Molecular Interactions in β-Lactoglobulin. VI. The Dissociation of the Genetic Species of β-Lactoglobulin at Acid pH's[2]

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A sedimentation and light scattering study of the dissociation of β-lactoglobulins A and B below pH 3.5 has been carried out. No great differences in the dissociation pattern of the two exist between pH 2 and 4. Detailed light scattering measurements at pH 2.7 revealed a difference of 0.6 kcal./mole in the free energies of dissociation of the two, β-lactoglobulin A dissociating more readily than β-B. The free energy of attraction between the two half-molecules has been calculated to be −9 to −10 kcal./mole over the pH range of 1.6 to 3.5.

Introduction

In previous communications[3,4] it has been shown that the 36,000 molecular weight species of β-lactoglobulin obtained from pooled milk undergoes a molecular dissociation into half-units in the pH region below 3.5. Since it is known that β-lactoglobulin contains two genetically different species,[5] which differ in their association behavior between pH 3.7 and 5.2,[6,7] it was of interest to establish whether these two closely related proteins differ with regards to the dissociation reaction. It is the purpose of this paper to present ultracentrifugal and light scattering studies on β-lactoglobulins A (β-A) and B (β-B) aimed at answering this question.

Experimental

Materials.—The genetically different β-lactoglobulins used in this study were prepared by the method of Aschaffenburg[8] from the milk of individual cows which had first been typed as β-A- or β-B-producing.[9]

Methods.—Ultracentrifuge experiments were performed in a Spinco Model E[10] analytical ultracentrifuge at 59,780 r.p.m., equipped with a phaseplate and a temperature control unit. All runs were done in Kel-F cells. Sedimentation constants were measured with a microcomparator.

Light scattering measurements were carried out in the Brice photometer,[11] using the previously described technique[12] for such studies as a function of temperature. Since no great differences in behavior were expected, the following steps were taken to assure internal consistency. Light scattering measurements on the two proteins were taken simultaneously, individual points being measured alternately on the two. The data were accumulated in several individual runs, and the two sets of cells used in these experiments were switched between β-A and β-B. It was considered that in this manner possible errors due to instrumental variations and individual cell differences could be eliminated.

Protein concentrations were determined spectrophotometrically at 278 mμ, using the absorptivity value[13] of 0.96 liter^{-1} cm.$^{-1}$ for both β-lactoglobulins. The value of the refractive increment for both proteins was taken as 0.1890,[14] and they were shown to be the same within experimental error. The pH's were measured at 25° using a Beckman Model G[10] pH meter.

Results and Discussion

The dependence of the sedimentation constant on pH at 2 and 25° for solutions of β-A and β-B (10 g./l.) in 0.1 *N* NaCl–HCl is shown in Fig. 1. For the purpose of comparison the best curve through the previously published points[4] obtained with pooled milk protein is shown by the dashed line. As was the case for the mixed protein, the sedimentation constants of both individual species decrease below pH 4 from a value of about 2.95 to 2.35 *S*. Comparison of the four sets of points reveals no significant difference in the pattern of this behavior. The values of the sedimentation constants (25°) of β-A and β-B at various pH's between 2 and 5.5 are in good agreement with the previous data on pooled milk protein.[15] The $s_{20,w}$ values obtained on β-A and β-B at 2 and 25° can also be considered as identical within experimental error, as the spread at any given pH is never greater than 3–5%. Thus, ultracentrifugal measurements show that the dissociation patterns of β-A and β-B between pH 2 and 4 are similar and do not differ within experimental error from that of the mixture of proteins.

In order to ascertain whether any differences, too small to be detected ultracentrifugally, exist between this dissociation of the two proteins, a light scattering investigation of the reaction was carried out as a function of temperature between 4.5 and 25°. Since it could be expected that most promising conditions for revealing small differences would be those at which the dissociation was sufficiently strong to give a well measurable effect, but not strong enough to be near completion in a moderate concentration range, the measurements were

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

(2) This work was presented in part at the 136th National Meeting of the American Chemical Society, Atlantic City, September 1959.

(3) R. Townend and S. N. Timasheff, This Journal, **79**, 3613 (1957).

(4) R. Townend, L. Weinberger and S. N. Timasheff, *ibid.*, **82**, 3175 (1960). (Paper IV).

(5) R. Aschaffenburg and J. Drewry, *Nature*, **176**, 218 (1955); R. D. Plowman, R. Townend, C. A. Kiddy and S. N. Timasheff, *J. Dairy Sci.*, **42**, 922 (1959) (Abst.).

(6) R. Townend and S. N. Timasheff, This Journal, **80**, 4433 (1958).

(7) S. N. Timasheff and R. Townend, *ibid.*, **83**, 464 (1961) (Paper V).

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

(9) This part of the work was carried out in collaboration with Drs. N. Bayley, R. D. Plowman and C. A. Kiddy of the Dairy Cattle Research Branch, U. S. Department of Agriculture, and will be reported separately.

(10) Mention of the above does not imply endorsement by the U. S. Department of Agriculture over others not mentioned.

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

(12) R. Townend and S. N. Timasheff, This Journal **82**, 3168 (1960) (Paper III).

(13) R. Townend, R. J. Winterbottom and S. N. Timasheff, *ibid.*, **82**, 3161 (1960) (Paper II).

(14) M. Halwer, G. C. Nutting and B. A. Brice, *ibid.*, **73**, 2789 (1951).

(15) The slight upward deviation of the individual protein $s_{20,w}$ values, which is never greater than 4%, may be due to different methods of temperature measurement. In the runs on β-A and β-B the temperature was controlled and measured with a Spinco RTIC unit.[10] In the earlier work[4] many of the points had been obtained without such a unit, the temperature of the rotor being measured immediately before and after the run.

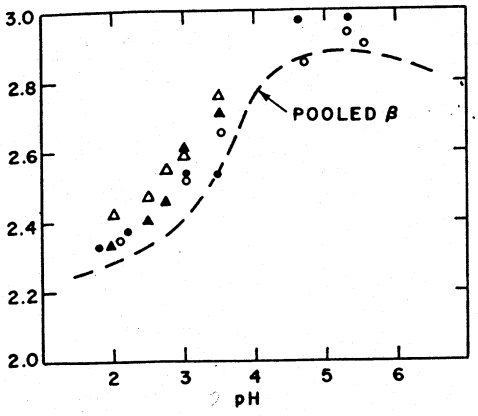

Fig. 1.—Ultracentrifugal data on β-lactoglobulins A and B at 10 g./l., 0.1 ionic strength NaCl–HCl. △, β-A 2°; ▲, β-B 2°; O, β-A 25°; ●, β-B 25°; dashed line, best line drawn through the points obtained at 25° with pooled milk protein.[4]

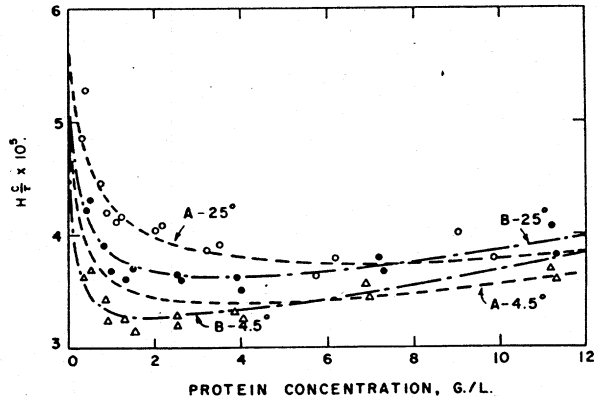

Fig. 2.—Light scattering data of β-lactoglobulins A and B at pH 2.7, 0.1 ionic strength NaCl–HCl. O, β-A at 25°; ●, β-B at 25°; △, β-B at 4.5°; lines calculated from the equilibrium constants and second virial coefficients as described in the text; dashed lines, β-A; dot-dash lines, β-A.

carried out at an intermediate pH in the zone of dissociation, *i.e.*, pH 2.7 at 0.1 ionic strength NaCl–HCl. For similar reasons this pH had been chosen in the previous study[4] for investigation of the effect of ionic strength and dielectric constant.

The light scattering results on the two proteins at 4.5 and 25° are shown in Fig. 2.[16] Although the general shape of the curves is similar to those obtained at room temperature with the pooled milk protein, two features are noteworthy: 1. In both cases the values of $HC_2/\Delta\tau$ at 4.5° fall below those obtained at identical concentrations at 25°; 2. The two proteins differ in their values of turbidity at both temperatures, this difference being larger at 25°. At both temperatures the β-B points fall below those of β-A in the low concentration region, indicating that here β-B dissociates to a somewhat lesser extent than β-A, while in the high concentration region the two sets of points are practically superimposable, indicating that β-B has a greater positive second virial coefficient in light scattering than β-A. This last observation is consistent[17] with the fact that at this pH β-B has a slightly higher net average charge than β-A.[18,19] (It should be recalled that β-A has two more titratable carboxyl groups per 36,000 molecular weight species than β-B.[18]) The data at 8° and 15° displayed a similar behavior. These data were analyzed by the previously described method,[4] and equilibrium constants and second virial coefficients were obtained.[20] These values are presented in Table I. Average light scattering curves were then calculated from the values of K_d and $2B_0/M_{1/2}$ as described previously.[4] These are shown by the curves in Fig. 2. The good coincidence of these curves with the experimental points verifies the consistency of the values of Table I with the experimental data.

As shown in Table I, the free energies of dissociation of the two proteins differ from each other by 0.6 kcal./mole over the temperature range studied. Since the data on Fig. 2 and Table I represent several independent experiments, this difference must be considered as real and outside of experimental error. The same must be true of the small changes in free energy with temperature observed for the two proteins. Comparison of the values of ΔF^0 of dissociation of β-A and β-B obtained at 25° with that previously reported for pooled milk β-lactoglobulin[4] (5.7 ± 0.1 kcal./mole) seems to indicate reasonable agreement. If one were to consider, however, that the two proteins dissociate independently and cannot enter into mixed reassociation forming hybrids, then the value of K_d obtained with the pooled protein is only an apparent equilibrium constant. In such a case, the amount of dissociated protein present under any given conditions is the sum of the two independently dissociated species, which, from the point of view of the data analysis, have identical molecular weights only as a coincidence. Using the equilibrium constants of Table I, the amount of total dissociated protein present at any total β-lactoglobulin concentration (using 69% β-A and 31% β-B, the composition of pooled β[19]) was calculated. From that an apparent K_d was evaluated. This constant has a value of 1.6×10^{-4} moles/liter and corresponds to an apparent ΔF^0 of dissociation of 5.2 kcal./mole. This is in fair agreement with the measured value of 5.7 kcal./mole, since an error of 3% in the assignment of the light scattering intercept would be sufficient to account for such a discrepancy.[21] Thus, comparison of the values of ΔF_0 of β-A and β-B with that of the pooled protein does not permit a conclusion as to whether mixed reassociation can take place.

From the equilibrium constants, values of ΔH^0

(16) For the sake of not overcrowding the figure the experimental points on β-A at 4.5° have been left out. For the same reason data at intermediate temperatures have not been included.

(17) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, This Journal, **79**, 782 (1957).

(18) C. Tanford and Y. Nozaki, *J. Biol. Chem.*, **234**, 2874 (1959).

(19) S. N. Timasheff and R. Townend, This Journal, **82**, 3157 (1960) (Paper I).

(20) It should be noted that for these calculations it is necessary to know the molecular weight of the dissociated species. 17,500 was taken as a reasonable value and the light scattering intercept at zero concentration was assigned a value of 5.71×10^{-5}.

(21) It should be stated that between the two studies, the light scattering instrument used had been greatly rebuilt and then recalibrated. A 3% uncertainty in calibration of light scattering is quite reasonable.

TABLE I

DISSOCIATION OF β-LACTOGLOBULINS A AND B AT pH 2.7

Protein	Temp., °C.	K_d, mole/l × 10^6	ΔF^0, kcal./mole	ΔH^0, kcal./mole	ΔS^0, e.u.	$2B_2/M_{1/2}$, l./g. × 10^{-7}
β-A	4.5	2.84 ± 0.6	5.8 ± 0.2	12.4 ± 1.4	23.8 ± 5	4.6
	8	4.08 ± 1.0	5.7 ± .3			
	15	7.50 ± 0.6	5.5 ± .1			
	25	13.0 ± 2	5.3 ± .1			4.8
β-B	4.5	1.00 ± 0.5	6.4 ± .3	12.8 ± 0.9	23.2 ± 3	7.0
	8	1.44 ± .6	6.2 ± .3			
	15	2.43 ± .9	6.1 ± .2			
	25	5.08 ± 1.2	5.9 ± .1			6.9

and ΔS^0 of dissociation of β-A and β-B into half-units were calculated. These are presented in Table I. In Fig. 3, the values of log K_d are plotted *vs.* $1/T$ for the two proteins. The slopes obtained are identical within experimental error, and differences in enthalpies and entropies of dissociation of the two proteins are too small to be discussed with significance.

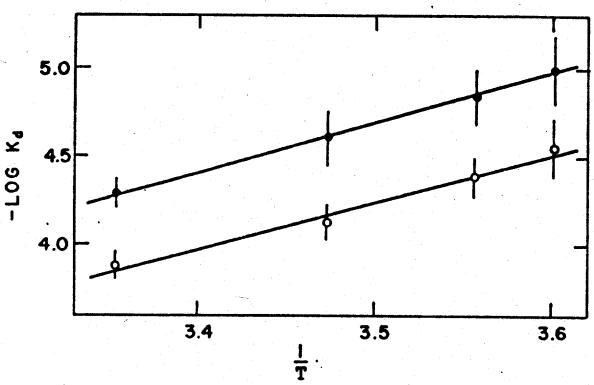

Fig. 3.—ΔH^0 plots for the dissociation of the genetic species of β-lactoglobulin at pH 2.7: O, β-A; ●, β-B.

In the previous study, it had been shown[4] that electrostatic repulsion plays an important role in the dissociation of β-lactoglobulin; therefore, the values of $\Delta(\Delta F^e)$, the change in electrostatic free energy of dissociation, were calulated for β-A and β-B. For the Green and Aschaffenburg spherical model[22] of the β-A and β-B sub units, the Verwey and Overbeek equation[23] for the repulsive energy between two touching spheres can be used directly, with the assumption of uniform charge distribution on the spheres. Then

$$-\Delta(\Delta F^e) = \frac{\psi_0^2 D b^2}{R} e^{-\kappa(R-2b)} \gamma \qquad (1)$$

where ψ_0 is the surface potential of the molecule and can be calculated from the Debye–Hückel theory,[24] D is the dielectric constant of the medium, b is the radius of the protein molecule, R is the distance between the centers of the two spheres, κ is the Debye–Hückel screening parameter and γ is a complicated function of κb and R/b, values of which have been tabulated by Verwey and Overbeek.[23] In the case of two touching spheres $R = 2b$. Using this equation and the titration data of Tanford[25] the values of $\Delta(\Delta F^e)$, calculated for β-A and β-B at pH 2.7 and 25°, turn out to be − 3.9 and − 4.0 kcal./mole, respectively. Combining these with the ΔF^0 values of Table I, one obtains free energies of attraction, ΔF^a, of −9.2 and −9.9 kcal./mole of β-A and β-B, respectively. Calculation of ΔF^a for pooled β-lactoglobulin at identical conditions results in a value of −9.6 kcal./mole. These calculations indicate that the attractive force which must be overcome in the dissociation of either species of β-lactoglobulin is of the order of − 9 to − 10 kcal./mole, the difference between the two proteins being too small to be regarded as significant. When a calculation of $\Delta(\Delta F^e)$ was performed according to equation 1 for pooled β-lactoglobulin, using previously reported data as a function of pH and ionic strength,[4] the free energy of attraction between the two half-molecules was found to be also between −9 and −10.5 kcal./mole and to be independent of pH and ionic strength.[26]

The data presented here show that both genetic species of β-lactoglobulin undergo a dissociation reaction in the pH region below 4. While the thermodynamic functions obtained with the two proteins are overlapping in their degree of uncertainty, the small differences in ΔF^0 observed are probably real but indicate the absence of large differences in the nature of the bonds formed between the two types of half-molecules. The difference in the light scattering second virial coefficients of the two proteins, reported in Table I, is too large to be wholly accountable in terms of the small charge difference. The less positive value in the case of β-A probably reflects the presence of stronger attraction between β-A molecules than between β-B molecules. It is interesting to note in this connection that at higher pH's (3.7 − 5.2), β-A passes through a region conducive to its strong aggregation while β-B does not.[7] It might be possible that at pH 2.7 this attractive force is already effective but is not sufficiently strong to overcome the non-specific electrostatic

(22) D. W. Green and R. Aschaffenburg, *J. Molecular Biology*, 1, 54 (1950).

(23) E. J. Verwey and J. Th. G. Overbeek, "Theory of the Stability of Lyphobic Colloids," Elsevier Publishing Co., Amsterdam, 1948.

(24) See, for example, G. Scatchard in E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Co., New York, N. Y., 1948.

(25) Y. Nozaki, L. G. Bunville and C. Tanford, THIS JOURNAL, 81, 5523 (1959).

(26) Calculation of the free energy of attraction between the two β-lactoglobulin subunits, using the previously published data on pooled β-lactoglobulin,[4] and the Verwey Overbeek potential:

Γ/2 = 0.1

pH	1.6	2.0	2.5	2.7	3.0	3.5
$\Delta(\Delta F^e)$	− 5.5	− 5.1	−4.4	−3.9	−3.2	−1.9
ΔF^a	−10.4	−10.0	−9.9	−9.6	−9.0	−9.2

pH = 2.7

Γ/2	0.03	0.3	0.1 (D = 69)
$\Delta(\Delta F^e)$	− 6.1	− 2.5	−4.2
ΔF^a	−10.0	−10.8	−9.2

repulsion between the molecules. The difference in second virial coefficients might also reflect a difference in binding of small ions by the two proteins.[27]

If β-lactoglobulin dissociates into the two equal subunits described by the Green and Aschaffenburg model,[28] the values of changes in entropies of rotation and translation can be estimated by the ideal gas equations.[28] The value obtained for their sum is *ca.* + 110 e.u. The difference between this value and the experimentally obtained value of 23–24 e.u. might be due possibly to a molecular rearrangement on dissociation which would introduce a higher degree of organization into the molecule or to a gain in water of hydration following dissociation. The immobilization of eight to ten water molecules per β-lactoglobulin submit would be sufficient to account for this. Since there is some evidence of appearance of a hydrophobic region on dissociation,[4] such an immobilization of water might occur by formation of clathrate[29] or ice-like[30] water structures. An alternate hypothesis is that the subunits of the protein are jointed by salt bridges which, upon rupture at low *p*H, allow water of electrostriction to be gained. A further contribution to the entropy term could arise from changes in proton binding during the dissociation.

The present experiments, although suggesting small differences between the dissociation patterns of the two β-lactoglobulins, leave unanswered questions on the structure of the subunits and on differences which may exist between them. Work has been initiated on this problem.

(27) S. N. Timasheff and B. D. Coleman, *Arch. Biochem. Biophys.*, **87** 63 (1960).

(28) See Glasstone, "Textbook of Physical Chemistry," 2nd Ed., D. Van Nostrand Company, New York, N. Y., page 873 ff.

(29) W. F. Claussen, *J. Chem. Phys.*, **19**, 1425 (1951); W. H. Rodebush and W. L. Masterson, *Proc. Natl. Acad. Sci., U. S.*, **40**, 17 (1954).

(30) I. M. Klotz, *Science*, **128**, 815 (1958).

[Contribution from the Palo Alto Medical Research Foundation, Palo Alto, California]

The Urea Denaturation of Chymotrypsinogen as Determined by Ultraviolet Spectral Changes. Evaluation of Additional Kinetic Constants[1]

By Charles H. Chervenka

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The investigation of the *p*H dependence of rates of urea denaturation of chymotrypsinogen has been extended in order to study the nature of three unusually acidic groups previously found. By employing lower temperatures and urea concentrations, data are obtained which allow the calculation of all of the kinetic constants of the Levy and Benaglia formula. The apparent dissociation constants of the three groups are found to be near 2, and these are postulated to be "masked" carboxylic acids. Some thermodynamic quantities are estimated.

In a previous report of studies of the urea denaturation of chymotrypsinogen,[2] the results indicated the presence in the protein of three prototropic groups with unusually low *pK* values. These dissociation constants could not be evaluated at 25° because the denaturation reactions were too rapid to measure in media more acid than about *p*H 3. We now wish to report the results of another series of experiments, in which the rates were measured over the *p*H range from 1 to 9.

Experimental

The methods and materials employed were the same as reported for the previous investigation.[2] Briefly, urea solutions containing hydrochloric acid or sodium hydroxide were equilibrated to the experimental temperature (± 0.1°) in the thermostated cuvette compartment of a Beckman model DU Spectrophotometer. A solution of five times recrystallized chymotrypsinogen was added at zero time, and the measured change in absorbance at 293 mμ with time was used to calculate first-order rate constants. All *p*H measurements were made at room temperature.

Results

Rates of Denaturation at Various Temperatures.—Figure 1 illustrates the *p*H dependence of the apparent first order rate constants of the denaturation of chymotrypsinogen at various temperatures. From the trend of these results it was obvious that further decreases of temperature alone would not reduce the rates sufficiently to allow extension of the *p*H range lower than *p*H 3. However, by using lower urea concentrations at 9.5° the range was extended to *p*H 1, as shown in Fig. 2A, data from which are plotted as the logarithms of rate constants *versus* the logarithm of the activity of urea in Fig. 2B, using the activity coefficients for urea at 25°.[3] From the relationship[4]

$$n = \frac{d \log k}{d \log U} \qquad (1)$$

the slopes of the lines in Fig. 2B give the apparent orders of reaction (n) with respect to the urea activity (U) at various *p*H values.

Composite Rate Data.—With these experimental values of the apparent order, eq. 1 was used to convert the rates of denaturation at various concentrations of urea to hypothetical values for 7.9 *M* urea. Fig. 3 shows the result of this computation and thus represents the variation in logarithm of the rate constant with *p*H from *p*H 1 to 9.

The special case of Levy and Benaglia's theory describing the *p*H dependence of denaturation rates[5] which was used previously[2] still applies to the new data at lower *p*H values; however, the

(1) This investigation was supported by research grant number A-2800 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service.

(2) C. H. Chervenka, This Journal, **82**, 582 (1960).

(3) G. Scatchard, W. J. Hamer and S. E. Wood, *ibid.*, **60**, 3061 (1938).

(4) R. B. Simpson and W. Kauzmann, *ibid.*, **75**, 5139 (1953).

(5) M. Levy and A. E. Benaglia, *J. Biol. Chem.*, **186**, 829 (1950).