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Fluorescence spectral resolution of tryptophan residues in bovine and human serum albumins

Nadim Tayeh, Tévamie Rungassamy, Jihad René Albani*

Laboratoire de Biophysique Moléculaire, Université des Sciences et Technologies de Lille, Bâtiment C6, 59655 Villeneuve d'Ascq Cédex, France

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ABSTRACT

Static quenching and time-resolved emission spectra of tryptophan residues of BSA (2 Trp residues) and HSA (1 Trp residue) were performed in the presence of high concentrations of calcofluor white, a fluorophore that is specific to both carbohydrate residues and to hydrophobic sites in proteins. In the absence of calcofluor white, BSA and HSA emit with a maximum at 340 and 330 nm, respectively. Also, tryptophan residues in both proteins fluoresce with three identical lifetimes. Time-resolved spectra of HSA show that the three lifetimes emit at a maximum equal to 330 nm while spectra obtained from BSA show different peak positions for the three lifetimes.

At high calcofluor concentrations, steady-state fluorescence emission spectrum of BSA displays a maximum at 330 nm instead of 340 nm in the absence of calcofluor. Fluorescence excitation spectra of the protein recorded in the absence and presence of calcofluor indicate the absence of protein conformational modification upon calcofluor white binding. Time-resolved emission spectra of the three lifetimes show identical peaks equal to 330 nm. Steady-state and time-resolved emission spectra performed on HSA in the presence of calcofluor do not show any modification in the emission peak (330 nm) indicating the absence of any conformational change and confirming the fact that the shift observed for tryptophan residues emission in BSA is the result of fluorescence quenching of Trp-134 residue.

1. Introduction

Bovine serum albumin (BSA), a large globular protein (66,000 Da), consists of a single chain of 583 amino acids residues, and forms sub-domains by paired 17 disulfide bonds [1]. BSA binds free fatty acids, other lipids and flavor compounds that can alter heat denaturation of the protein [2]. Also, BSA may play a role in lipid oxidation [3] and in the maintenance of blood pH [4].

BSA is formed from three domains I, II and III, divided into two sub-domains (A and B), and contains two tryptophan residues, Trp-134 and Trp-212, located respectively in domains I and II. Trp-212 residue is surrounded by a hydrophobic environment within a protein pocket while Trp-134 residue is located in a hydrophilic environment, close to the protein surface [1].

The primary sequence of HSA shows that the protein is a single polypeptide with 585 residues containing 17 pairs of disulfide bridges and one free cysteine [5]. Human serum albumin and many serum albumins from other species have been found to consist of three homologous domains probably derived through gene multiplication [6]. HSA contains only one tryptophan residue (Trp-214) that participates in additional hydrophobic packing interactions at the IIA–IIIA interface [1,7]. Trp-212 in BSA and Trp-214 in HSA are located in a similar hydrophobic microenvironment (sub-domain IIA) [1].

Structural and functional properties of bovine serum albumin have been performed following fluorescence emission of the Trp residues or of extrinsic fluorophores bound to the protein [8–11]. However, up to now, fluorescence spectrum of each of the two Trp residues has not been resolved yet and we know nothing on the possible relation between emission spectra and fluorescence lifetimes of BSA Trp residues. Therefore, in the present work, we attempted to resolve fluorescence emission spectrum of each of the two tryptophan residues in BSA by performing comparative studies with HSA. Time-resolved studies and static quenching were performed in the presence of high concentrations of calcofluor white, a fluorophore that is specific to both carbohydrate residues and to hydrophobic sites in proteins. In fact, calcofluor white is a fluorescent probe capable of making hydrogen bonds with β-(1 → 4) and β-(1 → 3) polysaccharides [12]. It is commonly used to study the mechanism by which cellulose and other carbohydrate structures are formed in vivo and is also widely used in clinical studies [13,14]. Also, calcofluor white binds to hydrophobic sites of proteins such as human serum albumin [15].

* Corresponding author. Tel.: +33 320337770.
E-mail address: Jihad-Rene.Albani@univ-lillel.fr (J.R. Albani).

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2. Materials and methods

Bovine and human serum albumins (purity >98%) were from Sigma. Proteins concentrations were determined at 278 nm with the following extinction coefficients 4.389×10^4 and $3.5219 \times 10^4\ M^{-1}\ cm^{-1}$ for bovine serum albumin and human serum albumin, respectively [16].

Absorbance data were obtained with a Shimadzu MPS-2000 double-beam spectrophotometer using 1-cm pathlength quartz cuvettes.

Steady-state fluorescence spectra were recorded with a PerkinElmer LS-5B spectrofluorometer. 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 [17,18].

Fluorescence lifetime measurements were performed with a Horiba Jobin Yvon FluoroMax-4-P, 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 and three lifetimes and then values of χ^2 were compared in order to determine the best fit. Since lifetimes data are based on the value of χ^2, it is important to make a description of the meaning and importance of this value. In the single photon counting method, the detection system measures the time between the excited pulse and the arrival of the first photon. The distribution of arrival times represents the decay curve also called the impulse response function. In order to obtain a decay curve that characterizes the sample only, excitation pulse should be infinitely sharp or should have a pulse much shorter than the decay time of the sample. Nevertheless, even if these two conditions are met, this is not always the case; the presence of the light pulse does not allow identifying the zero time point of the fluorescence decay. This will induce errors in number of lifetimes determination and calculation. The time distribution of the lamp pulse $L(t)$, also called the instrument response function, is measured in a separate experiment using scatter solution. The observed fluorescence decay is called $R(t)$ and the final response function is called $F(t)$.

$R(t)$ is given by the convolution of the lamp pulse with the impulse response of the sample [17,19,20]

$$R(t) = \int_0^t L(t')\, F(t - t')\, dt' \tag{1}$$

A method applied to estimate the impulse response function $F(t)$ is the least-squares analysis. The method calculates the expected value of $R(t)$ given assumed values of α_i and τ_i and the calculated value $[R_c(t)]$ is compared with the observed value $R(t)$. The α_i and τ_i values are varied until the best fit is obtained. The goodness of fit χ^2 is calculated from

$$\chi^2 = \sum_{i=1}^{n} w[R(t) - R_c(t)]^2 \tag{2}$$

where

$$w = \frac{1}{R(t)} \tag{3}$$

is a statistical weighting factor to account for the expected error in each value of $R(t)$. A minimal value of χ^2 indicates the best fit. A χ^2 value that approaches 1 indicates a good fit.

Differences between $R(t)$ and $R_c(t)$ is described by a function called the autocorrelation function of the differences and can be displayed by experimental curves. Close values of $R(t)$ and $R_c(t)$ yield experimental autocorrelation curves that are randomly distributed around zero, indicating by that a good fit. Finally, to find out whether a decay curve should be best analyzed with one, two or more lifetimes, values of χ^2 have to be compared. 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 two or three lifetimes [21].

The mean fluorescence lifetime is the second order mean [17]:

$$\tau_o = \sum f_i \tau_i \tag{4}$$

and

$$f_i = \frac{\beta_i \tau_i}{\sum \beta_i \tau_i} \tag{5}$$

where β_i are the preexponential terms, τ_i are the fluorescence lifetimes and f_i the fractional intensities.

All experiments on proteins were performed at 20 °C in 10 mM phosphate buffer and 0.143 M NaCl buffer (PBS buffer, pH 7).

3. Results and discussion

3.1. Binding effect of calcofluor on BSA fluorescence spectrum: static studies

Fig. 1 displays fluorescence emission spectra of bovine serum albumin in the presence of increased concentrations of calcofluor (λ_{ex} = 270 nm). Tryptophan residues emission peak located at 340 nm in the absence of calcofluor shifts to 328 nm in the presence of 54 μM of the extrinsic fluorophore. Emission decrease is the result of calcofluor binding to the protein and to the energy transfer from tryptophan residues to the extrinsic fluorophore. In fact, fluorescence intensity decrease of Trp residues is accompanied by an intensity increase of calcofluor (not shown). Also, addition of calcofluor white to BSA induces a decrease of the fluorescence excitation spectrum intensities. Fig. 2 shows fluorescence intensities of both excitation and emission spectra at different calcofluor

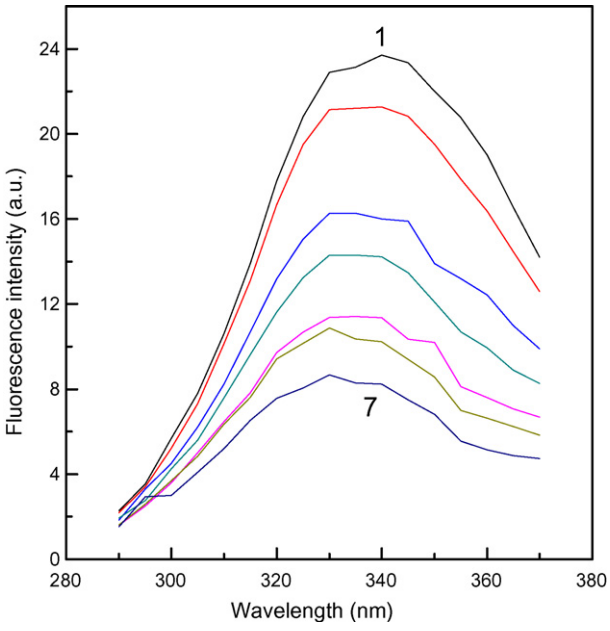

Fig. 1. Fluorescence intensity of 4.5 μM BSA in the presence of increased concentrations of calcofluor (curve 7: 54 μM). λ_{ex} = 270 nm. Spectra have been corrected for the inner filter effect.

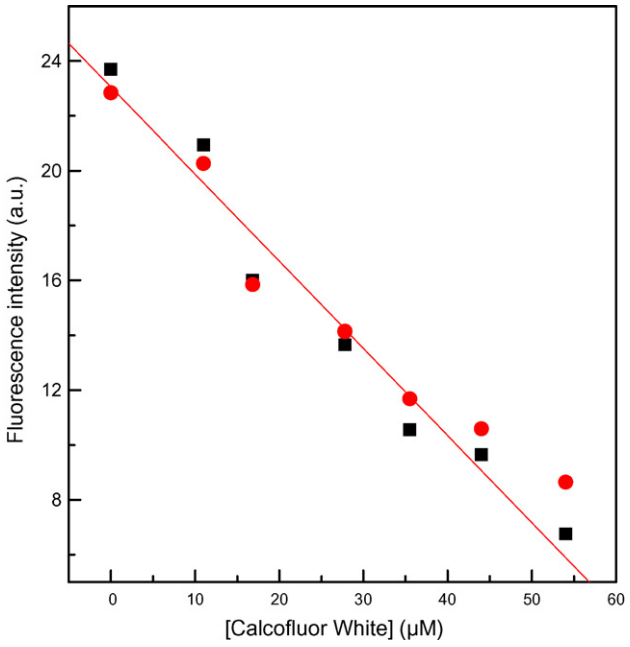

Fig. 2. Fluorescence intensity decrease of 4.5 μM BSA tryptophan emission (λ_{ex} = 270 nm and λ_{em} = 345 nm) (squares) and excitation (λ_{ex} = 285 nm and λ_{em} = 330 nm) (circles) with calcofluor concentration.

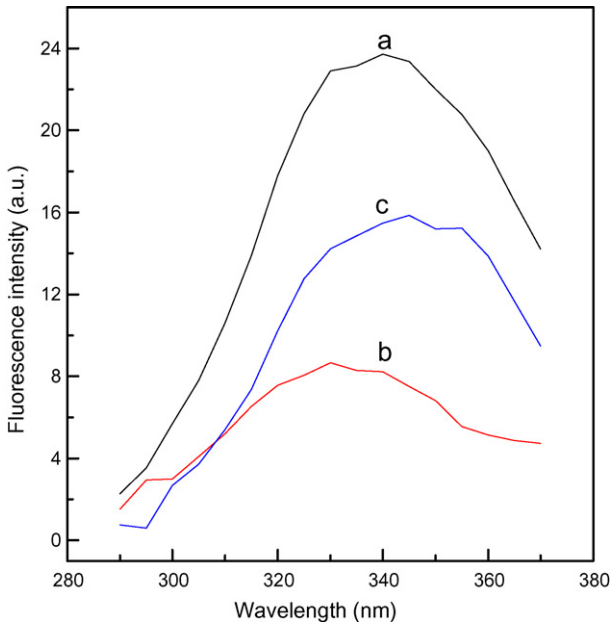

Fig. 3. Fluorescence spectra of 4.5 μM BSA (a), λ_{max} = 340 nm and of BSA in the presence of 54 μM calcofluor (b) λ_{max} = 330 nm. Spectrum c is the difference between spectra (a) and (b). λ_{max} = 345 nm.

concentrations. Analysis of the data, obtained from three different experiments, with equation

$$\frac{I_o}{I} = 1 + K_a[\text{calcofluor white}] \quad (6)$$

where I_o and I are the fluorescence intensities in the absence and presence of different calcofluor concentrations and K_a is the association constant, yields a value for K_a equal to $0.0287 \pm 0.0017\ \mu M^{-1}$ or a K_d equal to 35 ± 2 μM. This value is close to that (K_d = 40 μM) found for HSA–calcofluor complex [15].

Subtracting spectrum 7 from 1 of Fig. 1 yields an emission spectrum with a maximum located at 345 nm (Fig. 3). The shift in the fluorescence emission spectrum of BSA in the presence of calcofluor could be the result of (i) fluorescence quenching of the surface tryptophan residue, (ii) structural modification of BSA upon calcofluor binding or (iii) fluorescence quenching of the longest fluorescence lifetime accompanied by a decrease in the fractional intensity of this lifetime. Structural modification within the protein can be monitored by recording the fluorescence excitation spectrum of Trp residues in the absence and presence of low and high calcofluor concentrations. In fact, fluorescence excitation spectrum characterizes electron distribution of the molecule in the ground state. At λ_{em} = 330 nm, calcofluor does not emit and thus, only excitation spectrum of Trp residues would be recorded. Therefore, any modification of the fluorescence excitation spectrum in the presence of calcofluor would be the result of a structural modification of the protein in the ground state. Fig. 4 displays fluorescence excitation spectrum of BSA Trp residues in the absence (a) and presence of 54 μM calcofluor (b). We notice that in the presence of high calcofluor concentrations, excitation peak located at 280 nm is not shifted and the global shape of the excitation spectrum is not modified, i.e., binding of calcofluor has not affected local structure of BSA Trp residues. Nevertheless, structural modification of BSA could occur at higher calcofluor concentrations than the one used in our work (50 μM of calcofluor for 4.5 μM of BSA). Therefore, we recorded excitation spectra of BSA at different calcofluor concentrations using two protein concentrations 5 and 2.5 μM (Figs. 5 and 6, respectively). The results obtained clearly indicate that for 5 μM BSA, calcofluor added to the protein solution does not induce any structural modification of the protein. However, for 2.5 μM BSA, a significant modification of the excitation spectrum is observed at 150 μM calcofluor, indicating a protein structural perturbation. Therefore, in the conditions of our titration experiments (4.5 μM BSA and 54 μM calcofluor), structural modification of the protein is to be excluded as it is also shown in Fig. 4. Finally, it is important to notify that minimum calcofluor concentration, necessary to induce a protein structural modification, was also observed when interaction studies were performed with α_1-acid glycoprotein [22].

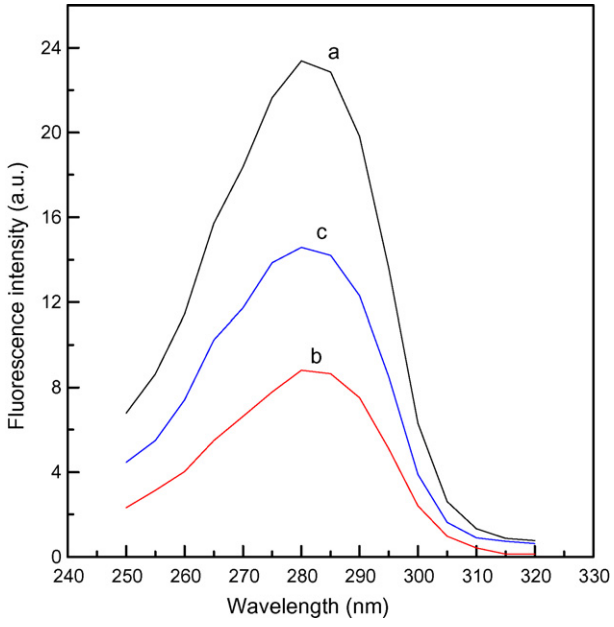

Fig. 4. Fluorescence excitation spectra of 4.5 μM BSA in the absence of calcofluor (a) and in the presence of 54 μM calcofluor (b). λ_{max} = 280 nm and λ_{em} = 330 nm. Spectrum c is the difference between spectra (a) and (b).

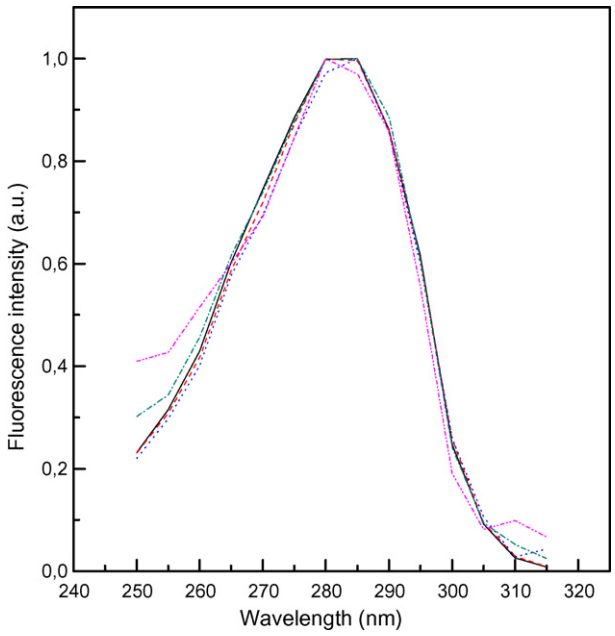

Fig. 5. Normalized fluorescence excitation spectra of 5 μM BSA in the absence (full line) and in the presence of 25 (dashed line), 50 (dotted line), 100 (dash dotted line) and 150 μM (dash dotted dotted line) calcofluor. λ_{em} = 330 nm.

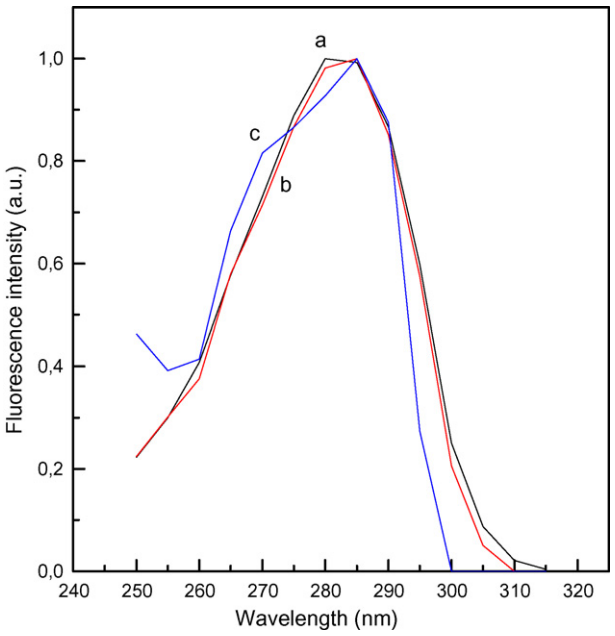

Fig. 6. Fluorescence excitation spectra of 2.5 μM BSA in the absence of calcofluor (a) and in the presence of 50 μM (b) and 150 μM (c) calcofluor. λ_{em} = 330 nm.

3.2. Fluorescence lifetime of BSA Trp residues

Fluorescence intensity decay of BSA Trp residues observed at 350 nm can be adequately described by a sum of three exponentials (Fig. 7)

$$I(\lambda, t) = 0.0473\, e^{-t/0.265} + 0.257\, e^{-t/3.055} + 0.6957\, e^{-t/6.577}$$

where 0.0473, 0.257 and 0.6957 are the preexponential factors and 0.265, 3.055 and 6.577 ns the decay times (χ^2 = 1.006). In the pres-

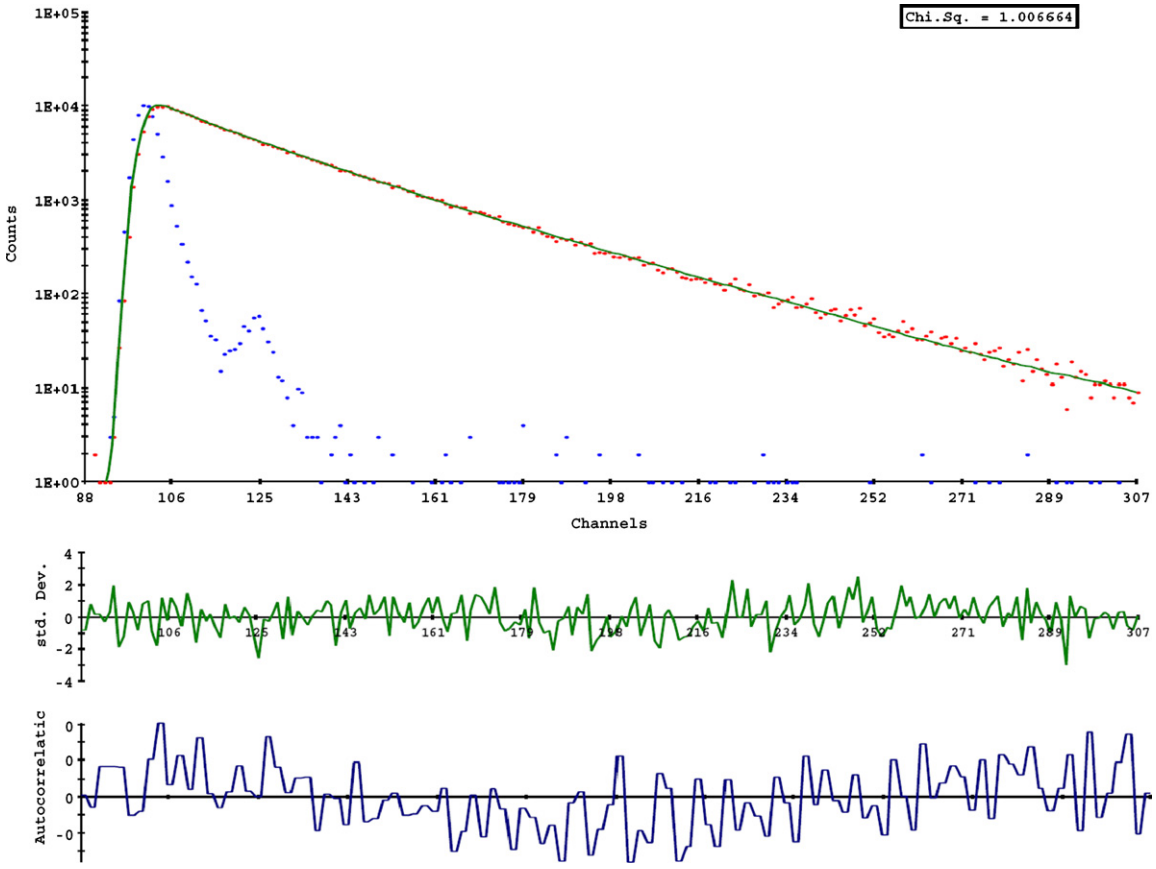

Fig. 7. Fluorescence intensity decay of Trp residues in bovine serum albumin. λ_{ex} = 296 nm and λ_{em} = 350 nm.

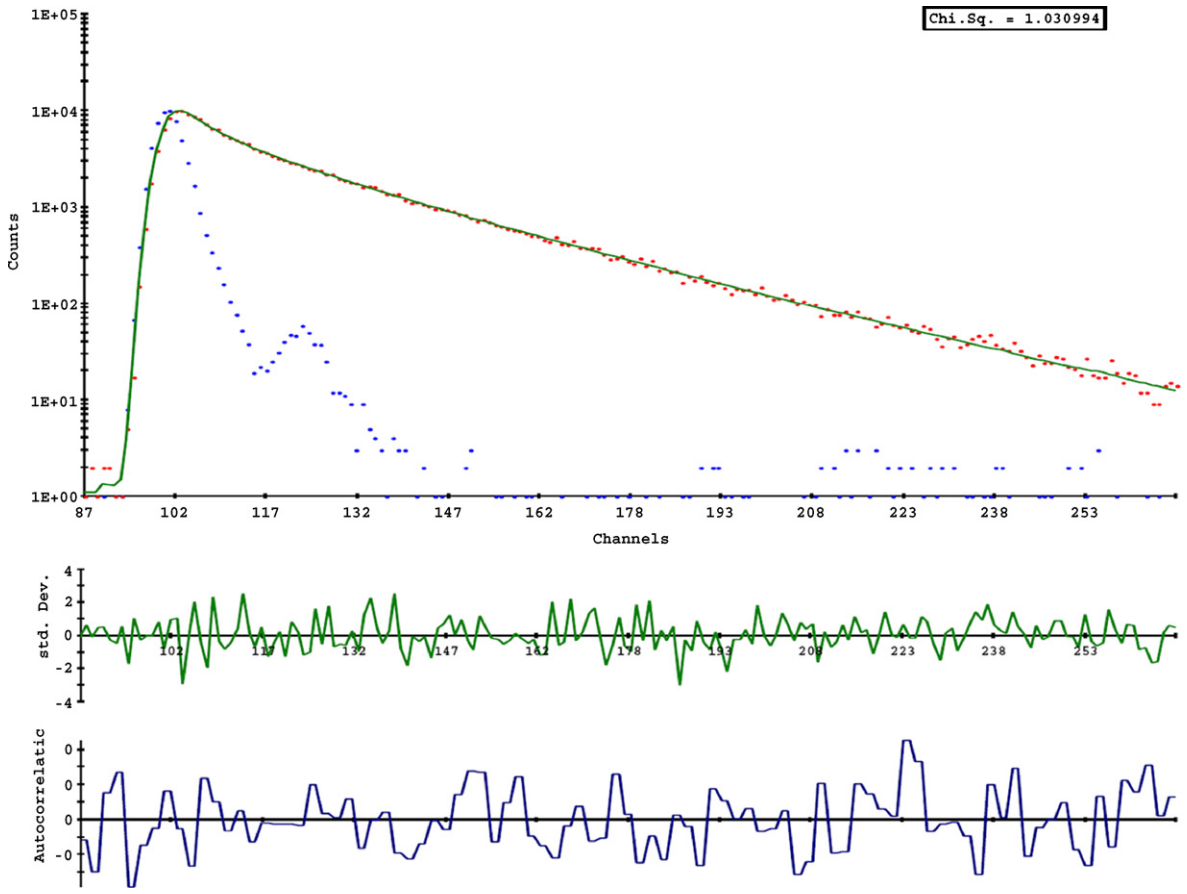

Fig. 8. Fluorescence intensity decay of Trp residues in bovine serum albumin in the presence of 54 μM calcofluor white. λ_{ex} = 296 nm and λ_{em} = 350 nm.

ence of 54 μM calcofluor, fluorescence intensity decay can still be characterized by a sum of three exponentials (Fig. 8)

$$I(\lambda, t) = 0.1375\,e^{-t/0.524} + 0.2572\,e^{-t/2.213} + 0.6053\,e^{-t/5.974}$$

where 0.1375, 0.2572 and 0.6053 are the preexponential factors and 0.524, 2.213 and 5.974 ns the decay times (χ^2 = 1.03). In the absence and presence of progesterone, the value of χ^2 increases to 1.265 and to 1.7, respectively, when we tried to fit the fluorescence decays with two fluorescence lifetimes instead of three. The increase of χ^2 values was observed for all the measurements performed for BSA and HSA at all wavelengths. Thus, data analysis with a three exponential model was the best for all our measurements.

Thus, binding of calcofluor to bovine serum albumin does not abolish any of the three fluorescence lifetimes of the protein Trp residues.

Fluorescence intensity and mean lifetime decreases as a function of calcofluor concentration are displayed in Fig. 9. Intensity quenching is much more important than lifetime indicating static nature of BSA–calcofluor interaction accompanied by energy transfer between Trp residues and calcofluor.

3.3. Time-resolved emission spectra of BSA

A very common method to put into evidence fluorescence emission spectrum of each component is the decay-associated spectra. This method allows combining dynamic time-resolved fluorescence data with steady-state emission spectrum [23]. Fluorescence decay profiles with excitation at 296 nm and measured from 315 to 370 nm, were best fitted with a three exponential decay model. The decay-associated emission spectra were constructed using the following equation

$$F_i(\lambda) = F_{ss}(\lambda)\frac{\beta_i(\lambda)\tau_i}{\sum \beta_i(\lambda)\tau_i} \quad (7)$$

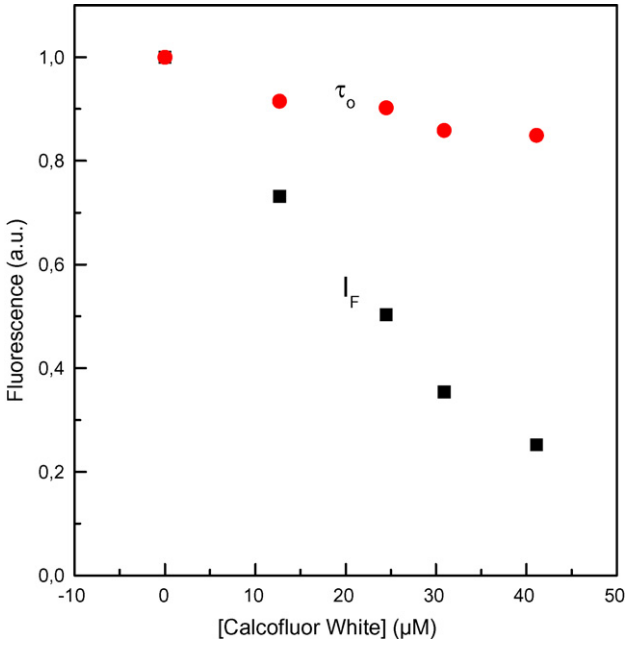

Fig. 9. Normalized fluorescence intensity and mean lifetime of 4.5 μM BSA, measured at 330 nm, in the presence of increased concentrations of calcofluor white.

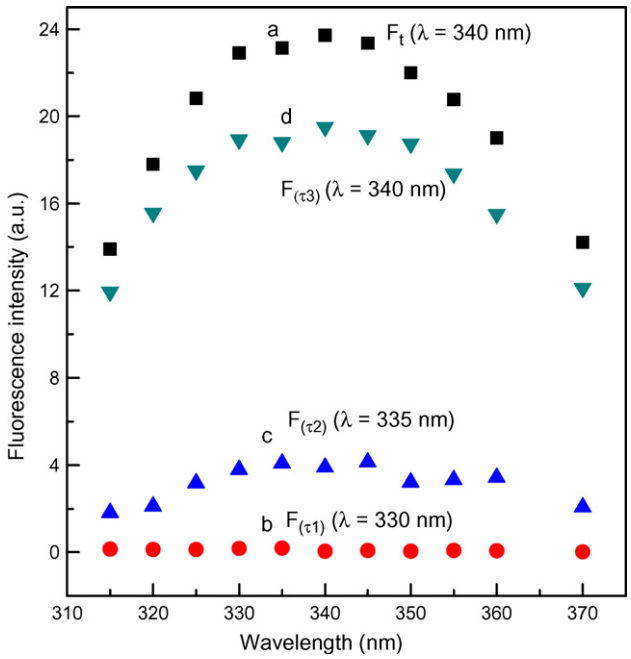

Fig. 10. Steady-state fluorescence emission spectrum of BSA (λ_{max} = 340 nm) (a) and decay-associated spectra of τ_1 (λ_{max} = 330 nm) (b), of τ_2 (λ_{max} = 335 nm) (c) and of τ_3 (λ_{max} = 340 nm) (d).

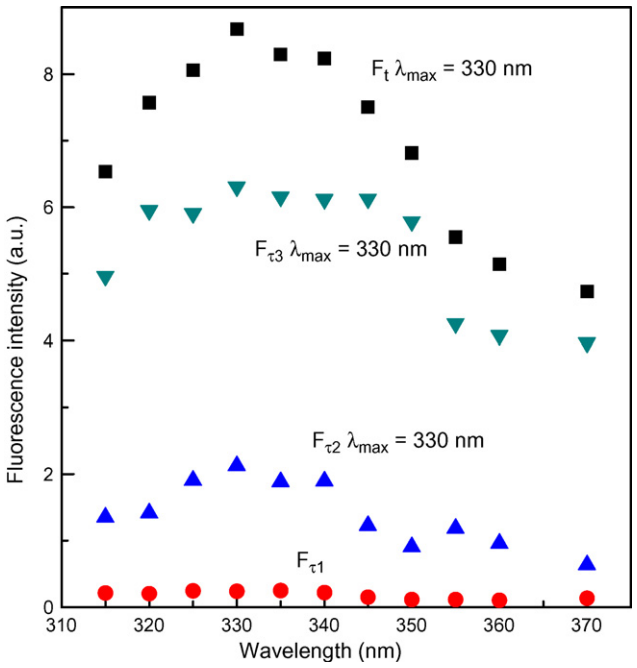

Fig. 11. Steady-state fluorescence emission spectrum of BSA in the presence of 54 μM calcofluor (λ_{max} = 330 nm) and decay-associated spectra of τ_1 (λ_{max} = 330 nm), of τ_2 (λ_{max} = 330 nm) and of τ_3 (λ_{max} = 330 nm).

where $F_i(\lambda)$ refers to the intensity of the *i*th component at a specific wavelength λ and $F_{ss}(\lambda)$ is the total intensity obtained from steady-state emission spectrum, which integrates the different weighted fluorescence lifetimes.

Fig. 10 displays decay-associated spectra of BSA Trp residues. We can notice that the three lifetimes display emission spectra which maxima are located at 330, 335 and 340 nm. This means that the two Trp residues are not necessarily surrounded by the same microenvironment, one tryptophan residue is surrounded by a hydrophobic environment while the second tryptophan residue would be in a more hydrophilic environment.

In the presence of 54 μM calcofluor, decay-associated spectra show that the three lifetimes emit with a maximum located at 330 nm (Fig. 11). Time-resolved spectra result is identical to that obtained with static fluorescence experiments (Figs. 1 and 3). Thus, the shift observed in the presence of calcofluor is the consequence of BSA fluorescence quenching of the surface tryptophan residue (Trp-134). However, this does not mean that calcofluor does not quench Trp-212 residue. Therefore, in order to see the effect of calcofluor binding on Trp-212 residue fluorescence, we performed static and dynamic quenching experiments on HSA in the absence and presence of calcofluor.

3.4. Binding of calcofluor to HSA: static fluorescence studies

Fig. 12 displays fluorescence emission spectrum of Trp-214 residue of HSA in the presence of increasing concentrations of calcofluor. We can notice that while fluorescence intensity decreases, emission peak located at 330 nm shifts for some spectra by 4 nm. Nevertheless, subtracting spectrum 7 (corresponding to the presence of 53 μM calcofluor) from spectrum 1 yields an emission spectrum with a maximum located at 330 nm (not shown).

These features are completely different from those observed for the interaction of calcofluor with BSA, favoring the fact that the shift (10–12 nm) observed in BSA tryptophan fluorescence in the presence of calcofluor is the result of Trp-134 quenching. Since Trp-212 is in the same position within BSA and HSA, Fig. 12 clearly indicates that in BSA both Trp residues are quenched by calcofluor.

Earlier studies have considered that Trp-214 of HSA and Trp-212 of BSA can possess similar fluorescence behavior [24,25]. In this case, subtracting fluorescence emission spectrum of HSA from that of the same concentration of BSA would yield emission spectrum of Trp-134 (Fig. 13). In fact, we notice that the difference spectrum (Fig. 13c) corresponds to that displayed in Fig. 3 (spectrum c). What is interesting to look at is the ratio of the intensities at the peaks of the spectrum corresponding to Trp-214 or -212 residue and BSA spectrum. With calcofluor addition, this ratio is equal to 0.367 while with simple subtraction of HSA spectrum from that of BSA, the ratio is equal to 0.388. Also, this ratio fits very well with that (0.379) calculated from the excitation spectra displayed in Fig. 4.

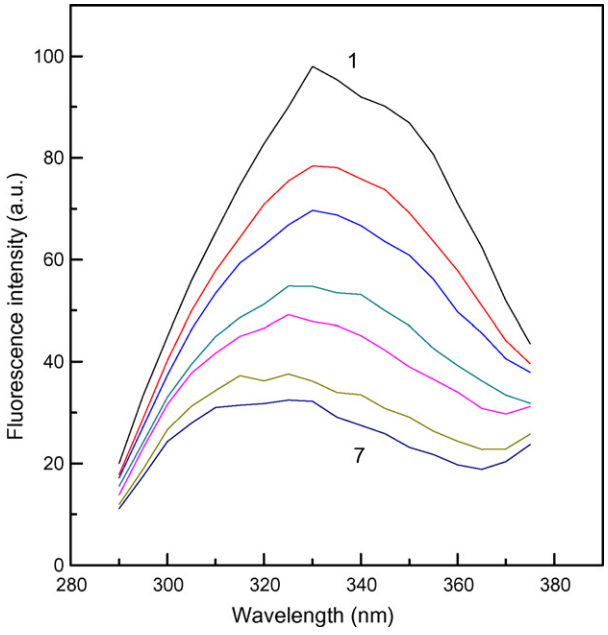

Fig. 12. Fluorescence emission spectra of 4.5 μM HSA in the presence of increased concentrations of calcofluor (curve 7, 53 μM calcofluor).

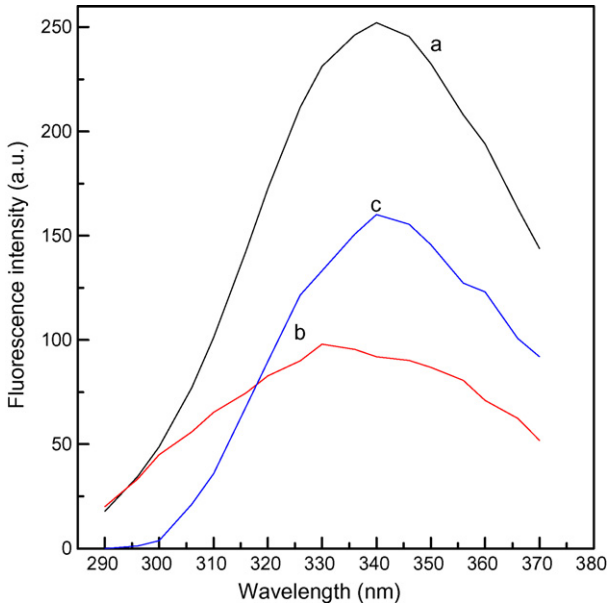

Fig. 13. Fluorescence emission spectra of 4.5 μM BSA (a), 4.5 μM HSA (b) and of the difference (c).

From the sum of the intensities along the emission wavelengths, we found that spectrum of Trp-134 is around 56% of BSA emission spectrum while that of Trp-212 residue is 44%. Considering the fact that fluorescence spectra of Trp-212 and Trp-134 residues are additive, implies that energy transfer between the two tryptophans is negligible, even absent. This means that the two Trp residues are perpendicular one to each other and/or located far from each others at a distance that does not allow energy transfer between them. Unfortunately, these two hypothesis cannot be confirmed by the three-dimensional structure of BSA since it has not been determined yet.

3.5. Fluorescence lifetime and time-resolved emission spectra of HSA Trp-214 residue

Fluorescence intensity decay of HSA Trp-214 residue observed at 325 nm can be adequately represented by a sum of three exponentials (Fig. 14)

$$I(\lambda, t) = 0.1235\,e^{-t/0.528} + 0.3104\,e^{-t/3.566} + 0.5661\,e^{-t/7.460}$$

where 0.1235, 0.3104 and 0.5661 are the preexponential factors and 0.528, 3.566 and 7.460 ns the decay times ($\chi^2 = 1.066$). Identical results were obtained by Flora et al. and Zolese et al. [26,27].

In the presence of 54 μM calcofluor, fluorescence intensity decay can still be characterized by a sum of three exponentials (Fig. 15)

$$I(\lambda, t) = 0.2350\,e^{-t/0.356} + 0.2760\,e^{-t/2.180} + 0.4890\,e^{-t/6.755}$$

where 0.2350, 0.2760 and 0.4890 are the preexponential factors and 0.356, 2.180 and 6.755 ns the decay times ($\chi^2 = 1.23$).

These results indicate that the longest fluorescence lifetime cannot be assigned to Trp-134 residue of BSA. Also, the data show that binding of calcofluor to human serum albumin does not abolish any of the three fluorescence lifetimes.

Fig. 16 displays decay-associated spectra of Trp-214 residue of HSA. The three lifetimes display emission spectra with maxima located at 330 nm. In the presence of 54 μM calcofluor, positions

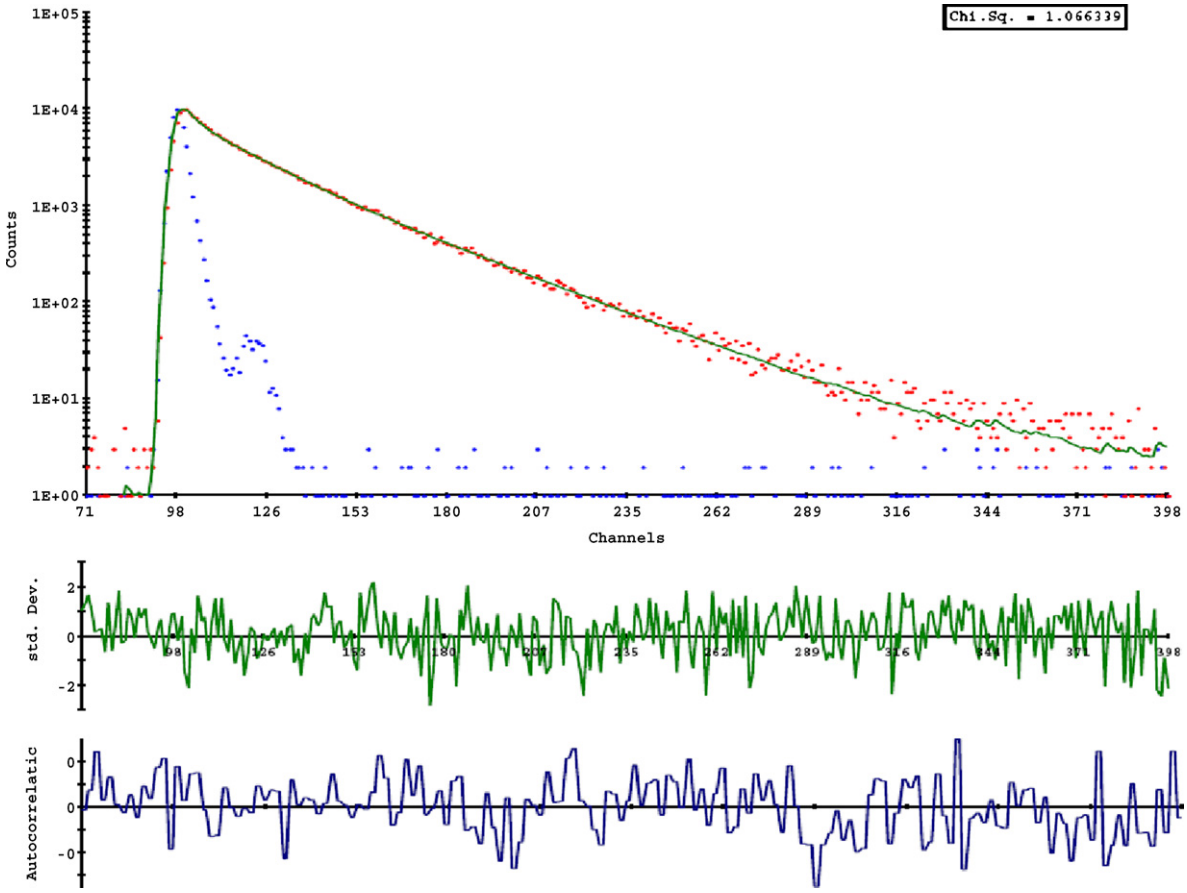

Fig. 14. Fluorescence intensity decay of Trp-212 residue in human serum albumin. λ_{ex} = 296 nm and λ_{em} = 325 nm.

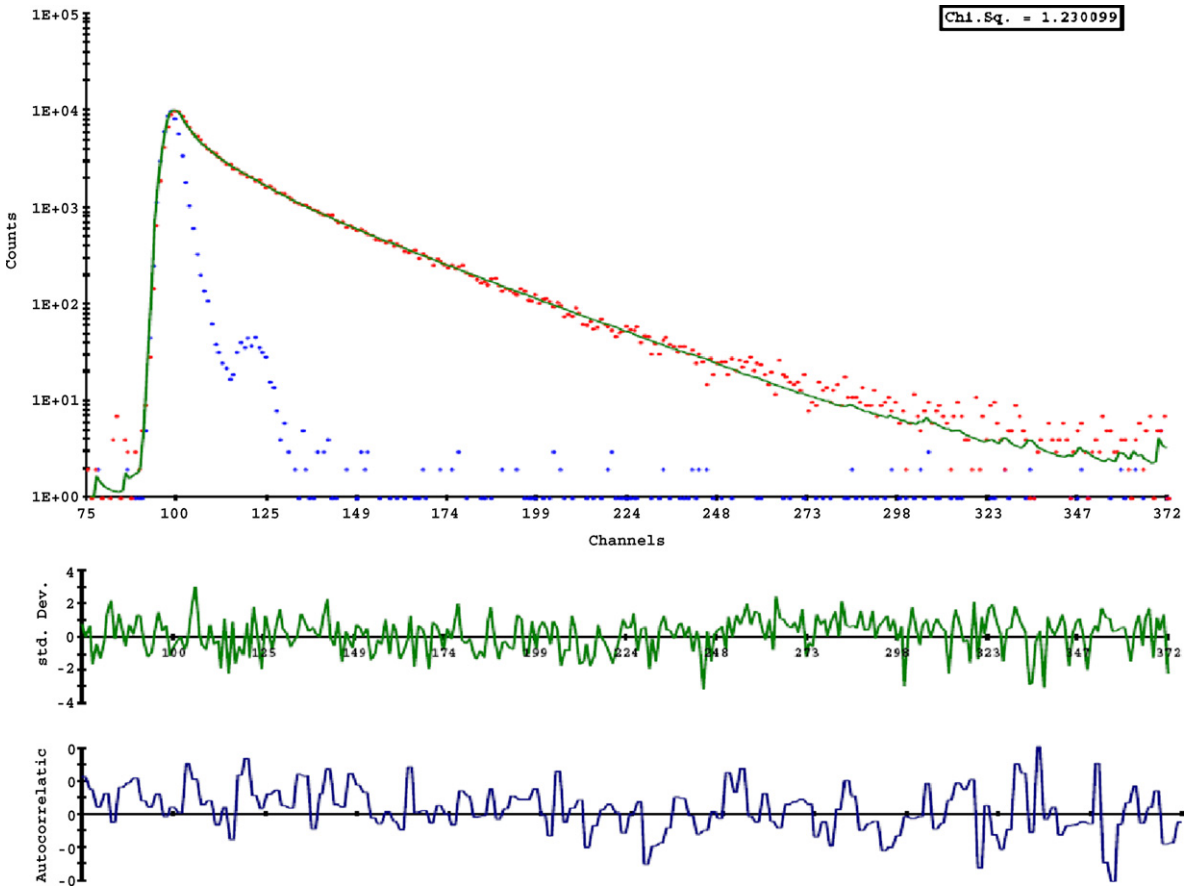

Fig. 15. Fluorescence intensity decay of Trp-212 residue in human serum albumin in the presence of 54 μM calcofluor. λ_{ex} = 296 nm and λ_{em} = 325 nm.

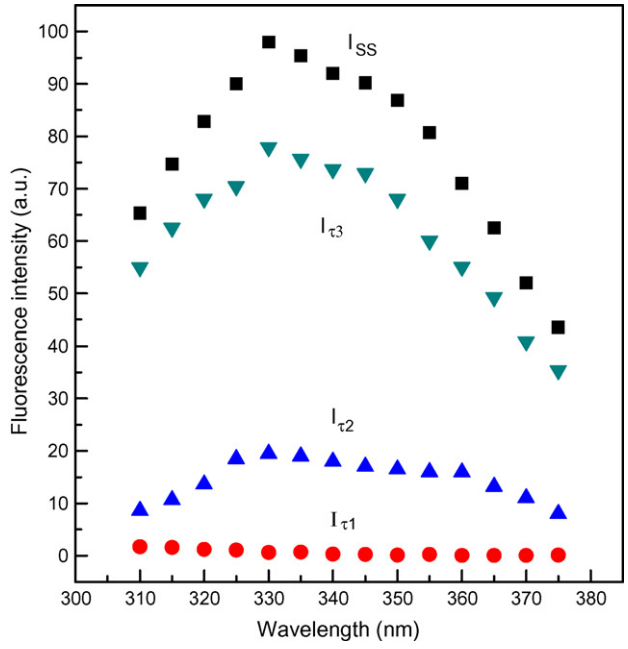

Fig. 16. Steady-state fluorescence emission spectrum of HSA in the presence of 54 μM calcofluor (λ_{max} = 330 nm) and decay-associated spectra of τ_1 (λ_{max} = 330 nm), τ_2 (λ_{max} = 330 nm) and τ_3 (λ_{max} = 330 nm).

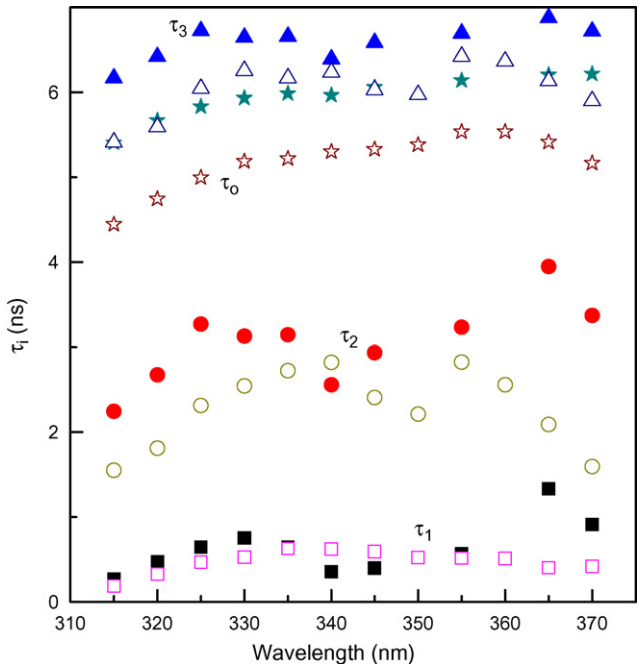

Fig. 17. Lifetimes variation of 4.5 μM BSA in the absence (full symbols) and presence of 54 μM calcofluor (empty symbols). Squares, circles, triangles and stars characterize τ_1, τ_2, τ_3 and τ_o, respectively.

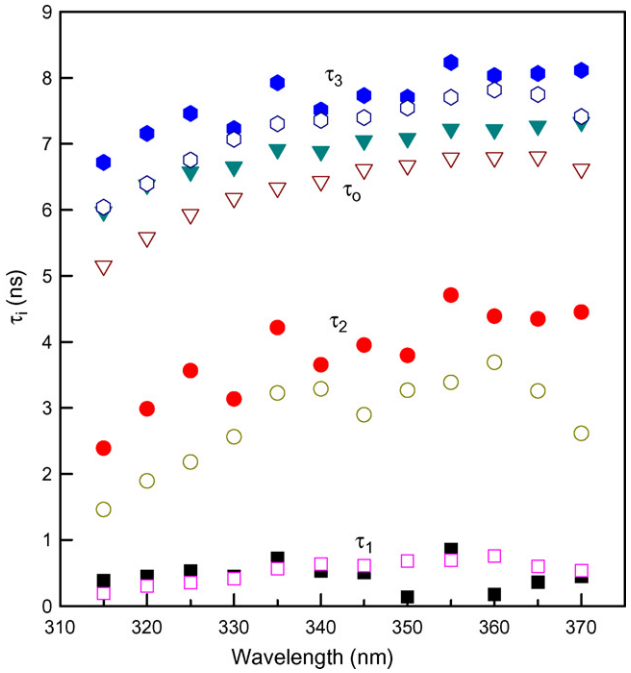

Fig. 18. Lifetime variation of 4.5 μM HSA in the absence (full symbols) and presence of 54 μM calcofluor (empty symbols). Squares, circles, lozenges and triangles characterize τ_1, τ_2, τ_3 and τ_o, respectively.

of the emission peaks are not modified (data not shown). This result is identical to that obtained with static analysis indicating that calcofluor binding to human serum albumin does not induce any structural modification within the protein.

3.6. Lifetimes and fractional intensities variation with emission wavelengths of Trp residues in BSA and HSA

Figs. 17 and 18, obtained in the absence and presence of calcofluor, display the three lifetimes variation with emission wavelength of BSA Trp residues and HSA Trp-214 residue, respectively. We can observe that fluorescence lifetimes decrease slightly in the presence of calcofluor as a result of energy transfer occurring between tryptophan residues and calcofluor. This decrease is identical whether it is occurring in BSA (2 Trp residues) or in HSA (1 Trp residue). Figs. 19 and 20 display fractional intensities variation for both BSA and HSA Trp residues in the absence (Fig. 19) and in the presence of 54 μM calcofluor (Fig. 20). Results obtained indicate that fractional intensities do not differ between HSA and BSA whether calcofluor is absent or present in the medium. Therefore, the shift observed in BSA fluorescence upon calcofluor binding (Fig. 1) is not the result of the longest fluorescence lifetime or of its fractional intensity quenching, but of fluorescence quenching of the surface Trp residue.

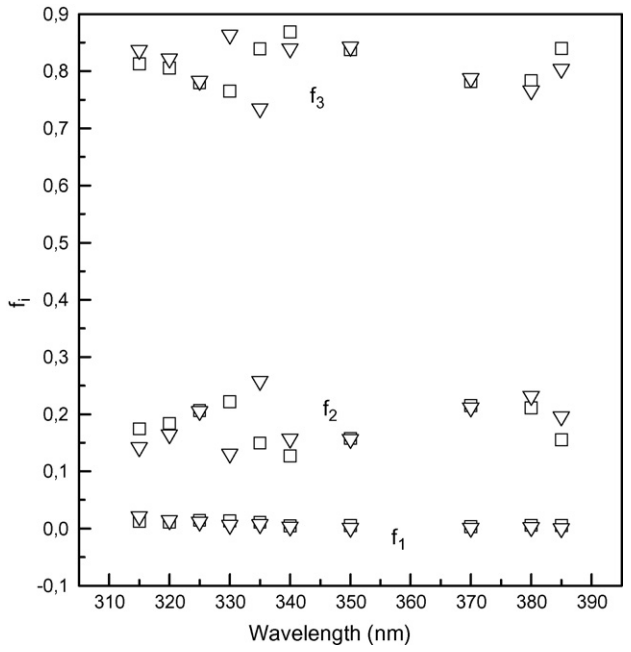

Fig. 19. Fractional intensities of Trp residues lifetimes in HSA (triangles) and in BSA (squares) in the absence of calcofluor.

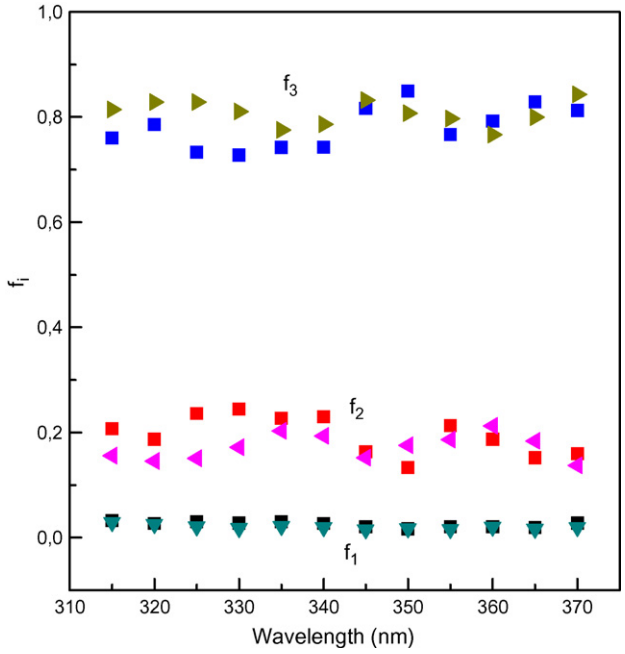

Fig. 20. Fractional intensities of Trp residues lifetimes in HSA (triangles) and in BSA (squares) in the presence of 54 μM calcofluor.

4. Conclusion

All the data displayed in this work fit well with the fact that binding of calcofluor to BSA induces a decrease in the fluorescence intensity of both Trp-134 and Trp-212 residues accompanied with a progressive blue shift of the peak as the result of total quenching of Trp-134 residue located near the protein surface as it is revealed by its position peak (345 nm). Our results show that tryptophan residues in both BSA and HSA fluoresce with three identical lifetimes. Time-resolved emission spectra of Trp-214 residue of HSA show that the three lifetimes emit at a maximum equal to 330 nm while spectra obtained from BSA Trp-212 and 134 residues show different peak positions for the three lifetimes. Addition of calcofluor to BSA decreases slightly fluorescence lifetimes values of the tryptophan residues. At high calcofluor concentrations, time-resolved emission spectra of the three lifetimes show identical peaks equal to 330 nm. Steady-state fluorescence emission spectrum of BSA recorded in the presence of high calcofluor concentrations displays a maximum at 330 nm instead of 340 nm in the absence of calcofluor. Fluorescence excitation spectra of BSA recorded in the absence and presence of calcofluor indicate the absence of protein conformational modification upon calcofluor white binding. Steady-state and time-resolved emission spectra performed on HSA in the absence and presence of calcofluor do not

show any modification in the emission peak (330 nm) indicating the absence of any conformational change and confirming the fact that the shift observed for tryptophan residues emission in BSA in the presence of calcofluor white is the result of fluorescence quenching of Trp-134 residue.

Our data reveal that one Trp residue emission can be described with three fluorescence lifetimes and that in BSA it is not possible to assign a specific lifetime to a specific Trp residue. Finally, it is important to mention that Trp-214 residue of HSA emits with three lifetimes and not two as it was mentioned many years ago (1.5 and 6.5 ns) [28] or (0.4 and 5.6 ns) [29].

What could be the origin of the three fluorescence lifetimes? Recent results obtained on free tryptophan in solution and on tryptophan present in proteins of different structures showed that the two lifetimes around 0.3–0.4 ns and 3–3.5 ns were measured for tryptophan free in solution and present within the proteins [30]. Thus, these two lifetimes seem to be independent of any structure around the tryptophan and simply reveal that they characterize an internal property or/and organization of the tryptophan structure independently of its environment [30]. These structures or sub-structures obtained in the excited state yield the two fluorescence lifetimes (0.4 and 5.6 ns) found for tryptophan whether free in solution or present within a protein. The third lifetime recorded in proteins could be attributed to interaction between the Trp residue (s) and the surrounding amino acids and to possible specific properties of the protein. Also, the 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 [30]. A recent result has shown that the fluorophore 6,P-toluidinyl-naphthalene-2-sulfonate (TNS) adopts in the excited state, conformations or sub-structures that differ whether it is dissolved in water, ethanol, bound to proteins or aggregated [21]. Fluorescence lifetimes (number, values and preexponential terms) characterize the observed sub-structures and their interactions with the surrounding environment while fluorescence spectra (excitation and emission) characterize the global state-structure of the fluorophore [21].

Also, it is important to indicate that time-resolved studies accompanied with static interactions are complementary tools to understand ligands binding to proteins.

Finally, the approach applied in the work, could be applied to other protein–drug combinations to yield mechanistic information on drug protein binding.

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