J Fluoresc (2011) 21:1683–1687
DOI 10.1007/s10895-011-0860-0

ORIGINAL PAPER

Sub-Structures Formed in the Excited State are Responsible for Tryptophan Residues Fluorescence in β-Lactoglobulin

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Received: 11 January 2011 / Accepted: 13 February 2011 / Published online: 25 February 2011

Abstract Origin of tryptophan residues fluorescence in β-lactoglobulin is analyzed. Fluorescence lifetimes and spectra of β-lactoglobulin solution are measured at pH going from 2 to 12 and in 6 M guanidine. Tryptophan residues emit with three lifetimes at all conditions. Two lifetimes (0.4–0.5 ns and 2–4 ns) are in the same range of those measured for tryptophan free in solution. Lifetimes in the denatured states are lower than those measured in the native state. Pre-exponential values are modified with the protein structure. Data are identical to those already obtained for other proteins. Fluorescence lifetimes characterize internal states of the tryptophan residues (Tryptophan sub-structures) independently of the tryptophan environments, the third lifetime results from the interaction that is occurring between the Trp residues and its environment. Pre-exponential values characterize substructures populations. In conclusion, tryptophan mission occurs from substates generated in the excited state. This is in good agreement with the theory we described in recent works.

Keywords β-lactoglobulin · Tryptophan residues · pH · Fluorescence lifetimes · Fluorescence emission spectra · Fluorophore sub-structures

Introduction

β-Lactoglobulin (lipocalin family proteins) is a small protein of 162 amino acid residues [1] (Mr=18,400) which tertiary structure possesses a pocket [calyx] where hydrophobic ligands can easily bind [2]. It normally exists as a dimer, each monomer is formed by 162 amino acids, with one free cysteine and two disulphide bridges [3]. However, dimerization percentage of β-lactoglobulin depends on the pH, although a disagreement exists on precise percentage of monomers and dimers at all studied pH [4, 5]. Nevertheless, at pH 2, β-lactoglobulin is in a native monomeric state and at pH 12, it is denatured. β-lactoglobulin contains two tryptophan residues, one (Trp-19) is surrounded by a hydrophobic environment and the second (Trp-61) in a hydrophilic one [6]. β-Lactoglobulin can bind fatty acids [7] and different hydrophobic molecules [8, 9].

Recently, we compared fluorescence lifetimes of tryptophan free in solution to those measured for tryptophan in proteins [10–14] in order to find out origin of tryptophan fluorescence lifetimes. Three lifetimes were found in all measured proteins. Our data showed that two lifetimes around 0.4–0.5 ns and 2–4 ns were measured for tryptophan free in solution and present within proteins, whether native or denatured. Thus, presence of these two lifetimes is independent of any structure around tryptophan and/or number of tryptophan residues in the protein, and characterizes an internal property or/and organization of the tryptophan structure formed in the excited state. The third lifetime recorded in proteins is attributed to interaction between Trp residue (s) and surrounding amino acids and to possible specific properties of the protein. Also, values of the relative amplitudes of the three fluorescence lifetimes can be dependent on the number of emitting Trp residues or/and on the type of interaction that is occurring between Trp residues and the surrounding environment. Lifetimes amplitudes will characterize populations of emitting species or tryptophan residues sub-structures. Fluorescence lifetimes and their populations (pre-exponential or amplitudes) values can be modified with the global structure of the protein.

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In the present work, we recorded fluorescence intensity decay of β-lactoglobulin solution at pH going from 2 to 12. We found that measured fluorescence decay parameters (lifetimes and amplitudes) are not dependent on the percentage of protein dimerization. At all pH, three fluorescence lifetimes characterizing internal states of the tryptophan residues were measured. Measurements displayed in 6 M guanidine solution yields three fluorescence lifetimes which values are lower than those obtained when the protein is in the native state. Also, in 6 M guanidine, values of the relative amplitudes differ from those measured in the native state, indicating that tryptophan sub-structures populations are dependent on protein tertiary structure. The results described in the present work are in good agreement with those we have recently published on the tryptophan fluorescence lifetimes origin.

Materials and Methods

Bovine β-lactoglobulin (purity >90%) was from Sigma – Aldrich (Saint Quentin Fallavier, France). Its concentration was determined at 278 nm with the following extinction coefficients: 17.6×10^3 M^{-1} cm^{-1} [15]. In all the experiments, the concentrations are expressed in monomers.

Absorbance data were obtained with a Varian DMS-100S (Les Ulis, France) spectrophotometer using 1-cm pathlength cuvettes. Fluorescence spectra were recorded with a Perkin–Elmer LS-5B spectrofluorometer (Perkin-Elmer, Courtaboeuf, France). The bandwidths used for the excitation and the emission were 5 nm. The quartz cuvettes had optical pathlengths equal to 1 and 0.4 cm for the emission and excitation wavelengths, respectively. Fluorescence spectra were corrected for the background intensities of the buffer solution. Observed fluorescence intensities were first corrected for the dilution, and then corrections were made for the inner filter effect as described [16, 17].

Fluorescence lifetime measurements were obtained with a Horiba Jobin Yvon FluoroMax-4-P, (Horiba- Jobin Yvon, Longjumeau, France) using the time correlated single photon counting method. A Ludox solution was used as scatter. Excitation was performed at 296 nm with a nanoLED. Each fluorescence decay was analyzed with one, two, three and four lifetimes and then values of $\chi 2$ were compared in order to determine the best fit. A minimal value of χ^2 indicates the best fit. A χ^2 value that approaches 1 indicates a good fit. For example, let us consider the value of χ^2 equal to 1.054, 1.06 and 1.1 when analysis is done with 1, 2 and 3 lifetimes, respectively. One lifetime should be considered as the best description of the decay curve since there was no real improvement in χ^2 value when the experimental decay was fitted with one, two, three or four lifetimes [16, 18–20].

All experiments were performed at 20 °C in 10 mM Tris buffer or/and 10 mM phosphate buffer and in 6 M guanidine pH 7.8.

Results and Discussion

Figure 1 displays fluorescence emission spectra of 10 μM β-lactoglobulin dissolved in 10 mM tris buffer at different pH. Displayed spectra indicate total absence of correlation between fluorescence emission and the pH of the protein solution. Thus, fluorescence intensity is not proportional to the percentage of protein monomers or dimers in solution. Positions of the emission peaks are very close at all pH except at pH 12 where β-lactoglobulin is denatured. In fact, emission peak is located at 330 nm at pH 2, 4, 6, 8 and 9 and at 335 nm at pH 7 and 10. Therefore, at all these pH, β-lactoglobulin is not denatured, emission occurs from tryptophan residues conserved within a native tertiary structure. Dissolved in pH 12 buffer, β-lactoglobulin emission occurs at a peak located at 350 nm indicating important structural modifications within the protein at this pH. Protein denaturation decreases the contact between Trp residues and their microenvironments and thus induces an

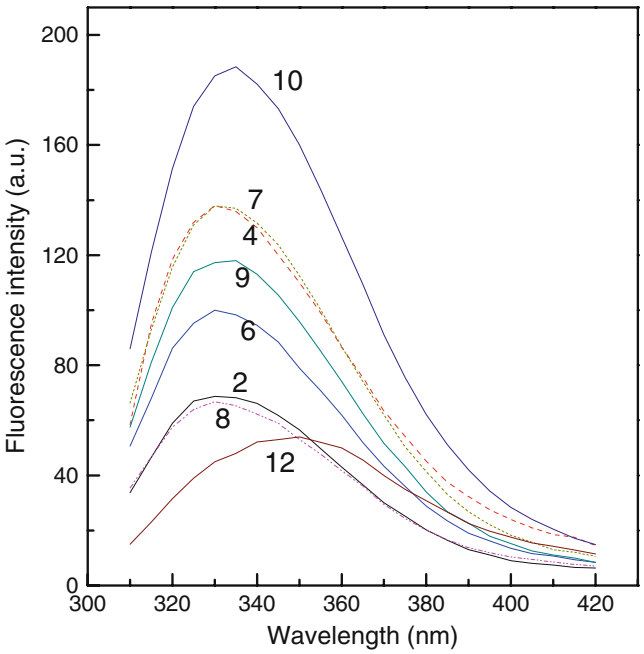

Fig. 1 Fluorescence intensity spectrum of 10 μM β-lactoglobulin with pH. λ_{ex}=295 nm. Percentages of dimers and monomers at the different pH are: pH 2: 100 monomers. pH 4: 85% monomers and 15% dimers. pH 6: 20% monomers and 80% dimers. pH 7: 35% monomers and 65% dimers. pH 8: 85% monomers and 15% dimers. pH 9: 90% monomers and 10% dimers. pH 10: 85% monomers, 7.5% dimers and 7.5% denaturated monomers. pH 12: 100% denaturated protein. Source of percentages of monomers and dimers: Invernizzi, G., Amalikova, M.S., Brocca, S., Lotti, M., Molinari, H. and Grandori, R. (2006) J. Mass. Spectrom. 41: 717–727

increase of the fluorescence intensity and of the radiative constant (k_r) value. However, Fig. 1 shows that at pH 12, fluorescence intensity is lower than that observed for other pH. This means that at pH 12, protein denaturation is not occurring but structural modifications. At this pH, Trp residues, although exposed to the solvent, are still in high contact with neighboring amino acids. At pH 2, β-lactoglobulin is in the molten globule state. The protein is stable although partially unfolded [21]. However, since emission peak occurs at 332 nm and not at 340 or 350 nm, protein unfolding, if any, appears to be not very important.

Figure 2 displays fluorescence lifetimes of β-lactoglobulin emission with pH. One can observe identical variation of the three fluorescence lifetimes between pH 4 and 10. Thus, β-lactoglobulin tertiary structure seems to display very identical tertiary structures at these pH but different from the structures at pH 2, 11 and 12. At pH 2, the protein seems to be more compact than at the other pH_s. At pH 11 and 12, fluorescence lifetimes decrease as the result of the low interaction between Trp residues and neighboring amino acids. Figure 3 shows that population of the three fluorescence lifetimes are pH dependent. This result is in good agreement with what we have already found: Pre-exponential factors or populations of fluorescence lifetimes are dependent on the interaction between tryptophan residues and their microenvironment [10; 13] and thus on the protein local conformation. From the data displayed in Fig. 3, β-lactoglobulin local conformations, at least around Trp residues, are not the same at all pH. While protein global conformation is the same at all pH, except at pH 12 as it is shown from the emission spectra (Fig. 1), local conformation around Trp residues differs from a pH to another.

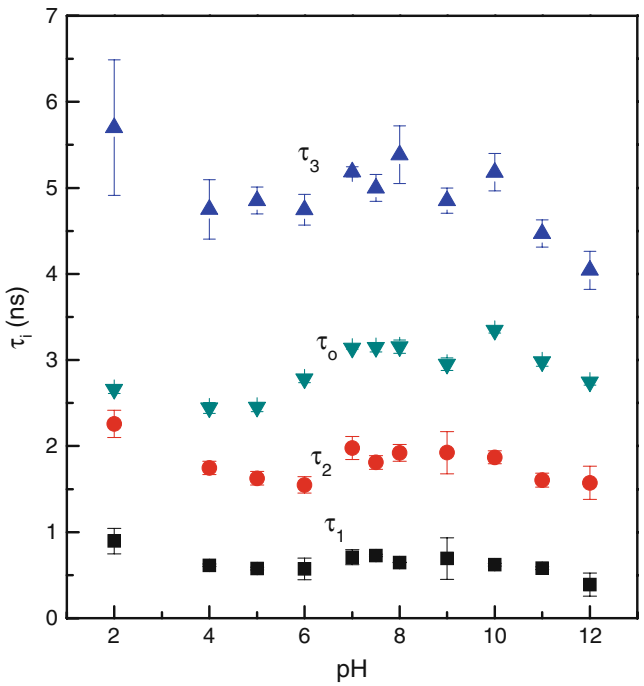

Fig. 2 Fluorescence lifetimes variation of Trp residues of β-lactoglobulin with pH. λ_{ex}=296 nm

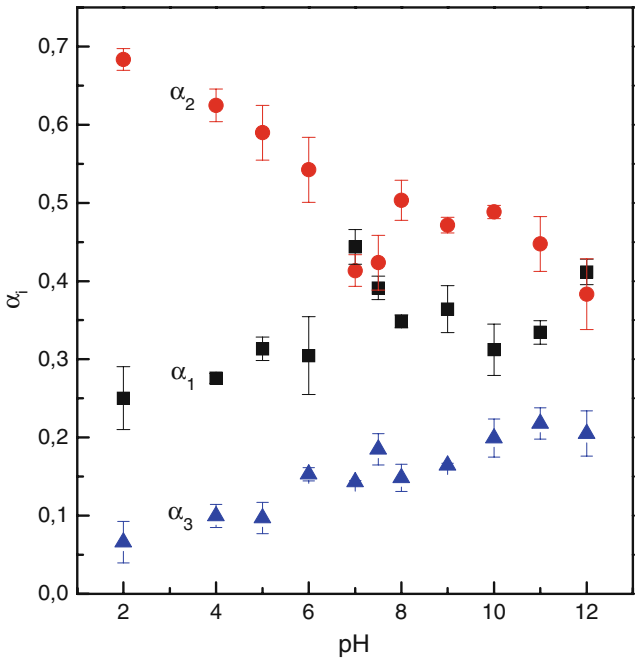

Fig. 3 Pre-exponential values of Trp residues of β-lactoglobulin at different pH. λ_{ex}=296 nm

Figure 4 displays fluorescence lifetimes variation of β-lactoglobulin with emission wavelength in native (pH 7) and denatured states (6 M guanidine). Protein denaturation

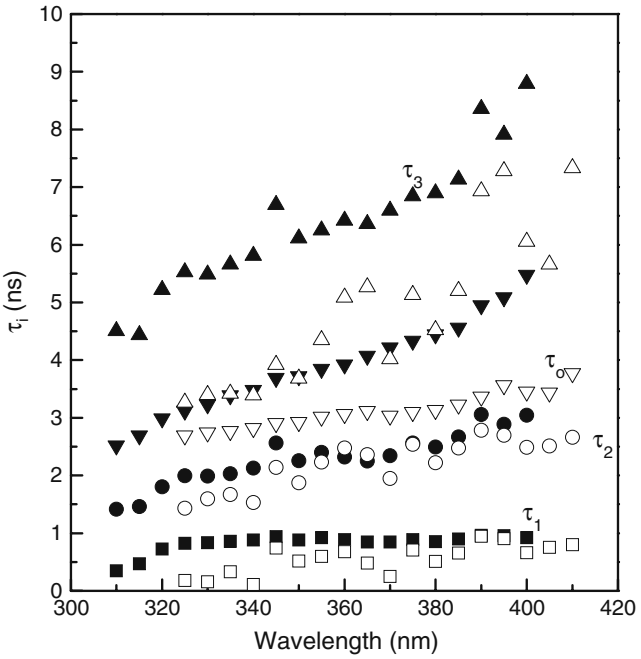

Fig. 4 Fluorescence lifetimes of β-lactoglobulin Trp residues in 10 mM phosphate buffer, pH 7 (closed symbols) and in 6 M guanidine pH 7.8 [open symbols]. Longest fluorescence lifetime τ_3 and mean one τ_o decrease significantly in the denatured protein. λ_{ex}=296 nm

induces a decrease in the longest and mean fluorescence lifetimes compared to those measured in the native state. At 350 nm, for example, fluorescence intensity decay at pH 7 of β-lactoglobulin can be adequately represented as

$$I[\lambda, t] = 0.579\, e^{-t/0.948} + 0.339\, e^{-t/2.572} + 0.082\, e^{-t/6.355}$$

where 0.579, 0.339 and 0.082 are the preexponential factors and 0.948, 2.572 and 6.355 ns the decay times ($\chi 2 = 1.045$). The mean fluorescence lifetime τ_o calculated from two experiments was found equal to 3.138±0.011 ns.

When β-lactoglobulin is dissolved in 6 M guanidine, its fluorescence intensity decay can be described as

$$I[\lambda, t] = 0.128\, e^{-t/0.515} + 0.479\, e^{-t/1.872} + 0.393\, e^{-t/3.685}$$

where 0.128, 0.479 and 0.393 are the preexponential factors and 0.515, 1.872 and 3.685 ns the decay times ($\chi 2 = 1.073$). The mean fluorescence lifetime calculated from two experiments was found equal to 2.923±0.04 ns.

Figure 4 indicates also that β-lactoglobulin denaturation does not yield a single fluorescence lifetime. This means that presence of three lifetimes for tryptophan residues in β-lactoglobulin does not depend on the protein structure only but also on the tryptophan itself. This decrease in the fluorescence lifetime can be explained by the fact that in the native state, interaction between β-lactoglobulin Trp residues and neighboring amino acids is important compared to that observed when the protein is totally denatured. Thus, in the native state, radiative rate constant decreases as the result of the important interaction between fluorophore and neighboring amino-acids. In the unfolded state, interaction between Trp residues and amino acids decreases, inducing by that an increase in the radiate rate constant and thus a decrease of the measured fluorescence lifetimes compared to that recorded in the native state.

Fluorescence lifetime decrease can also be explained by the difference in the local motions of the tryptophan residue between native and denatured states. In fact, upon denaturation, β-lactoglobulin tryptophan residues motions increase inducing an increase in the non-radiative rate constant k_i. This will decrease the value of fluorescence lifetime. Therefore, protein structure plays a role of gap in retaining and organizing photon emission energy. It has been suggested that fluorescence lifetimes of Trp residue[s] within random coil polypeptides [denatured proteins] are very close, the differences would come from the position of the tryptophan within the protein primary structure [end of the chain or in its middle] and/or presence of quencher group near Trp residue[s] [22–24]. Nevertheless, the data described in the present work indicate that fluorescence lifetimes characterize intrinsic properties of the fluorophore.

Global shape of lifetimes variation is identical in both native and denatured states, i.e., fluorescence lifetimes are the result of

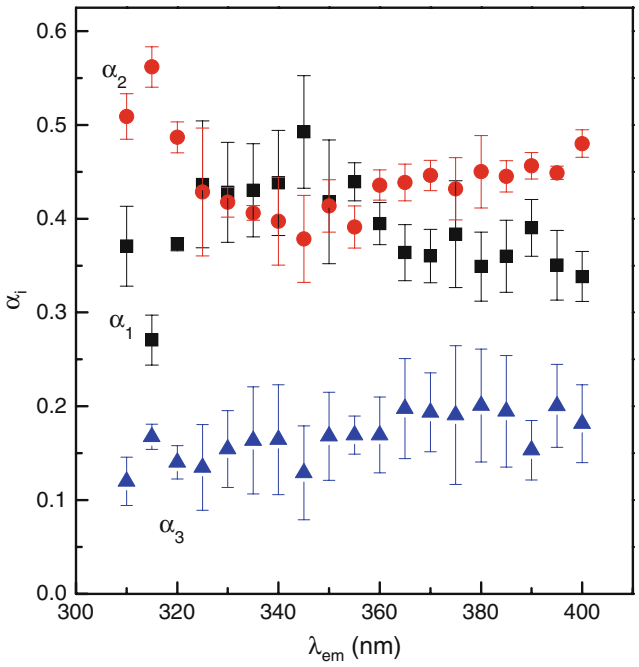

Fig. 5 Pre-exponentials variation with emission wavelength of β-lactoglobulin fluorescence lifetimes, measured at pH 7. λ_{ex}=296 nm

an emission from the same three tryptophan sub-structures. Protein denaturation would modify the population of the Trp lifetimes. In fact, despite the decrease of the three fluorescence lifetimes upon β-lactoglobulin denaturation, mean fluorescence lifetime of the denatured protein does not differ significantly

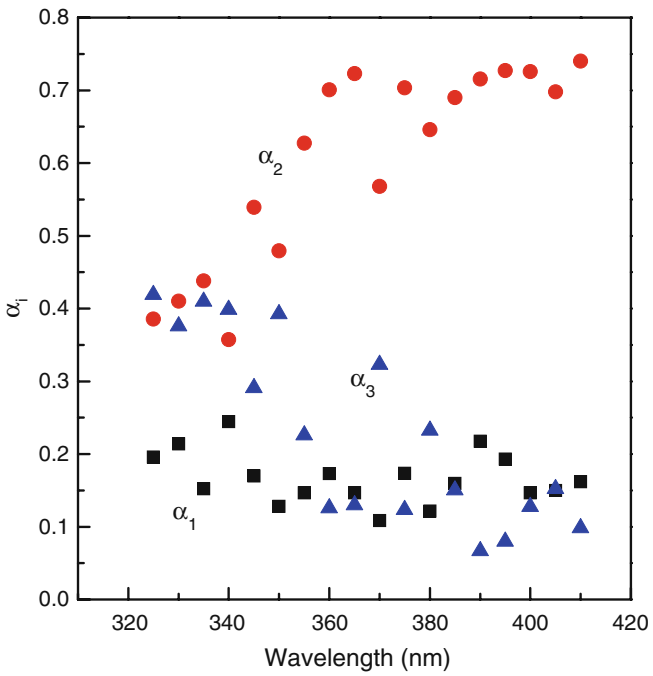

Fig. 6 Pre-exponentials variation with emission wavelength of β-lactoglobulin fluorescence lifetimes, measured in 6 M guanidine, pH 7.5. λ_{ex}=296 nm

from that measured for the native state as the result of the increase in the pre-exponential factors upon denaturation. Figures 5 and 6 display pre-exponential variations of the three fluorescence lifetimes with emission wavelengths at both native (pH 7) and denatured (6 M guanidine) states, respectively. First of all, the data show that we have three different fluorophore populations independent of the surrounding environment. We can notice that upon protein denaturation, population of each fluorescence lifetime is modified. Thus, local interactions around Trp residues differ with the state of β-lactoglobulin. Denaturation has modified the population of each of the three tryptophan substructures. This result is clear evidence that pre-exponential values characterize the type and nature of interaction existing between Trp residues and their environments, while number of lifetimes is an indication of the substructures that exist for the fluorophore in the excited state.

Results described in the present work are in good agreement with others already obtained on different proteins containing one Trp residue such as human serum albumin [11–13], two Trp residues such as bovin serum albumin [11], three or eight Trp residues such as α_1-acid glycoprotein and serotransferrin [10]. Therefore, number of fluorescence lifetimes in all these proteins was found equal to 3 and is independent of the number of tryptophan residues and/or of their locations within the proteins. Also, two fluorescence lifetimes are very close and identical to those found for free tryptophan in water. Therefore, these two lifetimes are independent of the protein structure and characterize internal properties of the tryptophan itself. These lifetimes characterize sub-structures of the tryptophan reached in the excited state. The third fluorescence lifetime measured in the proteins would characterize a new sub-structure that results from the interaction between tryptophan residues and surrounding environments. The populations of these sub-structures are dependent on local and global structural modifications of the protein and thus on the interaction of these sub-structures with the environment.

In conclusion, our data are in favour with our model already described previously for different fluorophores such as tryptophan, tyrosine and 6,p-toluidinyl-naphthalene-2-sulfonate [TNS] [10, 11, 20, 25]: Fluorophore excitation induces structural rearrangements different from those present in the ground state. The new sub-structures induced in the excited state will yield each a specific fluorescence lifetime. Our next goal now is to explain exact origin of the third lifetime observed in proteins.

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