8, 2.0, and 1.5, both salts acted as stabilizers, $MgSO_4$ being the stronger one by 2 kcal/mol. Preferential interaction measurements were carried out for both salts, and the results are given in Table III. For $MgCl_2$ the preferential hydration increased as the pH was lowered from 5.5, consistent with expectation from other proteins (Arakawa et al., 1990), the value remaining essentially unchanged below pH 2.8. For $MgSO_4$, the preferential hydration did not differ much between pH 5.5 and 2.8, again as expected from other proteins (Arakawa & Timasheff, 1982b). Measurements carried out at pH 1.5 on the native and unfolded RNase A in 0.6 M $MgCl_2$ did not show any large change in preferential interactions on denaturation. The values of the interaction parameter obtained with the unfolded protein, however, are subject to a 50% error, due to the technical difficulty of the experiment, which would conceal any moderate variation during protein unfolding.

The effects of the two salts on the unfolding of CTG, β-LG, and BSA are given in Table IV. Protein solubility was decreased by both 0.6 M $MgCl_2$ and 0.6 M $MgSO_4$, and a complete transition could not be observed in any case due to the onset of turbidity. Nevertheless, it is evident that $MgCl_2$ had little or no stabilizing power, whereas $MgSO_4$ was a strong stabilizer. For example, at pH 2.8, 0.6 M $MgCl_2$ did not affect the transition temperature of β-LG, whereas 0.6 M $MgSO_4$ increased it significantly, and at pH 5.5, $MgCl_2$ at both 0.6 and 1.2 M had essentially no effect on the transition of BSA, whereas $MgSO_4$ at the same concentrations raised the temperature of the onset of the transition by ~6–10 °C relative to the control.

In view of this difference in protein stabilization by $MgCl_2$ and $MgSO_4$, it seemed of interest to examine 2-methyl-2,4-pentanediol (MPD), which induces strong preferential hydration, the magnitude of which varies both with cosolvent concentration (Pittz & Timasheff, 1978) and solution pH (Pittz and Timasheff, unpublished) in a similar fashion to $MgCl_2$. The transition curves of RNase A as a function of MPD at pH 5.8 are shown in Figure 5 in the form of a van't

Table III: Preferential Interaction Parameters of RNase A in 0.6 and 1.2 M $MgCl_2$ and $MgSO_4$ at 20 °C

solvent	ϕ_2^0 (mL/g)	$\phi_2'^0$ (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial\mu_3/\partial m_3)_{T,P,m_2}$ [cal/(mol of salt·mol of salt)]	$(\partial\mu_2/\partial m_3)_{T,P,m_2}$ [cal/(mol of protein·mol of salt)]
			pH 5.5				
0.6 M $MgCl_2$	0.687 ± 0.002	0.699 ± 0.001	−0.0150 ± 0.0038	0.259 ± 0.066	−2.16 ± 0.55	3340	7200 ± 1800
1.2 M $MgCl_2$	0.693 ± 0.001	0.710 ± 0.002	−0.0230 ± 0.0040	0.197 ± 0.034	−3.31 ± 0.58	2370	7800 ± 1400
0.6 M $MgSO_4$	0.690 ± 0.002	0.719 ± 0.002	−0.0321 ± 0.0044	0.440 ± 0.060	−3.65 ± 0.50	971	3500 ± 500
1.2 M $MgSO_4$	0.693 ± 0.001	0.760 ± 0.001	−0.0816 ± 0.0024	0.559 ± 0.016	−9.29 ± 0.27	684	6400 ± 200
			pH 2.8				
0.6 M $MgCl_2$	0.691 ± 0.002	0.713 ± 0.001	−0.0278 ± 0.0038	0.483 ± 0.066	−4.00 ± 0.55	3340	13500 ± 1800
1.2 M $MgCl_2$	0.691 ± 0.001	0.715 ± 0.002	−0.0324 ± 0.0040	0.274 ± 0.034	−4.66 ± 0.58	2370	10900 ± 1400
0.6 M $MgSO_4$	0.693 ± 0.001	0.718 ± 0.001	−0.0276 ± 0.0022	0.384 ± 0.030	−3.14 ± 0.25	971	3100 ± 240
1.2 M $MgSO_4$	0.699 ± 0.001	0.753 ± 0.002	−0.0657 ± 0.0036	0.452 ± 0.025	−7.48 ± 0.41	684	5100 ± 280
			pH 2.0				
0.6 M $MgCl_2$	0.692 ± 0.002	0.711 ± 0.002	−0.0238 ± 0.0050	0.414 ± 0.086	−3.43 ± 0.72	3340	11400 ± 2400
			pH 1.5				
0.6 M $MgCl_2$(N)	0.692 ± 0.001	0.716 ± 0.002	−0.0301 ± 0.0038	0.517 ± 0.066	−4.33 ± 0.55	3340	14400 ± 1800
0.6 M $MgCl_2$(D)	0.690[a]	0.71 ± 0.01[b]	−0.025 ± 0.0125	0.431 ± 0.216	−3.60 ± 1.80	3340	12000 ± 6000

[a] Value in the unfolded state calculated from ϕ_2^0 in 6 M guanidine hydrochloride (Lee & Timasheff, 1974). [b] Partial specific volume obtained after dialyzing the protein solutions at 53 °C for 20 h against the solvent.

Table IV: Effect of $MgCl_2$ and $MgSO_4$ on the Thermal Denaturation of β-LG, BSA, and CTG

	onset of transition (°C)				
		$MgCl_2$		$MgSO_4$	
	control	0.6 M	1.2 M	0.6 M	1.2 M
β-LG, pH 2.8, 3 M Gdn-HCl	38–58	39[c]		45[b]	
BSA, pH 5.5[a]	51[a]	50[a]	48[a]	57[a]	60[a]
CTG, pH 2.8, 0.6 M Gdn-HCl	33[a]	36[b]		43[b]	
CTG, pH 2.8, 1.2 M Gdn-HCl	29[a]			39[b]	
CTG, pH 2.8, 3 M Gdn-HCl	<20[c]	<20[c]		23[b]	

[a] Turbidity occurred following the appearance of an initial transition (the number corresponds to the initial temperature of the transition). [b] Turbidity occurred before the appearance of a transition (the number given is the initial temperature of the absorbance increase). [c] No transition was observed when the temperature was raised from 20 °C.

Table V: Thermal Denaturation of RNase A in MPD at pH 5.8

	transition temp[a] (°C)			thermodynamic parameters at 20 °C		
	range					
	1	2	T_m	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ [cal/(mol·deg)]
control	54–67	54–66	59	13.0	106	320
30%	42–54	43–53	49	9.6	109	339
40%	39–52	51[b]	47	8.7	108	338

[a] The temperature was raised after preincubation at 30 °C (1) and 40 °C (2). [b] The start of the transition was not observed since the starting temperature of the experiment was higher than the temperature of the beginning of transition.

Hoff plot, and the data are summarized in Table V. It is evident that the protein is greatly destabilized by 30 and 40% (wt %) MPD, conditions at which native RNase A is preferentially hydrated to the extent of 0.6 and 0.8 g of H_2O/g of protein.[3] As also shown in Table V, the preincubation

[3] The final pH of the protein solutions, after addition of MPD, was 5.8 for the control, 6.1 for 30% MPD, and 6.3 for 40% MPD. This could affect protein stability. Considering the high isoelectric point of RNase A, pH 9.5 (Richards & Wyckoff, 1971), this protein should be more stable at higher pH. The lowered stability in MPD solution is due, therefore, to the interaction of the cosolvent with RNase A and not the small shift in pH.

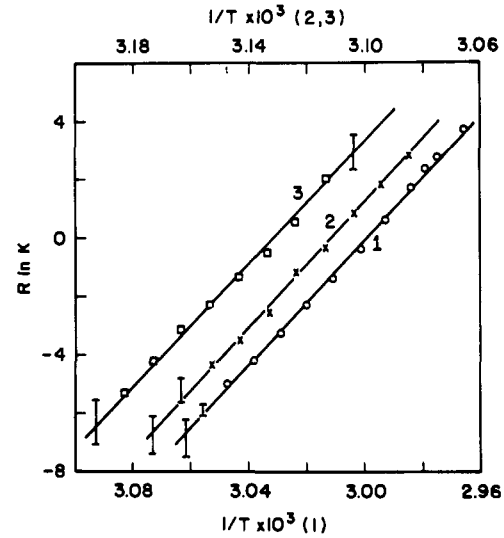

FIGURE 5: van't Hoff plots of the thermal transitions of RNase A in MPD at pH 5.8: 1, control (no MPD); 2, 30% MPD; 3, 40% MPD.

temperature had no effect on the transition, since the transition temperature was identical whether preincubation was at 30 or 40 °C.

Discussion

It is generally accepted that $MgCl_2$ is a salting-in agent and a structure destabilizer of proteins (von Hippel & Schleich, 1969; Collins & Washabaugh, 1985). Under some limited conditions of pH and concentration, however, it salts out proteins (Arakawa et al., 1990), reflecting their preferential hydration driven by the large unfavorable free energy of the interaction. It seemed reasonable to expect, therefore, that, under the same conditions, $MgCl_2$ might act as a protein stabilizer, in view of the good correlation that had been established between preferential hydration and protein structure stabilization (Lee et al., 1975; Lee & Timasheff, 1981; Gekko & Timasheff, 1981a,b; Gekko & Morikawa, 1981; Arakawa & Timasheff, 1982a,b, 1983; Leirmo et al., 1987). Contrary to this expectation, $MgCl_2$ either did not stabilize proteins or stabilized them to a much smaller extent than $MgSO_4$, even at conditions in which they displayed preferential hydrations of similar magnitudes. A similar situation is found with MPD and poly(ethylene glycol) (PEG), both of which induce preferential hydration of native proteins and behave normally

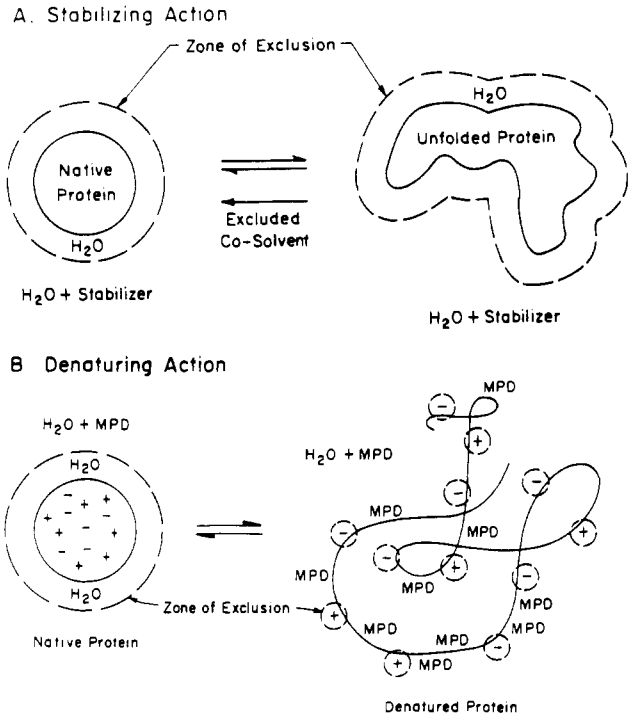

FIGURE 6: Patterns of protein–solvent interactions. (A) The predominant interaction with proteins is nonspecific exclusion due to the surface tension effect. Since in the asymmetric denatured state, it is greater per protein molecule than in the compact native state, the equilibrium is displaced to the left. (B) In the denaturation reaction, shown for the water–MPD system, MPD exclusion due to the high charge density on the native protein is replaced by MPD exclusion from individual charged sites and MPD binding to solvent-exposed nonpolar regions in the denatured state.

as precipitants, but can act as protein destabilizers (Arakawa & Timasheff, 1985; Lee & Lee, 1987).

These observations lead to the conclusion that, while the preferential interactions of native globular proteins with solvent components determine, possibly universally, the effect of the additives on protein solubility (Arakawa et al., 1990), the same correlation does not necessarily hold with protein stability. What is the difference between the preferentially excluded (salting-out) stabilizers and destabilizers? Their preferential hydration patterns lead to the identification of two distinct categories. In the first, which includes sugars, amino acids, glycerol, and certain salts, such as NaCl, $MgSO_4$, Na_2SO_4, and possibly $(NH_4)_2SO_4$ (Gerlsma, 1968, 1970; Gerlsma & Stuur, 1972, 1974; Lee & Timasheff, 1981; Gekko & Timasheff, 1981a,b; Arakawa & Timasheff, 1982a; Back et al., 1979; Tuengler et al., 1979), the preferential hydration is almost totally independent of the solvent pH and the cosolvent concentration.[4] These cosolvents act always as protein stabilizers. In the second category, which includes $MgCl_2$, PEG, and MPD, the preferential hydration is strongly dependent on either pH or concentration, or both, and their effect on protein stability cannot be predicted from their preferential interactions with the proteins in the native state.

The effect of cosolvents on protein stability is defined by the balance between their preferential interactions with the two end states of the protein unfolding reaction. This is depicted schematically in Figure 6 in which MPD was chosen as the example of a salting-out destabilizer. Quantitatively, this balance is expressed by the Wyman linkage relation (Wyman, 1964):

$$(\partial \ln K/\partial \ln a_3)_{T,P,m_2} = (\partial m_3/\partial m_2)^{D}_{T,\mu_1,\mu_3} - (\partial m_3/\partial m_2)^{N}_{T,\mu_1,\mu_3} = \Delta\bar{\nu} \quad (1)$$

where K is the equilibrium constant of the denaturation reaction, assumed to be two state, N $\rightleftharpoons$ D, N and D express the native and denatured states of the protein, subscripts 1, 2, and 3 are water, protein, and additive, m_i, μ_i, and a_i are the molal concentration, chemical potential, and activity of component i, T is the thermodynamic (Kelvin) temperature, and P is the pressure. The parameters $(\partial m_3/\partial m_2)^{D}_{T,\mu_1,\mu_3}$ and $(\partial m_3/\partial m_2)^{N}_{T,\mu_1,\mu_3}$ express the preferential interactions of the protein with the additive in the denatured and native states, respectively, and $\Delta\bar{\nu}$ is the difference in preferential interaction between the two states.

Let us take $MgSO_4$ and $MgCl_2$ as examples of the first and second categories of cosolvents, respectively. Their preferential interactions with proteins are determined by a fine balance between two factors (Arakawa & Timasheff, 1984a; Arakawa et al., 1990), salt binding due to the affinity of Mg^{2+} for anionic sites and peptide bonds (Robinson & Jencks, 1965), and salt exclusion due to its cohesive action on water, reflected in the large surface tension increment.[5] The change in preferential interaction during a denaturation reaction, $\Delta\bar{\nu}$, must reflect, therefore, changes in the balance between salt exclusion and salt binding in the two end states. A nonspecific salt-excluding effect, such as the increase of the surface tension of water by addition of the salt, being a function strictly of the size of the protein–solvent interface, must increase on protein denaturation, since an unfolded protein presents a greater surface area to contact with solvent than a native globular protein. The binding of Mg^{2+} ions must also increase on denaturation because of the increase in the number of exposed peptide bonds and the great reduction of the charge density of the expanded protein, which reduces the electrostatic repulsion between the cations and the net positive charge on the protein when working below the isoelectric point.

For $MgSO_4$ the dominant factor in determining $(\partial m_3/\partial m_2)^{N}_{T,\mu_1,\mu_3}$ is the very strong exclusion characteristic of SO_4^{2-} ions (Arakawa & Timasheff, 1984b). As a consequence, the effect of $MgSO_4$ on protein stability is determined overwhelmingly by the change in surface area during denaturation, and the relationship $(\partial m_3/\partial m_2)^{D}_{T,\mu_1,\mu_3} < (\partial m_3/\partial m_2)^{N}_{T,\mu_1,\mu_3} < 0$ is expected to hold independently of the solvent pH and salt concentration.

For $MgCl_2$ at the isoelectric pH of β-LG, the weak preferential interaction with the native protein (Arakawa et al., 1990) indicates that the two factors are of similar magnitude, consistent with the weaker exclusion of Cl^- ions (Arakawa & Timasheff, 1984b). The observed lack of an effect on the stability of lysozyme at pH 5.5 means that $(\partial \ln K/\partial \ln a_3)_{T,P,m_2} = \Delta\bar{\nu} \approx 0$, and hence $(\partial m_3/\partial m_2)^{D}_{T,\mu_1,\mu_3}$ must also be small. This is consistent with denaturation providing both a larger surface area and an increase in Mg^{2+} binding sites. At pH 2.8, $(\partial m_3/\partial m_2)^{N}_{T,\mu_1,\mu_3} < 0$ is the consequence of the smaller extent of salt binding than at pH 5.5 due to the electrostatic repulsion of Mg^{2+} by the large net positive charge of the protein, while the surface tension effect remains unchanged. The lack of an effect of $MgCl_2$ on the stability of lysozyme at pH 2.8 again indicates that $(\partial m_3/\partial m_2)^{D}_{T,\mu_1,\mu_3}$ must be essentially equal to

[4] It must be pointed out, however, that NaCl has displayed a concentration-dependent preferential binding to β-LG due to a specific effect (Arakawa & Timasheff, 1987) and especially strong binding to certain halophilic enzymes (Pundak & Eisenberg, 1981).

[5] For a discussion of uncertainties in the application of the surface tension increment to a protein–water interference, see the preceding paper (Arakawa et al., 1990).

$(\partial m_3/\partial m_2)^N_{T,\mu_1,\mu_3}$, i.e., the increase in salt binding in the denatured protein must be sufficient to balance out the increased surface tension effect at pH 2.8, as well. In the case of RNase A, a weak, but significant, stabilization by 1.2 M $MgCl_2$ was observed at pH 2.8, while the same salt had no effect at pH 5.5. Since the salt exclusion was large at low pH (Table III) and, hence, $(\partial m_3/\partial m_2)^N_{T,\mu_2,\mu_3} \ll 0$, the observed stabilization indicates that $(\partial m_3/\partial m_2)^D_{T,\mu_2,\mu_3} < (\partial m_3/\partial m_2)^N_{T,\mu_2,\mu_3} < 0$, meaning that the increase in salt exclusion on denaturation cannot be balanced out fully by an increase in salt binding. This suggests a strong contribution from the electrostatic repulsion of Mg^{2+} ions by the positive charge of the denatured protein for RNase A, stronger than for lysozyme, consistent with the much greater increase in positive charge density for RNase A than for lysozyme when the pH is changed from 5.5 to 2.8. The further stabilization as pH is lowered to 1.5 is probably related to the known partial unfolding of RNase A below pH 2.5 at 20 °C (Brandts & Hunt, 1967), since 0.1 M $MgCl_2$ and 0.3 M NaCl provide already significant stabilization by what must be a classical ionic strength effect (Tanford & Kirkwood, 1957; Tanford, 1957), while 0.3 M NaCl had no stabilizing effect at pH 2.8 (see Figure 1B,D).

A most remarkable example of the second class of cosolvents is the aqueous MPD system. The very great preferential hydration of RNase A in this solvent system has permitted its crystallization for structural studies (King et al., 1956). Yet it destabilizes strongly the structure of the same protein, as shown in Table V. Its interactions with the protein in the native $\rightleftharpoons$ denatured equilibrium are shown schematically in Figure 6B. MPD is repelled from charged groups (Pittz & Timasheff, 1978) and has an affinity for nonpolar residues (Pittz & Bello, 1971). It should, therefore, be excluded from charges on the protein surface and bind to proteins at hydrophobic sites. In the native protein the overwhelmingly predominant interaction is the repulsion of MPD from the high density of charges on the protein surface. Its decrease on denaturation permits MPD to penetrate to the protein surface and to bind to the many additional hydrophobic sites that become exposed to solvent on denaturation. The simultaneous decrease in exclusion and increase in binding on denaturation must have as a consequence that $(\partial m_3/\partial m_2)^D_{T,\mu_1,\mu_3} \gg (\partial m_3/\partial m_2)^N_{T,\mu_1,\mu_3}$; hence $\Delta\bar{\nu} \gg 0$, and K must increase on addition of MPD. As a result, $\Delta G°$ of denaturation is less positive by 3–4 kcal/mol in 30–40% MPD at 20 °C than in dilute buffer (Table V). Nevertheless, at both these solvent compositions the transition temperature is still considerably higher than room temperature, which permitted the protein to be crystallized without structural alterations (King et al., 1956). A similar situation is found with PEG. Its preferential interactions with native proteins are determined primarily by steric exclusion (Arakawa & Timasheff, 1985). Being hydrophobic in nature (Hammes & Schimmel, 1967), PEG interacts with nonpolar regions of the protein when these become exposed upon denaturation and, as a consequence, stabilizes the denatured form of proteins (Lee & Lee, 1987).

The classification of precipitant (salting-out) cosolvents into two categories is governed, therefore, by their mechanism of interactions with proteins. In the first category, which consists of the good stabilizers, the protein–solvent interactions are determined overwhelmingly by the properties of the solvent, and the role of proteins is almost fully restricted to presenting a surface. As illustrated in Figure 6A, the interaction is always that of preferential hydration, whether the protein is native or unfolded. The principal mechanism of interaction for these stabilizers is the strong increase in the surface free energy (surface tension) of water upon their addition. It is the increase in the surface of protein–solvent contact and, hence, in the surface free energy on unfolding that shifts the equilibrium toward the native compact state with resultant structure stabilization. In the second category (Figure 6B), it is the chemical nature of the protein surface that determines the interactions (repulsive or attractive). For these, the preferential interactions vary strongly with the state of the system, such as pH, cosolvent concentration, and state of folding of the protein. This class contains MPD (repelled by charges, bound to nonpolar regions), $MgCl_2$ (excluded by surface tension effect, attracted by Mg^{2+} binding to protein), PEG (excluded by steric effect, bound to nonpolar regions). In this category the pattern of interactions, therefore, is determined by shifts in the balance between exclusion and binding as the chemical nature of the protein surface changes with variations in the solution conditions. For MPD and PEG, binding to nonpolar regions increases greatly on unfolding; for $MgCl_2$ protein unfolding reduces the electrostatic free energy and, hence, repulsion of Mg^{2+} ions at acid pH.

Table VI: Effect of $MgSO_4$ on the Thermodynamic Parameters of Protein Denaturation at T_m

protein	$MgSO_4$ concn (M)	thermodynamic parameters at T_m: $\Delta H°$ (kcal/mol)	$\Delta S°$ [cal/(deg·mol)]
lysozyme, pH 2.8, 0.6 M Gdn-HCl	0	116	352
lysozyme, pH 2.8, 0.6 M Gdn-HCl	0.6	129	380
lysozyme, pH 2.8, 1.2 M Gdn-HCl	0	107	331
lysozyme, pH 2.8, 1.2 M Gdn-HCl	0.6	125	375
lysozyme, pH 5.5, 1.2 M Gdn-HCl	0	110	328
lysozyme, pH 5.5, 1.2 M Gdn-HCl	1.2	130	372
RNase, pH 1.5	0	65	215
RNase, pH 1.5	0.6	88	271
RNase, pH 2.0	0	74	241
RNase, pH 2.0	0.6	94	288
RNase, pH 2.8	0	80	250
RNase, pH 2.8	0.6	90	271
RNase, pH 2.8	1.2	88	259
RNase, pH 5.5	0	104	312
RNase, pH 5.5	0.6	133	392
RNase, pH 5.5	1.2	133	385

This difference in the character of protein–solvent interactions in the two categories of additives is further supported by the thermodynamic parameters of denaturation calculated from the transition curves. $MgSO_4$ increased both $\Delta H°$ and $\Delta S°$ of denaturation, as shown in Table VI. The protein structure stabilization by $MgSO_4$ is driven enthalpically, similarly to that by sucrose (Lee & Timasheff, 1981). Since both sucrose and $MgSO_4$ increase the surface free energy of water, the observed increase in the enthalpy of protein denaturation in the presence of these additives is consistent with the greater amount of work required for the increase in size of the protein-containing cavity on denaturation (Sinanoglu & Abdulnur, 1965). MPD, on the other hand, had an insignificant effect on $\Delta H°$ but increased $\Delta S°$. This entropically driven destabilization of protein structure by MPD is similar to that found for alcohols (Velicelebi & Sturtevant, 1979) and fully consistent with its known interaction with hydrophobic residues (Pittz & Bello, 1971). $MgCl_2$ had little effect on either parameter, reflecting the complexity of its preferential interactions with proteins.

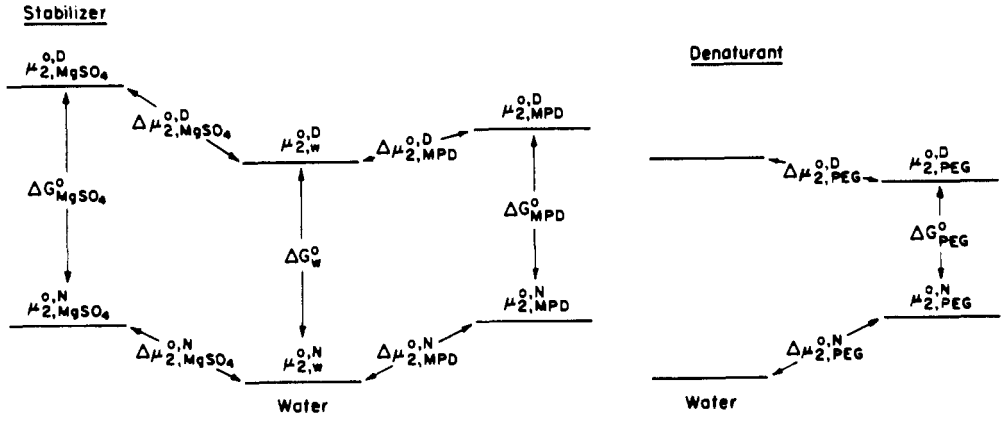

FIGURE 7: Free energy diagram of the effect of cosolvents on protein stability.

Quantitatively, the effect of preferential interactions on a denaturation reaction is given by the difference in the transfer free energies of the native and unfolded proteins from water (dilute buffer) to the solvent system, since

$$\Delta G^{\circ,N\text{-}D}_x - \Delta G^{\circ,N\text{-}D}_w = \Delta\mu^{\circ,N}_{2,x} - \Delta\mu^{\circ,D}_{2,x} \quad (2)$$

$$\Delta\mu^{\circ}_{2,x} = \mu^{\circ}_{2,x} - \mu^{\circ}_{2,w} = \int_w^x (\partial\mu_2/\partial m_3)\, dm_3 \quad (3)$$

where $\Delta G^{\circ,N\text{-}D}$ is the standard free energy of the N $\rightleftharpoons$ D equilibrium, $\Delta\mu^{\circ}_{2,x}$ is the transfer free energy of the protein from water to the cosolvent system, and the subscripts x and w indicate cosolvent and water, respectively. This is illustrated by the free energy diagram, shown in Figure 7, for a stabilizer ($MgSO_4$) and two salting-out denaturants (PEG and MPD). For the protein denaturation reaction in water (shown in the middle) $\Delta G^{\circ}_w = -RT \ln K_w$ is the difference between the chemical potentials of the denatured ($\mu^{\circ,D}_{2,w}$) and native ($\mu^{\circ,N}_{2,w}$) forms of the protein in water at constant temperature and pressure. Addition of any of these cosolvents to the protein solution in the native state raises the chemical potential of the protein, the magnitude of the increase being equal to the free energy of transfer of the native protein from water to the particular solvent system. In all the cases examined here, the transfer is thermodynamically unfavorable; i.e., all the cosolvents are precipitants. After denaturation, the chemical potentials of the proteins shift to the upper levels on the diagram. In the case of a stabilizer, such as $MgSO_4$, as is clear from Figure 6A, the exclusion is greater in the unfolded state than in the native state, with the consequence that $\Delta\mu^{\circ,D}_{2,MgSO_4} > \Delta\mu^{\circ,N}_{2,MgSO_4}$ and the free energy of denaturation, $\Delta G^{\circ}_{MgSO_4}$, becomes more positive than in water. The experimental results for RNase A in 1.2 M $MgSO_4$ at pH 2.8, given in Table II, are $\Delta G^{\circ,N\text{-}D}_{MgSO_4} - \Delta G^{\circ,N\text{-}D}_w = 3.8$ kcal/mol. Therefore, the transfer free energy of the protein is increased from 4.2[6] to 8.0 kcal/mol on unfolding.[7] A similar, but weaker, effect is evident for RNase A in $MgCl_2$ at acid pH. At pH 1.5, 0.6 M $MgCl_2$ increased $\Delta G^{\circ,N\text{-}D}$ of RNase A by 2.9 kcal/mol (Table II). This must be a reflection of a similar increase in $\Delta\mu^{\circ}_2$, which is consistent with the measured values, $(\partial\mu_2/\partial m_3)_{T,P,m_2} = 14.4 \pm 2$ and 12 ± 6 kcal/mol for the native and denatured forms, respectively, provided that the preferential interactions for these two states assume a similar salt concentration dependence. Furthermore, this comparison contains the assumption that the preferential hydration of the native protein, measured at 20 °C, does not change with temperature. This, in fact, may not be so, as this parameter has been shown to decrease with increasing temperature for BSA in aqueous glycerol and sorbitol solutions (Gekko & Morikawa, 1981).

For a structure destabilizer, the transfer free energy in the denatured state must be more favorable than in the native state. As an example of a denaturant that is strongly excluded from the native protein, let us take chymotrypsinogen in 20% PEG-1000, for which Lee and Lee (1987) have determined the preferential interactions in both the native and the denatured states. The standard free energies of denaturation in water and in the solvent system and the transfer free energies at 20 °C were calculated from the experimental denaturation and preferential interaction data[8] of Lee and Lee (1987), giving values of $\Delta G^{\circ}_w = 18.8$ kcal/mol, $\Delta G^{\circ}_{PEG} = 13.2$ kcal/mol, $\Delta\mu^N_2 = 2.7$ kcal/mol, and $\Delta\mu^D_2 = -3.1$ kcal/mol, which close the free energy box of Figure 7 to within 0.2 kcal/mol. In this system, therefore, the lowering of the transition temperature by 7.5 °C is the consequence of the transfer free energy decrease by 5.8 kcal/mol (from an unfavorable to a favorable interaction) when the protein is transformed from the native folded state to the unfolded one. For RNase A in 30% MPD, 20 °C, $\Delta G^{\circ,N\text{-}D}$ is lowered to 9.6 kcal/mol from 13.0 kcal/mol in dilute buffer, giving $\Delta\mu^N_2 - \Delta\mu^D_2 = 4.4$ kcal/mol.[7] In this system the interactions are thermodynamically unfavorable with both the native and the denatured states of the protein ($\Delta\mu^N_2 = 6.5$ kcal/mol), but less so with the unfolded protein.

The above analysis of the relations between preferential interactions and structural stabilization of proteins brings out clearly the reasons why the preferential interactions of native proteins with solvent additives do not necessarily correlate with protein stability, while such a correlation is true, apparently without exception, with protein solubility. Preferential hydration always induces salting-out, regardless of the mechanism of the preferential interaction. This is a necessary consequence of the identity of protein structure in the disperse and precipitated states (Arakawa et al., 1990). In the denaturation equilibrium the situation is exactly the converse, since, by definition, the protein structure must be different in the two end states of the process. Therefore, as illustrated in Figure

[6] This transfer free energy was calculated by using the two available values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, at 0.6 and 1.2 M, and assuming its linear dependence on m_3.

[7] This value can be obtained from measurements of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ as a function of m_3 for the unfolded form. For technical reasons we did not succeed in measuring $(\partial\mu_2/\partial m_3)_{T,P,m_2}$. In the case of salts, this was prevented by protein precipitation during the 24-h dialysis at temperatures above the transition.

[8] We thank Dr. J. C. Lee for giving us the original data. The transfer free energy was calculated by eq 3 with the assumption that the chemical potential perturbation is independent of PEG concentration (Arakawa & Timasheff, 1985).

6, when the protein–solvent interactions are independent of the chemical nature of the protein surface, the effect will always be that of stabilization. On the other hand, when the interactions are defined to a major extent by the chemical nature of the protein surface, addition of a cosolvent may either stabilize or destabilize the protein, even though it acts as a strong precipitant and, possibly, crystallizer of the native protein, and measurement of the interactions for the native protein gives no direct insight into the effect of the solvent on protein stability.

Registry No. MPD, 107-41-5; RNase A, 9001-99-4; CTG, 9035-75-0; $MgCl_2$, 7786-30-3; $MgSO_4$, 7487-88-9; lysozyme, 9001-63-2.

References

Arakawa, T., & Timasheff, S. N. (1982a) *Biochemistry 21*, 6536–6544.

Arakawa, T., & Timasheff, S. N. (1982b) *Biochemistry 21*, 6545–6552.

Arakawa, T., & Timasheff, S. N. (1983) *Arch. Biochem. Biophys. 224*, 169–177.

Arakawa, T., & Timasheff, S. N. (1984a) *Biochemistry 23*, 5912–5923.

Arakawa, T., & Timasheff, S. N. (1984b) *Biochemistry 23*, 5924–5929.

Arakawa, T., & Timasheff, S. N. (1985) *Biochemistry 24*, 6756–6762.

Arakawa, T., & Timasheff, S. N. (1987) *Biochemistry 26*, 5147–5153.

Arakawa, T., Bhat, R., & Timasheff, S. N. (1990) *Biochemistry* (preceding paper in this issue).

Back, J. F., Oakenfull, D., & Smith, M. B. (1979) *Biochemistry 18*, 5191–5196.

Bello, J., & Nowoswiat, E. F. (1965) *Biochim. Biophys. Acta 105*, 325–332.

Brandts, J. F., & Hunt, L. (1967) *J. Am. Chem. Soc. 89*, 4826–4838.

Collins, K. D., & Washabaugh, M. W. (1985) *Q. Rev. Biophys. 18*, 323–422.

Gekko, K., & Morikawa, T. (1981) *J. Biochem. 90*, 39–50.

Gekko, K., & Timasheff, S. N. (1981a) *Biochemistry 20*, 4667–4676.

Gekko, K., & Timasheff, S. N. (1981b) *Biochemistry 20*, 4677–4686.

Gerlsma, S. Y. (1968) *J. Biol. Chem. 243*, 957–961.

Gerlsma, S. Y. (1970) *Eur. J. Biochem. 14*, 150–153.

Gerlsma, S. Y., & Stuur, E. R. (1972) *Int. J. Peptide Protein Res. 4*, 377–383.

Gerlsma, S. Y., & Stuur, E. R. (1974) *Int. J. Peptide Protein Res. 6*, 65–74.

Hammes, G. G., & Schimmel, P. R. (1967) *J. Am. Chem. Soc. 89*, 442–446.

Jackson, W. M., & Brandts, J. F. (1970) *Biochemistry 9*, 2294–2301.

King, M. V., Magdoff, B. S., Adelman, M. B., & Harker, D. (1956) *Acta Crystallogr. 9*, 460–465.

Lee, J. C., & Timasheff, S. N. (1974) *Biochemistry 13*, 257–265.

Lee, J. C., & Timasheff, S. N. (1981) *J. Biol. Chem. 256*, 7193–7201.

Lee, J. C., & Lee, L. L. Y. (1987) *Biochemistry 26*, 7813–7819.

Lee, J. C., Frigon, R. P., & Timasheff, S. N. (1975) *Ann. N.Y. Acad. Sci. 253*, 284–291.

Leirmo, S., Harrison, C., Cayley, D. S., Burgess, R. R., & Record, M. T., Jr. (1987) *Biochemistry 26*, 2095–2101.

Noelken, M. E., & Timasheff, S. N. (1967) *J. Biol. Chem. 242*, 5080–5085.

Pittz, E. P., & Bello, J. (1971) *Arch. Biochem. Biophys. 146*, 513–524.

Pittz, E. P., & Timasheff, S. N. (1978) *Biochemistry 17*, 615–623.

Pundak, S., & Eisenberg, H. (1981) *Eur. J. Biochem. 118*, 463–470.

Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes (3rd Ed.) 4*, 647–806.

Robinson, D. R., & Jencks, W. P. (1965) *J. Am. Chem. Soc. 87*, 2470–2479.

Roxby, R., & Tanford, C. (1971) *Biochemistry 10*, 3348–3352.

Scott, R. A., & Scheraga, H. A. (1963) *J. Am. Chem. Soc. 85*, 3866–3873.

Sinanoglu, O., & Abdulnur, S. (1965) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24*, 12–23.

Tanford, C. (1957) *J. Am. Chem. Soc. 79*, 5340–5347.

Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.

Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.

Tanford, C., & Kirkwood, J. G. (1957) *J. Am. Chem. Soc. 79*, 5333–5339.

Townend, R., Winterbottom, R. J., & Timasheff, S. N. (1960) *J. Am. Chem. Soc. 82*, 3161–3168.

Tuengler, P., Long, G. L., & Durchschlag, H. (1979) *Anal. Biochem. 98*, 481–484.

Velicelebi, G., & Sturtevant, J. M. (1979) *Biochemistry 18*, 1180–1186.

von Hippel, P. H., & Schleich, T. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N., & Fasman, G. D., Eds.) Vol. 2, pp 417–574, Marcel Dekker, New York.

Wyman, J. (1964) *Adv. Protein Chem. 19*, 223–286.