Preferential Solvation of *S*-Cysteinyl Bovine Serum Albumin in Aqueous Solutions of Lithium Salts*

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ABSTRACT: The preferential interaction of the solvent components with *S*-cysteinyl bovine serum albumin was measured in aqueous solutions of seven lithium salts, sodium chloride, and potassium chloride by means of equilibrium dialysis and differential refractometry. The salt was preferentially bound in solutions of LiI, and LiSCN in the concentration range 2–7 M, in aqueous LiBr (2–8 M), and in aqueous LiCl (5–7 M).

There was little preferential interaction in aqueous $LiNO_3$ (2–6 M) and in 3.5 M $LiClO_4$. The protein was preferentially hydrated in 1.7 M Li_2SO_4, in 4.6 M NaCl, and in 3.2 M KCl. About 160 moles of salt was weakly bound at the highest concentrations of LiBr, LiI, and LiSCN used. These results, plus others, indicate that there is an approximate correlation between salt binding and unfolding. Since the salt–protein interactions are weak, it is likely that unfolding is not due to salt binding but rather to a general effect of the salts on the solvent. The preferential salt binding probably reflects the exposure of peptide groups upon denaturation.

The unfolding of proteins and polypeptides by aqueous solutions of neutral salts has been a subject of continuing interest. The mechanism of unfolding is likely to be complicated and to involve the mutual interaction of the salt, the macromolecule, and water (Bigelow and Geschwind, 1961; Von Hippel and Wong, 1964; Robinson and Jencks, 1965a,b; Ciferri *et al.*, 1967). Thus, a salt may cause unfolding by direct interaction with the macromolecule; *i.e.*, binding, or indirectly by altering the properties of the solvent. Evidence consistent with salt binding came from the finding of Mandelkern and Stewart (1964) that the change in the melting temperature of collagen with denaturant concentration could be fit by an equation derived by Flory (1957) that specifically included a binding term. In addition to this, Robinson and Jencks (1965b) concluded from indirect evidence obtained from a study of the solubilization of a tetrapeptide by neutral salts that binding of anions to the amide portion of the peptide group was of importance in protein denaturation. In spite of the simplicity of this concept, only a few studies have been made that gave direct evidence for the binding of denaturing salts to proteins or polypeptides. For example, Ciferri *et al.* (1967) found that KSCN, a denaturant, had a greater affinity for collagen than did KCl, which was not a denaturant. Kurtz and Harrington (1966) isolated complexes of LiBr and several polypeptides that contained 15–18% by weight of salt.

In view of the scarcity of information about binding of salts to proteins under unfolding conditions, it was considered desirable to make a study of the binding of lithium salts, at high concentration, to *S*-cysteinyl-BSA.[1]

No techniques are available for measuring the *total* amount of salt bound in concentrated salt solution, but preferential solvation can be readily measured. In this study equilibrium dialysis was combined with differential refractometry to measure the redistribution of solvent components that resulted from interaction with the protein. This technique was used recently to measure the preferential binding of Gd·HCl to BSA (Noelken and Timasheff, 1967), and 2-chloroethanol to β-lactoglobulin, BSA, and insulin (Timasheff and Inoue, 1968). A similar method was used by Gordon and Warren (1968) to study BSA in aqueous solutions of urea, urea derivatives, and dextrose. Preferential solvation measurements yield the difference between the solvent composition in the domain of the macromolecule and that farther away. If this is considered to be due to selective binding of one of the solvent components to the macromolecule, then the results would yield the minimum amount of that component bound.[2] This imposes some limitation on the interpretation of the results since the total amount bound will not be known.

Theoretical

Preferential solvation was determined, as previously (Noelken and Timasheff, 1967), through measurement of the difference between the refractive index increment of the protein at constant chemical potential of the solvent components and that at constant molarity of salt. The value is given by the following relationship

$$\left[\frac{\partial c_3}{\partial c_2}\right]^0_{T,\mu_1,\mu_3} = \frac{(\partial n/\partial c_2)^0_{T,\mu_1,\mu_3} - (\partial n/\partial c_2)^0_{T,P,c_3}}{(\partial n/\partial c_3)^0_{T,P,c_2}} \quad (1)$$

The components of the system are designated as: component 1 = water; 2 = protein; and 3 = salt. The term c is the concentration in grams per milliliter, T is the temperature, μ is the

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: BSA, bovine serum albumin; Gd·HCl, guanidine hydrochloride.

[2] The term "binding" will be used broadly to signify general thermodynamic interactions among the components of the system. The nature of the study does not allow conclusions to be drawn about stoichiometric complex formation at specific sites.

chemical potential, n is the refractive index, P is the pressure, and the superscript 0 indicates extrapolation to infinite dilution of the protein. The refractive index increment of the protein at constant molarity, $(\partial n/\partial c_2)_{T,P,c_3}$, is measured by comparing a protein solution with solvent that has the same molarity of salt. The value of the parameter $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$ is obtained by comparison of the protein solution and salt solution with which it is at osmotic equilibrium. This condition is closely met by use of the dialysate (Vrij and Overbeek, 1962; Casassa and Eisenberg, 1964). The term $(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$ represents the preferential binding of the salt to *S*-cysteinyl-BSA. Zero preferential binding on this scale corresponds to equal molarity of salt in a protein solution and in its dialysate. This, however, has the disadvantage of including the protein volume in its environment. It is more useful, therefore, to use a scale in which the protein volume is not included. The molal scale is one that is suitable for this purpose. Zero preferential binding on this scale corresponds to equal molarity of the third component for a protein solution and its dialysate. The preferential binding of a molal basis is related to that on a molar basis by the following equation (Casassa and Eisenberg, 1964; Noelken and Timasheff, 1967).

$$\left[\frac{\partial g_3}{\partial g_2}\right]^0_{T,\mu_1,\mu_3} = \frac{g_3}{\bar{v}_1 c_3}\left(\left[\frac{\partial c_3}{\partial c_2}\right]^0_{T,\mu_1,\mu_3} + c_3\bar{v}_2\right) \qquad (2)$$

where g_3 is the salt concentration in grams per gram of water, and $\bar{v}$ is the thermodynamic partial specific volume.

An alternative method eliminating effects due to protein volume is to use a corrected molar scale. This is given by

$$\left[\frac{\partial c_3}{\partial c_2}\right]^{cor}_{T,\mu_1,\mu_3} = \left[\frac{\partial c_3}{\partial c_2}\right]^0_{T,\mu_1,\mu_3} + c_3\bar{v}_2 \qquad (3)$$

The term $c_3\bar{v}_2$ in eq 2 and 3 is the negative of the partial derivative $(\partial c_3/\partial c_2)_{T,P,g_3}$.

A negative value of preferential salt binding signifies preferential hydration. The amount is given by

$$\left[\frac{\partial c_1}{\partial c_2}\right]^0_{T,\mu_1,\mu_3} = -\frac{c_1}{c_3}\left[\frac{\partial c_3}{\partial c_2}\right]^0_{T,\mu_1,\mu_3}$$
$$\left[\frac{\partial g_1}{\partial g_2}\right]^0_{T,\mu_1,\mu_3} = -\frac{1}{g_3}\left[\frac{\partial g_3}{\partial g_2}\right]^0_{T,\mu_1,\mu_3} \qquad (4)$$

Inspection of eq 2, 3, and 4 shows that under certain conditions it is possible to interpret results either as preferential salt binding or as preferential hydration depending on the choice of the scale. The same qualitative conclusions would result from use of either eq 2 or 3 but the values of the binding parameters would differ. Use of eq 2 yields results that can be applied to equilibrium sedimentation (Casassa and Eisenberg, 1961, 1964; Creeth and Pain, 1967; Reisler and Eisenberg, 1969). Otherwise, there is no reason to prefer either equation.

Experimental Section

S-Cysteinyl-BSA. This derivative of BSA was prepared as described previously (Noelken, 1970). Most of the experiments were done with material prepared from lot 9385 obtained from Nutritional Biochemicals. Essentially the same results were obtained with *S*-cysteinyl-BSA made from lot 8176. BSA lot 9385 was used in a previous study that dealt with preferential solvation in aqueous Gd·HCl.

Protein Concentration. The concentration of dialyzed *S*-cysteinyl-BSA was determined spectrophotometrically (Noelken, 1970) after dilution with about 15 volumes of 6 M Gd·HCl, using a value for $A^{1\%}_{1cm}$ of 6.14 at 278 mμ. This value was found to be the same in the presence (up to 0.4 M) of the lithium salts used, except $LiNO_3$. The value was 2% lower at a concentration of 0.4 M $LiNO_3$. Suitable control experiments showed that the dialysis tubing made only a negligible contribution to the ultraviolet absorption.

Reagents. The sources of the reagents were cited in a previous publication (Noelken, 1970).

Concentration of Lithium Salts. The concentration of LiCl, LiBr, LiI, and $LiNO_3$, used in the measurement of $(\partial n/\partial c_3)_{T,P,c_2}$ and $(\partial n/\partial c_2)_{T,P,c_3}$, was determined by a gravimetric procedure that involved conversion into Li_2SO_4 (Vogel, 1961; Erdey, 1965). Lithium perchlorate was determined by drying solutions to constant weight at 180° under vacuum; the salt loses its water of hydration above 130° (Walker, 1924). Lithium thiocyanate was determined gravimetrically by conversion into AgSCN (Pierce *et al.*, 1958). The salt concentration used in determining the value of $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$ as a function of c_3 was calculated from the density of the dialysate (Noelken, 1970). The additional density data needed for aqueous Li_2SO_4, NaCl, and KCl were obtained from the tables compiled by Beattie (1928).

Refractive Index Increment. Differences in the index of refraction at 436 mμ, 25°, were directly measured with a Brice-Phoenix differential refractometer. The cell had a permanently fused cover which prevented errors from creeping, evaporation, or condensation; it had two Teflon penny-head stoppers for covering the filling holes.

Pairs of salt solutions used to determine $(\partial n/\partial c_3)_{T,P,c_2}$ were prepared by diluting, by weight, a stock solution of which the weight fraction of salt was known. The concentration, in grams per milliliter, was determined through use of a least-squares-based equation that related the density to the weight fraction. The results are listed in Table I. A few values at low salt concentration were calculated from published tables of values of the refractive index. The parameter $(\partial n/\partial c_3)_{T,P,c_2}$ is the slope of a plot of the refractive index *vs.* concentration of salt in grams per milliliter. No protein was present in these measurements; the assumption was made that the results were identical with the results that would be obtained with the protein present, after extrapolating to zero protein concentration.

Pairs of solutions used in obtaining the value of the parameter $(\partial n/\partial c_2)_{T,P,c_3}$ were prepared by diluting, by weight, two portions of a stock salt, or urea, solution with water and a deionized stock solution of *S*-cysteinyl-BSA, respectively. The volume of the protein solution was calculated with the assumption that the contribution from salt (or urea) and water depended only on the salt molality and was unaffected by the presence of protein. This assumption is valid for BSA in 4–8 M urea (Katz and Ferris, 1966) and in 1.0–3.8 M Gd·HCl (Katz, 1968). The average of three recent values of the thermodynamic partial specific volume of native BSA, 0.734 ml/g of BSA, was used to calculate the volume contribution of *S*-cysteinyl-BSA (Van Holde and Sun, 1962; Hunter,

TABLE I: Refractive Index Increments of Several Salts at 436 mμ, 25°.

Concn (g/ml)	$(\partial n/\partial c_3)_{T,P,c_2}$	Concn (g/ml)	$(\partial n/\partial c_3)_{T,P,c_2}$
A. Lithium Bromide		D. Lithium Nitrate	
0.350	0.147	0.250	0.116
0.541	0.147	0.302	0.114
0.594	0.148	0.359	0.112
0.651	0.147	0.434	0.109
		0.494	0.107
B. Lithium Chloride			
0.079	0.203[a]	E. Lithium Perchlorate	
0.149	0.193[a]	0.225	0.059
0.220	0.186	0.291	0.059
0.279	0.184	0.300	0.059
0.294	0.184	0.362	0.059
0.348	0.180		
0.418	0.171	F. Lithium Sulfate	
0.470	0.164	0.085	0.153
0.478	0.162	0.108	0.147
		0.132	0.142
C. Lithium Iodide		0.182	0.133
0.661	0.167		
0.682	0.167	G. Lithium Thiocyanate	
0.778	0.167	0.126	0.268
0.792	0.168	0.170	0.266
0.911	0.168	0.215	0.265
0.924	0.169	0.335	0.260
1.008	0.170	0.462	0.254
1.056	0.166		
1.070	0.172	H. Sodium Chloride	
		0.266	0.143
		I. Potassium Chloride	
		0.238	0.114[b]

[a] Calculated from the data of Hüttig and Kukenthal (1928). [b] Extrapolated from the results of Pethica and Smart (1966).

1967; Reisler and Eisenberg, 1969). Reisler and Eisenberg found a lower value, 0.728 ml/g for unfolded BSA in 6 M Gd·HCl–0.2 M 2-mercaptoethanol, but use of this value would not significantly affect the results. The volume contribution of the salt and water was calculated in each case with the use of the appropriate least-squares-based equation that related density to weight fraction. The density *vs.* composition relationship for aqueous Gd·HCl was that determined by Kawahara and Tanford (1966). The observed refractive index differences were corrected for small amounts due to slightly unequal concentrations of salt (or urea) in the protein solution and the reference solution. The refractive index increments used for this purpose were obtained from the results of: Katz (1950) in the case of urea; Pethica and Smart (1966) for KCl and NaI; Noelken and Timasheff (1967) for Gd·HCl; and this study for LiBr (Table I).

The refractive index difference used to obtain the value of the parameter $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$ was obtained by comparison of a protein solution with its dialysate. A solution was usually dialyzed 2–4 days. The error due to failure to reach equilibrium and to transferring a solution to the cell was about 1%.

The apparent pH of the solutions used for the refractive index increment measurements was in the range 5.2–5.5 except for aqueous LiSCN and LiI. The LiSCN contained alkaline impurities and the pH obtained after dissolving *S*-cysteinyl-BSA in aqueous LiSCN was 7.6–7.7. At this pH the net charge would be about −16 according to the amino acid composition (Spahr and Edsall, 1964). The value of the parameter $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$ for *S*-cysteinyl-BSA in 6 M LiSCN was insensitive to pH. A value of 0.143 ml/g at pH 5.6 was found, compared to 0.144 ml/g at pH 7.7. These results would lead to values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ of 0.125 and 0.134 g of LiSCN per g *S*-cysteinyl-BSA, respectively; the difference is within the experimental error. The LiI solutions contained sodium thiosulfate (0.01 M) to prevent oxidation of the iodide ion. The apparent pH was kept at 6.6 instead of 5.2 to make the thiosulfate a more effective antioxidant. The net charge at this pH would be about −8.

Optical Rotatory Dispersion. Measurements of the optical rotatory dispersion of *S*-cysteinyl-BSA were made as before (Noelken, 1970).

Results

Preferential Solvation. The values of the refractive index increment of the salts are listed in Table I. The value of the partial derivative $(\partial n/\partial c_3)_{T,P,c_2}$ is obtained by extrapolation of the measured quantity $(\Delta n/\Delta c_3)_{T,P}$ to zero salt concentration. However, the variation with concentration was within the experimental error (about 1%) so the results were simply averaged. The values for LiCl agreed to within 3% of those obtained by taking the slope of a plot of refractive index *vs.* concentration, using the data of Hüttig and Kukenthal (1928). Their data were used to determine $(\partial n/\partial c_3)_{T,P,c_2}$ at LiCl concentrations below 0.22 g/ml.

The average value of the parameter $(\partial n/\partial c_2)_{T,P,c_3}$ for *S*-cysteinyl-BSA in water was 0.1924 ± 0.0007 ml/g (11 determinations). The variation of $(\partial n/\partial c_2)_{T,P,c_3}$ with the nature and concentration of the third component was determined by comparing the value for a given solution to that found for the same aqueous protein stock solution after dilution with water. Five different solutes were used. The results are shown in Figure 1. The ratio of the value of $(\partial n/\partial c_2)_{T,P,c_3}$ to that of $(\partial n/\partial c_2)_{T,P,H_2O}$ was found to be independent of the volume fraction of the third component. The average value for 31 data points was 1.00 with an average deviation of 0.009. It was assumed that the value of $(\partial n/\partial c_2)_{T,P,c_3}$ of 0.192 ml/g applied to *S*-cysteinyl-BSA in all of the salt solutions used in this study. The lack of dependence of $(\partial n/\partial c_2)_{T,P,c_3}$ on solvent composition differs slightly from the results of a previous study (Noelken and Timasheff, 1967) involving BSA. The dependence on Gd·HCl concentration in that study was given by the relationship

$$\left[\frac{\partial n}{\partial c_2}\right]_{T,P,c_3} = 0.192 + 3.94 \times 10^{-3}B - 7.33 \times 10^{-4}B^2$$

where B is the molarity of Gd·HCl. It was noted, however, that the experimental error arising from the volumetric technique used was 2–4%. All of the observed values were within experimental error of 0.192 ml/g. Furthermore, additional studies (M. Noelken, unpublished results) that employed the same volumetric method showed that $(\partial n/\partial c_2)_{T,P,c_3}$ for BSA

TABLE II: Preferential Solvation Parameters of *S*-Cysteinyl-BSA.

Salt Concn (g/ml)	$\left[\frac{\partial n}{\partial c_2}\right]_{T,\mu_1,\mu_3}$	$\left[\frac{\partial c_3}{\partial c_2}\right]^0_{T,\mu_1,\mu_3}$	$\left[\frac{\partial c_1}{\partial c_2}\right]^0_{T,\mu_1,\mu_3}$	$\left[\frac{\partial g_3}{\partial g_2}\right]^0_{T,\mu_1,\mu_3}$	$\left[\frac{\partial g_1}{\partial g_2}\right]^0_{T,\mu_1,\mu_3}$	$\left[\frac{\partial c_3}{\partial c_2}\right]^{cor}_{T,\mu_1,\mu_2}$
			A. In Aqueous Lithium Bromide			
0.164	0.175	−0.113	0.62	0.008		0.007
0.216	0.168	−0.159	0.68	0.000		0.000
0.372	0.159	−0.222	0.53	0.058		0.051
0.578	0.148	−0.298	0.42	0.152		0.126
0.631	0.144	−0.328	0.42	0.168		0.135
0.710	0.140	−0.350	0.38	0.219		0.171
			B. Lithium Chloride			
0.079	0.179	−0.064	0.78	−0.006	0.073	−0.006
0.149	0.169	−0.121	0.75	−0.013	0.081	−0.012
0.215	0.163	−0.154	0.64	0.008		0.004
0.281	0.160	−0.164	0.50	0.048		0.042
			C. Lithium Iodide			
0.233	0.168	−0.151	0.60	0.021		0.020
0.439	0.158	−0.209	0.42	0.130		0.113
0.633	0.142	−0.300	0.39	0.199		0.165
0.703	0.137	−0.329	0.38	0.233		0.187
0.920	0.120	−0.429	0.35	0.330		0.246
			D. Aqueous Lithium Nitrate			
0.140	0.178	−0.114	0.76	−0.012	0.08	−0.011
0.250	0.169	−0.200	0.71	−0.018	0.06	−0.016
0.324	0.164	−0.242	0.64	−0.006	0.01	−0.004
0.442	0.157	−0.321	0.58	0.004		0.003
			E. Lithium Thiocyanate			
0.117	0.175	−0.065	0.51	0.022		0.021
0.233	0.164	−0.107	0.39	0.077		0.064
0.355	0.146	−0.176	0.38	0.109		0.085
0.386	0.143	−0.190	0.37	0.125		0.093
0.476	0.132	−0.238	0.34	0.161		0.111
			F. Lithium Perchlorate			
0.372	0.177	−0.254	0.57	0.023		0.019
			G. Lithium Sulfate			
0.180	0.162	−0.228	1.21	−0.098	0.48	−0.096
			H. Sodium Chloride			
0.264	0.157	−0.238	0.82	−0.049	0.17	−0.044
			I. Potassium Chloride			
0.238	0.165	−0.236	0.90	−0.073	0.25	−0.061

and α_{s1}-casein B was independent of Gd·HCl concentration in the range 0–6 M. The average difference from the value obtained in water was 1.8% for 14 data points. The values of the refractive index increment of *S*-cysteinyl-BSA at constant chemical potential of the solvent components, $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$, are listed in Table II. The precision of the results is about 1% in most cases. As an example, the values in aqueous LiBr are plotted as a function of the salt concentration in Figure 2 (curve II). The upper curve (I) represents the value of $(\partial n/\partial c_2)_{T,P,c_3}$ as a function of the LiBr concentration. The value of the parameter $(\partial n/\partial c_3)_{T,P,c_2}$ was constant at 0.147 ml/g for the LiBr concentration range 0.16–0.63 g/ml. Appropriate values of the refractive index increment of the other salts were determined by extrapolation or interpolation of the results listed in Table I. Values of $(\partial c_3/\partial c_2)^0_{T,\mu_1,\mu_3}$ and the other binding parameters are listed in Table II. It can be seen from eq 1 that the error in $(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$ is inversely proportional to the magnitude of the parameter $(\partial n/\partial c_3)_{T,P,c_2}$ and proportional to the error in $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$ and $(\partial n/\partial c_2)_{T,P,c_3}$. For the results in LiBr the error is estimated at 0.02 g/g of protein regardless of the LiBr concentration. For the results in aqueous LiSCN and $LiNO_3$ it is 0.012 and 0.025 g per g of

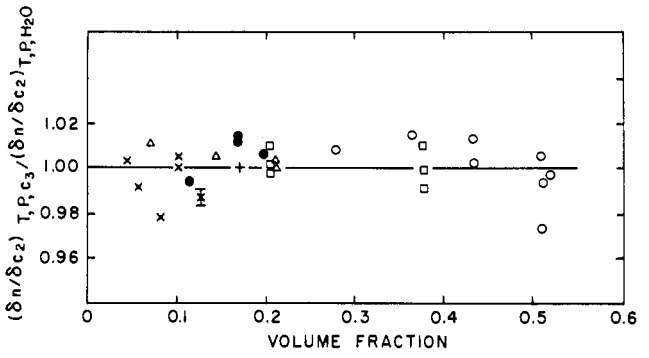

FIGURE 1: Dependence on solvent composition of the refractive index increment of BSA at constant molarity of the third component (×) KCl, (△) NaI, (●) LiBr, (+) average value of all the data points, (□) urea, and (○) Gd·HCl.

protein, respectively and between these limits for the other salts. The percentage error is magnified in the calculation of $(\partial g_3/\partial g_2)^0_{T,\mu_1,\mu_3}$ because this parameter is the difference between numbers of similar magnitude. Estimates of the error in this parameter are 0.02, 0.016, and 0.03 g per g of protein for the results in aqueous LiBr, LiSCN, and $LiNO_3$, respectively. An additional error would result from not knowing the exact value of $\bar{v}_2$. The error in the value of the parameter $(\partial g_3/\partial g_2)^0_{T,\mu_1,\mu_3}$ would be equal to the value of g_3 multiplied by the error in $\bar{v}_2$.

As a further check on the accuracy of the method, the preferential solvation of *S*-cysteinyl-BSA was measured in concentrated solutions of NaCl and KCl. The values found for $(\partial g_1/\partial g_2)^0_{T,\mu_1,\mu_3}$ were 0.17 and 0.25 g of H_2O per g of *S*-cysteinyl-BSA in 5.02 *m* NaCl and 3.96 *m* KCl, respectively, at pH 5.5, 25°. These values compare favorably to the corresponding values of 0.19, and 0.26 g of H_2O per g of BSA found by Cox and Schumaker (1961). They used a hydrodynamic method whereas the one used in this study is an equilibrium method. In their study preferential solvation was determined by comparison of the value of the sedimentation coefficient of the solvated particle to that expected for a hypothetical unsolvated particle.

The number of moles of salt preferentially bound to *S*-cysteinyl-BSA, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, was calculated to enable comparison of the salts (Figure 3). The binding parameter $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ is given by

$$\left[\frac{\partial m_3}{\partial m_2}\right]_{T,\mu_1,\mu_3} = \left[\frac{\partial g_3}{\partial g_2}\right]^0_{T,\mu_1,\mu_3} \times \frac{\text{mol wt of } S\text{-Cys-BSA}}{\text{mol wt of salt}} \quad (5)$$

The value 66,000 was used for the molecular weight of the protein (Spahr and Edsall, 1964).

Values of some of the preferential binding parameters for BSA in aqueous Gd·HCl are listed in Table III for the purpose of comparison. The relevant data were reported in a previous paper (Noelken and Timasheff, 1967). The values of $(\partial c_1/\partial c_2)^0_{T,\mu_1,\mu_3}$ and $(\partial g_3/\partial g_2)^0_{T,\mu_1,\mu_3}$ differ somewhat from those reported earlier because of the subsequent improvement in the determination of $(\partial n/\partial c_2)_{T,P,c_3}$. The previous conclusions concerning the effects of preferential binding of Gd·HCl on molecular weight determinations are not significantly changed, however. An erroneous comparison was inadvertently made

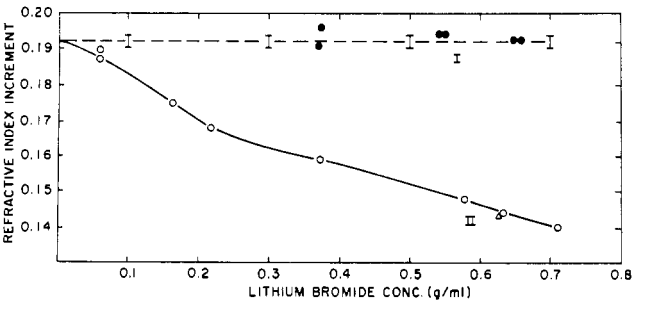

FIGURE 2: Refractive index increment of *S*-cysteinyl-BSA in aqueous lithium bromide. Curve I: the values obtained at constant molarity of LiBr. The dashed line represents the average value obtained in several solvent systems (Figure 1). Curve II: the values obtained at constant chemical potential of the solvent components. (○,●) BSA lot 9385 and (△) BSA lot 8176.

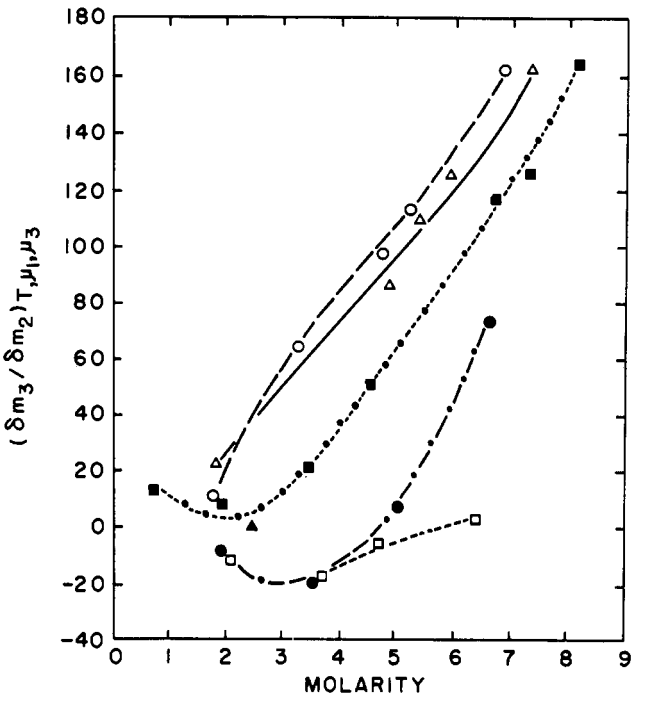

FIGURE 3: Dependence of the preferential solvation (molal scale) on solvent composition. (□) $LiNO_3$, (●) LiCl, (▲) $LiClO_4$, (■) LiBr, (○) LiI, and (△) LiSCN.

TABLE III: Preferential Binding Parameters of the System H_2O–BSA–Gd·HCl.

Gd·HCl (M)	$\left[\frac{\partial c_1}{\partial c_2}\right]_{T,\mu_1,\mu_3}$	$\left[\frac{\partial c_3}{\partial c_2}\right]^{cor}_{T,\mu_1,\mu_3}$	$\left[\frac{\partial g_3}{\partial g_2}\right]_{T,\mu_1,\mu_3}$
3	0.33	0.09	0.12
4	0.32	0.10	0.15
5	0.32	0.11	0.18
6	0.33	0.09	0.15
7.5	0.30	0.05	0.11

in the earlier study between the results for BSA in 5 M Gd·HCl and those of Kielley and Harrington (1960) for myosin in that solvent. They actually measured $(\partial c_3/\partial c_2)^{cor}_{T,\mu_1,\mu_3}$ rather than $(\partial g_3/\partial g_2)^0_{T,\mu_1,\mu_3}$ as was stated by Noelken and Timasheff. Their value of 0.05 g of Gd·HCl/g of myosin should have been compared to the value 0.11 g of Gd·HCl/g of BSA for $(\partial c_3/\partial c_2)^{cor}_{T,\mu_1,\mu_3}$ rather than the previously reported value of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ of 0.18 g/g of BSA. The agreement between the results is thus somewhat better than was stated, although still not good. The disagreement probably results from the different methodology used or from the fact that different proteins were used.

Optical Rotatory Dispersion. The values of the optical rotatory dispersion parameter, b_0, for *S*-cysteinyl-BSA in several solvents, are listed in Table IV. The value of b_0 is essentially the same in 0.14 M KCl, 1.7 M Li_2SO_4, 3.2 M KCl, or 4.6 M NaCl. This indicates the absence of an order–disorder transition involving the α-helical portion of the protein.

TABLE IV: Values of the Optical Rotatory Dispersion Parameter, b_0, and of Preferential Solvation Parameters for BSA and *S*-Cysteinyl BSA in Aqueous Salt Solutions.

Salt	M	$-b_0$	$\left[\frac{\partial m_3}{\partial m_2}\right]_{T,\mu_1,\mu_3}$	$\left[\frac{\partial m_1}{\partial m_2}\right]_{T,\mu_1,\mu_3}$
Gd·HCl	6	40[a]	100[b]	
LiSCN	7.2	65[a]	160	
LiBr	7	65[a]	120	
LiI	7	55[a]	160	
$LiNO_3$	6.4	140[a]	0	
LiCl	6.6	135[a]	65	
NaCl	4.6	320	−55	590
KCl	3.2	310	−65	870
Li_2SO_4	1.7	290	−60	1160
KCl	0.14	305	[c]	

[a] Taken from Noelken (1970). [b] The value is for BSA; the remainder are for *S*-cysteinyl-BSA. [c] Not measured.

Discussion

The dependence of the preferential solvation of *S*-cysteinyl-BSA upon the concentration of several lithium salts and Gd·HCl is given in Figure 3 and in Tables II and III. The inherent ambiguities in interpreting preferential solvation results are immediately obvious upon inspection of Tables II and III. If the results are expressed on a molal basis salt must be considered preferentially bound in the more concentrated solutions of LiCl, LiBr, LiI, LiSCN, and Gd·HCl, while the use of molar units without correction for the protein volume would lead to the conclusion that the protein is preferentially hydrated. Since binding is connected with changes in the environment of the protein, it is more realistic to consider the binding parameters that do not include contributions from the protein volume. Thus, further discussion will involve the parameters $(\partial g_3/\partial g_2)^0_{T,\mu_1,\mu_3}$ and $(\partial c_3/\partial c_2)^{cor}_{T,\mu_1,\mu_3}$.

A striking feature of the results is the large amount of preferential salt binding that occurs with several of the salts. About 160 moles of salt is bound per mole of *S*-cysteinyl-BSA (molal scale) at 7 M LiI, 7 M LiSCN, or 8 M LiBr. For BSA in 6 M Gd·HCl the value of $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ is 100 moles/mole. The value of $(\partial c_3/\partial c_2)^{cor}_{T,\mu_1,\mu_3}$ is only about 20% lower in each case. Furthermore, these values are less than the total amount of salt bound. If salt and water were bound in the same ratio as occurred in the dialysate, neither would be detected by this procedure.

The results of this paper and a preceeding paper (Noelken, 1970) suggest that there is a rough correlation between preferential solvation and protein unfolding (Table IV). Several of the lithium salts are denaturants, as can be ascertained from their effects on the value of the optical rotatory dispersion parameter, b_0, which is an indicator of α-helix content (Urnes and Doty, 1961). As a first approximation, systems in which *S*-cysteinyl-BSA, or BSA, is extensively unfolded are also characterized by preferential salt binding. On the other hand, preferential hydration is characteristic of the systems—aqueous NaCl, KCl, and Li_2SO_4—in which the native structure is retained. Consistent with this finding is the fact that the amount of preferential salt binding increases with increasing concentration of five lithium salts that denature *S*-cysteinyl-BSA (Figure 3). This correlation is also valid if total salt binding is considered. The total salt binding is given by the relation

$$\left[\frac{\partial m_3}{\partial m_2}\right]_{\text{Total}} = \left[\frac{\partial m_3}{\partial m_2}\right]_{T,\mu_1,\mu_3} + \frac{m_3}{55.5} \times \frac{\text{total moles of } H_2O}{\text{mole of protein}}$$

It would require that the total hydration of the native protein be about 0.5 g of H_2O/g of protein *greater* than that of the denatured protein for the correlation to be invalid. This possibility would seem to be very unlikely in view of the results of a study by Bull and Breeze (1968) in which the maximum hydration of BSA in a salt-free system was found to be 0.32 g/g of protein at a water activity of 0.92, and 0.49 g/g of protein at an activity of 0.98. The water activity in 1.7 M Li_2SO_4, 3.2 M KCl, and 4.6 M NaCl is 0.95, 0.87, and 0.81, respectively (Robinson and Stokes, 1965). Thus, values for the total hydration would be expected to be somewhat less than 0.5 g of H_2O/g of *S*-cysteinyl-BSA in these solvents. It is also significant that the degree of hydration of egg albumin, which is similar to that of BSA in salt-free systems, is decreased to 0.08 and 0.15 g per g of protein in 1–3 *m* NaCl and KCl, respectively (Bull and Breeze, 1970).

The results of studies with model compounds suggest that inorganic salts, including the lithium salts, interact weakly with peptide groups (Robinson and Jencks, 1965b; Bello *et al.*, 1966; Kurtz and Harrington, 1966; Schrier and Schrier, 1967; Von Hippel and Schleich, 1969). Gd·HCl interacts principally with aromatic side chains and pairs of adjacent peptide groups (Robinson and Jencks, 1965a; results of Nozaki and Tanford, reported by Aune and Tanford, 1969).

Denaturant binding (total) appears to be a general phenomenon that is not restricted to salts. Timasheff and Inoue (1968) have found that a large amount of organic solvent binding accompanies α-helix formation with proteins in aqueous chloroethanol, glycol, or methoxyethanol. They postulated that the organic solvent interacted weakly with nonpolar side chains. Gordon and Warren (1968) found that

urea, methylurea, and thiourea were bound to unfolded BSA whereas preferential hydration was observed in solutions of the nondenaturants, tetramethylurea and dextrose. Further evidence for the role of salt binding in the denaturation process comes from the study of Ciferri *et al.* (1967). They found that KSCN, a denaturant, had a greater binding affinity for collagen than KCl, which did not alter the collagen structure.

In conclusion, the results of this study and the preceeding one (Noelken, 1970) indicate that the unfolding of *S*-cysteinyl-BSA by Gd·HCl and several lithium salts is a thermodynamically reversible process that is accompanied by relatively nonspecific protein–denaturant interactions.

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