A Thermodynamic Analysis of the Monomer–Dimer Association of β-Lactoglobulin A at the Isoelectric Point*

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ABSTRACT: The monomer–dimer equilibrium of β-lactoglobulin A has been studied under isoelectric (pH 5.2) and alkaline (pH 7.0) conditions using the meniscus depletion sedimentation equilibrium technique. The auxiliary measurement of density increment was also made. Thermodynamic parameters for association in the temperature range 5–20° were determined from the association constants. The change in free energy accompanying association to dimer at the isoelectric point in 0.1 M NaCl and 20.0° was -8.08 ± 0.11 kcal mole^{-1}. The enthalpy and entropy changes were -6.91 ± 1.87 kcal mole^{-1} and 3.63 ± 1.00 eu, respectively. The free-energy change accompanying the association at pH 7.0 in a KH_2PO_4–Na_2HPO_4 buffer of ionic strength 0.1 at 20.0° was -6.27 ± 0.04 kcal mole^{-1}. The enthalpy change was -15.52 ± 0.30 kcal mole^{-1} and the entropy change was -31.65 ± 6.32 eu. The Verwey–Overbeek treatment of electrostatic repulsion between double-layered spheres of uniform charge was tested against the experimental evidence. It was found to account for only a fraction of the enthalpy change although it accounted for a third of the free-energy change in comparing the results at the isoelectric point with those at pH 7.0. The lack of agreement between experiment and plausible electrostatic theory was attributed to the possibility that the ion–dipole interactions of water with charged protein prevent formation of all possible hydrogen bonds between water molecules during association of the two subunits of protein.

The self-association equilibria of proteins have been investigated by a variety of techniques (Reithel, 1963; Klotz *et al.*, 1970). Despite the many investigations there remains a paucity of thermodynamic data required to formulate the distribution of the noncovalent forces involved. Various proposals concerning the forces and groups involved in self-association have been offered (Kauzmann, 1959; Tanford, 1964; Scheraga, 1963; Nemethy, 1967) from interpretations of the thermodynamic data available.

Previous studies of protein self-association have been made, with few exceptions, at pH values removed from the isoelectric point. The advantage of a net charge is that electrostatic repulsion lessens the association forces and potentiates dissociation at protein concentrations required for the techniques employed. In the present study it was considered prudent to make measurements at the isoelectric point in an effort to sort out less equivocally the contributions of the several possible types of forces.

Various theories have been proposed to aid in estimating the electrostatic repulsion between structural subunits and the contribution to the free energy of attraction (Linderstrøm-Lang, 1924; Verwey and Overbeek, 1948; Tanford, 1961). The approach of Verwey and Overbeek has been applied successfully to the monomer–dimer relation of β-lactoglobulin at low pH (Timasheff and Townend, 1961) and to the association–dissociation of insulin (Jeffrey and Coates, 1966). In each case there was a satisfactory semiquantitative description of the observed change in free energy with change in net charge. This treatment involved the assumption that nonspecific electrostatic repulsion was the sole force perturbing dissociation equilibria.

Verwey and Overbeek evaluated the electrostatic free-energy contribution to the change in free energy of attraction of two identical associating units (eq 1). The surface potential,

$$-\Delta(\Delta F^\circ_{elec}) = \psi_0^2 D b^2 R^{-1} e^{-(R-2b)\gamma} \quad (1)$$

ψ_0, of the idealized molecule can be calculated from the Debye–Hückel theory (see, *e.g.*, Steiner, 1953); D is the dielectric constant, b is the radius of the idealized protein sphere, R is the distance between the centers of the two spheres. The quantity, γ, is a function of κa and R/a, where κ is the Debye–Hückel screening parameter and a is the radius of exclusion for a small ion approaching the macromolecule. The surface potential is directly proportional to the net charge, $\bar{Z}$, and all calculations of $-\Delta(\Delta F^\circ_{elec})$ refer to the point of contact between spheres where $R = 2b$ and the exponential has a value of unity.

Assuming first, that electrostatic repulsion is the only perturbation at pH $\neq$ pI, and second, no change in conformation of the interacting units, we estimate the total free energy of attraction, ΔF°_a, between those units as

$$\Delta F^\circ_a = \Delta F^\circ_{exp} + \Delta(\Delta F^\circ_{elec})\text{pH} \quad (2)$$

The experimental evaluation of the free-energy change is through the relation

$$\Delta F^\circ_{exp} = -RT \ln K^\circ_a \quad (3)$$

The quantity, K°_a, is the experimentally determined association constant for a monomer–dimer equilibrium, the case under study here.

In the present investigation the meniscus depletion sedimentation equilibrium technique (Yphantis, 1964) was used

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to obtain data for the association equilibrium constants, at various pH and temperature values, for β-lactoglobulin A. This protein exists predominantly in the dimer form of 36,800 daltons (Piez *et al.*, 1961; Kelly, 1969) except at extremes of pH and dilution (McKenzie, 1967). Our results confirm that β-lactoglobulin dissociates into monomers at the isoelectric point (pH 5.2) as well as at pH extremes.

Theory

Calculation of the various apparent equilibrium constants, $K_a(r)$, requires the determination of the point weight-average molecular weights, $\bar{M}_w(r)$, at each radial position, r, in the centrifuge cell as indicated by

$$\bar{M}_w(r) = \frac{2RT}{(\partial\rho/\partial c)_\mu \omega^2} \frac{\mathrm{d} \ln c(r)}{\mathrm{d}r^2} \quad (4)$$

The symbols have the following meanings: R, gas constant; T, absolute temperature; $(\partial\rho/\partial c)_\mu$, change in density with respect to concentration of dialyzed protein; ω, rotor speed; $c(r)$, concentration of protein at any point; r, in the sedimenting column. The $\bar{M}_w(r)$ values were used in an equation, derived independently by Rao and Kegeles (1958) and Adams and Fujita (1963), descriptive for the association of monomers to dimer. The symbolism is: M_1, monomer molecular

$$K'_a = \frac{[\bar{M}_w(r) - M_1]\, M_1^2}{2\, c(r)\, [2\, M_1 - \bar{M}_w(r)]^2} = \frac{c'_2(r)}{[c'_1(r)]^2} \quad (5)$$

weight; c'_1 and c'_2, apparent molar concentrations of monomer and dimer.

When M_1 is known, as in the present case, an alternative calculation (Haschemeyer and Bowers, 1970) may serve to calculate the association constant. By rearranging eq 4, and integrating over a difference in radial position, dr, from the meniscus, eq 6 is obtained (Teller *et al.*, 1969; van Holde *et al.*, 1969). In this relation c_{im} is the concentration of species

$$c(r) = \Sigma_i c_{im} e^{H_i(r^2 - r_m^2)} \quad (6)$$

i at the meniscus, r_m is the radial position at the meniscus, and $H_i = \mathrm{d} \ln c_i/\mathrm{d}r^2 = M_i(\partial\rho/\partial c_i)\omega^2/2RT$. Using a least-squares matrix analysis for all concentration data, with the sum of two exponentials representing monomer and dimer, we may estimate the concentrations of monomer and dimer. The equilibrium constant is calculated from the values of concentrations at the meniscus.

Materials and Methods

Samples of β-lactoglobulin, A and AB, were kindly donated by Dr. S. N. Timasheff and some samples were purchased from Pentex. The samples used were recrystallized at least three times. All protein solutions were dialyzed against the appropriate buffers for at least 24 hr and with at least two changes of dialysate. Before use, the dialyzed solutions were filtered through prewashed Millipore filters (0.22–0.45 μ pore size).

Measurements of $\partial\rho/\partial c$ were performed, except where noted, by the technique of Goodrich *et al.* (1969).

The Spinco Model E analytical ultracentrifuge used was equipped with an electronic speed control, a Baird-Atomic B-9 filter over the light source, an RTIC temperature controller calibrated with a Bureau of Standards thermometer, and had an optical system capable of producing 40–47 Rayleigh interference fringes in the main optical envelope. The optical system was focused at the two-thirds plane of the cell (Svensson, 1954, 1956; Yphantis, 1964). Optical alignment was checked after each drive change by the procedures of Gropper (1964) and R. L. Baldwin (unpublished data). Temperature control was ±0.1° in precision.

A standard double-sector cell with sapphire windows and an aluminum-filled epon centerpiece was used. The solution column height was 3 mm in each experiment. Rotor speed varied from 34,000 to 44,000 rpm. A false column base of FC43 was not employed (Adams and Lewis, 1968).

The interference patterns recorded on Kodak II-G plates were measured with a Nikon microcomparator. Fringe displacements, the average over three black fringes at each radial position, were corrected by blank readings from a water–water run made after each experiment and before the cell was disassembled. Equilibrium was assumed when the fringe displacement at the majority of radial positions did not change more than ±5 μ in successive photographs. Molecular weights were calculated and data processed by computer programs developed at the University of Oregon (S. Lowe and M. J. Kelly, unpublished data) and at the University of Virginia (M. J. Kelly, unpublished data) for use on IBM 360/50 and Burroughs 5500 computers, respectively.

Concentration of protein in grams per liter were obtained from the vertical fringe displacement. The standard used was a synthetic boundary experiment (capillary-type cell) using protein samples whose concentration had been established by dry weight determinations (Goodrich and Reithel, 1970). The average of two separate series of determinations on different lots of β-lactoglobulin was 0.11806 ± 0.00008 cm/g per l. When the concentration, in terms of fringe displacement, was expressed as the number of fringes crossed (Schachman, 1963), dividing by an average fringe spacing value of 289 microns, the value 4.085 fringes/g per l. was obtained. This is in agreement with the averaged value of 4.10 ± 0.13 fringes/g per l. obtained for seven nonheme proteins (Babul and Stellwagen, 1969) and with the value of 4.02 fringes/g per l. obtained for β-lactoglobulin by Albright and Williams (1968).

Results

The values of $(\partial\rho/\partial c)_\mu$ for β-lactoglobulin A reflect the thermodynamic formalism of Casassa and Eisenberg (1961) in which the concentrations, c^*, and densities, ρ^*, are evaluated on the condition that, in the isopotential state, the concentration of diffusible components are assumed equal on both sides of the dialysis membrane. This convention ensures that all bound ions are included in the value of the protein molecular weight. The values of $(\partial\rho^*/\partial c^*)_\mu$ listed in Table I were determined using the assumption that a linear function obtained.

The apparent weight-average molecular weight as a function of concentration is presented in Figures 1 and 2 for representative systems. The individual points were calculated from the least-squares slopes of ln $c(r)$ plotted *vs.* r^2. The slope at each value of r was estimated by averaging seven adjacent points 100 μ apart. Figure 1 shows a plot of data obtained at various protein concentrations in 0.1 M NaCl (pH 5.2)–10^{-3} M DTT,[1]

[1] Abbreviation used is: DTT, dithiothreitol.

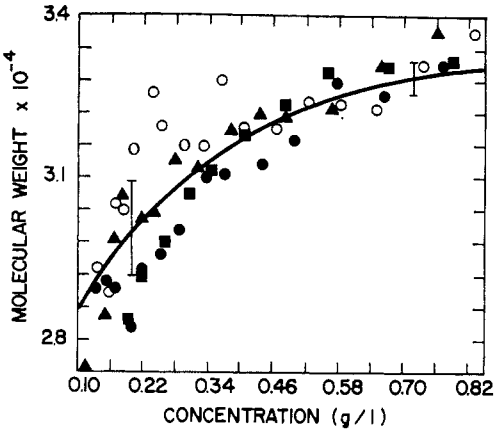

FIGURE 1: A plot of $\bar{M}_w(r)$ *vs.* the concentration of β-lactoglobulin A in 0.1 M NaCl + 10^{-3} M DTT, pH 5.2, 20.0°. The density of the solution was 1.0038 g/cm³ and the $(\partial\rho^*/\partial c^*)_\mu$ was 0.249 ml/g. The solid line through the data represents an apparent equilibrium constant of $1.12 \pm 0.20 \times 10^6$ M^{-1}.

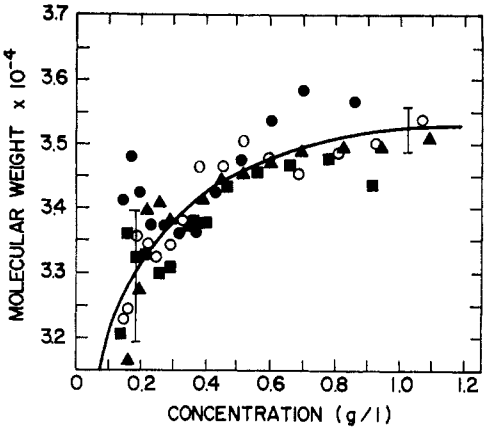

FIGURE 2: A plot of $\bar{M}_w(r)$ *vs.* the concentration of β-lactoglobulin A in Na_2HPO_4–KH_2PO_4 buffer, pH 7.0, I = 0.1, 5°. The closed circles represent runs with 10^{-3} M DTT added. The density of the solution was 1.0180 g/cm³ and the $(\partial\rho^*/\partial c^*)_\mu$ used was 0.245 ml/g. The solid line through the data represents an apparent equilibrium constant of $2.22 \pm 0.44 \times 10^5$ M^{-1}.

20.0°. Figure 2 shows a plot of data obtained when the buffer used was phosphate at an ionic strength of 0.1, pH 7.0, either in presence or absence of 10^{-3} M DTT, 5.0°. The use of DTT is explained later in the text.

A comparison, at the isoelectric point, of the calculated concentration distribution, using eq 6, with the measured values shows good agreement in Table II. The calculation assumed a simple monomer–dimer relation and was not improved by using three exponentials implying monomer–dimer–trimer.

Calculated equilibrium constants are listed in Table III. Standard deviations are given for individual concentration measurements in experiments employing 0.1 M NaCl and for those in phosphate buffer (pH 7.0) with DTT, at different temperatures. Equation 6 was used to determine monomer and dimer concentrations at the meniscus.

Discussion

Evaluation of the data in Table II may be made as follows. The measured values of concentration at any radial position in the solution column may be expected to have a standard deviation of $\pm 0.0003 + 1.25 \times 10^{-5} \times (dc/dx)_{cell}$ cm, where $(dc/dx)_{cell}$ is the concentration gradient in fringes per centimeter (Roark and Yphantis, 1969; Haschemeyer and Bowers, 1970). The standard deviation reported in Table II is 0.00027 cm, well within the limits of reading error.

Association Constants. The values of the equilibrium constants in Table III demonstrate that most of the data obtained are consonant with a monomer–dimer association model.

TABLE I: Values of $(\partial\rho^*/\partial c^*)_\mu$ for β-Lactoglobulin A.

Experimental Conditions	$(\partial\rho^*/\partial c^*)_\mu$ (ml/g)	ρ Solvent (g/ml)
KH_2PO_4–Na_2HPO_4, 20.00°		
pH 7.2, I = 0.1	0.249 ± 0.005	1.00441
pH 6.4, I = 0.1	0.2426 ± 0.0044	1.010978
pH 6.35, I = 0.2	0.2432 ± 0.0019	1.021921
NaCl, 20.00°		
pH 5.2, 0.1 M	0.2457 ± 0.0025	1.003841
same + 10^{-3} M DTT	0.2475 ± 0.0024	1.004238
NaCl, 5.00°		
pH 5.2, 0.1 M	0.2465 ± 0.0035	1.007295

TABLE II: Concentration Distributions of β-Lactoglobulin A in 0.1 M NaCl + 10^{-3} M DTT at 5° and 36,000 rpm.

Radius² − Meniscus² (cm²)	Concn Measured (cm)	Concn Calcd[a] (cm)	Difference (cm)
2.3408	0.0054	0.0053	−0.0001
2.4073	0.0062	0.0062	0
2.4739	0.0071	0.0073	0.0002
2.5405	0.0086	0.0086	0
2.6071	0.0100	0.0102	0.0002
2.6738	0.0120	0.0121	0.0001
2.7406	0.0144	0.0143	−0.0001
2.8074	0.0168	0.0170	0.0002
2.8742	0.0194	0.0202	0.0008
2.9411	0.0242	0.0240	−0.0002
3.0080	0.0283	0.0286	0.0003
3.0750	0.0342	0.0341	−0.0001
3.1420	0.0407	0.0406	−0.0001
3.2090	0.0487	0.0485	−0.0002
3.2761	0.0582	0.0580	−0.0002
3.3433	0.0698	0.0695	−0.0003
3.4105	0.0837	0.0832	−0.0005
3.4777	0.0996	0.0997	0.0001
3.5450	0.1193	0.1195	0.0002
3.6123	0.1432	0.1434	0.0002

[a] Calculated by least-squares matrix analysis of actual concentration data using eq 6 for the sum of two exponentials. The equilibrium constant, K'_a, for this distribution is 2.32×10^6 M^{-1}. The standard deviation, σ, for each individual concentration measurement using the sum of the squared residuals is ± 2.7 μ.

TABLE III: Equilibrium Constants for the Monomer–Dimer Association of β-Lactoglobulin A Using a Least-Squares Fit of the Sum of Two Exponentials.

KH_2PO_4–Na_2HPO_4 + 10^{-3} M DTT, pH 7.0, I = 0.1 K'_a (l./mole)					
	$\sigma^a \times$		$\sigma \times$		$\sigma \times$
5.00°	10^4	9.80°	10^4	20.00°	10^4
1.85×10^5	5	1.04×10^5	8	4.34×10^4	6.7
2.20×10^5	4.3			5.11×10^4	5.4
1.74×10^5	4.3			5.25×10^4	7.3
3.10×10^5	4.7				
$2.22 \pm .44 \times 10^5$ (av)				$4.96 \pm 0.35 \times 10^4$	
0.1 M NaCl + 10^{-3} M DTT, pH 5.2, K'_a (l./mole)					
1.93×10^6	5	1.04×10^6	6	1.13×10^6	3.75
2.17×10^6	6.5	7.60×10^5	6.5	7.41×10^5	4.4
2.32×10^6	2.7	9.00×10^5	5	9.29×10^5	4.0
2.03×10^6	8.0			1.30×10^6	4.8
				1.56×10^6	7.0
				1.06×10^6	5.0
$2.11 \pm 0.13 \times 10^6$		$9.00 \pm 0.90 \times 10^5$		$1.12 \pm 0.20 \times 10^6$	

[a] Standard deviation from the sum of squared residuals for each concentration measurement in the solution column.

Exceptions to this probably owe to a small amount of heterogeneity caused by a slow aggregation (Kelly, 1969). This slow aggregation initially presented difficulties in ultracentrifuge experiments of a duration greater than 21 hr. Since those experiments involving the lower concentrations of protein were especially divergent, a lability of the monomer was suspected. Inclusion of 10^{-3} M DTT virtually eliminated the irregularities and permitted runs of longer duration without difficulty. The use of this reagent did not cause any noticeable shift in the equilibrium constant and was used routinely in later experiments

The association constants of Table III may be compared by

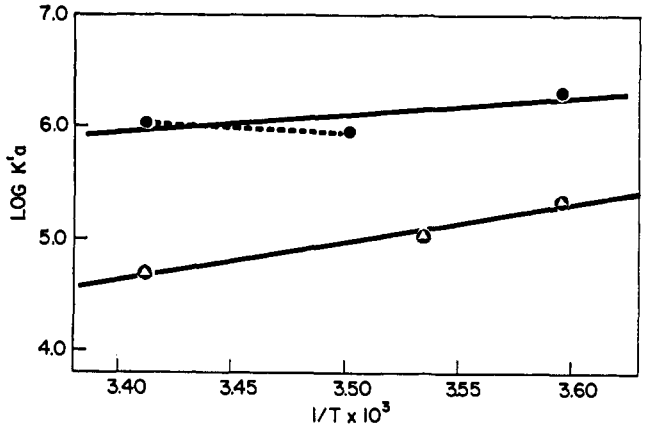

FIGURE 3: A plot of log K'_a *vs.* $1/T$ for runs in 0.1 M NaCl (upper curve) and phosphate buffer, pH 7.0 (lower curve). The solid lines represent a linear least-squares fit to the data points. The dotted line (upper curve) represents the best fit between the two data points to illustrate a possible transition.

TABLE IV: Thermodynamic Parameters of β-Lactoglobulin A Association at 20°, I = 0.1.

pH	$\Delta F°$ (kcal/mole)	$\Delta H°$ (kcal/mole)	$\Delta S°$ (cal/deg per mole)
7.0	-6.27 ± 0.04	-15.52 ± 0.30	-31.65 ± 6.32
6.0[a]	−6.8	−10	−11
5.2 (pI)	-8.08 ± 0.11	-6.91 ± 1.87	$+3.63 \pm 1.00$
2.7[b]	-5.4 ± 0.1	-12.4 ± 1.4	-23.8 ± 5.1

[a] Data of Georges and Guinand (1960) on β-lactoglobulin B. [b] Data of Timasheff and Townend (1961).

thermodynamic parameters. Using eq 3 to determine the free-energy change during association, we have made plots of log K'_a *vs.* T^{-1} to evaluate the enthalpy and entropy changes. A

$$\log K°_a = -\frac{\Delta H°}{2.303RT} + \frac{\Delta S°}{2.303R} \qquad (7)$$

comparison of plots of data obtained in NaCl with those obtained in phosphate buffer may be found in Figure 3. If a linear function is assumed, a least-squares treatment of the three points yields the values of Table IV. The upper line in Figure 3 might reflect a transition at 12.5° since this point appears to be outside experimental error. The points of 5 and 20° are consistent with a negative enthalpy change and a positive entropy change. The dotted line connecting the points at 20 and 12.5° leads to an estimate of a positive enthalpy of 5 kcal/mole. This transition might be the result of the compensation phenomenon discussed by Lumry and Rajender (1970) that may result in volume changes reflected in the partial specific volume. This quantity was not determined at 12.5° but was interpolated from measurements at 5 and 20°. The determination of the temperature coefficient of $(\partial\rho/\partial c)_\mu$ at several temperatures is now in progress. Until these data are at hand we assume that the values of K'_a at 12.5° are anomalous and that the slope of log K'_a plotted *vs.* T^{-1} is positive.

Free Energy of Association. Using eq 1 to calculate the electrostatic free-energy contribution to the total free energy of association at pH 7.0 yielded a value of −0.62 kcal/mole assuming a net charge of −13 (Basch and Timasheff, 1967). Substitution of this value in eq 2 resulted in a free energy of attraction, $\Delta F°_a$, of −6.89 kcal/mole, to be compared with the values of −9 to −10.8 kcal per mole obtained for low pH values (Timasheff and Townend, 1961). Justification for the use of this model was based upon the lack of evidence for conformational change in the pH range 2.0–6.0, excluding octamer formation in the pH region between 4 and 5. A small conformational change in the pH range 4.0–6.0 has been detected where dissociation is suppressed by high salt and octamer formation by high temperature (Timasheff *et al.*, 1966). This change is thought to be a minor rearrangement in the β structure.

Volume Changes. Evidence that there is not substantial conformational alteration in β-lactoglobulin when the pH is lowered may be adduced from studies on the apparent partial specific volume (Reithel and Sakura, 1963). Rasper and Kauzmann (1962) have measured the volume changes accompanying the ionization of the carboxyl group below pH 5. They noted a volume increase of 11 ml/mole of protons reacting.

Jacobsen and Linderstrøm-Lang (1949) calculated this value for β-lactoglobulin assuming a molecular weight of 40,000 (dimer) and 49 carboxyl groups. In our work 52 carboxyl groups were assumed to be present in the dimer. Using the value of 38 protons reacting (Basch and Timasheff, 1967) to calculate the change in volume that would accompany titration of β-lactoglobulin from pH 5.2 to 2.0 yields a $\Delta\phi^* = 0.01135$ ml/g. The value of $\Delta\phi^*$ obtained experimentally, after normalizing the data to 25° using a $d\phi/dt$ of 2.23×10^{-4} ml/(g deg), is 0.0115 ml/g. The agreement between these estimates indicates little conformational perturbation during titration to pH 2.0 beyond the electrostriction of water.

Changes in $\Delta F°$. Sedimentation equilibrium studies on β-lactoglobulin B (Albright and Williams, 1968) provided data which, by use of the Verwey–Overbeek equation, led to the value of $\Delta F°_a = -9.2$ kcal/mole. Comparison of values obtained on both sides of the isoelectric point ($Z = 0$) showed that the Verwey–Overbeek equation appeared to account for the free-energy change within ±1.5 kcal/mole. Since the model is highly simplified, the agreement is surprisingly good.

Changes in $\Delta H°$ *and* $\Delta S°$. With the experimentally determined thermodynamic data of Table IV at hand one may explore the probable combinations of forces perturbed when the pH is changed. It may be seen that $\Delta H°$ on both sides of the isoelectric point becomes more negative and the same thing is noted in the values of $\Delta S°$. If it is assumed that electrostatic repulsion is the only variable force, eq 1 can account for a minimal amount (~300 cal/mole) of the enthalpy change observed. Although the equation accounts roughly for the change in free energy the limitations are obvious.

The increasing negativity of $\Delta H°$ and $\Delta S°$ at pH values removed from the isoelectric point might reflect the change in electrostriction during titration. In the discussion above we have estimated the change in volume during the transition from pH 5.2 to 2.0 and found this attributable to electrostriction. This phenomenon is primarily an ion–dipole interaction possibly preventing water molecules from forming all possible hydrogen bonds (Gurney, 1962). If so, as carboxyl groups become uncharged, more water molecules closely associated with the protein can form hydrogen bonds with each other during association. Such a formation of extra hydrogen bonds would be consistent with the data (Schellman, 1955; Scheraga, 1961). Despite attempts to evaluate the energies of such bonds (Nemethy, 1963) the absolute magnitudes are uncertain.

Any model describing the forces involved in association at the isoelectric point must account for a negative $\Delta H°$ and a positive $\Delta S°$. In the association of two monomers there are at least two unfavorable entropy terms. The translational and rotational entropy losses have been estimated (Timasheff and Townend, 1961) to be −110 eu for β-lactoglobulin. Another method of estimation (Steinberg and Scheraga, 1963), assuming substantial rotational and vibrational freedom, led to −10 ± 8 eu for insulin. Thus, such estimates have yielded values that are negative but our data show a small positive value. Other studies yielding small $\Delta S°$ values are those for insulin dimerization, 0 to −12 eu (Doty and Myers, 1953; Steiner, 1953; Jeffrey and Coates, 1966), for antigen–antibody reaction, up to +30 eu (Epstein *et al.*, 1956; Singer and Campbell, 1955; Baker *et al.*, 1956), and a positive value for the polymerization of TMV protein (Smith and Lauffer, 1967).

Enthalpy changes in most cases of self-association are more negative than −5 kcal/mole. Kauzmann (1959) estimated the $\Delta H°$ for self-association to be slightly positive or zero. The formation of hydrophobic bonds in the association of β-lactoglobulin monomers would be consonant with a positive $\Delta S°$ but not a negative $\Delta H°$. On the other hand, formation of ion pair bonds might result because the dielectric constant of the protein domain is less than the bulk solvent. Were such a salt link formed (Schellman, 1953; Kauzmann, 1959) there would ensue a small negative $\Delta H°$ and a positive $\Delta S°$.

On the alkaline side of the isoelectric point the shift of $\Delta H°$ and $\Delta S°$ values are in the same direction but larger in magnitude. This may result from the conformational changes postulated to occur between pH 6 and 9. The values at pH 6.0 in Table IV (Georges and Guinand, 1960) correspond to a $K'_a = 1.25 \times 10^5$ M^{-1} determined by light-scattering techniques using β-lactoglobulin B. Values of 1.42×10^5 and 4.88×10^4 M^{-1} for β-lactoglobulins B and A, respectively, were calculated from sedimentation equilibrium data (Zimmerman *et al.*, 1970). The latter value is in agreement with our value listed in Table III.

The studies reported here emphasize the shortcomings of the present electrostatic models that do not predict the correct sign or magnitude of the thermodynamic parameters. Extension of these studies at higher ionic strength would be helpful in exploring the probabilities of a salt link.

Acknowledgments

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Abductin. Locus and Spectral Characteristics of a Brown, Fluorescent Chromophore*

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ABSTRACT: Abductin, the elastic protein of the internal hinge ligament of the scallop, exhibits both color and visible fluorescence. Neither of these spectral properties have previously been reported and they cannot be accounted for by the presently documented amino acid composition. The ultraviolet absorption spectrum is characterized by a shoulder at 280 nm which is independent of pH and unchanged after borohydride reduction, indicating that the chromophore is neither phenolic nor quinonoid. The visible absorption spectrum is characterized by a shoulder at 460 nm which disappears after borohydride reduction. Fluorescence of abductin is not observed in the native state because of strong quenching, but in dilute solution the protein exhibits a brilliant fluorescence of slightly greater intensity than that of elastin. The fluorescence spectrum is characterized by activation and emission at 380/490 nm in acid, and 360/450 nm in alkali (uncorrected). The variation with pH of the fluorescence intensity of the protein is characterized by changes in slope at pH 2 and pH 8 indicating that the fluorophore is different from that of resilin. Acid hydrolysis of abductin liberated a brown pigment that was rapidly adsorbed on charcoal, and had a fluorescence spectrum that approximated that of the intact protein. Abductin was subjected to extensive proteolysis by pronase and the digest examined by gel permeation chromatography. The colored, fluorescent, and ultraviolet-absorbing chromophores were confined to the enzyme-resistant regions of the protein molecule, which comprised 30% of the total weight. The location, in abductin, of the chromophores which may consist of one or several species, makes them possible candidates for cross-links.

The three rubber-like proteins elastin, resilin, and abductin, are insoluble in the generally accepted protein solvents. Since these proteins dissolve only in reagents that break peptide bonds, they must be cross-linked by covalent entities other than disulfide bridges. Hydrolysates of each of these proteins have yielded novel polyfunctional amino acids that are likely candidates for forming interchain cross-links in the intact proteins. The search for the cross-linking species in elastin was centered around that protein's unusual spectral characteristics, namely its color, fluorescence, and pH-independent ultraviolet absorbance, none of which could be accounted for by the then known amino acid composition. The search culminated in the isolation and characterization of the desmosine

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