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The State of Amino Acid Residues in β -Lactoglobulin

ROBERT TOWNEND, THEODORE T. HERSKOVITS,¹
SERGE N. TIMASHEFF²

*Eastern Regional Research Laboratory, United States Department of Agriculture,
Philadelphia, Pennsylvania 19118*

AND

MARINA J. GORBUNOFF

The extent of availability of the tryptophan, cysteine, and tyrosine residues of β -lactoglobulin has been examined under various conditions, using techniques of difference spectroscopy and chemical modification. It has been found that the solvent perturbation difference spectra of the tryptophans are independent of the state of association of this protein (monomer, dimer, octamer). Modification of the single cysteine residue per subunit affects the octamer formation to different extents, whether the derivative is neutral or carries a positive or negative charge. Of the four tyrosines per chain, two are readily available, one is hindered, and the fourth totally unavailable. These results are analyzed in terms of the known structural features of the protein, as well as of the various association reactions and conformational changes which it undergoes.

The single chain subunit of β -lactoglobulin, with a molecular weight close to 18,000 daltons, contains four tyrosine, four phenylalanine, two tryptophan, and one cysteine residues (1-3). Under isoelectric conditions, this protein exists in the state of a dimer (mol wt 36,000 daltons (4, 5)) which may associate to an octamer between pH 4 and 5, with low temperature favoring this association (6, 7). Of the known variants, the bovine genetic variant A (β -A) associates strongly to the octamer (6, 8), variant B (β -B) undergoes this reaction weakly (8), while neither variants C (β -C), D (β -D) (10), nor the goat protein (11) form octamers detectable in sedimentation velocity

¹ Present address: Chemistry Department, Fordham University, Bronx, New York.

² Present address: Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154.

experiments. Titration and conformational studies have revealed the existence in each monomer unit of a carboxylic residue buried in the hydrophobic interior of the molecule (12); this residue becomes ionized (12, 13) only after the molecule undergoes a conformational transition between pH 7 and 8, after which the dimeric molecule undergoes a dissociation into its subunits (14). Another small conformational change between pH 4 and 6 (15) involves the burial of a histidine residue in variant C in the acid form of this protein (16); this transition from the isoelectric to the acid form seems to precede the association of β -A to the octamer (17). The octamerization itself is accompanied by removal from titration of four carboxylic groups per subunit (18). Of the other reactive groups, it is known that the single sulfhydryl group does not react easily with all specific reagents

(for review, see Tilley, (19)) while the tyrosine residues in β -A are of three types with respect to reaction with cyanuric fluoride (20), namely, two react at pH 9.3, one at pH 10.8 or in the region of irreversible denaturation, and one is not reactive at all. Furthermore, three of the tyrosines ionize with an apparent pK value of 10.8, while the fourth group ionizes, in a time-dependent way, with an apparent pK of 12.3 (20).

In view of the availability of this rather detailed information on some structural features of these proteins, it seemed of interest to examine in greater detail the states of tryptophan, tyrosine, and cysteine residues and their relations to the association reactions and conformational transitions undergone by β -lactoglobulin.

MATERIALS AND METHODS

The bovine β -lactoglobulins A, B, and C were freshly recrystallized samples prepared from the milks of individual cows homozygous for these polymorphs (21, 22). The goat protein was the previously described sample (11). Acetyl tyrosine ethyl ester (ATyrE), acetyl tryptophan ethyl ester (ATrpE), and acetyl phenylalanine ethyl ester (APheE) were Mann-assayed products.³

The solvent perturbation difference spectroscopy (SPD) experiments were carried out using a Cary Model 14 spectrophotometer. Difference spectral measurements were made in matched pairs of two-compartment quartz cells of 2.00 cm and 2.00 mm total optical path, using the previously described procedure (23-26). Low temperature measurements ($2 \pm 1^\circ$) were carried out by circulating 0° water through the cell compartment. Moisture was prevented from condensing on the cell windows and instrument optics by passing a dry current of cold nitrogen over the optical faces of the cells and purging the instrument with dry nitrogen. The protein and model compound solutions employed for these experiments were precooled in an ice bath. After filling the double cells with the appropriate solutions and solvent blanks, further cooling and equilibration at the desired temperature was effected by use of the same ice bath. The final temperatures of the solutions were measured after the difference spectral scans by means of a small thermistor that could be inserted into the cells. pH values

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were measured with a Radiometer Model 4 pH meter with Radiometer glass electrodes. Analytical grade reagents were used throughout; dimethylsulfoxide was purified before use by shaking with NaHCO_3 followed by vacuum distillation and collection of the central fraction.

S-sulfo β -A was prepared by the method of Pechère *et al.* (27) and was readily soluble in 8 or 10 M urea at pH values between 2 and 7. All proteins, urea, and model compounds used were prepared as stock solutions and stored carefully stoppered. Solutions for solvent perturbation spectral measurements in 8 M urea were prepared from these stocks by using the same pipette for both the sample and reference, with complete pipette drainage into pairs of matched 5-ml volumetric flasks, as has been described (24, 28). Generally, 4 ml of protein stock in 10 M urea was mixed with 1 ml of liquid perturbant and, if necessary, made up to the 5.0-ml mark with water. In the case of sucrose, 1.080 g of carefully dried solid was added to the flask, then 4 ml of protein solution in 10 M urea and, after the sucrose had dissolved, the flask was filled to the line with water. Such a sucrose solution (21.6 g per 100-ml solution) is 20% by weight in pure water as solvent. The above solutions are called here 20% perturbant solutions.

The cysteine derivatives were prepared according to the method of Cunningham (29). Samples of β -A and β -B were reacted with iodine in KI solution at 0° and pH 6.5, and, after reaction, a 100-fold excess of either thioglycolic acid, 2-mercaptoethanol, or 2-mercaptoethylamine was added. The reaction mixtures were then dialyzed and lyophilized. The derivatives were found to be almost completely soluble⁴ in neutral or slightly acidic buffers at 0.1 ionic strength.

Acetylation of tyrosines with *N*-acetyl imidazole was carried out at pH 7.5 in a 0.05 M borate buffer according to the method of Riordan *et al.* (30). The concentration of the reagent was varied over a 100-fold range to permit differentiation between tyrosine residues exposed to different degrees; the extent of the reaction was followed by difference spectroscopy (30) on a Cary Model 14 spectrophotometer. The reaction of the goat β -lactoglobulin with cyanuric fluoride was carried out using the standard procedure (20, 31).

Ultracentrifugal experiments were carried out

⁴ The aminoethyl derivatives appeared partly denatured: the β -A derivative has a fair amount of insoluble material after lyophilization; the β -B derivative was essentially insoluble at pH 5. This comparative behavior is in agreement with the known greater susceptibility of the B variant to denaturation in the pH range above 6.

at 59,780 rpm on a Spinco Model E analytical ultracentrifuge. Circular dichroism (CD) spectra were obtained on a Cary Model 60 instrument equipped with the CD attachment. The optical rotatory dispersion curves of the carboxypeptidase derivative of β -A were obtained with a Durrum-Jasco ORD/UV instrument. The derivative itself was prepared according to the method of Wilcox (32).

RESULTS AND DISCUSSION

Tryptophans. The degree of exposure of tryptophan residues and its relation to the state of molecular association of β -A, B, and C was followed by solvent perturbation difference spectroscopy (SPD) between 250 and 350 nm.⁵ The SPD parameter $\Delta\epsilon_{\lambda}/\epsilon_{278}$ was followed at $\lambda = 291$ – 293 nm, which is the maximum of the tryptophan SPD spectrum; the reference ϵ_{278} corresponds to the maximum in the absorption spectrum of β -lactoglobulin in aqueous solution. This parameter (designated from here on as $\Delta\epsilon/\epsilon$) is a direct measure (24) of the solvent shift in the spectrum due to the fact that the spectra of exposed and partly buried aromatic groups are perturbed or shifted, usually to longer wavelengths, by the additives or perturbants employed in the SPD technique of difference spectroscopy. The ($\Delta\epsilon/\epsilon$) parameter varies, however, for a given protein or model compound system depending on the perturbant used, in a manner that bears no readily discernible relationship to the change in polarity of the solvent induced by the particular perturbant. Straightforward information can be obtained if the substance under study is compared with its standard (denatured protein or model compound analog, as the case may be) under the influence of the same perturbant and at the same temperature.

Table I presents data taken on S-sulfo β -A and the model compound analog of β -lactoglobulin, containing aromatic groups in the same proportion as they occur in the protein. Examination of the first two data columns (25°) shows that the model com-

⁵ Tyrosine difference spectra cannot be measured easily in a protein such as β -lactoglobulin, which contains significant numbers of tryptophan residues, because of the overpowering optical absorption of the latter (28, 33, 35).

TABLE I
SOLVENT PERTURBATION PARAMETERS OF
DENATURED DISULFIDE-CLEAVED β -A AND
ITS MODEL ANALOG

20% Perturbant	Analog ^a $\Delta\epsilon_{291-93}/\epsilon_{278}$		Cleaved protein ^b $\Delta\epsilon_{291-93}/\epsilon_{278}$	
25 ± 1°				
	Water	8 M urea	8 M urea	Corrected to water ^c
Sucrose	0.031	0.027	0.027	0.031
Ethylene glycol	0.042	.042	.035	.035
Methanol	.033	.034	.026	.025
Glycerol	.046	.038	.036	.044
Polyethylene glycol ^d	.071	.063	.038	.043
Dimethylsulfoxide	.087	.083	.057	.060
2 ± 1°				
Sucrose	0.031 ^e			0.031 ^f
Ethylene glycol	.047 ^e			.039 ^f

^a 1:2:2 molar ratios of ATrpE, ATyrE, and APhE, respectively.

^b S-sulfo β -A, $\Gamma/2$ 0.03, data independent of pH between 2.8 and 7.3.

^c Correction described in text.

^d Degree of polymerization 6.

^e Acetate buffer $\Gamma/2$ 0.1, pH 4.6; the rest of the data taken in $\Gamma/2$ 0.03 NaCl at pH values between 2.7 and 7.3.

^f Corrected to water at 2°.

pound is not affected, within the accuracy of the measurement, by the presence or absence of 8 M urea. Such a change would not, of course, be expected, but its absence should be experimentally verified. Comparison of the second and third columns shows that, with the exception of the cases of polyethylene glycol and dimethylsulfoxide,⁶ the model analog and the unfolded protein appear almost identical.

Since the S-sulfo β -A is insoluble in water except at extremes of alkaline pH, it is necessary to correct data obtained on this material in urea solution to a set of standard

⁶ These two perturbants are known to behave somewhat differently from the majority of substances used (24); dimethyl sulfoxide gives generally the highest value of $\Delta\epsilon/\epsilon$ of all common perturbants.

conditions. Furthermore, some experiments were done using native β -A in pH 4.6, 0.1 ionic strength acetate buffer at 2° (conditions under which the cyclic octamer is present to a maximal extent (6)) and these results, shown in the lower portion of Table I, also require correction to these conditions of low ionic strength and 2°. Urea solutions of 8 M cannot be prepared directly at 2°, because of the low solubility of urea at this temperature. Consequently, the reference parameters, required for the determination of the exposed chromophoric groups, were based on the data at 25° of the S-sulfo β -A protein, corrected for temperature with the data at 2° and 25° for the model compound. The required correction to conditions of water at 2° or 25° can be expressed as: $(\Delta\epsilon_\lambda/\epsilon_{278})_{P,U,25} \times (\Delta\epsilon_\lambda/\epsilon_{278})_{M,w,t}/(\Delta\epsilon_\lambda/\epsilon_{278})_{M,U,25}$. Here $\Delta\epsilon_\lambda$ is taken at the maximum of the tryptophan difference spectral curve, which is always between the limits of 291 and 293 nm; ϵ_{278} is measured at 278 nm, and the subscripts signify the following: P, S-sulfo protein; M, model compound analog; U, 8 M urea; w, water; and t, temperature (2° or 25° in this work). The corrected parameters are given in the last column of Table I and are used later to calculate R_P , the apparent fraction of the tryptophan side chains exposed to the perturbant in the state under study when the disulfide-cleaved protein in 8 M urea is taken as the reference state of complete exposure.

Figure 1 shows the SPD spectrum of β -A at 25° in two molecular states of association: at pH 2.4 and 0.03 ionic strength, where the protein is close to total dissociation (34) into the 18,000-dalton monomers (solid line), and at pH 4.6 and 0.1 ionic strength, where the number of molecules dissociated at any time approaches the vanishing point (dotted line). Also shown, for comparison, is the SPD spectrum of the unfolded, denatured protein (dashed line). Examination of the peak due solely to tryptophan (292–293 nm) shows that a sizeable fraction of these groups is unavailable to glycerol perturbant in both the monomer and dimer states, although there may possibly be slightly more contact of tryptophan with solvent when the molecule is in the dissociated state ($\Delta\epsilon/\epsilon$ goes from 0.019 to 0.023).

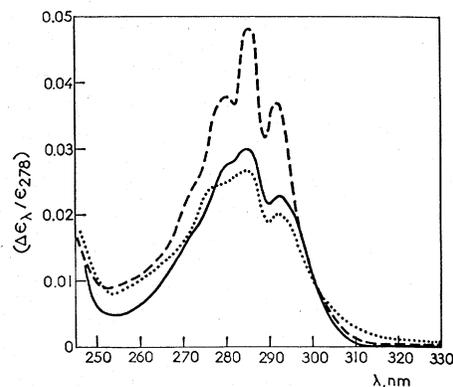


FIG. 1. Effect of the monomer-dimer transition on the SPD spectrum of β -A. Solid line: monomeric form at pH 2.4, 0.03 M NaCl; dotted line: dimeric (native) form, pH 4.6 1/2 0.1 acetic acid-sodium acetate buffer; dashed line: unfolded S-sulfo β -A in 8 M urea, pH 3.7, 0.03 M NaCl. Protein concentration 1.7–2.2 g/liter, 25 \pm 1°. Perturbant, 20% glycerol.

The major SPD spectral peak (at around 285 nm) contains contributions of both tryptophan and tyrosine absorption, and while no quantitative analysis of the data in terms of both exposed tryptophyls and tyrosyls (35) has been attempted, it is evident, however, that there is no significant change in intensity of the SPD spectrum in this spectral region, suggesting that the environment of the pertinent groups does not change significantly when the monomeric subunits combine to form the dimer.

Figure 2 presents the SPD spectrum of β -A with ethylene glycol as perturbant. This variant is the only one of the presently known four bovine β -lactoglobulin polymorphs (21, 22, 36) which forms the octamer in considerable amounts. The two lower curves in the figure were obtained at two temperatures. The solid line is the spectrum of the dimer at 25° (where the amount of octamer is very small; the amount of monomer is also negligible at pH 4.6, an ionic strength 0.1, and 23 g/liter protein concentration). When this solution is cooled to 2°, however, the protein associates strongly, resulting in a preponderance of the octamer (6). The data at this temperature are shown as the dotted line. Comparison of the two curves shows that the number of residues shielded from contact with the perturbant

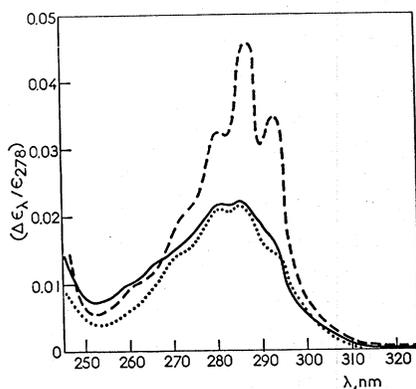


FIG. 2. Effect of the dimer-octamer transition on the SPD spectrum of β -A. Solid line: dimeric form, $25 \pm 1^\circ$, $\Gamma/2$ 0.1 acetate, pH 4.6, protein concentration 23 g/liter; dotted line: octamer form, $2 \pm 1^\circ$, same buffer and protein concentration; dashed line: S-sulfo β -A in 8 M urea, pH 3.8, 0.03 M NaCl concentration 1.8 g/liter. Perturbant 20% ethylene glycol.

is not significantly changed when the dimer molecule associates further to the octamer ($\Delta\epsilon/\epsilon$ goes from 0.016 to 0.015 as the temperature is lowered to 2°).

These data are also presented in Table II, where additional comparisons are made. The final column, R_P , is the ratio of the SPD parameter $\Delta\epsilon/\epsilon$ for the protein in the molecular state studied to $\Delta\epsilon/\epsilon$ of the reference state of complete exposure to solvent (here the subscript P indicates that the reference state is the S-sulfo derivative). As mentioned above, this ratio is the apparent fraction of the tryptophan side chains available to contact with perturbant, corrected as described. The corrected values (last column of Table I) are divided into the values of $\Delta\epsilon/\epsilon$ presented in column three of Table II and the results are tabulated in the fourth column of that table.

The first two sections of Table II give a comparison between the monomer and dimer states of β -A as seen by six perturbants. The data for glycerol (spectra shown in Fig. 1) show the largest change in the direction of more exposure of tryptophan to solvent as the molecule of β -A is dissociated, with regularly small changes shown for dimethylsulfoxide, polyethylene glycol, and ethylene glycol. In the case of sucrose, the data indicate less exposure in the dissociated state.

TABLE II
RESULTS OF SOLVENT PERTURBATION STUDIES ON β -A IN VARIOUS STATES OF ASSOCIATION

20% Perturbant	Protein concentration (g/liter)	$\Delta\epsilon_{291-92}/\epsilon_{278}$	R_P^a
Monomer, pH 3.4, $\Gamma/2$ 0.03, 25°			
Sucrose	2.1	0.013	0.42
Ethylene glycol	2.1	.016	.46
Methanol	2.1	.012	.48
Glycerol	2.1	.023	.52
Polyethylene glycol ^b	2.1	.016	.37
Dimethylsulfoxide	2.1	.023	.38
Dimer, pH 4.6, $\Gamma/2$ 0.1 ^c 25°			
Sucrose	23-26	0.016	0.52
Ethylene glycol	26	.016	.46
Methanol	2.6	.013	.52
Glycerol	2.6	.019	.43
Polyethylene glycol ^b	2.6	.015	.35
Dimethylsulfoxide	2.6	.020	.33
Dimer, pH 5.3, $\Gamma/2$ 0.1 ^c			
Ethylene glycol, 25°	26	0.018	0.51
Ethylene glycol, 2°	26	.021	.54
Octamer, pH 4.6, $\Gamma/2$ 0.1 ^c 2°			
Sucrose	23-26	0.015	0.48
Ethylene glycol	26	.015	.38

^a Ratio of $\Delta\epsilon/\epsilon_{278}$ to same parameter of S-sulfo protein. The latter is corrected for effect of urea and temperature (see text). The appropriate data for the S-sulfo protein are given in the last column of Table I.

^b Degree of polymerization 6.

^c Na acetate buffer.

These R_P values, as mentioned elsewhere (24, 28), are ratios of differences of small numbers, plus errors associated with corrections, and as such, have a fairly large error (on the order of 5-7%) associated with them. The conclusion can be drawn, therefore, from comparison of the first two sections, that any increase in exposure of tryptophans upon dissociation of the molecule is not very large.

The third section of Table II, for ethylene glycol perturbation only, shows that essentially no change in the SPD parameter is

caused by change of temperature alone. The pH (5.3) is too high for much octamer to be formed in aqueous solutions (16), and a separate ultracentrifugal experiment under these conditions (2°) showed no sign of octamer caused by the presence of 20% ethylene glycol. Comparing the second and last sections we see that, with ethylene glycol, the reaction dimer to octamer results in a drop of $\Delta\epsilon/\epsilon$ from 0.016 to 0.015 (shown also in Fig. 2). This is an insignificant change even though the resultant value of R_P drops from 0.46 to 0.38 at the same time, due to slight differences in the SPD parameter of the reference substance and the inherent uncertainties in the corrections. With sucrose there is a similar insignificant change in $\Delta\epsilon/\epsilon$ (0.016 to 0.015), and in this case the R_P actually increases. If the tryptophan side chains were located in the sites of contact when octamers are formed, a large decrease in the SPD parameter would certainly be evident due to the great increase in compactness⁷ and symmetry observed when octamers form (7, 37, 38). It is possible to conclude, therefore, that the side chains exposed to solvent are on an entirely different portion of the molecule than that which actually contacts the other units during the association reaction. Ultracentrifuge experiments at 2° in the presence of 20% ethylene glycol or sucrose show that the formation of the "fast peak" due to the octamer (6) is not visibly affected, and the percentages of the two "components" are unaltered.

Figure 3 shows the SPD parameter (ionic strength 0.003) as a function of pH for the native protein (open circles) and compares it with the unfolded molecule. In the pH range from 4 to 6, the A, B, and C polymorphs of β -lactoglobulin undergo a conformational change leading to an increased sedimentation coefficient and an increase in levorotation (15). Whatever the physical nature of these changes may be, the constancy of the SPD parameter through this region indicates that available—or partially available—tryptophan residues are not involved. The upper curve of Fig. 3 shows that

⁷ In the association to the octamer, experiments show that a large number of carboxyl side chains become buried and unavailable to titration (17, 18).

in the case of urea solutions of β -A or S-sulfo β -A, this parameter is likewise unchanged, as would be expected for a protein in a largely unfolded state.

Three of the four presently known genetic polymorphs are compared in Table III, with 20% sucrose a perturbant. None of the genetically controlled amino acid substitutions of these variants involves tryptophan (1–3, 40); so in spite of the differences between these three, which involve substitutions of charged amino acids, and lead to small differences in isoelectric points (21, 22, 36), optical rotatory behavior (9, 15, 38, 41) and solubility (42), the location of the tryptophan side chains can be specified as being in those portions of the molecules whose structures are highly similar, if not identical.

These solvent perturbation studies show that, in all probability, the tryptophan moieties of the bovine β -lactoglobulins are located in portions of the molecules that

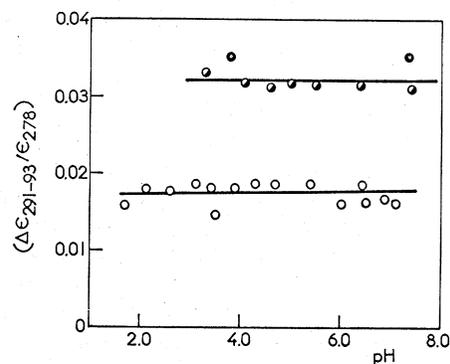


FIG. 3. SPD parameter of native and denatured β -A as a function of pH at $\Gamma/2$ 0.03. Open circles: native protein; half-filled circles: β -A in 8 M urea; filled circles: S-sulfo β -A in 8 M urea. $25 \pm 1^\circ$, protein concentration 1.8–2.7 g/liter. Perturbant, 20% ethylene glycol.

TABLE III
SOLVENT PERTURBATION PARAMETERS OF THE
GENETIC VARIANTS OF β -LACTOGLOBULIN

Variant	Perturbant: 20% sucrose ^a	
	$\Delta\epsilon_{291-93}/\epsilon_{278}$	R_P
β -A	$0.016 \pm .002$	0.52
β -B	.014	.45
β -C	.015	.48

^a $\Gamma/2$ 0.03, pH 2.0–5.5, 25° .

have little to do with association reactions or conformational transitions and confirm again the underlying similarity between the variants of this protein. Furthermore, from the data presented, it may be concluded that between pH 2 and 7, the tryptophan residues of the β -lactoglobulins are buried to an extent of about 50%. Whether this means that of the two tryptophan residues per chain, one is buried and the other one is exposed to solvent, or that the two are partly buried is impossible to say from the present data. The presence of optically active tryptophan bands in the circular dichroism spectrum of these proteins (43, 44) might favor the latter possibility.

Cysteine. All the variants of bovine β -lactoglobulin are believed to contain two free —SH groups per 36,000-dalton dimer (45–47), i.e., one sulfhydryl group per subunit. These groups are not, in general, equally reactive to all reagents (19), suggesting that they may be sterically hindered or partially buried. By taking advantage of the relative stability of the sulfenyl iodide derivative (48), Cunningham (29) was able to prepare soluble derivatives of mixed β -lactoglobulin (β -A plus β -B) in which the iodine atom of the sulfenyl iodide was further replaced by carboxymethyl, 2-hydroxyethyl, and 2-aminoethyl groups. These derivatives, in particular the first two, were assumed to retain a good deal of the native protein structure, as was to be inferred from the bimodality of the ultracentrifugal patterns obtained at pH 4.65 and 2° (47). In the present study, the three derivatives were prepared from β -A and β -B by the method of Cunningham and their ultracentrifugal properties were examined.

At conditions favoring maximal octamer formation of the native protein (pH 4.65, temperature <3°), 10 g/liter solutions of the carboxymethyl, 2-hydroxyethyl, and 2-aminoethyl derivatives of β -A (β A-S-COOH, β A-S-OH, and β A-S-NH₂, respectively) gave ultracentrifugal patterns like those reported by Cunningham and Nuenke (49). With the exception of β A-S-NH₂, the patterns are qualitatively similar to those produced by β -A (or mixed β -lactoglobulin) under such conditions of temperature and pH (50). Like their parent protein, the three derivatives of β -B (β B-S-COOH, β B-S-OH, and

β B-S-NH₂) showed little evidence of association in the ultracentrifuge at any conditions of pH or temperature investigated.

β A-S-COOH was somewhat less reactive at pH 4.65 than was native β -A. This lowered reactivity might possibly be due to a small amount of denatured material, since a portion of the lyophilized derivative is insoluble. In agreement with the results of Cunningham and Nuenke (49), β A-S-OH was associated still less, and β A-S-NH₂ did not show any bimodality in the ultracentrifuge.

It seemed possible that the presence of the additional carboxyl group in β A-S-COOH might have changed the pH dependence of the dimer \rightarrow octamer reaction, and that the lower percentage of fast component observed might be due to a shift in the maximum of the bell-shaped curve (6). A series of ultracentrifugal runs were carried out, therefore, between pH 3.8 and 5.0 and the percentage of the area sedimenting under the fast "component" was measured from tracings of the patterns. The results are compared with those of native β -A in Fig. 4. It is evident that no major differences exist between the two curves, although the derivative appears to associate to a somewhat smaller extent. The maximum of association lies, in both cases, between pH 4.4 and 4.6, and like β -A, the amount of "heavy component" decreases rapidly on both sides of the maximum. The β A-S-OH derivative, similarly, showed less fast component at pH 4.8 and pH 4.3 than at pH 4.65.

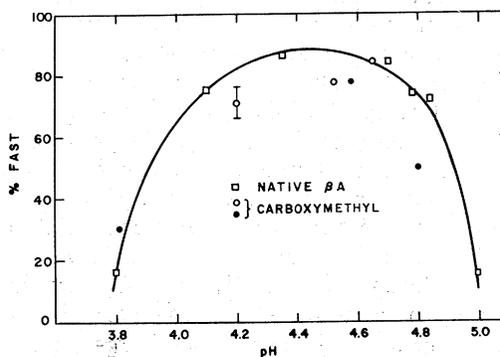


FIG. 4. pH dependence of the dimer \rightleftharpoons octamer reaction of native and S-carboxymethyl β -A. \square : native β -A; \circ , \bullet : carboxymethyl- β -A (two preparations). 10 g/liter protein; 2°; 0.1 ionic strength acetate buffer.

Below pH 3.5, all varieties of β -lactoglobulin show a decrease in sedimentation coefficient (at a constant ionic strength of 0.1), which progresses with a decrease in pH. This reflects the increasing dissociation into the 18,000-dalton monomer units, driven by increasing electrical charge (51). The sedimentation coefficients of the carboxymethyl, hydroxyethyl, and aminoethyl derivatives of both β A and β B were found to change with pH in a manner analogous to the native proteins. This indicates that the sulfhydryl groups, like the tryptophan side chains discussed above, are not located at the sites of subunit contact, and, regardless of the nature of the substituent group, do not affect the monomer-dimer equilibrium. Furthermore, from the Van der Waals dimensions of the substituents, it may be inferred that the —SH groups are located at a distance of more than 6 Å from the area of subunit contact.

With reference to the tetramerization reaction (dimer \rightarrow octamer), the situation possibly may be different. This reaction, which occurs strongly in β -A and only weakly in β -B (8), seems to involve the participation of carboxyl groups, since it is accompanied by the removal from titration of 32 carboxyls per octamer (18) and since the genetic substitution of a single aspartic acid residue in the primary structure of β -A for a glycine residue in β -B (39) increases the free energy of the reaction by 6 Kcal/mole at pH 4.6 and 2° (8). This residue substitution occurs in a portion of the chain particularly rich in carboxylic acid residues (39); clustering can be shown to have strong effects on the proton-donating ability of such groups (52). The different behavior of β A-S-COOH and β A-S-NH₂ with respect to the association reaction, and the slight decrease in the extent of this reaction when β -A is carboxymethylated, might suggest that the sulfhydryl group is not far removed from the tetramerization site. In such a case the addition of a positively charged amino group or a negatively charged carboxyl in this region could upset the charge balance needed for the over-all reaction to proceed readily. The electrical neutralization of a single carboxyl group by formation of an

internal ion pair with the -NH₃⁺ could leave the negative charge cluster appearing like that of β -B, which shows no large-scale octamer formation. Steric effects may also play a part. It seems reasonable that an additional carboxyl group (on either the β A-S-COOH or β B-S-COOH), which is not fixed in position by the primary structure, would tend to be repelled from the cluster site. The additional bulk of the group could account for the lower degree of association found with β A-S-COOH as compared with native β -A. The β A-S-OH derivative, being uncharged, would not be repelled, and could interfere sterically to a greater extent; this derivative is found to tetramerize to an intermediate degree.

A further possibility to be considered is that the chemical modification of the sulfhydryl group resulted in varying degrees of protein denaturation for the three derivatives. Circular dichroism spectra of the derivatives were obtained between 190 and 330 nm. All three derivatives displayed essentially identical dichroic spectra which, however, differed slightly from that of the native protein under identical conditions. In the native protein, the circular dichroism spectrum shows a broad negative extremum at 215 nm and a cross-over point at 204 nm (43, 44); all three derivatives displayed a shift to a negative maximum close to 210 nm, with no change in intensity, and a cross-over point at 198–200 nm. In the region between 260 and 330 nm, the only difference seems to be the disappearance of a weak negative band at 265 nm. Thus, while this small change in the optical activity of the protein might account for the diminution of octamerization of the β A-S-COOH derivative, it cannot be used to explain the difference in association of the three derivatives, since their spectra are identical.

It seems possible, therefore, that the sulfhydryl group in the β -lactoglobulin monomer is relatively near the sites of intermolecular contact when the dimer associates to form the cyclic (7) octamer. Preparation of other derivatives, with varying chain lengths, might well be expected to throw further light on the geometric details of this protein-protein interaction.

Tyrosines. The availability to solvent of the tyrosines was examined in the bovine β -A variant and in goat β -lactoglobulin by spectrophotometric titration, and reaction with cyanuric fluoride and *N*-acetylimidazole. The spectrophotometric titration, carried out in 1 M KHCO_3 buffer, showed that both variants contain three groups per chain that ionize above pH 9.3 with an apparent pK of 10.9; the fourth group ionizes only above pH 11 with an apparent pK of 12.3. In both variants, the electrostatic interaction parameter, w , is zero for all four groups, indicating that tyrosine ionization occurs concurrently with a considerable unfolding of the protein (53, 54). This is not surprising, since the bovine protein is known to undergo irreversible changes above pH 9.7 (54–56), accompanied by a very rapid increase in negative values of the ORD parameter a_0 (38, 41). Contrary to observation with β -A (20), in the goat protein, the ionization of the fourth group shows no time-dependence at pH 13. This is consistent with the reported higher lability of this variant (11).

Reaction with cyanuric fluoride (CYF) resulted in the cyanuration of two tyrosines per chain in bovine β -A at pH 9.3 and 25°, the third group reacting only at pH 10.8 (20). In the case of the goat variant, two residues reacted at pH 9.3 (both at 4° and 25°), the third residue becoming cyanurated at pH 10.0 and 25°. Since this protein starts undergoing irreversible changes at pH 8.7 (56), none of the goat lactoglobulin tyrosines are cyanurated when the protein is in a reversible conformational state. A further observation of interest with this protein is that, while no temperature effect has been found at pH 9.3, at pH 9.7 only two groups react at 4°, while increasing the temperature to 25° results in an increase of cyanuration to 2.6 groups.

The results of acetylation with *N*-acetylimidazole at pH 7.5 are shown on Fig. 5. It is evident that this reaction proceeds differently in the two proteins. As the mole ratio of reagent to protein was raised from 20:1 to 300:1, two tyrosines reacted in the bovine variant, the same extent of acetylation being attained only at twice that ratio in the goat variant. After reaction of the two groups,

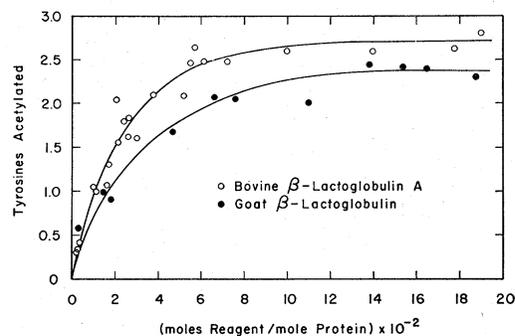


FIG. 5. Reaction of β -lactoglobulin A and goat β -lactoglobulin tyrosines with *N*-acetylimidazole in 0.1 M borate buffer, pH 7.5.

the acetylation continues very slowly, and tends asymptotically toward three groups per chain in the bovine proteins, while it lags considerably in the goat lactoglobulin. In general, the goat lactoglobulin tyrosines appear less susceptible to acetylation than those of β -A. After acetylation of the first group with essentially identical facility, the acetylation of the second residue proceeds at a much slower rate in the goat than in the bovine variant, the difference in total extent of reaction persisting at 0.4–0.5 groups above a reagent-to-protein ratio of 200:1. The difference between the acetylation of the second and third tyrosines of the two proteins suggests that, even though the goat protein is more labile at alkaline pH, its tyrosines may be located in a more restricted environment in the native state. In this respect it seems pertinent that the goat protein carries fewer negative charges than the bovine variant (11); as a result, the electrostatic contribution to molecular expansion at pH 7.5 should be less than in the bovine variants.

The acetylation reaction of the tyrosines is sluggish, since a large excess of reagent is necessary. While the result appears to be the same as with cyanuric fluoride (20), viz., two residues are more reactive than the third and the fourth is totally unreactive, even the reactive residues seem to be less accessible to *N*-acetylimidazole at pH 7.5 than to cyanuric fluoride at pH 9.3. The protein is known to undergo a reversible conformational transition between pH 7 and 9 (12), the structure being less compact in

the alkaline form of the native protein. Thus, it would appear that this conformational transition renders the tyrosines more available to chemical modification. This is consistent with the conclusion of Pantaloni (57) who reported that the $N \rightleftharpoons R$ transition described by Tanford *et al.* (12) results not only in the release of a carboxyl group to ionization (12) but also in the partial exposure of a previously hindered tyrosine residue (57). It appears that the tyrosine residues are exposed in the native protein only to a slightly greater extent than the tryptophans. This correspondence between solvent perturbation spectroscopy and reaction with cyanuric fluoride seems to hold for a number of proteins (31, 58).

The significance of conclusions drawn from chemical modification experiments depends to a large extent on the question whether the protein is altered in the course of the chemical reaction. Circular dichroism (CD) experiments were therefore carried out on β -A samples acetylated to various extents. The results are shown on Fig. 6 where the CD spectrum of an acetylated protein is compared with that of the native protein. It is evident that the two spectra are very similar. In both cases, the far ultraviolet region (below 250 nm), where the peptide transitions are observed, is characterized by a negative band at 216 nm and a positive one at 192 nm. The band intensities are essentially identical at 216 nm; at 192 nm, the acetylated protein displays somewhat weaker positive dichroism than the native

protein; in view of the high noise level in the CD spectrum at that wavelength, this difference is almost within experimental error. In the near ultraviolet region (between 260 and 320 nm), both spectra are characterized by negative bands at 293, 284–286, 277, and 267 nm.⁸ The spectra shown on Fig. 6 remain essentially unchanged up to an acetylation of 2.5 tyrosines in the rapid rise portion of the reaction curve (Fig. 5). Upon forced acetylation of the third group (which requires a reagent to protein ratio of 6000:1) the CD spectrum indicates that considerable conformational change occurs; the bands between 260 and 320 nm almost disappear, while in the lower wavelength region the negative maximum is displaced to 207–210 nm. Thus, the third group can be forced to react only after considerable disruption of the native structure, probably as a result of the acetylation of various other reactive residues. On the contrary, the lack of important changes in the CD spectrum after the acetylation of two tyrosines indicates that this reaction does not disturb the native conformation significantly. It would appear that these groups must be at least partly exposed. Furthermore, the lack of significant changes in the spectrum between 320 and 260 nm confirms the previous assignment of these bands (10, 43, 44, 59) to tryptophan and disulfide (60) transitions, with possibly some contribution from the inaccessible tyrosines. It is quite evident that the two most reactive tyrosines do not make significant contributions to these dichroic bands. It is reasonable to assert, therefore, that the degree of exposure of tyrosines in β -lactoglobulin A, deduced from the acetylation experiments, describes the situation as it exists in the native protein; viz., two groups are exposed, although somewhat hindered, and two are buried, with one becoming available on partial denaturation. Because

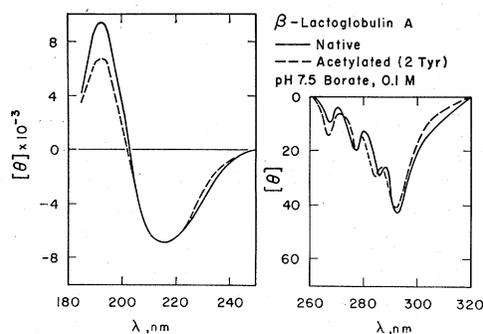


Fig. 6. Circular dichroism spectra of native β -lactoglobulin (solid line) and derivatives with one or two tyrosines acetylated (dashed line). pH 7.5; 0.1 M borate buffer.

⁸ While the positions of these bands are essentially identical with those observed at pH values between 1 and 5, their intensities are considerably lower. Furthermore, in the pH 7.5 borate buffer, the spectrum rejoins the baseline at 260 nm; in the lower pH region, this is not the case (43, 44), weak negative dichroism being observed between 265 and 250 nm, at which point the low wavelength negative absorption becomes dominant.

of the similar manner in which the cyanuration reaction proceeds at pH values above the $N \rightleftharpoons R$ transition, this conclusion can be extended to the reaction with cyanuric fluoride as well.

C-terminal end. Treatment of native β -lactoglobulin with carboxypeptidase is known to result in a crystalline derivative with two (and possibly more) terminal amino acid residues removed (3, 32, 61).⁹ In order to ascertain the contribution of the C-terminal portion of the polypeptide chain to the structural stability, β -A was treated with carboxypeptidase and the optical rotatory dispersion spectrum of the crystalline protein derivative was obtained; it is shown in Fig. 7. This spectrum exhibits the same features as that of the native protein (41); it has a bimodal trough with minima at 230 and 238 nm, a peak at 203 nm and a cross-over point at 222 nm. In the higher wavelength region, chromophoric side chain Cotton effects are evident with troughs at 294 and 285 nm and peaks at 289 and 282 nm. Both the positions and intensities of the ORD bands are identical with those of the native protein (41). It may be concluded, therefore, that the C-terminal portion of the polypeptide chain is most likely located on the surface of the globular structure and is not involved in the stabilization of any major features of the secondary and tertiary structure. It was ob-

⁹ While it is certain that the C-terminal sequence of Ile (162)-His (161) is cleaved off by this treatment, there is some uncertainty at present over the extent of the reaction. Greenberg and Kalan (61) reported that the C-terminal residue of the carboxypeptidase derivative of β -A is Leu; according to the sequence of Frank and Braunitzer (3), leucine cannot be closer to the C-terminal than the seventh residue, i.e., only in position 156. Kalan and Greenberg (62) did observe the release of considerable amounts of threonine and glutamine which occupy positions 157 (Thr), 158 (Glx), and 159 (Glx) in the sequence, and while these authors did not detect Cys-160, they did report the appearance of a precipitate during the carboxypeptidase cleavage. Furthermore, they could isolate the crystalline derivative in only 10% yield. For the purpose of the present study, we may conclude, therefore, that we are dealing with a derivative with at least the two C-terminal residues removed and a definite possibility that the degradation had been more extensive.

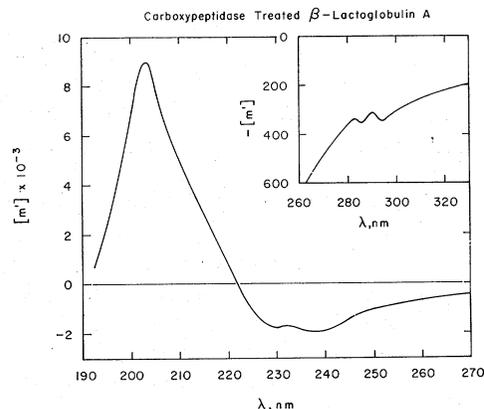


FIG. 7. Optical rotatory dispersion spectrum of carboxypeptidase treated β -lactoglobulin A. pH 4.5, 0.01 M NaCl, 25°.

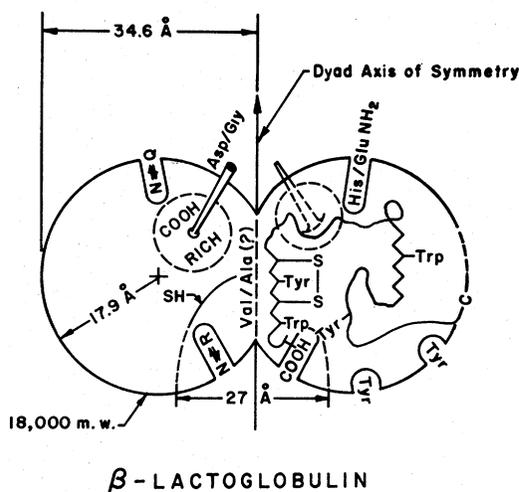


FIG. 8. Schematic representation of deduced structural features of the genetic variants of bovine β -lactoglobulin.

served furthermore that the carboxypeptidase derivative of β -A gives a bimodal ultracentrifugal pattern identical with that of the native protein at pH 4.6 and 2°; this suggests that the C-terminal sequence is not involved in the site of octamer formation and is possibly removed from that area of the molecule.

General considerations. From the data presented in this paper, as well as previously available information, the following structural features of β -lactoglobulin may be inferred. These are illustrated in Fig. 8.

The isoelectric protein consists of two identical subunits which may be represented best by spheres with a radius of 17.9 Å. In the dimer, these impinge on each other by 3.4 Å, and they are related by a dyad axis of symmetry (5, 7). The secondary structure contains a significant amount of antiparallel pleated-sheet β -structure (41, 44, 63, 64). The information available about some amino acid residues permits several conclusions about the structure of the protein. The two tryptophans are partly buried and probably attached to the β -structured region, since on denaturation the tryptophan ORD and CD bands disappear in a manner parallel to the decrease of β -structure, whether the denaturation is alkaline, leading to a disordered structure, or methanolic, leading to α -helix formation. One of the tryptophans is probably located in the area that contains the buried carboxyls (symbolized as a *cleft in* Fig. 8) (12), as has been shown by Pantaloni (57) by difference spectroscopy. Of the tyrosines, two are close to the surface, but slightly hindered; their availability to solvent increases in the course of the N \rightleftharpoons R transition (57); the third tyrosine is partly buried within the structural area that participates in the N \rightleftharpoons R transition. The fourth tyrosine is buried in the stable core of the molecule. This core probably contains β -structure (41, 65) and might possibly be stabilized by a disulfide bridge. The single cysteine residue is not fully available; it is located on a locus, such that the distance between the —SH groups of the two subunits is 27 Å (5); furthermore, it cannot be closer than 6 Å to the area of contact between the two subunits, but it is possibly in the vicinity of the tetramerization site involved in octamer formation. On Fig. 8 this locus is indicated by the dashed semicircle on the spheres. The solid portion indicates the most likely location of the —SH relative to other structural features. The C-terminal end lies on the surface of the protein in an unordered fashion and is removed from the site of octamer formation. Further known structural features are the location of the β -C difference histidine (for glutamine in β -A and β -B) in a mobile structural region that is involved in the pH 4–6 conformational transition (15). Since

this transition is believed to precede the octamerization (17), with only the acid form capable of aggregation, this residue is assigned a location close to the corresponding site of association. The N \rightleftharpoons R site, which contains a buried carboxyl (12), tyrosine (57), and probably tryptophan (57), can be assumed to be close to the area of monomer subunit contact since it triggers subunit dissociation. Finally, the tetramerization site itself, due to the symmetry requirements of the octamer geometry (422 symmetry) (37) must be located as shown on Fig. 8 where the bond directions are indicated by rods, one directed upward and to the front on the left subunit, the other one to the rear on the right subunit; the two are related by the dyad axis of symmetry. In view of the burial of four carboxyls per monomer in the course of this reaction (18), this site is considered to contain a cluster of carboxyls. By inference the Asp/Gly, A \leftrightarrow B substitution is assigned to this site. The other A \leftrightarrow B replacement (Val/Ala) cannot be located at present. It had appeared earlier from hybridization studies (66) that it was located in the area of subunit contact in the dimer (67). However, the conclusions on the lack of hybridization between the variants (9, 66) are now open to question (10, 68) and this previously made assignment can no longer be maintained with equal confidence

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