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Environment of tryptophan residues in various conformational states of α-lactalbumin studied by time-resolved and steady-state fluorescence spectroscopy

Alexander V. Ostrovsky, Lina P. Kalinichenko, Victor I. Emelyanenko, Alexander V. Klimanov and Eugene A. Permyakov

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Pushchino, Moscow Region 142292, U.S.S.R.

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Decay curves for tryptophan fluorescence of bovine and human α-lactalbumin in different states (metal-free and Ca^{2+} or Mg^{2+}-loaded states of the native and thermally denatured proteins) have been measured at different wavelengths. The curves are best fitted by a sum of three exponents assigned to emission of individual tryptophan residues. The result suggests that the red shift of the fluorescence spectrum of α-lactalbumin caused by release of the bound Ca^{2+} or thermal denaturation is due to changes in the environment of all emitting tryptophan residues.

1. Introduction

α-Lactalbumin is a small globular protein (molecular mass 14 kDa) which is a component of the lactose synthase system [1]. One of the most important characteristics of this protein is its high affinity for divalent cations [2–8]. It possesses one binding site for Ca^{2+}. Binding of Ca^{2+} causes structural rearrangements which can be detected through using various physical and physicochemical methods [2,4,6], among which the intrinsic fluorescence technique is one of the most convenient. Various α-lactalbumins contain three to five tryptophan residues per molecule. It has been shown that Ca^{2+} binding causes a shift of 10–15 nm in the tryptophan fluorescence spectrum of α-lactalbumin towards shorter wavelengths and a decrease in the value of the fluorescence quantum [6]. Thermal denaturation of α-lactalbumin results in a shift of the fluorescence spectrum maximum from 327–331 to 344–346 nm. However, the position of the shift on the temperature scale depends strongly upon the Ca^{2+} content of the protein: while the denaturation transition for metal-free α-lactalbumin has a midpoint temperature of 31 ± 1 °C, the transition for the Ca^{2+}-loaded protein occurs at above 45–50 °C [8].

The study of the tryptophan fluorescence of different states of proteins may provide a better understanding of the nature of structural changes induced by Ca^{2+} binding. Although α-lactalbumin has been investigated in steady-state fluorescence studies [3–8], few works have been devoted to investigation of the fluorescence decay of the protein [9,10]. These studies have shown that the fluorescence decay follows double-exponential kinetics or even a more complex process. The fluorescence decay time depends upon the Ca^{2+} content of the protein [10].

Correspondence address: E.A. Permyakov, Institute of Biological Physics, U.S.S.R. Academy of Sciences, Pushchino, Moscow Region 142292, U.S.S.R.

It is well known that the fluorescence decay of the tryptophan zwitterion in aqueous solution at normal pH values is best described by double-exponential kinetics [11–14]. The two exponential components correspond to different conformers (rotamers) of tryptophan in which the indole ring interacts differently with the carboxylate and amino groups [14]. In a polypeptide chain with both groups involved in peptide bonds, the interactions which take place in a free tryptophan are absent, therefore, it is reasonable to expect that the fluorescence decay would be monoexponential. Consequently, a multiexponential decay results from the fluorescence of tryptophan residues in different environments with different quenching properties, each exponential term corresponding to a particular type of environment.

Fluorescence decay being measured as a function of emission wavelength makes it possible to decompose the steady-state fluorescence spectrum of a protein into components associated with each particular lifetime. Wahl and Auchet [15] pioneered wavelength-resolved fluorescence decay measurements. By now perhaps the most thorough measurements of this kind have been carried out with horse liver alcohol dehydrogenase. It was shown that the fluorescence emission of this two-tryptophan-containing protein could be resolved into two decay-associated components which correspond to the emission of Trp-15 (emission maximum at 337 nm) and Trp-314 (maximum at 324 nm) [16]. This is a well proven result for it is in excellent agreement with other measurements performed using both pulse and phase methods [17–19] and with the spectral resolution achieved by selective quenching by I^- [20].

The reason for the fluorescence of separate Trp-15 and Trp-314 decaying monoexponentially is, as Ross et al. [16] suggested, that both residues are in highly restricted environments in which the indole ring experiences a sufficiently narrow range of interactions with the neighbouring side groups such that no kinetically distinct second environment exists.

It should be pointed out that most of the single-tryptophan-containing proteins exhibit nonexponential fluorescence decay which might be associated with a heterogeneous environment of the tryptophan chromophore in different protein conformers (see, for instance, refs. 21 and 22); nevertheless, examples of monoexponential decay are also known [21].

As a consequence of the above-mentioned properties, wavelength-resolved fluorescence decay measurements should be quite useful for monitoring the fluorescence of individual tryptophan residues in a protein, but care should be taken in the interpretation of results as some residues might exist in a heterogeneous environment and hence exhibit nonexponential fluorescence decay.

A rather complete and up-to-date survey by Beechem and Brand [23] describes attempts to resolve the fluorescence of individual tryptophan residues even in multitryptophan proteins. The most interesting example from the point of view of the present article is the work by Formoso and Forster [24] where they report on tryptophan fluorescence lifetime measurements in lysozyme (six tryptophan residues) which is homologous to α-lactalbumin [25,26]. In their study, Formoso and Forster along with intact lysozyme used lysozyme derivatives with nonfluorescent tryptophan residues which made it possible to assign particular lifetimes to tryptophan residues, although no wavelength-resolved decay measurements were made.

In the present work we have measured the fluorescence decay of different Ca^{2+}- and Mg^{2+}-loaded states of bovine (four tryptophan residues [27]) and human (three tryptophan residues [28]) α-lactalbumins at various temperatures. Each decay curve is best fitted by a sum of three exponentials. Decay-associated spectra were constructed from the decay measurements at different wavelengths. It was possible to assign the decay-associated spectra to individual tryptophan residues taking advantage of the presence of different numbers of tryptophan residues in the α-lactalbumins investigated and the data of Sommers and Kronman [29] who estimated the contribution of individual tryptophan residues to the total fluorescence of Ca^{2+}-loaded bovine α-lactalbumin. It has been concluded that Ca^{2+} and Mg^{2+} binding alters the environment of all fluorescent tryptophan residues.

2. Materials and methods

α-Lactalbumin was isolated from bovine and human milk and purified according to a preparative sequence similar to that described by Kaplanas and Antanavichius [30]. Protein concentrations were measured spectrophotometrically using $E_{1\%,1\ cm} = 20.1$ at 280 nm for bovine α-lactalbumin and $E_{1\%,1\ cm} = 18.2$ at 280 nm for human α-lactalbumin [31]. The purity of the proteins was confirmed by SDS gel electrophoresis.

Steady-state fluorescence measurements were carried out using a laboratory-built spectrofluorimeter described earlier [32]. All fluorescence spectra were corrected for instrumental spectral sensitivity. Fluorescence quantum yield was evaluated by comparing the areas under spectra of protein preparations and free tryptophan in water (fluorescence quantum yield 0.20 at 25°C [33]) with the same absorbance at the excitation wavelength (296.7 nm).

Fluorescence lifetimes were measured by means of the time-correlated single-photon counting technique using a PRA 3000 fluorescence lifetime instrument, modified to use frequency-doubled picosecond laser excitation pulses from a CR-590 day-laser with rhodamine 6G synchronously pumped by a CR-18 argon ion laser. The pulse duration was controlled with a CR-290 autocorrelator and amounted to less than 10 ps. The system is described in detail elsewhere [34,35]. Excitation was at 296 nm, emission being monitored via a Jobin Yvon H10 monochromator and additional cut-off filters. A magic-angle polarizer was used to check for polarization effects. No such effects were observed, therefore, measurements were carried out without polarizers.

A measured fluorescence decay curve $F(t)$ is the convolution product of the instrument response function $E(t)$ and the undistorted decay function of the fluorescent system $D(t)$:

$$F(t) = \int E(u-t)D(t)\,\mathrm{d}u. \tag{1}$$

$E(t)$ was measured at the excitation wavelength, with the fluorescent sample being substituted by an aqueous solution of glycogen as scatterer. The response of the Hamamatsu R 955K photomultiplier tube used for fluorescence registration remained unchanged over the wavelength range 285–400 nm. Fluorescence decay curves were assumed to be described by a sum of exponents:

$$D(t) = \sum_{i=1}^{N} A_i \exp(-t/\tau_i) \tag{2}$$

where $N = 1$, 2 or 3. The preexponentials A_i and decay lifetimes τ_i were obtained by means of the PRA iterative deconvolution program. A fit was accepted if χ^2 had a value within the interval 0.8–1.2 and the weighted residuals had a random distribution, which was checked using the statistical criteria of Durbin-Watson [36], run test [37] and autocorrelation of the weighted residuals [38]. Normally, data were collected until 80000 counts in a curve maximum had been reached.

The contribution of an i-th exponential term into the total fluorescence decay curve was calculated as

$$I_i = A_i\tau_i / \sum_{k=1}^{N} A_k\tau_k. \tag{3}$$

The mean fluorescence lifetime was calculated according to

$$\bar{\tau} = \sum_{i=1}^{N} I_i\tau_i. \tag{4}$$

Decay-associated spectra $S_i(\lambda)$ were constructed using a steady-state spectrum $S(\lambda)$ and the contributions of the corresponding exponential terms at different wavelengths $I_i(\lambda)$:

$$S_i(\lambda) = S_i(\lambda)I_i(\lambda). \tag{5}$$

The experimental points for $I_i(\lambda)$ were smoothed by linear or square regression polynomials.

3. Results and discussion

The mean fluorescence lifetime $\bar{\tau}$ in α-lactalbumin depends strongly on the emission wavelength. In the case of native bovine apo-α-lactalbumin, $\bar{\tau} = 1.2$ and 2.7 ns at 310 and 400 nm, respectively. Such an increase in $\bar{\tau}$ cannot be

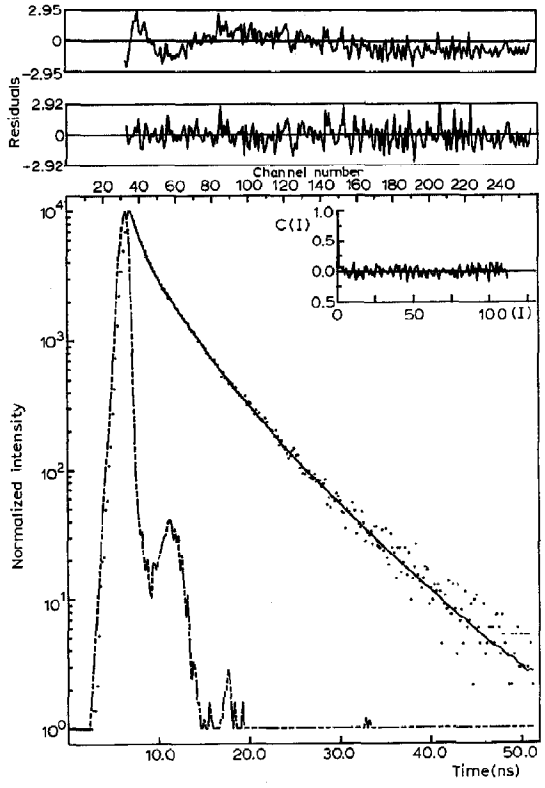

Fig. 1. Fluorescence decay for Ca^{2+}-loaded bovine α-lactalbumin in 10 mM Hepes (pH 8.1) at 12°C. (Top panels) Weighted residuals in the case of a double- and a triple-exponential fit. (Inset) Autocorrelation function for the triple-exponential weighted residuals. The instrument response function (— — —), data (·) and fitted curve (———) are shown on a semilogarithmic scale. Analysis is from data maximum (80000 counts), 223 channels, at 0.165 ns/channel. Excitation was at 296 nm and emission at 350 nm.

explained entirely by a relaxation process on the nanosecond time scale, since no term with a negative preexponential factor was observed at the red edge of the spectra [39]. Therefore, we have assumed that the increase in $\bar{\tau}$ is a result of heterogeneous emission by tryptophan chromophores located in different environments.

Fluorescence decay curves for all investigated forms of both bovine and human α-lactalbumins can be best fitted only by a sum of three exponentials. Representative curves for experimental laser excitation and tryptophan emission for bovine α-lactalbumin are shown in fig. 1. The excitation and emission are plotted on a semilogarithmic scale, while the weighted residuals and their autocorrelation function are shown on a linear scale. A random distribution of the latter two curves about the origin is indicative of a satisfactory fit. The randomly distributed autocorrelation function shows the absence of any systematic error that may be obscured by noise in the residuals themselves [38]. An attempt to fit the experimental data by a double-exponential curve yields a value of $\chi^2 \gg 1$ and an obvious systematic deviation in the weighted residuals plot (fig. 1, upper part). A three-exponential component analysis reduces the value of χ^2 to approx. 1. Fig. 1 shows that in this case the fitted curve is well superimposed upon the experimental points and the residuals and their correlation function appear to be distributed randomly, indicating a satisfactory fit to the data.

It is noteworthy that the lifetimes of the two components τ_2 and τ_3 are practically constant over the wavelength range studied (fig. 2, bottom panel). In the case of Ca^{2+}-loaded native proteins the longest lifetime constant τ_1 increases somewhat towards the red edge of the spectra. This exponential component contributes only about 10% to the total emission.

Fig. 2A (top panel) shows the decay-associated spectra for bovine α-lactalbumin, constructed from the steady-state fluorescence spectra according to ref. 5, using the experimentally obtained values of the contributions I_i of the exponential terms to the total emission at different wavelengths. The spectral positions (λ_i), lifetimes (τ_i) and contributions (I_i) of individual decay-associated spectra together with quantum yields (q) and spectral positions (λ) of the steady-state spectra for bovine and human α-lactalbumins are summarized in table 1.

It should be noted that our results are in quantitative agreement with those reported by Formoso and Forster [9]. In their work the fluorescence decay of bovine α-lactalbumin, most probably in the Ca^{2+}-loaded form, was analysed in terms of a double-exponential law. They found that the longer-lived component contributes about 10% to the total emission, from which it was concluded that the majority of protein molecules exist in a conformation characterized by strong

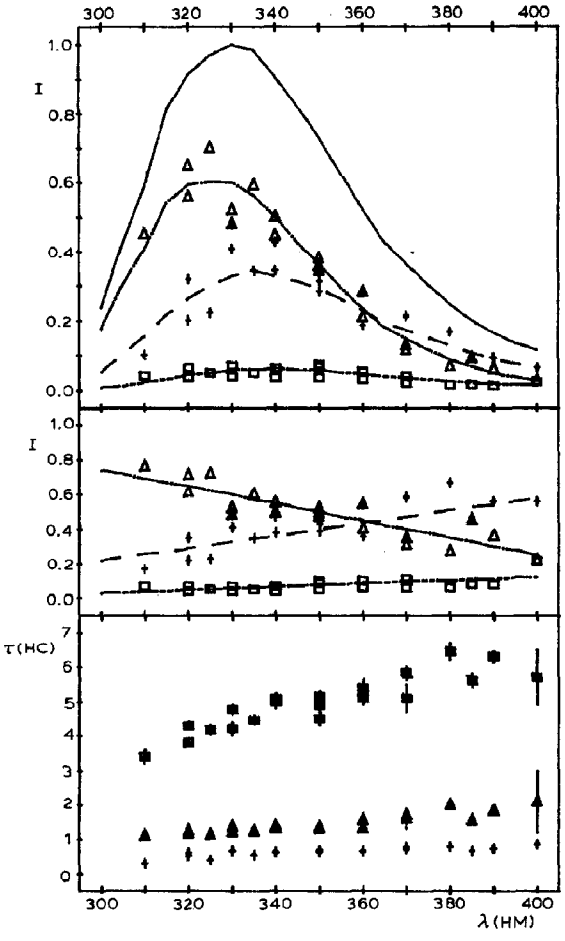

Fig. 2. Steady-state and decay-associated emission spectra (top panel) for native Ca^{2+}-loaded bovine α-lactalbumin in 10 mM Hepes (pH 8.1) at 12°C. Contributions of each exponential term into integral fluorescence I_i (center panel) and fluorescence lifetimes (bottom panel) measured at different emission wavelengths.

quenching of the fluorescence component with the longer lifetime. The shorter decay lifetime in their work is approximately the same as that obtained in our measurements, while the longer-lived component is the mean value of our two longer decay terms.

Although the two α-lactalbumins that we studied contain different numbers of tryptophan residues, the fluorescence decays of both proteins can be adequately fitted with a sum of three exponentials, the corresponding lifetimes being similar (see table 1). Table 1 clearly demonstrates that the contribution of the component with the shortest lifetime $\tau_3 = 0.6–0.7$ ns to the total emission S_3, for native Ca^{2+}-loaded bovine α-lactalbumin is almost 2-fold lower than that for native Ca^{2+}-loaded human α-lactalbumin. This can be explained by the lack of Trp-26 in the human protein. Thus, the contribution of Trp-26 to the total fluorescence can be estimated as half of S_3 in the bovine protein, i.e., 15–20%. Sommers and Kronman [29] have estimated the contributions of different tryptophan residues to the total fluorescence of Ca^{2+}-loaded bovine α-lactalbumin to be 50% for Trp-118, 10% for Trp-60 and 20% each for Trp-104 and Trp-26. These values are very similar to S_i for native Ca^{2+}-loaded bovine α-lactalbumin obtained in this study, therefore it is possible to assign the decay-associated spectra to emission from distinct tryptophan residues. Thus, the slowest decay component with $S_1 = 7\%$ can be assigned to the emission of Trp-60 ($\lambda_1 = 340$ nm), the intermediate decay term with a contribution of 60% is most likely due to the emission of Trp-118 ($\lambda_3 = 326$ nm) and finally, the shortest exponential term seems to arise from the emission of Trp-104 and Trp-26. According to the model for the tertiary structure of α-lactalbumin constructed by Brown et al. [25] and Warme et al. [26], who used as a starting point X-ray data for hen egg lysozyme, the distance between Trp-26 and Trp-104 is about 4 Å. Such a small distance leads to highly efficient energy transfer between these residues [40]. Due to intensive energy transfer the emission of these residues can be characterized by a single lifetime as if it arose from a single chromophore.

Sommers and Kronman [29] have put forward the proposal that the excitation energy transferred from Trp-26 and Trp-104 is channeled into Trp-60 with an efficiency of about 70%. This seems to result in a considerable decrease in the fluorescence lifetime of Trp-26 and Trp-104 to 0.6–0.7 ns. On the other hand, the fluoresence of Trp-60 can be significantly quenched by two disulfide bridges, which according to the model structure may be in contact with Trp-60 [25,26]. Taking into account the relatively long lifetime (about 5 ns),

Table 1

Quantum yields (q) and spectral positions (λ) of steady-state fluorescence and contributions (S_i), spectral positions (λ_i), and lifetimes (τ_i) of decay-associated spectra for various forms of bovine and human α-lactalbumins

Fluorescence parameters	α-Lactalbumin									
	Bovine					Human				
	Ca^{2+}		Apo		Mg^{2+}	Ca^{2+}		Apo		Mg^{2+}
	12°C	72°C	5°C	45°C	5°C	5°C	73°C	5°C	45°C	5°C
λ(nm)	327	343	333	344	331	328	341	332	342	331
q	0.040	0.020	0.072	0.066	0.063	0.037	0.020	0.054	0.030	
Trp-60										
S_1 (%)	7	10	7	30	5	15	20	13	30	22
λ_1 (nm)	340	345	347	340	341	337	343	341	336	338
τ_1 (ns)	5.0	3.4	6.3	4.3	5.9	5.3	3.2	5.9	4.1	5.9
Trp-118										
S_2 (%)	60	55	43	50	30	70	58	70	51	46
λ_2 (nm)	326	343	330	346	330	327	343	330	343	330
τ_2 (ns)	1.5	1.2	1.9	1.9	2.0	1.9	1.3	2.1	1.9	2.5
Trp-104 (Trp-26)										
S_3 (%)	33	35	50	20	65	15	22	17	19	32
λ_3 (nm)	335	345	334	345	330	330	338	336	346	326
τ_3 (ns)	0.7	0.5	0.9	0.5	0.9	0.6	0.45	0.9	0.6	0.9

one can conclude that this is a static quenching process.

According to the proposed model structure for α-lactalbumin, Trp-118 is located at too great a distance from the rest of the tryptophan residues to be able to interact with them. Warme et al. [26] have predicted three possible conformations, T2, T3 and T4 for the end chain of α-lactalbumin. In the T2 conformation, Trp-118 is exposed to the solvent, otherwise it is blocked by the end chain and is believed to be in a hydrophobic environment. Our results rule out this possibility since the position of the spectrum assigned to Trp-118 is at very short wavelengths (326–327 nm), characteristic of the hydrophobic environment [40].

The long-wavelength position of the decay-associated spectrum assigned to Trp-60 ($\lambda_1 = 340$ nm) suggests its location on the surface of the protein in contact with bound water molecules [40]. This is in good agreement with the data of Sommers and Kronman [29], who found that the fluorescence of Trp-60 is quenched by I^- with higher efficiency as compared to the emission of other tryptophan residues. Moreover, the fluorescence spectrum of guinea pig α-lactalbumin which lacks Trp-60 is slightly blue-shifted relative to bovine, goat and human α-lactalbumins in which Trp-60 is present.

The proposed assignment of decay-associated spectra to individual tryptophan residues in α-lactalbumins allows one to monitor changes in their environment induced by the binding of cations and thermal denaturation.

Ca^{2+} binding causes pronounced changes in the parameters of the decay-associated spectra, namely a 4–6 nm blue shift of the decay-associated spectra and a decrease in individual lifetimes by a factor of about 1.1–1.5 in the human protein and of 1.25 in the bovine form. At the same time, Ca^{2+} binding increases the contribution of Trp-26 and Trp-104 emission, S_3. In other words, the Ca^{2+}-induced shift of the steady-state fluorescence spectrum is due not only to the blue shift of the fluorescence spectra of individual tryptophan residues, but also to the increased contribution from S_3.

The apo forms of both bovine and human α-lactalbumins retain differences in the position of the fluorescence spectrum for their tryptophan chromophores. This means that the changes in the environment of tryptophan residues induced by Ca^{2+} removal are not very pronounced. This observation is supported by the results obtained by Koga and Berliner [41] who have demonstrated that the 'hydrophobic box' in bovine α-lactalbumin containing tryptophan residues remains almost unchanged by Ca^{2+} removal.

Thermal denaturation of α-lactalbumin causes significant changes in fluorescence. For the thermally denatured proteins the maxima of almost all the decay-associated spectra are within the interval 343–346 nm. Only the fluorescence spectra for Trp-60 of bovine and human apo-α-lactalbumins and Trp-104 of Ca^{2+}-loaded human α-lactalbumin are at shorter wavelengths. Temperature-dependent quenching processes result in a decrease of the fluorescence lifetimes of tryptophan chromophores. The transition temperature for the thermal denaturation of Ca^{2+}-loaded α-lactalbumins is more than 30 °C higher than that for the apo-proteins, therefore, the fluorescence lifetimes of the Ca^{2+}-loaded forms are shorter than those of the apo-proteins. One can see from table 1 that the thermally induced decrease in fluorescence lifetimes for various tryptophan residues is different, however, the lifetime shortenings for the same residues in bovine and human α-lactalbumins correlate with each other. This does not hold true for Trp-118, the fluoroscence lifetimes of which are considerably reduced by heating in the Ca^{2+}-loaded protein although the corresponding decay-associated spectrum is shifted by 13–16 nm.

The extent of most of the contributions of different decay-associated spectra is changed only slightly by heating except for S_1 (Trp-60) in the apo-α-lactalbumins.

The results presented here support the suggestion of Sommers and Kronman [29] that in thermally denatured α-lactalbumins the environment of all tryptophan residues is uniform. According to our data, thermally induced conformational changes involve all tryptophan chromophores but do not make them fully accessible to the solvent. The positions of the maxima in their fluorescence spectra (343–346 nm) indicate that their indole rings are in contact with bound water molecules [40].

The binding site of α-lactalbumin is capable of binding Mg^{2+}, but the equilibrium binding constant for Mg^{2+} is 5–6 orders of magnitude lower than that for Ca^{2+}. Mg^{2+}-loaded human and bovine α-lactalbumins have fluorescence spectra positioned at somewhat longer wavelengths in comparison with the Ca^{2+}-loaded proteins. This shows that the structure of the protein is more relaxed in the Mg^{2+}-loaded state. The fluorescence lifetimes of chromophores in the Mg^{2+}-loaded proteins and in the apo state of α-lactalbumins are almost equal.

The contributions of the decay-associated spectra for the Mg^{2+} state are quite different from those in the case of the Ca^{2+} form and their values are closer to those for the apo state. However, it is surprising that the spectral position λ_3 (Trp-104 and Trp-26 in bovine and Trp-104 in human α-lactalbumin) in Mg^{2+}-loaded proteins is blue-shifted by 4–5 nm as compared to those for the Ca^{2+} forms. Thus, according to the above, the environments of the tryptophan residues in Mg^{2+}-loaded α-lactalbumins differ from those of the Ca^{2+}-loaded and apo proteins.

In this paper we have attempted to demonstrate that it is possible to monitor changes in the environments of separate tryptophan residues with the help of wavelength-resolved fluorescence decay measurements. In general, the data obtained in the present study show that the shift of the fluorescence spectrum for both human and bovine α-lactalbumins towards longer wavelengths induced by release of the bound Ca^{2+} or Mg^{2+} or by thermal denaturation is caused by changes in the environment of all the emitting tryptophan chromophores.

We would like to note that though a triple-exponential fit to the experimental decay curves is quite satisfactory, a more subtle analysis might provide us with more parameters, especially concerning the exponential term with the smallest amplitude, however, we believe that it would not affect the main conclusions drawn from the proposed model.

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