

Conformational Transitions of Bovine β -Lactoglobulins A, B, and C

(Received for publication, November 19, 1965)

SERGE N. TIMASHEFF, LINDA MESCANTI, JAY J. BASCH, AND ROBERT TOWNEND

From the Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, Philadelphia, Pennsylvania 19118

SUMMARY

The optical rotatory dispersion and proton-binding properties of bovine β -lactoglobulins A, B, and C have been examined as a function of pH. It was found that the b_0 parameter remains constant between pH 2.5 and 10.5, while a_0 undergoes large changes, from values of approximately -150 at pH 4 and below to -600 at pH 12. Two conformational changes occur in all three genetic variants, between pH 4 and 6, and pH 6.5 and 9.5, respectively. The first is accompanied by the liberation to titration of 1 cationic residue per chain in the C variant, with no similar effect in variants A and B. In the second transition an abnormal carboxyl per chain becomes ionizable in all three variants, as has been shown to occur in the A and B proteins.

EXPERIMENTAL PROCEDURE¹

β -Lactoglobulins A, B, and C were prepared from the milk of previously typed homozygous cows by standard techniques and recrystallized before use.

Optical rotation dispersion measurements were made in a Rudolph model 200S spectropolarimeter with the same techniques and method of data analysis as reported previously (6). Ultracentrifugal experiments were carried out with a Spinco model E analytical ultracentrifuge. Titrations were performed in a point by point manner, with the use of a Radiometer model 4 pH meter with a glass electrode, type G202, and a calomel reference electrode, type K100, in which contact to the solution is made by an inverted capillary KCl bridge. The vessel and electrodes were enclosed in a Faraday cage. Concentration measurements were made spectrophotometrically in a Zeiss model PMQ II instrument at $278\text{ m}\mu$, with an absorptivity value of 9.6 dl per g previously determined for all three proteins (7, 9).

Titration measurements were carried out at 25° in KCl, ionic strength 0.15, sedimentation runs were performed in 1.0 M NaCl , and the optical rotation was studied at 25° in NaCl-HCl or NaCl-NaOH of 0.03 or 0.1 ionic strength or, alternatively, in acetate buffers ($\Gamma/2 = 0.1$), since it had been shown previously that the optical rotations of the lactoglobulins are unchanged in these three media (6).

RESULTS

The results of the ORD² measurements between pH 3.5 and 11.5 on the three variants are summarized in Fig. 1, where the Moffitt-Yang (10) a_0 parameter is plotted as a function of pH. The b_0 parameter remains essentially constant for all three proteins between pH 2.5 and 10.5, having values of 79 ± 5 for β -A, 75 ± 5 for β -B, and 74 ± 3 for β -C. On the other hand, a_0 undergoes a series of changes above pH 4 with the optical rotation becoming more negative as pH increases. Between pH 4 and 7.5 this change in a_0 is greatest in β -A (from -135 to -225) and least pronounced in β -C (from -160 to -225). At any pH in this range, the a_0 values of the three proteins are different, with β -C being most negative, as reported previously (6). These values approach each other at pH 7.5 and remain essentially

In the course of a hydrogen ion equilibrium study of β -lactoglobulin C (β -C), it was found that the titration curve of this protein displays anomalies in the pH regions of 4.5 to 6 and 6.5 to 9 (1). In the case of β -lactoglobulins A and B (β -A and β -B), the anomalous titration in the higher pH region has been shown by Tanford *et al.* (2-4) to be linked to a conformational transition. Therefore, it seemed of interest to see whether a similar transformation occurred in the lower pH region as well. A number of earlier observations have suggested that this might be the case. Pedersen (5), in 1936, observed a small change in the sedimentation coefficient of mixed β -lactoglobulin near pH 5, the value of $s_{20,w}$ decreasing by about 0.25 S with a 0.8 decrease in pH. Tanford and Taggart (3) have reported an unusually large dependence of optical rotation on charge between pH 4.5 and 6, while studies of the three variants carried out at low temperature in our laboratory (6, 7) showed that the Moffitt-Yang optical rotatory dispersion constant a_0 becomes more negative with increase in pH in this range, at least in the case of β -B and β -C. Furthermore, the strikingly anomalous Tiselius electrophoresis patterns given by β -A and β -B below pH 6 suggest the presence of a molecular isomerization (8). It is the purpose of this paper to report on such a conformational transition in all three genetic variants of β -lactoglobulin, as well as to show that β -C undergoes the same transition as β -A and β -B at the higher pH (near 7.5).

¹ It is not implied that the United States Department of Agriculture recommends the companies mentioned in this section, or their products, to the exclusion of others in the same business.

² The abbreviation used is: ORD, optical rotatory dispersion.

together as pH is increased further. The large change observed between pH 6.5 and 9.5 corresponds, in β -A and β -B, to the conformational transition previously examined by Tanford *et al.* (2, 3). That study was carried out by optical rotation on mixed β -lactoglobulin³ and by titration on β -A and β -B separately (4). The curve drawn through the β -A points between pH 6 and 10 (Fig. 1B, solid line) is a theoretical one derived from the optical rotation data on a mixture of β -A and β -B (3). The good agreement between the two sets of data indicates that the changes in ORD resulting from the pH 6.5 to 9.5 conformational transition of β -A and β -B have essentially identical pH dependences, a result which is not surprising in view of the similarities in titration behavior. The further steep increase in $-a_0$ above pH 10 in all three variants reflects the irreversible denaturation at these pH values, which seems to be identical in the A and C variants, although β -B is somewhat more prone to denaturation between pH 9 and 10.5. The fact that b_0 does not change with pH in any of the three genetic variants agrees with our previous observation on β -A (6) and underlines further the similarity between them. Furthermore, the constancy of b_0 over the pH range of interest makes permissible the analysis of the transformation in terms of a_0 , since, if b_0 does not change, changes in a_0 become directly proportional to changes in specific rotation at any wave length. When the effect is small, as is the case with β -A between pH 4 and 6 (levorotation increases by 9° at 405 m μ and 12° at 365 m μ), this analysis results in the magnification of the phenomenon under investigation (for β -A between pH 4 and 6, a_0 changes by 25°), along with a smoothing out of the random experimental errors of measurements taken at the various wave lengths.

pH 6.5 to 9.5 Transformation—In β -A and β -B, a_0 starts to assume increasingly negative values with increased pH starting from pH 6.5. β -C, however, first passes through a plateau region, its change in a_0 with pH becoming pronounced only above pH 7.5. The change in optical rotation of β -C above this pH can be described by the same 1-proton transition equation which describes β -A and β -B (3, 11),

$$y = \frac{K^*/[H^+]}{1 + K^*/[H^+]} \quad (1)$$

where y is the apparent extent of conversion as defined by Tanford (11), and K^* is the over-all equilibrium constant of the transition. K^* is a function of the pK of the ionizable group involved, as well as of the equilibrium constants of the transition with the particular ionizable group in both the basic and the acidic forms. From the data of Fig. 1B, pK* appears to have a value somewhat higher than that of β -A and β -B.

The titration curves of β -lactoglobulins A, B, and C between pH 6.5 and 9 are compared in Fig. 2 (upper). The three curves are parallel, and their mutual displacement is consistent with the charge differences resulting from the differences in amino acid composition; β -A has 2 more aspartyl carboxyls than β -B or β -C (12, 13) and β -C 2 more imidazoles than β -A or β -B (14). The solid line through the β -C data is calculated for the ionization of 48 normal side chain carboxyls (the same as β -B) and 6 im-

³ It can be assumed safely that the mixed lactoglobulin used by Tanford *et al.* contained only variants A and B. C is a rare variant and has been found so far only in Jersey cattle; it is not found in Holstein cattle, which are generally the main source of commercial milk from which mixed β -lactoglobulin is prepared.

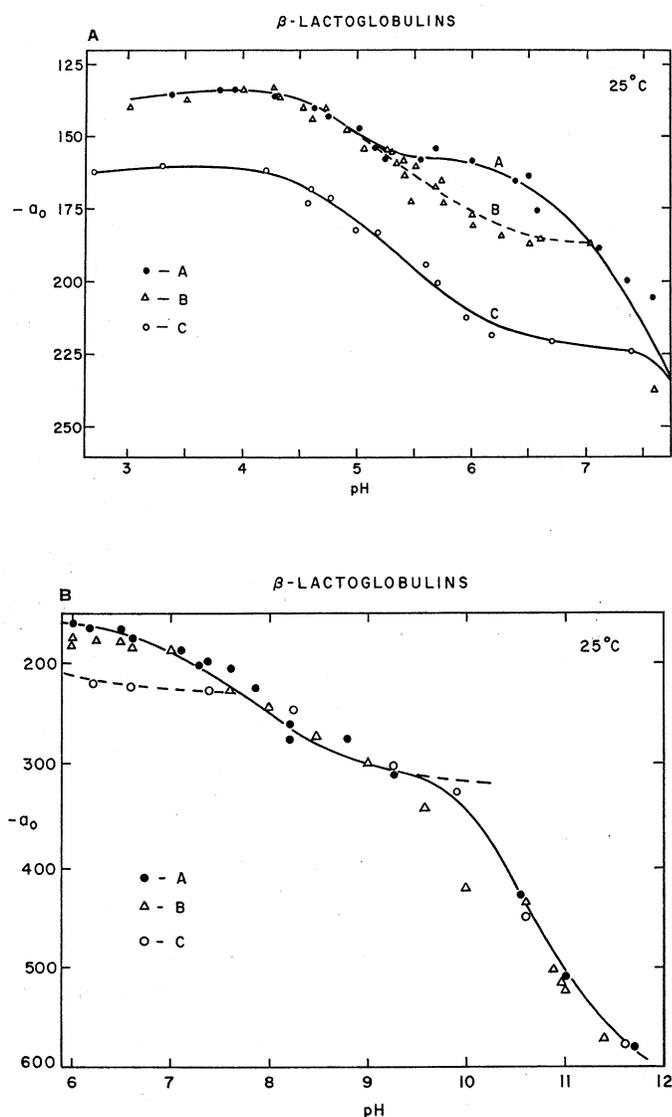


FIG. 1. Changes of optical rotation (a_0) with pH of β -lactoglobulins A (\bullet), B (Δ), and C (\circ). Curves are calculated according to Equations 1 and 2, as explained in the text.

idazoles, plus 2 abnormal carboxyls identical with those found in β -lactoglobulins A and B. The dashed line is for the same number of groups, less the 2 abnormal carboxyls, while the dotted line is the titration curve that would be obtained if all the 50 side chain carboxyls ionized with a normal pK. (In the case of β -A and β -B, the curves drawn through the points of Fig. 2 (upper) are theoretical for the appropriate number of ionizable groups, including the 2 abnormal carboxyls; these are in excellent agreement with the results of Tanford and Nozaki (4) and Nozaki, Bunville, and Tanford (15) for these two proteins.) From the good agreement between the solid curve and the experimental points, it is evident that β -C also contains the 2 buried carboxyls and that these become available to titration in the same pH region in all the proteins. It is possible to conclude, therefore, that all three presently known genetic variants of bovine β -lactoglobulin undergo an identical transition between pH 6.5 and 9.5, indicating a strong similarity between their over-all native structures.

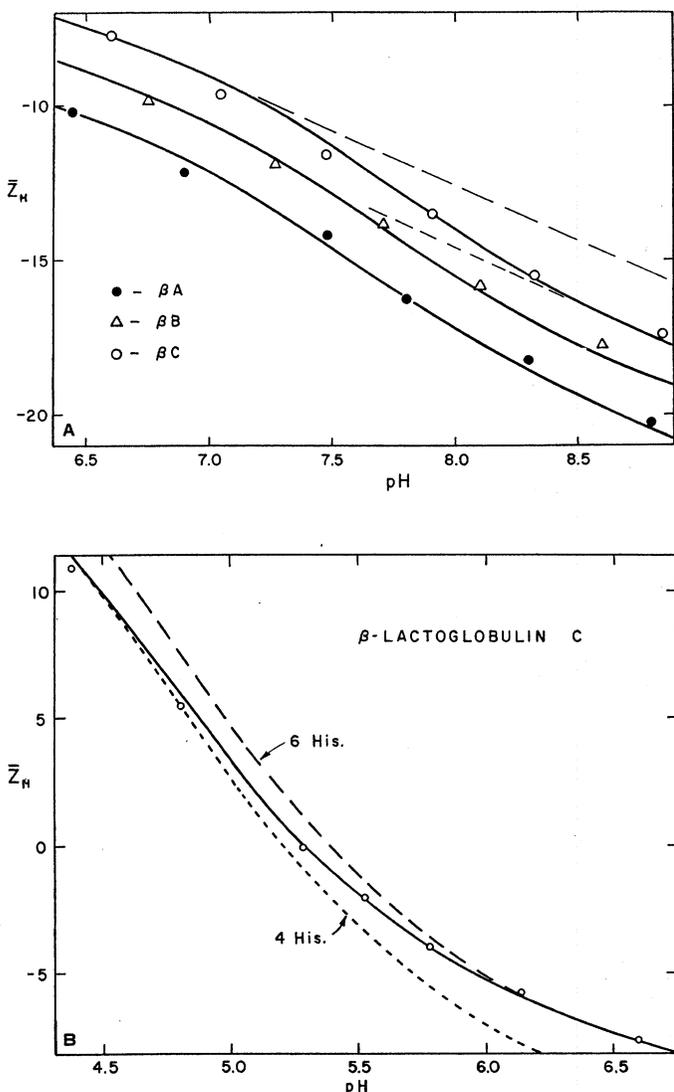


FIG. 2. Hydrogen ion equilibria of the β -lactoglobulins in the transition regions. Curves are theoretical for the appropriate numbers of titratable groups, as explained in the text.

pH 4 to 6 Transformation—The change of the Moffitt-Yang a_0 constant between pH 4 and 6, shown in Fig. 1A for the three variants, has been compared with the change in the β -C titration curve, shown in Fig. 2 (lower), as well as with the hydrodynamic changes common to all three variants, and the equilibria have been analyzed with the assumption that the conformational transitions taking place between the native isoelectric species, N , and the acid forms, Q , of the proteins are rapidly reversible. The latter condition is evident from the fact that the ORD values are identical whether a given pH is approached from the acid or alkaline side, in this pH range.

In order to determine the thermodynamic parameters of the $N \rightarrow Q$ transition, it is necessary to establish first a base-line, *i.e.* the pH dependence of the optical rotation of each species. For this purpose it was assumed that the optical rotation of both the Q and N forms of all three proteins changes in identical linear fashion with respect to specific charge, $z = \bar{Z}_H/\text{molecular weight}$. With the use of the data below pH 4.2 and the β -C points between pH 6.0 and 7.4, a value of $da_0/dz = 0.9 \times 10^4$ was obtained, which

compares reasonably well with the value of 0.7×10^4 , calculated from the 400 $m\mu$ data of Tanford and Taggart (3) measured below pH 4.5 on a mixture of β -A and β -B.

With this value of da_0/dz , the base-lines shown for β -A, β -B, and β -C on Figs. 3, 4, and 5 were obtained, and the theoretical curves were calculated for transitions involving one ionizable group (Equation 1) and two ionizable groups (Equation 2),

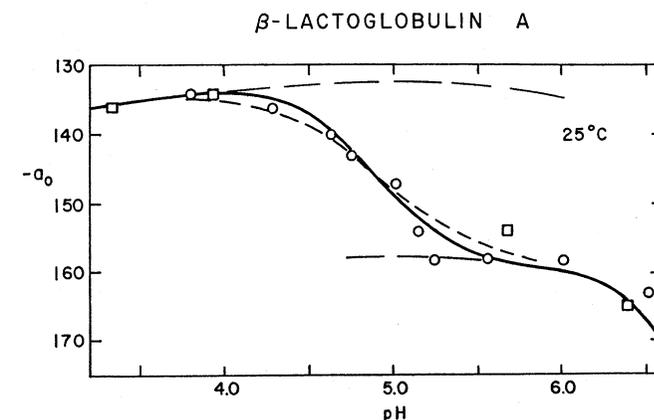


FIG. 3. pH 4 to 6 transition of β -A. Protein, 5 g per liter; 25°; \circ , $\Gamma/2 = 0.1$ acetate buffers; \square , $\Gamma/2 = 0.03$ NaCl-HCl (NaOH); —, Equation 2; ---, Equation 7. Long dashes represent base-line for calculations.

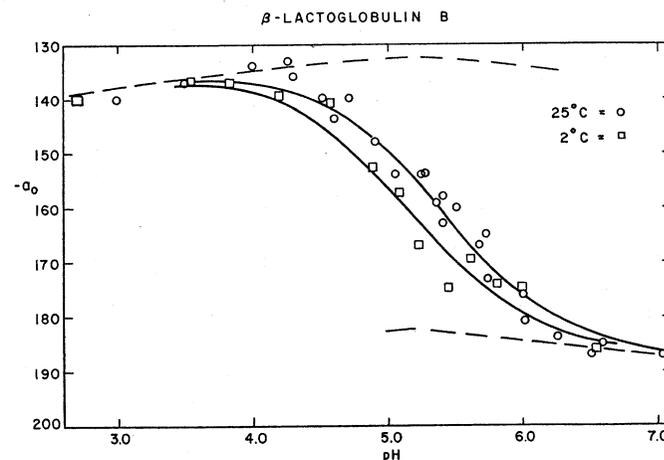


FIG. 4. pH 4 to 6 transition of β -B. \circ , 25°; \square , 2°. Lines are calculated according to Equation 1.

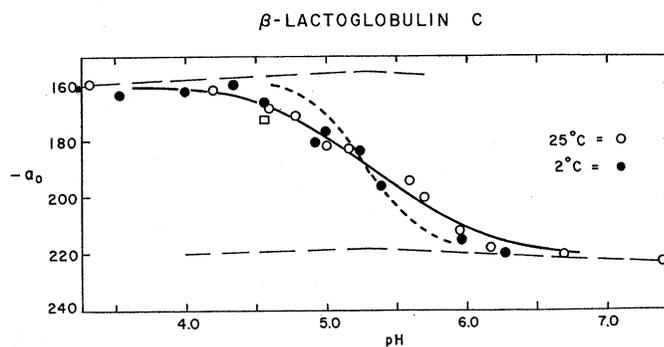


FIG. 5. pH 4 to 6 transition of β -C. \circ , 25°; \bullet , 2°; —, Equation 1; ----, Equation 2.

respectively (11).

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

where the symbols have the same meaning as in Equation 1. Although the total magnitudes of the changes in a_0 during the transition are small ($\Delta a_0 = 25$ for β -A, 40 for β -B, and 45 for β -C) and experimental errors are relatively large ($\pm 3^\circ$), the results indicate definitely that β -B and β -C (see Figs. 4 and 5) follow the equation for a single proton transition (Equation 1). The pK^* values are 5.30 and 5.05 for β -B at 25° and 2°, respectively, and 5.25 for β -C at both temperatures. The β -A points at 25° (Fig. 3) fall on a much steeper curve and approach the pH dependence described by Equation 2, with a midpoint at pH 4.85, *i.e.* $pK^* = 9.70$. No corresponding data could be obtained for β -A at 2° since, in this pH range, β -A undergoes a reversible tetramerization in the cold (9), its a_0 value being a direct function of its degree of aggregation between 3.7 and 5.1 (6). The pH dependence of pK^* , resulting from electrostatic interaction of ionizable groups, may be neglected, since the transitions occur near the isoelectric points (pH 5.1 to 5.3) and the net average charge on all the proteins is quite small over the entire pH range considered. The data on β -B and β -C yield ΔH^* values of -4.9 and 0 kcal per mole, respectively.

Since the changes in a_0 between pH 4 and 6 indicate conformational changes, the region was examined further by velocity sedimentation experiments. The pH dependence of $s_{20,w}$ for β -A in 1.0 M NaCl is shown in Fig. 6. For the sake of comparison, Pedersen's (5) data on the mixed protein are also shown. These runs were carried out at high ionic strength in order to suppress dissociation into subunits, which is favored by low pH (16, 17). It can be seen that, as pH decreases, sedimentation coefficient decreases, and the corresponding equivalent spheres expand from a radius of 25.8 Å in the *N* form to 27.2 Å in the *Q* or acid protein. This is in agreement with Pedersen's conclusion on the change in frictional coefficient (5). In each case, the corresponding theoretical transition curve, derived from ORD data, also describes satisfactorily the transition in hydrodynamic behavior.

According to amino acid analysis (14), β -C contains 42 cationic groups, while β -A and β -B have 40 (β -C has 6 histidine residues, β -A and β -B, 4 each), yet the titration curve of β -C reveals a total acid-binding capacity, \bar{Z}_H , of only 40. The *dashed line* in Fig. 2 (*lower*) is the calculated titration curve for β -C, in the pH range 4.5 to 6, with a \bar{Z}_H of 42. The *dotted line* is a similar curve

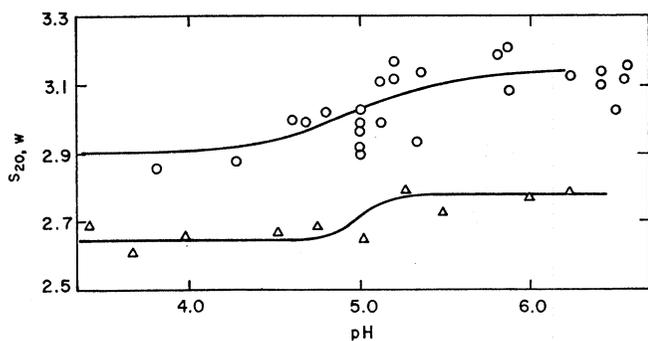


FIG. 6. Sedimentation coefficient in transition region. O, mixed variants of β -lactoglobulin (data of Pedersen, Reference 5); Δ , β -A. Curves are calculated according to Equations 1 and 2, respectively.

with $\bar{Z}_H = 40$. It is clear that, as the pH decreases between 6.5 and 4.5, the experimental points shift anomalously from the curve for 42 bound protons to that for 40. The *solid line* of Fig. 2 (*lower*) linking the two theoretical curves is derived from the change in a_0 shown in Fig. 5 for the same pH range. It is evident, then, that as β -C undergoes the *Q* \rightarrow *N* transition, its capability of binding protons increases by two groups per 36,000 molecular weight, or one group per single polypeptide chain.

DISCUSSION

The change in ORD properties of β -lactoglobulins A, B, and C between pH 4 and 6, reported here, is consistent with the findings of Schellman (18), who observed a change in $[\alpha]_D$ of mixed β -lactoglobulin between pH 4 and 6, as well as with those of Tanford and Taggart (3), who reported a considerable change in the specific rotation at 400 and 589 $m\mu$ between pH 4.5 and 6.0. These findings disagree with results alluded to by Bell and McKenzie (19) that there is no difference between the ORD properties at pH 4.7 and those at 6.9 for either β -B or β -C. The presently described transition explains the unusually large change of $[\alpha]$ with charge found by Tanford and Taggart (3); there is excellent agreement between their value of $d[\alpha]/dz = 4.0 \times 10^4$ at 365 $m\mu$ and our value of 3.2×10^4 at 405 $m\mu$. The latter value was obtained by drawing the best straight line through the data at that wave length in the zone of transition.⁴ This structural change, furthermore, accounts for the change in sedimentation behavior noticed by Pedersen (5) and may be used to explain in part the complex electrophoretic patterns given by the β -lactoglobulins in the region acid to pH 6 (8).

The results of Fig. 1 indicate clearly that all three genetic variants of β -lactoglobulin undergo two discrete conformational transitions between pH 4 and 9.5. Between pH 6.5 and 9.5 the transition is essentially identical in the three proteins and involves the participation of a single abnormal carboxyl per polypeptide chain. This case has been analyzed in detail by Tanford *et al.* (2-4). The pH 4 to 6 transition involves the ionization of one group in β -B and β -C, while in β -A the pH dependence points to a 2-proton ionization. Throughout this pH region, the β -lactoglobulins exist as dimers of two identical chains; thus, in β -A, the pH dependence might reflect either a cooperative simultaneous transition involving the two chains, with one carboxylic group acting in each, or a transition in which each chain is independent of the other and two groups are affected in each, giving a total of four groups per 35,500 molecular weight unit. Although the β -A data do not permit differentiation between the two mechanisms, one may infer from comparison with β -B and β -C that the second possibility is more likely. In β -B and β -C the pH dependence of the transition follows the curve of the ionization of a single group. It is evident, then, that in these variants the two identical chains undergo the conformational transition independently. In view of the generally strong similarities between the three β -lactoglobulins, it is reasonable to assume that the two chains undergo the transition independently in β -A as well with 2 protons being released from each subunit.

What is the nature of the ionizable groups involved in the iso-

⁴ The fact that Tanford and Taggart found a straight line fit of their ORD data can be attributed to the fact that they used a mixture of β -A and β -B. Since the pH dependence of the transition differs for the two variants, the change observed with a mixture must be a smeared out summation of the separate transitions.

electric transition? In the pH 7.5 transition, the ionizable group involved has been shown unequivocally to be an anomalously titrating carboxyl (2-4, 20). It is not possible, however, to establish with equal certainty the nature of the groups involved in the pH 5 transition. The titration curves of β -A and β -B show that all ionizable groups, with the exception of the anomalous carboxyl mentioned above, titrate with essentially normal pK values, and the value of the electrostatic interaction parameter, w , remains constant over the entire pH range investigated. The transition occurs within the range of carboxyl ionization; in this region the carboxyls are 60 to 90% ionized. If, out of 25 dicarboxylic amino acids per chain, one or two had pK values increased by 0.5 unit above the average value of 4.7 as a result of the transition, the net effect on the titration curve would be negligible (it should be remembered, furthermore, that the pK value calculated from a titration curve is the average for all the groups of a given family, with ionization constants of individual groups being distributed about that value (21)). These considerations point again to carboxyls as the ionizable groups involved.

One of the differences in primary structure between β -A and the two other genetic variants is the substitution of 1 aspartyl residue per chain in β -A for a glycyl residue in β -B and β -C. The occurrence of a two-carboxyl transition in β -A, while only one carboxyl participates in β -B and β -C, suggests the involvement of this difference amino acid. In β -B and β -C, the parts of the primary structure participating in the conformational change would include, then, 1 dicarboxylic acid residue plus the difference glycine. In β -A, however, the corresponding sequences would include two dicarboxylic acids, one of them being the difference aspartic acid. It is of interest to note that the tryptic undecapeptide containing the Asp-Gly substitution contains 4 other dicarboxylic acid residues in its sequence (22).

In the case of β -C, in addition to giving rise to the change in optical rotation, the conformational transition is reflected as well by an anomaly in the titration curve (Fig. 2B), which in the pH interval in question passes from a curve with 42 cationic groups to one with only 40 such titratable groups. Since no such titration anomaly is found in β -A or β -B, it seems reasonable to assume that the cationic group involved in the β -C transition is the difference histidine residue (in β -A and β -B the corresponding residue is glutamine). This basic group may be involved in the structural transition in two possible ways. First, it may be drawn into the hydrophobic interior of the molecule as the molecular folding changes during the $N \rightarrow Q$ transformation. It would lose its proton in the process, since the burial of a charge in the low dielectric interior of a protein would involve the expenditure of a prohibitive amount of energy (23). This proton could, however, be simply transferred to a carboxyl titrating in this pH range with the possible formation of a carboxyl-imidazole hydrogen bond. The second way in which the histidine could be involved in the transition is by means of the formation of an ion pair with the carboxylate form of a nearby aspartyl or glutamyl residue. The ion pair then could become buried in the hydrophobic interior of the molecule; such a structure would be stable at the low pH extreme of the titration and would result in a net decrease in the acid-binding capacity of two groups per 35,500 molecular weight unit. Both mechanisms would affect the titration curve in an identical manner and change a 42-cationic group titration curve to a curve with 40 groups. This result is observed in the transition region (Fig. 2, lower). The present data do not permit us

to distinguish between these two mechanisms, although the second one would appear to be more plausible.

The pH dependence of the transition is a direct function of the equilibrium constants of the reaction, k_0 and k_1 , before and after the dissociation of the protons from the groups involved (11). Tanford and Taggart (3) have calculated k_0 for the pH 7.5 transition, making use of approximations which are valid when pK^* is highly displaced from the normal ionization pK of the group involved. In the case of the pH 4 to 6 transition, pK^* is close to the normal pK of carboxyl groups ($\text{pK} \sim 4.7$), and no such approximation can be made. However, with β -B and β -C, only one ionizable group is involved, and limiting values of k_0 may be calculated. According to Tanford (11),

$$\frac{K^*}{K_i} = \frac{k_0(1 + k_1)}{k_1(1 + k_0)} \quad (3)$$

where k_1 must be greater than 1. If k_1 is set equal to 1 and ∞ , in turn, with $\text{pK}_i = 4.65$ and $\text{pK}^* = 5.25$, then k_0 is found to vary within the limits of 0.143 and 0.333, giving a free energy of the transition, ΔF^0 , between 0.66 and 1.15 kcal per mole.⁵ For β -B the enthalpy of the transition, ΔH^0 , is given by $\Delta H^0 = \Delta H_B^* - \Delta H_{\text{COOH}}^0 = -4.1 - 1 = -5.1$ kcal per mole, where ΔH_{COOH}^0 is the heat of dissociation of a normal carboxyl which, in the case of β -lactoglobulin, is found to be 1 kcal per mole (24). Combination of this value of ΔH^0 with the possible limits of ΔF^0 gives an entropy for the $Q \rightarrow N$ transition, ΔS^0 , of between -19 and -21 cal per degree mole. In β -C, where the transition to the acid form is accompanied by the removal from titration of one basic group per chain, the enthalpy of the $Q \rightarrow N$ reaction is given by

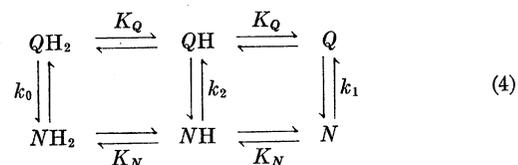
$$\Delta H^0 = \Delta H^* - \Delta H_{\text{COOH}}^0 - \Delta H_{\text{cat}}^0$$

where ΔH_{cat}^0 is the heat related to the liberation to solvent of the cationic group unavailable to titration in the acid region. With the use of the experimental value of $\Delta H_{\text{cat}}^* = 0$, and the above deduced value of $\Delta H^0 = -5.1$ kcal per mole, we obtain

$$\Delta H_{\text{cat}}^0 = 0 - 1 + 5.1 = 4.1 \text{ kcal per mole}$$

This positive value of the enthalpy of release of the buried group is consistent with the concept of its burial as part of a salt bridge (25).

Analysis of the β -A data becomes more complicated. As pointed out above, the isoelectric transition in this protein follows closely the curve for a 2-proton transition. From the similarity with β -B and β -C, it is quite probable that k_0 for β -A is not very small. Thus, Equation 2 cannot be used directly, but the complete expression must be applied. Since the two groups involved in the β -A transition are assumed to be identical (two carboxyls), it is simplest to assign them identical pK values. With the use of Tanford's notation (11), the β -A transition can be expressed as



Since the two ionization constants are equal in each of the con-

⁵ Lowering of the pK does not change these results drastically; i.e. if $\text{pK}_i = 4.25$, ΔF^0 is found to vary between 1.3 and 1.7 kcal per mole.

formational states, we obtain

$$\frac{k_0}{k_1} = \frac{K_N^2}{K_Q^2} \quad (5)$$

and

$$k_2^2 = k_0 k_1 \equiv k_{01} \quad (6)$$

Then, the relation of the apparent extent of conversion reduces to

$$y = \frac{\frac{k_0(1+k_1)}{k_1(1+k_0)} \left[\frac{k_1(k_{01}-k_0)}{k_{01}(k_1-k_0)} \frac{K_i}{[H^+]} + \frac{K_i^2}{[H^+]^2} \right]}{1 + 2 \frac{k_0(1+k_{01})}{k_{01}(1+k_0)} \frac{K_i}{[H^+]} + \frac{k_0(1+k_0)}{k_0(1+k_0)} \frac{K_i^2}{[H^+]^2}} \quad (7)$$

The upper limit of the β -B, β -C k_0 value (0.333) results in a situation amenable to treatment with this equation. Setting $k_1 = 10^3$ and $pK_i = 4.65$, Equation 7 results in the *dashed line* of Fig. 3. This is in as good agreement with the data as the curve (*solid line*) calculated from Equation 2.

The situation represented by the above calculation is a limiting case. If one of the carboxyls in β -A is indeed the difference aspartic acid, then it is known to be located next to two lysines in the primary structure of this protein (22). With the proper orientation of charges, such an environment could greatly lower the normal pK of this group. In this case, the value of 4.65 is certainly an upper limit. A lower value of pK would result in a smaller k_0 and a closer approach to Equation 2.

It seems necessary also to examine the possibility that the transitions observed at pH 4 to 6 and 6.5 to 9.5 are parts of a single two-step transformation. In order for this to be true, however, the normal pK values of the groups involved must be greatly different (11). Here, the groups involved in both transitions are carboxyls, which have essentially identical normal pK values. This eliminates a two-step transition from consideration and establishes that the two transitions must be independent events.

On the assumption of the validity of the calculated thermodynamic parameters, at least as indicators of the signs and orders of magnitude of the changes, a few comments might be made about the possible nature of the pH 4 to 6 transition. Sedimentation data indicate that, as protons are dissociated from the protein in its isoelectric region, the structure becomes more compact (the radius of the equivalent sphere changes from 27.2 to 25.8 Å during the transition). The a_0 ORD parameter, on the other hand, becomes more negative, indicating either (a) less order (26), (b) more groups exposed to solvent (27), or (c) a small decrease in the amount of β structure in the molecule (28) (such a structure has been shown to be present in β -lactoglobulin (29)). The b_0 parameter, on the other hand, does not change, but retains a small negative value. While a and b are in apparent contradiction with the hydrodynamic data, they are consistent with the third alternative, although certainly not proving it. The observed negative heat of transition from the acid to the native form suggests that the stability of the acid protein (Q) is caused, at least in part, by hydrophobic effects (30-32). This hypothesis is also consistent with the increase in entropy accompanying the generation of the acid form of β -lactoglobulin.

A final remark seems desirable on the significance of this transition in the β -A case. In the pH 4 to 5 region this variant undergoes, in the cold, a reversible tetramerization (9, 33) (an equilibrium between the 35,500 molecular weight dimer and an oc-

tamer). This reaction is maximal between pH 4.4 and 4.7. The occurrence of this association in a pH region in which the protein possesses a net charge and nonspecific electrostatic repulsion exists suggests that the association must be preceded by the generation of a specific structure in which key groups assume a conformation proper for complementarity (34). It is quite possible that, as the pH decreases from the isoelectric and the protein undergoes the transition, a conformation capable of forming the specific octamer is formed, this conformation being the acid form, Q, of β -lactoglobulin. Experiments aimed at an answer to this question are presently in progress in our laboratory.

REFERENCES

1. BASCH, J. J., AND TIMASHEFF, S. N., *Arch. Biochem. Biophys.*, in press.
2. TANFORD, C., BUNVILLE, L. G., AND NOZAKI, Y., *J. Am. Chem. Soc.*, **81**, 4032 (1959).
3. TANFORD, C., AND TAGGART, V. G., *J. Am. Chem. Soc.*, **83**, 1634 (1961).
4. TANFORD, C., AND NOZAKI, Y., *J. Biol. Chem.*, **234**, 2874 (1959).
5. PEDERSEN, K. O., *Biochem. J.*, **30**, 961 (1936).
6. HERSKOVITS, T. T., TOWNEND, R., AND TIMASHEFF, S. N., *J. Am. Chem. Soc.*, **86**, 4445 (1964).
7. TOWNEND, R., HERSKOVITS, T. T., SWAISGOOD, H. E., AND TIMASHEFF, S. N., *J. Biol. Chem.*, **239**, 4196 (1964).
8. TIMASHEFF, S. N., AND TOWNEND, R., *J. Am. Chem. Soc.*, **82**, 3157 (1960).
9. TIMASHEFF, S. N., AND TOWNEND, R., *J. Am. Chem. Soc.*, **83**, 464 (1961).
10. MOFFITT, W., AND YANG, J. T., *Proc. Natl. Acad. Sci. U. S.*, **42**, 596 (1956).
11. TANFORD, C., *J. Am. Chem. Soc.*, **83**, 1628 (1961).
12. GORDON, W. G., BASCH, J. J., AND KALAN, E. B., *J. Biol. Chem.*, **236**, 2908 (1961).
13. PIEZ, K. A., DAVIE, E. W., FOLK, J. E., AND GLADNER, J. A., *J. Biol. Chem.*, **236**, 2912 (1961).
14. KALAN, E. B., GREENBERG, R., WALTER, M., AND GORDON, W. G., *Biochem. Biophys. Res. Commun.*, **16**, 199 (1964).
15. NOZAKI, Y., BUNVILLE, L. G., AND TANFORD, C., *J. Am. Chem. Soc.*, **81**, 5523 (1959).
16. TOWNEND, R., WEINBERGER, L., AND TIMASHEFF, S. N., *J. Am. Chem. Soc.*, **82**, 3175 (1960).
17. TIMASHEFF, S. N., AND TOWNEND, R., *J. Am. Chem. Soc.*, **83**, 470 (1961).
18. SCHELLMAN, J. A., *Compt. Rend. Trav. Lab. Carlsberg, Sér. Chim.*, **30**, 395 (1958).
19. BELL, K., AND MCKENZIE, H. A., *Nature*, **204**, 1275 (1964).
20. SUSI, H., ZELL, T., AND TIMASHEFF, S. N., *Arch. Biochem. Biophys.*, **85**, 437 (1959).
21. EDSALL, J. T., AND WYMAN, J., *Biophysical chemistry, Vol. I*, Academic Press, Inc., New York, 1958, Chapter 9.
22. TOWNEND, R., *Arch. Biochem. Biophys.*, **109**, 1 (1965).
23. TANFORD, C., *J. Am. Chem. Soc.*, **79**, 5348 (1957).
24. TANFORD, C., *Advan. Protein Chem.*, **17**, 69 (1962).
25. TANFORD, C., SWANSON, S. A., AND SHORE, W. S., *J. Am. Chem. Soc.*, **77**, 6414 (1955).
26. URNES, P., AND DOTY, P., *Advan. Protein Chem.*, **16**, 401 (1961).
27. TANFORD, C., DE, P. K., AND TAGGART, V. G., *J. Am. Chem. Soc.*, **82**, 6028 (1960).
28. SCHELLMAN, J. A., AND SCHELLMAN, C. G., *J. Polymer Sci.*, **49**, 129 (1961).
29. TIMASHEFF, S. N., AND SUSI, H., *J. Biol. Chem.*, **241**, 249 (1966).
30. KAUZMANN, W., *Advan. Protein Chem.*, **14**, 1 (1959).
31. TANFORD, C., *J. Am. Chem. Soc.*, **84**, 4240 (1962).
32. NEMETHY, G., AND SCHERAGA, H. A., *J. Phys. Chem.*, **66**, 1773 (1962).
33. TIMASHEFF, S. N., in H. W. SCHULTZ AND A. F. ANGLEMIER (Editors), *Proteins and their reactions*, Avi Publishing Company, Inc., Westport, Conn., 1964, p. 179.
34. TIMASHEFF, S. N., AND TOWNEND, R., *Nature*, **203**, 517 (1964).