Abnormal Solubility Behavior of β-Lactoglobulin: Salting-In by Glycine and NaCl†

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Received January 30, 1987

ABSTRACT: The causes of the salting-in of β-lactoglobulin by glycine and NaCl, a solubility behavior contrary to expectations, were probed by a detailed study of the interactions between these solvent components and the protein. The preferential interactions of β-lactoglobulin with solvent components in aqueous glycine and NaCl systems have been compared with those of bovine serum albumin and lysozyme. At neutral pH, β-lactoglobulin exhibited insignificant preferential interactions in glycine and NaCl at low cosolvent concentrations and an increasing preferential hydration at higher concentrations, the levels approaching the values expected from the other two proteins. These results indicate considerable binding of the electrolytes to β-lactoglobulin, sufficient to compensate for the exclusion due to perturbation of the solvent surface tension. The difference between the preferential interactions of β-lactoglobulin and the other proteins with these two solvent additives was shown to be the cause of the increase of β-lactoglobulin solubility even at high concentrations of the additives, at which they have salting-out effects on the other proteins. The preferential interactions of NaCl with the three proteins were examined as a function of pH. The results showed no pH dependence of the preferential hydration for bovine serum albumin and lysozyme, while this parameter increased significantly for β-lactoglobulin at lower pH. This suggests that the binding of electrolytes to β-lactoglobulin is due to a unique charge distribution on the surface of the protein around neutral pH, which imparts to this protein a large dipole moment.

The solubility of β-lactoglobulin (β-LG)[1] at its isoelectric point (pH 5.1) in water is very low. It can be increased sharply by addition of NaCl or glycine to the medium, the salt being more effective than the amino acid (Cohn & Ferry, 1943; Edsall & Wyman, 1958). The salting-in of β-LG by NaCl and glycine has been a puzzle since its discovery (Cohn & Ferry, 1943; Kirkwood, 1943). It is quite unexpected, since these two additives belong to the salting-out class (von Hippel & Schleich, 1969; Robinson & Jencks, 1965). Cohn and Ferry (1943) have proposed that this behavior is a consequence of the large dipole moment of β-LG (Ferry & Oncley, 1941) and the resultant electrostatic interactions between the protein and the additives. To gain an understanding of the causes of this unusual solubility pattern of a globular protein, a study was carried out of the nature of the interactions between NaCl and glycine and β-lactoglobulin.

The preferential interactions of proteins with solvent components are known to be closely related to protein stability and solubility. Thus, those substances that have a stabilizing and salting-out effect on proteins in aqueous solutions normally lead to large protein preferential hydrations (Aune & Timasheff, 1970; Pittz & Timasheff, 1978; Lee & Timasheff, 1981; Lee & Lee, 1979, 1981; Gekko & Timasheff, 1981; Arakawa & Timasheff, 1982a,b, 1983, 1984a,b, 1985), as found for sucrose, glycerol, Na_2SO_4, and poly(ethylene glycol). To the contrary, destabilizing and salting-in substances are preferentially bound to proteins (Timasheff & Inoue, 1968; Lee & Timasheff, 1974; Izumi et al., 1980; Arakawa & Timasheff, 1982b, 1984a,b). Since glycine and NaCl are preferentially excluded from bovine serum albumin (BSA) and lysozyme, which they salt out and stabilize (Arakawa & Timasheff, 1983), the solubility of β-LG in the presence of these additives suggested a pattern of interactions different from those of the other two proteins. In the present study, this hypothesis was tested and shown to be true, the interactions of β-LG with glycine and NaCl being fully consistent with the explanation proposed by Cohn and Ferry (1943) for the unexpected solubility behavior of this protein.

Materials and Methods

β-LG (119C-8015, 106C-8070), BSA (80F-9340, 89C-9300), and lysozyme (57C-8025) were from Sigma; lysozyme (30C671) was from Worthington. The proteins were dialyzed against distilled deionized water, passed through a sintered-glass filter, and lyophilized. For β-LG, the solvents were prepared by adjusting the pH of the aqueous amino acid or NaCl solutions with NaOH or HCl without additional buffer components to avoid extra interactions between the protein and ionic species from the buffer. For lysozyme and BSA, the solvents were 0.02 M buffers.

Density measurements were carried out as described previously (Lee & Timasheff, 1974, Lee et al., 1979). In cases where the protein solution showed precipitation in the dialysis bags or test tubes, it was shaken gently to make it homogeneous just before transfer to the densimeter cell, or it was taken up into a disposable syringe, allowed to stand for about 30 min, during which time the large protein aggregates sedimented into the tip of the syringe, and transferred into the cell via the same syringe after removal of the sedimented aggregates. Both methods were found to be effective, since no change of the solution density was observed during the measurements after

† This is Publication No. 1620 from the Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254. This work was supported by grants from the National Institutes of Health (CA-16707 and GM-14603).
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[1] Abbreviations: β-LG, β-lactoglobulin; BSA, bovine serum albumin; Gdn·HCl, guanidine hydrochloride.

0006-2960/87/0426-5147$01.50/0

Table I: Partial Specific Volumes and Preferential Interaction Parameters of β-Lactoglobulin in Aqueous Amino Acid System[a]

	conditions	ϕ_2^0 (mL/g)	$\phi_2'^0$ (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	g_3 (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	appearance[b]
			pH 5.1				
1	dilute salt	0.745 ± 0.0004	0.744 ± 0.003				P
2	0.5 M glycine	0.743 ± 0.001	0.742 ± 0.001	0.0025 ± 0.0050	0.0389	−0.064 ± 0.128	P
3	1 M glycine	0.744 ± 0.001	0.749 ± 0.002	−0.0133 ± 0.0080	0.0787	0.169 ± 0.102	T
4	2 M glycine	0.743 ± 0.002	0.759 ± 0.001	−0.0485 ± 0.0091	0.165	0.294 ± 0.055	C
5	0.5 M glycine + 0.05 M NaCl	0.740 ± 0.002	0.745 ± 0.001				T
6	1 M glycine + 0.05 M NaCl	0.741 ± 0.002	0.750 ± 0.001				T
			pH 6.2				
7	0.5 M glycine, no salt	0.746 ± 0.001	0.744 ± 0.001	0.0050 ± 0.0050	0.0389	−0.128 ± 0.128	T
			pH 7.0				
8	1 M glycine	0.740 ± 0.002	0.742 ± 0.001	−0.0053 ± 0.0080	0.0787	0.068 ± 0.102	C
9	1 M β-alanine	0.744 ± 0.001	0.746 ± 0.001	−0.0066 ± 0.0066	0.0948	0.069 ± 0.069	C
10	1 M α-alanine	0.747 ± 0.001	0.751 ± 0.001	−0.0142 ± 0.0071	0.0951	0.150 ± 0.075	C
			1.4 M Glycine, pH 6.17				
11	lysozyme	0.697 ± 0.002	0.721 ± 0.001	−0.0685 ± 0.0084	0.112	0.610 ± 0.075	
12	BSA	0.736	0.752 ± 0.001	−0.0463 ± 0.0028	0.112	0.412 ± 0.025	

[a] In all cases for β-LG, protein concentration was determined in 6 M Gdn·HCl. [b] Appearance of experimental protein solution: P, precipitate; T, turbid; C, clear.

thermal equilibrium was reached, which indicated that aggregates are not sedimented out in the cell. This was also confirmed by the values of the partial specific volumes obtained. Protein concentrations were determined spectrophotometrically on a Cary Model 118 spectrophotometer, using absorbance values of 6.58 dL/(g·cm) at 278 nm for BSA (Noelken & Timasheff, 1967), 27.4 dL/(g·cm) at 281 nm for lysozyme (Roxby & Tanford, 1971), and 9.6 dL/(g·cm) at 278 nm for β-LG (Townend et al., 1960). For those samples which contained precipitates, the concentration was measured in 6 M Gdn·HCl, using absorbance values of 6.25 dL/(g·cm) at 278 nm for BSA, 26.9 dL/(g·cm) at 281 nm for lysozyme, and 9.6 dL/(g·cm) at 276 nm for β-LG.

The preferential binding parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ or $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, and the preferential hydration parameter, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, were calculated from the isomolal (ϕ_2^0) and isopotential ($\phi_2'^0$) partial specific volumes determined in each solvent system, as described by Lee et al. (1979), where g_i is the concentration of component i in grams per gram of water, m_i and μ_i are its molality and chemical potential, T is the thermodynamic (kelvin) temperature, and components 1, 2, and 3 refer to water, protein, and additive, respectively.

The thermodynamic interaction parameter was calculated from

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = -(\partial \mu_2/\partial m_3)_{T,P,m_2}/(\partial \mu_3/\partial m_3)_{T,P,m_2} \quad (1)$$

$$\mu_i = \mu_i^0 + RT \ln a_i$$

where P is pressure, a_i is the activity of component i, and R is the universal gas constant. The self-interaction term, $(\partial \mu_3/\partial m_3)_{T,P,m_2}$, defined as $RT(\partial \ln a_3/\partial m_3)_{T,P,m_2}$, was calculated from a_3 by using the mean ionic activity coefficient, $\gamma_\pm$, for NaCl (Robinson & Stokes, 1959) and the activity coefficient, γ, for glycine (Ellerton et al., 1964). For α- and β-alanine, no corrections for the activity coefficient were made, since no data are available. It is evident however, from the results for glycine, that this correction becomes significant only at high amino acid concentrations, i.e., 2 M.

Results

Typical plots of the apparent partial specific volume, ϕ_{app}, as a function of protein concentration, are shown in Figure 1. The scatter in ϕ_{app} falls within the usual experimental error in densimetry, i.e., ±0.001 mL/g, and duplicate experiments

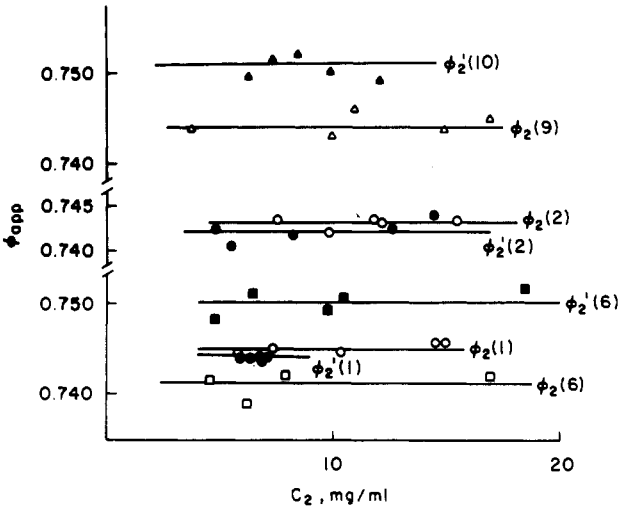


FIGURE 1: Protein concentration dependence of ϕ_{app} of β-LG in aqueous amino acid systems. The indicated numbers refer to Table I.

gave identical values of the partial specific volumes within experimental error. Since some of the systems contained precipitates, these results demonstrate that the densimetry method can provide reliable data even under such circumstances if special precautions are taken. This is an advantage over optical techniques, which can be used only for transparent solutions. For most cases there was no significant concentration dependence of ϕ_{app}. Values of the partial specific volume of β-LG at pH 5.1 in the absence of salt and glycine determined at constant chemical potential, $\phi_2'^0$, and constant molality, ϕ_2^0, were found to be identical within experimental error, indicating that the experimental conditions are satisfactory for undertaking a preferential interaction study, since the interaction parameters are obtained from the difference between ϕ_2^0 and $\phi_2'^0$ induced by the addition of component 3.

The values of the partial specific volumes of β-LG, measured in glycine and α- and β-alanine solutions at various conditions, and the resulting preferential interaction parameters are listed in Tables I and II, where they are compared with the results for lysozyme and BSA taken from Arakawa and Timasheff (1983). At pH 5.1, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was close to zero in 0.5 M glycine and became increasingly negative as the glycine con-

Table II: Interaction Parameters in Aqueous Amino Acid Systems

	conditions	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial\mu_2/\partial m_3)_{T,P,m_2}^{\text{exptl}}$ [cal (mol of protein)$^{-1}$ (mol of salt)$^{-1}$]	$(\partial\mu_3/\partial m_3)_{T,P,m_2}$ [cal (mol of salt)$^{-1}$ (mol of salt)$^{-1}$]	$(\partial\mu_2/\partial m_3)_{T,P,m_2}^{\text{exptl}}/(\partial\mu_2/\partial m_3)_{T,P,m_2}^{\text{calcd}}$
			pH 5.1		
2	0.5 M glycine	1.22 ± 2.44	−1300 ± 2500	1060	−0.11
3	1 M glycine	−6.52 ± 3.92	3200 ± 1900	490	0.25
4	2 M glycine	−23.8 ± 4.5	5400 ± 1000	230	0.42
			pH 6.2		
7	0.5 M glycine	2.43 ± 2.43	−2500 ± 2500	1060	−0.20
			pH 7.0		
8	1 M glycine	−2.60 ± 3.92	1300 ± 1900	490	0.10
9	1 M β-alanine	−2.71 ± 2.71	1500 ± 1500	550	
10	1 M α-alanine	−5.88 ± 2.94	3200 ± 1600	540	0.29
			1.4 M Glycine, pH 6.17		
11	lysozyme	−13.0 ± 1.6	5100 ± 600	350	0.78
12	BSA	−41.9 ± 2.5	16300 ± 1000	350	0.87

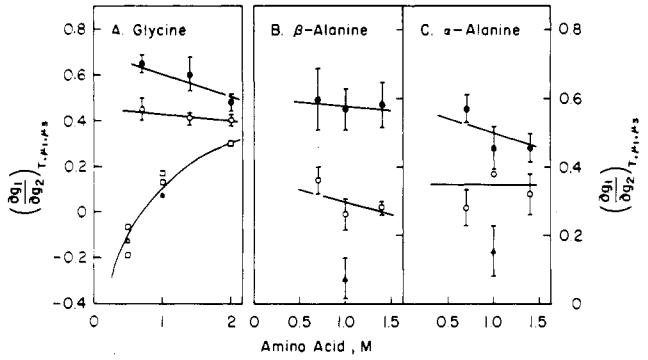


FIGURE 2: Comparison of the preferential hydrations, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, of β-LG, BSA, and lysozyme in aqueous glycine (A), β-alanine (B), and α-alanine (C) systems: (●) BSA; (○) lysozyme; (□) pH 5.1, β-LG; (△) pH 6.2, β-LG; (▲) pH 7.0, β-LG.

centration increased. Addition of 0.05 M NaCl to 0.5 and 1 M glycine increased the difference between ϕ_2^0 and $\phi_2'^0$. The β-LG solution, which was turbid in 0.5 and 1 M glycine, became transparent in 2 M amino acid, showing the expected increase in solubility. The extent of preferential interaction remained essentially the same at pH 6.2 and 7.0, at which conditions β-LG is much more soluble than at pH 5.1, nor did it change significantly when glycine was replaced by α- or β-alanine. In fact, at all conditions used, this parameter was drastically less negative for β-LG than for lysozyme and BSA.

The preferential hydration parameter, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, calculated from the above values, was found to be independent of pH, whether the protein was fully soluble or not, as shown in Table I and Figure 2A. The glycine concentration dependence of this parameter for β-LG differed totally from those for BSA and lysozyme, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ for β-LG being negative at low glycine concentration and becoming positive as the concentration increased, while it was always positive, decreasing slightly with amino acid concentration, for the other two proteins. Since the difference in preferential interactions of glycine with lysozyme and BSA is due to the difference in their surface areas (Arakawa & Timasheff, 1983), the value of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ for β-LG could be expected to lie between them if the mechanism of protein–glycine interaction is the same for all three proteins. As seen in Figure 2A, this is totally at variance with the observation. Therefore, factors other than protein size must determine the pattern of β-LG interaction with glycine. In 1 M α- and β-alanine solutions, β-LG showed a small or zero preferential hydration, which again differs from the results for lysozyme and BSA, shown in parts B and C of Figure 2.

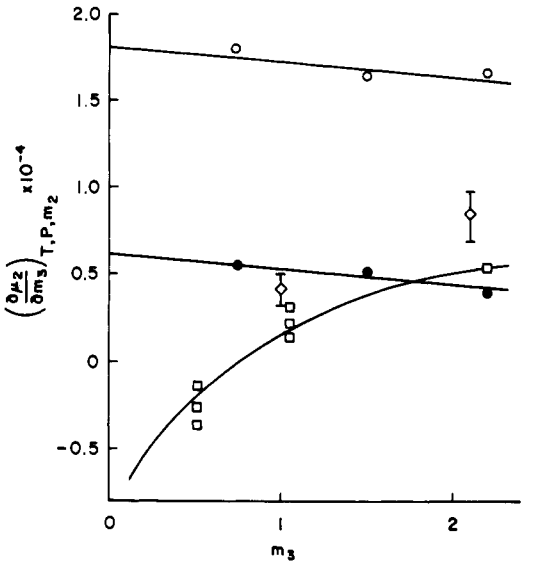


FIGURE 3: Dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ on additive concentration, m_3, for BSA (○), lysozyme (●), and β-LG (□) in the aqueous glycine system and for β-LG (◇) in the aqueous NaCl system.

The preferential interaction parameter is related to the total binding of solvent components to a protein by (Inoue & Timasheff, 1972)

$$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = (A_1 - A_3)/g_3 \qquad (2)$$

where A_1 and A_3 are the total bindings of additive and water, respectively, in grams per gram of protein. Setting $A_3 = 0$, A_1 becomes equal to $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$. In the case of lysozyme and BSA, A_1 is greater than the hydration values for most proteins (Table I). This large hydration had been shown to be due to the exclusion of the amino acids from the protein surface, which is proportional to the surface area of the proteins. Assuming that the force driving the exclusion of amino acids from the protein surface operates in β-LG just as in the other two proteins, the observed lower preferential hydration for β-LG must be ascribed to compensating binding of amino acid.

The chemical potential change of the protein induced by the addition of glycine, $(\partial\mu_2/\partial m_3)_{T,P,m_2}^{\text{exptl}}$, calculated with eq 1, is given in column 3 of Table II and plotted vs. m_3 in Figure 3. For β-LG, the values were not much different from zero at 0.5 and 1 M amino acid, but increased with m_3. The positive value at 2 M glycine indicates that, at this concentration, glycine increases the activity of the protein and hence decreases its solubility. These results are again totally at variance with

Table III: Partial Specific Volumes and Preferential Interaction Parameters of β-LG in the Aqueous NaCl System

conditions	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)	appearance[b]
pH 1.55,[c] 1 M	0.754 ± 0.001	0.762 ± 0.001	−0.0127 ± 0.0032	0.213 ± 0.054	P
pH 2.0,[c] 1 M	0.749 ± 0.001	0.760	−0.0174 ± 0.0016	0.292 ± 0.027	P
pH 2.5,[c] 1 M	0.747 ± 0.001	0.760 ± 0.001	−0.0206 ± 0.0032	0.346 ± 0.054	P
pH 3.0,[c] 1 M	0.747 ± 0.001	0.760 ± 0.001	−0.0206 ± 0.0032	0.346 ± 0.054	P
pH 3.0, 2 M	0.746 ± 0.001	0.769 ± 0.001	−0.0385 ± 0.0035	0.316 ± 0.029	P
pH 4.0, 1 M	0.746 ± 0.001	0.756 ± 0.001	−0.0158 ± 0.0032	0.265 ± 0.054	T
pH 5.1, 1 M	0.751 ± 0.001	0.756 ± 0.001	−0.0079 ± 0.0032	0.133 ± 0.053	C
pH 5.1, 2 M	0.752 ± 0.001	0.766 ± 0.002	−0.0245 ± 0.0052	0.201 ± 0.043	C
pH 7.0, 1 M	0.747 ± 0.001	0.751 ± 0.001	−0.0063 ± 0.0032	0.106 ± 0.053	C
pH 10.0, 1 M	0.748 ± 0.001	0.751 ± 0.001	−0.0048 ± 0.0032	0.080 ± 0.053	C
pH 10.0, 2 M	0.748 ± 0.001	0.759	−0.0193 ± 0.0018	0.158 ± 0.015	C

[a] The value of g_3 was 0.0596 at 1 M and 0.122 at 2 M. [b] Appearance of experimental protein solutions: P, precipitate; T, turbid; C, clear. [c] Protein concentration was determined in 6 M Gdn·HCl.

Table IV: Partial Specific Volumes and Preferential Interaction Parameters of BSA and Lysozyme in the 1 M NaCl System

pH	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)
		BSA		
4.5	0.734 ± 0.001	0.744 ± 0.001	−0.0158 ± 0.0032	0.265 ± 0.053
5.6	0.735 ± 0.001	0.744 ± 0.001	−0.0145 ± 0.0032	0.243 ± 0.054
7.0	0.739 ± 0.001	0.748	−0.0142 ± 0.0015	0.239 ± 0.027
9.0	0.738 ± 0.001	0.748	−0.0158 ± 0.0021	0.266 ± 0.035
		Lysozyme		
3.0[b]	0.703 ± 0.001	0.720 ± 0.001	−0.0269 ± 0.0032	0.451 ± 0.0049
4.5	0.707 ± 0.002	0.723 ± 0.002	−0.0253 ± 0.0063	0.424 ± 0.106
7.0[b]	0.701 ± 0.002	0.716 ± 0.002	−0.0237 ± 0.0063	0.398 ± 0.106

[a] $g_3 = 0.0596$. [b] Protein concentration was determined in 6 M Gdn·HCl.

those found for lysozyme and BSA, which gave nearly constant, positive values at all amino acid concentrations.

For BSA and lysozyme, the principal source of the exclusion is the perturbation of the surface free energy (surface tension) of the proteins by the amino acids (Arakawa & Timasheff, 1983). This factor was, therefore, evaluated for β-LG by using the relation (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a,b, 1983)

$$(\partial\mu_2/\partial m_3)_{T,P,m_2}^{\text{calcd}} = N_{\text{AV}} s_2 (\partial\sigma/\partial m_3)_{T,P,m_2} \qquad (3)$$

where N_{AV} is Avogadro's number, s_2 is the protein surface area,[2] and σ is the surface tension of the aqueous amino acid solution. Since all the amino acids used increase the surface tension of water (Pappenheimer et al., 1936), eq 3 predicts positive values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ independent of the amino acid concentration, provided that s_2 and $(\partial\sigma/\partial m_3)_{T,P,m_2}$ remain constant. The calculated values for glycine and α-alanine were $(\partial\mu_2/\partial m_3)_{T,P,m_2}^{\text{calcd}} = 12\,800$ and 10 900 cal (mol of β-LG)$^{-1}$ (mol of amino acid)$^{-1}$, respectively. The deviation of the actual exclusion from that expected from the surface tension perturbation can be expressed best by the ratio between the experimental value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ and the calculated one. Values of this ratio, given in the last column of Table II, show it to be low or even negative for β-LG, indicating that the observed preferential interaction is not an expression principally of the perturbation of the surface free energy induced by the addition of these amino acids, as is the case for lysozyme and BSA, which showed the usually observed ratios of ca. 0.8. Since the surface free energy perturbation is a general nonspecific thermodynamic property of the solvent, these results point to significant amino acid binding by β-LG at low glycine concentrations which compensate the nonspecific exclusion. At 2 M glycine, this ratio increases for β-LG, pointing to a greater manifestation of the surface tension effect and a decreased contribution of amino acid binding.

This analysis of the β-LG results in terms of a combination of surface tension perturbation with amino acid binding is similar to that developed for the divalent cation salt systems (Arakawa & Timasheff, 1984a), in which salt binding had been found to be a function of solvent pH, i.e., of protein charge. A comparable examination of the pH dependence of the amino acid interactions with β-LG could not be undertaken since addition of the required large amounts of acid or base would introduce counterion species into the system, which, in turn, would contribute to the preferential interaction. To circumvent this difficulty, the preferential interaction measurements were extended to NaCl, which also increases the solubility of β-LG, similarly to glycine (Cohn & Ferry, 1943). The results, summarized in Table III, show negative $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ and positive $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ values at all conditions, namely, the protein is preferentially hydrated just as in 1 and 2 M glycine. For NaCl, the preferential interaction parameters are strongly dependent on pH. As shown in Figure 4, for 1 M NaCl $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ decreased with increasing pH after an initial increase below pH 2.5 and varied little above pH 5. For the 2 M salt system, the tendency appeared to be the same, but the preferential hydration was significantly higher than in 1 M salt at pH >4. The solubility of β-LG increased as pH increased above 3 at both NaCl concentrations and was high enough at pH 5.1 to give a clear protein solution.

As in the amino acid systems, measurements were carried out with lysozyme and BSA for comparison. The results are presented in Table IV and Figure 4. For these proteins, the preferential interaction parameters were nearly independent of solvent pH. In this solvent system as well, the preferential

[2] The surface areas of BSA and lysozyme were calculated as described previously (Arakawa & Timasheff, 1983, 1984a). The surface area of β-LG was calculated from its partial specific volume and molecular weight of 36 800 as dimer by using the surface-to-volume ratio for BSA determined by small-angle X-ray scattering (Luzzati et al., 1961).

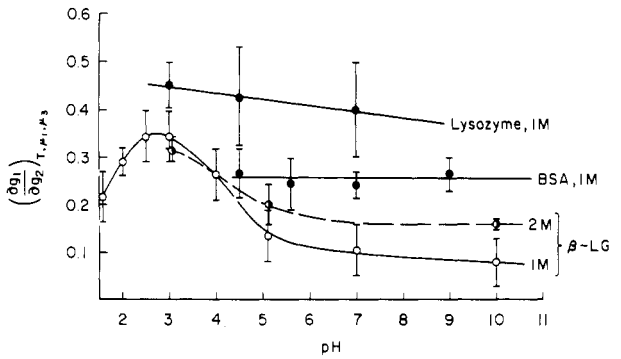


FIGURE 4: pH dependence of the preferential hydrations of lysozyme, BSA, and β-LG in the aqueous NaCl system.

hydration of β-LG was significantly lower than those of lysozyme and BSA, whereas it should be between the two if the surface free energy mechanism is applicable (Arakawa & Timasheff, 1984a). At pH 3.0, however, it approached the expected value, indicating that the compensation of salt exclusion by salt binding, which is prevalent at higher pH, becomes insignificant at acid conditions. Since the ionic properties of NaCl are independent of pH, this pH profile must be ascribed to the variation of the charged state of β-LG. The decrease in $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ observed below pH 3.0 may be explained, however, by increased aggregation.

The surface exclusion–salt binding competition was further analyzed in terms of the ratio of the experimental values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ to those calculated from surface tension perturbation. The results are given in Table V. The values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}^{\text{calcd}}$, calculated with $(\partial\sigma/\partial m_3)_{T,P,m_2} = 1.64$ (Melander & Horvath, 1977), where 19400 cal (mol of protein)$^{-1}$ (mol of salt)$^{-1}$ for β-LG, 9950 cal (mol of protein)$^{-1}$ (mol of salt)$^{-1}$ for lysozyme, and 28700 cal (mol of protein)$^{-1}$ (mol of salt)$^{-1}$ for BSA. The ratio was the one expected from surface free energy perturbation for lysozyme and BSA at all pH values and for β-LG at pH 3.0. Otherwise, this ratio was low, just as in the glycine system, indicating NaCl binding to β-LG. At pH 3.0 in 1 M NaCl, the value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}^{\text{exptl}}$ for β-LG was intermediate between those of BSA and lysozyme, indicating an interaction with salt in a manner similar to the other proteins. The decreased preferential hydration of β-LG at higher pH must be ascribed, therefore, to a pH-induced alteration of a property of β-LG, which is specific for this protein. The measured preferential interactions predict that at pH 3 NaCl should have the same effect on β-LG solubility as on those of BSA and lysozyme, i.e., it should behave as a salting-out salt. This is seen to be true from the appearance of the solutions: β-LG formed a precipitate at acid conditions, whereas its solutions were transparent at neutral pH, as shown in Table III.

Table V: Interaction Parameters of the Proteins in the Aqueous NaCl System

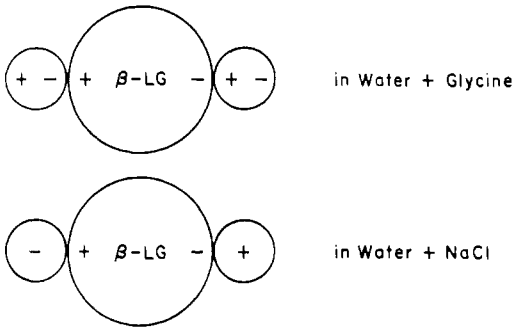
conditions	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial\mu_2/\partial m_3)_{T,P,m_2}^{\text{exptl}}$ [cal (mol of protein)$^{-1}$ (mol of salt)$^{-1}$]	$(\partial\mu_2/\partial m_3)^{\text{exptl}}/(\partial\mu_2/\partial m_3)^{\text{calcd}}$
		β-LG	
pH 1.55, 1 M	−8.00 ± 2.02	8700 ± 2200	0.45
pH 3.0, 1 M	−13.0 ± 2.0	14100 ± 2200	0.73
pH 3.0, 2 M	−24.2 ± 2.2	14700 ± 1300	0.76
pH 4.0, 1 M	−9.95 ± 2.02	10800 ± 2200	0.56
pH 5.1, 1 M	−4.97 ± 2.02	5400 ± 2200	0.28
pH 5.1, 2 M	−15.4 ± 3.3	9400 ± 2000	0.48
pH 7.0, 1 M	−3.97 ± 2.02	4300 ± 2200	0.22
pH 10.0, 1 M	−3.02 ± 2.02	3300 ± 2200	0.17
pH 10.0, 2 M	−12.2 ± 1.1	7400 ± 700	0.38
		BSA	
pH 4.5, 1 M	−18.4 ± 3.7	20000 ± 4000	0.70
pH 7.0, 1 M	−16.5 ± 1.7	18000 ± 1800	0.63
		Lysozyme	
pH 3.0, 1 M	−6.58 ± 0.78	7200 ± 800	0.72
pH 7.0, 1 M	−5.80 ± 1.54	6300 ± 1000	0.63

Discussion

The pattern of interactions of β-LG with glycine and NaCl is markedly different from those of lysozyme and BSA. One possible reason for such a difference could be aggregation of β-LG in glycine solutions at pH 5.1 (see Table I), which would decrease the surface area per unit mass of protein. If protein preferential hydration in the NaCl and glycine systems is a function mainly of the protein surface area (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a,b, 1983), then the value of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ for β-LG in 1 M glycine at pH 5.1, calculated from the BSA results, should be ~0.5 g/g. The observed value is only 0.17 g/g, implying an aggregate with a specific surface area 0.34 that of the monomer. When a spherical approximation for monomer and aggregate is used, this ratio leads to an average degree of aggregation of 25. This is highly unlikely since (1) the preferential interaction parameter was determined by extrapolation to zero protein concentration and (2) identical preferential interaction results were obtained at conditions at which β-LG was fully soluble (see Table I and Figure 2). Identical interaction patterns were also found in NaCl at neutral pH. Therefore, the low preferential hydration of β-LG in the amino acid and NaCl systems must be ascribed to factors other than the value of its surface area. This means that the β-LG preferential hydrations in these solvent systems cannot be determined predominantly by the protein surface area, as is true of a number of solvent systems with a variety of proteins. In the sugar systems, the preferential hydrations observed for chymotrypsinogen, α-chymotrypsin, ribonuclease A, BSA, lysozyme, tubulin, and β-LG could be related to their surface areas and the perturbation of the surface free energy (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a). Since sugars are not electrolytes, these observations suggest the electrolyte nature of the amino acids and NaCl as the factor responsible for the peculiar preferential interaction of β-LG with these two systems.

For BSA and lysozyme, the exclusion of the additive from the protein surface, i.e., the preferential hydration, is due mainly to the increase in the surface free energy of the protein induced by addition of glycine or NaCl. The surface tension effect, being a general and nonspecific property of the solvent, should also apply to β-LG. Therefore, the low preferential hydration must result from a compensating binding of NaCl and glycine specific to this protein. The binding profile of the additives as a function of pH and ligand concentration reveals some important features that give insight into the binding mechanism. Measurements of β-LG solubility (Cohn & Ferry, 1943) have led to the postulate that the large dipole moment of the β-LG molecule, which is 730 Debye units (Ferry &

Chart I



Oncley, 1941), plays an important role in its low solubility in water at pH 5.1 and that the roles of glycine and NaCl in its solubility enter at this level. Amino acids have large permanent dipole moments (Edsall, 1943), which for neutral amino acids such as glycine and alanine, do not change significantly between pH 4 and 9. It seems reasonable then that β-LG should bind glycine through dipole–dipole and NaCl through dipole–ion interactions (Edsall & Wyman, 1958) in the general manner depicted in Chart I.[3] This would reduce the excess free energy of β-LG due to its dipole moment. Since the electrostatic force is generally large, the standard free energy of binding could be significant. Furthermore, the bindings of the various amino acids should not be much different from each other in view of the similarity of their dipole moments. This is in agreement with observation. Nor should there be any pH effect on the binding of glycine between pH 5.1 and 7.0, since the glycine dipole moment does not change. This is again consistent with observation.

In the NaCl system, the ionic properties of the salt should not depend on pH. Therefore, the observed variation of NaCl binding must be ascribed to variations of the electrostatic properties of β-LG. The major change occurs between pH 5.0 and 3.0, i.e., in the pH zone in which the carboxyl groups are titrated. This leads to a decrease of negative charges as the protein becomes a positive macroion and a reduction of its dipole moment. Therefore, at low pH, β-LG should lose its major force for electrolyte binding and its interactions with salts and amino acids should become similar to those of BSA and lysozyme, exactly as observed at pH 3.0 for the NaCl system.

Now that it is established that the preferential interaction of glycine and NaCl with β-LG arises from a fine balance between additive binding and hydration (a part of which is due to the surface free energy exclusion of the additives), the increased solubility of β-LG by the additives can now be explained in terms of this interaction mechanism. According to the Wyman linkage theory (Wyman, 1964), a change in protein solubility induced by the additive must be related to the interactions of solvent components with the protein in solution and in the solid phase, as expressed by

$$\begin{aligned}\partial \ln S/\partial \ln a_3 &= (A_3^{l} - A_3^{s}) - (m_3/55.5)(A_1^{l} - A_1^{s}) \\ &= \Delta A_3 - (m_3/55.5)\Delta A_1 \\ &= \Delta\nu \end{aligned} \quad (4)$$

where S is protein solubility, A_i is the total binding of component i in moles per mole of protein, and superscripts l and s refer to the solution (liquid) and solid phases, respectively. For BSA and lysozyme, the bindings of glycine and NaCl were small, while protein hydration was very large, i.e., $A_3^{l} \approx 0$ and $A_1^{l} > 0$. Since the protein hydration (and exclusion of the additive) should decrease in the solid phase due to protein–protein contacts, $\Delta A_1 > 0$ and hence $\Delta\nu < 0$ with $\Delta A_3 \approx 0$. As a result, addition of glycine and NaCl should decrease the solubility of BSA and lysozyme. On the other hand, A_3^{l} was shown to be significant for β-LG, particularly at lower glycine and NaCl concentrations. In the solid phase, the protein is expected to have decreased bindings of glycine and NaCl due to the formation of protein–protein contacts that decrease the accessible surface area and possibly also the dipole moment. This gives $\Delta A_3 > 0$. The protein solubility is determined, then, by a fine balance between positive values of ΔA_3 and ΔA_1. Qualitatively, it can be expected that the solubility of β-LG will increase at lower glycine and NaCl concentrations where binding is predominant, whereas the solubility will decrease at higher concentrations of additives where the exclusion of the additive by the surface free energy effect becomes predominant. This explains the salting-in effects of glycine and NaCl at the lower additive concentrations.

More quantitatively, the effect of additives can be analyzed from the chemical potential change of the protein induced by the additives. The change in protein solubility is related to the chemical potentials of the protein in water, $\mu_{2,w}$, and in the additive solution, μ_2, since (Arakawa & Timasheff, 1985)

$$\Delta\mu_2 = \mu_2 - \mu_{2,w} = -\ln (S/S_w) \quad (5)$$

where the subscript w refers to water. This is based on the usual assumption that the chemical potential of the protein in the solid phase is independent of the presence of additive. The value of $\Delta\mu_2$ can be calculated from $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ by

$$\Delta\mu_2 = \int_0 (\partial\mu_2/\partial m_3)_{T,P,m_2} m_3 \quad (6)$$

This can be obtained from the area under the plot of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ vs. m_3. As seen in Figure 3, it is evident that $\Delta\mu_2$ is positive for BSA and lysozyme and hence $S_w > S$, indicating decreased protein solubility in the presence of glycine. On the other hand, the plots for β-LG appear to extrapolate to negative values as the glycine and NaCl concentrations decrease. This leads to negative $\Delta\mu_2$ at the lower concentrations of additive. As a result, $S > S_w$, and the protein solubility should be increased by the addition of glycine and NaCl, explaining the original observation of Cohn and Ferry (1943).

Registry No. NaCl, 7647-14-5; glycine, 56-40-6; lysozyme, 9001-63-2.

[3] The apparent increased value of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, manifested through a larger difference $(\phi_2^0 - \phi_2'^0)$ when 0.05 M NaCl is added to the glycine solutions, can be understood in terms of the interactions of NaCl with β-LG in competition with glycine. For a four-component system the difference $(\phi_2^0 - \phi_2'^0)$ expresses interactions of all components, as (Casassa & Eisenberg, 1964; Nieuwenhuysen, 1979)

$$\rho_0(\phi_2^0 - \phi_2'^0) = (\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}(1 - \rho\bar{v}_3) + (A_4' - g_4 A_1')(1 - \rho_0\bar{v}_4)$$

where component 3 = glycine and component 4 = NaCl. Thus, if we set $A_4 \approx 0$, the second terms on the right of the equation becomes negligible with $g_4 \approx 0$, and $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ assumes a more negative value, suggesting a decrease in glycine binding in the presence of NaCl. This could reflect either screening of charges on the protein by the NaCl or competition with glycine for the same loci on the protein.

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Trypsinogen–Trypsin Transition: A Molecular Dynamics Study of Induced Conformational Change in the Activation Domain†

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Received July 1, 1986; Revised Manuscript Received May 8, 1987

ABSTRACT: The trypsinogen to trypsin transition has been investigated by a stochastic boundary molecular dynamics simulation that included a major portion of the trypsin molecule and the surrounding solvent. Attention focused on the "activation domain", which crystallographic studies have shown to be ordered in trypsin and disordered in its zymogen, trypsinogen. The chain segments that form the activation domain were found to exhibit large fluctuations during the simulation of trypsin. To model a difference between trypsin and trypsinogen, the N-terminal residues Ile-16 and Val-17 were removed in the former and replaced by water molecules. As a result of the perturbation, a structural drift of 1–2 Å occurred that is limited to the activation domain. Glycine residues are found to act as hinges for the displaced chain segments.

Trypsin is an enzyme for which the functional significance of flexibility has been established. It is known that activation of the zymogen, trypsinogen, by removal of the N-terminal hexapeptide is directly coupled to a flexible to rigid transition in a relatively localized region of the trypsin molecule (Huber & Bennett, 1983; Bennett & Huber, 1985). X-ray structures of trypsin, trypsinogen, and a number of inhibitor-bound complexes solved at high resolution (Huber & Bode, 1978; Kossiakoff et al., 1977) provide information concerning the transition. Also, equilibrium and kinetic data are available (Keil, 1971; Bode & Huber, 1976). However, the detailed dynamics of the transition, including the essential interactions

† This work was supported by the National Science Foundation (A.T.B. and M.K.) and the Max-Planck-Institut für Biochemie (A.T.B. and R.H.). The CRAY time was supplied by a grant from the Office of Advanced Scientific Computing of the National Science Foundation.
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0006-2960/87/0426-5153$01.50/0