The Interaction of β-Lactoglobulin with Solvent Components in Mixed Water–Organic Solvent Systems[1]

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Abstract: A light-scattering study has been carried out on solutions of β-lactoglobulin A in mixtures of water with methanol or 2-chloroethanol in 0.02 *M* NaCl and 0.01 *M* HCl. It has been found that the protein remains in monomeric form in both solvents. Preferential binding of solvent components to β-lactoglobulin has been calculated from data obtained at a constant concentration of 2-chloroethanol. Preferential interaction of the protein with chloroethanol changes gradually to preferential hydration with an increase in concentration of the alcohol. Simultaneously, it has been shown by optical rotatory dispersion and circular dichroism that a progressive increase in solvent composition from aqueous to 2-chloroethanol induces a gradual change from the native globular structure to one rich in α helix.

It has become evident recently that the solution structure of proteins is, to a great extent, a function of the structure of the solvent and, thus, of the interactions between the protein and the solvent. Optical rotatory dispersion studies on β-lactoglobulin have shown[4–6] that when the solvent composition is changed progressively from aqueous to nonaqueous (*e.g.*, water–methanol mixtures) a conformational change occurs frequently from the native globular structure to one much richer in α-helical content. Such changes in protein conformation may be accompanied by changes in the state of aggregation of the protein, as well as in the degree of interaction of the protein with solvent components. Light-scattering measurements and the proper application of multicomponent theory make possible the determination both of the degree of molecular association and of the extent of interaction between the macromolecule and solvent components, if these have nonidentical refractive indices. On the other hand, if the two solvent components have practically identical refractive indices, the interpretation of light-scattering measurements is reduced to the treatment of a two-component system. The two cases can be exemplified by water–2-chloroethanol and water–methanol mixtures, respectively.

In this paper, we describe a light-scattering study on the solution behavior of β-lactoglobulin A in mixtures of water with 2-chloroethanol together with optical rotatory dispersion and circular dichroism data, and compare the protein–solvent interactions obtained from the light-scattering study with the conformational changes in β-lactoglobulin A (β-Lg A). In addition, the state of aggregation of β-Lg A in aqueous methanol solutions is investigated by light-scattering measurements.

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(3) Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(4) C. Tanford, P. K. De, and V. G. Taggart, *J. Am. Chem. Soc.*, **82**, 6028 (1960).

(5) C. Tanford and P. K. De, *J. Biol. Chem.*, **236**, 1711 (1961).

(6) S. N. Timasheff, R. Townend, and L. Mescanti, *ibid.*, **241**, 1863 (1966).

Theory

Since the original theoretical treatment of light scattering in multicomponent systems, given by Zernike,[7] a number of studies[8–20] have developed extensively the theory of multicomponent systems. The practical equations for use with a three-component system (where the principal solvent is component 1, the additional solvent or electrolyte is component 3, and the macromolecule is component 2)[21] have been developed[23] in the Scatchard–Stockmayer notation; with the use of these, it is possible to characterize the thermodynamic parameters of the protein–solvent interaction, as well as to obtain the molecular weight of the protein. Two types of measurement are necessary.[11–18,23,24] In the first type, the light-scattering and the refractive index increments are measured on protein solutions in which the molality of component 3 is kept identical with that of the reference solvent. The excess turbidity of the solution over the solvent, $\Delta\tau$, is described by eq 1.

In eq 1 n is the refractive index of the solution, N is Avogadro's number, λ is the wavelength of the

(7) F. Zernike, *Arch. Neerland, Sci.* (IIIA), **4**, 74 (1918).

(8) H. C. Brinkman and J. J. Hermans, *J. Chem. Phys.*, **17**, 574 1949).

(9) J. G. Kirkwood and R. J. Goldberg, *ibid.*, **18**, 54 (1950).

(10) W. H. Stockmayer, *ibid.*, **18**, 58 (1950).

(11) H. Shogenji, *Busseiron Kenkyu*, **62**, 1 (1953).

(12) T. Ooi, *J. Polymer Sci.*, **28**, 459 (1958).

(13) A. Vrij, Doctoral Dissertation, University of Utrecht, 1959.

(14) A. Vrij and J. Th. G. Overbeek, *J. Colloid Sci.*, **17**, 570 (1962).

(15) D. Stigter, *J. Phys. Chem.*, **64**, 842 (1960).

(16) D. Stigter in "Electromagnetic Scattering," M. Kerker, Ed., Pergamon Press Inc., New York, N. Y., 1963, p 303.

(17) E. F. Casassa and H. Eisenberg, *J. Phys. Chem.*, **64**, 753 (1960).

(18) E. F. Casassa and H. Eisenberg, *Advan. Protein Chem.*, **19**, 287 (1964).

(19) C. Strazielle and H. Benoit, *J. Chim. Phys.*, **58**, 678 (1961).

(20) S. N. Timasheff and M. J. Kronman, *Arch. Biochem. Biophys.*, **83**, 60 (1959).

(21) This notation, adopted by Scatchard[22] and Stockmayer,[10] appears to be the one most generally used at present. Kirkwood and Goldberg[9] call the principal solvent component 0, and the added solvent component 1; Vrij and Overbeek[14] call the principal solvent component 0, the macromolecular component 1, and the added solvent component 2.

(22) G. Scatchard, *J. Am. Chem. Soc.*, **68**, 2315 (1946).

(23) H. Inoue and S. N. Timasheff, Advances in Chemistry Series, American Chemical Society, Washington, D. C., in press.

(24) M. Noelken and S. N. Timasheff, *J. Biol. Chem.*, **242**, 5080 (1967).

$$H'\left[\frac{\partial n}{\partial C_2}\right]^2_{m_3}\frac{C_2}{\Delta\tau} = \frac{1}{(1+D)^2}\left[\frac{1}{M_2} + 2B^0C_2\right] \quad (1)$$

$$H' = \frac{32\pi^3n^2}{3N\lambda^4} \quad (1a)$$

$$D = \frac{(\partial n/\partial m_3)_{m_2}}{(\partial n/\partial m_2)_{m_3}}\left[\frac{\partial m_3}{\partial m_2}\right]_{T,p,\mu_3} \quad (1b)$$

$$= \frac{(1 - C_2\bar{V}_3)(\partial n/\partial C_3)_{m_2}}{(1 - C_2\bar{V}_2)(\partial n/\partial C_2)_{m_3}}\frac{M_3}{M_2}\left[\frac{\partial m_3}{\partial m_2}\right]_{T,p,\mu_3} \quad (1c)[25]$$

$$B^0 = \frac{1}{M_2^2}\left[\frac{V}{2}\left\{\beta_{22} - \frac{\beta_{23}^2}{(\Sigma\nu_3/m_3) + \beta_{33}}\right\} + \bar{V}_2M_2\right] \quad (1d)$$

$$\left[\frac{\partial m_3}{\partial m_2}\right]_{T,p,\mu_3} = -\frac{\beta_{32}}{(\Sigma\nu_3/m_3) + \beta_{33}} \quad (1e)$$

$$\beta_{ij} = \frac{1}{RT}\left[\frac{\partial\mu_i^{(e)}}{\partial m_j}\right]_{T,p,m} \quad (1f)$$

light *in vacuo*, $\bar{V}_i$ is the partial specific volume of component i, C_i is the concentration of component i in grams per milliliter, m_i is the molality of component i (moles/1000 g of principal solvent), M_i is the molecular weight, p is the pressure, μ is the chemical potential, V is the volume of solution (in milliliters) containing 1000 g of principal solvent, R is the gas constant, T is the thermodynamic temperature, and $\mu_i^{(e)}$ is the excess chemical potential of component i when the chemical potential is $\mu_i = RT\Sigma\nu_i \ln m_i + \mu_i^{(e)} + \mu_i^0(T,p)$ and $\Sigma\nu_i$ is the number of particles into which component i dissociates.

In the second type of experiment, the light-scattering and the refractive index increments are measured on solutions which had been brought to a state of osmotic equilibrium with solvent;[26] the light-scattering equation then reduces to a simple pseudo-two-component form[12,14,15,17,23]

$$H'\left[\frac{\partial n}{\partial C_2}\right]_{T,p,\mu_3}\frac{C_2}{\Delta\tau} = \frac{1}{M_2} + 2B'C_2 \quad (2)$$

$$B' = B^0 + \frac{M_3}{M_2^2}\bar{V}_3\left[\frac{\partial m_3}{\partial m_2}\right]_{T,p,\mu_3} \quad (2a)$$

Thus, it is possible to determine the molecular weight of the protein, M_2, and the degree of molecular association, if any, from experiments at a constant chemical potential of component 3. With a knowledge of M_2 (if two solvent components have nonidentical refractive indices), the degree of preferential interaction of the protein with the third component, $(\partial m_3/\partial m_2)_{T,p,\mu_3} \approx (\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$,[26] can be obtained from experiments at a constant concentration of component 3 by using eq 1 and 1c. The nonideality term, β_{ij}, can be calculated, then, from eq 1d and 1e, making the assumption that β_{33} is zero as a first approximation if its value is not known from auxiliary measurements.

(25) On extrapolation of the concentration C_2 to zero, the term $(1 - C_2\bar{V}_2)$ reduces to 1.

(26) The rigorously correct procedure requires that the light-scattering and differential refractometry measurements be carried out under a hydrostatic pressure equal to the osmotic pressure of the solution.[17] Normally these measurements are performed after establishment of dialysis equilibrium, giving $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ rather than $(\partial m_3/\partial m_2)_{T,p,\mu_3}$; it has been shown by Stigter,[15] however, that the resulting error is negligibly small.

Experimental Section

Material. β-Lactoglobulin A was prepared from the milk of previously typed homozygous cows by standard techniques[27] and recrystallized before use. The solvents used in the light-scattering measurements were doubly distilled through all-Pyrex stills just before use.

Light Scattering. Light-scattering measurements were carried out on the Brice photometer[28] at 25° using 2-mm slit optics and the 436-mμ mercury line. Stock solutions (*ca.* 3 g/100 ml) of β-Lg A were made up in 0.02 *M* NaCl and 0.01 *M* HCl aqueous solvent, and cleared for light scattering by centrifuging in a Spinco Model L centrifuge[29] at 40,000 rpm for 30 min, followed by filtration through an ultrafine sintered-glass filter of special design.[30,31] In experiments without dialysis, extreme caution was taken to keep constant the molality of the third component, m_3.[32] The volume ratio necessary to prepare a water–chloroethanol mixture (or a water–methanol mixture) of a given m_3 was determined in a preliminary experiment. A blank light-scattering measurement was first carried out in each Dintzis-type cell[33] filled with a solvent mixture of a given m_3. The working solutions were then made up by adding increments of the stock protein solution and the proper amount of chloroethanol (or methanol) by volume to the solvent blank, using ultramicro burets. The amount of alcohol which must be added to maintain the same value of m_3 in the solution was calculated from the predetermined volume ratio. Mixing was accomplished by gentle inversion and rocking of the Teflon-stoppered cell. This procedure was repeated until the β-Lg A concentration had reached about 6 g/l. Concentrations were measured on the stock solutions and the cell contents were weighed at the beginning and end of each series of measurements to check for evaporation or leakage. The solvents used were also filtered through an ultrafine sintered-glass filter. In the case of refractive index increment measurements, a β-Lg A solution (*ca.* 5 g/l.) at a given m_3 was gradually diluted with solvent of the same m_3, each dilution being used for a measurement on the differential refractometer. This procedure was repeated until the protein concentration had reached about 0.5 g/l.

In the experiments with dialysis, the several solutions of β-Lg A at different concentrations (*ca.* 5 ml each) were prepared in a given water–chloroethanol mixture, dialyzed overnight against a large excess of the same solvent, and passed through the sintered-glass filter after centrifugation. The light-scattering measurements were carried out in a Dintzis-type cell on these solutions, using the dialysate as a blank. The refractive index increments were measured on the same solutions following the light-scattering measurements.

Specific refractive index increments were measured in the Brice differential refractometer[34] (25.0°) at 436 mμ. Absolute refractive indices of water–2-chloroethanol mixtures were measured at the

(27) R. Aschaffenburg and J. Drewry, *Biochem. J.*, **65**, 273 (1957).

(28) B. A. Brice, M. Halwer, and R. Speiser, *J. Opt. Soc. Am.*, **40** 768 (1950).

(29) Mention of specific manufacturers does not imply endorsement by the U. S. Department of Agriculture over others not mentioned.

(30) F. F. Nord, M. Bier, and S. N. Timasheff, *J. Am. Chem. Soc.*, **73**, 289 (1951).

(31) M. Bier in "Methods in Enzymology," Vol. 4, S. P. Colowick and N. O. Kaplan, Ed., Academic Press Inc., New York, N. Y., 1957, p 165.

(32) An alternate way of carrying out such experiments is to keep the volume concentration (molarity) of the third component constant.[14] In such a case, eq 1 becomes[24]

$$H'\left[\frac{\partial n}{\partial C_2}\right]^2_{T,p,C_3}\frac{C_2}{\Delta\tau} = \frac{1}{(1+D)^2M_2}\left\{1 + \left[\left[\frac{\partial\mu_2^{(e)}}{\partial C_2}\right]_{T,p,C_3} - \frac{M_2(\partial\mu_3/\partial C_2)^2_{T,p,C_3}}{M_3(\partial\mu_3/\partial C_3)_{T,p,C_2}}\right]\frac{C_2}{RT}\right\}$$

$$D = \frac{(\partial n/\partial C_3)_{T,p,C_2}}{(\partial n/\partial C_2)_{T,p,C_3}}\left[\frac{\partial C_3}{\partial C_2}\right]_{T,\mu_1,\mu_3} \quad (1')$$

The preferential binding on a molal basis, $(\partial g_3/\partial g_2)^0_{T,\mu_1,\mu_3}$, is then[24]

$$\left[\frac{\partial g_3}{\partial g_2}\right]^0_{T,\mu_1,\mu_3} = \frac{M_3}{M_2}\left[\frac{\partial m_3}{\partial m_2}\right]^0_{T,\mu_1,\mu_3} = \frac{g_3}{\bar{V}_3C_3}\left[\left[\frac{\partial C_3}{\partial C_2}\right]^0_{T,\mu_1,\mu_3} + C_2\bar{V}_2^\circ\right]$$

It is important to note that preferential binding expressed in molal (weight) and molar (volume) concentration units may differ, not only in magnitude, but even in sign.[18,24] The superscript 0 refers to extrapolation to zero of the concentration of the macromolecular component.

(33) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood, and B. D. Coleman, *J. Am. Chem. Soc.*, **79**, 782 (1957).

(34) B. A. Brice and M. Halwer, *J. Opt. Soc. Am.*, **41**, 1033 (1951).

same temperature and wavelength at several concentrations using an Abbe refractometer. The values of $(\partial n/\partial C_3)m_2$ were obtained from slopes of the plots of the refractive indices against the concentration, C_3.

Concentrations were measured with a Zeiss Model PMQ II spectrophotometer at 278 mμ, using an absorptivity value of 0.96 l./cm g for β-Lg A.[35]

Optical Rotatory Dispersion. Stock solutions of β-Lg A were prepared by the same procedure as used in the light-scattering measurements. The working solutions were then made up by dilution with a mixed water–2-chloroethanol solvent containing 0.02 *M* NaCl and 0.01 *M* HCl. Sodium chloride was not included in the 100% chloroethanol solution.[36] All the solutions were used as soon as possible after preparation. The optical rotatory dispersion (ORD) measurements were made on a Durrum-Jasco ORD/uv 5 apparatus. The approximate concentrations and the cells used were as follows: 0.20 g/100 ml in a 5.0-cm light-path cell between 600 and 300 mμ, 0.20 g/100 ml in a 1.0-mm cell between 350 and 250 mμ, 0.20 g/100 ml in a 0.11-mm cell between 270 and 200 mμ, and 0.020 g/100 ml in a 0.11-mm cell between 210 and 185 mμ. With an increase in 2-chloroethanol contents, the limit of measurement was at a higher wavelength, because of the strong uv absorption of this solvent. In the case of 100% chloroethanol, ORD spectra could be obtained only down to 200 mμ.

The molar residue rotations $[m']_\lambda$ were calculated by

$$[m']_\lambda = [\alpha]_\lambda \frac{M_0}{100} \frac{3}{n^2 + 2} \tag{3}$$

where $[\alpha]_\lambda$ is the specific rotation, M_0 is the mean residue weight which is taken to be 112 for β-lactoglobulin,[38] and n is the refractive index at a given wavelength λ, obtained by interpolation and extrapolation from the data of Foss and Schellman.[39] Using the data of $[m']_\lambda$ above 320 mμ, the rotatory dispersion parameters, a_0 and b_0, of the Moffitt–Yang equation[40] were calculated from

$$[m']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \tag{4}$$

where λ_0 is taken as 212 mμ.[40]

Circular Dichroism. The circular dichroism (CD) spectra were measured from 270 to 185 mμ (where possible) with the same apparatus, solutions, and cells as used in ORD measurements.

The molar ellipticity $[\theta]$ (deg cm²/dmol) was calculated by

$$[\theta] = \frac{3300}{dc_2}(S \times 10^{-2})R \tag{5}$$

where d is the cell thickness in centimeters, c_2 is the protein concentration in moles of residues per liter, S is the instrument setting (0.005 or 0.002° for 10 cm per deflection), and R is the reading on the chart in millimeters.

Results

Light Scattering. The first organic solvent used was methanol. Since its refractive index is very close to that of water, the term D in eq 1 vanishes and the molecular weight of the protein is obtained even in a mixed solvent by the conventional light-scattering method without dialysis. Experiments on β-Lg A were carried out varying the methanol contents from 0 to 80% by volume; all mixed solvents contained 0.02 *M* NaCl and 0.01 *M* HCl. The results are summarized in Table I, and indicate that below 80% methanol, the protein exists in monomeric form.

(35) R. Townend, R. J. Winterbottom, and S. N. Timasheff, *J. Am. Chem. Soc.*, **82**, 3161 (1960).

(36) In this case, the concentration of β-Lg A was measured by micro-Kjeldahl nitrogen analysis, using the value of 15.46% for the nitrogen content.[37]

(37) B. D. Polis, H. W. Schmukler, J. H. Custer, and T. L. McMeekin, *J. Am. Chem. Soc.*, **72**, 4965 (1950).

(38) T. T. Herskovits, R. Townend, and S. N. Timasheff, *J. Am. Chem. Soc.*, **86**, 4445 (1964).

(39) J. G. Foss and J. A. Schellman, *J. Chem. Eng. Data*, **9**, 551 (1964).

(40) W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci. U. S.*, **42**, 596 (1956).

Table I. Light-Scattering Results of β-Lactoglobulin A in Water–Methanol at Constant Concentration of Methanol

Methanol, vol %	$M_2 \times 10^{-4}$	$B^0 \times 10^4$ ml/g
0	1.85	16.9
20	1.86	16.2
30	1.82	16.7
40	1.91	18.5
50	1.81	−7.0
60	1.79	−6.5
80	2.55	−6.0

2-Chloroethanol is also known to be a structure-forming denaturant for proteins.[4,41,42] Its refractive index at 436 mμ, 1.447, is quite different from that of water, 1.340, giving a large value of $(\partial n/\partial C_3)$. When this solvent is used as the third component, it becomes possible to measure both the degree of association and the preferential interactions of protein with solvent components. First, light-scattering measurements were carried out on β-Lg A solutions which had been dialyzed just before the measurement; this made it possible to obtain the molecular weight of β-Lg A in different water–2-chloroethanol mixtures. The 2-chloroethanol contents were varied between 5 and 60%. When the protein concentration was greater than 2 g/l., the scattered intensity gradually increased, showing aggregation. Below this concentration, the scattered intensity remained essentially unchanged over a 24-hr period. The time dependence did not occur until a protein concentration of 4 g/l. when the concentration of 2-chloroethanol was less than 20% by volume. Plots of $H'(\partial n/\partial C_2)^2{}_{T,\mu_1,\mu_3}C_2/\Delta\tau$ against C_2 in this system are shown in Figure 1 and the resulting data, such as molecular weight and virial coefficient, are listed in Table II; the average molecular weight is found to be 18,700 ± 100. It is well known[43] that at pH's below 3.5 β-lactoglobulin A molecules dissociate to a monomer with a molecular weight of 18,000. Considering that light-scattering measurements on dialyzed solutions are more difficult and, thus, involve a larger experimental error than those on solutions prepared without dialysis, these results indicate that the β-Lg A dissociates completely to a monomer in water–2-chloroethanol mixtures in the presence of 0.02 *M* NaCl and 0.01 *M* HCl. At elevated concentrations of 2-chloroethanol, the error in the light-scattering points obtained with dialyzed solutions was considerably greater than that with similar solutions without dialysis. The least-squares plots through these points, however, extrapolated to the known molecular weight of the monomer (see Figure 1 and Table II), indicating that neither aggregation nor degradation had occurred. The sole purpose of the measurements with dialysis was to detect such potential changes. Thus, the poorer precision of the data after dialysis should not have any significant effect on the values of the preferential binding, since the latter quantity is obtained solely from light-scattering experiments without dialysis (see below).

Light-scattering measurements were also carried out on β-Lg A solutions without dialysis in the same mixtures; the concentration of 2-chloroethanol was varied

(41) P. Doty, *Rev. Mod. Phys.*, **31**, 107 (1959).

(42) P. Callaghan and N. H. Martin, *Biochem. J.*, **83**, 144 (1962).

(43) R. Townend, L. Weinberger, and S. N. Timasheff. *J. Am Chem Soc.*, **82**, 3175 (1960).

Table II. Molecular Parameters of β-Lactoglobulin A in Water–2-Chloroethanol Mixtures Obtained from Light-Scattering Measurements at Constant Chemical Potential and at Constant Concentration of Chloroethanol

Chloroethanol, vol %	At const chem potential $M_2 \times 10^{-4}$	$B^\circ \times 10^3$ ml/g	At constant concentration $I^b \times 10^5$	$B^0 \times 10^3$ ml/g	β_{23}	$\beta_{22} \times 10^{-3}$	$(\partial m_3/\partial m_2)_{\mu_1,\mu_3}$, mol/mol	$(\partial g_3/\partial g_2)_{\mu_1,\mu_3}$, g/g
0	1.85[a]	1.69	5.40	1.69		1.15		
5	1.82	1.92	4.75	1.46	−33.8	1.90	26.5	0.114
10	1.90	1.47	4.55	1.95	−22.7	2.05	37.5	0.161
20	1.88	1.80	3.57	2.67	−29.4	4.67	108.2	0.466
30	1.85	1.53	3.18	2.14	−26.1	5.33	164.0	0.706
40	1.88	1.33	3.35	1.64	−17.1	3.44	165.9	0.714
50	1.88	−0.59	3.71	1.67	−10.2	2.09	148.0	0.637
60	1.90	−1.88	4.72	1.51	−2.6	0.57	57.3	0.247
80			6.30	0.78	2.5	0.47	−145.0	−0.624
							(139.7)[c]	(0.135)[c]

[a] The solutions at 0% chloroethanol were made without dialysis because of absence of chloroethanol. [b] I is the intercept at $C_2 = 0$ of $H' \cdot (\partial n/\partial C_2)_{m_3}{}^2 C_2/\Delta\tau$ *vs.* C_2 plot. [c] The values in parentheses correspond to $(\partial m_1/\partial m_2)_{\mu_1,\mu_3}$ and $(\partial g_1/\partial g_2)_{\mu_1,\mu_3}$, respectively.

from 0 to 80% by volume. In this series, the light-scattering experiments were completed within 1 day for a given chloroethanol concentration, during which time there was no time dependence of the scattering. Even in the case of 80% chloroethanol, the intensity remained constant at protein concentrations less than 2 g/l. Plots of $H'(\partial n/\partial C_2)^2{}_{m_3}C_2/\Delta\tau$ *vs.* C_2 for this series are illustrated in Figure 2. Since the molecular weight of component 2 had been determined already in the light-scattering experiments carried out in identical solvent mixtures at constant chemical potential, the values of B^0 and D in eq 1 were obtained

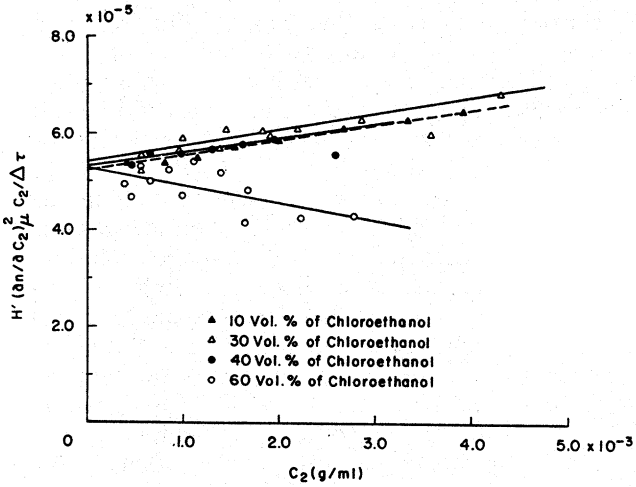

Figure 1. Light-scattering data of β-lactoglobulin A in various concentrations of 2-chloroethanol at constant chemical potential. The solvent components are water, 2-chloroethanol, 0.02 *M* NaCl, and 0.01 *M* HCl.

from the slopes and the intercepts at $C_2 = 0$ of the plots of Figure 2. From the deviations of the intercepts from the true molecular weight, the preferential solvation and thermodynamic interaction parameters were calculated using eq 1c, 1d, and 1e, assuming that β_{33} is zero as a first approximation. The partial specific volumes of 2-chloroethanol in aqueous solutions used in eq 1c were obtained from density measurements and a graphical method of intercepts;[44] these values are listed in Table III. (For the partial specific volume of

(44) G. N. Lewis and M. Randall, "Thermodynamics and the Free Energy of Chemical Substances," McGraw-Hill Book Co., Inc., New York, N. Y., 1923, p 38.

Table III. Density and Partial Specific Volume of Components in Water–2-Chloroethanol Mixtures at 25°

Chloroethanol, vol %	Density, g/ml	$\bar{V}_1$[a]	$\bar{V}_3$[a]
0	0.9971		
5	1.0098	1.0025	0.7934
10	1.0226	1.0018	0.7965
20	1.0476	1.0003	0.8001
30	1.0708	0.9950	0.8125
40	1.0915	0.9898	0.8220
50	1.1106	0.9853	0.8271
60	1.1296	0.9813	0.8297
80	1.1652	0.9710	0.8340
100	1.1982		

[a] Subscripts 1 and 3 correspond to water and 2-chloroethanol, respectively.

β-Lg A, that in aqueous solution, 0.751,[45] was used.) The preferential solvation data are given in Table II, where the results are expressed both as $(\partial m_3/\partial m_2)_{\mu_1,\mu_3}$, *i.e.*, the number of moles of component 3 bound to a

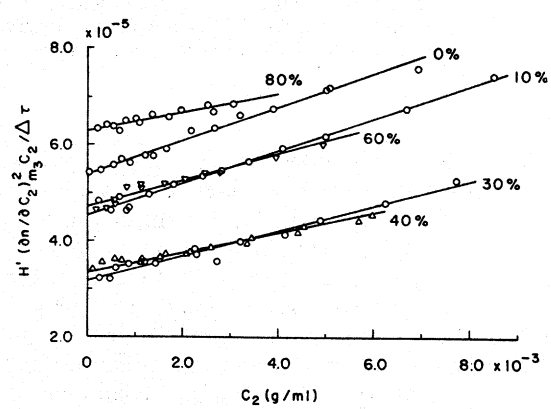

Figure 2. Light-scattering data of β-lactoglobulin A in various concentrations of 2-chloroethanol at constant concentration of the latter. The solvent components are water, 2-chloroethanol, 0.02 *M* NaCl, and 0.01 *M* HCl.

mole of component 2, and as $(\partial g_3/\partial g_2)_{\mu_1,\mu_3}$, *i.e.*, the number of grams of component 3 bound per gram of component 2, where g_i is the concentration of component i in grams per gram of component 1. A negative value of $(\partial m_3/\partial m_2)_{\mu_2,\mu_3}$ indicates a deficiency of component 3 in the immediate vicinity of molecule 2,

(45) K. O. Pedersen, *Biochem. J.*, **30**, 961 (1936).

Table IV. Optical Rotatory Dispersion and Circular Dichroism Parameters of β-Lactoglobulin A in Water–2-Chloroethanol, 0.02 *M* NaCl, 0.01 *M* HCl

Chloroethanol, vol %	$-[m']_{233.5} \times 10^{-3}$	$+[m']_{199} \times 10^{-3}$	$-a_0$	$-b_0$	$-[\theta]_{221} \times 10^{-3}$	$-[\theta]_{208} \times 10^{-}$
0	1.7 (237)[a]	10.9 (202)[a]	159	66	6.0 (217)[a]	
10	1.7 (237)[a]	10.8	165	65	7.5 (217)[a]	
20	6.1	28.7	259	226	16.9	19.3
30	7.1	32.5	191	288	19.6	21.9
40	7.3	35.6	135	314	22.1	23.2
60	7.5	39.9	112	333	22.7	23.4
80	8.0	47.9	46	362	25.8	26.9
100[b]	8.6	49.6	11	400	26.9	26.9

[a] The numbers in parentheses refer to the actual wavelength at which the maximum or minimum is located and at which the reported rotation was measured. [b] No sodium chloride is included.

i.e., preferential hydration of component 2; the extent of hydration is given by eq[46] 6.

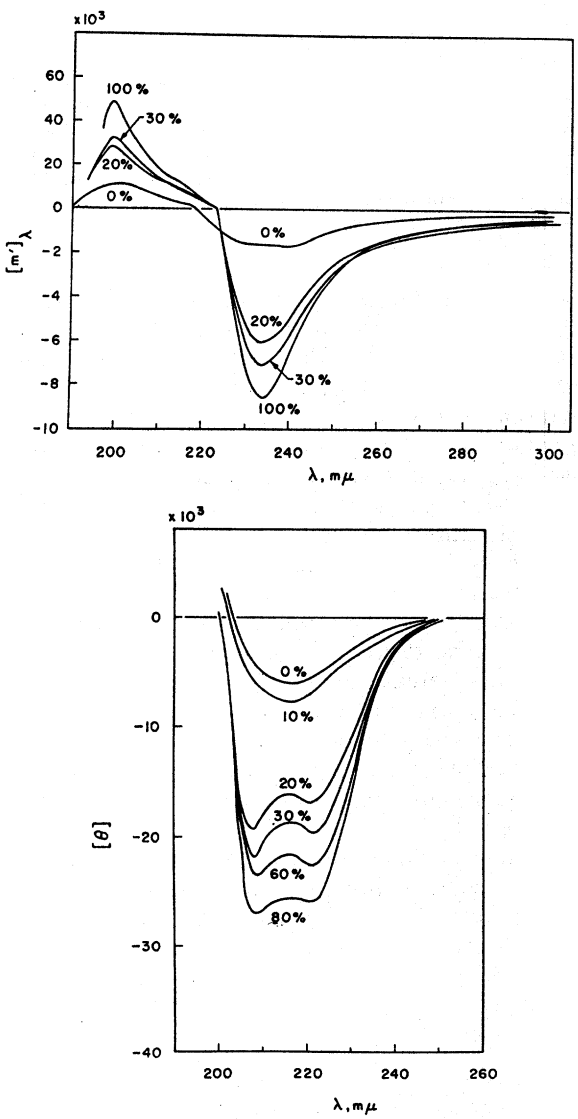

Figure 3. (A, top) Effect of variation of 2-chloroethanol concentration on the optical rotatory dispersion of β-lactoglobulin A, and (B, bottom) on the circular dichroism of β-lactoglobulin A.

ORD and CD. Since aggregation of macromolecules has an effect on their ORD and CD curves,[47–49] extreme care should be taken in interpreting these data in terms of configuration. Our measurements of ORD and CD were carried out in the concentration range where it is confirmed by light-scattering measurements that β-Lg

$$\left[\frac{\partial m_1}{\partial m_2}\right]_{\mu_1,\mu_3} = -\frac{m_1}{m_3}\left[\frac{\partial m_2}{\partial m_3}\right]_{\mu_1,\mu_3} \tag{6}$$

A does not aggregate substantially. The Moffitt–Yang parameters, a_0 and b_0, were calculated using eq 4 from the ORD data between 320 and 600 mμ. In the cases of 0 and 10% chloroethanol, mean residue rotations ([*m'*]) were used only above 350 mμ, because of the presence of aromatic Cotton effects in the region between 280 and 300 mμ for these concentrations.[6,50] The values of a_0 and b_0 obtained are given in Table IV. The effects of increasing the amount of 2-chloroethanol (0.02 *M* NaCl and 0.01 *M* HCl) on the ORD of β-Lg A below 270 mμ are shown in Figure 3a. The 0 and 10% chloroethanol curves are identical for all practical purposes, except for the displacement of the 202-mμ peak to 199 mμ in 10% chloroethanol. The results of the CD measurements are shown in Figure 3b. Addition of 10% chloroethanol also has little effect on the circular dichroism curve. The trough at 217 mμ increases slightly from −6000 to −7500. Although a positive peak was observed around 195 mμ, we will not consider this positive spectrum because the absorption of 2-chloroethanol makes it quite difficult to get a circular dichroism curve much below 198 mμ.

As the concentration of chloroethanol is increased from 10 to 20%, a drastic change is observed in the ORD curve.[4] This change corresponds to one which takes place as the methanol concentration is increased from 30 to 40%.[6] The broad trough is sharply deepened to an [*m'*] of −6100 at 233.5 mμ, and the positive peak at 199 mμ increases about three times in height ([*m'*]) = +28,700). At the same time, the broad negative CD band splits into two, with increases in intensity to −16,900 at 221 mμ and −19,300 at 208 mμ, as shown in Figure 3b. The changes both in ORD and CD suggest that addition of 2-chloroethanol increases greatly the content of a right-handed α-helix in β-Lg A in the region between 10 and 20% chloroethanol. Further increase in chloroethanol concentration does not result in any additional changes in the general shape of the ORD and CD curves, but the optical rotations at 233.5

(46) S. M. Timasheff in "Electromagnetic Scattering," M. Kerker, Ed., Pergamon Press Inc., New York, N. Y., 1963, p 337.
(47) T. M. Schuster, *Biopolymers*, 3, 681 (1965).
(48) Y. Tomimatsu, L. Vitello, and W. Gaffield, *ibid.*, 4, 653 (1966).
(49) J. Y. Cassim and J. T. Yang, *Biochem. Biophys. Res. Commun.*, 26, 58 (1967).
(50) R. Townend, T. F. Kumosinski, and S. N. Timasheff. *J. Biol. Chem.*, 242, 4538 (1967).

and 199 mμ and the ellipticities at 221 and 208 mμ increase progressively. The exact values are summarized in Table IV.

Discussion

Aggregation. It has been shown by light-scattering measurements that β-Lg A undergoes a monomer–dimer equilibrium reaction in 0.09 *M* NaCl–0.01 *M* HCl aqueous solution at 25°, and the light-scattering intensities do not change appreciably during the experiments.[43] Addition of methanol, while maintaining the concentrations of NaCl and HCl at 0.09 *M* and 0.01 *M*, respectively, resulted in progressive time-dependent increases of the scattered intensities when the methanol concentration had reached 30%, showing a time-dependent aggregation. On the contrary, removal of sodium chloride resulted in no change in the light scattered by β-Lg A at 30% methanol and 0.01 *M* HCl up to a β-Lg A concentration of 10 g/l., even after standing at room temperature for 6 days. The plot of $H'(\partial n/\partial C_2)^2_{m_3}C_2/\Delta\tau$ against C_2, however, is concave upward at higher concentrations of C_2, reflecting the increasing charge on the β-Lg A molecules and the resulting electrostatic repulsion, in the absence of screening. This makes it difficult to derive thermodynamic parameters from the light-scattering measurements under these essentially salt-free conditions.

The following conditions were chosen: NaCl 0.02 *M*, HCl 0.01 *M*. At 30 vol % methanol, the light-scattering intensity of β-Lg A (up to 7 g/l.) does not change in 1 day, indicating absence of aggregation. Higher methanol concentrations induce a time-dependent aggregation at concentrations above 2 g/l. Below this concentration of β-Lg A, however, the scattered intensity is almost invariable. Considering the experimental error of the light-scattering measurements, addition of methanol up to 60% by volume has no effect on the molecular weight of β-Lg A. This is shown in the second column of Table I. The average molecular weight of β-Lg A between 0 and 60% methanol is 18,400, indicating that β-Lg A dissociates to the monomer state under these conditions. At 80% methanol, however, the molecular weight of 25,500 suggests that some subsequent aggregation occurs. The second virial coefficient does not change appreciably below 40% methanol, while it drops to a negative value above this concentration.

In the case of water–2-chloroethanol mixtures, containing 0.02 *M* NaCl and 0.01 *M* HCl, no aggregation at all occurs below 2 g/l. of protein for at least 24 hr, irrespective of dialysis.

Solvation. While the intercept of $H'(\partial n/\partial C_2)^2_{\mu_1,\mu_3}\cdot C_2/\Delta\tau$ *vs.* C_2 plots at $C_2 = 0$ corresponds to the reciprocal of the molecular weight in the case of measurements at constant chemical potential of the third component, the reciprocal of such an intercept, in the case of measurements at a constant *concentration* of the third component, does not correspond to the true molecular weight but to the apparent molecular weight, *i.e.*, the molecular weight multiplied by $(1 + D)^2$ if the refractive indices of the two components of the solvent mixture are different from each other. Such is the case where water and 2-chloroethanol make up the mixed solvent. The ratio of the apparent molecular weight, M_{app}, to the true molecular weight, M_w, is

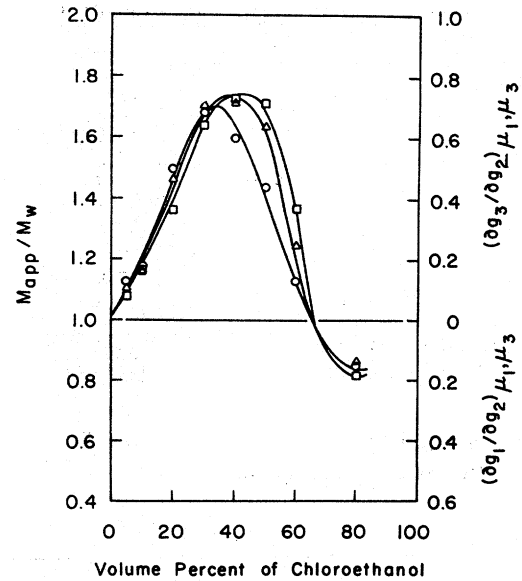

Figure 4. Variation of ratio of the apparent molecular weight to the true molecular weight, M_{app}/M_w, and the preferential solvation to β-lactoglobulin A with increasing concentration of 2-chloroethanol: ○, M_{app}/M_w; △, $(\partial g_i/\partial g_2)_{\mu_1,\mu_3}$ from light-scattering data; □, $(\partial g_i/\partial g_2)_{\mu_1,\mu_3}$ from refractive index increment data; *i* corresponds to component 3 (2-chloroethanol) and component 1 (water) above and below abscissa, respectively.

plotted against the concentration of chloroethanol in Figure 4. From this ratio, which is equal to $(1 + D)^2$, it is possible to calculate the preferential solvation, $(\partial m_3/\partial m_2)_{\mu_1,\mu_3}$ or $(\partial g_3/\partial g_2)_{\mu_1,\mu_3}$, and the thermodynamic parameter of interaction between β-Lg A and 2 chloroethanol, β_{32}, as is shown in the Results. Plots of $(\partial g_3/\partial g_2)_{\mu_1,\mu_3}$ against the concentration of chloroethanol are also presented in Figure 4. At alcohol concentrations above 65 vol %, the plot represents grams of water bound per gram of β-Lg A, $(\partial g_1/\partial g_2)_{\mu_1,\mu_3}$ instead of $(\partial g_3/\partial g_2)_{\mu_1,\mu_3}$. β-Lg A preferentially adsorbs 2-chloroethanol at low concentrations of the latter. The degree of preferential solvation increases with an increase in the alcohol concentration, and reaches a maximum around 40 vol %. It then decreases monotonely to negative preferential adsorption of chloroethanol, passing through zero at about 65 vol %. Above this concentration, β-Lg A adsorbs water preferentially.

The preferential adsorption of solvent components to a macromolecule is directly related to the difference between the refractive index increment at constant concentration and at constant chemical potential of component 3.[14,23,24] The amount of preferential solvation is given by eq 7 when C_2 is extrapolated to zero.

$$\left[\frac{\partial m_3}{\partial m_2}\right]_{\mu_1,\mu_3} = \frac{M_2}{M_3(1 - \bar{V}_3C_3)}\left\{\left[\frac{\partial n}{\partial C_2}\right]_{\mu_1,\mu_3} - \left[\frac{\partial n}{\partial C_2}\right]_{m_3}\right\}\Big/\left[\frac{\partial n}{\partial C_3}\right]_{m_2} \quad (7)$$

The difference between the two types of refractive index increments is so small by comparison with their absolute values that the derived preferential solvation is not too accurate.[14] The preferential adsorption of 2-chloroethanol to β-Lg A derived from refractive index increments, however, agrees fairly well with that derived

Table V. Preferential Solvation of β-Lactoglobulin A in Mixtures of Water with 2-Chloroethanol, Calculated from Refractive Index Increments

Chloroethanol, vol %	$(\partial n/\partial C_2)_{\mu_1,\mu_3}$, ml/g	$(\partial n/\partial C_2)_{m_3}$, ml/g	$(\partial n/\partial C_3)_{m_2}$, ml/g	$(\partial m_3/\partial m_2)_{\mu_1,\mu_3}$, mol/mol	$(\partial g_3/\partial g_2)_{\mu_1,\mu_3}$, g/g	$(\partial m_1/\partial m_2)_{\mu_1,\mu_3}$, mol/mol	$(\partial g_1/\partial g_2)_{\mu_1,\mu_3}$, g/g
0		0.189					
5	0.192	0.184	0.103	18.9	0.081		
10	0.195	0.179	0.103	39.8	0.171		
20	0.198	0.169	0.100	83.4	0.359		
30	0.202	0.159	0.094	149.5	0.643		
40	0.192	0.152	0.092	166.8	0.718		
50	0.175	0.143	0.089	165.2	0.711		
60	0.150	0.137	0.088	85.0	0.366		
80	0.117	0.131	0.082	−197.0	−0.848	189.7	0.183

from light-scattering measurements. This is shown in Table V and Figure 4. In the absence of molecular weight changes, the light-scattering approach seems to be preferable to differential refractometry for measuring solute–solvent interactions. In light scattering, measurement of such interactions does not involve the use of membranes, since interaction with solvent components is obtained directly from the deviation of the apparent molecular weight from the true value; in differential refractometry, however, the interaction is measured by the difference between refractive index increments obtained with and without dialysis (see eq 7). Furthermore, comparison of eq 1 and 2 with 7 shows that macromolecule–solvent component interactions result in larger relative changes of the measured parameters in light scattering than in differential refractometry: in the first technique, the measured effect corresponds essentially to the difference between the squares of the refractive increments measured in the two ways.

The value of B^0, which is directly related to the protein–protein interaction parameter, β_{22}, obtained from light scattering, at a constant concentration of the third component, should correspond to B^0 calculated from the light scattering at constant chemical potential. Agreement between these two parameters is not too good, especially in high concentrations of chloroethanol. This may be due to the following. The slope is more influenced by experimental error than the intercept when $H'(\partial n/\partial C_2)^2C_2/\Delta\tau$ is plotted against C_2. Furthermore, our system has been treated as a three-component system; it consists, however, of five components, *i.e.*, water, 2-chloroethanol, NaCl, HCl, and β-Lg A. We have disregarded electrostatic interactions between the protein and the electrolytes in the three-component treatment; this probably has a greater effect on protein–protein interaction coefficients than on protein–solvent interactions.

It is worthwhile to add a relation between $(H'C_2/\Delta\tau)_{m_3}$ and $(H'C_2/\Delta\tau)_{\mu_1,\mu_3}$ which is measured on extrapolation of C_2 to zero. Using eq 1, 2, and 7, we obtain

$$\lim_{C_2\to 0}\{(H'C_2/\Delta\tau)_{m_3} - (H'C_2/\Delta\tau)_{\mu_1,\mu_3}\} = 0$$

This means that when values of $(H'C_2/\Delta\tau)_{m_3}$ and of $(H'C_2/\Delta\tau)_{\mu_1,\mu_3}$ are plotted againt C_2 at the same concentration of 2-chloroethanol, the two lines should extrapolate to the same point at $C_2 = 0$. Our light-scattering data do extrapolate to nearly the same point in each series of mixed solvents.

Conformational Change. The optical rotatory dispersion and circular dichroism studies show that addition of 2-chloroethanol to β-Lg A solution (0.02 *M* NaCl and 0.01 *M* HCl) results in almost no change in the conformation of the protein below 10% by volume. When the concentration is increased to 20%, both curves change suddenly, and the detailed features characteristic of the native structure disappear. The ORD curve is quite close to that of β-Lg A in 40% acidic methanol[6] and the CD curve is also similar to that given by an α-helical poly-γ-benzyl-L-glutamate at pH 4[51] and poly-L-lysine at pH 11.[51–53] Further increases in 2-chloroethanol do not induce substantial changes in the shape of the ORD and CD curves, but continue to cause a slight increase in the intensity of the troughs and peaks. Figure 5 shows a plot of the optical parameters a_0, b_0, $[m']_{233.5}$, $[m']_{199}$, and $[\theta]_{221}$ against the concentration of 2-chloroethanol.[54] A sharp transition is observed in the range between 10 and 20%. By comparing Figure 5 to Figure 7 of Timasheff, *et al.*,[6] this transition corresponds to the conformational change in β-Lg A from the native α-helix-poor structure to the denatured α-helix-rich structure; above 20% chloroethanol the contents of α-helix in β-Lg A are increasing much more slowly. The change in a_0 and b_0 of β-Lg A agrees with that observed by Tanford, De, and Taggart[4] on mixed β-A- and β-B-lactoglobulin in the same organic solvent. Since β-lactoglobulin has been shown to contain a structure other than α helical and aperiodic (or disordered), namely the antiparallel-chain pleated-sheet (or β) structure,[6,50,55,56] it seemed of interest to enquire whether such structures persisted even after the α helix-promoting denaturation with 2-chloroethanol. For this purpose, the apparent degree of right-handed α helicity was calculated as a function of 2-chloroethanol concentration from the Moffitt–Yang b_0 parameter[40] (with the assumption that b_0 intrinsic is −630° for the right-handed α helix and zero for the disordered and β structures[6,50]) and from the related Shechter–Blout A_{193} and A_{225} two-term Drude

(51) G. Holzwarth and P. Doty, *J. Am. Chem. Soc.*, **87**, 218 (1965).
(52) R. Townend, T. F. Kumosinski, S. N. Timasheff, G. D. Fasman, and B. Davidson, *Biochem. Biophys. Res. Commun.*, **23**, 163 (1966).
(53) P. K. Sarkar and P. Doty, *Proc. Natl. Acad. Sci. U. S.*, **55**, 981 (1966).
(54) A more appropriate parameter to use would be the rotational strength of the 221-mμ dichroic band, rather than the ellipticity at its apex. Because of the uncertainty involved in the resolution of overlapping bands and the general qualitative similarity of the CD curves above 20% chloroethanol, it was considered that a plot of $[\theta]_{221}$ as a function of alcohol concentration would be a sufficient qualitative indication of the conformational change involved.
(55) S. N. Timasheff and H. Susi, *J. Biol. Chem.*, **241**, 249 (1966).
(56) S. N. Timasheff, H. Susi, and L. Stevens, *ibid.*, **242**, 5467 (1967).

Table VI. Apparent Helix Contents of β-Lactoglobulin A in Water–2-Chloroethanol Mixtures

2-Chloroethanol, vol %	Apparent per cent right handed α helix from b_0	H_{193}	H_{225}
0	11	26	11
10	10	26	11
20	36	42	37
30	46	48	45
40	50	53	47
60	53	56	50
80	58	60	52
100	64	65	57

equation parameters.[57,58] The results are summarized in Table VI. We see that, by all criteria, the apparent contents of α helix increase sharply between 10 and 20% 2-chloroethanol. Above this solvent composition, a slow rise in a α helix contents continues, reaching a maximum of about 65% in pure 2-chloroethanol, in a manner similar to the earlier observations in acidic methanol.[6] Comparison of the H_{193} and H_{225} values obtained in the presence of 2-chloroethanol (Table VI) shows that, according to the criteria of Shechter and Blout,[57] the optical rotatory dispersion properties of β-lactoglobulin in 2-chloroethanol cannot be accounted for in terms of α-helical and unordered regions alone, but that another non-α-helical ordered structure persists. When the S–S bridges in β-lactoglobulin are broken by S-sulfonation, the H_{193} and H_{225} values obtained in methanol become 87 and 88, respectively, indicating the disappearance of the "third" structure; this suggests that there is in this protein a structurally stable region constrained by an S–S bridge. In the earlier study with methanol,[6] the conclusion had been reached that this stable region is rich in β structure. It is interesting to note that the large electrophoretically

(57) E. Shechter and E. R. Blout, *Proc. Natl. Acad. Sci. U. S.*, **51**, 695, 794 (1964).
(58) J. P. Carver, E. Shechter, and E. R. Blout, *J. Am. Chem. Soc.*, **88**, 2562 (1966).

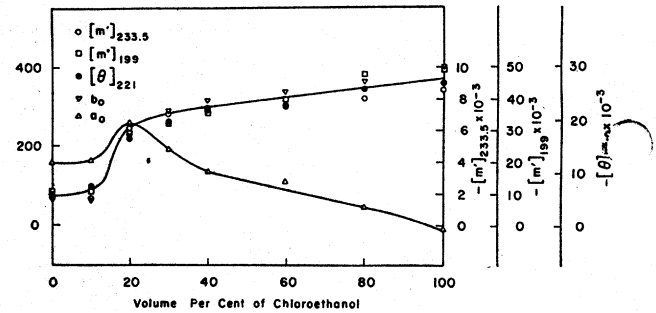

Figure 5. Dependence of a_0, b_0, $[m']_{233.5}$, $[m']_{199}$, and $[\theta]_{221}$ of β-lactoglobulin A on 2-chloroethanol concentration in the mixed solvents of water with 2-chloroethanol.

immobile tryptic peptide of β-lactoglobulin[59] gives an infrared spectrum in the amide I band region typical for an antiparallel-chain pleated-sheet structure.[56]

The dependence of the preferential binding of 2-chloroethanol to β-Lg A on the concentration of the former is very similar to that observed in the case of the water–chloroethanol–bovine serum albumin system.[60] It is, however, quite different from that found with other organic solvents, for example, with β-Lg A in ethylene glycol and methyl Cellosolve,[61] and bovine serum albumin in glycerol.[60] In addition, this concentration dependence of preferential interaction with solvent components does not seem to have any simple relation to the conformational change in β-Lg A induced by the same mixed solvents. Further investigations on the effect of this and other solvent components on several proteins are now in progress.

Acknowledgment. This work was supported in part by Grant GB-5186 from the National Science Foundation and Grant GM 14603-02 from the National Institutes of Health.

(59) R. Townend, *Arch. Biochem. Biophys.*, **109**, 1 (1965).
(60) J. Stauff and K. N. Mehrotra, *Kolloid-Z.*, **176**, 1 (1961).
(61) H. Inoue and S. N. Timasheff, in preparation.