Biophysical Chemistry 4 (1976) 203–213

A COMPARATIVE STUDY ON BOVINE α-LACTALBUMIN AND LYSOZYME BY NANOSECOND FLUOROMETRY

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Received 2 October 1975

Analysis of the time decay of fluorescence anisotropy of 1-dimethylaminonaphthalene-5-sulfonyl (DNS) and fluorescamine derivatives of bovine α-lactalbumin and lysozyme reveals that no significant differences in mean rotational relaxation times are present. While fluorescamine molecules appear to orient randomly on these proteins, DNS is bound with a preferential orientation. Other fluorescence characteristics of the labels are also cited.

1. Introduction

The time decay of fluorescence anisotropy has been used to obtain useful information as to the size and shape parameters, as well as the internal rigidity, of biopolymers conjugated with a fluorescent label. The value of the technique in detecting internal degrees of rotational freedom or conformational changes reflected by changes in rigidity is well-established. However, many uncertainties remain as to the relationship of the computed relaxation times to the actual shape of the molecule. For this reason, further study of this technique as applied to proteins of known three-dimensional structure, such as lysozyme, seems justified.

A second motivation for this study is the question of the conformational similarity of lysozyme and α-lactalbumin, which have extensive homology of their amino acid sequences [1]. The two proteins show similar optical rotatory dispersion, circular dichroism, and infrared spectra [2]. Model-building studies have demonstrated that the amino acid sequence of α-lactalbumin is compatible with a conformation similar to that of lysozyme [3,4]. The molecular weights, frictional ratios, and partial specific volumes of the two proteins are almost identical [5,6].

The only direct physical evidence against the structural similarity of the two is the recent report by Rawitch [7] of significantly different relaxation times for lysozyme and α-lactalbumin, as obtained from static measurements of polarization of fluorescence. These data were interpreted in terms of a significant difference in shape [7]. While low angle X-ray scattering experiments [8] appeared to indicate a significantly larger radius of gyration for α-lactalbumin, these were subsequently reinterpreted in terms of a small amount (< 5%) of dimerization of α-lactalbumin [9], with the conclusion that the actual radii of gyration were closely similar [9].

It thus is of some interest to reexamine the fluorescence polarization behavior of α-lactalbumin and lysozyme to determine whether the conclusion of significantly different shapes in solution is justified.

The technique of nanosecond fluorometry has been successfully applied to the study of proteins in recent years [10–14]. From the time decay of anisotropy the average rotational relaxation time, which is related to the size and shape of the protein, may be computed.

In this paper, we present the results of the application of nanosecond fluorometry to the study of α-lactalbumin and lysozyme, using the fluorescent probes 1-dimethylaminonaphthalene-5-sulfonate (DNS)* and 4-phenylspiro [furan-2(3H),1′-phthalan] 3,3′ dione (fluorescamine). Fluorescamine is a new re-

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* See following page.

agent for preparing fluorescent derivatives of primary amines introduced by Udenfriend, Weigele, and coworkers [15], while DNS is a commonly used fluorescent probe, reacting primarily with α- and ε-amino groups and occasionally with phenolic hydroxyls in proteins.

The aims of this study are: (1) to compare the observed rotational relaxation times obtained with different labels with those expected for lysozyme, whose three-dimensional structure is well-established, (2) to compare the properties of the different labels, and (3) to compare the observed relaxation times of α-lactalbumin and lysozyme in order to determine whether there is evidence for a difference in shape.

2. Experimental: Materials

α-Lactalbumin was prepared from fresh (unpasteurized) milk according to the procedure described by Robbins and Kronman [16]. The resulting α-lactalbumin was recrystallized three times. Purified protein was stored at −20°C; it showed a single band on polyacrylamide gel electrophoresis. Twice recrystallized lysozyme and dansyl chloride were purchased from the Sigma Chemical Co. Fluorescamine was obtained from Hoffman-La Roche, Inc. Protein concentrations were determined using specific absorptivities, $E^{1\%}_{280} = 20.1$ for α-lactalbumin [17] and 26.9 for lysozyme [18].

3. Experimental: Methods

3.1. Preparation of conjugates

Dansyl-protein conjugates were prepared as follows: dansyl chloride, 6% solution in acetone, was diluted 10× with 0.1 M sodium bicarbonate buffer, pH 8.3. Then, the resulting mixture was added dropwise with stirring to a 1% protein solution in the above buffer. After storing overnight at 4°C the resulting conjugate was centrifuged at 8000 rpm for 15 min and then fractionated on a 2 × 50 cm column of Sephadex G-100. Elution was accomplished with 50 mM cacodylate buffer, pH 7.4 or 50 mM acetate buffer, pH 5.5. The molarity of dye in the final preparation was determined by employing a molar extinction coefficient of 3400 at 340 nm [19]. The contribution of dye at 280 nm was estimated from the ultraviolet spectra of the free dye [20]. The dye/protein (D/P) ratio of the protein conjugates ranged from 0.31 to 0.42 moles of dye per mole of protein.

Fluorescamine-treated proteins were prepared at room temperature by addition of fluorescamine, 0.12% solution in acetone, to proteins in 0.2 M sodium borate; the pH of the solution was then adjusted to 9.0. Labeled proteins were passed through a 1 × 50 cm Sephadex G-25 column developed with 50 mM cacodylate buffer, pH 7.4 or 50 mM acetate buffer, pH 5.5. The molarity of labeling was determined by using a molar extinction coefficient of 5450 at 380 nm [21]. The contribution of the dye at 280 nm was corrected by assuming a molar extinction coefficient of 15 000 [21]. The degree of labeling ranged from 0.54 to 3.40 moles of dye per mole of protein.

3.2. Preparation of modified protein-dye conjugates [22,23]

Tyrosine residues on proteins were nitrated by addition of tetranitromethane (TNM), 20% solution in ethanol, to proteins in 0.1 M tris buffer, pH 8.5. After standing for 30 min, the solution was passed through a 1 × 25 cm Sephadex G-25 column developed with 0.3 M KCl, 0.01 M KOAc, pH 5.0. Pooled fractions were then passed through another 1 × 25 cm Sephadex G-25 column; elution was accomplished with the same buffer. Dansyl conjugated modified proteins were prepared first by reducing the nitrated protein, in 0.3 M KCl, 0.01 M KOAc, pH 5.0, with 0.1% sodium dithionite; then, dansyl chloride, 0.4% solution in acetone/H_2O was added. After 30 min, 0.1 M cysteine (pH 5.0) was added. The resulting conjugate was dialyzed against working buffer overnight before placing on a 2 × 50 cm Sephadex G-25 column.

* Abbreviations: DNS-, 1-dimethylaminonaphthalene-5-sulfonyl-;
FL-, fluorescamine-labeled;
LAC, α-lactalbumin;
LYS, lysozyme;
LAC(M), modified α-lactalbumin;
LYS(M), modified lysozyme

3.3 Fluorescence measurements

The steady-state intensity and spectral distribution of fluorescence were measured with an Aminco spectrophotofluorometer. Fluorescence spectra were corrected for the spectral sensitivity of the optical system consisting of lenses, a grating monochromator, and a photomultiplier according to the method of Lippert et al. [24]. Relative fluorescence quantum yields were determined by the method of Parker and Rees [25]. Measurements of fluorescence lifetime and nanosecond anisotropy were made with an Ortec 9200 single photon counting nanosecond fluorometer. Appropriate Corning filters or Baird Atomic interference filters were used to select the excitation and emission wavelengths.

In the measurements of fluorescence anisotropy, the exciting light was vertically polarized. A polarizer was also placed in the emission beam, which could be rotated by 90° to permit the measurement of the intensities of vertically and horizontally polarized components. Polacoat disks were used as the polarizers. All the measurements were done at room temperature (22 ± 1°C). Appropriate controls were examined to correct for the scattered light and possible intrinsic fluorescence arising from the quartz cuvette and the interference filter. Typically $4 - 5 \times 10^6$ counts were accumulated.

3.4. Data analysis

The time-dependent anisotropy, $A(t)$, is defined as

$$A(t) = \frac{I_{\parallel}(t) - GI_{\perp}(t)}{I_{\parallel}(t) + 2GI_{\perp}(t)}. \tag{1}$$

Here, $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the fluorescence intensities observed with the analyzer parallel and perpendicular to the vertically polarized exciting beam. The instrument correction factor, G, which is equal to $I_{\parallel}/I_{\perp}$ for aqueous solutions of 9-aminoacridine, is introduced to correct for any unequal transmission by the system for, or any unequal response of the photomultiplier to, light of varying polarization. 9-Aminoacridine has a fluorescence lifetime of 17 ns, high quantum yield of about 1 [26] and a very low relaxation time. Thus, it is ideal for the determination of the instrument correction factor.

Two methods were employed to compute the anisotropy from raw data. The first method (Method I) was to assume an exciting light pulse having the shape of a delta function and to confine the analysis to times after decay of the initial exciting pulse. The second method (Method II) was to obtain the quantities $I_{\parallel}(t) + 2I_{\perp}(t)$ and $I_{\parallel}(t) - I_{\perp}(t)$ separately by deconvolution. In this case,

$$S(t) = I_{\parallel}(t) + 2I_{\perp}(t), \tag{2}$$

$$D(t) = I_{\parallel}(t) - I_{\perp}(t) = A(t)\,S(t). \tag{3}$$

We have also measured the quantity $I_{\parallel}(t) + 2I_{\perp}(t)$ by adjusting the analyzer at 35° with the vertical and using unpolarized exciting light. In the simplest cases, both $S(t)$ and $A(t)$ decay as a single exponential, i.e.,

$$S(t) = \alpha_0 e^{-t/\tau}, \tag{4}$$

$$A(t) = A_0 e^{-t/\phi} \tag{5}$$

$$D(t) = \alpha_0 A_0 \exp\left\{-\left(\frac{1}{\tau} + \frac{1}{\phi}\right)t\right\}, \tag{6}$$

where α_0 is the amplitude and τ the fluorescence lifetime of the component; A_0 is the initial emission anisotropy, i.e., the anisotropy in the absence of rotary Brownian motion; and ϕ is the correlation time which is related to the rotational relaxation time by

$$\phi = \rho/3. \tag{7}$$

If $S(t)$ is composed of two exponentials, and $A(t)$ is a single exponential, we have

$$S(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}, \tag{8}$$

$$A(t) = A_0 e^{-t/\phi},$$

$$D(t) = A_0\alpha_1 \exp\left\{-\left(\frac{1}{\tau_1} + \frac{1}{\phi}\right)t\right\}$$

$$+ A_0\alpha_2 \exp\left\{-\left(\frac{1}{\tau_2} + \frac{1}{\phi}\right)t\right\}. \tag{9}$$

In both cases, the correlation times can be computed from eqs. (4) and (6) or from eqs. (8) and (9).

The rotational relaxation time can be calculated theoretically. For a rigid sphere, $A(t)$ decays exponentially,

$$A(t) = A_0 e^{-t/\phi}.$$

ϕ is related to the volume V of the hydrated sphere,

the viscosity η of the solution, the absolute temperature T, and the Boltzman constant k by

$$\phi = V\eta/kT. \tag{10}$$

The time-course of the emission anisotropy for particles other than rigid spheres is complex. Explicit equations have been derived only for rigid ellipsoids of revolution [10], for which $A(t)$ decays as the sum of three exponential terms, provided that the absorption and emission transition dipoles of the chromophore are parallel,

$$A(t) = A_0 \sum_{i=1}^{3} A_i \exp(-t/\phi_i). \tag{11}$$

The rotational correlation times ϕ_i, corresponding to the three principal axes, depend on the axial ratio of the ellipsoid as well as on the factors given in eq. (10), whereas the coefficients A_i depend on the orientation of the emission transition moment of the chromophore with respect to the ellipsoid axes. For a prolate ellipsoid, the following three cases are considered:

Case I, if the emission transition moments are randomly oriented with respect to the ellipsoid axes, $A(t)$ reduces to

$$A(t) = \tfrac{1}{5}A_0(e^{-t/\phi_1} + 2e^{-t/\phi_2} + 2e^{-t/\phi_3}). \tag{12}$$

Case II, if they are perpendicularly oriented to the major axis of the ellipsoid, we have

$$A(t) = \tfrac{1}{4}A_0(e^{-t/\phi_1} + 3e^{-t/\phi_3}). \tag{13}$$

Case III, when they are oriented parallel to the major axis of the ellipsoid, $A(t)$ has the form

$$A(t) = A_0 e^{-t/\phi_1}. \tag{14}$$

The mean rotational correlation time, $\bar{\phi}$ can be obtained from the slope at the initial stage of the plot of $A(t)$ versus t as

$$\text{Case I} \quad \frac{1}{\bar{\phi}} = \frac{1}{5}\left(\frac{1}{\phi_1} + \frac{2}{\phi_2} + \frac{2}{\phi_3}\right), \tag{15}$$

$$\text{Case II} \quad \frac{1}{\bar{\phi}} = \frac{1}{4}\left(\frac{1}{\phi_1} + \frac{3}{\phi_3}\right), \tag{16}$$

and

$$\text{Case III} \quad \frac{1}{\bar{\phi}} = \frac{1}{\phi_1}. \tag{17}$$

Deconvolution was done with the method of moments [27,28]. A copy of the Flortran program, based on the method of moments, was kindly supplied to us by Dr. Dyson. All the analyses were done on a Univac 1108 computer.

4. Results

4.1. Fluorescence spectra and fluorescence lifetime of the conjugates

Fluorescence spectra of FL-conjugated proteins were almost the same for α-lactalbumin and lysozyme, peaking around 480 nm; corrected spectra were identical with those reported in the literature [29]. As shown in fig. 1, fluorescence spectra of DNS-conjugated proteins showed a marked difference between α-lactalbumin and lysozyme for similar degrees of labeling. In general, the peak of the fluorescence spectrum of DNS–LAC was shifted about 20–30 nm to the shorter wavelength compared to that of DNS–LYS and had a shoulder around the fluorescence peak of DNS–LYS; this shoulder was pronounced on corrected fluorescence spectra. On the other hand, DNS–conjugated modified proteins gave almost the same fluorescence spectra as that of DNS–LAC. Table 1 summarizes the peaks of observed and corrected fluorescence spectra and fluorescence lifetimes of all the conjugates studied.

To make the spectral properties of DNS–LAC clearer, the time decay of fluorescence intensity was measured as a function of the fluorescence wavelength. The analysis showed that there exists two different decays with the magnitude of approximately 20 and 7 ns and that the contribution of the component with shorter lifetime increases with an increase in the fluorescence wavelength (table 2).

4.2. Decay of fluorescence anisotropy and rotational relaxation times

Typical time decays of fluorescence anisotropy for dye-conjugated proteins are shown in figs. 2 and 3. These figures indicate that fluorescence anisotropy decays as a single exponential at the initial stage and that the initial slope of FL-conjugated protein is larger than that of DNS-conjugated ones, reflecting different

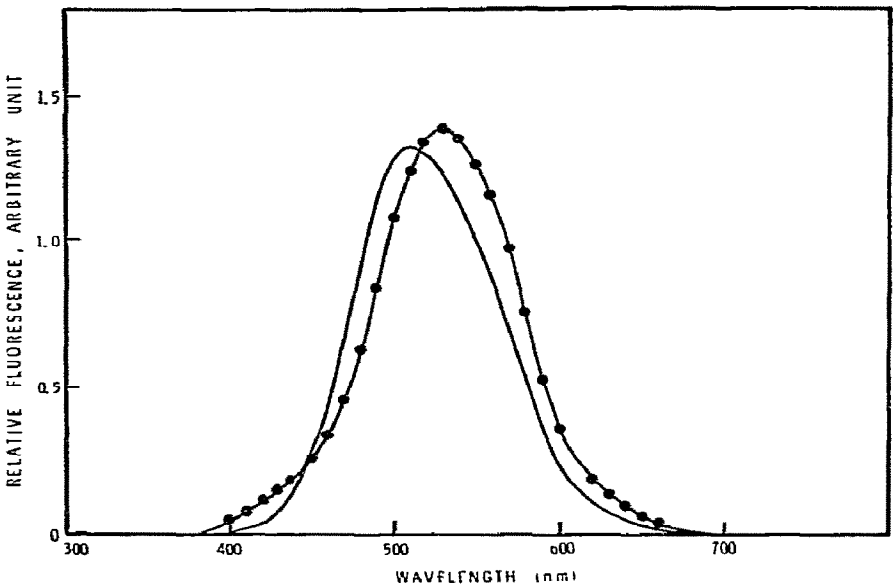

Fig. 1. Emission spectra of DNS–LAC (——) and DNS–LYS (–●–) conjugates. Both conjugates contain 0.42 fluorescent group/mole of protein. The spectra were measured in 50 mM cacodylate buffer, pH 7.4, with excitation monitored at 340 nm. The spectra are in arbitrary units.

Table 1
Fluorescence of dye–protein conjugates

Sample	Buffer[a]	D/P	λ_{em}^{max} (nm)[b] (obs.)	λ_{em}^{max} (nm)[c] (corr.)	α_1[d]	τ_1 (ns)[e]	α_2[d]	τ_2 (ns)[e]
FL–LYS	acetate	1.18	478	491	0.262	8.5	0.738	3.4
FL–LAC	acetate	3.40	–	–	0.357	8.1	0.643	2.8
FL–LYS	cacodylate	1.00	484	494	0.165	10.2	0.835	4.3
FL–LAC	cacodylate	0.98	478	492	0.252	8.9	0.748	4.4
DNS–LYS	acetate	0.41	537	590(540)				
DNS–LAC	acetate	0.31	506	538(580)				
DNS–LYS	cacodylate	0.42	532	563		7.1[f]		
DNS–LAC	cacodylate	0.42	510	540(568)	0.380	20.6	0.620	6.1
DNS–LYS(M)	acetate	0.95	508	540(580)	0.453	19.5	0.547	5.4
DNS–LAC(M)	acetate	3.33	500	536(580)	0.311	18.3	0.689	3.6

a) Buffer compositions are given in the experimental section.
b) Excitation wavelength for DNS-conjugated proteins was 358 nm and that for FL-conjugated proteins was 385 nm; emission was monitored at 540 nm for DNS-conjugated proteins and at 500 nm for FL-conjugated proteins and DNS–conjugated modified proteins.
c) Values in parentheses are the shoulder positions on the corrected fluorescence spectra.
d) These values have been normalized.
e) The precision of the fluorescence decay measurements is about 10%.
f) One-component analysis best fitted the data.

Table 2
Dependence of the decay characteristics of DNS–LAC[a] on emission wavelength

λ_{em} (nm)	α_1[b]	τ_1 (ns)	α_2[b]	τ_2 (ns)
480	0.412	21.2	0.588	7.2
500	0.380	20.6	0.620	6.1
540	0.334	20.2	0.666	7.7
580	0.232	19.9	0.768	7.0

a) DNS–LAC (D/P = 0.42) is in 50 mM cacodylate, pH 7.4.
b) These values have been normalized. Excitation wavelength was 358 nm.

In the three-component analysis, the same two lifetimes found in the two-component analysis recurred plus a third component of negligibly small amplitude or negative decay time. This is a strong indication that the decay curves can be represented as the summed contribution of two species of different lifetimes.

rotational motions. The scattering of anisotropy data after channel 120–130 seems to be due to the effect of the after pulse of the light source.

Two methods (Method I and II) were applied to obtain the mean rotational relaxation times, $\bar{\rho}$. In Method I, the value of $\bar{\rho}$ was calculated from the slope at the initial stage of the plot of ln $A(t)$ versus t. In Method II, the quantity $D(t)$ was obtained as the sum of two exponentials after deconvolution; then, values of $\bar{\rho}$ were calculated using eqs. (8) and (9). As listed in table 3, the $\bar{\rho}$ value calculated by the two methods are in fairly good agreement.

The $\bar{\rho}$ values reported here are the average values of at least two experiments for each sample; the reproducibility was very good. The D/P ratios were kept low to avoid any occurrence of energy transfer.

Our $\bar{\rho}$ values for FL–LYS are in good agreement with the value (25 ns) reported for fluorescein isothiocyanate–LYS [30]; however, they are a little smaller than the values (28–29 ns) reported by Chen [29] for the same FL–LYS conjugate. This difference might lie in the fact that the two groups have used different techniques, which, in turn, have yielded different lifetimes for the conjugates. While Chen used static polarization techniques in his study, we have employed the single photon counting techniques. By application of the method of moments to the analysis of the time decay curve, two lifetimes were found, 10.2 and 4.3 ns. If the same fluorescence decay curve had been treated as a single exponential, a lifetime of 6.2 ns would have been obtained, which is the value Chen reported for fluorescamine labeled lysozyme.

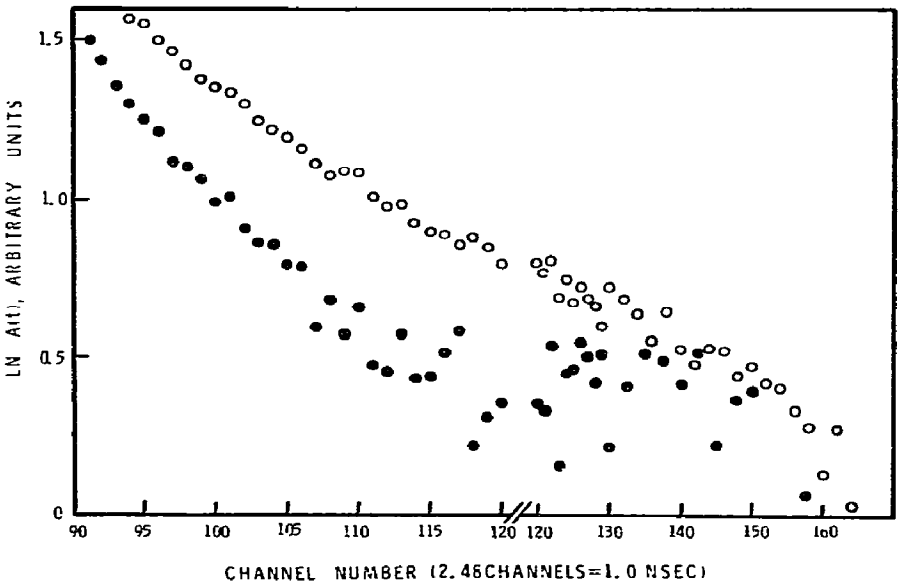

Fig. 2. Decay of the fluorescence anisotropy for the DNS–LAC (open circles; D/P = 0.42; λ_{ex} = 358 nm; λ_{em} = 540 nm) and FL–LAC conjugates (dots; D/P = 0.54; λ_{ex} = 385 nm; λ_{em} = 500 nm). Both conjugates were in 50 mM cacodylate buffer, pH 7.4.

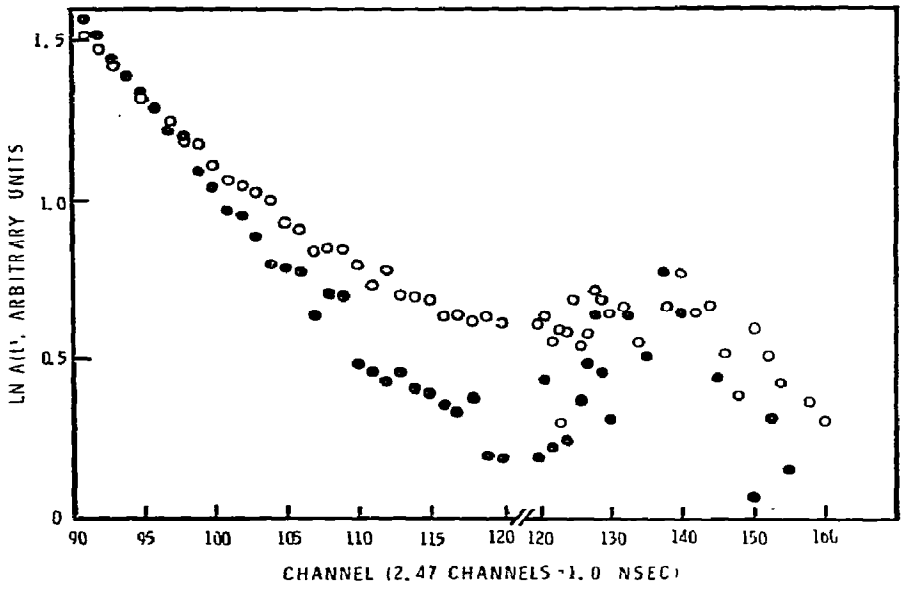

Fig. 3. Decay of the fluorescence anisotropy for the DNS–LYS (open circles; D/P = 0.42; λ_{ex} = 358 nm; λ_{em} = 540 nm) and FL–LYS conjugates (dots; D/P = 0.95; λ_{ex} = 385 nm; λ_{em} = 500 nm). Both conjugates were in 50 mM cacodylate buffer, pH 7.4.

While we obtained approximately the same $\bar{\rho}$ values for dansyl conjugated α-lactalbumin and lysozyme, Rawitch [7] reported significantly different values for the same conjugates ($\bar{\rho}$ = 35 ns for α-lactalbumin, $\bar{\rho}$ = 25 ns for lysozyme). This discrepancy may possibly be explained by the different techniques used.

Table 3
Rotational relaxation times of dye-labeled proteins

Sample	Buffer[a]	$\bar{\rho}$ (ns)[b]	
		Method I	Method II
FL–LYS	acetate	20.0	23.1
FL–LAC	acetate	24.2	23.0
FL–LYS	cacodylate	21.0	23.3
FL–LAC	cacodylate	22.4	22.9
DNS–LYS	acetate	29.8	
DNS–LAC	acetate	33.0	
DNS–LYS	cacodylate	29.5	
DNS–LAC	cacodylate	31.9	
DNS–LYS(M)	acetate	26.4	26.9
DNS–LAC(M)	acetate	33.3	33.8

a) Buffer compositions are given in the experimental section.
b) $\bar{\rho}$ was calculated for T/η = 333 deg/cp, which is the value for aqueous solutions at 25°; The uncertainty of the $\bar{\rho}$ values is about 10%.

4.3. Elution profiles of conjugates

The difference in the mean rotational relaxation times between DNS- and FL-conjugated proteins urged us to check whether it is due to the aggregation of the conjugates. Conjugates were prepared as usual. Additional amounts of protein were added to the conjugate solution in order to minimize the contribution of absorption of dye at 280 nm. The resulting solution was passed through a 2 X 50 cm Sephadex G-100 column. Absorption at 280 nm and the relative fluorescence intensity were measured for each fraction. Comparison of the absorption and fluorescence intensity profiles indicates that, within the accuracy of measurements, the conjugate does not aggregate more than the protein itself. Since both DNS- and FL-labeled proteins gave similar elution profiles, aggregation of the DNS-labeled proteins has been ruled out in the interpretation of the difference in rotational relaxation times between DNS- and FL-conjugated proteins.

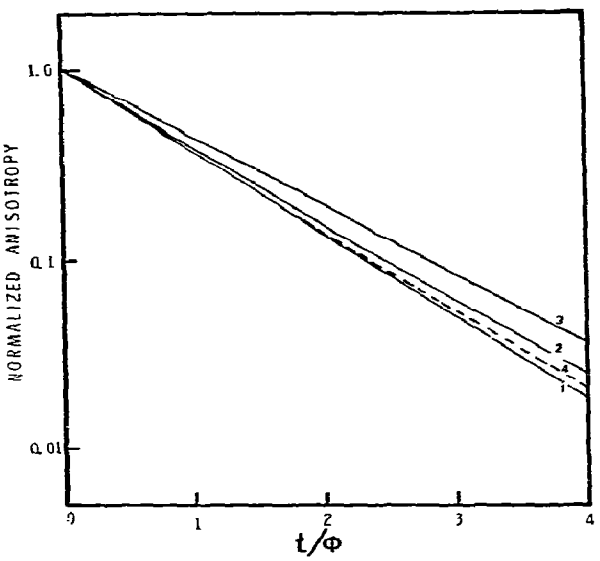

Fig. 4. Time-dependence of the normalized anisotropy for a rigid prolate ellipsoid of axial ratio 1.5. The chromophore is assumed to be a sphere (1), randomly oriented relative to the ellipsoid axes (2), oriented parallel to the major axis of the ellipsoid (3), and oriented perpendicular to the major axis of the ellipsoid (4).

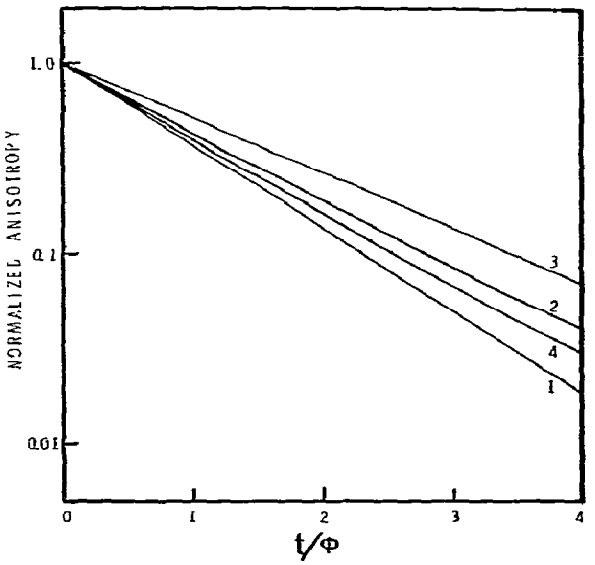

Fig. 5. Time dependence of the normalized anisotropy for a rigid prolate ellipsoid of axial ratio 2.0. Orientation of the chromophore same as in fig. 4.

5. Discussion

Within experimental uncertainty, the rotational relaxation times, $\bar{\rho}$, of α-lactalbumin and lysozyme are of the same magnitude whether fluorescamine or dansyl was used as the probe; however, $\bar{\rho}$ values obtained with DNS–LAC and DNS–LYS conjugates were significantly larger in both cases. This discrepancy could be interpreted by dansyl induced aggregation, by rotation of the dye with respect to protein, or by preferential orientation of the dye with respect to the coordinate axes of the protein molecules.

Kronman and Andreotti [17] found that even at protein concentrations as high as 5.85% at pH 5.24 and 6.00, α-lactalbumin did not aggregate. Adams and Filmer [31] reported that at 25°, pH 6.70, lysozyme associated to a significant extent. Comparison of the absorption and fluorescence intensity gel filtration elution profiles indicated that both DNS- and FL-labeled proteins do not aggregate more than the proteins themselves. Protein concentrations in this study were normally ~0.1%; therefore, DNS- and FL-conjugated protein solutions should be monodisperse.

Rotation of the label with respect to protein seems to be unlikely. If this should be true, a rotational relaxation time component with the magnitude of 1–2 ns would have been obtained.

Anderson and Weber [32] proposed a model of preferential orientation of the ANS molecules in planes parallel to the equator of a prolate ellipsoid of axial ratio 4 in their investigation on the complexes of ANS with bovine serum albumin. Mihalyi and Albert [33] also suggested that the dansyl groups were not distributed randomly on the bovine fibrinogen molecules, but were bound preferentially to a few specific sites of the protein. We have done some theoretical calculations for the mean rotational relaxation time of lysozyme, assuming that it is a prolate ellipsoid and that the absorption and emission dipoles of the chromophore are parallel, based on eqs. (5)–(14). Fig. 4 and fig. 5 show that, at fixed axial ratio, p, of the prolate ellipsoid, the plot of the logarithm of the normalized anisotropy ($A(t)/A_0$) versus t/ϕ, depends only on θ, the orientation of the emission transition moment of the chromophore. It is apparent from these figures

Table 4
Theoretical calculations on the mean rotational relaxation times of lysozyme[a)]

p(1/p)	Hydration[b)]	$\theta = 90°$	$\bar{\rho}$(ns) random orientation	$\theta = 0°$
1.0	0.22		14.0[c)]	
	0.52		18.6	
1.5	0.22	14.2 (15.9)	14.9 (15.4)	16.9 (14.6)
	0.52	18.9 (20.9)	19.7 (20.4)	22.4 (19.3)
2.0	0.22	15.3 (18.1)	16.4 (17.5)	21.1 (16.3)
	0.52	20.3 (23.9)	21.8 (23.1)	28.0 (21.5)
3.0	0.22	18.4 (23.5)	21.7 (22.6)	32.8 (21.1)
	0.52	24.4 (31.0)	28.8 (29.8)	43.6 (27.8)

a) Definitions of p, θ, and $\bar{\rho}$ are given in the text.
The partial specific volume of lysozyme is assumed to be 0.702 ml/g [6] and its molecular weight 14 100 [34]; calculations were done for 25°C.
Values in parentheses are the mean rotational relaxation times of lysozyme, assuming that it is an oblate ellipsoid.

b) Based on hydrodynamic results (diffusion and sedimentation), a value of 0.52 was obtained for hydration; a value of 0.22 was obtained from thermodynamic methods (absorption isotherms) [35].

c) This is the rotational relaxation time for a sphere of the same volume as the ellipsoid.

that a protein would have a larger mean rotational relaxation time if the chromophores were oriented along the major axis of the ellipsoid, i.e., $\theta = 0°$. Random orientation or orientation perpendicular to the major axis ($\theta = 90°$) of the ellipsoid would result in smaller $\bar{\rho}$ values. Table 4 presents the calculated $\bar{\rho}$ values according to eqs. (15)–(17); theoretical calculations for the mean rotational relaxation time of lysozyme, assuming that it is an oblate ellipsoid, are also included in this table. X-ray crystallographic studies by Blake et al. [4] showed that lysozyme is roughly ellipsoidal with an axial ratio of 1.5. However, the axial ratio may be larger than 1.5 for lysozyme in solution. In table 4 calculations were done for axial ratios of 1.5, 2.0, and 3.0 using two extreme values of hydration, 0.22 and 0.52 g/g protein [35]. Our experimental results would fit the calculated $\bar{\rho}$ values for FL–LYS for random orientation of the label if it is assumed that lysozyme is a prolate ellipsoid and that $p = 2$–3 and hydration is between 0.22 and 0.52 g/g protein.

Fluorescamine is known to react with primary amines. While there are several amino groups available on the surfaces of α-lactalbumin and lysozyme, it is likely that fluorescamine molecules are bound randomly on their surfaces. The fluorescence lifetime results of fluorescamine conjugated proteins showed two fluorescence decay times. It clearly reflects the presence of two different kinds of microenvironment; thus, at least two different sites are present on α-lactalbumin and lysozyme molecules.

Fluorescence lifetime results showed that DNS–LYS decays as a single exponential, whereas DNS–LAC decays as the sum of two exponential terms. The emission maximum of DNS–LAC shifts to a shorter wavelength [$\lambda_{em}^{max(obs)}$ 530 → 510 nm], with a shoulder at the emission maximum of DNS–LYS (~530 nm). Results presented in table 2 indicate that the component with a shorter fluorescence lifetime corresponds to the shoulder of the DNS–LAC fluorescence spectra, while the other component corresponds to the emission maximum. Furthermore, the fluorescence lifetime of DNS–LYS has the same magnitude as the lifetime of the component of DNS–LAC which corresponds to the shoulder of the DNS–LAC fluorescence spectra. A well-known feature of the tertiary structure of lysozyme is the presence of a predominantly hydrophobic "spine" [4]. It has been suggested that a similar hydrophobic zone occurs in α-lactalbumin and that it may include a cavity [36]. It is not unlikely that covalent attachment of the dansyl label may be preceded by binding to a hydrophobic region. If this is the case, some degree of selective orientation of the label with respect to the axes of the protein is to be expected. It is therefore plausible to speculate that, in the dansyl conjugates of α-lactalbumin and lysozyme, the label is covalently attached to an amino group while its non-polar portion is bound by the hydrophobic region with the direction of its emission axis roughly parallel to the long axis of the protein. The composite character of the emission from the α-lactalbumin conjugate could be explained in terms of two alternate sites of binding within the hydrophobic cavity, possibly differing in the extent of contact with solvent.

The $\bar{\rho}$ values obtained with the dansyl conjugated modified α-lactalbumin and lysozyme were somewhat unexpected. The $\bar{\rho}$ value of modified lysozyme is between those of FL–LYS and DNS–LYS, while the $\bar{\rho}$ value of modified α-lactalbumin is unchanged; furthermore, their fluorescence characteristics (spectra and

lifetime) are almost the same as those of DNS–LAC at pH 7.4. Dansyl molecules are expected to react exclusively with amino-tyrosine groups at pH 5.0 [23]. In view of our finding, it seems to be possible that dansyl molecules bound to the modified tyrosine residue within the hydrophobic zone orient parallel to the major axis, while those bound elsewhere are oriented more randomly. This would be consistent with the observed value of $\bar{\rho}$ intermediate to those of FL–LYS and DNS–LYS. While the location of the tyrosine residues of α-lactalbumin is unknown, it is of course possible to explain the results in terms of a preferential attachment of dansyl labels to tyrosine groups placed within hydrophobic regions in the cavity.

In any event it is clear that the time decay of anisotropy does not provide support for a significant difference in shape between lysozyme and α-lactalbumin. Our values of relaxation times for FL lysozyme and DNS-modified lysozyme are in reasonable agreement with the literature values obtained by static polarization measurements [7,29,30], as do our values for DNS–lactalbumin. While our values for DNS–lysozyme are smaller than those for DNS–lactalbumin, the difference is much less than reported previously [7] and probably does not exceed experimental uncertainty. The difference between the two proteins almost disappears for fluorescamine conjugates. Any differences in shape or conformation must lie within the resolving power of the method. It thus appears that all of the physical techniques applicable to solutions have failed to establish differences in the shape parameters of the two proteins.

It is equally clear that the time decay of fluorescence anisotropy yields rotational relaxation times for lysozyme which correspond to *smooth* prolate ellipsoids of larger axial ratio than expected from the known structure deduced from X-ray crystallography. This may reflect either a significant change in shape in solution or, more probably, the effects of surface irregularities upon the measured relaxation time. The fluorescamine conjugates have consistently yielded values of $\bar{\rho}$ more in harmony with the known structure than have the DNS conjugates, probably because of the greater importance of specific orientation in the latter case.

Acknowledgement

The authors are indebted to Mr. Charles C. Bowen for computer programming and to Dr. Ming S. Tung for valuable advice and many helpful discussions.

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