The effects of N or S substitution on Ag^+ complexing are exactly the opposite; it is greatly increased. Evidently it is not electrostatic forces which matter here, but rather the type of covalent bonding which is involved in the many well-known complexes of Ag^+ with amines. Indeed the stability constants of noncyclic analogs are of comparable magnitude, *e.g.*, for $H_2NCH_2CH_2OCH_2CH_2OCH_2CH_2NH_2$ log K_1' is about 7.9[12] compared to 7.8 for the cyclic diaza compound; for $CH_3OCH_2CH_2NH_2$ log K_1' is 3.2[12] compared to 3.3 for the cyclic monoaza compound. That the stability constant for the dithia compound lies between those of the diaza and the unsubstituted polyethers is in agreement with the conclusion of Lotz, *et al.*,[12] that the relative stability of the silver–nonmetal bond increases in the order O < S < N.

The stability constants for the silver complexes of nitrogen- and sulfur-containing cyclic polyethers, therefore, provide the first evidence of complexing by other than purely electrostatic forces. However, it cannot be determined from the present evidence whether silver complexing of the straight polyethers is exclusively electrostatic or whether covalent bonding contributes to the stability. On the other hand, there is nothing to indicate that covalent bonding plays any part in the alkali metal complexes.

(12) J. R. Lotz, B. P. Block, and W. C. Fernelius, *J. Phys. Chem.*, **63**, 541 (1959).

Acknowledgments. This work would not have been possible without C. J. Pedersen, who laid the foundations for it, synthesized most of the polyether samples, and, though now retired, freely shared his wide experience. The author is indebted to the following coworkers at the Du Pont Experimental Station: M. H. Bromels, R. N. Greene, and C. H. Park for providing polyethers; D. J. Sam for devising the isomer separation; E. G. Brame for nmr interpretation; and M. J. Coverdale for technical assistance. He acknowledges many helpful discussions with Professors G. Eisenman, University of California, Los Angeles, as well as R. M. Izatt and J. J. Christensen, Brigham Young University.

Dissociation Equilibrium and Potentiometric Titration of β-Lactoglobulin in Acidic Solutions[1]

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Abstract: The nonelectrostatic part of the standard free energy change accompanying dissociation of (charged) β-lactoglobulin molecules into two (charged) subunits in acidic solutions is estimated from literature values of the dissociation equilibrium constants by subtraction of electrostatic free energies obtained by numerical solution of the nonlinearized Poisson–Boltzmann equation. The nonelectrostatic portion of the standard free energy change (molality units, infinitely dilute reference state) is 10.6 ± 1 kcal/ru and is independent of pH and ionic strength. The standard free energy change in the dissociation of uncharged (*i.e.*, isoionic) β-lactoglobulin into two uncharged (isoionic) subunits is estimated from the experimental potentiometric titration curves using a method analogous to that previously applied to the helix–coil transition of poly(L-glutamic acid). The result agrees with the nonelectrostatic part of the standard free energy of dissociation of the charged molecules into charged subunits. When the dissociation is taken into account, the calculated electrostatic free energies yield theoretical potentiometric titration curves that agree rather well with experiment. The counterion radii needed to obtain a good fit are more realistic than those required when dissociation is ignored.

Aggregation of protein molecules is commonly encountered and frequently has important implications when seen in a biological context. Examination of the forces responsible is therefore of considerable interest. In some cases, dissociation of ionizable groups on the protein leads to charge repulsions that cause disaggregation of the subunits. Titration of the protein with appropriate acids or bases then enables an investigator to sweep through the entire range of states of aggregation from completely associated to completely dissociated subunits. Such a reaction is particularly susceptible to experimental study, and, since direct electrostatic interactions are the kind that are most precisely estimated, particularly susceptible to exact analysis.

The protein β-lactoglobulin displays such a dissociation equilibrium, and it has been the subject of extensive and thorough experimental investigation by Timasheff, Townend, and their coworkers.[2-8] They were able to show by various methods that the protein

(1) This investigation was supported by Research Grant No. GM-05488 from the Division of General Medical Sciences, U. S. Public Health Service.

(2) S. N. Timasheff and R. Townend, *J. Amer. Chem. Soc.*, **82**, 3157 (1960).
(3) R. Townend, R. J. Winterbottom, and S. N. Timasheff, *ibid.*, **82**, 3161 (1960).
(4) T. Townend and S. N. Timasheff, *ibid.*, **82**, 3168 (1960).
(5) R. Townend, L. Weinberger, and S. N. Timasheff, *ibid.*, **82**, 3175 (1960).
(6) S. N. Timasheff and R. Townend, *ibid.*, **83**, 464 (1961).
(7) S. N. Timasheff and R. Townend, *ibid.*, **83**, 470 (1961).
(8) R. Townend, C. A. Kiddy, and S. N. Timasheff, *ibid.*, **83**, 1419 (1961).

molecule (mol wt 35,500) is dissociated in sufficiently acidic solutions into two identical subunits. By determining the weight-average molecular weight as a function of pH and ionic strength, these workers were able to assess the weight fraction of protein present as dimer and as monomer and thus to determine the numerical value of the "macroscopic" equilibrium constant for dissociation[5]

$$K = \frac{4\beta^2 C}{(1 - \beta)} \quad (1)$$

where C = total formality of protein (formula weight 35,000, *i.e.*, as dimer) and β = weight fraction of subunits (monomer) in the solution.

To analyze their results further, Timasheff, Townend, *et al.*, assumed that the system at any given pH and ionic strength can be described as an equilibrium mixture of dimers of charge $Z+$ protonic units and subunits of charge $Z/2$, *i.e.*

$$P_2^{Z+} \rightleftharpoons 2P^{Z/2+} \quad (2)$$

and they calculated the standard free energy change (molality basis, infinitely dilute reference state) for this process using the measured K and the relation

$$\Delta G^\infty = -RT \ln K \quad (3)$$

They then divided this standard free energy change into two parts, an electrostatic part (ΔG_{el}^∞) and a remainder (ΔG_a^∞)

$$\Delta G^\infty = \Delta G_a^\infty + \Delta G_{el}^\infty \quad (4)$$

The nonelectrostatic part of the free energy change (ΔG_a^∞), of course, includes the attractive forces responsible for the aggregation, and it is this quantity that needs to be scrutinized to obtain insight into the nature of such forces. Timasheff, *et al.*, therefore estimated ΔG_{el}^∞ from Linderstrøm-Lang's equation (*i.e.*, in the Debye–Hückel approximation) for the electrostatic free energy and computed the nonelectrostatic contribution by subtraction. They found, somewhat surprisingly, that the nonelectrostatic part of the standard free energy (ΔG_a^∞) decreases with increasing pH or ionic strength.[5]

Although there may be other causes for this reported variation of ΔG_a^∞,[5] it seems reasonable to examine the hypothesis that it is artifactual and caused by some combination of (1) the use of the Debye–Hückel approximation, which is inadequate for highly charged proteins,[9] and (2) the imprecision with which a complex system containing a wide variety of charged macromolecular species of both monomer and dimer can be represented simply by eq 2 and 3 and use of the macroscopic quantity K as determined from experimental data by use of eq 1.

To check the first possible difficulty we computed, on the basis of eq 2, the electrostatic free energy both of the dimer and of its subunits from numerical solutions of the nonlinearized Poisson–Boltzmann equation (*i.e.*, without use of the Debye–Hückel approximation).[9] The difference between the two is ΔG_{el}^∞ and this was used in (4) along with the ΔG^∞ provided by Timasheff, *et al.*, to calculate ΔG_a.

The second possible difficulty was examined by a simple extension of the "area" method used earlier in studies of the helix–coil transition in poly(glutamic acid).[10,11] In the present context, this approach allows an estimate to be made, from the titration curves, of the standard free energy change accompanying dissociation of *uncharged* (*i.e.*, isoionic) β-lactoglobulin into two *uncharged* subunits. This quantity should be the same, if our analysis is correct, as ΔG_a.

Since computation of the electrostatic free energy requires knowledge of the molecular charge and since the area method requires precise knowledge of the full potentiometric titration curve, we performed potentiometric titration experiments on β-lactoglobulin in order to extend our information over a wider range of charge and ionic strength. With these, and the results of Timasheff *et al.*,[5] in hand, we are also able to improve our previous comparison of existing potentiometric titration data on β-lactoglobulin with the numerical solutions of the Poisson–Boltzmann equation by taking dissociation into account. The reliability of such calculations of electrostatic free energy of proteins is thus better assessed.

Experimental Section

Protein Samples. The β-lactoglobulin used was obtained from pooled milk and three times crystallized; it was purchased from Pentex Inc., Kankakee, Ill. (Lot 29). About 1 g of the sample was dissolved in 100 ml of deionized water, and the solution was purified by centrifugation at 10,000 rpm (no. 21 rotor) in the Spinco Model L for 45 min.

Isoionic solutions were prepared by passage through a mixed-bed ion-exchange resin column, Amberlite MB-1. The isoionic pH was found to be 5.26 ± 0.02; the change in the isoionic pH with addition of NaCl is reported in a separate paper.[12]

Several potentiometric titration experiments were carried out by using the isoionic solutions. However, since isoionic β-lactoglobulin is not very soluble, most experiments were carried out using the original sample solution without ion exchange. By comparison of numerous potentiometric titration curves for isoionic solutions with those for nondeionized solutions, it was found that the nondeionized sample consisted of molecules with +3.3 protonic charges per 35,500 amu. If we take this charge number into account, no difference is found between the potentiometric titration data for the two solutions. The original sample simply contained a small amount of salt. This was confirmed by converting the ash of the sample into the sulfate in a platinum dish; the resulting salt was completely water soluble and showed the strong flame test characteristic of sodium; we felt it safe to assume, therefore, that the salt contained no calcium but was mostly the sodium salt.

Potentiometric Titration. Measurements of pH were carried out at 25° ± 2° with a Radiometer M4 instrument. NBS or Beckman pH standards were used.

The sample solution (15 ml, *ca.* 1%) was diluted to 30 ml by addition of a NaCl solution appropriate to yield the desired salt concentration. The solution was titrated with 0.05 N HCl or NaOH standard solutions containing the same concentration of NaCl, using a TESA microburet of 0.0001-ml sensitivity. The effect on the experimental data of the dilution of protein solution resulting from the titration itself is negligible.

Since Townend, Timasheff, *et al.*, reported the values of ΔG^∞ as a function of pH, it was necessary to determine the values of Z at these pH's from the potentiometric titration data. The titration curve for 0.1 M NaCl solutions is shown in Figure 1 as an example. The charge number (Z) of the protein molecule can be calculated from curve 3 as a function of pH. For a detailed explanation, the reader is referred to the legend of Figure 1 and to a previous paper.[12] The charge numbers so obtained are listed in Table I along with the values of ΔG^∞ from ref 5. The degree of dissociation (α) of the carboxyl groups was also calculated from curve 3 of Figure 1.

The Model and the Computation of Theoretical Electrical Potentials. The method employed follows closely our earlier work,[9]

(9) N. Nagasawa and A. Holtzer, *J. Amer. Chem. Soc.*, **86**, 531 (1964).

(10) M. Nagasawa and A. Holtzer, *ibid.*, **86**, 538 (1964).
(11) D. Olander and A. Holtzer, *ibid.*, **90**, 4549 (1968).
(12) M. Nagasawa and I. Noda, *ibid.*, **90**, 7200 (1968).

Table I

[NaCl], M	0.1	0.1	0.1	0.1	0.1	0.1	0.03	0.3	
pH	1.6	2.0	2.5	2.7	3.0	3.5	2.7	2.7	
Z	39.1	38.4	36.3	34.8	31.6	24.4	33.0	36.8	
ΔG^{∞}, ref 5, kcal/ru	4.9	4.9	5.5	5.7	5.8	7.3	3.9	8.3	
$-\Delta G_{el}^{\infty}$, kcal/ru	5.7	5.6	5.1	4.2	3.8	2.6	6.9	4.0	
ΔG_a^{∞}, kcal/ru	10.6	10.5	10.6	10.3	9.7	9.9	10.8	12.3	Av 10.6 ± 1

to which the reader is referred for procedural details and primary references. As is discussed in that work the pH of a protein solution is given by

$$pK_{app} \equiv pH - \log [\alpha/(1 - \alpha)] = pK_0 - 0.434\phi_b \quad (5)$$

in which the "apparent" pK is defined by the left-hand equality, pK_0 is the intrinsic ionization constant of (in the present instance) carboxyl groups, α is their degree of ionization, and $\phi_b = \epsilon\psi_b/kT$, where ϵ is the magnitude of the protonic charge and ϕ_b is the electrostatic potential at the point on the molecule from which the ionizable protons are replaced or removed, *i.e.*, a point at the molecular surface. Computation of the titration curve hence requires computation of ϕ_b.

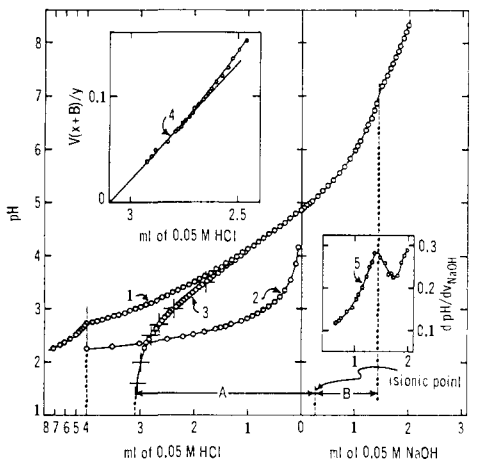

Figure 1. Example of the potentiometric titration data for β-lactoglobulin: NaCl concentration, 0.100 M; protein concentration, 0.500%. Curves 1, 2, and 3 denote the observed pH, a blank experiment, and the amount of bound hydrogen ion, respectively. The maximum amount of bound hydrogen ion (A) was determined by extrapolation of curve 4 to zero ordinate. The relevent relationship is[12] $V[(x + B)/y] = (A - x)/K$, where x and y are the numbers of moles of bound and of free hydrogen ion, respectively, in a solution of volume V and K is the carboxyl ionization constant. The meaning of B and the method for its determination are clear from the figure. It should not be confused with the B of eq 6; the context should make clear which B is under discussion. Curve 5 shows the ratio of the difference in two successive ordinates ("dpH") to the corresponding difference of abscissas ("dV_{NaOH}"). The maximum in this differential curve gives the end point.

To this end, the protein molecule (be it dimer or subunit) is assumed to be a sphere of radius b uniformly smeared with charge. The distance of closest approach of counterions (*i.e.*, the quantity a, the radius of protein sphere plus the counterion radius), is chosen as 26.2 Å[9,13] for the dimer and, on the assumption that dissociation is isochoric, $26.2/2^{1/3}$ Å for the subunit.[14] The quantity ϕ_a, the reduced potential at the distance of closest approach, is then obtained from computer solutions to the nonlinearized Poisson–Boltzmann equation[9] and converted to the desired (molecular surface) potentials using

$$\phi_b = \phi_a + BZ \quad (6)$$

$$B = (\epsilon^2/DkT)[(a - b)/ab]$$

where D is the dielectric constant of the medium. The quantity B is adjusted to provide the best fit to the data.[9] As explained in the caption to Figure 1, this new B should not be confused with the B in that figure.

Ideally, one would fit the data for pure dimer (at low charge) and monomer (at high charge) independently and find that the same value of B is required for both. At high ionic strength, since no dissociation is observed during the titration, experimental data can be compared with the theoretical values for pure dimer. Even at the lowest ionic strength in this work, however, complete dissociation does not obtain over a significantly large segment of the experimental titration curve to make the comparison between experiments and theory practical for pure monomer.

The data at low ionic strength, therefore, can only be fit directly if measurements of the relative amounts of monomer and dimer are already available. The degree of dissociation into monomers, β, can be determined by various methods. The light scattering measurements of Timasheff, Townend, *et al.*, provide data at some ionic strengths. If such information is not at hand, as, for example, at 0.01 M NaCl where light scattering measurements of β have not been made, the dissociation constant K can be calculated from eq 3 and 4, using the values of ΔG_{el}^{∞} and ΔG_a^{∞} (obtained as shown below), and then β calculated from eq 1.

The value of ΔG_{el}^{∞} is estimated as the difference in the electrical work required to charge up the original molecule, assumed to be spherical and uniformly smeared with charge, and that required for the two subunits each assumed to be a sphere uniformly smeared with half that charge

$$\Delta G_{el}^{\infty}/RT = 2\int_0^{Z/2} \phi_b' dZ - \int_0^{Z} \phi_b dZ \quad (7)$$

where the prime denotes the subunit. The actual computation of ΔG_{el}^{∞} from the ϕ_b's was done graphically. In order to estimate ϕ_b from computer results, however, we must know the value of B, as is clear from eq 6. In this paper, successive approximations were used. That is, the value $B = 0$ was first used to compute an approximate ΔG_{el}^{∞}. This, combined with the value of ΔG_a^{∞} obtained as shown below, provides ΔG^{∞} and, through eq 3 and 1, the value of β. Changes in B are then made until the most satisfactory curve is obtained. The value of ϕ_b for the equilibrium *mixture* of monomer and dimer is determined as the weighted numerical average of the values for monomer and dimer, and then the titration curve is computed from eq 5.

In our previous paper, the degree of helix in the helix–coil transition of poly(glutamic acid) was calculated from the potentiometric titration data. In this work, the analogous quantity β could be calculated if both experimental potentiometric titration curves for pure monomer and pure dimer were at hand. The method, unfortunately, could not be used in this work, since as noted above, the titration curves for monomer are not experimentally accessible over a sufficiently wide range of charge.

Results

Analysis of Potentiometric Titration Curves. The experimental values of pK_{app} are plotted against Z (by which is meant, in this case, the average number of protonic charges *per 35,500 amu*) in Figures 2 and 3 and are to be compared with those calculated from theoretical values of ϕ_b. The estimated error for the ex-

(13) Y. Nozaki, L. G. Bunville, and C. Tanford, *J. Amer. Chem. Soc.*, 81, 5523 (1959).

(14) Strictly speaking, the assumption of constant volume requires that the molecular radii (b) be in the ratio $2^{1/3}$, leading to the relationship $a' = (a/2^{1/3} + [(2^{1/3} - 1)r/2^{1/3}]$ between the distances of closest approach, where r = the radius of the counterion. Since the second term is so small, however, and since it is considerably more convenient to choose the approach distance first and then adjust r ($= a - b$) to obtain the best b, we have used $a' \simeq a/2^{1/3}$. The effect on the results is immaterial.

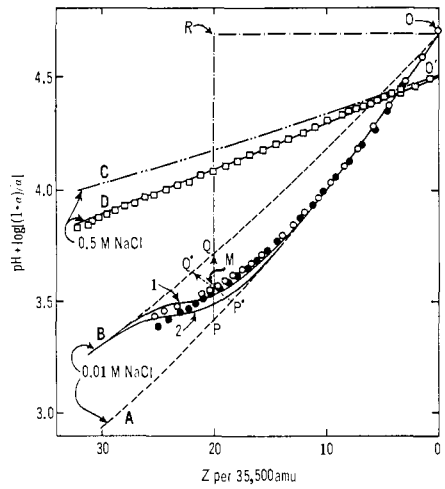

Figure 2. Theoretical and experimental curves of pK_{app} *vs.* Z for the carboxyl groups of β-lactoglobulin in 0.5 and 0.01 *M* NaCl solutions. Theoretical curves O'C and O'D are for dimer in 0.5 *M* NaCl with $a = 26.2$ Å. For curve O'C, $a - b = 0$; for O'D, $a - b = 1.2$ Å. Squares are data points for 0.469% protein in 0.5 *M* NaCl. Dashed curve OP'PA (OQQ'B) is calculated from theory for dimer (subunit) in 0.01 *M* NaCl with $a = 26.2$ Å ($26.2/2^{1/3}$ Å) and $a - b = 1.4$ Å. Solid curves are those calculated as described in the text for mixtures of dimer and subunits using the dashed curves as a basis. The upper solid curve (1) is for 0.194% protein, the lower (2) for 0.469%. Circles denote experimental points for 0.194% protein, the lower (2) for 0.469%. Circles denote experimental points for 0.194% protein and filled circles for 0.469% in 0.01 *M* NaCl. Points M, P, P', Q, and Q' correspond to those so designated in Figure 4. There was a difference of 0.02 pH unit between pK_0's for the experiments with 0.194 and 0.469% in 0.01 *M* NaCl. To make comparison easier, the experimental points for 0.469% in 0.01 NaCl were shifted toward the experimental points for 0.194% by 0.02 pH unit.

perimental points at the highest charge in 0.01 *M* NaCl of Figure 2 is ±0.025 pH unit. For solutions at high ionic strength, the agreement between the observed values and those calculated for the undissociated original molecule is very good, though the constant B of eq 6 must be chosen arbitrarily. One example is shown in Figure 2. O'C is the graph of pK_{app} *vs.* Z calculated for the dimer in 0.5 *M* NaCl with $a - b = 0$, while O'D was calculated with $a - b = 1.2$ Å. In 0.5 *M* NaCl solution, dissociation of protein into its subunits is inappreciable.

In 0.01 *M* NaCl, however, there is a considerable amount of dissociation at high charge densities. When we fit calculated graphs of pK_{app} *vs.* Z with experimental data, choosing the same value for $a - b$ as for the 0.5 *M* data, clear deviations from theory are observed if Z is higher than about 16. The agreement can be extended, however, by adjusting somewhat the constant B (*i.e.*, $a - b$). In our previous paper,[9] neglect of dissociation led to $a - b = 0$ as the most appropriate value. Taking dissociation into account, we now find that $a - b = 1.4$ Å provides the best fit with the experimental results, as is shown in Figure 2. Although agreement is still not perfect, it seems to us that the potentiometric titration data of β-lactoglobulin are well represented by the calculated graphs, at least up to $Z = 25$. Similar findings at intermediate ionic strengths are shown in Figure 3.

Determination of ΔG_a. This quantity was determined in two ways. The first method is quite analogous to that of Timasheff, *et al.* That is, the equilibrium of eq

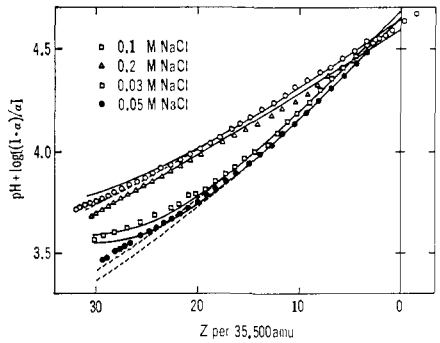

Figure 3. Theoretical and experimental curves of pK_{app} *vs.* Z for the carboxyl groups of β-lactoglobulin in various NaCl solutions. Broken lines are the curves calculated for the dimer, *i.e.*, the molecule of 35,300 amu. Solid lines are the curves calculated when dissociation of the samples is taken into account. The values of $a - b$ used are 1.6 Å for 0.03 *M* NaCl, 0.7 Å for 0.05 *M*, 0.4 Å for 0.1 *M*, and 1.6 Å for 0.2 *M*.

2 is assumed to describe the system and the electrostatic part of the standard free energy change, ΔG_{el}^{∞} for the dissociation is estimated from eq 7. For example, in Figure 2 the dashed curve OP'PA represents the pK_{app} *vs.* Z plot for the dimer in 0.01 *M* NaCl, and the area OP'PQRO, for example, gives the electrostatic free energy of the dimer having $Z = 20$. The dashed line OQQ'B represents pK_{app}', the apparent pK of a subunit, plotted *vs.* Z *of the original molecule* (*i.e.*, per 35,500 amu) for the same ionic strength; the area OQRO is therefore the electrostatic free energy of *two* subunits, each with charge $Z = 10$. Therefore, ΔG_{el}^{∞} for the process of eq 2 with $Z = 20$ is given by the area OPQO. The pH of the solution has already been connected with Z by the titration curve, so the corresponding values of ΔG^{∞} may be selected from the results of ref 5, and the value of ΔG_a^{∞} computed using eq 4. All three quantities are listed in Table I, which clearly shows that the present computation, which differs from the work of Timasheff, Townend, and coworkers only in its avoidance of the Debye–Hückel approximation, provides values of ΔG_a^{∞} that are almost independent of pH and ionic strength, the average value being +10.6 ± 1 kcal/ru.

The second method used to determine ΔG_a^{∞} is based on the same principle as the determination of the standard free energy of the helix–coil transition described earlier.[10] In this method, the area OMBQO (*i.e.*, the area defined by the experimental points and the subunit curve) is related to ΔG_a^{∞} by a line of reasoning closely analogous to that outlined for the helix–coil transition in Appendix I of the earlier work.[10] The only differences are that, in the present case, protons are successively added to a positively charged macromolecule instead of being removed from a negatively charged one, and that the quantity G_{ion} in the equation

$$pK_{app} - pK_0 = -(0.434/RT)(\partial G_{ion}/\partial Z) \qquad (8)$$

must include as "other free energy changes" accompanying titration, the mass action contribution[15]

(15) The terminology we employ here requires clarification. When one stoichiometric unit of a dissociation reaction is carried out in a very large volume of solution at constant temperature and pressure, the Gibbs free energy change contains terms like $RT \ln [C_1^2/C_2]$, where C_1 is the concentration of monomer and C_2 of dimer in whatever units are preferred. We refer to such terms as the "mass action" contribution to the free energy change. If mole fraction units are used, this contribu-

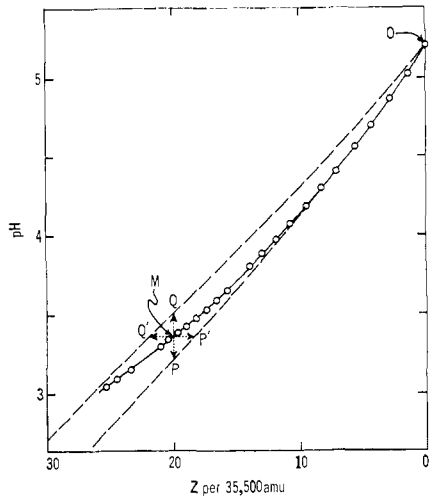

Figure 4. Theoretical and experimental curves of pH *vs.* Z for β-lactoglobulin in 0.01 M NaCl. Curve OP′P is the theoretical curve for the dimer and OQQ′ for the monomer, both with $a - b =$ 1.4 Å. Experimental points are for 0.196% protein in 0.01 M NaCl. Points M, P, P′, Q, and Q′ correspond to those so designated in Figure 2.

caused by dissociation. Consequently, integration of the experimental curve provides an area A_1 which is

$$A_1 = -(0.434/RT)\int_{Z=0}^{Z_m}(\partial G_{ion}/\partial Z)dZ = -(0.434/RT)\Delta G_1 \quad (9)$$

where Z_m is the maximum charge possible on a dimer and ΔG_1 is the protein part of the total *standard* free energy change (except that associated intrinsically or combinatorially with carboxyl ionization) plus the protein part of the electrical free energy change plus the protein part of the mass actional free energy change associated with the process

$$Z_mH^+ + P_2^0 \longrightarrow 2P^{Z_m/2+}$$

If we *could* back-titrate the system to the discharged state while disallowing dimerization, integration would provide an analogous quantity (ΔG_2) for the subunits

$$A_2 = -(0.434/RT)\int_{Z_m}^{0}(\partial G_{s,ion}/\partial Z)\,dZ = -(0.434/RT)\Delta G_2 \quad (10)$$

referring, in the same way, to the process $2P^{Z_m/2+} \rightarrow 2P^0 + Z_mH^+$. The sum gives

$$A = A_1 + A_2 = -(0.434/RT)(\Delta G_1 + \Delta G_2) = -(0.434/RT)\Delta G \quad (11)$$

for the process $P_2^0 \rightarrow 2P^0$ where, since all electrical contributions must come to naught, ΔG is seen to be the *standard* free energy change *plus* the mass actional free energy change for the transformation of uncharged dimer to uncharged monomer at the concentration of the experiment. Thus

$$\Delta G = \Delta G_a^\infty + RT \ln [(2C)^2/C] = \Delta G_a^\infty + RT \ln (4C) \quad (12)$$

where C is the total formality (as dimer) of protein in the experiment. In eq 12 we identify the standard free energy for the dissociation of uncharged molecules with the nonelectrostatic part of the standard free energy for the dissociation of charged species. The values of ΔG_a^∞ so calculated from the area (which is algebraically negative) OMBQO = $A_1 + A_2$ of Figure 2 and the total concentration are given in Table II. The average value is 10.0 kcal/ru.

Table II. ΔG_a^∞ by the "Area" Method from Data for 0.01 M NaCl

[Protein], %	ΔG^∞, kcal/ru	ΔG_a^∞, kcal/ru
0.194	4.7	9.7
0.469	5.7	10.2

Discussion

In all cases, the fit of theoretical to experimental curves is, it seems to us, satisfactory if small adjustments in B are allowed. The values of B required are more meaningful than they are if dissociation is left out of account, as was done originally.[9]

Some of the discrepancy that remains between theory and experiment may be caused by the assumption of spherical shape for both dimer and its subunits. According to Timasheff, Townend, *et al.*, the subunits are almost spherical while the dimer may be approximated by a prolate ellipsoid of revolution having axial ratio 2:1. Although the nonspherical shape of the dimer is not apparent from the titration curve (*i.e.*, good agreement is obtained between theory and experiment at high ionic strengths), this might simply be a result of compensation of the effects of shape by proper choice of B. It may be, then, that slightly different values of B ought to be used for the dimer and for its subunits. We have chosen to ignore this possible difference because it is only possible to obtain data for the completely dissociated system over a very narrow range of charge. Such a difference cannot produce any great change in values of ΔG_a^∞, however, because the term BZ almost cancels out in the computation, as is plain from eq 6 and 7.

It is clear from Table I that values of ΔG_a^∞ obtained without the use of the Debye–Hückel approximation are independent of salt concentration and pH. We believe this to be more likely, and ascribe the earlier, contrary finding[5] to use of this approximation beyond its applicable range.

The area method provides a numerical value for ΔG_a^∞ (10.0 kcal/ru) that is in close agreement with that provided by the use of eq 2 as a model for the reaction (10.6 kcal/ru). While this would appear to justify eq 2, we believe that the logical superiority of the area method recommends it as the method of choice wherever the data allow. The conceptual difficulties attending eq 2 become more apparent when pH is plotted directly *vs.* charge as in Figure 4. It is clear from the figure that the system at point M (a 0.194% solution of β-lactoglobulin in 0.01 M NaCl at pH 3.42) is not a mixture of dimers of charge $Z = 20$ and subunits each of charge 10 (*i.e.*, of P and Q), but rather a mixture of dimers with mean charge 18 (P′) and subunits each with mean charge 11 (Q′). Numerically, however, the difference is negligible. The points P, P′, Q, Q′, and M of Figure 4 are also shown in Figure 2. Moreover,

tion is also called the "cratic" term; see R. W. Gurney, "Ionic Processes in Solution," McGraw-Hill, New York, N. Y., 1953.

as was explained in the section on the computation of ϕ_b, ϕ_b for the mixture M was obtained as the average of the values for P and Q. Strictly, it should be given by the average of the values for P′ and Q′. Numerically, however, the difference between these average values is negligible.

About the numerical value of ΔG_a^∞ little can be said at present since the chemical groupings on the surface of contact between subunits are not known. If such information becomes available, through crystal structure determinations, the value of ΔG_a^∞ will serve as a test of our ability to calculate intermolecular forces. In that connection, of course, it would be advantageous to determine ΔH_a^∞ and ΔS_a^∞ from, say, the temperature dependence of ΔG_a^∞. To that end, however, extensive further experimentation is required.

Characterization and Triplet-State Electron Paramagnetic Resonance Spectra of the Binuclear [Molybdenum(V)]$_2$–Glutathione Complex. An Example of Molybdenum–Polypeptide Complexes

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Abstract: A diamagnetic binuclear [Mo(V)]$_2$–glutathione complex, $Na[Mo_2O_4(glutat)(H_2O)]\cdot 3H_2O$, has been isolated and characterized. Infrared analysis indicates that glutathione serves as a pentadentate ligand. Epr studies in 0.2 *M* phosphate buffer solution over a pH range from 8 to 10 show that Mo(V) with glutathione forms a diamagnetic binuclear species in which the Mo_2O_4 group dissociates to form a paramagnetic binuclear species. An equilibrium between these two species exists in solution. The 11-line hyperfine splitting observed using isotopically enriched ^{95}Mo(V) has given unambiguous indication of electron-exchange coupling in the paramagnetic binuclear [Mo(V)]$_2$–glutathione complex. Under the imperfect assumption of axial symmetry, the relevant epr parameters of this paramagnetic complex are $g = 1.962$, $g_{||} = 1.966$, $g_\perp = 1.960$, $a = 0.0029$ cm^{-1}, $A = 0.0047$ cm^{-1}, $B = 0.0020$ cm^{-1}, and $D = 0.0083$ cm^{-1}. From the zero-field splitting constant, D, and molecular models, the Mo–Mo distance is estimated as ~6.0 Å. A possible structure based on the intermetallic distance is proposed for the paramagnetic [Mo(V)]$_2$–glutathione complex. The biological implications for molybdenum-containing enzymes is also discussed.

Molybdenum–sulfur bonding[1–6] has been of great interest to scientists because of its biological implications. As part of our study on the molybdenum–amino acid complexes[5,6] as possible models for molybdenum–enzyme interaction, we have used glutathione, a cysteine-containing short polypeptide (γ-L-glutamyl-L-cysteinylglycine), as a ligand for our present investigation. Our previous study[5,6] on the Mo(V)–cysteine complex, $Na_2Mo_2O_4(cyst)_2\cdot 5H_2O$, indicated that the dioxo bridge can be broken by the attack of OH^- in solution, or by heat in the solid state, to form a monomeric species which gives rise to an epr signal. The lability of the dioxo bridge is created by the sulfhydryl group of the polydentate ligands and enhanced by increasing the chain length of the ligand. According to these results, glutathione appears to be a better simple ligand for this type of study.

A recent study of molybdenum(V)–thiol complexes[2] reported that no epr signal could be observed from an aqueous solution containing Mo(V) and glutathione. On the contrary, we have obtained well-defined signals from this system. Moreover, although considerable attention has been devoted to the epr study of paramagnetic binuclear complexes, such as vanadyl–tartrate[7–11] and copper–tartrate complexes,[9] no report has been made on paramagnetic molybdenum binuclear species. Here, we report the existence of a paramagnetic binuclear [Mo(V)]$_2$–glutathione complex for the first time.

In this study, a diamagnetic binuclear [Mo(V)]$_2$–glutathione complex has been isolated and characterized. The ir analysis indicates that glutathione serves as a pentadentate ligand. Epr studies show that glutathione with Mo(V) forms a diamagnetic dimer in which the Mo_2O_4 group dissociates to form a paramagnetic dimer. Its electron paramagnetic resonance spectra show that electron-exchange coupling occurs in this complex. Hyperfine splitting observed using isotopically enriched ^{95}Mo gives unambiguous indication of such coupling. The triplet state of this complex is shown to exist both in the frozen-solution epr spectrum and in the liquid-phase epr spectrum.

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