Preferential Interactions of Proteins with Salts in Concentrated Solutions†

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ABSTRACT: The preferential interactions of proteins with solvent components were studied in concentrated salt by densimetric measurements. Proteins were found to be preferentially hydrated in NaCl, $NaCH_3COO$, and Na_2SO_4. The resulting unfavorable free-energy change was related to the effects of these salts on solubility and stability of the proteins. This unfavorable free-energy change was correlated with the large, positive surface tension increment of these salts, i.e., their perturbation of surface free energy. On the other hand, KSCN, $CaCl_2$, and $MgCl_2$ showed considerable binding to bovine serum albumin, which could be related to their destabilizing and salting-in effects on macromolecules. Since the last two salts have high surface tension increments, it was concluded that this does not necessarily lead to protein preferential hydration and stabilization.

The conformational stability and solubility of proteins are sensitive functions of solvent composition. The effect of salts on these properties of proteins in aqueous solution is a strong function of the ionic species present. For example, $CaCl_2$ and NaSCN are known to decrease the conformational stability of macromolecules (von Hippel & Wong, 1965) and to have a salting-in effect (von Hippel & Schleich, 1969; Klotz, 1965), while high concentrations of $(NH_4)_2SO_4$ and Na_2SO_4 are commonly used to precipitate or crystallize proteins in the native form. This topic has been reviewed in detail by von Hippel & Schleich (1969).

Previous studies from our laboratory have shown that knowledge of preferential interactions of solvent components with proteins in three-component systems can lead to an understanding of the mechanism of the effect of solvent components on the stability and solubility of proteins (Lee & Timasheff, 1974, 1981; Timasheff & Inoue, 1968; Lee et al., 1975; Timasheff et al., 1976; Gekko & Timasheff, 1981; Pittz & Timasheff, 1978). It seemed of interest, therefore, to investigate whether the various salt effects on proteins can also be related to the preferential interaction patterns of salts with proteins. Kuntz & Kauzmann (1974) have reviewed the available data on the effect of salt on protein hydration, but the information is insufficient to interpret the widely different salt effects in terms of preferential interactions.

In their analysis of the effect of solvents on the stability of DNA, Sinanoglu & Abdulnur (1964, 1965) have proposed an important role for the free energy of cavity formation reflected in the surface tension of the solvent. This effect has been found (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982) to make a significant contribution to protein stabilization by sugars in aqueous solution, the preferential interaction being a strong function of the increase in the surface tension of water by the addition of sucrose (International Critical Tables, 1928). Recently, Melander & Horvath (1977) have concluded on the basis of the cavity theory that the solubility of proteins in aqueous salt solutions can be corrected with the surface tension increment of water induced by the addition of salts. We have undertaken, therefore, a study of the preferential interactions of proteins with solvent components in aqueous salt solutions in order to explore possible relationships between such interactions and the stability and solubility of proteins as well as with the surface tension effects, and the results are presented in this paper.

Materials and Methods

Bovine serum albumin (BSA)[1] (lot 65C-7533) and lysozyme (lot 57C 8025) were purchased from Sigma. All the salts used, CH_3COONa, NaCl, Na_2SO_4, KSCN, $MgCl_2$, $CaCl_2$, and $MgSO_4$, were of reagent grade and were used without further purification.

The preferential interactions of the solvent components with proteins were obtained from the partial specific volumes of the proteins, measured with a Precision DMA-02 density meter (Anton Paar, Gratz), using previously described procedures (Lee & Timasheff, 1974; Lee et al., 1979; Gekko & Timasheff, 1981) and the protocol of the preceding paper (Arakawa & Timasheff, 1982). The preferential interaction parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, is obtained from the data by (Casassa & Eisenberg, 1964)

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} = \frac{(1 - \rho_0\phi'^0_2) - (1 - \rho_0\phi_2^0)}{1 - \rho_0\bar{v}_3} \quad (1)$$

where g_i is the concentration of component i in grams of i per gram of water, μ_i is its chemical potential, T is the Kelvin temperature, ρ_0 is the density of the reference solvent, $\bar{v}_3$ is the partial specific volume of component 3 (the salt), and ϕ_2^0 and ϕ'^0_2 are the apparent partial specific volumes of the protein determined at conditions at which the molality and the chemical potential of component 3 are, in turn, kept identical in the solution and the reference solvent. The preferential interaction parameter is a direct expression of changes in the free energy of the system induced by addition of component 3, since

$$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2} = \left(\frac{\partial \mu_3}{\partial m_2}\right)_{T,P,m_3} = -\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}\left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2} \quad (2)$$

where P is pressure and m_i is the molal concentration of component i related to g_i by $m_i = 1000g_i/M_i$, where M_i is its molecular weight. The preferential solvation by component 3 is related to preferential hydration by

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

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[1] Abbreviation: BSA, bovine serum albumin.

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Table I: Interaction Parameters

condition	g_3 (g/g)	$\bar{v}_3$ (mL/g)	ϕ_2^0 (mL/g)	ϕ'^0_2 (mL/g)	$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	A_3 (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)
				Lysozyme			
0.02 M acetate, pH 4.5			0.714 ± 0.002	0.712 ± 0.001			
0.5 M acetate, pH 4.68	0.0431	0.502	0.712 ± 0.002	0.726 ± 0.001	−0.0295 ± 0.0063		0.684 ± 0.147
1 M acetate, pH 4.71	0.0906	0.516	0.712 ± 0.002	0.731 ± 0.001	−0.0433 ± 0.0068		0.478 ± 0.075
1 M NaCl, pH 4.5	0.0597	0.331	0.707 ± 0.002	0.723 ± 0.002	−0.0253 ± 0.0063		0.424 ± 0.106
				Bovine Serum Albumin			
0.02 M acetate, pH 4.5			0.735 ± 0.001	0.736 ± 0.001			
0.5 M Na_2SO_4, pH 4.5	0.0718	0.165	0.734 ± 0.001	0.750 ± 0.001	−0.0206 ± 0.0026		0.287 ± 0.036
0.5 M Na_2SO_4, pH 7.0	0.719	0.165	0.735 ± 0.002	0.758 ± 0.001	−0.0298 ± 0.0039		0.414 ± 0.054
1 M Na_2SO_4, pH 4.5	0.145	0.208	0.735 ± 0.002	0.781 ± 0.001	−0.0669 ± 0.0044		0.459 ± 0.030
1 M Na_2SO_4, pH 5.6	0.141	0.208	0.735 ± 0.001	0.788 ± 0.001	−0.0739 ± 0.0028		0.524 ± 0.020
1 M NaCl, pH 4.5	0.0596	0.331	0.734 ± 0.001	0.744 ± 0.001	−0.0158 ± 0.0032		0.265 ± 0.053
1 M NaCl, pH 5.6	0.0597	0.331	0.735 ± 0.001	0.744 ± 0.001	−0.0145 ± 0.0032		0.243 ± 0.054
1 M $CaCl_2$, pH 5.6	0.114	0.216 [a]	0.734 ± 0.001	0.731 ± 0.001	0.00367 ± 0.00245	0.0380	−0.0321 ± 0.0214
1 M KSCN, pH 5.6	0.102	0.530	0.738 ± 0.001	0.735 ± 0.001	0.00706 ± 0.00471	0.0378	−0.0689 ± 0.0459
0.5 M $MgCl_2$, pH 4.5	0.0479	0.150	0.733 ± 0.001	0.737 ± 0.001	−0.00491 ± 0.00245	0.0095	0.102 ± 0.051
1 M $MgCl_2$, pH 5.6	0.0975	0.176	0.737 ± 0.001	0.740 ± 0.001	−0.00416 ± 0.00277	0.0251	0.0427 ± 0.0285
1 M $MgSO_4$, pH 4.5	0.121	0.136	0.734 ± 0.001	0.769 ± 0.001	−0.0469 ± 0.0027		0.388 ± 0.0222

[a] Dunn (1966).

The cosolvent self-interaction term, $(\partial\mu_3/\partial m_3)_{T,P,m_2}$, can be calculated from

$$\left(\frac{\partial\mu_3}{\partial m_3}\right)_{T,P,m_2} = RT\left(\frac{\partial \ln a_3}{\partial m_3}\right)_{T,P,m_2} \quad (3)$$

and the activity of salt, a_3, can, in turn, be calculated from the mean molal activity coefficient, $\gamma_\pm$, of salt with eq 4 for NaCl, CH_3COONa, KSCN, and $MgSO_4$ and with eq 5 for $CaCl_2$, Na_2SO_4, and $MgCl_2$. The partial specific volumes of

$$a_3 = m_3^2\gamma_\pm^2 \quad (4)$$

$$a_3 = 4m_3^3\gamma_\pm^3 \quad (5)$$

the proteins were measured in a 0.02 M acetate or phosphate buffer, except for the CH_3COONa and $CaCl_2$ systems. The acetate solutions were equimolal in CH_3COONa and CH_3COOH. For example, the 0.5 M acetate solution contained 0.5 M CH_3COONa and 0.5 M CH_3COOH. The pH of a 1 M $CaCl_2$ solution was adjusted with HCl.

Protein concentrations were determined on a Cary Model 118 spectrophotometer. The absorbance values used were 27.4 dL/(g cm) at 281 nm for lysozyme (Roxby & Tanford, 1971) and 6.58 dL/(g cm) at 278 nm for BSA (Noelken & Timasheff, 1967) in dilute salt solutions. The absorbance values of the proteins in concentrated salts are expected to be somewhat different from those of the native proteins in dilute salt. For lysozyme, these were determined by diluting volumetrically two aliquots of aqueous protein solution with identical amounts of a dilute buffer in which the absorptivity of the protein is known and of the salt solution in question and then measuring their absorbances at 281 nm. The resulting absorbance values were 27.2 dL/(g cm) in 0.5 and 1 M acetate and 27.7 dL/(g cm) in 1 M NaCl. For BSA, the dilute salt absorbance value was used throughout. While this may affect slightly the absolute values of the partial specific volumes, it should have no effect on the preferential interaction parameters.[2] The light scattering contribution was found to be insignificant and was neglected. Where needed, the partial specific volumes, $\bar{v}_3$, of the salts were determined by the same procedure as those of proteins at constant molality. The $\bar{v}_3$ value for $CaCl_2$ was taken from the literature (Dunn, 1966).

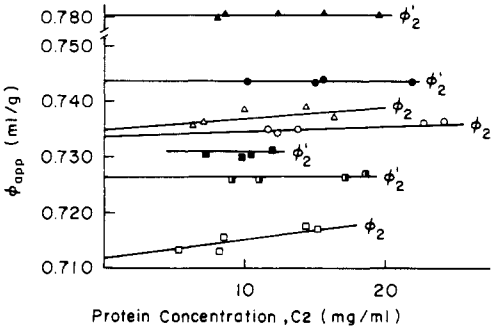

FIGURE 1: Protein concentration dependence of the apparent specific volume: lysozyme, 1 M acetate (□, ■), 0.5 M acetate (right-side solid square); BSA, 1 M NaCl, pH 4.5 (○, ●), 1 M Na_2SO_4, pH 4.5 (△, ▲).

Results

The partial specific volumes of BSA and lysozyme were measured in dilute buffer, both at constant molality and at constant chemical potential. The results, listed in Table I, show that the two values were close to identical in both cases, indicating that the experimental procedures were satisfactory for an examination of the preferential interactions of the salts with proteins, since the interaction parameter is obtained from the difference between the partial specific volumes at the two conditions in the presence of the salt. Typical plots of the apparent specific volume, ϕ_{app}, vs. protein concentration are shown in Figure 1. While for lysozyme, ϕ_2 increased slightly with protein concentration at all conditions, BSA showed little concentration dependence. In no case was there a concentration dependence of ϕ'_2.

Results of the preferential interactions are given in Table I. For salting-out salts, namely, CH_3COONa, NaCl, and Na_2SO_4, ϕ'^0_2 was considerably higher than ϕ_2^0. The preferential interaction parameters of the salts with the proteins, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, were calculated with eq 1. In the case of acetate, the interaction of CH_3COOH with protein was neglected even though it was present at high concentration and may bind somewhat to the proteins. This could be done since

[2] The identity, within experimental error, of the ϕ_2^0 values in various salts with the value in dilute buffer strongly supports the assumption on the identity of the absorptivity values. This is further supported by the measured values for lysozyme cited above.

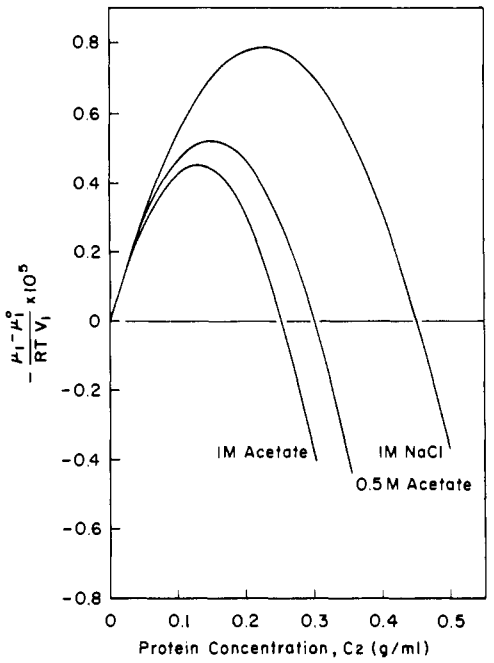

FIGURE 2: Effect of protein concentration on the chemical potential of water in salt solutions for lysozyme.

the contribution of the preferential interactions of CH_3COOH with the proteins to the difference between ϕ_2^0 and ϕ'^0_2 should be much smaller than that of CH_3COONa, the partial specific volume of CH_3COOH (0.864 mL/g) being much closer to that of water than that of CH_3COONa (0.516 mL/g) at 1 M. Assuming 0.04 g/g for the preferential binding of CH_3COOH in 1 M acetate, which would correspond to a very strong interaction, we obtain 0.708 mL/g for ϕ'^0_2 which is not much different from the experimental value of 0.712 mL/g for ϕ_2^0. It may be reasonably concluded, therefore, that the observed difference is mostly due to interaction of CH_3COONa with the protein. The value of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was negative in all cases for CH_3COONa, NaCl, and Na_2SO_4, indicating a deficiency of the salt in the immediate domain of the protein, namely, a preferential hydration of the protein, as shown in the last column of Table I. In the case of $MgCl_2$, the measured preferential hydration was very small and essentially within experimental error from zero. To the contrary, $MgSO_4$, known as a strong salting-out agent, showed a large preferential hydration, the magnitude of which was between those of NaCl and Na_2SO_4. For KSCN and $CaCl_2$, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was small and positive, but within experimental error from zero, indicating that there is probably a small excess of these two salts in the domain of the protein.

The preferential interaction parameter is related to the total values of the binding of water and salt to the proteins by (Inoue & Timasheff, 1972)

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} = A_3 - g_3 A_1 \qquad (6)$$

where A_1 and A_3 are the total amounts of water and salt bound to protein, respectively, expressed as grams of each component per gram of protein. For CH_3COONa, NaCl, and Na_2SO_4, assuming total exclusion of the salt from the domain of the protein, namely, setting $A_3 = 0$, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ is equal to the total hydration, A_1. All the values given in the last column of Table I are close to, or greater than, the usual protein hydration value of 0.2–0.4 g of water per g of protein (Kuntz & Kauzmann, 1974; Kuntz, 1971; Bull & Breese, 1968), indicating that these salts may be largely excluded from the domain of the protein. For KSCN, $MgCl_2$, and $CaCl_2$, the amount of salt binding to the protein, A_3, was calculated with the assumption of a hydration of 0.3 g/g of protein ($A_1 = 0.3$). The results are shown in the seventh column of Table I and in the third column of Table II. They indicate a significant salt binding to proteins.

The effect of the salts on the chemical potentials of the proteins, $(\partial\mu_2/\partial m_3)^{exptl}_{T,P,m_2} = (\partial\mu_3/\partial m_2)^{exptl}_{T,P,m_3}$, was calculated with eq 2 and is given in the fifth column of Table II. The cosolvent self-interaction term, $(\partial\mu_3/\partial m_3)_{T,P,m_2}$, listed in the sixth column, was calculated with eq 3–5, by using values of $\gamma_\pm$ taken from Robinson & Stokes (1955). The large positive change in chemical potential seen for CH_3COONa, NaCl, and Na_2SO_4 shows that introduction of the salt into an aqueous solution of the proteins is thermodynamically unfavorable, destabilizing the system. This may lead to association or aggregation of the protein, or even to phase separation in the form of amorphous precipitation or crystallization. Quantitatively, the last effect may be expressed in terms of a phase isotherm, which is the dependence of the chemical potential of the principal solvent on protein concentration (Pittz & Timasheff, 1978; Timasheff et al., 1976):

$$\frac{\mu_1 - \mu_1^0}{RTV_1} = -\frac{C_2}{M_2}\left\{1 + \frac{V_m}{2RTM_2}C_2\left[\left(\frac{\partial\mu_2^{(e)}}{\partial m_2}\right)_{T,P,m_3} + \left(\frac{\partial\mu_2}{\partial m_3}\right)_{T,P,m_2}\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}\right] + 0(C_2^2)\right\} \qquad (7)$$

where $\mu_2^{(e)}$ is the excess chemical potential of the protein, R is the universal gas constant, V_1 is the molar volume of component 1, C_2 is the protein concentration in grams per milliliter, and V_m is the volume of solution containing 1 kg of principal solvent. The self-interaction term, $(\partial\mu_2^{(e)}/\partial m_2)_{T,P,m_3}$, was assumed to be due solely to the excluded volume of the protein and was calculated from (Tanford, 1961)

$$\left(\frac{\partial\mu_2^{(e)}}{\partial m_2}\right)_{T,P,m_3} = \frac{RT}{V_m}(M_2\bar{v}_2) \qquad (8)$$

The results of these calculations, using the data on the partial specific volumes of the proteins and the interaction parameters, are illustrated in Figures 2 and 3 which compare the phase isotherms of lysozyme and BSA, respectively, for different solvent systems. In Figure 3, the phase isotherm for 1 M glucose is presented for comparison (Arakawa & Timasheff, 1982). In all the systems, the chemical potential of the water first decreases as the protein concentration increases. Then, except for $CaCl_2$ and $MgCl_2$, after passing through a minimum, it starts to increase and finally changes its sign to a positive value at a given protein concentration. This protein concentration, which would be close to the saturation concentration of the protein in the corresponding solvent, is given in the last column of Table II as C_{satn}. As is evident from the figures and Table II, these values depend strongly on the concentration of the additive, the protein, and the nature of the additive. For 0.5 M Na_2SO_4, C_{satn} also shows a dependence on the solvent pH.

It may be of interest to compare these calculated solubilities of proteins with actual experimental values. It is known that, for a salting-out system, the solubility of a protein first increases with salt concentration and then, after passing through a maximum, decreases sharply (Dixon & Webb, 1961). At sufficiently high salt concentrations, the concentration dependence of solubility can be expressed as

$$\log S = \beta - K_s C_s \qquad (9)$$

Table II: Thermodynamics of Preferential Interactions

condition	$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$	Λ_3' (mol/mol)	$\left(\frac{\partial \sigma}{\partial m_3}\right)_{T,P,m_2}$ [a]	$\left(\frac{\partial \mu_3}{\partial m_2}\right)_{T,P,m_3}^{\text{exptl}}$ [b]	$\left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2}$ [b]	$\left(\frac{\partial \mu_3}{\partial m_2}\right)_{T,P,m_3}^{\text{calcd}}$ [b]	$\frac{\left(\frac{\partial \mu_3}{\partial m_2}\right)^{\text{exptl}}}{\left(\frac{\partial \mu_3}{\partial m_2}\right)^{\text{calcd}}}$	C_{satn} (g/mL)
				Lysozyme				
0.5 M acetate, pH 4.68	−5.14 ± 1.10			11380 ± 2440	2214			0.30
1 M acetate, pH 4.71	−7.55 ± 1.19			8500 ± 1340	1126			0.25
1 M NaCl, pH 4.5	−6.20 ± 1.54		1.64	6750 ± 1680	1088	9950	0.678	0.45
				Bovine Serum Albumin				
0.5 M Na_2SO_4, pH 4.5	−9.86 ± 1.24		2.73	21480 ± 2700	2179	47500	0.452	0.43
0.5 M Na_2SO_4, pH 7.0	−14.2 ± 1.86		2.73	30940 ± 4050	2179	47500	0.651	0.19
1 M Na_2SO_4, pH 4.5	−32.0 ± 2.1		2.73	33630 ± 2210	1051	47500	0.708	0.074
1 M Na_2SO_4, pH 5.6	−35.4 ± 1.3		2.73	37210 ± 1370	1051	47500	0.783	0.060
1 M NaCl, pH 4.5	−18.4 ± 3.7		1.64	20020 ± 4030	1088	28700	0.698	0.23
1 M NaCl, pH 5.6	−16.8 ± 3.7		1.64	18280 ± 4030	1088	28700	0.637	0.28
1 M $CaCl_2$, pH 5.6	2.25 ± 1.50	23.3	3.66	−5160 ± 3440	2292	63700	−0.081	
1 M KSCN, pH 5.6	4.94 ± 3.29	26.4	0.45	−4910 ± 3270	994	7800	−0.629	
0.5 M $MgCl_2$, pH 4.5	−3.51 ± 1.80	6.8	3.16	13210 ± 6780	3764	55300	0.239	
1 M $MgCl_2$, pH 5.6	−2.97 ± 1.98	17.9	3.16	7650 ± 5100	2577	55300	0.139	
1 M $MgSO_4$, pH 4.5	−26.5 ± 1.5		2.10	20030 ± 1130	756	36500	0.549	0.18

[a] In units of dynes per centimeter per mole of salt in 1000 g of H_2O. [b] In units calories per mole of salt per moles of i in 1000 g of H_2O.

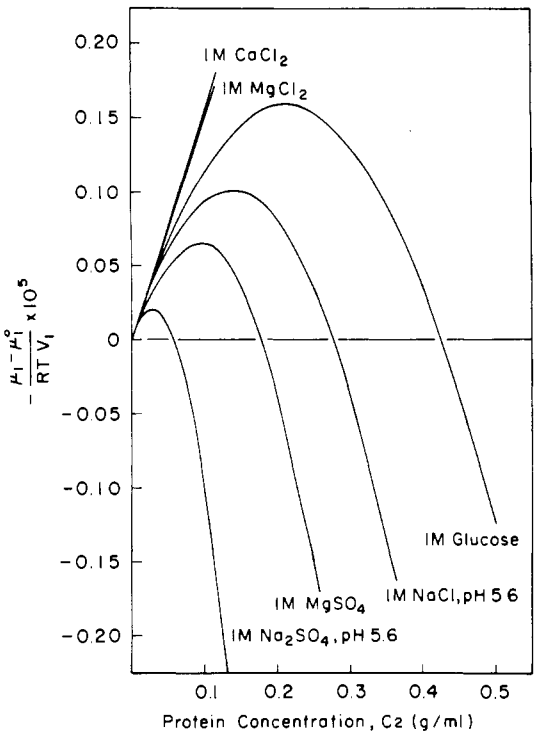

FIGURE 3: Effect of protein concentration on the chemical potential of water in 1 M salt solutions for BSA.

where S is the protein solubility in grams per milliliter, C_s is the salt concentration in moles per liter, K_s is the salting-out constant, and β is the solubility extrapolated to zero salt concentration, but which does not correspond to the actual solubility in the absence of salt. For serum albumin in the $(NH_4)_2SO_4$ system, the relation is (Cohn & Edsall, 1943)

$$\log S = 1.2 - 2.1C_s \qquad (10)$$

This leads to estimates of $S = 1.4$ g/mL at 0.5 M $(NH_4)_2SO_4$, $S = 0.13$ g/mL at 1 M $(NH_4)_2SO_4$, and $S = 0.011$ g/mL at 1.5 M $(NH_4)_2SO_4$. Since Na_2SO_4 is known to be a stronger salting-out agent than $(NH_4)_2SO_4$, the above value at 1 M salt is comparable with our calculated value, 0.06 to ~0.07 g/mL at 1 M Na_2SO_4. As was shown by Dixon & Webb (1961), the above equation no longer holds at 0.5 M salt, and, therefore, the extrapolation to 0.5 M salt would not give an actual solubility, but a higher value. It is of interest to note that the effect of 1 M glucose on the solubility of the protein was of the same order of magnitude as that of 1 M NaCl. On the other hand, in the cases of KSCN and $CaCl_2$, introduction of the salt resulted in a negative change in the chemical potential of the system. Thus, the thermodynamic effect is favorable, resulting in an increase in the solubility of the protein. In fact, the phase isotherm of BSA in 1 M $CaCl_2$ solution shows a monotone decrease of $\mu_1 - \mu_1^0$ with protein concentration, indicating no phase separation of the protein (Figure 3). For 1 M $MgCl_2$, $(\partial\mu_3/\partial m_2)_{T,P,m_3}$ is positive, and hence the thermodynamic effect is unfavorable, but too small to bring about a phase separation. On the other hand, a phase separation of BSA was found in the 1 M $MgSO_4$ system, C_{satn} being again intermediate between the values in 1 M NaCl and 1 M Na_2SO_4.

All the salts used in this study increase the surface tension of water, just as do sucrose (International Critical Tables, 1928) and glucose (Landt, 1931). In view of this and the correlation by Melander & Horvath (1977) of protein solubility with surface tension, it seemed of interest to examine the relation between this property of the solution and the preferential interactions. By definition, the surface tension of the solvent, σ, is related to the surface free energy by

$$dG = \sigma ds \qquad (11)$$

where s is the surface area. The change in the chemical potential of the protein due to the increase of the surface tension of the solvent by the salt, $(\partial\mu_2/\partial m_3)^{calcd}_{T,P,m_2}$, can be calculated formally from

$$\left(\frac{\partial\mu_2}{\partial m_3}\right)^{calcd}_{T,P,m_2} = N_{Av}s_2\left(\frac{\partial\sigma}{\partial m_3}\right)_{T,P,m_2}\left[1 + \left(\frac{\partial \ln \gamma_3}{\partial \ln m_3}\right)_{T,P,m_2}\right] \qquad (12)$$

where N_{Av} is Avogadro's number, s_2 is the surface area of the protein, and $(\partial\sigma/\partial m_3)_{T,P,m_2}$ is the molal surface tension increment. The values of s_2 were calculated from the partial specific volumes of the proteins, their molecular weights, and the surface to volume ratios determined by small-angle X-ray scattering (Pessen et al., 1971; Luzzati et al., 1961). The values of $(\partial\sigma/\partial m_3)_{T,P,m_2}$ were taken from the table in the paper of Melander & Horvath (1977); they are listed in the fourth column of Table II. The results of the calculations and the ratio of the experimental values to the calculated ones are given in the seventh and eighth columns of Table II, respectively. In the cases of NaCl and Na_2SO_4, this ratio does not vary much from a value of 0.7, independent of the nature of both the protein and the salt. For $MgCl_2$, the experimental value, 7650 cal/mol, is only 14% of the calculated one, and, for KSCN and $CaCl_2$, the calculated and experimental values are of opposite signs. In view of the constancy of this ratio over a variety of conditions, deviations from this ratio may be taken as a measure of the solvent stabilization–ion binding balance. A strong downward deviation may be regarded as the overcoming of preferential exclusion by specific binding of the ionic species.

The effects of pH and nature of the salts on the interaction parameter for BSA seem particularly striking. BSA is known to undergo a partial unfolding in acid pH (Yang & Foster, 1954; Aoki & Foster, 1957; Vigai & Foster, 1967). Comparison of the values of the ratio in column 8 of Table II shows that at pH 4.5 and 0.5 M Na_2SO_4 this ratio is lowered to 0.452, suggesting penetration of the salt into the domain of the protein and possible salt binding. This effect is not observed in 0.5 M Na_2SO_4 at pH 7.0, in 1 M Na_2SO_4 at pH 4.5, or in 1 M NaCl at pH 4.5, suggesting that it is the anion which is mainly responsible for the thermodynamic stabilization and that it is required at a high molar level. In the case of the magnesium salts, the preferential exclusion is perturbed by the binding of the Mg^{2+} ion to BSA (Robinson & Jencks, 1965). As a result, the ratio is lowered. Comparing 0.5 M $MgCl_2$ with 1 M NaCl, both at pH 4.5 and 1 M in Cl^-, shows that the presence of Mg^{2+} ions greatly reduces the ratio. The same situation is true of the pair of 1 M $MgSO_4$–1 M Na_2SO_4, where the levels of the stabilizing sulfate ion are identical. Yet in the magnesium salt, the SO_4^{2-} ion is not able to counteract fully the direct binding effect of Mg^{2+} to BSA. The $MgSO_4$–$MgCl_2$ pair shows that the SO_4^{2-} ion is a greater structure stabilizer than Cl^-, since in this case the ratio is greater for $MgSO_4$, even though the measurement was done at lower pH. This is consistent with the comparison of Na_2SO_4 with NaCl. Both at identical anion concentrations (1 M) and at identical Na^+ concentrations (1 M), and at pHs where the protein is quite stable, the exclusion of the salt from the protein is stronger for SO_4^{2-}, as is evident from column 5 of Table II.

Discussion

The results presented in this paper show that the preferential interaction with proteins of NaCl, CH_3COONa, and Na_2SO_4 is largely negative; i.e., the proteins are preferentially hydrated. Protein preferential hydration in concentrated salts has also been found for ribonuclease in 1.33 M Na_2SO_4 (Timasheff et al., 1976) and for chicken heart glyceraldehyde-3-phosphate dehydrogenase in potassium phosphate (Aune & Timasheff, 1970), which is known to be a strong salting-out salt. The universality of this observation leads to the conclusion that protein preferential hydration is a general phenomenon in salting-out systems. The introduction of these salts into an aqueous solution of the protein results in a positive change in the chemical potential of the protein, which should lead to an eventual phase separation. The phase isotherms and the values of C_{satn} in Table II show that, at the protein concentrations commonly used, lysozyme and BSA cannot be precipitated by high concentrations of these salts except for 1 M Na_2SO_4. For example, lysozyme would precipitate at a concentration of more than 0.45 g/mL in 1 M NaCl, as shown in Figure 2. This is in agreement with the known fact that both proteins are highly soluble in aqueous media. Comparison of Figures 2 and 3 shows that lysozyme is more soluble than BSA. For both proteins, an increase in salt concentration predicts a lowering of the solubility, consistent with the salting-out precipitation of proteins. The salt concentration dependence of protein solubility appears to be stronger for Na_2SO_4 than for CH_3COONa, which might suggest that, in the 1 M acetate system, the interaction of CH_3COOH with the protein can no longer be neglected. As shown in Figures 2 and 3, and Table II, the protein solubility increases in the orders CH_3COONa < NaCl and Na_2SO_4 < NaCl < glucose when compared at the same molarity of the cosolvent. Figure 3 shows that, in 1 M cosolvent, BSA should precipitate at concentrations above 60 mg/mL for Na_2SO_4, 280 mg/mL for NaCl, and 430 mg/mL for glucose. The values of C_{satn} indicate that 1 M Na_2SO_4 and 1 M acetate are, respectively, 4 times and twice as effective on protein solubility as 1 M NaCl, giving a salting-out order of effectiveness of NaCl < CH_3COONa < Na_2SO_4. These results are fully consistent with the known facts that Na_2SO_4 is a much stronger protein precipitant than NaCl and that CH_3COONa is intermediate between the two. There are no solubility data for proteins in aqueous sugar solutions. We may use, however, the data of Klotz (1965) on the effects of various substances on the clouding point of poly(vinyloxazolidinone) in aqueous solution. In this system, sucrose was intermediate between $(NH_4)_2SO_4$ and NaCl. Considering the higher effectiveness of Na_2SO_4 as a precipitant than of $(NH_4)_2SO_4$, it seems reasonable to assume that mono- and disaccharides, such as glucose and sucrose, respectively, should be close to NaCl in their effectiveness as protein precipitants.

It has been reported by many authors (von Hippel & Schleich, 1969; Klotz, 1965) that the addition of KSCN, $MgCl_2$, or $CaCl_2$ to an aqueous solution of macromolecules results in the destabilization of the macromolecules, for example, in a decrease of their transition temperature. For these salts, the preferential interaction parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, was found to be very small, in contrast to that for NaCl, CH_3COONa, and Na_2SO_4, permitting extensive total binding of these salts when calculated with the assumption of a hydration of 0.3 g of water per g of protein. Scatchard and co-workers have measured the binding of various salts to human serum albumin by different methods (Scatchard & Black, 1949; Scatchard et al., 1950, 1957, 1959). From measurements of the pH displacement of isoionic protein solutions induced by the addition of salts, they found that as many as 25 mol of SCN^- was bound per mol of protein at 0.2 M salt. Our calculated value of 26 mol of salt/mol of protein for the total binding of KSCN to BSA is, therefore, quite consistent with these results, although we have used higher concentrations of the salt. Edsall et al. (1950) have also reported the binding of several Ca^{2+} ions to BSA at a low $CaCl_2$ concentration and considerable more Ca^{2+} binding sites than 10 at high concentration. Since in the present paper, the $CaCl_2$ interaction was measured at high salt concentration, the obtained result of 23 mol of salt/mol of protein again seems to agree with the direct measurement. Under the conditions used, it is highly unlikely that these salts bind to the proteins in the form of ion pairs; rather, it must be the cation or the anion which binds at specific sites, with the counterions distributed properly in the electrical double layer to satisfy the electroneutrality of the protein component. In the case of $MgCl_2$ and $CaCl_2$, the ions bound predominantly to the protein should be Mg^{2+} and Ca^{2+}, since Cl^- is also a constituent of NaCl, which is mostly excluded from the domain of the protein, although chloride is known to bind to proteins (Scatchard et al., 1957). In the case of KSCN, both K^+ and SCN^- are known to bind to proteins, but it is likely that it is the SCN^- which binds predominantly at specific sites on BSA. For these anions, the binding constants for human serum albumin (Scatchard et al., 1957) were reported to be 2400 (mol/1000 g of H_2O)$^{-1}$ for Cl^- and 46 200 (mol/1000 g of H_2O)$^{-1}$ for SCN^- on the first class of binding sites and to decrease for the second and third classes, but always remaining greater for SCN^- than for Cl^-. There seem to be no reliable data for cation binding constants for both bovine and human serum albumins. It might be possible then to assign the destabilizing effects of KSCN, $MgCl_2$, and $CaCl_2$ to the binding of their constituent ion species to proteins. The increased net charge of the protein due to the binding of the ion should increase the electrostatic free energy of the protein, the resulting repulsive forces causing a decrease in the stability of the protein (Linderstrøom-Lang, 1924). This electrostatic repulsive force should also prevent protein association or aggregation; in other words, it should increase the solubility of the proteins in aqueous solutions of these salts, which is fully in agreement with the known protein salting-in property of these salts.

Since surface tension has been invoked as an important factor contributing to protein solubility (Melander & Horvath, 1977), it seemed of interest to examine whether it could make a significant contribution to the preferential interaction of salts with proteins, as it does in the sucrose and glucose systems (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982). In the NaCl and Na_2SO_4 solvent systems, the value of $(\partial\mu_3/\partial m_2)^{calcd}_{T,P,m_3}$, calculated from the surface tension increment induced by the salt, was found to be of the same order of magnitude as $(\partial\mu_3/\partial m_2)^{exptl}_{T,P,m_3}$ obtained from interaction data. It would seem, therefore, that, in these salts, the increase of the surface tension of water by addition of the salt can make a significant contribution to preferential interaction, just as in the case of sucrose and glucose (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982). The surface area of a protein, however, may not necessarily contribute uniformly to the interface free energy. While $(\partial\mu_3/\partial m_2)^{exptl}_{T,P,m_3}$ for 1 M NaCl and 1 M Na_2SO_4 does not depend on the solvent pH, that for 0.5 M Na_2SO_4 at pH 4.5 is lower than those for the same salt concentration at pH 7.0 or for 1 M Na_2SO_4 at both pHs, indicating the contribution of other factors to $(\partial\mu_3/\partial m_2)^{exptl}_{T,P,m_3}$, e.g., the charge density on the surface of the protein.

In the case of $CaCl_2$, $MgCl_2$, and KSCN, there was no relationship between $(\partial\mu_3/\partial m_3)^{\text{exptl}}_{T,P,m_3}$ and $(\partial\mu_3/\partial m_2)^{\text{calcd}}_{T,P,m_3}$, these quantities being of opposite signs for KSCN and $CaCl_2$. For the KSCN system, this result is reasonable, because, for this salt, the parameter $(\partial\sigma/\partial m_3)_{T,P,m_2}$ is much lower than that for the other salts. The results with the $MgCl_2$ and $CaCl_2$ systems clearly point to the danger of assigning preferential hydration solely to the perturbation of the surface free energy by the salt, since both $MgCl_2$ and $CaCl_2$ have large values of $(\partial\sigma/\partial m_3)_{T,P,m_2}$, and evidently other factors must be predominant.

It was J. Traube (Traube, 1910) who first pointed out the relation between the surface tension of solvents and the solubility of gases, nonelectrolytes, colloids, and suspensions in aqueous salt solution. He arranged anions and cations into separate series in order of their effectiveness in increasing the surface tension of the solvent and found a close relation between this effectiveness and the salt effect on the solubility of such substances. Although he did not give any theoretical interpretation for this relation, his finding for anions is quite reliable because the relative effectiveness of anions on solubility, assigned by him, agrees with current knowledge. Recently, Melander & Horvath (1977) applied the cavity formation theory to aqueous salt solutions and proposed that protein solubility in aqueous salt solutions can be explained in terms of the surface tension of the solvent, as there is a good correlation between the molal surface tension increments of salts and the anionic lyotropic series. From the above two studies and our experiments, it seems evident that, in the salts with identical cations (in this study, CH_3COONa, NaCl, Na_2SO_4, and probably KSCN because of the similarity of K^+ to Na^+), there is a distinct relation between surface tension, solubility, and preferential interactions. This relation, however, does not hold fully, since $CaCl_2$ and $MgCl_2$ are classified as having a salting-in or destabilizing effect on proteins in spite of their higher surface tension increments than Na_2SO_4. Our finding that $CaCl_2$ is preferentially bound to proteins while $MgCl_2$ is only weakly excluded is fully consistent with their known salting-in properties. Since the increase in the surface tension of the solvent induced by the salts should lead to the exclusion of the salt components from contact with the protein, there must be some attractive forces between the salt and the protein which overcome the repulsive force due to the surface tension increment.

In this respect, the result for $MgSO_4$ is very suggestive. This salt, contrary to $MgCl_2$, showed a large preferential hydration, consistent with its high surface tension increment. Its value of the $(\partial\mu_3/\partial m_2)^{\text{exptl}}_{T,P,m_3}/(\partial\mu_3/\partial m_2)^{\text{calcd}}_{T,P,m_3}$ ratio, however, was significantly lower than those for NaCl and Na_2SO_4, indicating an important role of Mg^{2+} in the decrease of this ratio for $MgCl_2$ and $MgSO_4$. Thus, our results provide further evidence that the preferential interaction parameter is a good measure of the effectiveness of a solvent component as a protein structure stabilizer or destabilizer, as well as a salting-in or salting-out agent.

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Isolation of Neuronal Parvalbumin by High-Performance Liquid Chromatography. Characterization and Comparison with Muscle Parvalbumin†

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ABSTRACT: Neuronal parvalbumin has been isolated from rat brain and purified to homogeneity by high-performance liquid chromatography (HPLC) on reverse-phase supports. This procedure includes four consecutive chromatographic steps with an overall protein recovery of 74% and a 26 400-fold purification. The concentration of parvalbumin was found to be approximately 10 mg/kg wet weight in brain tissue, which is about 100 times lower than that in rat muscle. The physical properties of brain parvalbumin are described and compared with those of the muscle counterpart. These proteins were identical in their molecular weights (12 000), isoelectric points (4.9), retention times on C-18 reverse-phase HPLC columns, Ca^{2+} content (two per molecule), amino acid compositions, and immunological properties. A comparison of the tryptic peptide maps of brain and muscle parvalbumin by analytical HPLC also revealed identity and showed that the isolation method described here did not alter the chemical structure of the protein.

The role of calcium in cellular regulation is thought to be mediated by Ca^{2+} receptors such as calmodulin, troponin C, S-100 protein, vitamin D dependent Ca^{2+}-binding protein (CaBP),[1] and parvalbumin. Their Ca^{2+}-binding sites display significant internal structural homology, a fact that has suggested evolution from a smaller ancestral precursor by gene duplication [for reviews, see Kretsinger (1980) and Siegel et al. (1980)]. Whereas calmodulin is involved in regulating a broad spectrum of cellular activities and troponin in regulating muscle contraction, the physiological role(s) of S-100, vitamin D dependent CaBP, and parvalbumin are less clear. The physical properties of muscle parvalbumins have been mainly described for lower vertebrates and more recently for mammals [for reviews, see Pechère et al. (1973), Hamoir (1974), and Kretsinger (1980)]. When antibodies against rat muscle parvalbumin are used, immunological cross-reactivity has only been detected in a few organs, including the brain from lower vertebrates (Gosselin-Rey et al., 1978; Gerday et al., 1979), mammals (Baron et al., 1975), and chicken (Heizmann & Strehler, 1979). Using a monospecific antiserum against rat muscle parvalbumin, Celio & Heizmann (1981) found that the immunologically active material in rat brains is restricted to a distinct subpopulation of neurons.

Characterization of the immunological active components present in brain preparations has not been attempted probably because of the small quantitites present, e.g., estimated to be as low as 2 mg/kg of rabbit brain (Baron et al., 1975). Due to the usual poor overall yields experienced with the more "conventional" isolation methods (ion exchange, gel filtration, etc.), quite large amounts of starting material would be required for the preparation of milligram quantities of such proteins. Based on our previous observations concerning the chromatographic behavior of peptides (Hughes et al., 1979; Wilson et al., 1981a) and proteins (Wilson et al., 1982a,b) on reverse-phase supports, we felt that such methodology might well offer the possibility to prepare enough of the material to carry out a comparative study with muscle parvalbumin. The isolation of rat brain parvalbumin by high-performance liquid chromatography (HPLC) is described, and the amino acid analysis, two-dimensional gel pattern, and Ca^{2+} content as well as the immunological properties are compared with those of the muscle counterpart. In addition, HPLC, a sensitive and efficient tool to carry out peptide analysis in the subnanomole range (Wilson et al., 1981b), was chosen to prove the similarity (if not identity) of both proteins.

Materials and Methods

Proteins and Chemicals. Parvalbumin from leg muscles of 60-day-old rats (SIV-50) was prepared as described for the chicken protein (Strehler et al., 1977). The single alteration in the procedure was the addition of the following protease inhibitors to the homogenization medium: pepstatin (1 μM), PMSF (0.4 mM), TPCK (0.15 mM), leupeptin (1 μM), all

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[1] Abbreviations: HPLC, high-performance liquid chromatography; CaBP, vitamin D dependent Ca^{2+}-binding protein; PMSF, phenylmethanesulfonyl fluoride; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; troponin C, Ca^{2+}-binding subunit of troponin; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; Tris, tris(hydroxymethyl)aminomethane.

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