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Polarized Fluorescence Decay Curves for β-Lactoglobulin A in Various States of Association*

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With the Technical Assistance of J. C. Auchet

ABSTRACT: The decay curves of the parallel and perpendicular components of fluorescence have been determined on β-lactoglobulin A solutions, at conditions in which this protein exists mainly in the form of a monomer, dimer, or octamer. The measured relaxation times are in fair agreement with molecular parameters determined by small angle X-ray scattering.

The recent development of a technique for measuring separately the decay curves of the parallelly and perpendicularly polarized components of fluorescence has permitted the determination of the relaxation times of macromolecules under various conditions (Wahl, 1965, 1966, 1969). This approach has given the possibility of examining directly the shapes of macromolecules by fluorescence polarization without varying the environment. It seemed of interest, therefore, to apply this technique to a protein which can exist in various well-characterized states of aggregation. For this purpose, we have selected β-lactoglobulin A and have determined its fluorescence depolarization curves under conditions where this protein exists predominantly as a monomer, dimer, or octamer. The results of this study are reported in the present paper.

β-Lactoglobulin is a globular protein, the basic subunit of which consists of a single polypeptide chain with a molecular

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weight of 18,400 (Townend *et al.*, 1960). This chain is folded into a compact structure cross-linked by two S–S bonds. The single-chain subunits are known to associate to form specific aggregates, the size of which is a function of pH and temperature. In the pH region between 4 and 7.5, this protein exists predominantly in the form of a dimer (Timasheff, 1964). Below pH 4 (Townend *et al.*, 1960) and above pH 7.5 (Georges and Guinand, 1962) the dimer undergoes a reversible dissociation into monomers. Between pH 4 and 5, genetic variant A undergoes a further association to an octamer, this reaction being promoted by low temperatures (Timasheff and Townend, 1961). X-Ray diffraction studies on crystals of this protein have shown that the gross structure of the dimer is best described by a model of two spheres, 17.9 Å in radius, which impinge upon each other by 3.4 Å and are related by a dyad axis of symmetry (Green and Aschaffenburg, 1959). This model of the dimer was found to account as well for small angle X-ray scattering data obtained in solution (Witz *et al.*, 1964). These small angle X-ray scattering studies have also led to the conclusion that the octamer is best described by a pseudo-cubic compact structure with 422 symmetry (Timasheff and Townend, 1964).

Materials and Methods

The β-lactoglobulin A used was a sample prepared from the milk of a homozygous A/A cow. The fluorescent group, dimethylamino-1-sulfonyl-5-naphthalene (Fluka), was attached to the protein by reacting 100 mg of β-lactoglobulin A dissolved in 10 ml of pH 7.8 phosphate buffer (ionic strength, $\Gamma/2 = 0.1$) with 0.2 ml of an acetone solution containing varying amounts of the dye. In the preparation of the two derivatives used in this study, the amount of dye added was 2 and 0.6 mg, respectively. In each case the reaction mixture was kept at room temperature for 0.5 hr; it was then passed through a Dowex 2 column that had been equilibrated with pH 5.5 acetate buffer ($\Gamma/2 = 0.1$) to remove excess dye and then dialyzed against the pH 5.5 acetate buffer. The amounts of dye fixed were 0.68 mole of dye/mole of protein monomer (preparation I) and 0.18 mole of dye/mole of protein monomer (preparation II). Ultracentrifugal experiments carried out on preparation I in pH 2.0 NaCl–HCl ($\Gamma/2 = 0.03$), 20°, pH 5.5 acetate ($\Gamma/2 = 0.1$), 20° and pH 4.5 acetate ($\Gamma/2 = 0.1$), 9°, resulted in patterns identical with those of the native protein, indicating that the modified β-lactoglobulin A undergoes the same association reactions as the native protein.

The fluorescence depolarization experiments were carried out under three sets of conditions: (1) in pH 2.0 NaCl–HCl ($\Gamma/2 = 0.03$), 20° on preparation I, using a protein concentration of 3.2 g/l.; at these conditions mostly monomer is present; (2) in pH 4.6 acetate ($\Gamma/2 = 0.1$), 2°, on preparation I diluted eightfold with unreacted protein, using a total protein concentration of 20 g/l.; at these conditions the predominant species is the octamer; and (3) in pH 5.5 acetate ($\Gamma/2 = 0.1$), 20°, on preparation I diluted eightfold with unreacted protein, after initial adjustment to pH 2.0 at a total protein concentration of 20 g/l. and on preparation II directly at a protein concentration of 9.6 g/l.; at these conditions essentially only dimer exists. Preparation I was diluted with unreacted protein in the last two cases in order to keep the average amount of dye per kinetic unit at less than one molecule.

The fluorescence experiments were carried out on the previously described instrument (Wahl, 1969) using an exciting flash of short duration. The principle of this instrument is based on the single photoelectron method (Pfeffer, 1965; Lami *et al.*, 1966). Since the dye has an absorption maximum at 330 mμ, the exciting band was selected at 334 mμ. The exciting ray is vertically polarized and the decay curves of parallelly and perpendicularly polarized fluorescence are measured alternately many times and averaged.

The data were analyzed as described before (Wahl, 1969). The recorded decay curves, $i_{||}(t)$ and $i_{\perp}(t)$ are used to calculate the sum, $s(t)$, and difference, $d(t)$, decay curves defined as

$$s(t) = i_{||}(t) + 2i_{\perp}(t) \tag{1a}$$

$$d(t) = i_{||}(t) - i_{\perp}(t) \tag{1b}$$

The shapes of the $s(t)$ and $d(t)$ curves are a function of the shape of the exciting flash and the resolving power of the apparatus. They are related to the decay curves, $S(t)$ and $D(t)$, which would be obtained with an ideal infinitely short flash, by (Wahl, 1969)

$$s(t) = \int_0^t g(t')S(t - t')dt' \tag{2a}$$

$$d(t) = \int_0^t g(t')D(t - t')dt' \tag{2b}$$

where $g(t')$ is the apparatus response to the exciting flash; this is measured directly by introducing a mirror in the sample compartment in place of the fluorescent solution.

The decay curve of the emission anisotropy, $R(t)$, is obtained from the ratio of the two decay curves

$$R(t) = \frac{D(t)}{S(t)} \tag{3}$$

The shape of the $R(t)$ curve is a function of size, shape, and structure of the fluorescent molecule. For a spherical molecule (Jablonski, 1961)

$$R(t) = R_0 e^{-3t/\rho_0} \tag{4}$$

where

$$\rho_0 = \frac{3\eta V}{kT} \tag{5}$$

V is the volume of the molecule, k is Boltzmann's constant, η is the solution viscosity, T is the thermodynamic temperature, and ρ is the relaxation time. For the general case

$$R(t) = R_0 \sum_i A_i e^{-3t/\rho_i} \tag{6}$$

where $\Sigma A_i = 1$. This equation describes, for example, the case of symmetrical ellipsoids of revolution (Perrin, 1936). If the assumption is made that the fluorescent groups are randomly oriented on the protein molecule (Weber, 1952), it is possible to calculate the harmonic mean of the relaxation

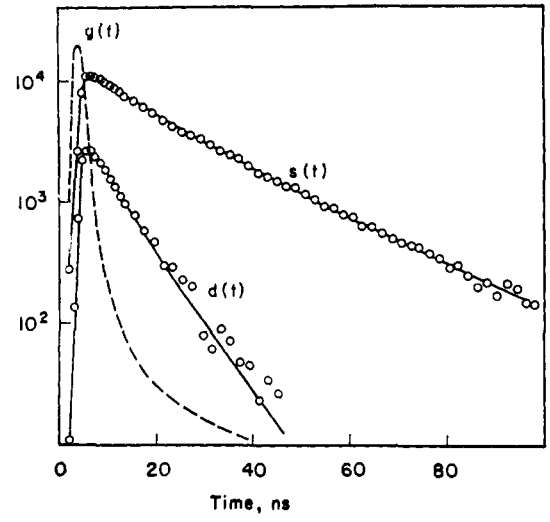

FIGURE 1: Decay curves, $s(t)$, and $d(t)$ for the monomer. The circles are experimental values. The solid lines are calculated convolution products. The dotted line is the flash response, $g(t)$.

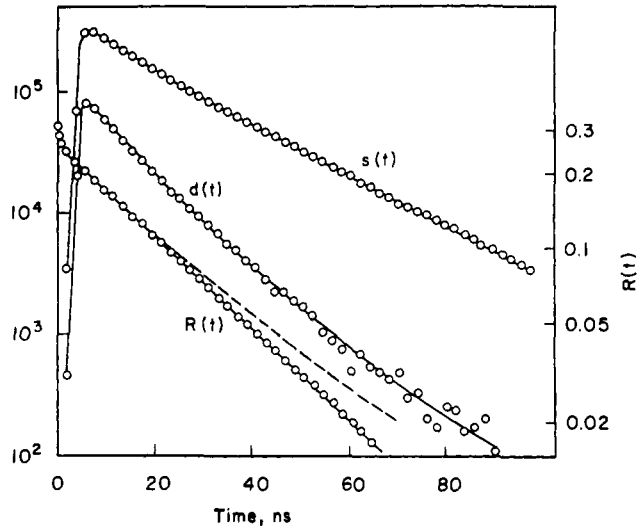

FIGURE 2: Decay curves, $s(t)$, and $d(t)$ for the dimer. The circles are experimental values. The solid lines are calculated convolution products. Anisotropy of the dimer as shown by the $R(t)$ curve; the dotted line has been calculated for $p = 3$.

times, ρ_h, from the initial slope of the $R(t)$ curve

$$\rho_h = \rho_0 f(p) \tag{7}$$

where ρ_0 is given by eq 5, and $f(p)$ is a function of the axial ratio, p, which may be calculated from Perrin's equations (Perrin, 1936).

Furthermore, a mean relaxation time, $\langle\rho\rangle$, may be calculated from the mean decay time constants, $\langle\tau_s\rangle$ and $\langle\tau_D\rangle$; the latter are obtained from the moments of $g(t)$, $s(t)$, and $d(t)$ curves; thus (Wahl and Lami, 1967)

$$\langle\tau_s\rangle = \frac{M_1[s(t)]}{M_0[s(t)]} - \frac{M_1[g(t)]}{M_0[g(t)]} \tag{8}$$

$$\langle\tau_D\rangle = \frac{M_1[d(t)]}{M_0[d(t)]} - \frac{M_1[g(t)]}{M_0[g(t)]}$$

M_1 and M_0 are the first and zero-order moments. Then

$$\langle\rho\rangle = \frac{3\langle\tau_s\rangle\langle\tau_D\rangle}{\langle\tau_s\rangle - \langle\tau_D\rangle} \tag{9}$$

The average, $\langle\rho\rangle$, is not simple; it is, however, weighted in favor of large relaxation times.

Results and Discussion

β-Lactoglobulin A was examined under the three sets of conditions described in the Experimental Section which correspond to a predominance of (1) monomer, (2) octamer, and (3) dimer.

The obtained $s(t)$ curves were almost identical in all our experiments. Their shapes over the time interval of interest can be calculated from the convolution product eq 2a, setting $s(t)$ as the sum of two exponential functions. The coefficients, a_i, and time constants, τ_i, of the $s(t)$ curve are: $a_1 = 0.27$, $a_2 = 0.73$, $\tau_1 = 1.96$ nsec, and $\tau_2 = 15.2$ nsec. The sum $a_1 +$ a_2 is set arbitrarily at unity. On the other hand, the $d(t)$ curves differ greatly for the monomer, dimer, and octamer.

The results of a typical experiment carried out at conditions where monomer predominates are presented in Figure 1. The $d(t)$ curve was analyzed by calculating the convolution product of eq 2b, setting

$$D(t) = R_0 S(t) e^{-3t/\rho_0} \tag{10}$$

A good fit of the experimental curve was obtained with the constants $\rho_0 = 29.5$ nsec and $R_0 = 0.315$. It is found that the thermal rotation is a function of a single relaxation time, in agreement with the model of a rigid sphere. From this value of the relaxation time, eq 5 gives a radius of 21 Å, or 20% higher than that determined by X-ray diffraction (Green and Aschaffenburg, 1959). This higher value may be due to a high degree of hydration of the protein in solution; it could also reflect the presence of some dimer in equilibrium with the β-lactoglobulin subunit (Townend *et al.*, 1960).

Identical results were obtained for the dimer whether preparation I or II was used. The results for preparation II are shown on Figure 2. The $d(t)$ curve was analyzed in terms of a $D(t)$ curve which is the sum of two exponentials. The data of Figure 2 were found to fit best the parameters: $a_1 = 0.117$, $a_2 = 0.199$, $\tau_1 = 1.22$ nsec, and $\tau_2 = 10.3$ nsec. $R(t)$ was then calculated using eq 3, giving the curve shown on a semilogarithmic scale in Figure 2. Except at very short times, this curve is linear, with a slope corresponding to a relaxation time of 70 nsec. This relaxation time is for the rotation of the whole kinetic unit and, within the model of an ellipsoid of revolution, it corresponds to ρ_h. The axial ratio, p, of the dimer must be less than 3, since for $p = 3$, the $R(t)$ plot of Figure 2 is curved, as shown by the dotted line. Taking $p = 2$ gives $f(p) = 1.168$ and $\rho_0 = 60$ nsec (eq 7). This value is twice that of the monomer and corresponds to twice the volume of the monomer since the two measurements have been carried out at identical temperatures. At small times, a slight

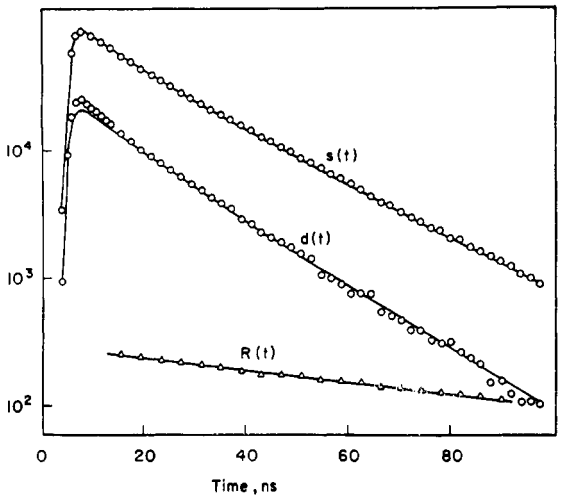

FIGURE 3: Decay curves, $s(t)$, and $d(t)$ for the octamer. The circles are experimental values. The solid lines are calculated convolution products.

upward curvature is observed in the experimental $R(t)$ curve of Figure 2. This could be due either to depolarization because of energy transfer between dimethylamino-1-sulfonyl-5-naphthalene molecules or to the presence of small relaxation times within the protein. The first possibility may probably be ruled out, since in that case preparations I and II should have different $d(t)$ curves because of different contents of fluorescent groups. This, however, is not so. Furthermore, energy transfer between dimethylamino-1-sulfonyl-5-naphthalene molecules is very inefficient (André-Frey and Wahl, 1969). It would seem then that small relaxation times are present. From the initial slope of the $R(t)$ curve of Figure 2 it is possible to calculate a relaxation time of order -1 (Wahl, 1966), $\rho_{-1} = 22$ nsec. The small relaxation times are determined by eliminating ρ_h from ρ_{-1} according to

$$\frac{1}{\rho_{-1}} = \frac{(1 - A)}{\rho_1} + \frac{A}{\rho_h} \qquad (11)$$

where $A = R_0'/R_0$, R_0 is the value of $R(t)$ at $t = 0$, and R_0' is the extrapolation of the linear part of the $R(t)$ curve of Figure 2. The results are $R_0 = 0.317$, $R_0' = 0.270$, $A = 0.85$, and $\rho_1 = 4.4$ nsec. The small value of ρ_1 could be due to the relaxation of a mobile segment of the molecule. It should be recalled that the structure of β-lactoglobulin contains several crevices with groups buried within them and which open as a result of reversible conformational transitions (Tanford and Taggart, 1961; Timasheff *et al.*, 1966; Townend *et al.*, 1969).

The results of experiments at conditions under which octamer is prevalent are shown on Figure 3. In these experiments, the form of the exciting flash has a negligibly small effect between 15 and 70 nsec. In this time interval the $s(t)$ and $d(t)$ decays are slow and the intensities are high relative to the flash. It is then possible to obtain $R(t)$ simply as the ratio $d(t)/s(t)$. This curve, shown by the triangles of Figure 3, is linear on a semilogarithmic scale and results in a relaxation time of 234 nsec. This gives a radius of 35 Å for the equivalent sphere, taking into account the temperature of the experiment (2°) and the viscosity of the solution. Assuming that the octamer has spherical symmetry and is formed of rigid monomers, its radius should be twice that of the monomer. The observed ratio is 1.67. This lower value could be due either to the presence of some internal freedom of rotation or to the presence in the system of lower associated species, such as dimers, tetramers, and hexamers in equilibrium with octamer. That such intermediates are present in the β-lactoglobulin A association system is known from thermodynamic studies (Kumosinski and Timasheff, 1966). The presence of these smaller kinetic units is further supported by the analysis of the portion of the $d(t)$ curve near the origin, using the convolution product of eq 2b and 10, with $\rho_0 = 234$ nsec. This results in good agreement between the experimental and calculated curves except near the origin. Near the origin the experimental curve has a higher slope which would correspond to smaller relaxation times.

Finally, values of $\langle\tau_s\rangle$, $\langle\tau_D\rangle$, and $\langle\rho\rangle$ were calculated for the various experiments using eq 8 and 9. The resulting mean relaxation times, $\langle\rho\rangle$, were found to be 31, 67, and 195 nsec for monomer, dimer, and octamer, respectively; these are the magnitudes expected for the three species.

In conclusion, the present experiments indicate that the method of fluorescence depolarization may be used successfully to characterize the geometry of associating protein systems.

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Interaction of Dodecyl Sulfate with Native and Modified β-Lactoglobulin*

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ABSTRACT: β-Lactoglobulin polymorphs A, B, and C (mol wt 36,000) interact with dodecyl sulfate yielding complexes containing 2 moles of dodecyl sulfate/mole of protein. Neither S-carboxymethylation, alkylation of tryptophan residues, nor limited hydrolysis of β-lactoglobulin with carboxypeptidase A affected the dodecyl sulfate complexing ability. However, low levels of photooxidation in the presence of methylene blue, which destroys histidine, primarily, does abolish the ability to complex.

The results indicate that the binding site conformation is critically influenced by a histidine residue located in the interior of each polypeptide chain.

A number of studies have been made concerning the capacity of β-lactoglobulin to bind a variety of organic compounds. Lundgren (1945) and Bull (1946) correlated the binding of anionic detergents with the sum of the positive groups in β-lactoglobulin. Klotz and Urquhart (1949) found that the protein displayed only weak or slight affinity for anionic methyl orange. McMeekin *et al.* (1949) succeeded in isolating a crystalline complex of bovine β-lactoglobulin and dodecyl sulfate. Hill and Briggs (1956) were able to demonstrate different stages of β-lactoglobulin–*n*-octylbenzene-*p*-sulfonate interaction by equilibrium dialysis and electrophoresis.

The early investigations were carried out using pooled bovine β-lactoglobulin. Recently, however, four different genetic forms of β-lactoglobulin have been detected (Aschaffenburg and Drewry, 1955; Bell, 1962; Grosclaude *et al.*, 1966). These have been designated as β-lactoglobulins A, B, C, and D. With the discovery and availability of the β-lactoglobulin polymorphs, there has developed an interest in reexamining the binding characteristics of these related proteins. The interaction of these proteins with dodecyl sulfate might detect structural differences among closely related molecular species. The alkane binding characteristics of β-lactoglobulins A and B have been studied by Wishnia and Pinder (1966), while Ray and Chatterjee (1967) investigated conformational changes and binding characteristics of bovine β-lactoglobulins A and B, and also goat and buffalo β-lactoglobulins using methyl orange, dodecyl sulfate, and dodecylpyridinium bromide.

This report examines the binding characteristics of bovine β-lactoglobulins A, B, C, and also β-lactoglobulin from pooled milks (AB). I used dodecyl [^{35}S]sulfate in order to study more precisely the quantitative aspects of the protein–detergent interaction. This is an improvement on the method of McMeekin *et al.* (1949) where the binding was measured by gravimetric sulfur determinations. This report also includes studies undertaken to identify those amino acids of the polypeptide chain which are involved in the rather specific binding of the detergent molecule. These studies include photooxidation and other chemical modifications of the protein molecule prior to interaction with dodecyl [^{35}S]sulfate.

Materials and Methods

Bovine β-lactoglobulins A, B, and C and carboxypeptidase A modified β-lactoglobulin B were kindly supplied by Dr. E. B. Kalan. Crystalline sodium dodecyl [^{35}S]sulfate (specific activity 1.87 mCi/mmole) was obtained from New England Nuclear Corp.[1]

β-Lactoglobulin–dodecyl [^{35}S]sulfate derivatives were prepared by a method similar to that of McMeekin *et al.* (1949). Typically, 4.2 ml of 0.2 M dodecyl [^{35}S]sulfate was added to 50 ml of a 1% solution of β-lactoglobulin at pH 4.2, giving a 6:1 detergent to protein molar ratio in the reaction mixture. After standing overnight, the crystalline protein–dodecyl sulfate complex was separated from the mother liquor by centrifugation and recrystallized by dialysis from 0.1 M sodium chloride until a constant specific activity was obtained.

A 6:1 detergent to protein molar ratio was also used in preparing the dodecyl [^{35}S]sulfate derivative of the chemically modified β-lactoglobulins. In these instances the binding experiments were carried out at pH 6.6 owing to the differences

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