J Fluoresc (2011) 21:1301–1309
DOI 10.1007/s10895-010-0813-z

ORIGINAL PAPER

Relation Between Proteins Tertiary Structure, Tryptophan Fluorescence Lifetimes and Tryptophan $S_o \rightarrow {}^1L_b$ and $S_o \rightarrow {}^1L_a$ Transitions. Studies on α_1-acid Glycoprotein and β-lactoglobulin

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Received: 20 May 2010 / Accepted: 29 December 2010 / Published online: 1 February 2011

Abstract We measured fluorescence lifetimes and fluorescence spectra (excitation and emission) of tryptophan residues of α_1-acid glycoprotein (three Trp residues) and β-lactoglobulin (two Trp residues) in absence and presence of 450 μM progesterone. Progesterone binds only to α_1-acid glycoprotein. In absence of progesterone, each of the two proteins displays three fluorescence lifetimes. Addition of progesterone induces a partial inhibition of the $S_o \rightarrow {}^1L_a$ transition without affecting fluorescence lifetimes. The same experiments performed in presence of denatured proteins in 6 M guanidine show that addition of progesterone inhibits partially the $S_o \rightarrow {}^1L_a$ transition and its peak is 15 nm shifted to the red compared to that obtained for native proteins. However, the $S_o \rightarrow {}^1L_b$ transition position peak is not affected by protein denaturation. Thus, the tertiary structure of the protein plays an important role by modulating the tryptophan electronic transitions. Fluorescence emission decay recorded in absence and presence of progesterone yields three fluorescence lifetimes whether proteins are denatured or not. Thus, protein tertiary structure is not responsible for the presence of three fluorescence lifetimes. These characterize tryptophan substructures reached at the excited states and which population (pre-exponential values) depend on the tryptophan residues interaction with their microenvironment(s) and thus on the global conformation of the protein.

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Keywords α_1-acid glycoprotein · β-lactoglobulin · Progesterone · Tryptophan residues · Fluorescence lifetime · Tryptophan $S_o \rightarrow {}^1L_b$ and $S_o \rightarrow {}^1L_a$ transitions · Fluorescence excitation spectrum

Introduction

Tryptophan absorbs with two transitions $S_o \rightarrow {}^1L_a$ and $S_o \rightarrow {}^1L_b$. In polar solvents, ${}^1L_a$ has lower energy than ${}^1L_b$ and emission is supposed to be observed from this lower state [1], although experiments demonstrating this assumption have never been showed. Recently, performing fluorescence excitation spectra on free tryptophan in solution (ethanol or buffer) we showed that high concentrations of progesterone abolish completely the $S_o \rightarrow {}^1L_a$ transition [2]. The hormone absorbs between 220 and 280 with a peak around 250 nm, while its absorption is equal to zero beyond 280 nm. Thus, progesterone absorbs at the same wavelengths as $S_o \rightarrow {}^1L_a$ transition, while its absorption is negligible in the $S_o \rightarrow {}^1L_b$ transition domain. Nevertheless, presence of progesterone did not modify tryptophan fluorescence lifetimes, as if they do not depend directly on the two tryptophan transitions. This filter effect of high progesterone concentrations, observed on tryptophan free in solution, occurs independently of any binding process since progesterone does not bind to free tryptophan in solution.

Since tryptophan fluorescence observables are used to study proteins structure and dynamics and interaction of macromolecules with different ligands [3–6], it was interesting to find out whether proteins structure play a role in the two tryptophan transitions $S_o \rightarrow {}^1L_a$ and $S_o \rightarrow {}^1L_b$. Thus, fluorescence excitation spectra and lifetimes measurements were performed on α_1-acid glycoprotein

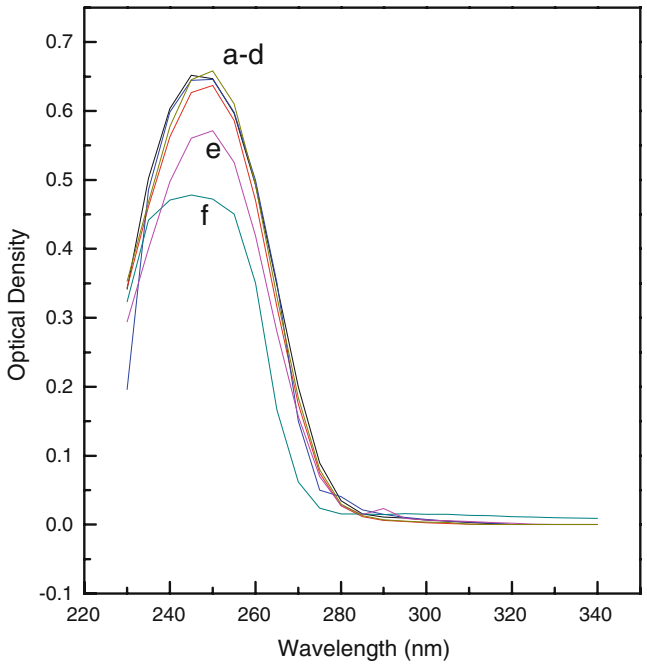

Fig. 1 Absorption spectra of 250 μM progesterone on 0.4 cm pathlength in presence of L-Trp dissolved in Tris pH 2, pH 7.5, pH 11 (**a**–**c**), in ethanol (**f**), in presence of 10 μM β-lactoglobulin (**e**) and of 3.6 μM α_1- acid glycoprotein (**d**)

and bovine β-lactoglobulin in absence and presence of high concentrations of progesterone. Both proteins belong to the lipocalin family proteins characterized by the presence of a calyx (identical eight antiparallel β strands) [7]. Nevertheless, progesterone binds specifically to α_1-acid glycoprotein only.

α_1-Acid glycoprotein (orosomucoid) is a small acute-phase glycoprotein (Mr=41,000) that is negatively charged at physiological pH. It consists of a chain of 183 amino acids [8] contains 40% carbohydrate by weight and has up to 16 sialic acid residues (10–14% by weight) [9]. Five heteropolysaccharide groups are linked via an N-glycosidic bond to the asparaginyl residues of the protein [10]. The N-terminal fragment of α_1-acid glycoprotein adopts a spatial conformation so that a pocket in contact with the buffer is induced [11–13]. A number of activities of possible significance have been described for α_1-acid glycoprotein such as, the ability to bind different drugs such as warfarin [14] and the β-drug adrenergic blocker, propranolol [15] and certain steroid hormones such as progesterone [9]. Many of these activities have been shown to be dependent on the glycoforms of α_1-acid glycoprotein [16]. α_1-Acid glycoprotein contains three Trp residues, one residue, Trp-160, is at the surface of the protein [9, 10, 17], a second tryptophan Trp-122 residue is partially buried in the protein matrix and the third residue Trp-25 is in a hydrophobic environment in the pocket [17, 18]. The three Trp residues contribute to the fluorescence of α_1-acid glycoprotein [18]. Progesterone-binding site is within the pocket near Trp-25 residue [17].

β-Lactoglobulin is a small protein of 162 amino acid residues [18] (Mr =18,400) which tertiary structure possesses a pocket (calyx) where hydrophobic ligands can easily bind [7]. It normally exists as a dimer, each monomer is formed by 162 amino acids, with one free cysteine and two disulphide bridges [19]. Tertiary structure of the protein varies with the pH. For example, at pH 2, β-lactoglobulin is in a native monomeric state and at pH 12, it is denatured. At some pH such as pH 7.5, mixture of both monomeric and dimeric forms could be find [20]. β-lactoglobulin contains two tryptophan residues, one (Trp-19) is surrounded by a hydrophobic environment and the second (Trp-61) in a hydrophilic one [21]. Only Trp 19 residue contributes to the protein fluorescence. β-Lactoglobulin can bind fatty acids [22] and different hydrophobic molecules [23–25].

Results obtained in the present work show that addition of progesterone to the proteins solutions inhibits partially $S_o \rightarrow {}^1L_a$ transition, to the difference of the experiments performed on tryptophan free in solution where $S_o \rightarrow {}^1L_a$ transition was completely abolished. However, in both cases, none of the decay parameters (fluorescence lifetimes and their pre-exponential values) was affected. Experiments performed in 6 M guanidine solution indicate that emission decay of tryptophan residues within the proteins are still analyzed with three lifetimes in absence and presence of progesterone. Also, the $S_o \rightarrow {}^1L_a$ transition is partly inhibited and its peak is 15 nm shifted to the red compared to that obtained for the native proteins. However, the $S_o \rightarrow {}^1L_b$ transition position peak is not affected by protein denaturation. Therefore, protein structure plays an important role in the $S_o \rightarrow {}^1L_a$ transition modulation, i.e., the $S_o \rightarrow {}^1L_a$ transition is sensitive to structural modification occurring within proteins.

Materials and Methods

L-Tryptophan was from Sigma and was used as received. The fluorophore was dissolved in deionised water.

Table 1 Values of the molar extinction coefficient of progesterone calculated at 245 nm in the different media

Solution	pH 2	pH 7.5	pH 11	Ethanol	α_1-acid glycoprotein	β-lactoglobulin
ε (mM^{-1} cm^{-1})	6.516	6.268	6.444	4.784	6.452	5.608

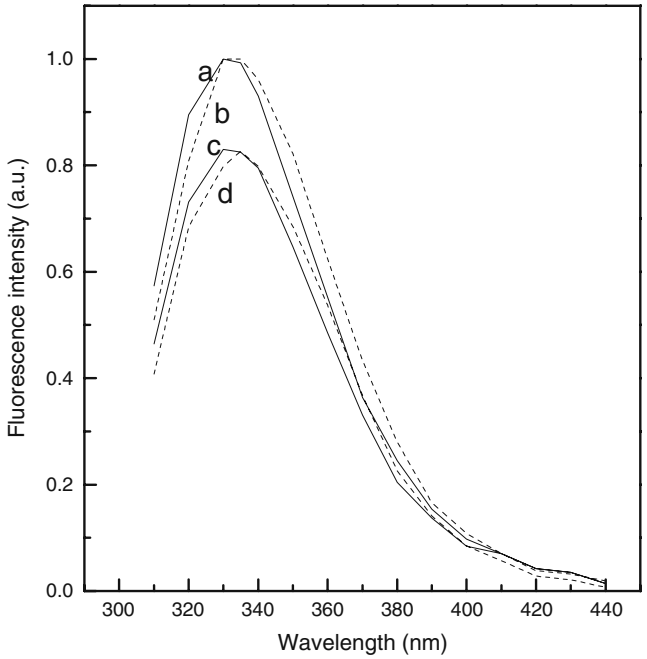

Fig. 2 Fluorescence emission spectra of 3.6 μM α_1- acid glycoprotein (**a** and **c**) and of 10 μM β-lactoglobulin (**b** and **d**) in absence (**a** and **b**) and presence of 250 μM progesterone (**c** and **d**). λ_{ex}=295 nm Spectra of the two proteins recorded in absence of progesterone are normalized

α_1-Acid glycoprotein was purified as already described [26]. Its concentration was determined spectrophotometrically using an extinction coefficient of 29.7 mM^{-1} cm^{-1} at 278 nm [27]. Bovine β-lactoglobulin was from Sigma. Its concentration was determined spectrophotometrically at 278 nm, $\varepsilon_{278nm} = 17.6 \times 10^3\ M^{-1}\ cm^{-1}$ [28]. Progesterone (from Sigma) was dissolved in spectroscopic grade EtOH from Fluka. Two stock solutions (100 and 50 mM) were prepared. The stock volume added to the proteins solutions was 5 μL, inducing final concentrations in the cuvette around 500 or 250 μM, respectively.

Absorbance data were obtained with a Varian DMS-100S spectrophotometer using 1-cm pathlength cuvettes.

Fluorescence spectra were recorded with a Perkin-Elmer LS-5B spectrofluorometer. Bandwidths used for the excitation and the emission were 10 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, then corrections were made for the absorption at the excitation and emission wavelengths as already described [29, 30]. Finally, fluorescence spectra were corrected for the background intensities of the buffer solution.

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 and emission was observed at 330 nm for the proteins in the native state and at 350 nm in 6 M guanidine solution.

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 $\chi 2$ indicates the best fit. A $\chi 2$ value that approaches 1 indicates a good fit [29, 31–33].

All experiments were performed at 20 °C in 10 mM phosphate buffer pH 7 or in 10 mM Tris buffer pH 7.5.

Results

Figure 1 displays absorption spectra of 250 μM progesterone in presence of L-Trp dissolved in Tris pH 2, pH 7.5, pH 11 (a–c), in ethanol (f), in presence of 10 μM β-lactoglobulin (e) and in presence of 3.6 μM α_1-acid glycoprotein (d). Spectra were recorded with 0.4 cm pathlength. Optical density of progesterone in presence of α_1-acid glycoprotein is identical to that observed in presence of L-Trp dissolved in the polar solvents. Table 1 displays the values of the molar extinction coefficient of progesterone calculated at 245 nm in the different media.

Figure 2 displays normalized fluorescence emission spectra of 10 μM β-lactoglobulin and 3.6 μM α_1-acid glycoprotein in the absence and presence of 250 μM

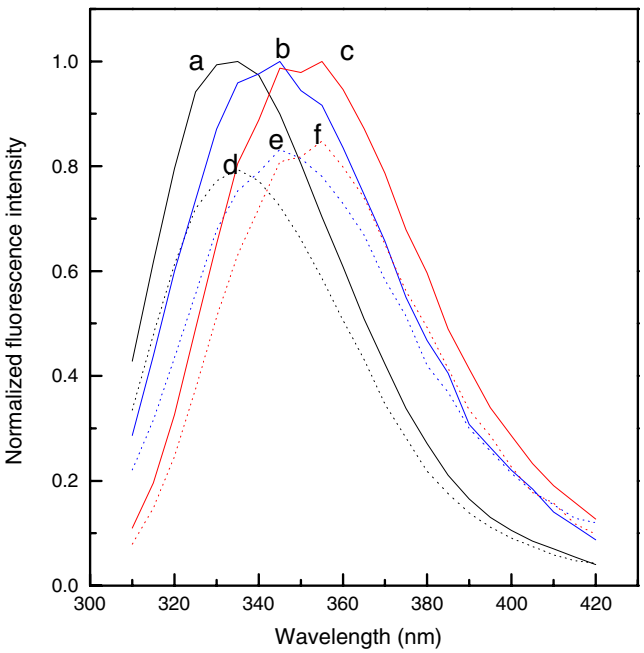

Fig. 3 Normalized fluorescence emission spectra of β-lactoglobulin in phosphate buffer, at pH 2 (**a** and **d**), pH 11 (**b** and **e**) and in 6 M guanidine pH 7.8 (**c** and **f**), in absence (**a**, **b** and **c**) and presence of 250 μM progesterone (**d**, **e** and **f**). Intensity decrease in presence of progesterone is identical whether the protein is in the native monomeric state (pH 2), in the perturbed monomeric state (pH 11) and in the totally denatured state (in 6 M guanidine)

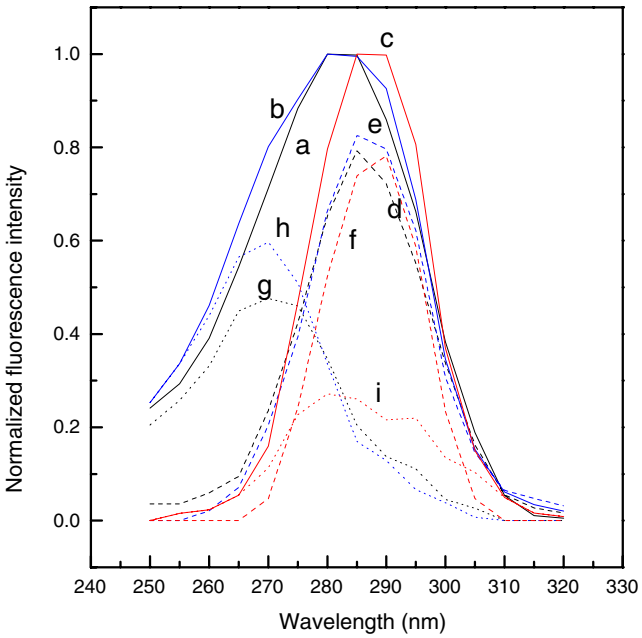

Fig. 4 Fluorescence excitation spectra of β-lactoglobulin in phosphate buffer, at pH 2 (**a**, **d** and **g**), pH 11 (**b**, **e** and **h**) and in 6 M guanidine pH 7.8 (**c**, **f** and **i**), in absence (**a**, **b** and **c**) and presence of 250 μM progesterone (**d** to **i**). Addition of progesterone yields spectra d, **e** and **f** ($S_o \rightarrow {}^1L_b$ transitions). $S_o \rightarrow {}^1L_a$ transition spectra are the difference between spectra in absence (**a**, **b** and **c**) and those obtained in presence of progesterone (**d**, **e** and **f**). $S_o \rightarrow {}^1L_a$ transition (spectra **g**, **h** and **i**) has a peak equal to 280 or 265 nm and $S_o \rightarrow {}^1L_b$ transition (spectra **d**, **e** and **f**) has a peak equal to 290 nm. In presence of guanidine, the $S_o \rightarrow {}^1L_a$ transition is perturbed compared to that observed at pH 2 and 11

progesterone. The results clearly indicate that addition of progesterone induces identical decrease in the fluorescence intensity spectra of both protein solutions. Progesterone does not bind to β-lactoglobulin and thus this decrease is simply the result of high progesterone concentration which binds non-specifically to the proteins. Identical results were obtained when experiments were performed on β-lactoglobulin dissolved in pH 2 (native monomer form), pH 11 (partly denatured monomer form) buffers, and in 6 M guanidine solution (Fig. 3), showing that binding of progesterone to β-lactoglobulin is not specific. Decrease of the fluorescence intensity is the same for the three protein states and thus results from the presence of high progesterone concentration added to the protein solutions and not necessarily to specific binding (see also discussion).

Tryptophan absorbs with two transitions $S_o \rightarrow {}^1L_a$ and $S_o \rightarrow {}^1L_a$ [1]. Recently, by performing fluorescence excitation spectra on free tryptophan in solution (ethanol or buffer), we showed that high progesterone concentrations abolish completely the $S_o \rightarrow {}^1L_a$ transition, without any modification in the fluorescence decay parameters (lifetimes and pre-exponentials) [2]. Also, our data did not allow us to find direct correlation between tryptophan fluorescence lifetimes and the two transitions. But what about tryptophans in proteins: i.e., does the structure surrounding the fluorophore affect any of the two tryptophan transitions? Also, how are affected fluorescence lifetimes?

Figure 4 displays fluorescence excitation spectra of β-lactoglobulin in phosphate buffer, at pH 2 (a, d and g), pH 11 (b, e and h) and in 6 M guanidine pH 7.8 (c, f and i), in absence (a to c) and presence of 250 μM progesterone (d to i). Addition of progesterone yields spectra d, e and f ($S_o \rightarrow {}^1L_b$ transitions). $S_o \rightarrow {}^1L_a$ transition spectra are the difference between spectra in absence (a, b and c) and presence of progesterone (d, e and f).

The data show that addition of progesterone to the β-lactoglobulin solutions does not abolish $S_o \rightarrow {}^1L_a$ transition completely as it is the case for free tryptophan in solution [2]. Thus, protein structure plays important role in maintaining partially the $S_o \rightarrow {}^1L_a$ transition.

Figure 4 indicates that in the three protein states, $S_o \rightarrow {}^1L_b$ transitions display a maximum at 290 nm and show equal intensities (spectra d, e and f). However, $S_o \rightarrow {}^1L_a$ transition in the phosphate buffer displays a peak equal to 265 nm. The position of the peak is identical to that observed when tryptophan was dissolved in ethanol [2]. When the protein is dissolved in 6 M guanidine solution, position of the $S_o \rightarrow {}^1L_a$ transition peak shifts to 280 nm, accompanied with a decrease in its fluorescence intensity

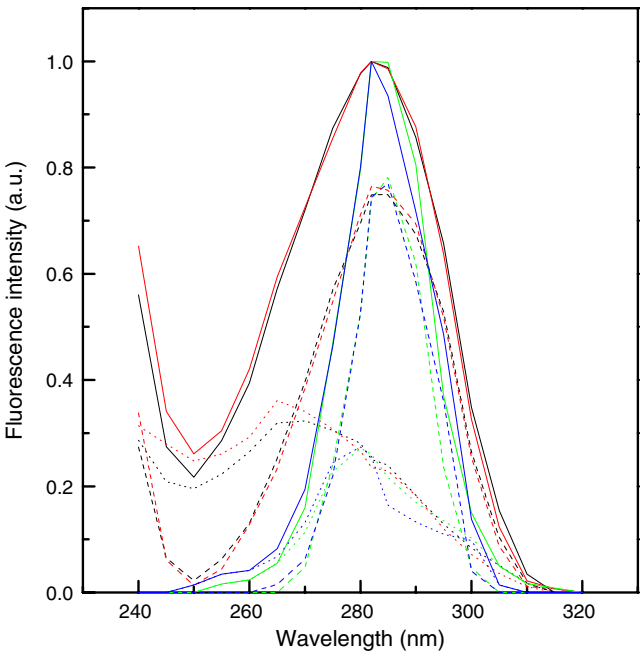

Fig. 5 Fluorescence excitation spectrum of β-lactoglobulin in Tris buffer, pH 7.5 (*black lines*), α_1- acid glycoprotein in Tris buffer pH 7.5 (*red lines*), β-lactoglobulin in 6 M guanidine (*green lines*) and α_1-acid glycoprotein in 6 M guanidine (*blue line*). So–– > ${}^1L_b$ transition (*dashed spectra*) has a peak equal to 285 nm (native) and 288 nm (denatured protein). So–– > ${}^1L_a$ transition (*dotted spectra*) has a peak equal to 265 nm (native) and 280 nm (denatured protein)

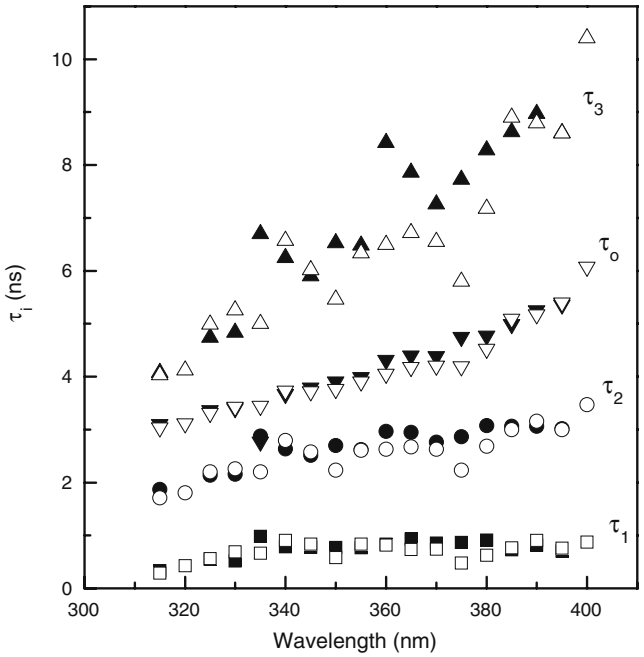

Fig. 6 Fluorescence lifetimes values of α_1-acid glycoprotein with emission wavelength in absence (*closed symbols*) and presence (*open symbols*) of 250 μM progesterone. λ_{ex}=296 nm

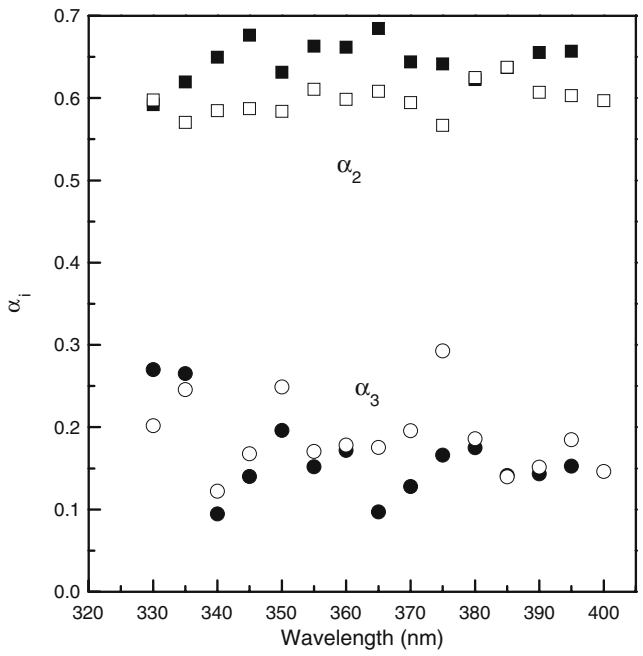

Fig. 8 Fluorescence lifetimes pre-exponential values of α_1-acid glycoprotein with emission wavelength in absence (*closed symbols*) and presence (*open symbols*) of 250 μM progesterone. λ_{ex}=296 nm. Here, only α_2 and α_3 are displayed for clarity reason

compared to that obtained in phosphate buffer. Thus, in presence of guanidine, the $S_o \rightarrow {}^1L_a$ transition is perturbed compared to that observed at pH 2 and 11. This phenomenon is clearly related to the strong structural modification of the protein (see also discussion). To the difference of α_1-acid glycoprotein, β-Lactoglobulin does not bind progesterone; therefore we compared data obtained on β-lactoglobulin to those obtained with the glycoprotein.

Figure 5 displays fluorescence excitation spectrum of β-lactoglobulin in Tris-buffer buffer, pH 7.5 (black

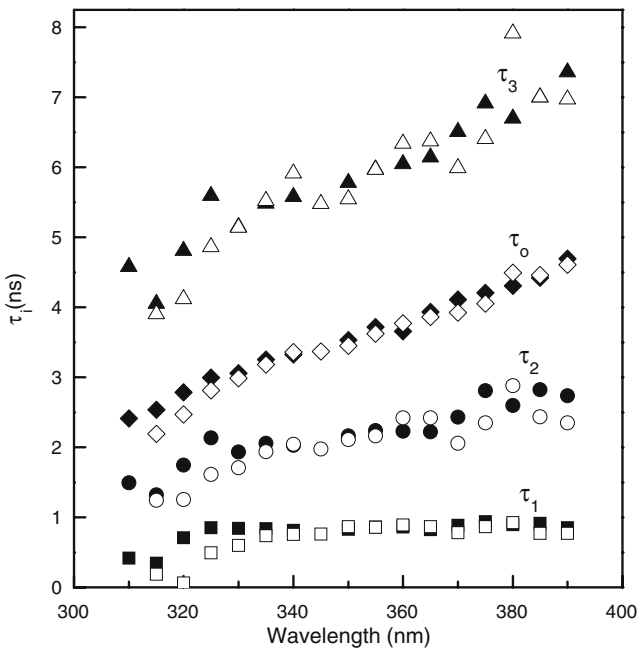

Fig. 7 Fluorescence lifetimes values of β-lactoglobulin with emission wavelength in absence (*closed symbols*) and presence (*open symbols*) of 450 μM progesterone. λ_{ex}=296 nm

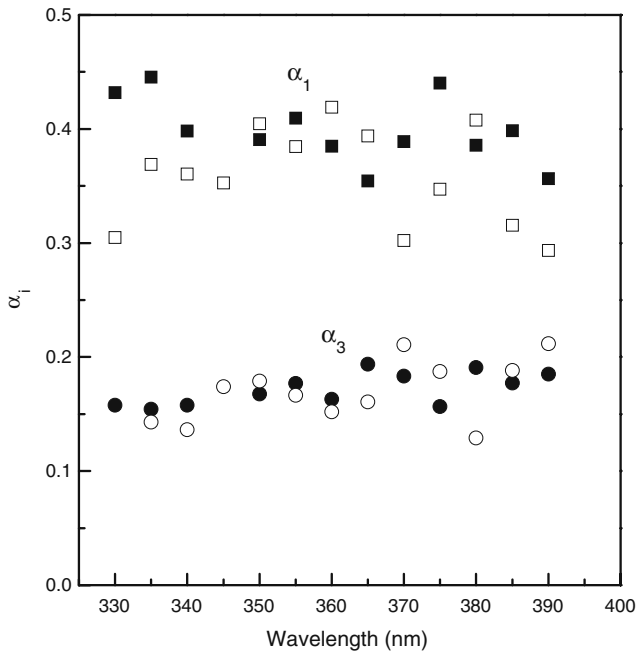

Fig. 9 Fluorescence lifetimes pre-exponential values of β-lactoglobulin with emission wavelength in absence (*closed symbols*) and presence (*open symbols*) of 450 μM progesterone. λ_{ex}=296 nm. Here, only α_1 and α_3 are displayed for clarity reason

lines), α_1-acid glycoprotein in Tris- buffer pH 7.5 (red lines), of β-lactoglobulin in 6 M guanidine (green lines) and of α_1-acid glycoprotein in 6 M guanidine (blue line). The results show that in both proteins, S_o—> ^1L_b transition (dashed spectra) has a peak equal to 285 nm (native) and 288 nm (denatured). Thus, the shift in the peak is not significant between the two states. Moreover, intensity of the S_o—> ^1L_b transition in both states is not modified. Thus, S_o—> ^1L_b transition of tryptophan does not depend on the protein structure. However, this is not the case for the S_o—> ^1L_a transition. In fact, Fig. 5 shows that S_o—> ^1L_a transition (dotted spectra) has a peak equal to 265 nm (native) and 280 nm (denatured protein). Therefore, this transition is clearly dependent on the protein structure and mainly the tertiary structure. Since experiments obtained with α_1- acid glycoprotein are identical to those obtained with β-lactoglobulin, then there is no correlation between progesterone binding on α_1- acid glycoprotein and partial inhibition of the S_o—> ^1L_a transition.

Figures 6 and 7 display, in the native state, fluorescence lifetimes variation along the emission wavelengths of α_1-acid glycoprotein and β-lactoglobulin tryptophan residues, respectively, in absence and presence of high progesterone concentrations (250 μM with α_1- acid glycoprotein and 450 μM with β-lactoglobulin). The data displayed indicate that binding / presence of progesterone in the protein solution does not modify any of the fluorescence lifetimes. Also, pre-exponential values which characterize population of each fluorescence lifetime are not affected by progesterone addition to the proteins solutions (Figs. 8 and 9).

Figures 10 and 11 display fluorescence intensity decays of β-lactoglobulin dissolved in 6 M guanidine solution in absence (Fig. 10) and presence (Fig. 11) of 450 μM progesterone. In both cases, intensity decay can be best analyzed with three fluorescence lifetimes.

In absence of progesterone, fluorescence intensity decay I(λ,t), of β-lactoglobulin can be adequately represented as

$$I(\lambda, t) = 0.143\, e^{-t/0.41} + 0.607\, e^{-t/2.017} + 0.250\, e^{-t/4.462}$$

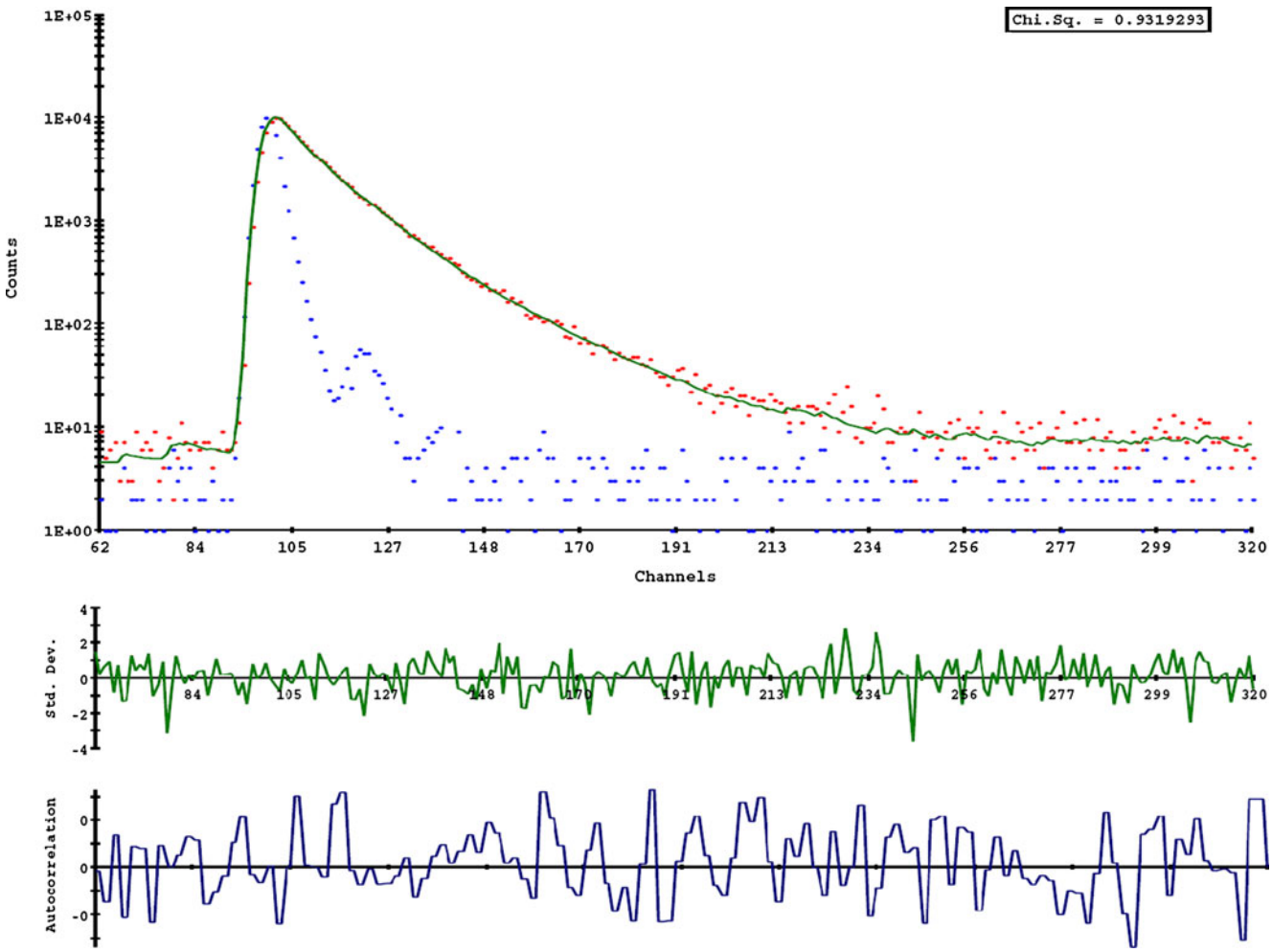

Fig. 10 Fluorescence intensity decay of 5 μM β-lactoglobulin in 6 M guanidine solution in absence of progesterone. λ_{ex}=296 nm and λ_{em}=350 nm

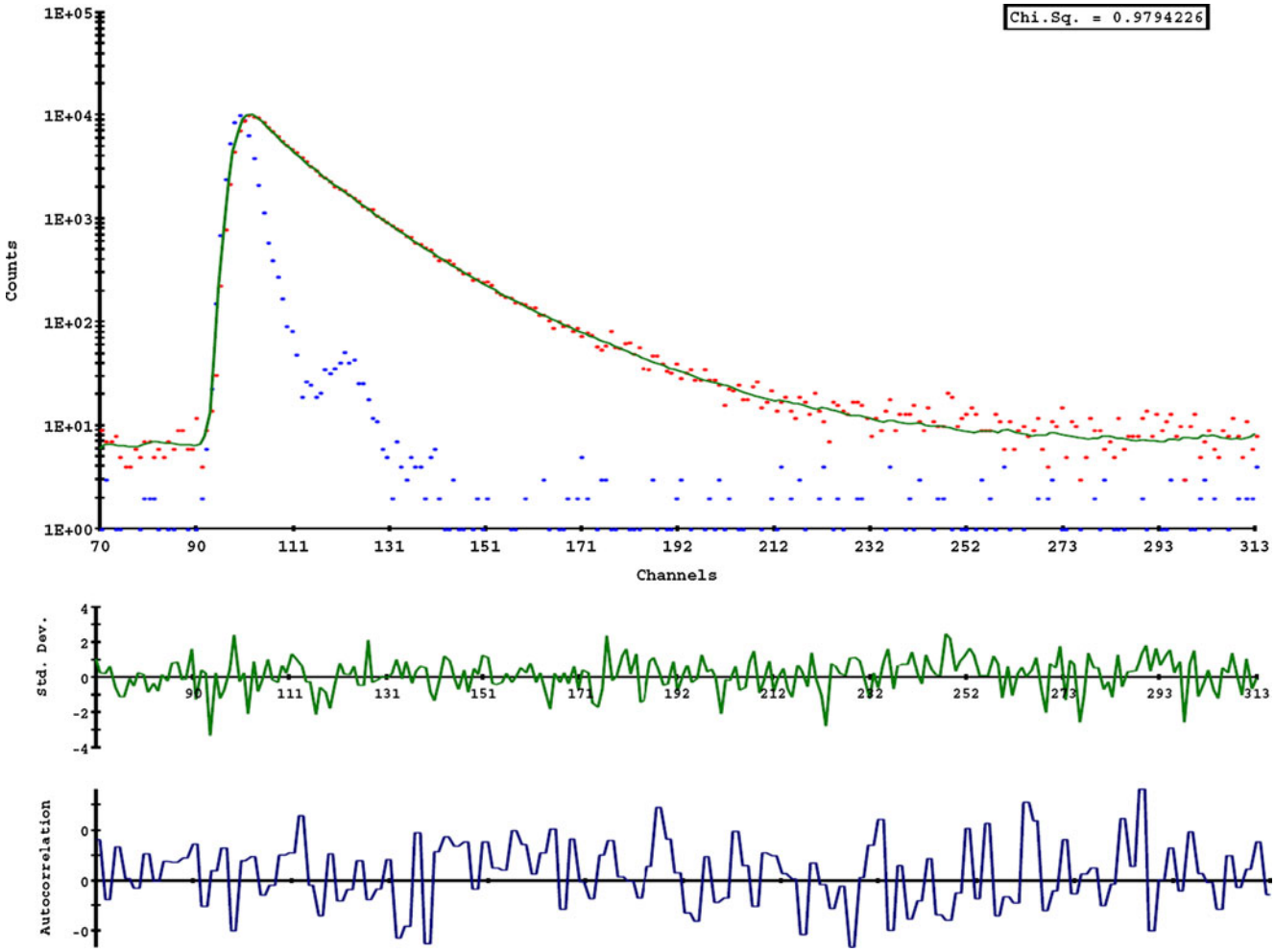

Fig. 11 Fluorescence intensity decay of 5 μM β-lactoglobulin in 6 M guanidine solution in presence of 450 μM progesterone. λ_{ex}=296 nm and λ_{em}=350 nm

where 0.143, 0.607 and 0.25 are the pre-exponential factors, 0.41, 2.017 and 4.462 ns are the decay times and λ is the emission wavelength (350 nm) ($\chi 2$=0.932).

In presence of 450 μM progesterone, fluorescence intensity decay I(λ,t) of β-lactoglobulin can be adequately represented also by a sum of three exponentials

$$I(\lambda, t) = 0.147\, e^{-t/0.492} + 0.647\, e^{-t/2.234} + 0.206\, e^{-t/5.0}$$

where 0.147, 0.647 and 0.206 are the pre-exponential factors, 0.492, 2.234 and 5.0 ns are the decay times and λ is the emission wavelength (350 nm) ($\chi 2$=0.979).

The measurements indicate that addition of progesterone to a denatured solution of β-lactoglobulin does not affect significantly any of the fluorescence decay parameters (lifetimes and pre-exponentials). The same results were obtained for denatured solution of α_1- acid glycoprotein in 6 M guanidine (data not shown).

Discussion

Although progesterone concentrations are important in the fluorescence cuvettes, we added only small volume (5 μl to the 1 ml protein solutions) of progesterone stock solutions so that to avoid interference, if any, of added volumes. The same precautions have been taken when we performed the same experiments with free tryptophan in solution [2].

The present work shows for the first time the possibility of resolving the $S_o \rightarrow {}^1L_a$ and $S_o \rightarrow {}^1L_b$ Transitions of tryptophan in proteins, directly from the fluorescence excitation spectrum. To the difference of free tryptophan in solution, high progesterone concentrations added to proteins solutions, do not abolish totally the tryptophan residues $S_o \rightarrow {}^1L_a$ transition. Thus, proteins structure plays an important role in the modulation of this transition, i.e., it plays a role of gap in retaining and organizing excitation energy, as it is the case for the emission energy [34]. Upon protein denaturation with 6 M guanidine and thus upon

protein tertiary structure loss, a red shift of 15 nm is observed in the $S_o \rightarrow {}^1L_a$ transition. Its peak position moves from 265 nm in the native state to 280 nm in the denatured one accompanied with an intensity decrease. However, the $S_o \rightarrow {}^1L_b$ transition parameters (intensity and position maximum) are not affected by the protein denaturation. Thus, only $S_o \rightarrow {}^1L_a$ transition is sensitive to protein structural modification. Our results are in good agreement with earlier studies performed on indole derivatives in solution and showing that $S_o \rightarrow {}^1L_a$ transition is highly sensitive to solvent interactions [35–37]. Nevertheless, since the present work describes experiments performed within proteins, it is more judicious to say that $S_o \rightarrow {}^1L_a$ transition is highly sensitive to environmental modification. Also, our data clearly show that $S_o \rightarrow {}^1L_b$ transition is not sensitive to tryptophan environment modifications. In fact, proteins denaturation does not induce any modification in the peak position and intensity of $S_o \rightarrow {}^1L_b$ transition.

Fluorescence lifetimes measured in absence and presence of progesterone are identical (Figs. 6 and 7) indicating that they do not depend on any of the two transitions. This result was also observed on free tryptophan in solution [2] even in the complete inhibition of the $S_o \rightarrow {}^1L_a$ transition. Tryptophan free in water emits with two lifetimes equal to 0.5 and 2.78 ns, these two lifetimes are observed for tryptophan residues in proteins, whether the macromolecules contain one or more Trp residues (this result and Ref. [2, 3, 38–40]). Thus, these two lifetimes characterize two substates or substructures of the tryptophan, reached in the excited state, independently of the environment (solvent and / or Trp-amino acids interactions). These substructures differ from an environment to another, modifying the values of the two lifetimes and of their corresponding amplitudes (Figs. 6, 7, 8 and 9). Each of both $S_o \rightarrow {}^1L_a$ and $S_o \rightarrow {}^1L_b$ transitions can induce the excited state of tryptophan, then afterward, reorganization of the fluorophore into two (in water) or three (in proteins) structures, occurs. Therefore, this structural reorganization in the excited state is independent of the two transitions.

The third lifetime observed in proteins is the result of the interaction between tryptophan residue(s) and amino acids environments inducing a third substructure with specific emission decay parameters. The fact that denatured proteins fluorescence decay can be analysed with three fluorescence lifetimes too, indicates the absence of any correlation between protein tertiary structure and the three fluorescence lifetimes presence. However, protein denaturation modifies the values of fluorescence decay parameters as the result of the new interaction between the fluorophore with its environment. For β-lactoglobulin and α_1-acid glycoprotein, these modifications are not very important (the present work). However, upon denaturation of human serum albumin, fluorescence lifetimes of the only tryptophan decrease significantly [40]. Protein denaturation induces important modification in the $S_o \rightarrow {}^1L_a$ transition parameters (peak position and intensity) without affecting the $S_o \rightarrow {}^1L_b$ transition. Nevertheless, since three fluorescence lifetimes are still observed in the denatured state, there is no direct relation between $S_o \rightarrow {}^1L_a$ transition and fluorescence decay parameters. Excitation of tryptophan molecules induces an excited state where fluorophore molecules retain two or different conformations different from those observed in the ground state. These conformations in the excited states will yield specific fluorescence decay parameters which values depend on the state of the protein. Also, nature of the protein influences the values of the tryptophan decay parameters [34].

Results described in the present work are obtained on α_1-acid glycoprotein where progesterone can bind and on β-lactoglobulin where progesterone binding is not specific. Thus, progesterone effect observed is not the result of specific binding of the hormone to the proteins. Also, we obtained the same data on Cyclophilin B (results not shown). Therefore, the positions of the $S_o \rightarrow {}^1L_a$ and $S_o \rightarrow {}^1L_b$ transitions do not vary with the type of globular protein.

In conclusion, this work puts into evidence that protein structure plays important role in the modulation of the $S_o \rightarrow {}^1L_a$ transition. This simply results from the fact that excited electrons are part of the fluorophore making their transition dependent on the fluorophore structure itself and on the surrounding amino acids. Measured lifetimes are the consequence of one or both transition states, which yield a reorganization of the tryptophan in the excited state into two or three interrelated structures.

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