Preferential Interactions Determine Protein Solubility in Three-Component Solutions: The $MgCl_2$ System[†,‡]

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ABSTRACT: The correlation between protein solubility and the preferential interactions of proteins with solvent components was critically examined with aqueous $MgCl_2$ as the solvent system. Preferential interaction and solubility measurements with three proteins, β-lactoglobulin, bovine serum albumin, and lysozyme, resulted in similar patterns of interaction. At acid pH (pH 2–3) and lower salt concentrations (<2 M), the proteins were preferentially hydrated, while at higher salt concentrations, the interaction was either that of preferential salt binding or low salt exclusion. At pH 4.5–5, all three proteins exhibited either very low preferential hydration or preferential binding of $MgCl_2$. These results were analyzed in terms of the balance between salt binding and salt exclusion attributed to the increase in the surface tension of water by salts, which is invariant with conditions. It was shown that the increase in salt binding at high salt concentration is a reflection of mass action, while its decrease at acid pH is due to the electrostatic repulsion between Mg^{2+} ions and the high net positive charge on the protein. The preferential interaction pattern was paralleled by the variation of protein solubility with solvent conditions. Calculation of the transfer free energies from water to the salt solutions for proteins in solution and in the precipitate showed parallel dependencies on salt concentration. This indicates that the nature of interactions between proteins and solvent components is the same in solution and in the solid state, which implies no change in protein structure during precipitation. Analysis of the transfer free energies and preferential interaction parameter in terms of the salting-in, salting-out, and weak ion binding contributions has led to the conclusions that, when the weak ion binding contribution is small, the predominant protein–salt interaction must be that of preferential salt exclusion most probably caused by the increase of the surface tension of water by addition of the salt. A necessary consequence of this is salting-out of the protein, if the protein structure is to remain unaltered.

Previous studies on the effect of salts on protein solubility and stability have shown that salts, such as Na_2SO_4, $NaCH_3COO$, and $MgSO_4$, that at high salt concentration stabilize proteins or decrease their solubility (von Hippel & Wong, 1965; Collins & Washabaugh, 1985; Cohn & Ferry, 1943) are preferentially excluded from the surface of the native protein (Arakawa & Timasheff, 1982b, 1984; Tuengler et al., 1979; Pundak & Eisenberg, 1981), while salts, such as $MgCl_2$, $CaCl_2$, and KSCN, that denature proteins or increase their solubilities (von Hippel & Schleich, 1969) are either preferentially bound to proteins or display little interaction with them (Arakawa & Timasheff, 1982b, 1984). For divalent cation salts, it was proposed that the propensity of a salt to act as a salting-out or salting-in agent was governed by a fine balance between preferential hydration (preferential salt exclusion) due to the effect of the salt on the surface free energy of water and the binding of the cation to the protein (Arakawa & Timasheff, 1984).

The observed variation with pH, i.e., with the charge state of the protein, of the preferential interactions of $MgCl_2$ in aqueous solutions, as well as its protein precipitating action at pH 3 at 1 M salt, but not 2 M (Arakawa & Timasheff, 1984), which is contrary to normal salting-out, identified this system as a sensitive probe of the correlations between preferential interactions and the effect of solvent components on protein solubility. Therefore, a detailed examination of the aqueous $MgCl_2$ solvent system was carried out as a function of salt concentration at different pH values. For comparison, the preferential interactions of the good salting-in salt, KSCN, with proteins were also examined.

Materials and Methods

The proteins used were bovine serum albumin (BSA)[1] (89C-9300, 80F-9340) and β-lactoglobulin (119C-8015) from Sigma and lysozyme (39J847P) from Worthington. They were dissolved in distilled–deionized water, thoroughly dialyzed against it, passed through a sintered glass filter, and lyophilized. The salts used were of reagent grade.

$MgCl_2$ solutions were prepared by dilution of a concentrated stock solution made from a fresh bottle. The pH of the stock solution was adjusted to 2 or 3 with HCl. After dilution to the final salt concentration, the solution pH was readjusted. The pH 4.5 or 5.1 $MgCl_2$ solutions were made in 0.02 M acetate buffer. KSCN solutions were made in a 0.04 M glycine–NaOH buffer, pH 10, or 0.02 M phosphate, pH 5.6, or just by titration.

Preferential interaction measurements were carried out by densimetry at 20 °C on an Anton Paar precision density meter, DMA-02, as described previously (Lee & Timasheff, 1974; Lee et al., 1979; Gekko & Timasheff, 1981; Arakawa &

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[‡] This paper and the following one are dedicated to Professor Charles Tanford on the occasion of his retirement.
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[1] Abbreviations: BSA, bovine serum albumin; β-LG, β-lactoglobulin; Gdn-HCl, guanidine hydrochloride.

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Timasheff, 1984). The apparent protein partial specific volumes, ϕ_2^0 and $\phi_2'^0$, were determined at conditions at which the molalities of solvent components and their chemical potentials were, in turn, kept identical in the protein solution and in the reference solvent. Setting component 1 = water, component 2 = protein, and component 3 = additive (here salt), according to the notation of Scatchard (1946) and Stockmayer (1950), the preferential interaction of component 3 with protein, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, is calculated from (Cohen & Eisenberg, 1968)

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

where g_i is the concentration of component i in grams per gram of water, T is the thermodynamic temperature, μ_i is the chemical potential of component, i, $\bar{v}_3$ is the partial specific volume of component 3, and the superscript 0 indicates extrapolation to zero protein concentration. The preferential hydration parameter, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, is obtained from (Timasheff & Kronman, 1959; Reisler et al., 1977)

$$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = -(1/g_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (2)$$

These parameters are converted to those on the molal basis by multiplying by a molecular weight factor.

The preferential interaction parameter is a direct expression of the mutual perturbation of the chemical potentials of components 2 and 3 (Casassa & Eisenberg, 1961, 1964):

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = -(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_3/\partial m_3)_{T,P,m_2} \quad (3)$$

where m_i is the molal concentration of component i and P is the pressure. The self-interaction term is given by

$$(\partial\mu_3/\partial m_3)_{T,P,m_2} = nRT/m_3 + nRT(\partial \ln \gamma_\pm/\partial m_3)_{T,P,m_2} \quad (4)$$

where R is the universal gas constant, n is 2 for KSCN and 3 for $MgCl_2$, and $\gamma_\pm$ is the mean ionic activity coefficient of the salt. The last quantity was obtained from Robinson and Stokes (1955). The preferential interaction is related to total bindings of components 1 and 3 to protein by (Inoue & Timasheff, 1972)

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = A_3 - g_3A_1 \quad (5)$$

where A_i is the total binding of component i in grams per gram of protein.[2]

The partial specific volume of component 3, $\bar{v}_3$, was determined by densimetry. The values (in mL/g) found were as follows: for $MgCl_2$, 0.150 at 0.5 M, 0.175 at 1 M, 0.195 at 1.5 M, 0.200 at 2 M, and 0.235 at 3 M; for KSCN, 0.528 at 1 M and 0.543 at 2 M; and for NaCl, 0.368 at 3.0 M.

Protein solubilities were determined by dissolving the protein in the desired solvent and adding protein until the solution turned turbid or viscous. It was then dialyzed against several changes of the same solvent for ca. 20 h at 20 °C. The protein (solution plus precipitate) was then removed from the dialysis bags and centrifuged for 5 min in a Beckman Airfuge at 95000 rpm. The concentration of the supernatant was measured spectrophotometrically by diluting it with the dialyzing solvent. This was defined as the solubility.

Protein concentrations were determined spectrophotometrically on Cary Model 118 and Perkin-Elmer Lambda 3B instruments. The absorptivities [dL/(g·cm)] used were 6.58 at 278 nm for BSA (Noelken & Timasheff, 1967), 9.6 at 278 nm for β-LG (Townend et al., 1960), and 27.4 at 281 nm for lysozyme (Roxby & Tanford, 1971). In concentrated salt solution, absorptivity values were determined by the method of Gekko and Timasheff (1981). These were 9.7, 9.8, and 9.85 for β-LG, 6.60, 6.63, and 6.68 for BSA, and 27.8, 28.2, and 28.6 for lysozyme, respectively, in 1, 2, and 3 M $MgCl_2$. For intermediate salt concentrations, the absorptivity values were calculated by assuming a linear dependence on salt concentration. When the protein concentrations were determined in 6 M Gdn-HCl, the absorptivities used were 6.25 at 278 nm for BSA, 9.6 at 276 nm for β-LG, and 26.9 at 281 nm for lysozyme.

[2] Schellman (1987a,b) has recently presented an elegant analysis of the thermodynamic meaning of preferential binding in terms of contacts of the protein with water and ligand molecules.

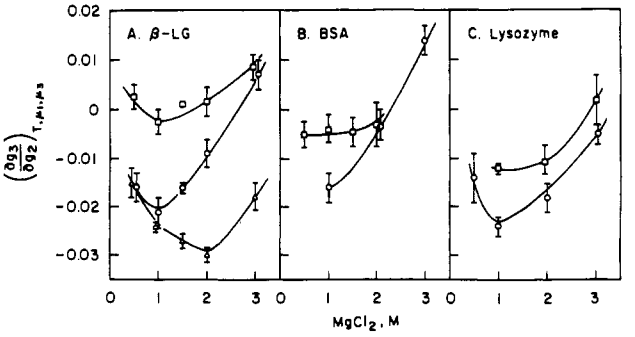

FIGURE 1: Preferential interaction of $MgCl_2$ with β-lactoglobulin, BSA, and lysozyme as a function of pH: pH 2.0 (△), pH 3.0 (○), and pH 4.5 (□) for BSA and lysozyme and 5.1 (□) for β-LG. The data for BSA at pH 4.5 are taken from Arakawa and Timasheff (1984).

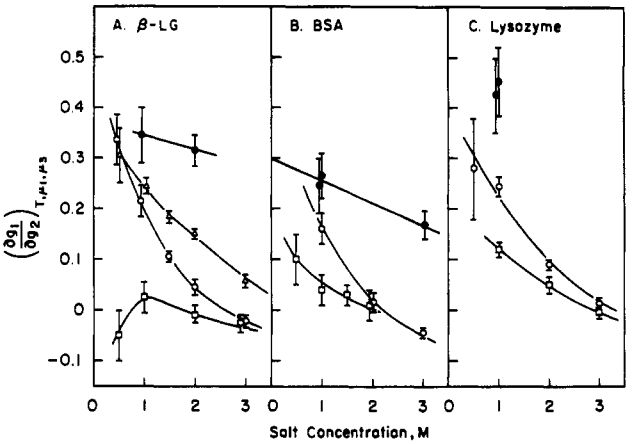

FIGURE 2: Salt concentration dependence of the preferential hydration of proteins at various pH values: (△) pH 2.0 in $MgCl_2$, (○) pH 3.0 in $MgCl_2$, (□) pH 4.5 or 5.1 in $MgCl_2$; (●) in NaCl (pH 3.0 for β-LG; pH 4.5 and 5.6 for BSA; pH 4.5 for lysozyme). The NaCl data are taken from Arakawa and Timasheff (1982b, 1987).

Results

The results of the preferential interaction measurements in $MgCl_2$ solutions are listed in Table I. The preferential salt-binding parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, is plotted against salt concentration in Figure 1. For β-LG, at pH 5.1, which is the isoelectric point of this protein, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ is not much different from zero at low $MgCl_2$ concentrations and becomes positive at 3 M salt. At acid conditions, it is negative. It decreases with an increase in salt concentration up to 2 M at pH 2.0, and 1 M at pH 3.0, at which points the trend is reversed. Its value becomes weakly positive at pH 3.0 in 3 M salt but remains strongly negative at pH 2.0. The pattern of preferential exclusion from the protein surface at salt concentrations below 3 M at acid pH, and negligible interactions at the isoelectric pH (5.1) and at high salt concen-

Table I: Interaction Parameters of Proteins in $MgCl_2$ Solutions

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)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$[a] (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial\mu_2/\partial m_3)^{exp}_{T,P,m_2}$[b] [cal/(mol of protein·mol of salt)]	ratio[c] (R)	$MgCl_2$ binding[d] (mol/mol)
				β-Lactoglobulin[e]				
				pH 2.0				
0.5[f]	0.750 ± 0.001	0.762 ± 0.001	−0.0147 ± 0.0025	0.304 ± 0.052	−5.68 ± 0.97	21 600 ± 3 700	0.58	4
1.0[f]	0.750 ± 0.001	0.768	−0.0238 ± 0.0013	0.244 ± 0.013	−9.20 ± 0.50	23 920 ± 1 300	0.64	5
1.5[f]	0.752 ± 0.001	0.771	−0.0269 ± 0.0014	0.182 ± 0.009	−10.4 ± 0.5	22 900 ± 1 100	0.61	7
2.0[f]	0.750 ± 0.001	0.770	−0.0296 ± 0.0015	0.148 ± 0.007	−11.4 ± 0.6	23 900 ± 1 300	0.64	7
3.0[f]	0.753 ± 0.001	0.764 ± 0.001	−0.0183 ± 0.0033	0.058 ± 0.011	−7.07 ± 1.28	14 700 ± 2 700	0.39	11
				pH 3.0				
0.5[f]	0.745 ± 0.001	0.758 ± 0.001	−0.0159 ± 0.0025	0.331 ± 0.052	−6.14 ± 0.97	23 300 ± 3 700	0.62	4
1.0[f]	0.745 ± 0.001	0.761 ± 0.001	−0.0211 ± 0.0026	0.216 ± 0.027	−8.15 ± 1.00	21 200 ± 2 600	0.57	6
1.5[f]	0.750 ± 0.001	0.761	−0.0155 ± 0.0014	0.105 ± 0.010	−5.99 ± 0.54	13 200 ± 1 200	0.36	11
2.0	0.750 ± 0.001	0.756 ± 0.001	−0.0089 ± 0.0030	0.044 ± 0.015	−3.44 ± 1.16	7 200 ± 2 400	0.19	14
3.0	0.751 ± 0.001	0.747 ± 0.001	0.0067 ± 0.0033	−0.022 ± 0.011	2.59 ± 1.29	−5 400 ± 2 700	−0.14	21
				pH 5.1				
0.5	0.746 ± 0.001	0.744 ± 0.001	0.0024 ± 0.0024	−0.051 ± 0.051	0.95 ± 0.95	−3 600 ± 3 600	−0.10	11
1.0	0.748 ± 0.001	0.750 ± 0.001	−0.0026 ± 0.0026	0.027 ± 0.027	−1.00 ± 1.00	2 600 ± 2 600	0.07	13
2.0	0.751 ± 0.001	0.750 ± 0.001	0.0015 ± 0.0030	−0.007 ± 0.015	0.58 ± 1.16	−1 200 ± 2 400	−0.03	19
3.0	0.752 ± 0.001	0.747 ± 0.001	0.0083 ± 0.0030	−0.026 ± 0.011	3.21 ± 1.28	−6 700 ± 2 700	−0.18	21
				BSA				
				pH 3.0				
1.0[f]	0.734 ± 0.001	0.746 ± 0.001	−0.0156 ± 0.0026	0.162 ± 0.027	−11.3 ± 1.9	29 400 ± 4 000	0.53	10
2.0	0.740 ± 0.001	0.742 ± 0.001	−0.0029 ± 0.0029	0.015 ± 0.015	−2.07 ± 2.07	4 300 ± 4 300	0.08	24
3.0	0.737 ± 0.001	0.729 ± 0.001	0.0135 ± 0.0034	−0.044 ± 0.011	9.63 ± 2.43	−20 000 ± 5 100	−0.36	36
3 M NaCl, pH 4.5	0.734 ± 0.001	0.749 ± 0.001	−0.0278 ± 0.023	0.170 ± 0.023	−32.3 ± 4.3	16 200 ± 2 200	0.56	
				Lysozyme				
				pH 3.0				
0.5[f,g]	0.706 ± 0.002	0.717 ± 0.002	−0.0135 ± 0.0049	0.280 ± 0.102	−2.03 ± 0.74	7 700 ± 2 800	0.40	3
1.0[f,g]	0.707 ± 0.001	0.725 ± 0.001	−0.0238 ± 0.0020	0.244 ± 0.022	−3.57 ± 0.30	9 300 ± 800	0.48	4
2.0[d]	0.713 ± 0.001	0.725 ± 0.001	−0.0176 ± 0.0029	0.087 ± 0.014	−2.64 ± 0.44	5 500 ± 900	0.29	6
3.0[d]	0.719 ± 0.001	0.722	−0.0050 ± 0.0017	0.016 ± 0.005	−0.75 ± 0.26	1 600 ± 500	0.08	8
				pH 4.5				
1.0	0.713	0.722 ± 0.001	−0.0119 ± 0.0013	0.122 ± 0.013	−1.79 ± 0.19	4 600 ± 500	0.24	6
2.0	0.714 ± 0.001	0.721 ± 0.001	−0.0103 ± 0.0030	0.051 ± 0.015	−1.55 ± 0.45	3 200 ± 900	0.17	8
3.0	0.716 ± 0.001	0.715 ± 0.002	0.0017 ± 0.0050	−0.005 ± 0.016	0.25 ± 0.76	−500 ± 1 600	−0.03	9

[a] The values of g_3 used in the calculation were 0.0482 at 0.5 M, 0.0975 at 1 M, 0.148 at 1.5 M, 0.202 at 2 M, and 0.310 at 3 M. [b] The values of $(\partial\mu_3/\partial m_3)_{T,P,m_2}$ were 3800 at 0.5 M, 2600 at 1 M, 2200 at 1.5 M, 2080 at 2 M, and 2080 at 3 M. [c] The value of $(\partial\mu_2/\partial m_3)^{cal}_{T,P,m_2}$ calculated from the surface tension effect was 37 300 for β-LG, 55 100 for BSA, and 19 200 for lysozyme. [d] Calculated from the surface tension effect. [e] The molecular weight of β-LG was taken as 36 800, since at high salt concentration, the acid dissociation of this protein should be greatly suppressed (Townend et al., 1960). [f] Protein concentration measured in 6 M Gdn-HCl. [g] Lysozyme from Sigma (57C-8025) was used in these measurements.

tration at low pH, is reflected by the preferential hydration, plotted in Figure 2A. At acid conditions, this parameter is positive, more so at pH 2.0 than at pH 3.0, but it decreases sharply with salt concentration. At pH 5.1, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ is little different from zero.

The large values of preferential hydration at acid pH and low salt concentrations, which reach 0.3 g of H_2O/g of protein, are similar in magnitude to the generally observed hydration values of most proteins (Bull & Breese, 1968; Kuntz, 1971; Kuntz & Kauzmann, 1974), indicating a close to total exclusion of the salt from the immediate domain of the protein.[3] The decrease of the exclusion of the salt from the protein domain with an increase in $MgCl_2$ concentration is in contrast to the salting-out NaCl system, for which the large preferential hydration, shown for comparison in Figure 2, is little dependent on salt concentration.

The patterns of preferential interactions of $MgCl_2$ with BSA and lysozyme at pH 3.0 and 4.5, plotted in panels B and C of Figure 1, were found to be similar to that of β-LG. A similar situation was found for the preferential hydration parameter, plotted in Figure 2B,C. At both pH 3.0 and 4.5, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ for lysozyme was more positive than for BSA and β-LG, reflecting the difference in the isoelectric points of the proteins, ca. 11 for lysozyme (Alderton et al., 1945) and near 5 for the other two (Foster, 1960; Treece et al., 1964).

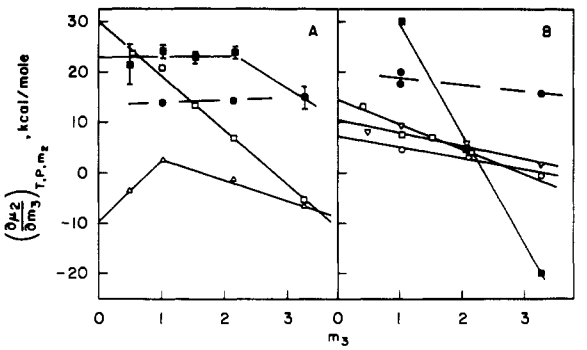

FIGURE 3: Dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ on $MgCl_2$ concentration for the three proteins at different pH values. (A) β-LG: pH 2.0 (■), pH 3.0 (□), pH 5.1 (△). (B) BSA: pH 3.0 (■); pH 4.5 (□). Lysozyme: pH 3.0 (▽), pH 4.5 (○). The values in NaCl for β-LG and BSA are given by (●).

[3] As shown by eqs 2 and 5, the measured preferential hydration is the balance between the total hydration and the total salt binding to the protein. It may, therefore, assume both positive and negative values. The measured preferential hydration approaches in value that of the total hydration when the total binding of component 3 (the salt in the present case) approaches zero.

The preferential hydration in the presence of NaCl again assumed the large values expected of a salting-out salt.[4]

The above pattern of variations of preferential interactions is a reflection of the mutual perturbations of the chemical potentials of the proteins and the salt. Their values, plotted in Figure 3, show that, for β-LG at pH 2, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is positive and essentially independent of salt concentration between 0.5 and 2 M $MgCl_2$; i.e., the system is increasingly thermodynamically destabilized. At pH 3.0, this parameter becomes a decreasing function of salt concentration and the interaction of the protein with the salt progresses from strongly thermodynamically unfavorable to weakly favorable in the given medium. At pH 5.1, it is very weak, with a possible maximum at 1 M salt, which may be explained by ion–dipole interactions as in the β-LG–NaCl system (Arakawa & Timasheff, 1987).

For BSA and lysozyme the values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ also decreased linearly with m_3, the decrease for BSA at pH 3.0 being very sharp, just as for β-LG. On the other hand, the same parameter for the NaCl system, plotted for comparison in Figure 3, showed no variation with salt concentration, both for β-LG at pH 3.0 and for BSA at the isoionic pH. This behavior is characteristic of normal salting-out salts. In the $MgCl_2$ system it was found only for β-LG at pH 2, below 2 M salt.

The parameter $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is a measure of the change of the thermodynamic stability of the system induced by a change in solvent composition at a given composition. It reflects, therefore, the relative affinities of salt and water for protein at the given solvent composition. The difference between the affinities of a protein for the given solvent system and for pure water is expressed by the transfer free energy, $\Delta\mu_2$, of the protein from water to the salt medium. The two are related by (Vlachy & Lapanje, 1978; Arakawa & Timasheff, 1985)

$$\Delta\mu_2 = \mu_{2,m_3} - \mu_{2,w} = \int_0^{m_3} (\partial\mu_2/\partial m_3)_{T,P,m_2}\, dm_3 \quad (6)$$

where the subscript w refers to water. This parameter is totally independent both of the mechanism of interaction between protein and solvent components and of the state of the protein, whether native or denatured.

This integration was carried out for all the solvent systems examined with the assumption of a linear dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ on salt molality, except for β-lactoglobulin at pH 5.1 and 2.0 where the data were treated as two straight lines intersecting at 1.02 and 2.12 *m* $MgCl_2$, respectively. The values of $\Delta\mu_2$ calculated in this manner are presented in Table II and plotted in Figure 4.[5] Except for β-LG at pH 5.1, the transfer free energy was found to be positive for all three proteins at all pH values and all $MgCl_2$ concentrations studied. Therefore, the transfer of the proteins from water to the salt solutions is thermodynamically unfavorable. It becomes even more so as the salt concentration increases, but the rate of increase diminishes with increasing salt. It reaches a maximum at 2.8 *m* $MgCl_2$ for β-LG at pH 3.0 and 2.2 *m* $MgCl_2$ for BSA at pH 3.0. Application to these results of the usual solubility equation

$$-\ln (S_{2,m_3}/S_{2,w}) = \Delta\mu_2/RT \quad (7)$$

where S_2 is protein solubility in molal units, would lead to the expectation that addition of $MgCl_2$ should generally decrease protein solubility, i.e., $MgCl_2$ would essentially be an salting-out agent.[6] This, however, disagrees with its general classification as a salting-in salt (von Hippel & Schleich, 1969) and the observation that, at pH 3.0, 20 mg/mL solutions of β-LG and BSA were precipitated by addition of 1 M $MgCl_2$, but not 2 M (Arakawa & Timasheff, 1984).

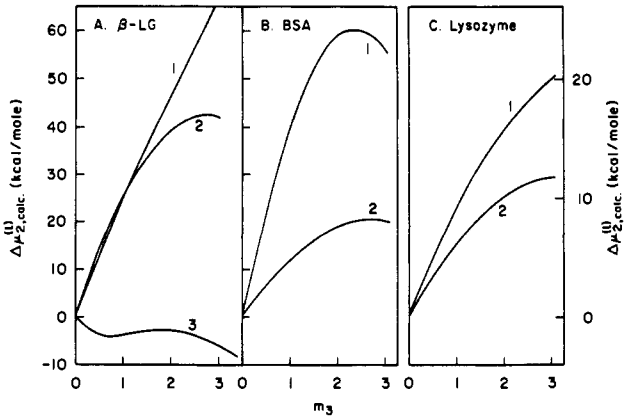

FIGURE 4: Dependence on salt concentration of the transfer free energy of proteins from water to $MgCl_2$ solution at various pH values, calculated from the data of Figure 3 as described in the text. (A) β-Lactoglobulin: 1, pH 2.0; 2, pH 3.0; 3, pH 5.1. (B) BSA: 1, pH 3.0; 2, pH 4.5. (C) Lysozyme: 1, pH 3.0; 2, pH 4.5.

To resolve this apparent ambiguity, the solubilities of the three proteins in $MgCl_2$ solutions at the various pH values were determined in 0.5, 1.0, 1.5, 2.0, and 3.0 M salt. The results, presented in Table II, were totally in disagreement with the simple expectation derived from eq 7. For all three proteins, the solubility was greater at high salt concentration than at low concentration. Particularly striking are the solubility values of BSA and lysozyme at pH 3, for both of which addition of $MgCl_2$ to a concentration of 0.5 or 1.0 M reduced dramatically the solubility relative to that in water. For example, at pH 3.0, the solubility of lysozyme was decreased by a factor of 25 by making the solution 0.5 M in $MgCl_2$, with a further decrease to less than 1% of that in water in 1 M $MgCl_2$. This trend was sharply reversed at 1.5 M salt, and the solubility regained a very high value in 2 M $MgCl_2$. Furthermore, in the presence of $MgCl_2$ (0.5–1.5 M) the solubility of both β-LG and BSA decreased as the pH was lowered from the isoionic value. In fact, in the case of BSA, solubility could not be measured at pH 4.5, since saturation could not be attained, while it was low at pH 3.0, in particular, at 0.5 and 1.0 M $MgCl_2$. This behavior is diametrically opposite to that of normal salting-out salts, such as $MgSO_4$. Similar measurements on β-LG and lysozyme in $MgSO_4$ at pH 3.0 and 4.5–5.1 showed a strong decrease in protein solubility as the salt concentration was increased from 1 to 2 M (see Table II).

Consideration of the results obtained with $MgCl_2$ leads to the conclusion that the assumption inherent in eq 7, namely,

[4] For lysozyme this parameter could not be measured in 2 M NaCl because of protein precipitation.

[5] These calculated values contain an uncertainty due to the assumption that $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ has the same dependence on m_3 below the lowest salt concentration point measured as above it, in the present case, 0.5 M $MgCl_2$. In salt systems, however, there is a negative contribution to $\Delta\mu_2$ from salting-in terms. As a result, as shown in the Appendix, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ must have negative values at low salt concentrations. Their neglect in the integration renders the calculated $\Delta\mu_2$ values too positive. This uncertainty in the absolute values of $\Delta\mu_2$, however, does not introduce any qualitative errors into the discussion of results obtained at salt concentrations >0.5 M $MgCl_2$. A detailed analysis is given in the Appendix.

[6] The decrease of $\Delta\mu_2$ above 2.2 *m* salt in the case of BSA at pH 3 suggests a deviation from normal behavior.

Table II: Solubility and Transfer Free Energy of Proteins in $MgCl_2$ and $MgSO_4$ Solutions

concentration		solubility				
M	*m*	mg/mL	g of protein/g of H_2O	$\Delta\mu_2^l$ (kcal/mol)	$\Delta\mu_2^s$ (kcal/mol)	$\Delta\mu_2^l - \Delta\mu_2^s$ (kcal/mol)
			$MgCl_2$			
			β-LG (pH 2.0)			
0	0	150	>0.1693			
0.5	0.504	12.6	0.0128	11.66	10.16	1.50
1.0	1.019	13.1	0.0135	23.53	22.06	1.47
1.5	1.546	14.2	0.0148	35.74	34.32	1.42
2.0	2.083	13.0	0.0137	48.78	47.32	1.46
3.0	3.221	16.4	0.0178	70.53	69.22	1.31
			β-LG (pH 3.0)			
0	0	>150	>0.1693			
0.5	0.504	9.9	0.0101	13.92	12.28	1.64
1.0	1.019	14.7	0.0151	25.24	23.83	1.41
1.5	1.546	14.9	0.0155	33.89	32.50	1.39
2.0	2.083	21.9	0.0232	39.76	38.60	1.16
3.0	3.221	>62		40.87		
			β-LG (pH 5.1)			
0	0	75.0	0.0796			
0.5	0.504	68.7	0.0731	−3.60	−3.65	0.05
1.0	1.019	70.2	0.0756	−3.60	−3.63	0.03
1.5	1.546	80.9	0.0889	−3.01	−2.95	−0.06
2.0	2.083	83.1	0.0925	−3.01	−2.92	−0.09
3.0	3.221	105.8	0.1242	−7.70	−7.44	−0.26
			BSA (pH 3.0)			
0	0	>200	>0.2349			
0.5	0.504	0.8	0.0008	23.21	19.90	3.31
1.0	1.019	0.6	0.0006	41.07	37.59	3.48
1.5	1.546	73.3	0.0800	53.40	52.77	0.63
2.0	2.083	>85		59.77		
3.0	3.221	>95		50.65		
			Lysozyme (pH 3.0)			
0	0	>250	>0.3038			
0.5	0.504	11.1	0.0113	4.95	3.03	1.92
1.0	1.019	2.2	0.0022	9.33	6.46	2.87
1.5	1.546	25.4	0.0267	13.14	11.72	1.42
2.0	2.083	>80		16.44		
3.0	3.221	>105		20.70		
			Lysozyme (pH 4.5)			
0	0	>250	>0.3038			
0.5	0.504	19.7	0.0202	3.40	1.82	1.58
1.0	1.019	26.0	0.0270	6.26	4.85	1.41
1.5	1.546	36.3	0.0384	8.58	7.38	1.20
2.0	2.083	>100		10.36		
3.0	3.221	>110		11.72		
			$MgSO_4$			
			β-LG (pH 3.0)			
1.0	1.010	2.3	0.0023	13.8[a]	11.3	2.50
2.0	2.052	0.8	0.0008			3.12
			β-LG (pH 5.1)			
1.0	1.010	2.1	0.0021	10.8[a]	8.7	2.12
2.0	2.052	1.3	0.0013			2.40
			Lysozyme (pH 3.0)			
1.0	1.010	29.7	0.0306	4.75[a]	3.40	1.34
2.0	2.052	1.3	0.0013			3.18
			Lysozyme (pH 4.5)			
1.0	1.010	40.3	0.0418	4.04[a]	2.88	1.16
2.0	2.052	4.1	0.0042			2.49

[a] Calculated by using $(\partial\mu_2/\partial m_3)$ values from Arakawa and Timasheff (1984).

that the chemical potential of proteins in precipitated form is independent of solvent composition, is not valid. Taking into account the transfer free energy of the protein from water to salt containing solvent in the solid state, the solubility equation becomes (Arakawa & Timasheff, 1985)

$$-RT(\ln S_{2,m_3} - \ln S_{2,w}) = \Delta\mu_2^l - \Delta\mu_2^s \quad (8)$$

where the superscripts l and s refer to the solution (liquid) and solid phases, respectively. The transfer free energies of the proteins in the precipitated state from an aqueous to a salt medium ($\Delta\mu_2^s = \mu_{2,m_3}^s - \mu_{2,w}^s$) were calculated with eq 8 from the solubility and the preferential interaction in solution data. They are listed in Table II.[7] The variations with salt concentration of the values of $\Delta\mu_2^s$ are strikingly similar to those of $\Delta\mu_2^l$ (see Table II). In all cases, except for β-LG at pH 5.1, $\Delta\mu_2^s$ was less positive than $\Delta\mu_2^l$; in the presence of $MgCl_2$ the proteins are in thermodynamically less unfavorable state

[7] These values differ from the absolute ones by the carry-over of the error in the evaluation of $\Delta\mu_2^l$.

Table III: Interaction Parameters of Proteins in KSCN Solutions

condition	ϕ_2^0 (mL/g)	$\phi_2'^0$ (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial \mu_2/\partial m_3)_{T,P,m_2}$ [cal/(mol of protein·mol of salt)]
			β-LG			
1 M						
pH 5.1	0.750 ± 0.001	0.743 ± 0.001	0.0163 ± 0.0047	−0.158 ± 0.045	6.17 ± 1.78	−6200 ± 1800
pH 10.0[a,b]	0.735 ± 0.001	0.727 ± 0.002	0.0187 ± 0.0070	−0.182 ± 0.068	7.08 ± 2.65	−7100 ± 2700
2 M						
pH 5.1	0.754 ± 0.001	0.752	0.0053 ± 0.0027	−0.024 ± 0.012	2.01 ± 1.02	−1000 ± 500
pH 10.0[a,b]	0.735 ± 0.001	0.734 ± 0.001	0.0027 ± 0.0054	−0.012 ± 0.024	1.02 ± 2.04	−500 ± 1000
			BSA			
1 M						
pH 5.6[a]	0.739 ± 0.001	0.737 ± 0.001	0.0047 ± 0.0047	−0.045 ± 0.045	3.29 ± 3.29	−3300 ± 3300
pH 10.0[a]	0.737 ± 0.001	0.735 ± 0.001	0.0047 ± 0.0047	−0.045 ± 0.045	3.29 ± 3.29	−3300 ± 3300
2 M						
pH 5.6[a]	0.743 ± 0.001	0.744 ± 0.001	−0.0027 ± 0.0054	0.012 ± 0.024	−1.89 ± 3.78	900 ± 1900
pH 10.0[a]	0.742	0.742 ± 0.001	0 ± 0.0027	0 ± 0.012	0 ± 1.89	0 ± 900

[a] Concentration measured in 6 M Gdn-HCl. [b] The low value of ϕ_2 reflects the partial unfolding of the protein at high pH (Townend et al., 1967), which is known to be accompanied by a large decrease in partial specific volume (Lee & Timasheff, 1974).

relative to water when they are in precipitated form than in free solution. These results demonstrate clearly that the chemical potential of proteins in precipitated form (μ^s_{2,m_3}) is a function of solvent composition, which is fully consistent with the known fact that protein crystals contain both water and other solvent components (Rupley, 1969; Snape et al., 1974). The similarity of the patterns of variation of the transfer free energies in the solid and solution states also shows that the precipitation of proteins in their native form does not alter the nature of their interactions with solvent components.

What are the factors which determine these interactions? In the case of salts, they consist of a balance between salt binding and the exclusion of salt. In the case of good salting-out salts, as well as sugars and some amino acids, a strong correlation has been found between the preferential exclusion and the increase in the surface tension of water induced by dissolution of these substances (Timasheff et al., 1976; Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a,b, 1983, 1984). Following this correlation, a very useful measure of the contribution of the surface free energy perturbation to the preferential interaction[8] has been found to be the ratio, R, between the values of $(\partial \mu_2/\partial m_3)_{T,P,m_2}$ obtained experimentally and calculated from the surface tension increment, since from the Gibbs adsorption isotherm (Gibbs, 1878) it is clear that (Lee & Timasheff, 1981)

$$(\partial \mu_2/\partial m_3)^{\text{calc}}_{T,P,m_2} = N_{\text{av}} s_2 (\partial \sigma/\partial m_3)_{T,P,m_2} \qquad (9)$$

where N_{av} is Avogadro's number, s_2 is the surface area of the protein molecule and σ is the surface tension of the aqueous solution. By use of the value of $(\partial \sigma/\partial m_3)_{T,P,m_2} = 3.16$ for $MgCl_2$ (Melander & Horvath, 1977), $(\partial \mu_2/\partial m_3)^{\text{calc}}_{T,P,m_2}$ was calculated for the three proteins by the procedure described previously (Arakawa & Timasheff, 1984). The values obtained were 37 300 cal/(mol of protein·mol of salt) for β-LG, 55 100 for BSA, and 19 200 for lysozyme. The values of the ratio R listed in Table I approach those of good salting-out salts, such as NaCl and Na_2SO_4 (R = 0.5–0.6) (Arakawa & Timasheff, 1982b), only at acid pH and low $MgCl_2$ concentrations, in fact, at conditions at which $MgCl_2$ acts as a salting-out agent (see Table II). This indicates that the surface tension effect is dominant at these conditions. The low values of R found for the $MgCl_2$ system at the other solvent conditions indicate the balancing of the nonspecific exclusion by a significant binding of $MgCl_2$ to the proteins. The extent of the required binding of $MgCl_2$ was calculated with eq 5 by defining A_1 as the preferential hydration induced by the surface tension perturbation. The results, given in the last column of Table I, show that the calculated binding is small at low solvent pH and low salt concentrations and probably reflects the contribution of salting-in to the interaction free energy (see the Appendix), as well as the uncertainty in calculating the magnitude of the surface tension perturbation at a water–protein interface from values measured at a water–air interface. The values of the salt binding increase with an increase in salt concentration and pH and tend to become independent of conditions. These trends reflect the following: (i) The law of mass action becomes important as the salt concentration increases. For example, for the very simple model of independent sites, and a binding constant of 0.5 M^{-1}, reasonable for binding requiring >1 M ligand concentration, the extent of binding increases by a factor of 3 for a salt concentration change from 0.5 to 3 M. (ii) The electrostatic repulsion between the cation and the protein, which is strongly positively charged at acid pH, decreases at higher pH values, and hence the effective binding constant increases. Using a simple Debye–Hückel electrostatic interaction term (Tanford, 1961), one can estimate an increase in the effective binding constant by a factor of ca. 5 when the net protein charge decreases from +50 to +10. Evidently, this is only an indication of the expected change, since the theory used is adequate neither for highly charged molecules nor at high salt concentrations (Harned & Owen, 1950).

For comparison, a preferential interaction study was carried out with KSCN, a salting-in salt (von Hippel & Schleich, 1969) in which the preponderant binding is that of the anion. The results obtained with BSA and β-LG, presented in Table III, show $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ values close to zero or positive, with no dependence on pH. The essentially zero or slightly negative preferential hydration parameter reflects salt binding rather than a decrease of hydration. Values of $(\partial \mu_2/\partial m_3)_{T,P,m_2}$, calculated with $(\partial \mu_3/\partial m_3)_{T,P,m_2}$ = 1000 and 500 cal/mol[2] at 1 and 2 M KSCN (Robinson & Stokes, 1955), were near zero or negative. The effect of KSCN on the surface tension of water is weak, $(\partial \sigma/\partial m_3)_{T,P,m_3} = 0.45$ (Melander & Horvath, 1977). The resulting weak exclusion of the salt from the

[8] The great difference between the natures of the water–air interface and the water–protein interface, which is highly heterogeneous, suggests the exercise of caution in the unequivocal acceptance of this model. Nevertheless, the good correlation between the value of the chemical potential perturbation measured in preferential binding experiments and that calculated from the surface tension increment gives strong support to the use of this model.

protein–water interface is, therefore, easily overwhelmed by the extensive salt binding.

Discussion

Correlation between Protein Solubility and Preferential Interactions. The detailed examination of the preferential interactions of proteins in $MgCl_2$ solution as a function of salt concentration and pH has shown that the preferential hydration parameter (Figure 2) is a strong function of both variables. These results clearly manifest the particular character of $MgCl_2$ in its interactions with proteins. Although $MgCl_2$ is generally classified as a salting-in and denaturation-inducing salt (von Hippel & Schleich, 1969), at some conditions (acid pH and lower salt concentrations) it salted out the proteins studied here. The salting-out pattern was totally at variance with that of normal salting-out salts, such as NaCl, Na_2SO_4, and $MgSO_4$. For these, the solubility is increased by departure from the isoelectric pH and it is decreased by an increase in salt concentration. For $MgCl_2$, both correlations are reversed. Since the three proteins studied here are very different from each other in molecular weight, isoelectric point, and other physical properties, the observed behavior of $MgCl_2$ may well be general.

Contrary to this, the salting-in salt, KSCN, was shown to have preferential interaction characteristics independent of pH, just like NaCl, which is a salting-out salt. Consistently with this, NaCl is preferentially excluded from proteins, while KSCN is preferentially bound.

The results of the present study are in accord with the earlier proposal that for divalent cation salts the preferential interactions observed constitute a balance between protein hydration and cation binding (Arakawa & Timasheff, 1984). The protein preferential hydration in $MgCl_2$ becomes significant only when the Mg^{2+} binding is suppressed by an increase in the effective positive charges on the proteins, such as at low pH. In the case of KSCN, the preferential interaction was essentially independent of pH both in 1 and 2 M salt, which is consistent with the high binding affinity of the SCN^- ion for both proteins (Scatchard et al., 1950, 1957) and model peptides (Robinson & Jencks, 1965; Schrier & Schrier, 1967). For $MgSO_4$, preferential hydration was dominant at all conditions (Arakawa & Timasheff, 1982b, 1984).

Comparison of the solvent dependencies of protein solubility and preferential interactions shows a general correlation between the two. When the protein was strongly preferentially hydrated, as β-LG in $MgCl_2$ at pH 2.0, protein solubility was decreased. When the salt was not preferentially excluded from the protein, as is true of all three proteins in 3 M $MgCl_2$, pH 3.0, and of BSA in the presence of KSCN, the protein was highly soluble at the experimental concentrations. This complex pattern of protein solubility and preferential interactions in $MgCl_2$ solutions renders this solvent system an excellent tool for testing critically the earlier proposal that, in three-component systems, protein solubility is determined by the preferential interaction parameter, with preferential hydration always leading to salting out (Timasheff et al., 1976; Pittz & Timasheff, 1978; Arakawa & Timasheff, 1982b). The complete correlation between the variation of solubility and $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ with salt concentration and pH found in this study strongly supports the validity of this proposal.

The Meaning of Salting-Out. The observation without exceptions that preferential hydration reduces the solubility of globular proteins is the necessary consequence of the fact that preferential interactions are strictly a measure of the change in the chemical potential of proteins induced by the additive and, therefore, of the effect of the additive on protein activity, as stated by eq 3. Protein salting-out is defined by the difference between the transfer free energies of protein from water to the additive-containing solvent in solution and in the precipitate. Therefore, the universally observed relation leads to the conclusion that, in salting-out systems, the chemical potentials of native globular proteins in the solution and precipitated states must have similar dependencies on solvent composition, i.e., that the interactions between proteins and solvent components must be of identical chemical nature in these two states and that the interactions of proteins with salting-out solvents must be less unfavorable in the precipitated state than in solution. This implies that there are no changes in protein structure during precipitation. In salting-out systems, the predominant protein–solvent interaction is that of the preferential exclusion of additive, i.e., preferential hydration. In the case of salts, this exclusion can be explained best as the direct and necessary consequence of the increase by the salt of the surface free energy (surface tension) of water. As shown in Figure 5, the only change in the system during precipitation is a reduction of the protein surface exposed to solvent due to the formation of protein–protein contacts. Since preferential exclusion is a nonspecific effect related to the area of the protein–solvent interface, the formation of these contacts reduces the extent of cosolvent exclusion per monomer. As a result, $(\partial g_1/\partial g_2)^s_{T,\mu_1,\mu_3} < (\partial g_1/\partial g_2)^l_{T,\mu_1,\mu_3}$ and $(\partial\mu_2/\partial m_3)^s_{T,P,m_2} < (\partial\mu_2/\partial m_3)^l_{T,P,m_2}$. This leads to the necessary consequence that $\Delta\mu_2^s < \Delta\mu_2^l$, which is the experimental observation (Table II).

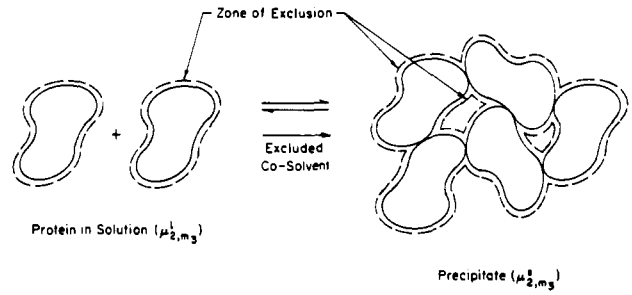

FIGURE 5: Schematic representation of the salting-out reaction. The preferential exclusion of cosolvent is reduced in the precipitated state.

Competition between Salting-Out and Ion Binding. What is the cause of the increase in protein solubility at high $MgCl_2$ concentration (2–3 M) relative to that at lower salt concentration (0.5–1 M)? As discussed in the Appendix, in salt systems, the protein–solvent interactions are determined by three factors: the thermodynamically unfavorable salt exclusion, most probably due to the surface tension increase, which is the cause of salting-out, and the thermodynamically favorable salting-in and weak ion binding processes. The solubility is determined by a balance of these factors in the solution and solid states. The salting-in phenomenon is a general electrostatic effect on protein molecules in solution. It is essentially a Debye–Hückel type of screening and is saturated at low salt concentration (<0.5 M $MgCl_2$). Therefore, its contribution to solubility is invariant at ionic strengths >1.0. As shown above, preferential salt exclusion, which is determined by the area of protein–solvent contact (eq 6), is always greater in solution than in the precipitate. Since this contact area is independent of solvent composition both in the solution and the precipitate, the difference of $\Delta\mu_2$ between the two states must increase monotonely with an increase in salt concentration, which leads to monotonely increasing salting-out once salting-in is saturated (see Appendix). Weak ion binding, as shown above and in the Appendix, is a phenomenon that increases with salt concentration due to mass

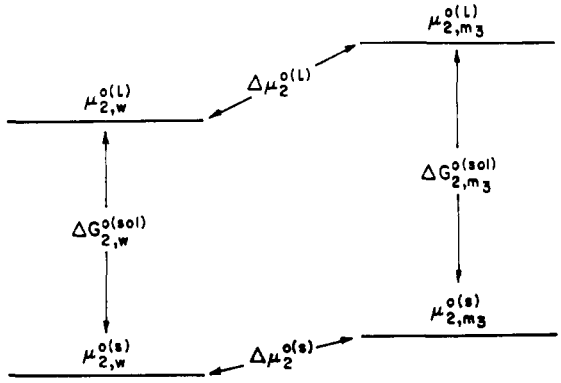

FIGURE 6: Diagrammatic illustration of the chemical potential and free energy changes during protein precipitation in water and in the presence of a salting-out component. For a detailed description, see the text.

action and to screening of the net charge of the protein. For example, in BSA at pH 3, the calculated binding of Mg^{2+} in solution is 10 mol of ions/mol of protein and 24 mol of ion/mol of protein at 1 and 2 M salt, respectively. This binding can be expected to attain greater values in solution than in the precipitate simply as a consequence of the sequestration of potential binding sites within regions of interprotein contacts, resulting in a greater negative contribution to $\Delta\mu_2^l$ than to $\Delta\mu_2^s$. It is this negative contribution which led to the observed decrease of $\Delta\mu_2^l$ at the higher $MgCl_2$ concentrations (Figure 4). On the other hand, the smaller contribution to $\Delta\mu_2^s$ should affect less the monotone increase of this parameter. The difference between the two, which determines the protein solubility, could then have a maximum and finally become zero or even negative, leading to salting-in behavior at high salt concentration (see Appendix). This is what has been observed in this study. For example, $\Delta\mu_2^l - \Delta\mu_2^s$ for lysozyme at pH 3 changes from 2.9 kcal/mol in 1 M $MgCl_2$ to 1.4 kcal/mol in 1.5 M $MgCl_2$, and the solubility jumps from 2.2 mg/mL in 1 M $MgCl_2$ to 25.4 mg/mL in 1.5 M salt (it becomes unmeasurable in 2 M $MgCl_2$). As a contrast, for the good salting-out salt $MgSO_4$, the same parameter changes from 1.3 kcal/mol in 1 M to 3.2 kcal/mol in 2 M, with the solubility decreasing from 29.7 to 1.3 mg/mL.

Salting-Out as a Necessary Consequence of Cosolvent Exclusion. Why is salting-out a necessary consequence of the preferential exclusion of a cosolvent from a native globular protein in solution? This becomes clear from the diagram of chemical potential changes in the salting-out process, shown in Figure 6. The standard chemical potentials of a protein in aqueous solution in the precipitated (s), solid state, $\mu_{2,w}^{0(s)}$, and in the solution (l), liquid state, $\mu_{2,w}^{0(l)}$, are depicted by the horizontal lines on the left. Their difference gives the standard free energy of solution of the protein in water, $\Delta G_{2,w}^{0(sol)} = -RT \ln S_{2,w}$, within the approximation that activity coefficients may be neglected. This quantity contains all the specific and nonspecific interactions involved in the interprotein contacts, as well as all changes in entropy related to the incorporation of a single protein molecule into the large structure. As the salting-out material is added to the system at concentrations sufficiently high to overwhelm salting-in, e.g., >0.5 M $MgCl_2$, the environment of the protein, in both the solution and the precipitate, becomes less favorable than in water and its standard chemical potentials in the two states, $\mu_{2,m_3}^{0(s)}$ and $\mu_{2,m_3}^{0(l)}$, depicted by the horizontal lines at the right, become more positive, the magnitude of the change being equal to the transfer free energy in each state. Since $\Delta\mu_2^s < \Delta\mu_2^l$, $\Delta G_{2,m_3}^{0(sol)}$ must be more positive than $\Delta G_{2,w}^{0(sol)}$ and the solubility in the salting-out medium must be reduced relative to that in water. This difference between the transfer free energies is the consequence solely of the general nonspecific effect of the precipitant, since the interprotein contact interactions remain the same as those in water.

Figure 6 also explains why salting-in cannot occur without a change in protein structure when the predominant interaction is that of cosolvent exclusion. A decrease of the free energy of solution on addition of the cosolvent would require that $\mu_{2,m_3}^{0(s)}$ become more positive, since $\mu_{2,m_3}^{0(l)}$ is a fixed value. For this to occur, the free energy of transfer in the solid state must be more positive than that in solution. Since preferential exclusion is proportional to the total area of protein–solvent contact, this could be satisfied only by an increase of this area during precipitation, which, in turn, could be accomplished only by a change in protein structure. Conversely, if the interaction is that of preferential binding of the cosolvent ($\Delta\mu_2^{0(l)}$ negative), salting-in would require only that its binding to protein be greater in solution than in the solid state, which can be accomplished without any structural change in the macromolecule.

Appendix

Significance of $\Delta\mu_2$ and $(\partial\mu_2/\partial m_3)_{T,P,m_3}$. The transfer free energy of a protein from water to a salt solution, $\Delta\mu_2$, is made up of three principal contributions, salting-in, salting-out, and salt binding:

$$\Delta\mu_2 = f(Z,m_3^{1/2}) + \Delta\mu_2 \text{ (salt exclusion)} + f(km_3,Z) \quad \text{(A1)}$$

where Z is the net charge on the protein and k is the effective binding constant of an ion (e.g., Mg^{2+}) to a site on the protein. The first and third terms are favorable interactions and, therefore, negative; the second term is an unfavorable interaction and, therefore, positive. The relative contributions of the three parameters to the transfer free energies have been evaluated and are shown in the upper part of Figure A1 for two cases, one in which the extent of ion binding is small (case 1) and the other one in which it is extensive (case 2). In these calculations, the magnitude of the salting-in contribution to $\Delta\mu_2$ was taken from the solubility data on hemoglobin (Cohn, 1936). This term is negative and it becomes saturated at an ionic strength of ~0.8 (0.3 M $MgCl_2$). The salting-out contribution was set as a linear function of m_3, as expected from the surface tension perturbation effect. The value used was that found for proteins as a result of the surface tension increment of salting-out salts. The ion binding contribution was estimated by assuming very weak binding at independent sites, $\Delta\mu_2^{\text{binding}} = nRT \ln [1/(1 + ke^{-\phi(z)}[X])]$, where n is the number of sites, [X] is salt concentration, and $\phi(z)$ is the electrostatic work term, such as the Linderstrom–Lang relation (Tanford, 1961). The results of the calculations are shown in Figure A1 for high (i.e., little binding, solid curve 1) and low (i.e., extensive binding, solid curve 2) protein charge. While these calculations are inexact due to the inapplicability of the Debye–Hückel theory at the conditions of protein charge and ionic strength encountered in these studies, they indicate the order of magnitude of the effects and the nature of the trends.

As seen in Figure A1, for salts, $\Delta\mu_2$ assumes negative values at ionic strengths below 0.9 M, after which it increases monotonely with salt concentration. This increase is linear for salts that are good salting-out agents, such as $MgSO_4$ and Na_2SO_4, but it becomes concave downward for those salts that bind to protein, such as $MgCl_2$, and can actually pass through a maximum (curve 2). Curves 1 and 2 closely depict the data obtained with β-LG at pH 2 and 3. The values calculated from

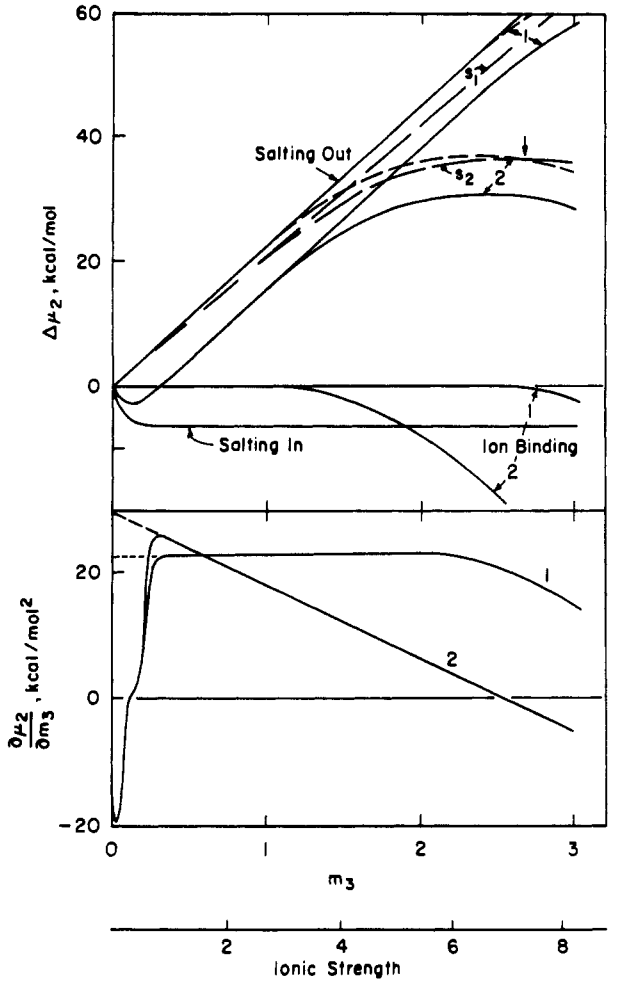

FIGURE A1: Contributions of preferential exclusion (salting-out), salting-in, and ion binding to the transfer free energy ($\Delta\mu_2$) and the perturbation of the chemical potential of the protein by the salt ($\partial\mu_2/\partial m_3$) in the solution state. Curves 1 and 2 correspond to small and extensive binding of salt to protein, respectively. The dashed lines in the $\Delta\mu_2$ plot are the values obtained by integration of ($\partial\mu_2/\partial m_3$) as described in the text. The long dashed lines s_1 and s_2 are the values for the solid phase, corresponding to low and extensive salt bindings.

the experimental preferential interactions, described in this paper, are given by the dashed lines 1 and 2. These lines are displaced from the theoretical values to more positive ones by a constant increment which is equal to the salting-in contribution, once it becomes invariant with m_3. This displacement is the consequence of the neglect of its contribution in the integration of eq 6, as explained in the lower part of Figure A1.

The lower part of Figure A1 gives the dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ on m_3 for these two cases. The values above 0.5 M $MgCl_2$ are the experimental ones obtained with β-lactoglobulin at pH 2 (curve 1) and pH 3 (curve 2). Below that salt concentration, the variation of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ with m_3 was derived from the slope of the theoretical $\Delta\mu_2$ curve. As is evident, $(\partial\mu_2/\partial m_3)_{T,P,m_3}$ assumes negative values at low salt concentrations (<0.1 M). The calculation of $\Delta\mu_2$ values from the preferential interaction data by integration of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ neglected the variation of that parameter below 0.5 M $MgCl_2$, which led to a value that is too positive by the area contained between the dashed lines extrapolated to $m_3 = 0$ and the actual curve below $m_3 = 0.3$. This, in fact, is equal to $\Delta\mu_2$ (salting-in) once it reaches its plateau.

The calculations summarized in Figure A1 can also serve to define the difference between the interactions expressed by $\Delta\mu_2$ and $(\partial\mu_2/\partial m_3)_{T,P,m_2}$. Comparison of their variations with m_3 (e.g., Figures 4 and 3) shows that the trends are frequently in opposite directions: $\Delta\mu_2$ in most cases increases with salt concentration, indicating that transfer from water to salt solution is progressively more unfavorable thermodynamically. On the other hand, the interaction parameter might seem to indicate the opposite. This is due to the difference in reference states: $\Delta\mu_2$ refers always to pure water, while $(\partial\mu_2/\partial m_3)$ refers to water in a solvent with salt concentration m_3. Thus, the negative values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ at pH 3 in 3 M $MgCl_2$ for β-LG and BSA reflect a stronger affinity for the salt than for water in a solvent of that given composition. On the other hand, the transfer free energy compares the state of the protein in the salt solution to that in pure water. The interaction parameter is the rate of change of that comparison; i.e., it is a comparison of the state of the protein in a given concentration of the salt relative to its state at a salt concentration lower by an infinitesimally small increment. Thus, a constant positive value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ corresponds to a constant increase in the unfavorable thermodynamic interactions between protein and solvent (curves 1 of Figure A1, β-LG at pH 2) while a decreasing value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ indicates that these interactions are becoming more favorable with respect to solvent, while they may still be unfavorable with respect to pure water, even though decreasingly so. For example, we see in Figure 4 that, in 3 M $MgCl_2$, BSA at pH 3 is in an environment thermodynamically less unfavorable than in 2 M salt, even though both environments are unfavorable relative to water. Therefore, a negative value of $(\partial\mu_2/\partial m_3)_{T,P,m_3}$, following positive values, does not mean salting-in relative to water, but only relative to a salt solution of a slightly lower concentration. This analysis can also explain the increase in protein solubility at high salt concentrations when salt binding is extensive. By eq 8, solubility is defined by the difference between $\Delta\mu_2$ in the dissolved and precipitated states. Precipitation has the consequence that both the positive contribution to $\Delta\mu_2$ from a general effect, such as the increase in surface tenson, and the negative contribution from salt binding are reduced due to the reduction in the protein–solvent interface. Calculated $\Delta\mu_2^s$ vs m_3 curves are shown in Figure A1 for a good salting-out salt (long dashed line s_1, derived from the solubility data of β-LG in $MgSO_4$ at pH 3.0) and for a binding salt, such as $MgCl_2$ (long dashed line s_2, obtained by decreasing by 20% the extent of binding from that in the solution state). It is evident that, for the salting-out salt, $\Delta\mu_2^l - \Delta\mu_2^s$ becomes progressively more positive, leading to the progressive decrease in solubility (compare s_1 with dashed curve 1). In the case where extensive salt binding occurs, $\Delta\mu_2^l - \Delta\mu_2^s$ is also increasingly positive up to 1 *m* salt, at which point the difference starts to decrease, and the two curves cross at 2.6 *m* (compare s_2 with dashed curve 2). This becomes translated into a progressive decrease in protein solubility up to 1.4 *m* $MgCl_2$, above which the trend reverses. At 2.6 *m* salt, the solubility regains that in water and becomes greater than in water at higher salt concentrations. The same analysis can be applied to the low salt concentration range for classical salting-out salts, such as Na_2SO_4, Na_3 citrate, or $MgSO_4$ (Green, 1932). Since, at low salt concentrations, the salting-in effect is stronger than salting-out, which reflects the variation of $\Delta\mu_2$ with m_3 of Figure A1, the decrease in solubility sets in at a point at which the solubility is greater than in water (solid curve 1 or 2 at low salt concentration where the value is negative). This is the reason why salt precipitation of proteins must be performed at high salt concentrations. In fact, since by eqs 6 and 8 the salting out constant, $K_s \equiv (\partial \log S_2/\partial m_3)_{T,P,m_2}$, is equal to $(\partial\mu_2/\partial m_3^l)_{T,P,m_2} - (\partial\mu_2/\partial m_3)^s_{T,P,m_2}$

(Timasheff & Arakawa, 1988), the linear decrease of solubility (normally identified with salting-out) starts at solubility values in salt which are greater than that in water, even though the chemical potential increment is positive, i.e., the net interaction is progressively more unfavorable.

Registry No. $MgCl_2$, 7786-30-3; KSCN, 333-20-0; $MgSO_4$, 7487-88-9; lysozyme, 9001-63-2.

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