Contribution of the Surface Free Energy Perturbation to Protein−Solvent Interactions†

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ABSTRACT: Surface tension measurements were carried out at 20 °C by a capillary drop−weight method on aqueous solutions of sodium glutamate (NaGlu), lysine hydrochloride (LysHCl), potassium aspartate (KAsp), arginine hydrochloride (ArgHCl), lysylglutamate (LysGlu), argininylglutamate (ArgGlu), guanidinium sulfate, trehalose, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide, 2-methyl-2,4-pentanediol (hexylene glycol), and poly(ethylene glycol)s of molecular weights 200, 400, 600, and 1000. All of the salts and the sugar increased the surface tension of water, while the last four compounds decreased it, with 2-methyl-2,4-pentanediol lowering it most effectively and TMAO being the least effective. The preferential hydration of bovine serum albumin (BSA) and lysozyme was measured in KAsp, ArgHCl, LysGlu, and ArgGlu. The high values of preferential hydration found in all cases, except for BSA in ArgHCl, suggest that they should stabilize protein structure, as had been found for lysine hydrochloride and monosodium glutamate [Arakawa, T., & Timasheff, S. N. (1984) *J. Biol. Chem. 259,* 4979−4986]. A correlation was found for both BSA and lysozyme in KAsp, NaGlu, LysHCl, ArgGlu, and LysGlu between the surface tension effect and the observed preferential interactions, indicating that the change in the surface free energy of the protein-containing cavity due to the surface tension increase for water by these amino acid salts contributes dominantly to the observed increase in the chemical potential of the protein by their addition. The lack of a correlation observed for BSA, but not lysozyme, in ArgHCl at low concentrations where preferential binding is close to zero suggests, however, that the surface tension effect is not the sole factor involved in the protein−solvent interactions in these amino acid salts. Binding of ArgHCl to BSA, probably through hydrogen bonds between the Arg guanidinium group and peptide bonds, was proposed to occur, the affinity of Arg^+ being reduced by electrostatic repulsion when proteins carry a net positive charge, such as is the case with lysozyme. Since the four organic solvent additives also lead to protein preferential hydration, no correlation exists between their preferential interactions and the surface free energy perturbation. Therefore, in their case, the preferential hydration must be ascribed to other factors that overcome the preferential binding expected from the Gibbs adsorption isotherm. The surface tension results, however, are consistent with the binding of the organic solvents to proteins through hydrophobic interactions, explaining, at least in part, the observed concentration dependence of the interactions.

In previous studies the conclusion had been reached that the preferential hydration of proteins in the presence of a number of protein-precipitating and structure-stabilizing cosolvents is the consequence of the cohesive force exerted by these additives on water molecules. Such substances include sugars (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a), a number of salts (Arakawa & Timasheff, 1982b, 1984b), and some amino acids (Arakawa & Timasheff, 1983, 1984a). This cohesive effect, manifested by an increase in the surface free energy of water, is the origin of the measured positive free energy of interaction between these additives and proteins and the consequent preferential hydration of proteins in their presence. These considerations are consistent with the proposal by Sinanoglu and co-workers (Sinanoglu, 1968; Sinanoglu & Abdulnur, 1964, 1965; Halicioglu & Sinanoglu, 1969) that the surface free energy of the formation of a cavity to accommodate a solute molecule in a solvent plays an important role in the stability and self-association of macromolecules. The direct correlation between the increase in the surface tension of water by salts and their effect on protein solubility, found by Melander and Horvath (1977), was an early demonstration of this effect, with the exception of such salts as $MgCl_2$ and $CaCl_2$ for which this correlation failed. Furthermore, Honig and co-workers (Nicholls et al., 1991; Sharp et al., 1991) have argued that surface tension can be used as a measure of hydrophobicity.

While this correlation is maintained for a number of the substances listed earlier, several exceptions have pointed to a more complex situation. Thus, urea and $MgCl_2$, which increase the surface free energy of water (*International Critical Tables*, 1928), are preferentially bound to proteins at high cosolvent concentrations (Prakash et al., 1981; Arakawa & Timasheff, 1984b, 1985a). Conversely, betaine and glycerol, which decrease the surface free energy of water (*International Critical Tables*, 1928; Pappenheimer et al., 1936), induce protein preferential hydration (Gekko & Timasheff 1981a; Arakawa & Timasheff, 1983). An expla-

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nation offered for this apparent contradiction is that it is a consequence of the fact that those additives that showed preferential cosolvent binding, in spite of their raising the surface free energy of water, have a strong affinity for particular groups present on proteins (Prakash et al., 1981; Arakawa & Timasheff, 1984a−c; Arakawa et al., 1990b), while those that lowered the surface free energy were excluded by other mechanisms; e.g., glycerol is excluded as a result of the solvophobic effect (Gekko & Timasheff, 1981a).

The observed net interactions are the resultant of a balance between forces that attract the additive to the protein (negative free energy of interaction, binding of the additive) and those that repel the additive from the protein (positive free energy of interaction and exclusion of the additive, i.e., preferential hydration). Formally, this can be expressed, at constant temperature and pressure, as (Timasheff & Inoue, 1968; Inoue & Timasheff, 1972; Timasheff & Arakawa, 1988; Timasheff, 1992b)

$$-(\partial\mu_2/\partial m_3)_{m_2}/RT = \left(B_3 - \frac{m_3}{55.56}B_1\right)[(1/m_3) + (\partial \ln \gamma_3/\partial m_3)_{m_2}] \quad (1)$$

where components 1, 2, and 3 are water, protein, and cosolvent, respectively (Scatchard, 1946; Stockmayer, 1950), μ_i, m_i, and γ_i are the chemical potential, molal concentration, and activity coefficient of component i, respectively, T is the thermodynamic (kelvin) temperature, and B_3 and B_1 are the effective numbers of cosolvent and water molecules occupying sites on (bound to) the protein, expressed as moles bound per mole of protein. B_1 and B_3 are not thermodynamic quantities, but a molecular description of the interaction equilibrium. If B_1 is equated with the hydration resulting from interfacial effects (manifested as preferential exclusion of cosolvent), and remembering that the protein cosolvent interaction, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, is measured at equilibrium dialysis, i.e., it is the preferential interaction with protein, eq 1 is seen to be the thermodynamic expression of the balance between the molecular binding of the cosolvent (B_3) and its exclusion (B_1) (Timasheff, 1993). It is this balance that determines whether a particular cosolvent, taken at the given concentration m_3, will salt out or salt in proteins, as well as whether it will stabilize or destabilize structures. If the two interactions (binding and exclusion) vary differently with changes in conditions (e.g., pH, temperature, concentration of cosolvent), their resultant may lead to a particular substance having opposite effects on protein solubility and stability at different conditions. An example of this may be found in the case of $MgCl_2$ (Arakawa et al., 1990a,b).

In order to extend our understanding of the role of surface free energy perturbation in the protein precipitation and stabilization processes, surface tension measurements were carried out for a number of substances known to be preferentially excluded from globular proteins. These were the protein crystallizers (McPherson, 1976; King et al., 1956), but denaturants (Lee & Lee, 1987; Arakawa et al., 1990b) 2-methyl-2,4-pentanediol (MPD,[1] hexylene glycol) and the poly(ethylene glycol)s (PEGs), the universal solvent dimethyl sulfoxide (DMSO), the stabilizing salt guanidinium sulfate (von Hippel & Wong, 1965; Arakawa & Timasheff, 1984c), the stabilizer trimethylamine *N*-oxide (TMAO), the disaccharide, trehalose, and several amino acid salts, including monosodium glutamate and lysine hydrochloride, have been shown to stabilize proteins (Arakawa & Timasheff, 1984a). Preferential interaction measurements were also extended to those amino acid salts for which such data were not available. The results of these studies are discussed in this paper in terms of the balance between the exclusion from and the binding to proteins of these substances.

MATERIALS AND METHODS

Materials. The proteins used were bovine serum albumin (BSA) from Sigma and lysozyme from Worthington. ArgHCl, NaGlu, KAsp, LysHCl, L-Lys-L-Glu, L-Arg-L-Glu, and trimethylamine *N*-oxide (TMAO) were of reagent grade from Sigma. The PEGs and DMSO were obtained from Sigma; MPD and $(Gu)_2SO_4$ were from Eastman; trehalose was from Pfanstiehl Laboratories. The proteins were dialyzed thoroughly against distilled, deionized water, passed through a sintered-glass filter, and lyophilized. Aqueous solutions of the amino acid salts were made without additional electrolytes. Their pH values are listed in Table 1.

Surface Tension. Surface tension measurements were carried out by the drop-weight method with capillaries prepared in our laboratory. The bottoms of the capillaries were polished on a rotating lathe so that the sample liquid could wet them readily. The diameters of the capillary bottoms were determined with a screw micrometer by taking the average of between 5 and 14 readings. The capillary was set up in a vertical position in a constant temperature room controlled at 20 °C and surrounded by sheets of plastic to avoid perturbation by air flow. Care was also taken to avoid vibration of the capillary during measurements. The aqueous solutions were dropped at a rate of approximately 8 drops/min through the capillary, and a constant number of drops was collected in weighing bottles that had been kept overnight in the same room. The weight was measured on a Mettler sensitive balance, and to convert the weight to volume, the density was determined on an Anton Paar densimeter (DMA-02) kept in the same room. The surface tension of water, determined in this manner from the measured dimension of the capillary, was found to be close to the literature value (72.75 dyn/cm), indicating that the experimental system was satisfactory. The more exact dimensions of the capillary and the instrument constant were determined according to the method of Harkins and Brown (1919) and Harkins and Humphery (1916) using water as a standard. This instrument constant and the experimental conditions were further tested with NaCl solutions. The surface tension values obtained were identical to literature values within experimental error. The measured values of 73.0 and 80.9 dyn/cm for 0.58% and 22.13% (w/w) NaCl solutions compared with the literature values of 72.9 and 80.7 dyn/cm. The instrument constant was routinely checked with water and found to be more or less constant, at least

[1] Abbreviations: BSA, bovine serum albumin; KAsp, potassium L-aspartate; ArgHCl, L-arginine hydrochloride; LysGlu, L-lysyl-L-glutamate; ArgGlu, L-argininyl-L-glutamate; NaGlu, monosodium L-glutamate; LysHCl, L-lysine hydrochloride; LysAsp, L-lysyl-L-aspartate; PEG, poly(ethylene glycol); DMSO, dimethyl sulfoxide; MPD, 2-methyl-2,4-pentanediol (hexylene glycol); $(Gu)_2SO_4$, guanidinium sulfate; GuHCl, guanidine hydrochloride; TMAO, trimethylamine *N*-oxide; BSA, bovine serum albumin; β-LG, β-lactoglobulin; RNaseA, ribonuclease A.

Table 1: Partial Specific Volumes and Interaction Parameters for BSA in Amino Acid Salts

concn (M)	pH	$\bar{v}_3$ (mL/g)	g_3 (g/g)	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)	$(\partial\mu_3/\partial m_3)_{T,P,m_2}$ (cal/mol^2)	$(\partial\mu_2/\partial m_3)^{exp}_{T,P,m_2}$ (cal/mol^2)	$(\partial\sigma/\partial m_3)$	$(\partial\mu_2/\partial m_3)^{exp}/(\partial\mu_2/\partial m_3)^{cal}$
						Arginine Hydrochloride					
0.2	5.6	0.671	0.0435	0.737 ± 0.003	0.734 ± 0.001	0.0095 ± 0.0130	−0.218 ± 0.290	4536	−13900 ± 18000	2.23	−0.39 ± 0.59
0.5	5.7	0.681	0.114	0.737	0.736	0.0035	−0.031	1542	−1740	1.91	−0.05
0.7	5.7	0.685	0.164	0.739	0.742	−0.011	0.068	992	3550	1.71	0.12
1.0	5.7	0.690	0.247	0.737	0.744	−0.028	0.114	623	5670	1.42	0.19
1.5	5.8	0.696	0.404	0.738 ± 0.002	0.753	−0.070 ± 0.0141	0.173 ± 0.035	407	9170 ± 6900	1.00	0.53 ± 0.39
						Potassium Aspartate					
0.5	6.3	0.482	0.0894	0.738 ± 0.0005	0.758 ± 0.001	−0.0421 ± 0.0030	0.470 ± 0.035	1866	31100 ± 2700	2.05	0.87 ± 0.08
1	6.5	0.498	0.187	0.736	0.772	−0.0851	0.456	883	29900	2.05	0.81
2	6.7	0.521	0.414	0.737	0.802	−0.195	0.471		37300[a]	2.05	
2.5	6.8	0.528	0.547	0.739 ± 0.002	0.812 ± 0.003	−0.245 ± 0.017	0.447 ± 0.031		35500[a]	2.05	
						Lysylglutamate					
0.2	5.8	0.655	0.0612	0.738 ± 0.0005	0.744 ± 0.0002	−0.0181 ± 0.0021	0.297 ± 0.035		23600 ± 2700[a]		
0.4	5.8	0.662	0.128	0.736	0.755	−0.062	0.487		38600		
0.5	5.9	0.666	0.164	0.738	0.759	−0.072	0.437		34600		
0.7	5.9	0.674	0.240	0.738	0.768	−0.111	0.464		36600		
1.0	6.0	0.683	0.371	0.736 ± 0.001	0.777 ± 0.0005	−0.171 ± 0.006	0.461 ± 0.017		36500 ± 3600		
						Argininylglutamate					
0.2	6.7	0.649	0.0671	0.736 ± 0.003	0.744 ± 0.001	−0.024 ± 0.012	0.361 ± 0.160	4540	23300 ± 10000	2.50	0.54 ± 0.24
0.3	6.7	0.654	0.103	0.736	0.755	−0.0601	0.586	2846	36300	2.50	0.83
0.5	6.7	0.660	0.180	0.738	0.759	−0.073	0.404	1568	24100	2.50	0.55
0.77	6.8	0.668	0.295	0.739 ± 0.001	0.768 ± 0.002	−0.114 ± 0.012	0.387 ± 0.040	1003	24200 ± 3200	2.50	0.55 ± 0.07

[a] Values not corrected for the activity coefficient due to a lack of necessary data.

within the period of use at a fixed capillary setting. The standard deviation of surface tension determined in this way generally did not exceed 0.1 dyn/cm, allowing quantitative comparison between the various solvent systems.

Preferential Interactions. Preferential interaction measurements were carried out by dialysis equilibrium coupled with high-precision densimetry, as described previously (Lee & Timasheff, 1974; Lee et al., 1979; Gekko & Timasheff, 1981a; Arakawa & Timasheff, 1982a).[2] The preferential binding of additive (component 3) to protein, expressed as grams of additive per gram of protein at dialysis equilibrium, is given by:

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = M_3/M_2(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (\phi_2^0 - \phi_2'^0)/(1/\varrho_o - \bar{v}_3) \quad (2)$$

where g_i and M_i are the concentration of component i in grams per gram of water and its molecular weight, respectively, ϱ_o is the density of the solvent, and $\bar{v}_3$ is the partial specific volume of the additive. ϕ_2^0 and $\phi_2'^0$ are the apparent partial specific volumes of the protein, extrapolated to zero protein concentration and measured at isomolal (protein dissolved in the solvent with no dialysis) and isopotential (protein dialyzed against solvent to equilibrium) conditions, respectively. The preferential hydration is (Timasheff, 1963; Reisler et al., 1977)

$$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = -1/g_3(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (3)$$

The perturbation of the chemical potential of the protein induced by the addition of component 3 can be calculated from dialysis equilibrium measurements by (Kirkwood & Goldberg, 1950; Casassa & Eisenberg, 1964)

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = -(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_3/\partial m_3)_{T,P,m_2} \quad (4)$$

where P is pressure, and the solvent additive self-interaction term, $(\partial\mu_3/\partial m_3)_{T,P,m_2}$, for salts is given by

$$(\partial\mu_3/\partial m_3)_{T,P,m_2} = nRT/m_3 + nRT(\partial \ln \gamma_\pm/\partial m_3)_{T,P,m_2} \quad (5)$$

For all of the amino acid salts studied, n, the number of ions in one salt equivalent is 2. The variation in the mean molal ionic activity coefficient, $\gamma_\pm$, with amino acid concentration was calculated from activity coefficient data for ArgHCl, KAsp, and ArgGlu; it was neglected for LysGlu due to the unavailability of data. The partial specific volumes of components 3 were determined by densimetry as described previously (Arakawa & Timasheff, 1984b). Comparison of eqs 1, 4, and 5 shows that

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = B_3 - \frac{m_3}{55.56}B_1 \quad (6)$$

Protein Concentration. These were determined spectrophotometrically using absorptivity values of 6.58 dL/(g·cm) at 278 nm for BSA (Noelken & Timasheff, 1967) and 27.4 at 281 nm for lysozyme (Roxby & Tanford, 1971) in dilute buffer. Absorptivities in concentrated salts, determined as described previously (Gekko & Timasheff, 1981a), were 6.66 for BSA in 1.5 M ArgHCl and 6.59 in 1 M KAsp and were 27.6 and 28.2 for lysozyme in 0.7 and 1.5 M ArgHCl, respectively, and 27.5 in 1 M KAsp. Since these values did not differ much from those in dilute buffer, absorptivities at intermediate salt concentrations were estimated by assuming a linear concentration dependence. This was verified for lysozyme in ArgHCl. When the concentration was measured in 6 M guanidine hydrochloride, the absorptivity values used were 6.25 for BSA and 26.9 for lysozyme.

RESULTS

Surface Tension Increments. The results of the surface tension measurements are given in Figure 1A−D. For all of the amino acid salts, the surface tension of the aqueous solution increased with increasing salt concentration, which is similar to the known effect of inorganic salts on the surface tension of water (*International Critical Tables*, 1928; Melander & Horvath, 1977) and consistent with previous measurements on zwitterionic amino acids (Bull & Breese, 1974). For NaGlu, LysHCl, KAsp, ArgGlu, and LysGlu, the surface tension increased almost linearly with the molal concentration, giving values for $(\partial\sigma/\partial m_3)_{T,P,m_2}$, the molal increment of surface tension, σ, of 2.58, 2.32, 2.05, 2.50, and 2.50 (dyn/cm)/(mol/1000 g of H_2O), respectively. For ArgHCl, the dependence was nonlinear, and therefore $(\partial\sigma/\partial m_3)_{T,P,m_2}$ was obtained by measuring the tangent as a function of salt concentration. The values are listed in Table 1. The high values of these increments indicate that these salts exert a strong cohesive force on water, which can be expected from their high charge densities. Trehalose increased the surface tension of water similarly to sucrose, with $(\partial\sigma/\partial m_3)_{T,P,m_2} = 1.34$. Guanidinium sulfate $[(Gu)_2SO_4]$ also increased the surface tension of water, but to a much smaller extent than the amino acid salts. For $(Gu)_2SO_4$, the concentration dependence of the surface tension displayed curvature below $m_3 \approx 0.5$, after which it became linear with salt concentration. The slope measured above 0.5 m had a value of 1.3 (dyn/cm)/(mol/1000 g of H_2O). This must be compared with that of GuHCl, for which the increase is much shallower (Breslow & Guo, 1990).

The surface tensions of aqueous PEG, MPD, and DMSO solutions are presented in Figure 1C as a function of the organic solvent concentration, expressed as grams per gram of water, g_3.[3] That of TMAO is in Figure 1D. All four organic cosolvents decreased the surface tension of water, more sharply at lower concentrations. Among them, MPD is the most effective surface tension depressant. It lowered the value from 72.75 dyn/cm at $g_3 = 0$ to 39 dyn/cm at $g_3 = 0.2$ ($m_3 = 1.69$). After this, the decrease continued slowly. In contrast, the surface tension decreased gradually for DMSO, PEG, and TMAO. TMAO was the least effective at lowering the surface tension of water. For DMSO, its shallow effect on surface tension is consistent with its strong tendency to associate with water, as manifested by a variety of physical properties of aqueous DMSO solutions (Jacob et al., 1971). A similar situation can be expected for the

[2] A simplified and more detailed explanation of the interpretation of these types of results can be found in, for example, Timasheff and Arakawa (1988).

[3] These units were chosen instead of molarity or molality, since the concentrations of organic solvent−water mixtures usually are expressed in volume or weight percent.

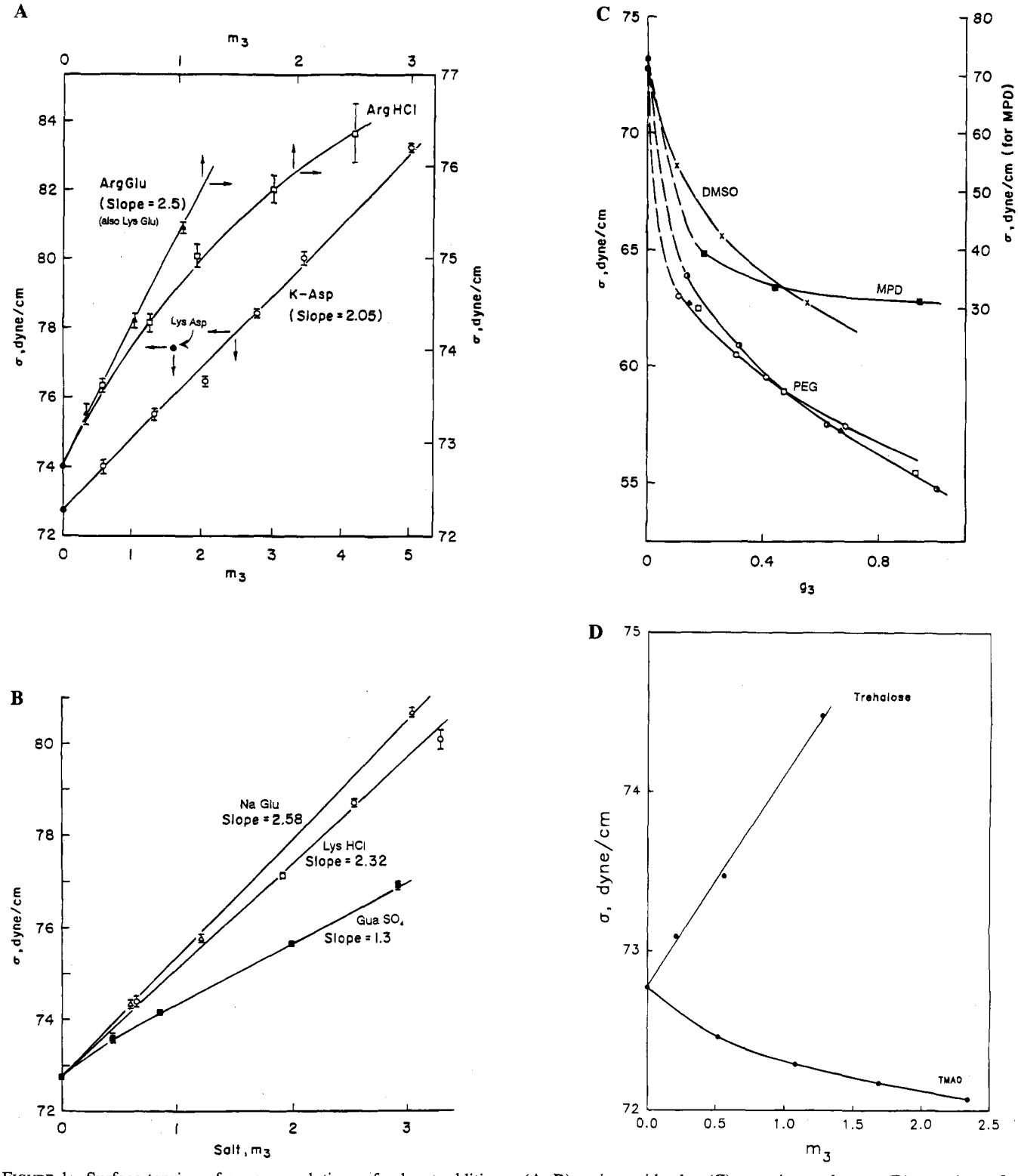

FIGURE 1: Surface tension of aqueous solutions of solvent additives: (A, B) amino acid salts; (C) organic cosolvents; (D) osmolytes. In A, the left ordinate and the lower abscissa refer to KAsp and LysAsp; the right ordinate and the upper abscissa refer to ArgGlu, LysGlu, and ArgHCl. In C, the left ordinate refers to DMSO and the PEGs (200, ◑; 400, □; 600, ▲; and 1000, ○); the right ordinate refers to MPD. The error bars indicate the standard deviations of measurements.

amine. For the series of PEGs,[4] i.e., PEG 200, 400, 600, and 1000, the effect on surface tension was nearly identical on a gram concentration basis. This means that, on a molar basis, their effectiveness as surface tension depressors increases in the order of 200 < 400 < 600 < 1000. Since the molecular weight should be nearly proportional to the degree of polymerization of the repeating unit, CH_2CH_2O, their nearly identical effectiveness on a gram basis suggests that each repeating unit has the same capability of lowering the surface tension, regardless of the degree of polymerization. This probably reflects a preferred surface orientation

[4] The numbers show the average molecular weights of the PEGs, given by Sigma. The exact value is not very important in the preferential interaction and surface tension studies. The important information is the effect on these properties of the differences in average molecular size.

Table 2: Partial Specific Volumes and Preferential Interaction Parameters for Lysozyme in Amino Acid Salts

concn (M)	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)	$(\partial \mu_2/\partial m_3)^{exp}_{T,P,m_2}$ (cal/mol^2)	$(\partial \mu_2/\partial m_3)^{exp}/(\partial \mu_2/\partial m_3)^{cal}$
			Arginine Hydrochloride			
0.2	0.712 ± 0.001	0.723	−0.0347 ± 0.0038	0.798 ± 0.087	10700 ± 1500	0.79 ± 0.11
0.4	0.715 ± 0.002	0.723 ± 0.003	−0.0273 ± 0.0170	0.305 ± 0.191	3730 ± 3200	0.31 ± 0.26
0.5	0.716 ± 0.001	0.724 ± 0.002	−0.0279 ± 0.0105	0.244 ± 0.092	2900 ± 1500	0.25 ± 0.13
0.7	0.716 ± 0.001	0.726 ± 0.001	−0.0369 ± 0.0074	0.225 ± 0.045	2480 ± 800	0.24 ± 0.07
1.5	0.716 ± 0.002	0.736 ± 0.002	−0.0931 ± 0.019	0.230 ± 0.046	2570 ± 800	0.41 ± 0.13
			Potassium Aspartate			
0.5	0.713 ± 0.001	0.722	−0.0189 ± 0.0031	0.211 ± 0.035	2950 ± 600	0.24 ± 0.05
1.0	0.716 ± 0.001	0.740 ± 0.001	−0.0570 ± 0.0047	0.305 ± 0.025	4200 ± 400	0.34 ± 0.03

of the repeating units, with the $(CH_2)_2$ units in contact with air while the oxygens are turned toward water.

Preferential Interactions. The partial specific volumes of BSA in ArgHCl, KAsp, LysGlu, and ArgGlu and of lysozyme in ArgHCl and KAsp are listed in Tables 1 and 2. Their essential invariance with solvent conditions when determined at constant molality of solvent components, ϕ_2^0, and near identity to those reported previously (Gekko & Timasheff, 1981a) indicate that the protein structures were not altered by these amino acid salts at high concentrations. Their combination according to eq 2, with apparent partial specific volumes determined at constant chemical potential, $\phi_2^{'0}$, listed in Tables 1 and 2, resulted in the values of preferential binding, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$. These were negative for all systems, except for BSA in 0.2 and 0.5 M ArgHCl, signifying that, in the presence of these amino acid salts, the proteins are preferentially hydrated. This large preferential exclusion of the additive is very similar to that previously measured for BSA, lysozyme, and β-LG in NaGlu and LysHCl (Arakawa & Timasheff, 1984a), which suggests that the preferential interactions of these amino acid salts with proteins are determined by a common mechanism. The values of the preferential hydration parameter, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, calculated by eq 3, were found to be identical to or higher than the hydration values normally observed for most proteins, 0.2–0.4 g/g (Bull & Breese, 1968; Kuntz, 1971; Kuntz & Kauzmann, 1974). This, by eq 6, indicates that the additives are indeed strongly excluded from the protein domain. The results for BSA in ArgHCl point to penetration by that salt of the solvation layer of the protein (Arakawa & Timasheff, 1984b, 1987).

The dependence on salt concentration of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ for BSA in KAsp, NaGlu, LysHCl, and ArgHCl is plotted in Figure 2. A qualitative difference is apparent between ArgHCl and the salts of the two acidic amino acids. In ArgHCl, this parameter increases from zero, or even negative values at low salt concentrations, to significant positive values at higher concentrations. In the case of NaGlu and KAsp, the preferential hydration remains essentially constant at a value close to 0.45 g of H_2O/g of protein over the entire concentration range studied, while for LysHCl, this parameter follows intermediate behavior.

In contrast, for lysozyme, the preferential hydration values for KAsp and ArgHCl, listed in Table 2, and that for NaGlu (Arakawa & Timasheff, 1984a) are nearly identical. The preferential hydration in the acidic amino acid salts is greater for BSA than for lysozyme, while the opposite is true in the basic ones. Such a result is consistent with the conclusion drawn previously for the LysHCl and NaGlu systems (Arakawa & Timasheff, 1984a): that the net charge on a protein is one of the factors that determines its preferential interactions with solvent components in amino acid salt

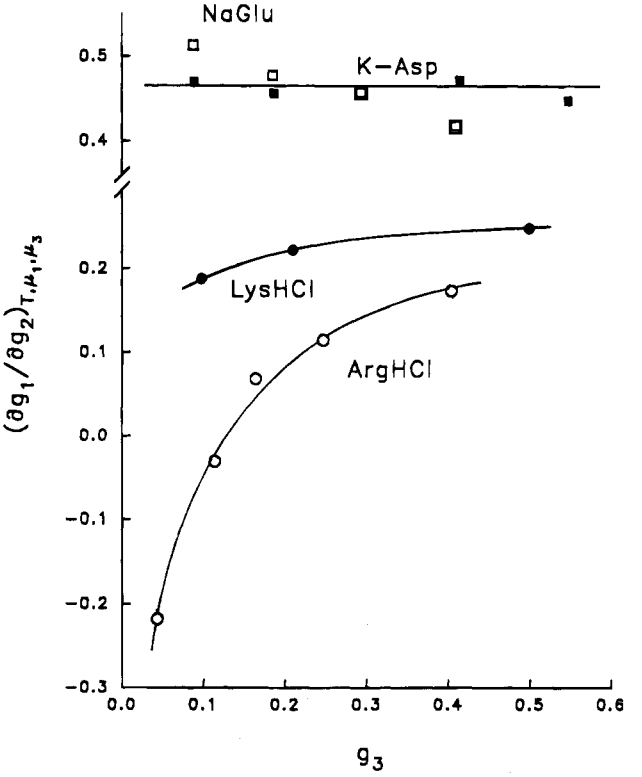

FIGURE 2: Preferential hydration of BSA in NaGlu, KAsp, LysHCl, and ArgHCl.

systems since, in the pH range of this study, BSA should be slightly negative (Foster, 1960) while lysozyme should carry a considerable net positive charge, owing to its high isoelectric point (Alderton et al., 1945).

The extensive exclusion of NaGlu from BSA, with considerably lower exclusions of ArgHCl and LysHCl, suggested that a study of LysGlu and ArgGlu should shed additional light on the nature of the interactions. For these two salts, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ was found to be essentially independent of the salt concentration. The values of ca. 0.4–0.5 g of water/g of protein (see Table 1) are of the same order of magnitude as those of BSA in NaGlu or KAsp. The Glu^- ion appears, therefore, to dominate in the interactions of LysGlu and ArgGlu with the protein.

Surface Tension and Preferential Exclusion. The relation between the perturbation of the surface free energy of water, G^s, by solvent additives and their excess (positive or negative) at an interface is given by the Gibbs adsorption isotherm (Gibbs, 1878):

$$d\sigma = -RT\Gamma_3 \, d \ln a_3 \qquad (7)$$

where $\sigma = dG^s/ds$ is surface tension and s is the area of the interface. The parameter Γ_3 is the excess amount (positive

or negative) of additive (here salt or organic cosolvent) per unit surface area in the interface at its activity, $a_3 = m_3\gamma_3$, in the bulk. When this equation is applied to the particular system of a water−protein interface, Γ_3 becomes equal to $(\partial m_3/\partial m_2)^{\sigma}_{T,\mu_1,\mu_3}/N_{Av}s_2$ (Lee & Timasheff, 1981), i.e., the excess of additive at the solvent−protein interface over its concentration in the bulk at dialysis equilibrium, where s_2 is the surface area of a protein molecule[5] and N_{Av} is Avogadro's number. Written out specifically for a protein, eq 7 becomes

$$(\partial\sigma/\partial \ln a_3)_{T,P,m_2} = -(RT/N_{Av}s_2)(\partial m_3/\partial m_2)^{\sigma}_{T,\mu_1,\mu_3} \quad (8)$$

When this is combined with eq 4, we obtain

$$(\partial\mu_2/\partial m_3)^{\sigma}_{T,P,m_2} = N_{Av}s_2(\partial\sigma/\partial m_3)_{T,P,m_2} \quad (9)$$

where the superscript σ means that this value is calculated from the surface tension increment.

Equation 9 expresses the perturbation of the chemical potential of a protein by the additive due to the change in the surface free energy of the protein-containing cavity caused by the introduction of the additive into the water. It is evident that additives that increase the surface tension of water must increase the chemical potential of the protein and be preferentially excluded from the surface of the protein. The converse is true for those additives that decrease the surface tension. Examination of the preferential interaction data (Arakawa & Timasheff, 1984b,c; this paper) shows that this rule is obeyed by the amino acid salts, disaccharides, and $(Gu)_2SO_4$. The results with the organic solvents (Pittz & Timasheff, 1978; Arakawa & Timasheff, 1985b; Bhat & Timasheff, 1992), however, are inconsistent with the simple prediction of eq 9, since for these compounds the measured values of the thermodynamic interaction parameter, $(\partial\mu_2/\partial m_3)^{exp}_{T,P,m_2}$, were positive (Pittz & Timasheff, 1978; Arakawa & Timasheff, 1985b; Bhat & Timasheff, 1992), whereas those calculated from $(\partial\sigma/\partial m_3)_{T,P,m_2}$ were negative, indicating that, in these systems, other types of interactions must be dominant.

An effective measure of the contribution of the increase in surface tension by an additive to its preferential exclusion is given by the ratio, R, of the experimentally measured value of the preferential interaction parameter, $(\partial\mu_2/\partial m_3)^{exp}_{T,P,m_2}$, given by eq 4, to that calculated from the surface tension increment by eq 9:[6]

$$R = (\partial\mu_2/\partial m_3)^{exp}_{T,P,m_2}/(\partial\mu_2/\partial m_3)^{\sigma}_{T,P,m_2} \quad (10)$$

Values of the ratio for the various amino acids are listed in Tables 1 and 2 and compared in Figure 3A−C. Taking the acidic amino acids first, BSA and β-lactoglobulin, this ratio was high, the values of 0.7−0.9 being similar to those found for the sugar and salting-out salt systems (Arakawa & Timasheff, 1982a,b, 1984b). For tubulin the ratio is somewhat lower, possibly reflecting the lack of knowledge of tubulin geometry in the calculation. For the BSA−KAsp system (Figure 3C), the ratio was again similar to that of NaGlu. For lysozyme, the values of the ratio were much smaller both for KAsp (Figure 3C) and NaGlu (Figure 3A). This may be caused by electrostatic attraction between the positively charged protein and Asp^- ions that would permit their penetration of the water layer at the surface of lysozyme. In the case of the basic amino acids, the pattern is reversed. In LysHCl, shown in Figure 3B, the ratios for BSA and β-LG (0.3−0.5) are considerably lower than those for NaGlu and KAsp. For lysozyme, on the other hand, the ratio was high at low concentrations and decreased as the salt concentration increased. A similar pattern was obtained for lysozyme in ArgHCl (Figure 3C). In the BSA−ArgHCl system, shown in Figure 3C, the salt concentration dependence of the ratio had a qualitatively different character, in that the ratio was negative at low salt concentrations, but became positive at 0.55 M and increased with an increase in salt concentration. These observations suggest a significant role for the surface tension increment in determining the preferential interactions at high salt concentrations, but its compensation by another factor at low concentrations. In ArgGlu, the ratio had a value of 0.55 that was essentially independent of concentration, which reflects the dominance of the Glu^- ion.

DISCUSSION

Protein−Solvent Interface. The concepts discussed in this paper are based on considerations of interactions at the protein surface, i.e., the interface between solvent (water) and the protein, and surface tension variations at that surface. Classically, surface tension is measured at the water−air interface. It reflects the cohesive forces in liquid water. In water−oil mixtures, this becomes the interfacial tension at the water−oil interface, and its numerical value is a function of the cohesive forces of the two phases, as well as of the weak interactions between them. The protein−water interface differs from the classical situation in that (1) this interface is not flat, as is the water−air interface at which surface measurements are made, and (2) it is not chemically homogeneous, as is, for example, an oil−water interface. The first difference, i.e., the overall convex curvature of the interface between water and a protein molecule, leads to a decrease in the surface tension values from those measured at a flat interface (Choi et al., 1970; Tanford, 1979; Sharp et al., 1991; Nicholls et al., 1991). Honig and co-workers (Sharp et al., 1991; Nicholls et al., 1991) have, in fact, calculated by geometric considerations the decreases in surface tension by factors similar to the R values that are reported here (0.6−0.8) for cosolvents that are totally excluded from the protein surface.

The second factor, the chemical nonhomogeneity of the protein surface, leads to competition between exclusion due to the increase in the surface tension and attractive interactions between cosolvent and protein surface. The surface that the protein exposes to solvent can be regarded as a mosaic of loci with different chemical characteristics, e.g., charged and other polar groups, and nonpolar patches with different degrees of hydrophobicity. Its interaction with solvent components will, therefore, be heterogeneous over the surface. First, introduction of the protein molecule into the solvent must form a cavity in the latter. The energy of the cavity formation will be reflected by the surface free energy at the interface, namely, by the interfacial tension.

[5] In this calculation, the values of s_2 were calculated from the partial specific volumes and molecular weights of the proteins, together with their surface to volume ratios determined by small angle X-ray scattering (Pessen et al., 1971; Luzzati et al., 1961).

[6] In this comparison, we must point out uncertainties in the calculation of the preferential interaction from the surface tension increment due to the lack of exact knowledge of the protein−solvent interface area, the neglect of surface curvature, and the assumption that the surface tension increment at a protein−water interface will be the same as that measured at the air−water interface.

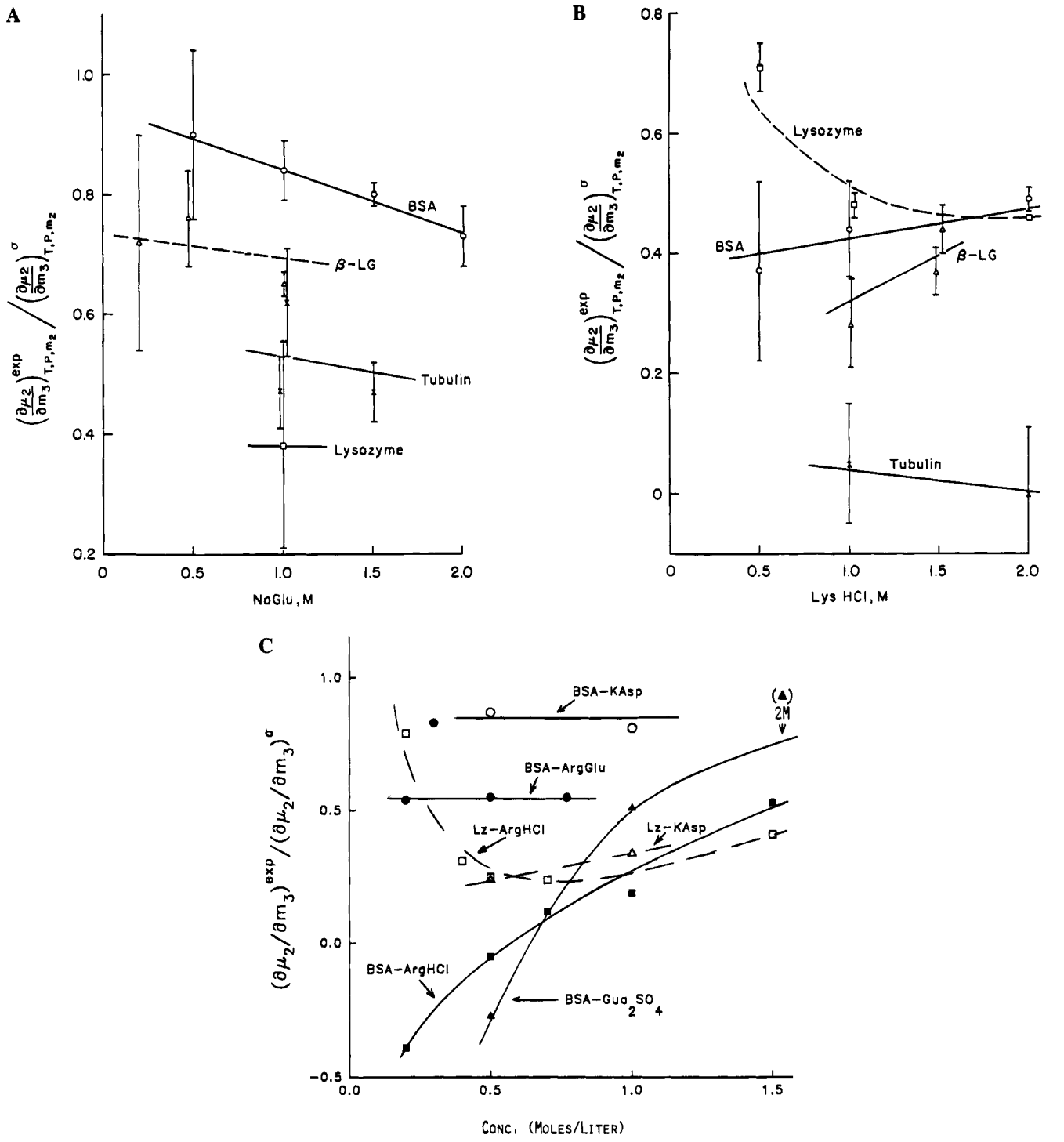

FIGURE 3: Ratio, R, of the experimentally measured values of the preferential interaction parameter to that calculated from the surface tension increment: (A) sodium glutamate and various proteins; (B) lysine hydrochloride and various proteins; (C) comparison of the interactions of BSA and lysozyme (Lz) with the salts. Symbols: BSA with KAsp (○), ArgHCl (■), ArgGlu (●), $(Gu)_2SO_4$ (▲); lysozyme (Lz) with KAsp (△), ArgHCl (□). The error bars indicate the standard deviations in single density measurements. Each value of $(\partial\mu_2/\partial m_3)_{T,P,\mu_3}$, however, is an extrapolation to zero protein concentration of densities measured at different protein concentrations. As a consequence, the points are not scattered and reflect real trends.

For an aqueous medium, this will be equal to the surface tension of water, properly corrected for curvature of the protein surface (Sharp et al., 1991; Nicholls et al., 1991), that is, reflected in the ratio, R, discussed earlier. As a consequence, if the attractive interactions between the protein surface and the cosolvent molecules are much weaker than those with water, the measured exclusion from the interface of a cosolvent that raises the surface tension of water will be that calculated according to the Gibbs isotherm (eqs 7 and 8) modified by the ratio R. This is true, for example, of sucrose (Lee & Timasheff, 1981). Molecules that have an affinity for loci on the protein molecule, e.g., by the formation of nonpolar contacts such as those between the valine side chain and hydrophobic patches on the protein

surface, will penetrate to the protein surface and displace some water molecules. This manifests itself in compensation of the exclusion (preferential hydration) due to the surface tension increment and the consequent reduction in the measured exclusion from that expected from the surface tension increment. Molecules that have a much higher affinity for protein loci than water, such as urea, will penetrate to the protein surface to a much greater extent and displace many water molecules. This exchange with water molecules, which are occupying protein loci in a nonthermodynamically neutral manner (Schellman, 1987; Timasheff, 1992b, 1994) as a consequence of the surface tension increment, will shift the balance in favor of excess occupancy by the cosolvent which, phenomenologically, manifests itself in cosolvent (e.g., urea) preferential binding, as measured in dialysis equilibrium experiments.

Stabilization or destabilization of a protein structure by a cosolvent is determined by the difference between the preferential interactions of the mixed solvent with the protein in the unfolded (denatured) and native (compact) states. Unfolding of a protein increases the protein−solvent interface. Hence, on a mole basis, the preferential exclusion of a cosolvent that raises the surface tension of water will increase, since it is determined only by the total area of protein−solvent contact. On the other hand, unfolding exposes new groups on the protein to contact with solvent. If these have little affinity for the cosolvent molecules (e.g., sucrose) relative to water, the net effect will be one of increased preferential exclusion, and the native structure will be stabilized [see Timasheff (1992, 1993)]. On the other hand, if the newly exposed groups have affinities for the cosolvent molecules, they will effectively decrease the preferential exclusion and lower the stabilizing capacity of the cosolvent (e.g., ArgHCl). Should the newly exposed groups have a strong affinity for cosolvent molecules, the extensive interactions (e.g., newly exposed peptide groups with urea molecules) could completely overwhelm the increase in the exclusion due to the surface tension increment and render contact of the cosolvent with the unfolded protein even more favorable than with the native protein. This will render the denatured form thermodynamically more favorable than the native form and result in protein structure destabilization. This complex balance between cosolvent exclusion in the native and denatured states due to the surface tension increment and favorable interactions at loci in the two end states of the protein determines whether a particular cosolvent will be a stabilizer or a destabilizer. It is clear, then, that the entire pattern of interactions at the protein−solvent interface is a continuum from strong stabilization to strong unfolding. Each cosolvent finds its place along this spectrum (or fan) as a consequence of the described balance for its interactions with the protein surface. In this manner, the stabilization−destabilization is determined by the protein−solvent interface and its variations during protein transitions.

Preferential Binding−Exclusion Balance. Phenomenologically, the results of the present studies permit the classification of the correlation between the perturbation of the surface tension of water by solvent additives and the preferential interactions into three categories: (i) those in which there is no correlation and the surface tension is lowered, but the interaction is that of preferential exclusion (this is found with MPD, PEG, and the methylamines); (ii) those for which the ratio, R, of the experimentally measured preferential exclusion and that calculated from the surface tension effect maintain a value of 0.5−0.8 and do not change with concentration of the additive; and (iii) those for which this ratio varies with additive concentration. These classes of interactions may be explained in terms of the molecular definition of preferential interactions, given in eqs 1 and 6. As stated earlier, preferential binding, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, as measured by dialysis equilibrium, is an expression of the fine balance between the binding of the cosolvent (additive), B_3, i.e., its presence at loci on the surface of the protein,[7] and the binding of water.[7] The latter, in fact, is the source of the observed exclusion of the additive. This becomes evident by setting, in turn, B_1 and B_3 equal to zero. Thus, if in eq 6 we set $B_3 = 0$ and apply eq 3 to the result, we find that

$$B_1 = -\frac{55.56}{m_3}(\partial m_3/\partial m_2)^{\text{exclusion}}_{T,\mu_1,\mu_3} \tag{11}$$

This permits us to rewrite eq 6 as (Timasheff & Inoue, 1968; Timasheff, 1992b, 1993)

$$(\partial m_3/\partial m_2)^{\text{dial equil}}_{T,\mu_1,\mu_3} = (\partial m_3/\partial m_2)^{\text{binding}}_{T,\mu_1,\mu_3} + (\partial m_3/\partial m_2)^{\text{exclusion}}_{T,\mu_1,\mu_3} \tag{12}$$

This situation is analogous to that described by Breslow and Guo (1990) for the effect of chaotropic salts on the solubility of nonpolar molecules in water, which involves competition between cavitation and solute solvation.

Analysis of interactions in terms of this balance has led to the conclusion (Arakawa et al., 1990a) that when exclusion is dominant and does not vary with solution conditions, protein salting out and stabilizing actions always follow. In situations where binding is sufficiently extensive so that at some conditions it can overcome the exclusion, the correlation between that effect and protein stability disappears, although salting out still takes place (Arakawa et al., 1990a,b). Let us now analyze the three classes identified in the present study in terms of the balance between binding and exclusion and the expected contribution of the surface tension perturbation.

MPD and PEG. In the first class, MPD and the PEGs both strongly lower the surface tension of water. The Gibbs adsorption isotherm (eq 8) would then predict a large excess of these solvent components in the vicinity of proteins. This is contrary to the experimental observations. Both MPD (Pittz & Timasheff, 1978) and the PEGs (Lee & Lee, 1979, 1981; Arakawa & Timasheff, 1987; Bhat & Timasheff, 1992) are preferentially excluded from proteins: MPD due to its strong repulsion from charges on the surface of the native protein, and PEGs due to their bulkiness, which leads to preferential hydration by the steric hindrance mechanism first proposed by Kauzmann, as quoted by Schachman and Lauffer (1949). Consistent with their depression of the surface tension of water, both MPD and the PEGs have

[7] Binding in the current context does not mean complexation at specific sites on the protein molecule. Binding means the number of water or additive molecules in contact with the protein surface as a time average or their thermodynamic perturbation by the protein at the given solvent composition. This includes thermodynamically neutral, statistical contacts [as discussed by Schellman (1987, 1990)], as well as a whole spectrum of weak interactions (Timasheff, 1992a, 1993), many of which may be strictly entropic in nature. The numerical values of B_1 and B_3, therefore, are only effective values and are neither hydration numbers nor binding stoichiometries.

strong nonpolar character (Pittz & Bello, 1971; Hammes & Schimmel, 1967), which should make them surface active and cause them to seek contact with nonpolar residues in proteins. This is the origin of their protein-denaturing character (Lee & Lee, 1987; Arakawa et al., 1990b), since their affinity for the newly exposed nonpolar regions leads to thermodynamic stabilization of the denatured form. When the proteins are in the native globular state, however, the interactions of MPD and the PEGs that lead to exclusion are evidently stronger than the binding ones, which is the source of the negative values of preferential binding measured by dialysis equilibrium. Stated formally, $(\partial\mu_3/\partial m_2)^{\text{exclusion}}_{T,P,m_3}$ is $>|(\partial\mu_3/\partial m_2)^{\text{binding}}_{T,P,m_3}|$, since, similar to eq 12,

$$(\partial\mu_3/\partial m_2)^{\text{dial equil}}_{T,P,m_3} = (\partial\mu_3/\partial m_2)^{\text{binding}}_{T,P,m_3} + (\partial\mu_3/\partial m_2)^{\text{exclusion}}_{T,P,m_3} \quad (13)$$

Amino Acid Salts. In the case of the amino acid salts, the ratios, R, obtained for NaGlu and KAsp, shown in Figure 3A,C, assign them to the second class, namely, the observed preferential exclusion is close to that predicted from the surface tension perturbation, especially for BSA and β-LG. The lysozyme data indicate that the contribution of the surface tension effect to the preferential interactions is somewhat compensated by binding of the Glu^- and Asp^- ions to the positively charged protein. For LysHCl, exclusion due to the surface tension effect is again the dominant factor. The pattern, however, is reversed. The lower ratios for BSA and β-LG indicate significant binding to these proteins, while for lysozyme, the high value of R at low salt concentrations suggests electrostatic repulsion of the Lys^+ ion from the positively charged protein. This gradually becomes screened out as the ionic strength increases, which causes R to approach the common value of 0.5. The extremely low ratio obtained with tubulin is consistent with the concept of electrostatic interactions. Tubulin has a large negatively charged domain in its C-terminal region (Ponstingl et al., 1981; Krauhs et al., 1981). This should exert a strong attraction for Lys^+ ions and lead to the near-zero preferential interactions observed (Arakawa & Timasheff, 1984c).

The large values of preferential hydration found for KAsp, NaGlu, LysGlu, and ArgGlu and, except for tubulin, LysHCl, which range from 0.2 to 0.4 g of water/g of protein, suggest that these amino acid salts can be classified into the category of substances that always exhibit preferential hydration (Arakawa & Timasheff, 1990b). Since similar results had been obtained for glycine and alanine salts (Arakawa & Timasheff, 1983), it might be tempting to draw the general conclusion that amino acid salts always lead to preferential hydration. That such a sweeping conclusion is dangerous can be seen from the results obtained with ArgHCl, which were totally different when the protein used was BSA or lysozyme.

For the BSA−ArgHCl system, the interaction pattern is strikingly similar to that observed for BSA in the guanidinium sulfate system (see Figure 3C) and that for β-LG in the glycine system, which had been shown to conform to the exclusion−binding balance mechanism (Arakawa & Timasheff, 1984c, 1987). Let us examine, then, the BSA−ArgHCl system in terms of the same mechanism. The total binding of ArgHCl to BSA, calculated by eq 12, with the exclusion term calculated from the surface tension perturbation, gave values of 10, 17, 16, 16, and 7 mol of salt/mol of protein at 0.2, 0.5, 0.7, 1.0, and 1.5 M salt, respectively. It is clear that the binding becomes saturated at low salt concentrations and falls off at high concentrations, as the ratio R reaches a value of 0.53. The Arg^+ ion can bind to proteins not only electrostatically but also through hydrogen bonds between its guanidinium group and protein peptide bonds, similarly to guanidinium ions (Robinson & Jencks, 1965; Nozaki & Tanford, 1970; Lee & Timasheff, 1974). The monotone increase in R with salt concentration can reflect both the saturation of binding and possibly its reduction by the screening effect at high ionic strength. For the lysozyme−ArgHCl system, the pattern was reversed. The ratio was high at low salt concentrations, but decreased to values similar to those with BSA as the concentration of ArgHCl was raised. This can be ascribed to the gradual screening of the strong repulsion of Arg^+ from the net positive charge of the protein, which should reduce the electrostatic repulsion, raise the effective binding affinity, and, as a consequence, lower the ratio R. The essential identity of the ratio R for lysozyme and BSA at the highest salt concentrations, then, testifies to the total screening out of the difference in net charge between the two proteins.

The concentration independence of the ratio R for ArgGlu, with a value approaching those of salting-out salts (Arakawa & Timasheff, 1982b), shows that Glu^- ion exclusion is dominant over the binding of Arg^+ and Lys^+. Its overwhelming of the attraction of Arg^+ and Lys^+ seen in ArgHCl and LysHCl at low concentrations means that Glu^- has a much stronger exclusion capacity than Cl^-, which is qualitatively equivalent to the observation that substitution of SO_4^{2-} or CH_3COO^- for Cl^- in inorganic salts enhances the exclusion of the salt from protein (Arakawa & Timasheff, 1984b,c). Since this order is identical to that of the lyotropic series of anions, Glu^- must be classified as a stronger protein precipitant than Cl^-. Examination of Figure 3 as a whole shows that, at the higher salt concentrations, the ratio R tends to approach a value of 0.5−0.8. This value of the ratio is the same as that found for sugars and a number of salts and other amino acids; the perturbation of the surface free energy for all of these has been shown to determine the extent of preferential hydration (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a,b, 1983, 1984b). It can be concluded, therefore, that for these amino acid salts as well, the surface tension effect is the major factor in determining the pattern of the preferential interactions of proteins with solvent components.

Guanidinium Salts. A situation similar to that of ArgHCl−BSA is found with $(Gu)_2SO_4$, for which the measured preferential binding varies from zero or positive at low salt concentrations to exclusion at high concentrations, which renders that salt a protein stabilizer (von Hippel & Wong, 1965; Arakawa & Timasheff, 1984c). The corresponding values of the ratio R obtained for BSA in $(Gu)_2SO_4$ were −0.27 at 0.5 M salt, 0.51 at 1 M, and 0.92 at 2 M. The values of binding calculated by eqs 9 and 12 were 16, 27, and 27 mol of salt bound/mol of BSA at 0.5, 1.0, and 2.0 M $(Gu)_2SO_4$, respectively, which reflects the affinity of guanidinium ions for aromatic residues and peptide groups (Robinson & Jencks, 1965; Nozaki & Tanford, 1971; Lee & Timasheff, 1974). The rapid increase of the ratio R to the same level as that of other salts at high $(Gu)_2SO_4$ concentrations, however, reflects the constant total hydration due to the surface tension effect, which assumes progressively more importance as the $(Gu)_2SO_4$ concentration

increases (see eq 6). When the anion is changed from SO_4^{2-} to Cl^-, the guanidinium salt becomes a strong protein denaturant. GuHCl has a much smaller effect on the surface tension of water than $(Gu)_2SO_4$ (Breslow & Guo, 1990), as $(\partial\sigma/\partial m_3)$ varies from 1.0 (dyn/cm)/(mol/1000 g of H_2O) below 2 M GuHCl to 0.3 at higher concentrations. This means that the cohesive force exercised by the anions is different in the two salts, similar to NaCl and Na_2SO_4, for which the surface tension increments are 1.64 and 2.74, respectively. In the case of the inorganic salts, both are preferentially excluded from proteins[8] and both are precipitants and stabilizers, the SO_4^{2-} salt being the stronger. For the guanidine salts, sulfate exclusion can overwhelm guanidinium binding, while chloride cannot. Hence, GuHCl is a denaturant, while $(Gu)_2SO_4$ is a globular protein structure stabilizer (von Hippel & Wong, 1963).

Osmolytes and DMSO. Finally, DMSO and the methylamines are other examples of the complexities of the balance between binding and exclusion. The surface tension data predict preferential binding of these solvent additives to proteins at all concentrations. The experimental results, however, are preferential hydration of proteins at low DMSO concentrations and preferential binding as the DMSO concentration is increased.[9] The binding at high concentrations may be expected because of both the surface tension depression and the hydrophobic interactions between the two methyl groups of DMSO and nonpolar groups in proteins. It is also true, as shown in Figure 1C, that the effectiveness of DMSO as a surface tension depressant is much smaller than those of MPD and the PEGs. This is probably a consequence of its strong affinity for water via the sulfoxide group (Jacob et al., 1971). DMSO then should undergo two competing reactions in aqueous protein solution: binding to the nonpolar surface regions of proteins and association with water in the bulk solvent. The observed preferential hydration of proteins at low DMSO concentrations, then, could be due to the large excess of water, which would permit strong hydration of DMSO and greatly increase the distance of closest approach of the hydrated molecule to surfaces.

The observations with the methylamines can find a similar explanation. Their weak lowering of the surface tension of water should result in their weak accumulation in the surface layer, which should be reinforced at nonpolar regions of the protein surface owing to the methyl groups, in particular in TMAO. On the other hand, the interaction of polar regions on the protein surface with water molecules could be sufficiently strong to render their replacement by methylamines highly unfavorable in the water–ligand exchange equilibrium. This would result in preferential exclusion (Timasheff & Kronman, 1959; Schellman, 1987, 1990; Timasheff, 1992b, 1993). The binding of methylamines due to nonpolar interactions, however, never becomes sufficiently strong to overcome their effective exclusion from polar loci, at least in the case of hydrophilic globular proteins, hence the observed weak preferential exclusion (Arakawa & Timasheff, 1983; Lin & Timasheff, 1994). In the case of hydrophobic proteins, this balance may shift, which should render these natural osmolytes (Somero, 1986; Timasheff, 1992c) solubilizers of hydrophobic proteins, e.g., membrane proteins.

Conclusion. The present discussion offers a rationale for the general observation that additives, for which protein preferential hydration is independent of both cosolvent concentration and solvent conditions, act as protein structure stabilizers (Arakawa et al., 1990b), while those for which the preferential interactions vary with solvent conditions, such as pH and cosolvent concentration, either do not stabilize protein structure or actually act as destabilizers. This is true of poly(ethylene glycol) (Lee & Lee, 1979, 1981; Arakawa & Timasheff, 1985b; Bhat & Timasheff, 1992), 2-methyl-2,4-pentanediol (Pittz & Timasheff, 1978), and $MgCl_2$ (Arakawa & Timasheff, 1984b; Arakawa et al., 1990a,b). In this light, it may be inferred that protein structure stabilization should be expected for BSA and lysozyme in NaGlu and KAsp, for lysozyme in ArgHCl, and for BSA in LysGlu and ArgGlu, in view of the large protein preferential hydration in these amino acid salts. Furthermore, the observed protein structure stabilization by LysHCl and NaGlu (Arakawa & Timasheff, 1984a) suggests that amino acid salts may be useful as protein-stabilizing agents, just as sucrose and glycerol. Their practical limitation in that respect is their nature as polyvalent weak electrolytes, which restricts their use to a limited pH region and could lead to perturbation of the electrostatic properties of macromolecules. On the other hand, the surface tension analysis offers a fundamental explanation for the destabilizing actions of the PEGs and MPD and the complex behavior of DMSO, the guanidinium salts, and the methylamines.

[8] An exception to this universal observation is the finding by Pundak and Eisenberg (1981) of a large preferential binding of NaCl to certain halophilic enzymes with their stabilization. This can be ascribed to the unique natures of these enzymes, which are extremely unstable in low ionic strength buffers, and to the particular protein–salt complexes that are formed (Zaccai & Eisenberg, 1990).

[9] T. Arakawa and S. N. Timasheff, unpublished results.

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