with $m \sim 3$. The curve for $\bar{x} = \bar{\nu}/n$ approximately follows a similar curve with $m \sim 2$. While these results apply only to the particular situation treated, we may expect that their qualitative aspects are typical, *i.e.*, we shall often expect close correspondence between the titration curve and the transition curve and shall usually find both to be steep, such that m of equation 44 lies somewhere between 1 and n. For n titratable groups buried in the interior of a protein molecule we can expect ρ to be large, which leads to equation 44 with $m \rightarrow n$ for both y_β and $\bar{\nu}$.

The equations of this section may be applied to the expansion of a compact conformation to a more loosely coiled one under the influence of electrostatic repulsion. In this case the change in conformation is associated with *all* titratable groups. However, the effect of electrostatic interaction on K_α and K_β, and, hence, on ρ and σ, now plays the major role in the process, in contrast to the examples discussed explicitly in this paper. We shall postpone consideration of this situation to a later paper.

Discussion

An example of an ionization-linked change in conformation is the N → R transition in β-lactoglobulin, which is discussed in the following paper.[13] It will be seen that this transition is associated with a single titratable group. An example of a transition associated with several titratable groups is the acid denaturation of hemoglobin.[14] This transition obeys equation 44 with $m \simeq 5$ and it is accompanied by the titration of a large number of anomalous groups. However, both intrinsic and electrostatic factors are involved, so that the present treatment is not directly applicable.

It should be observed that the general approach we have formulated is not restricted to changes in conformation. For example, α could represent a free protein molecule and β a protein molecule to which a small molecule has been bound. The parameter y_β would represent the extent of binding, and the conclusion would be that if the extent of binding depends on pH, then there must be titratable groups which change their pK values when binding occurs. This particular situation has been discussed in considerable detail in connection with the binding of O_2 by hemoglobin.[15,16] Another possibility is that β represents a polymer of α, rather than a new conformation. In this case, if the degree of polymerization depends on pH, then the titration curve of the polymer must differ from that of the monomer. A well-known example is the polymerization of fibrin monomer.[17,18]

In conclusion, it must be noted that the general approach of this paper is not original. It should properly be regarded as an extension of the principles already formulated by Wyman[15] in connection with the hemoglobin–oxygen reaction.

Acknowledgments.—This investigation was supported by research grants G-5829 from the National Science Foundation and A-3114 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

(13) C. Tanford and V. G. Taggart, *J. Am. Chem. Soc.*, **83**, 1634 (1961).

(14) S. Beychok and J. Steinhardt, *ibid.*, **81**, 5679 (1959); references to many earlier papers on this subject are given in this paper.

(15) J. Wyman, *Advances in Protein Chem.*, **4**, 407 (1948).

(16) R. A. Alberty, *J. Am. Chem. Soc.*, **77**, 4522 (1955).

(17) H. A. Scheraga and M. Laskowski, Jr., *Advances in Protein Chem.*, **12**, 1 (1957).

(18) E. Mihalyi, *J. Biol. Chem.*, **209**, 733 (1954).

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Ionization-linked Changes in Protein Conformation. II. The N → R Transition in β-Lactoglobulin

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Near pH 7.5 native β-lactoglobulin (N) undergoes a transition to a new conformation (R), as previously described. The reaction satisfies the criteria for a conformational change involving a single titratable group. This group is a carboxyl group which appears to be buried in the hydrophobic interior of the protein molecule in conformation N. Our earlier study of this reaction showed two rather than one anomalous carboxyl group per molecule, so that there must be two locations in the protein molecule where the N → R transition occurs independently. There is presumably one location in each of β-lactoglobulin's two polypeptide chains. The thermodynamic parameters of the transition, at 25°, at each of the two locations before titration of the buried groups, are $k_0 = (\mathrm{R})/(\mathrm{N}) = 0.0025$, $\Delta F^0 = 3500$ cal./mole, $\Delta H^0 = 7000$ cal./mole, $\Delta S^0 = 12$ cal./deg. mole. These estimates depend on assignment of normal values to the thermodynamic parameters for dissociation of the carboxyl group in conformation R, so that ΔF^0 has an uncertainty of perhaps 1000 cal./mole. The uncertainty in ΔH^0 is somewhat greater than this, while ΔS^0 has an uncertainty of the order of ±7 cal./deg. mole.

A previous paper from this Laboratory[1] has described a reversible change in conformation which β-lactoglobulin undergoes near pH 7.5. It was shown that this transition is accompanied by a change in specific rotation (but not in the dispersion parameter b_0[2]), by a small change in sedimentation coefficient,[3] (but not in molecular weight[4]) and by the titration of two anomalous carboxyl groups. The curves describing the changes in optical rotation and in sedimentation coefficient are essentially superimposable, and both coincide within experimental error with the titration curve of the two anomalous groups.[5]

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(1) C. Tanford, L. G. Bunville and Y. Nozaki, *J. Am. Chem. Soc.*, **81**, 4032 (1959).

(2) C. Tanford, P. K. De and V. G. Taggart, *ibid.*, **82**, 6028 (1960).

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

(4) S. N. Timasheff, personal communication.

(5) Figure 1 of ref. 1 shows the titration curve to be somewhat steeper.

This transition is thus one which is amenable to treatment by the theory of the preceding paper.[6] It was shown in that paper that a *p*H-dependent change in conformation is inevitably associated with anomalous titration of one or more groups. We are fortunate in this case that the anomalous groups already have been identified by direct means.

Since our earlier paper on this reaction was published, we have shown[2] that β-lactoglobulin possesses other conformations apart from the two involved here and apart from the completely unfolded or denatured protein. Moreover, the molecule has been shown capable of existing in different states of aggregation.[7,8] Some convenient method for labelling these various possible conformations is therefore required. It has been customary in the past to use N to designate the native conformation and D the completely unfolded molecule. We shall use other capital letters to designate other conformations. As A and B are already in use to label the genetic isomers of β-lactoglobulin,[9,10] there would seem to be no uniquely preferable system. We shall arbitrarily use R to label the conformation which is the product of the present transition, *i.e.*, the reaction will be described as $N \rightleftarrows R$.[11,12]

Experimental

The β-lactoglobulin used in this work was purchased from the Pentex Corp., Kankakee, Ill. Stock solutions were prepared by dissolving the protein in dilute KCl and removing insoluble material by centrifugation. The concentration of protein in these solutions was determined by drying at 107–108° or by spectrophotometric measurements, as previously described.[13] In some cases the concentration was determined by measuring the specific rotation under conditions where its value was known from previous data.

Solutions for measurement were prepared by dilution of these stock solutions and by addition of standard HCl, KOH and KCl to achieve the desired *p*H and ionic strength, the latter being 0.15 for all of the data of this paper. The protein concentration varied between 0.4 and 1.0 g. per 100 ml. A single solution sometimes was used for measurements at all three of the temperatures at which data were obtained. At other times separate aliquots or separate solutions were employed at each temperature.

At high *p*H (above *p*H 9) there is a slow irreversible change in optical rotation[14] which follows the reversible change of interest here. Where this reaction was important, data were obtained at various intervals of time and extrapolated to $t = 0$. Some interference in achieving this result arose from convection currents in the polarimeter tube which often produced erratic data for the first two or three readings. The fluctuations due to convection currents represented a larger fraction of the total rotation at higher wave lengths. They were much less important at 25° than at 13 or 38°.

Optical rotation measurements were made in jacketed polarimeter cells of length 20 cm., in a housing which was also jacketed. Water from constant temperature baths was circulated first through the cell jacket and then through the housing. The polarimeter used was the Rudolph photoelectric spectropolarimeter, equipped with zirconium and mercury lamps. Most of the measurements described were obtained with the zirconium lamp, using wave lengths of 400 and 589 mμ. Others were obtained with the mercury lamp, using 405 and 578 mμ. These latter data were converted to 400 and 589 mμ, respectively, by use of dispersion curves which we have measured under a variety of conditions. The correction factors are $[\alpha]_{589}/[\alpha]_{578} = 0.955$ independent of *p*H; $[\alpha]_{400}/[\alpha]_{405} = 1.040$ for conformation N and slightly less (1.037) toward the end of the transition.

The flow method used to determine some of the data of Fig. 2 was based on the technique of Rehm, *et al.*[15] A protein solution was circulated from a titration vessel through a polarimeter tube with entrance and exit vents located near the ends. Acid or base was added by increments, and *p*H and specific rotation were determined continuously. These data were obtained at 365 mμ only, using the mercury lamp, and were adjusted to 400 mμ by use of appropriate dispersion data. The factor $[\alpha]_{400}/[\alpha]_{365}$ varies from 0.735 (below *p*H 6) to 0.76 (above *p*H 9). The flow method was judged less precise than the point-by-point method and was used only to determine the variation of $[\alpha]$ for the native protein outside the *p*H range of the transition.

All *p*H measurements were made in jacketed vessels, using Beckman Model G or GS *p*H meters with external glass electrodes in the apparatus previously described.[16]

Results

Figure 1 shows the experimental data at 25°, including points at relatively low *p*H which were obtained after exposure to *p*H 9.5. These points fall on the same curves as the rest, proving the reversibility of the reaction and permitting use of the data to determine thermodynamic parameters of the reaction. Before these parameters can be determined, it is, however, necessary to know how the values of $[\alpha]$ of pure N and pure R vary with *p*H.[17]

Considering N first, we show in Fig. 2 the relation between $[\alpha]$ and the average molecular charge $\bar{Z}_H$ due to binding or dissociation of protons, the value of $\bar{Z}_H$ at any *p*H being taken from the titration curve of β-lactoglobulin determined in this Laboratory.[13,18] The data of Fig. 2 suggest that $[\alpha]_N$ may be represented as a linear function of $\bar{Z}_H$. Extending this linear relation to more negative $\bar{Z}_H$, and then reconverting to the *p*H

It must be noted, however, that the titration curve of the anomalous groups is obtained as the difference between an experimental total titration curve and two calculated curves (one for each conformation). The precise course of the titration curve for the anomalous groups thus depends on the value used for the molecular weight, as group numbers are adjusted to be integral numbers per mole, and on the assumed equivalence of the electrostatic interaction in the two conformations. It would be unrealistic to say more than that the curves "coincide within experimental error." It might be noted also that no simple mechanism would lead to a titration curve for the anomalous groups which is steeper than the curve describing the course of the transition.

(6) C. Tanford, *J. Am. Chem. Soc.*, **83**, 1628 (1961).

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

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

(9) R. Aschaffenburg and J. Drewry, *Nature*, **176**, 218 (1955); **180**, 376 (1957).

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

(11) The letter R stands for "reversible," but we shall show in subsequent papers that there are other conformations readily reached by reversible paths from N.

(12) It may be desirable at some future date to indicate on the conformation label the number of polypeptide chains per molecule. This number is two for both N and R, so that the transition could be described as $N_{(2)} \rightleftarrows R_{(2)}$.

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

(14) M. L. Groves, N. J. Hipp and T. L. McMeekin, *ibid.*, **73**, 2790 (1951).

(15) C. Rehm, J. I. Bodin, K. A. Connors and T. Higuchi, *Anal. Chem.*, **31**, 483 (1959).

(16) C. Tanford, "Electrochemistry in Biology and Medicine," T. Shedlovsky, ed., John Wiley and Sons, Inc., New York, N. Y., 1955.

(17) As explained in the preceding paper, what *appears* to be pure N or R may actually be a mixture of constant composition. From the equilibrium constant to be determined below we conclude that the starting material in the present transition contains less than 1% of R. We shall point out that there is good reason for believing that the product is also virtually pure.

(18) In this case we use the calculated titration curve for pure N (ref. 13) rather than the experimental curve. Below, where we consider $[\alpha]$ of R as a function of $\bar{Z}_H$ we similarly use the calculated curve for R.

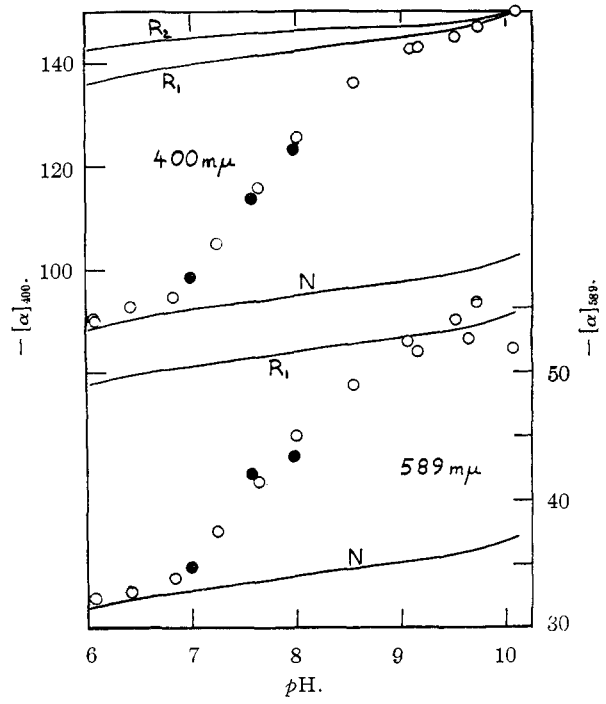

Fig. 1.—Experimental data at 25° and ionic strength 0.15, at 400 and at 589 mμ. Filled circles represent reversed points, obtained after exposure to *p*H 9.5; the curves labelled N represent the optical rotation of pure N, the curves labelled R_1 and R_2 represent the optical rotation of pure R, using alternative assumptions discussed in the text.

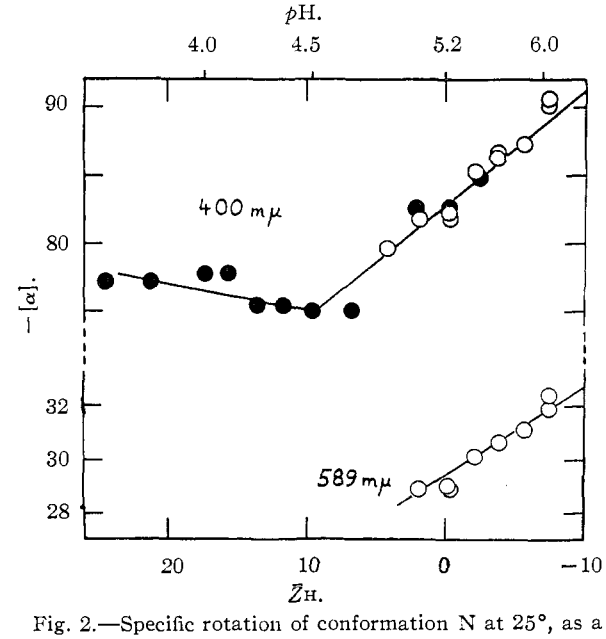

Fig. 2.—Specific rotation of conformation N at 25°, as a function of the charge due to titration. Filled circles were obtained by a flow method as described in the Experimental section; the scale at the top of the figure shows the *p*H values (25°, ionic strength 0.15) at several values of $\bar{Z}_H$.

scale, gives the values of $[\alpha]_N$ used in the calculation and shown in Fig. 1.

Because of the onset of irreversible denaturation above *p*H 9.5, it is not possible to obtain experimental data like those of Fig. 2 for conformation

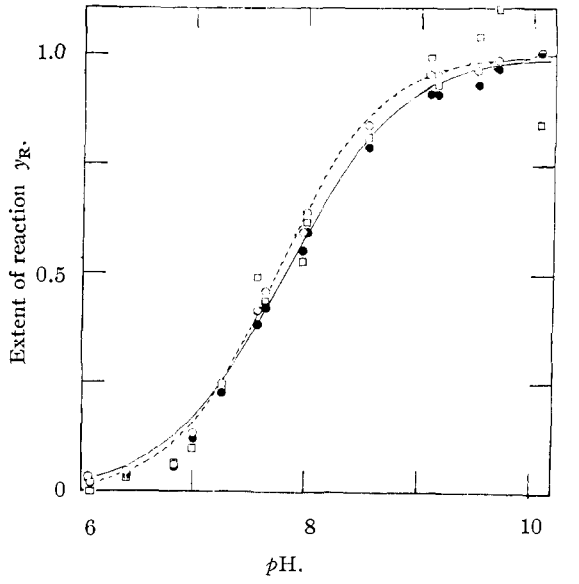

Fig. 3.—Apparent degree of conversion of N to R at 25°, ionic strength 0.15 as determined from Fig. 1. Open circles represent data at 400 mμ, using curves R_1 and N, filled circles represent the same data, using curves R_2 and N; squares represent the data at 589 mμ; the dashed line is drawn according to equation 2 with pK^* constant; the solid line is equation 2 with pK^* slightly *p*H-dependent, as given by equation 3.

R in its pure state. It is thus necessary to make an arbitrary assumption concerning $[\alpha]_R$ during the course of the transition. The simplest assumption (curves labelled R_1 in Fig. 1) is that the relation between $[\alpha]_R$ and $\bar{Z}_H$ is the same as that between $[\alpha]_N$ and $\bar{Z}_H$.

The change of $[\alpha]_N$ with $\bar{Z}_H$ observed for conformation N is, however, unusually large. Where z is the specific charge due to titration, $z = \bar{Z}_H$/mol. wt., we have $\partial[\alpha]/\partial z = 4.0 \times 10^4$ at 365 mμ and 1.1×10^4 at 589 mμ. For native ribonuclease, we have observed in similar studies, at 365 mμ, $\partial[\alpha]/\partial z = 0.6 \times 10^4$. For lysozyme, at 578 mμ, $\partial[\alpha]/\partial z = 0.2 \times 10^4$. For serum albumin also our values of $\partial[\alpha]/\partial z$ are exceedingly small between *p*H 4.5 and *p*H 7.5 where no transitions occur. (Jirgensons,[19] however, has obtained some data with this protein which show $\partial[\alpha]/\partial z$ in this same *p*H range to be as large as our value for β-lactoglobulin N.) It seems advisable, in view of these results with other proteins, to consider the possibility that $[\alpha]_R$ may vary with $\bar{Z}_H$ less steeply than $[\alpha]_N$, and we have thus drawn the curve R_2 in Fig. 1 to correspond to a 50% reduction in $\partial[\alpha]_R/\partial\bar{Z}_H$. This curve has been drawn for the data at 400 mμ only: at 589 mμ the data at high *p*H are so scattered that the determination of the value of $[\alpha]_R$ in the region of *p*H 10 is quite uncertain, introducing an uncertainty considerably greater than that which might be ascribed to uncertainty in the effect of *p*H upon this value.

As will be seen below, our final conclusions are relatively insensitive to these assumptions concerning $[\alpha]_N$ and $[\alpha]_R$.

(19) B. Jirgensons, *Arch. Biochem. Biophys.*, **78**, 227 (1958).

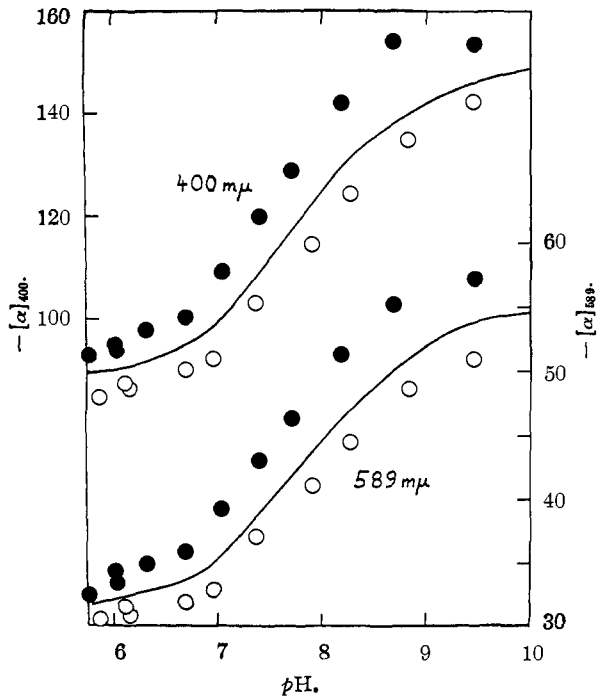

Fig. 4.—Experimental data at 38° (filled circles) and at 13° (open circles); the lines drawn are experimental curves through the data at 25° shown in Fig. 1.

We now proceed to compute the apparent extent of conversion from N to R as

$$y_R = ([\alpha] - [\alpha]_N)/([\alpha]_R - [\alpha]_N) \quad (1)$$

obtaining the results shown in Fig. 3. The open circles show the results obtained (at 400 mμ) using curves N and R_1 of Fig. 1. They follow closely the curve

$$y_R = \frac{K^*/(H^+)}{1 + K^*/(H^+)} \quad (2)$$

with $pK^* = 7.74$, which is the dashed line shown in the figure. The closed circles were obtained using the data at 400 mμ with curves N and R_2. They fall closer to the solid line of Fig. 3, which is drawn according to equation 2, but with K^* varying with Z_H according to the relation

$$K^* = K^*_{int}\, e^{2w'Z_H} \quad (3)$$

with $w' = 0.039$ and $pK^*_{int} = 7.30$. If $[\alpha]_R$ in Fig. 1 had been assumed entirely independent of $\bar{Z}_H$, then an even flatter curve than the solid line of Fig. 3 would have been obtained.

The squares of Fig. 3 show the value of y_R obtained from the data at 589 mμ. They show more scatter than the values obtained from the data at 400 mμ, for the reasons already given, but within experimental error follow the same course. Any other result would of course be incompatible with the supposition that we are observing here a single step change in conformation.

In Fig. 4 are shown data similar to those of Fig. 1, obtained at 13 and 38°. No data are shown in the region of irreversible denaturation because the time required for attainment of temperature equilibrium and elimination of convection currents in the polarimeter tube was in these cases too large to permit a meaningful extrapolation to zero time. Thus, while $[\alpha]_N$ could be obtained for these data

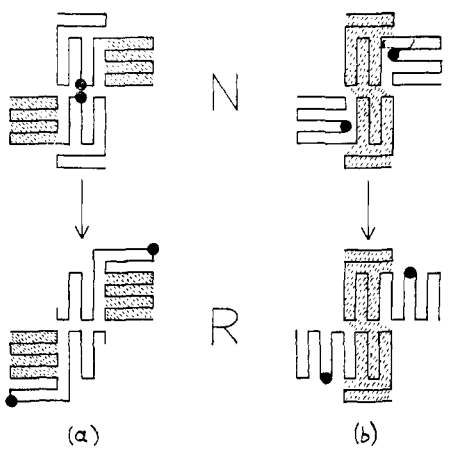

Fig. 5.—Schematic representations of the N → R transition; the dark circles represent the anomalous carboxyl groups buried in the interior of the molecule in N and exposed to the surface in R. In representation (a) these groups (in N) are at the surface of contact between the two polypeptide chains, in representation (b) they are not; in both cases the shaded regions represent portions of the polypeptide chains whose folding is not altered by the transition. The form of the equation for the transition appears to exclude possibility (a).

in the same way as at 25°, the values of $[\alpha]_R$ had to be estimated from the corresponding values at 25°. The simplest assumption to make is that the effect of temperature on $[\alpha]_R$ is the same as its effect on $[\alpha]_N$. With this assumption one finds that the data at 38 and 13° again fit equation 2 and that $pK^*_{13°} - pK^*_{25°} = pK^*_{25°} - pK^*_{38°} = 0.25$, with a probable error of the order of 0.05. The corresponding value of $\Delta H^* = 2.303R\ d pK^*/d(1/T)$ is 8 kcal./mole.

Discussion

The titration curve of β-lactoglobulin indicates the presence of two anomalous carboxyl groups. If these groups are associated together with a single change in conformation, then the change in conformation should follow equation 20 of the preceding paper[6]

$$y_R = \frac{K^*/(H^+)^2}{1 + K^*/(H^+)^2} \quad (4)$$

if both N and R represent pure conformations. If either N or R represents a mixture rather than a pure conformation, then the curves of Fig. 3 should be flatter than that given by equation 4, but steeper than that given by equation 2.[20] This is clearly not the observed result, and one can thus conclude from Fig. 3 that the reaction N → R is associated with just a single anomalous carboxyl group. This result is easily understandable in view of the fact that β-lactoglobulin consists of two polypeptide chains, which all available data indicate to be identical or nearly so.[21] The conformational change described in this paper would thus appear to occur independently in each chain, following the equation for a transition associated with a single titratable group. What this means in terms of structure is illustrated, entirely schematically, by Fig. 5. The conforma-

(20) Ref. 6, Fig. 3.

(21) R. Townend and S. N. Timasheff, *J. Am. Chem. Soc.*, **79**, 3613 (1957).

tional change N → R appears to involve parts of the two chains not in immediate contact with each other. The area of contact between the two chains might also be affected, but it is known that the molecular weight of β-lactoglobulin is not changed by the N → R transition[4] so that it is simpler to assume no change in the area of contact.

Having established that the transition follows equation 2, we may without further assumption replace K^* in that equation by equation 8 of the preceding paper[6]

$$K^* = \frac{k_0(1 + k_1)}{k_1(1 + k_0)} K_R = \frac{1 + k_1}{1 + k_0} K_N \qquad (5)$$

where K_R and K_N are the dissociation constants of the anomalous carboxyl group in R and N, respectively, and k_0 and k_1 represent the equilibrium constants (R)/(N) before and after dissociation of the proton from the anomalous group.

The value of K^* we have found is about 10^{-3} of the K of a normal carboxyl group. Moreover, $(1 + k_1)/(1 + k_0)$ is necessarily greater than 1—unless k_1 is relatively small it is much greater than 1. Thus K_N is even smaller than K^*, perhaps very much smaller. We can conclude, without assuming any particular model, that pK_N is of order 7.5 or larger. We do not know of any weak local forces which can lead to so large a pK for a carboxyl group and conclude that the only possible explanation is that the anomalous group is, in conformation N, buried in the interior of the protein molecule (see Fig. 5). For the reasons given in the preceding paper a group so buried would have a very large pK, and, correspondingly, there would be a large value of k_1.

In conformation R the previously buried carboxyl group is exposed to the solvent. We shall assume that it now possesses a normal pK, equal to that of the other carboxyl groups of β-lactoglobulin, which we shall call pK_{COOH}. With $K_R = K_{COOH}$ and k_1 large, we get

$$K^* = \frac{k_0}{1 + k_0} K_{COOH} = k_0 K_{COOH} \qquad (6)$$

since $K^*/K_{COOH} \sim 10^{-3}$ requires that $k_0 \ll 1$. For any normal carboxyl group of β-lactoglobulin, we have[22]

$$K_{COOH} = K' e^{2w'\bar{Z}_H} \qquad (7)$$

where $pK' = 4.69$ and $w' = 0.039$. Combining this value with the value of K^* which corresponds to equation 3 (solid line of Fig. 3), we obtain for k_0 the value $\log k_0 = -2.6$.

If the dashed line of Fig. 3 were used rather than the solid line, we would have $pK^* = 7.74$ independent of pH. Equation 6 would still apply. Equation 7 is, of course, an approximation, but we know of no better one to make unless the exact positions of all charged groups are known. In any event, K_{COOH} must be pH-dependent and therefore a constant value of pK^* requires that k_0 be pH-dependent. The value $\log k_0 = -2.6$ would now apply only to the region about pH 7.74. At lower pH $\log k_0$ would be smaller, the value obtained by extrapolation of all data to the isoelectric point being $\log k_0 = -3.05$.

(22) Ref. 13, equations 7 to 9 and following text.

The value of ΔH^0 for the reaction N → R is obtained directly from equation 6

$$\Delta H^* = \Delta H^0 + \Delta H^0_{COOH} \qquad (8)$$

where ΔH^0_{COOH} is the heat of dissociation of a normal carboxyl group in β-lactoglobulin. We have determined the latter as + 1.0 kcal./mole. With the value of ΔH^* given above, this leads to $\Delta H^0 = 7$ kcal./mole. These data and the corresponding values of ΔF^0 and ΔS^0 are summarized in Table I.

TABLE I

THERMODYNAMIC DATA FOR THE N → R TRANSITION WITH UNDISSOCIATED COOH GROUPS, AT 25°, IONIC STRENGTH 0.15

$k_0 = (R)/(N)$	0.0025
ΔF^0 (cal./mole)	3550
ΔH^0 (cal./mole)	7000
ΔS^0 (e.u.)	+12 (±7)[a]

[a] As indicated above, k_0 may be pH-dependent, in which case the present value applies only to the region near pH 7.74. Furthermore, equation 7 is an approximation, assuming all normal carboxyl groups to be identical. The anomalous COOH group here under discussion is assumed to behave as a normal group in conformation R. It is impossible to estimate how large an error this might introduce: an error of 0.5 in log K_{COOH} is perhaps the maximum. If this is coupled with an uncertainty of 1 to 2 kcal. in ΔH^0 we arrive at an uncertainty of 4 to 7 e.u. in ΔS^0.

These additional remarks may be made concerning the transition.

(1) The statement made earlier,[1] that the N → R transition may be triggered by titration of an imidazole group, is certainly false. If this were the case, then an imidazole group would have an anomalous pK. The fact that the anomalous carboxyl group has the titration characteristics of an imidazole group is purely fortuitous.

(2) It is not possible to make any definite statement about the environment of the buried carboxyl group. The thermodynamic parameters which have been determined apply to the N → R transition as a whole, including the contribution made by transfer of the buried group to the surface. However, the positive value of ΔH^0 leads to a preference for model structures which give N a low enthalpy. Thus it is likely that the buried carboxyl group is hydrogen-bonded.

(3) The hydrophobic regions most probably contain no buried carboxyl groups apart from the two involved directly in the N → R transition. Moreover, no basic groups are likely to be buried because the imidazole groups show no titration anomalies and all the other basic groups are charged in the pH range of the transition, and, if buried, would have a prohibitively large self-energy. On the other hand, phenolic groups may be buried along with the carboxyl groups. The present procedure can provide no information on this subject, as was discussed in the preceding paper.[6]

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