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ORIGINAL PAPER

Fluorescence Lifetimes of Tryptophan: Structural Origin and Relation with $S_o \rightarrow {}^1L_b$ and $S_o \rightarrow {}^1L_a$ Transitions

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Abstract We measured fluorescence lifetimes of L-Tryptophan dissolved in de-ionized water and in ethanol in the absence and the presence of high progesterone concentrations. The hormone absorbs between 220 and 280 with a peak around 250 nm, while its absorption is equal to zero beyond 280 nm. Tryptophan excitation spectrum recorded in presence of progesterone shows that the $S_o \rightarrow {}^1L_a$ transition is completely abolished while the $S_o \rightarrow {}^1L_b$ transition is not affected. Emission of L-tryptophan in water occurs with two fluorescence lifetimes, 0.40 and 2.8 ns. In ethanol, three fluorescence lifetimes equal to around 0.2, 1.8 and 4.8 ns were observed. Addition of progesterone to the medium does not affect any of the fluorescence lifetimes indicating clearly that both transitions could induce tryptophan excitation and that recorded fluorescence lifetimes could be assigned to sub-structures generated in the excited state.

Keywords Fluorescence lifetime · Tryptophan residues · $S_o \rightarrow {}^1L_b$ and $S_o \rightarrow {}^1L_a$ transitions

Introduction

Tryptophan is the main fluorophore within proteins. Its fluorescence properties have been studied and applied to understand proteins structure and dynamics and interaction of macromolecules with different ligands [1–5]. 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. However, up to now, there are no studies revealing the possible correlation between the two transitions and fluorescence lifetimes. Also, in the literature, there is not a single fluorescence experiment on only one of the two transitions, the reason for that is the absence of a method that permits to abolish one transition without affecting the second. In the present work, we measured fluorescence lifetimes and we recorded fluorescence spectra (emission and excitation) of L-tryptophan dissolved in water and in ethanol in absence and presence of high progesterone concentrations. 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. The results we obtained showed that in presence of high progesterone concentrations, $S_o \rightarrow {}^1L_a$ transition was completely abolished as it is shown by the excitation spectrum while fluorescence lifetimes were not modified whatever the emission wavelength was. Thus, $S_o \rightarrow {}^1L_b$ transition alone excites tryptophan in solution. Both transitions could induce tryptophan excitation and fluorescence lifetimes emanate from tryptophan substructures generated in the excited state.

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Materials and methods

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

Progesterone (from Sigma) was dissolved in twice distilled ethanol. Concentration of the stock solution was equal to 60 mM. Final progesterone concentrations in the fluorescence cuvettes (1 ml tryptophan solution) were equal

to around 650 and 240 μM, for tryptophan dissolved in water and in ethanol, 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. The 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 emission and excitation wavelengths, respectively. 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 [6, 7]. 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, using the time correlated single photon counting method. Excitation was performed at 296 nm with a nanoLED and lifetimes were measured each 5 nm from 310 to 390 nm.

The mean fluorescence lifetime calculated is the second order mean [6]:

$$\tau_o = \sum f_i \tau_I \quad (1)$$

and

$$f_i = \alpha_i \tau i / \sum \alpha_i \tau i \quad (2)$$

where α_i are the preexponential terms, τ_i are the fluorescence lifetime and f_i the fractional intensities.

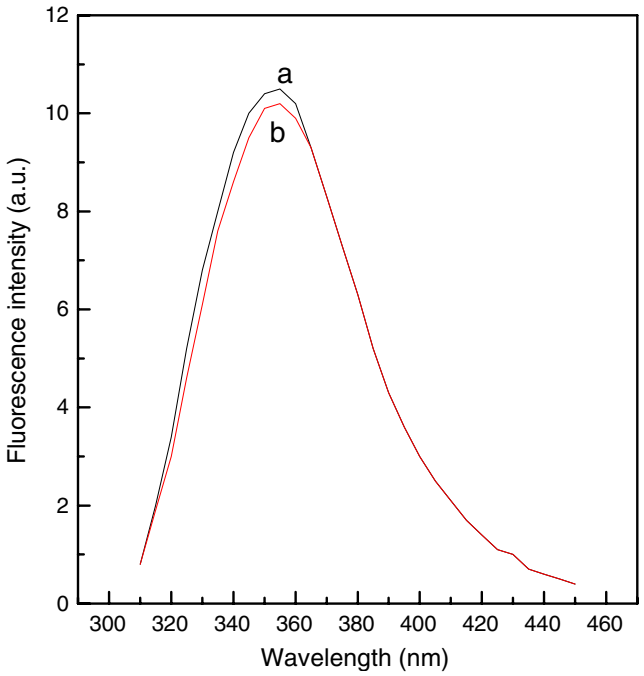

Fig. 1 Fluorescence emission spectra of L-Tryptophan in deionised water in the absence (a) and in the presence (b) of 650 μM progesterone. λ_{ex}=295 nm

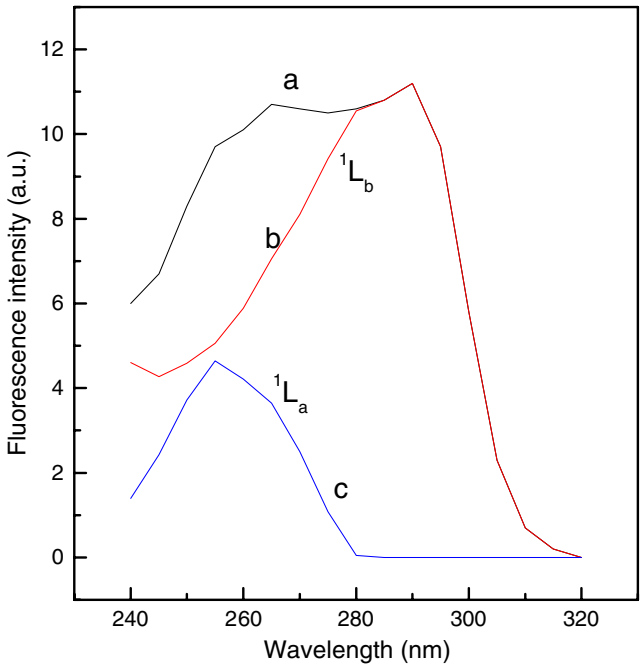

Fig. 2 Fluorescence excitation spectra of L-tryptophan dissolved in deionised water in absence (spectrum a) and in presence of 650 μM progesterone (spectrum b). Excitation spectrum obtained in presence of progesterone corresponds to the $S_o \rightarrow {}^1L_b$ transition. Spectrum c which corresponds to the $S_o \rightarrow {}^1L_a$ transition is obtained by subtracting (b) from (a). λ_{max} of ^1L_a and of ^1L_b are equal to 255 and 290 nm, respectively

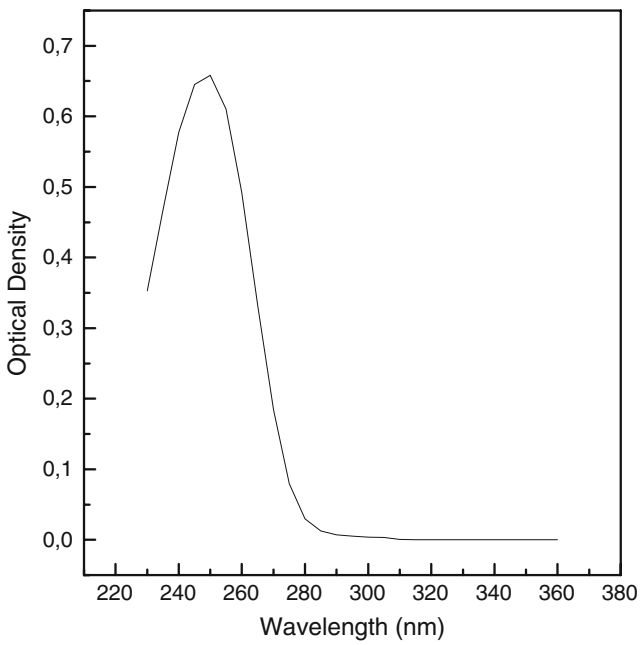

Fig. 3 Absorption spectrum of 240 μM progesterone in twice distilled ethanol measured with optical pathlength of 0.4 cm

All experiments were performed at 20°C.

Results

Figure 1 displays fluorescence emission spectrum of L-Tryptophan in water in absence (a) and presence of 650 µM progesterone (b). λ_{ex}=295 nm. Presence of progesterone at high concentration does not induce any significant decrease of tryptophan fluorescence intensity.

Figure 2 displays fluorescence excitation spectrum of L-tryptophan in water in absence (a) and presence of 650 µM progesterone (b) (λ_{em}=350 nm). In absence of progesterone, we observe a significant decrease in the intensity of the excitation spectrum, the peak of the recorded spectrum being at 290 nm. Subtracting spectrum (b) from (a) yields spectrum (c) with a peak around 255 nm. Spectra (b) and (c) are similar to the ^1L_b and ^1L_a transitions that characterize the two singlet states of Tryptophan [8]. Transition dipoles of these two states are oriented almost perpendicular to each other. Emission of tryptophan in solution and in most proteins is supposed to be unstructured and thus would take place from the ^1L_a state which is considered to be the first excited state S_1 [6]. Addition of progesterone to L-tryptophan in water inhibits completely the ^1L_a state and thus ^1L_b state would be the main responsible of tryptophan emission. At 295 nm, the ^1L_b state is preferentially selected since at this wavelength, the $S_o \rightarrow {}^1L_a$ transition is completely abolished.

Figure 3 shows absorption spectrum of 240 µM progesterone dissolved in ethanol. Progesterone absorbs mainly between 220 and 280 nm. Beyond this wavelength, hormone absorption is close to zero. Thus, high progesterone concentrations act as a filter of the ^1L_a state, excitation at wavelength equal to 295 nm would select ^1L_b state.

Figures 4 and 5 display fluorescence intensity decay curve (in Ln) of L-tryptophan in water in absence and in presence of 650 µM progesterone recorded at 310 nm. In

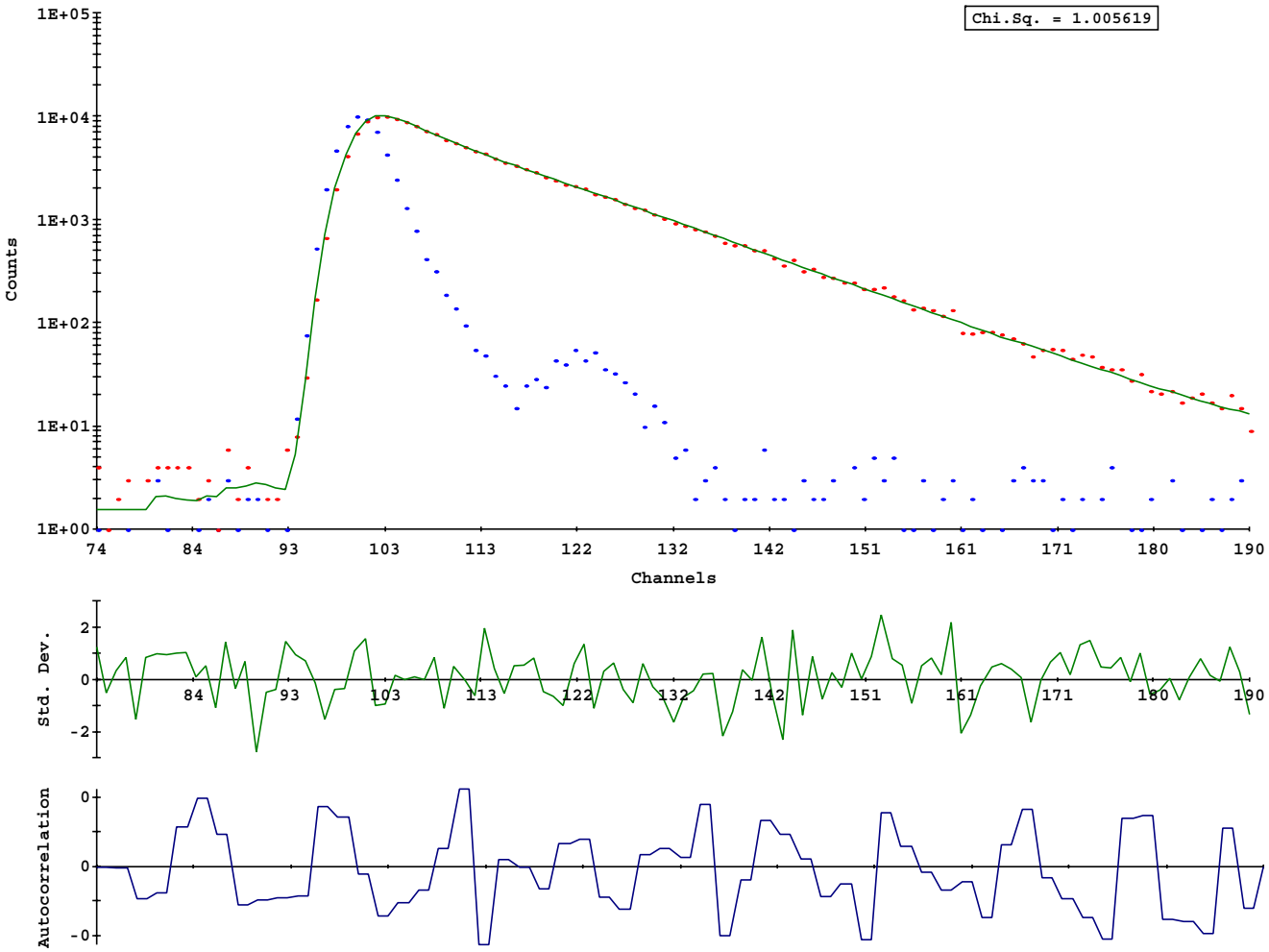

Fig. 4 Fluorescence intensity decay of L-tryptophan in deionised water. λ_{ex}=296 nm and λ_{em}=310 nm

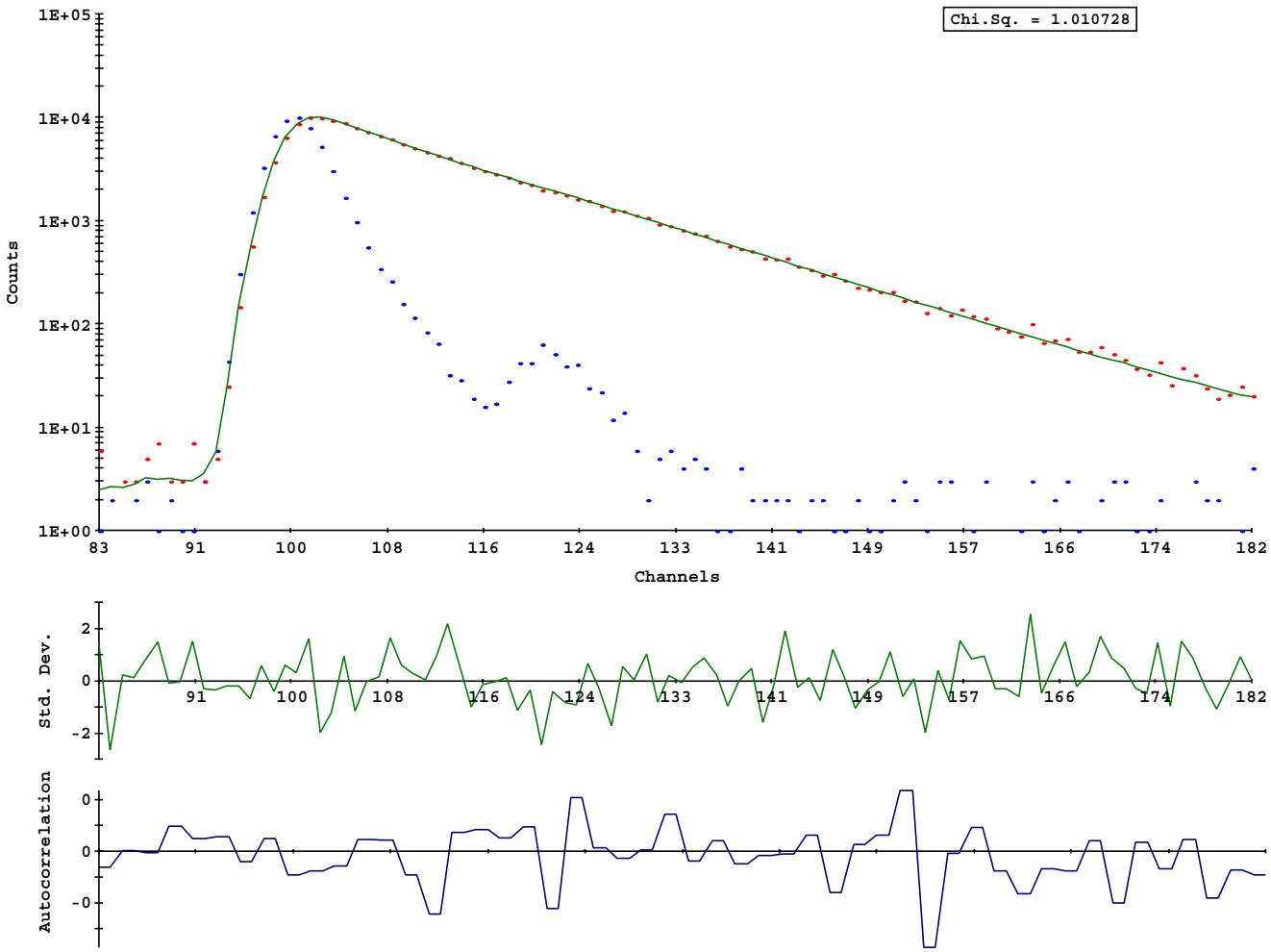

Fig. 5 Fluorescence intensity decay of L-tryptophan in deionised water in presence of 650 µM progesterone. λ_{ex}=296 nm and λ_{em}=310 nm

absence of progesterone, recorded fluorescence lifetimes were equal to 0.50386±0.05425 and 2.775±0.01158 ns (χ2= 1.0056) and in presence of progesterone, recorded lifetimes were equal to 0.46024±0.0330 and 2.752±0.0111 ns (χ2= 1.0107). We can notice that both short and long lifetimes do not vary significantly in presence of progesterone.

Figure 6 displays fluorescence lifetimes variation in water in absence and presence of progesterone along the emission wavelengths. We can notice that presence of progesterone does not modify any of the two lifetimes. Also, mean fluorescence lifetime is not affected by the presence of progesterone (Fig. 7).

Figure 8 shows pre-exponential lifetimes variation of tryptophan in water in absence and presence of progesterone. The results clearly indicate the absence of any effect of progesterone presence in solution on the pre-exponential values.

Time resolved emission spectra of tryptophan in water indicate that the two lifetimes contribute to the global emission spectrum of the fluorophore (Fig. 9). However, it is evident that contribution of the long fluorescence lifetime to the global emission spectrum is much more important than that of the short lifetime.

Figure 10 displays fluorescence excitation spectra of L-tryptophan in ethanol in absence and presence of 240 µM progesterone. λ_{max} of ^1L_a and of ^1L_b are equal to 265 and 282 nm, respectively. Thus, excitation at 295 nm in presence of high progesterone concentrations allows selecting the $S_o \rightarrow {}^1L_b$ transition only, the $S_o \rightarrow {}^1L_a$ transition being completely abolished by high progesterone absorption. Figures 11 and 12 display fluorescence intensity decay of L-Tryptophan in ethanol in absence and presence of 240 µM progesterone, respectively. In absence of progesterone, fluorescence intensity decay I(λ,t), of L-tryptophan can be adequately represented by a sum of three exponentials

$$I(\lambda, t) = 0.12385\, e^{-t/0.3475} + 0.79295\, e^{-t/1.768} + 0.0832\, e^{-t/4.77}$$

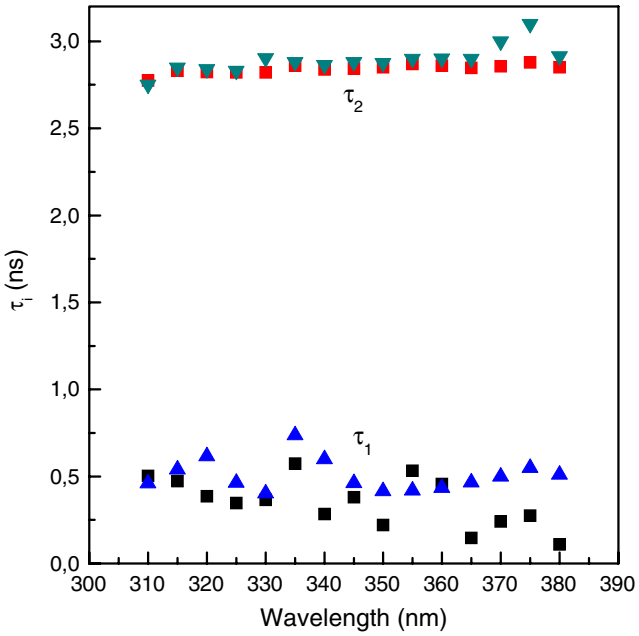

Fig. 6 Fluorescence lifetimes variation of L-tryptophan in deionised water with emission wavelength in absence (*squares*) and presence (*triangles*) of 650 μM progesterone. λ_{ex}=296 nm

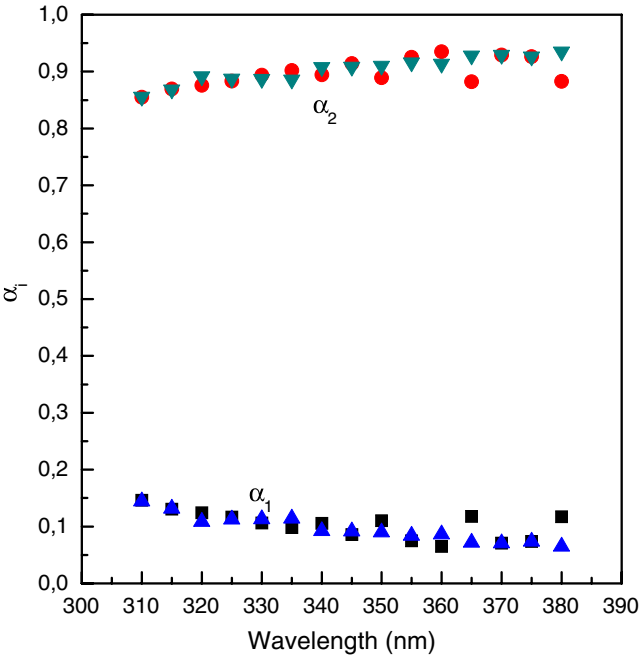

Fig. 8 Pre-exponential variation of L-tryptophan fluorescence lifetimes in deionised water in absence (*squares and circles*) and in presence of 650 μM progesterone (*triangles*). λ_{ex}=296 nm

where 0.12385, 0.70295 and 0.0832 are the pre-exponential factors, 0.3475±0.0305, 1.768±0.042 and 4.77±0.026 ns are the decay times and λ is the emission wavelength (315 nm) ($\chi 2$=1.073).

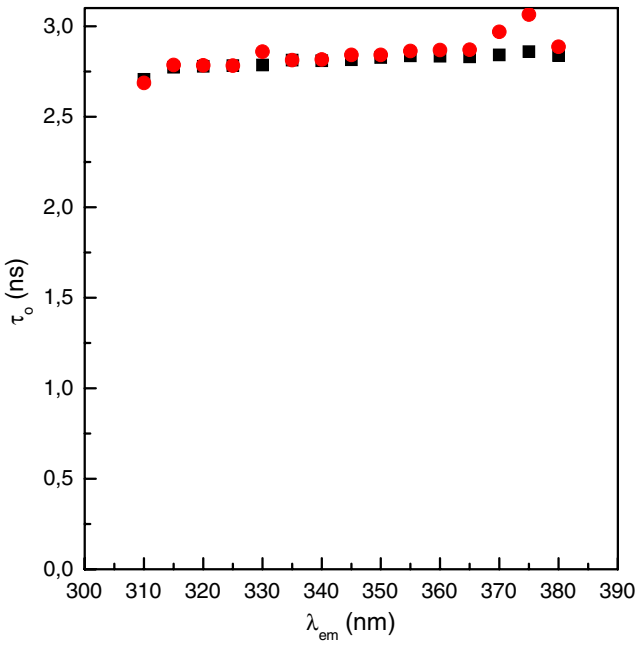

Fig. 7 Mean fluorescence lifetime variation of L-tryptophan in deionised water with emission wavelength in absence (*squares*) and presence (*circles*) of 650 μM progesterone. λ_{ex}=296 nm

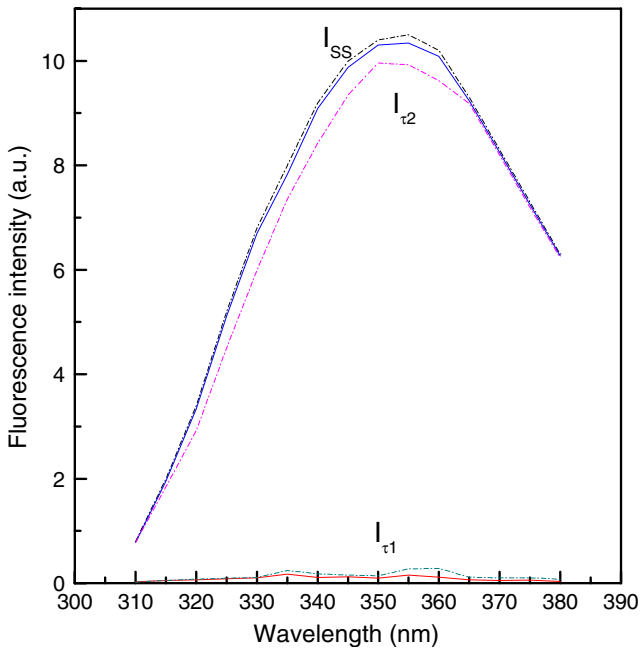

Fig. 9 Fluorescence emission spectra of the two lifetimes of L-Tryptophan in water in absence (*solid lines*) and in presence of 650 μM progesterone (*dashed lines*). Since progesterone presence does not affect significantly the recorded steady state spectrum (Spectrum I_{SS}), we have considered in the figure the emission spectra recorded in absence and presence of progesterone as of equal intensities

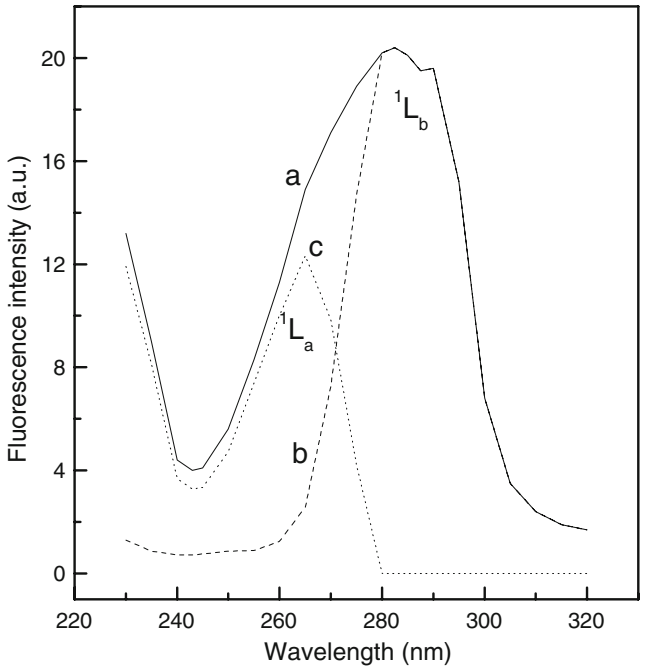

Fig. 10 Fluorescence excitation spectra of L-tryptophan dissolved in distilled ethanol in absence (spectrum a) and in presence of 240 μM progesterone (spectrum b). Excitation spectrum obtained in presence of progesterone corresponds to the $S_o \rightarrow {}^1L_b$ transition. Spectrum c which corresponds to the $S_o \rightarrow {}^1L_a$ transition is obtained by subtracting (b) from (a). λ_{max} of ^1L_a and of ^1L_b are equal to 265 and 282 nm, respectively

In presence of 240 μM progesterone, fluorescence intensity decay I(λ,t) of L-tryptophan can be adequately represented also by a sum of three exponentials

$$I(\lambda, t) = 0.1398\, e^{-t/0.378} + 0.7719\, e^{-t/1.81} + 0.0883\, e^{-t/4.796}$$

where 0.1398, 0.7719 and 0.0883 are the pre-exponential factors, 0.378±0.0402, 1.81±0.065 and 4.796±0.035 ns are the decay times and λ is the emission wavelength (315 nm) ($\chi 2 = 1.014$).

The measurements indicate that addition of progesterone to a solution of L-tryptophan–ethanol does not affect any of the fluorescence decay parameters (lifetimes and pre-exponentials). In absence and presence of 240 μM progesterone, analysing fluorescence decays in ethanol with two lifetimes increases the value of χ2 at all the emission wavelengths (Tables 1 and 2). The same result was also obtained in water when fluorescence intensity decays were analysed with three lifetimes instead of two (not shown).

Figure 13 displays lifetimes variation along emission wavelengths of L-tryptophan dissolved in ethanol in absence and presence of 240 μM progesterone. We notice that the three lifetimes and the mean one (τ_o) are not affected by the presence of progesterone. Also, pre-exponential values of the three lifetimes are not affected by the presence of progesterone as it is clearly indicated in Figs. 14 and 15. The fact that progesterone presence abolishes completely the $S_o \rightarrow {}^1L_a$ transition without modifying any of the fluorescence lifetimes and their pre-exponentials values indicate that the three lifetimes result from excited state induced by the $S_o \rightarrow {}^1L_b$ transition.

Figure 16 displays time resolved emission spectra of the three lifetimes in the absence and presence of 250 μM progesterone. We can notice that the two longest lifetimes are the main contributors to tryptophan emission spectrum whether progesterone is absent or present in solution.

Discussion

It is important here to remind that although progesterone concentrations are important in the fluorescence cuvettes, we added only small volumes (11 μl to the 1 ml tryptophan solution in water and 4 μl to the 1 ml tryptophan solution in ethanol) of progesterone stock solution so that to avoid interference, if any, of added volumes. Tryptophan free in water emits with two lifetimes equal to 0.5 and 2.78 ns. Tryptophan absorbs with two transitions $S_o \rightarrow {}^1L_a$ and $S_o \rightarrow {}^1L_b$. Our data clearly rule out the axiom which considers that in polar solvents, emission occurs principally from ^1L_a state. Also, our results rule out the fact that the 0.5 ns would be the result of the $S_o \rightarrow {}^1L_b$ transition while the 2.78 ns would be induced by the $S_o \rightarrow {}^1L_a$ transition.

Progesterone does not bind to tryptophan free in solution although its presence at high concentrations abolishes completely the ^1L_a state. Thus, upon excitation at 295 nm and in presence of high progesterone concentrations, the ^1L_b state is the primary contribution to tryptophan emission.

In presence of high progesterone concentrations and thus in the absence of the $S_o \rightarrow {}^1L_a$ transition, two fluorescence lifetimes are still observed, 0.46 and 2.75 ns, values equal to those measured in the absence of progesterone. Also, presence of the hormone in solution does not affect lifetimes pre-exponential values which characterize the contribution of each lifetime to the global emission decay. Therefore, in a first approximation, one could conclude that $S_o \rightarrow {}^1L_b$ transition is the main transition for tryptophan excitation. However, in the absence of experiments on the $S_o \rightarrow {}^1L_a$ transition alone, it is more serious to say that combination of both transition states induce the excited state of tryptophan and the reorganization of the fluorophore into two interrelated structures. The 0.5 and 2.75 ns lifetimes are associated to these two structures. Finally, it is clear from our data that we cannot assign a specific fluorescence lifetime to each of ^1L_a and ^1L_b states.

Dissolved in ethanol, tryptophan emits with three lifetimes 0.3475, 1.768 and 4.77 ns. Thus, tryptophan in ethanol would adopt in the excited state sub-structures

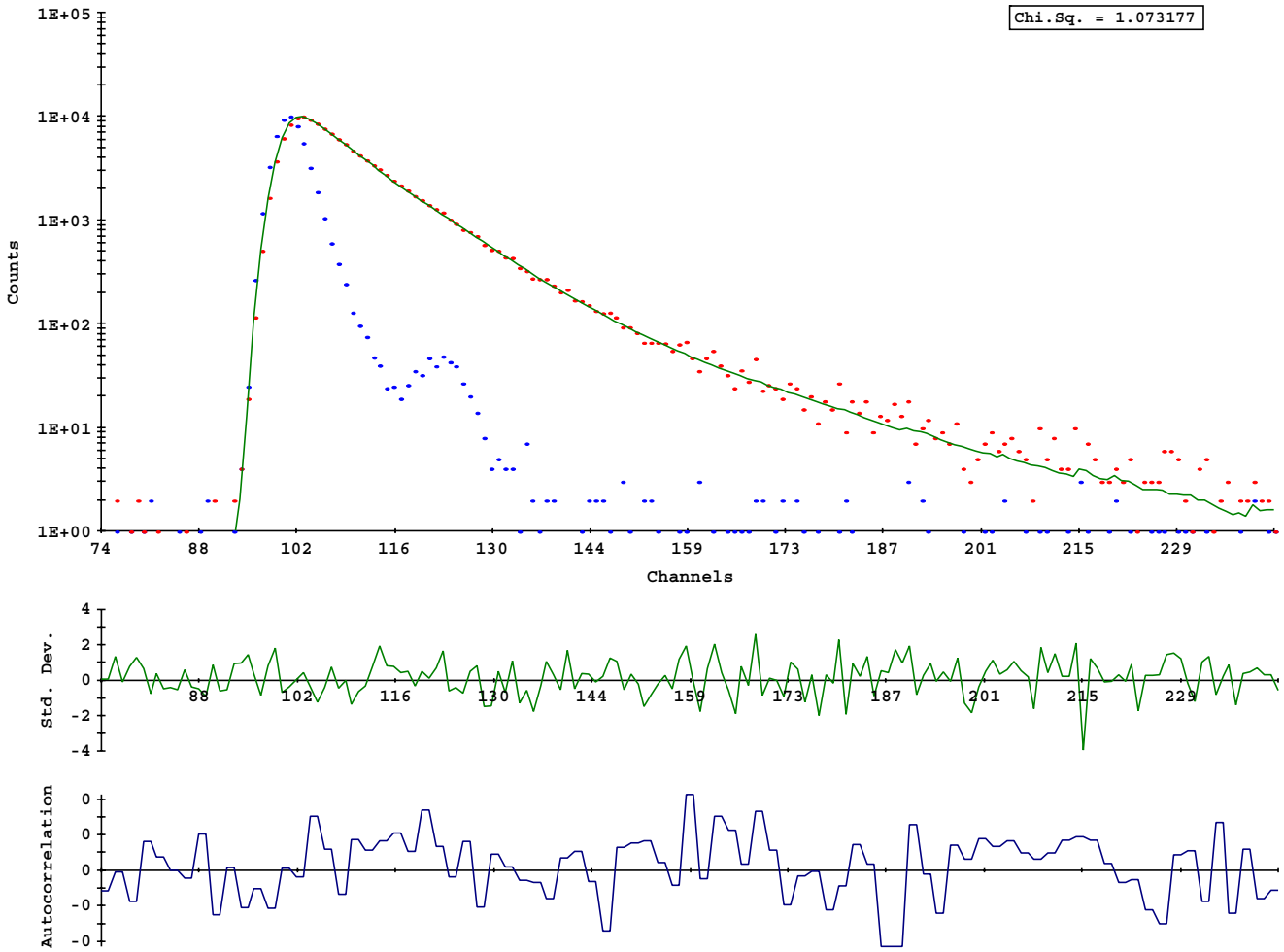

Fig. 11 Fluorescence intensity decay of L-tryptophan in distilled ethanol. λ_{ex}=296 nm and λ_{em}=315 nm

different from those observed in water. Three lifetimes have already been observed for tryptophan dissolved in non aqueous solvents [9]. Addition of progesterone at high concentrations abolishes $S_o \rightarrow {}^1L_a$ transition, as it is revealed from the excitation spectrum, without affecting fluorescence lifetimes or their pre-exponential values. Therefore, the three lifetimes observed in ethanol would be generated by the $S_o \rightarrow {}^1L_b$ transition. If this is the case, what would be the role of the $S_o \rightarrow {}^1L_a$ transition in the excitation process? Also, on the light of our data, an important question could be asked. What is the real relation between the two transitions and the fluorescence lifetimes observed in solution (two lifetimes in water and three lifetimes in ethanol)? In other words, is there any correlation between the two transitions and the fluorescence lifetimes? Excitation spectrum characterizes electrons distribution within a molecule at the ground state while fluorescence lifetimes occur from the excited state and thus reflect electron distribution within the molecule in this state. Therefore, possibility that fluorescence lifetimes are not directly correlated with any of the two transitions is not to be excluded. In fact, we notice that while high progesterone concentrations abolish $S_o \rightarrow {}^1L_a$ transition, fluorescence lifetimes are not modified. Also, this could simply mean that excitation of tryptophan molecules in solution induces an excited state where fluorophore molecules retain two or different conformations different from those observed in the ground state. In this case, rotamers model as it was suggested does not fit well with our data. In fact, rotamers are considered to be static structures, within the nanosecond time scale, existing in the ground state and responsible, in the excited state, of the tryptophan emission. Thus, this model excludes any conformational change within the tryptophan molecules when they reach the excited state. Rotamers model was described for polypeptides in order to explain the multi-exponential decay of tryptophan in these macromolecules. Then, the same model was extended to describe the bi-exponential decay of tryptophan free in water. In polypeptides, lifetime of each rotamer is explained as the result of the quenching interactions between indole and quenchers groups. Charge transfer from the excited indole moiety to the carbonyl group of the peptide bond,

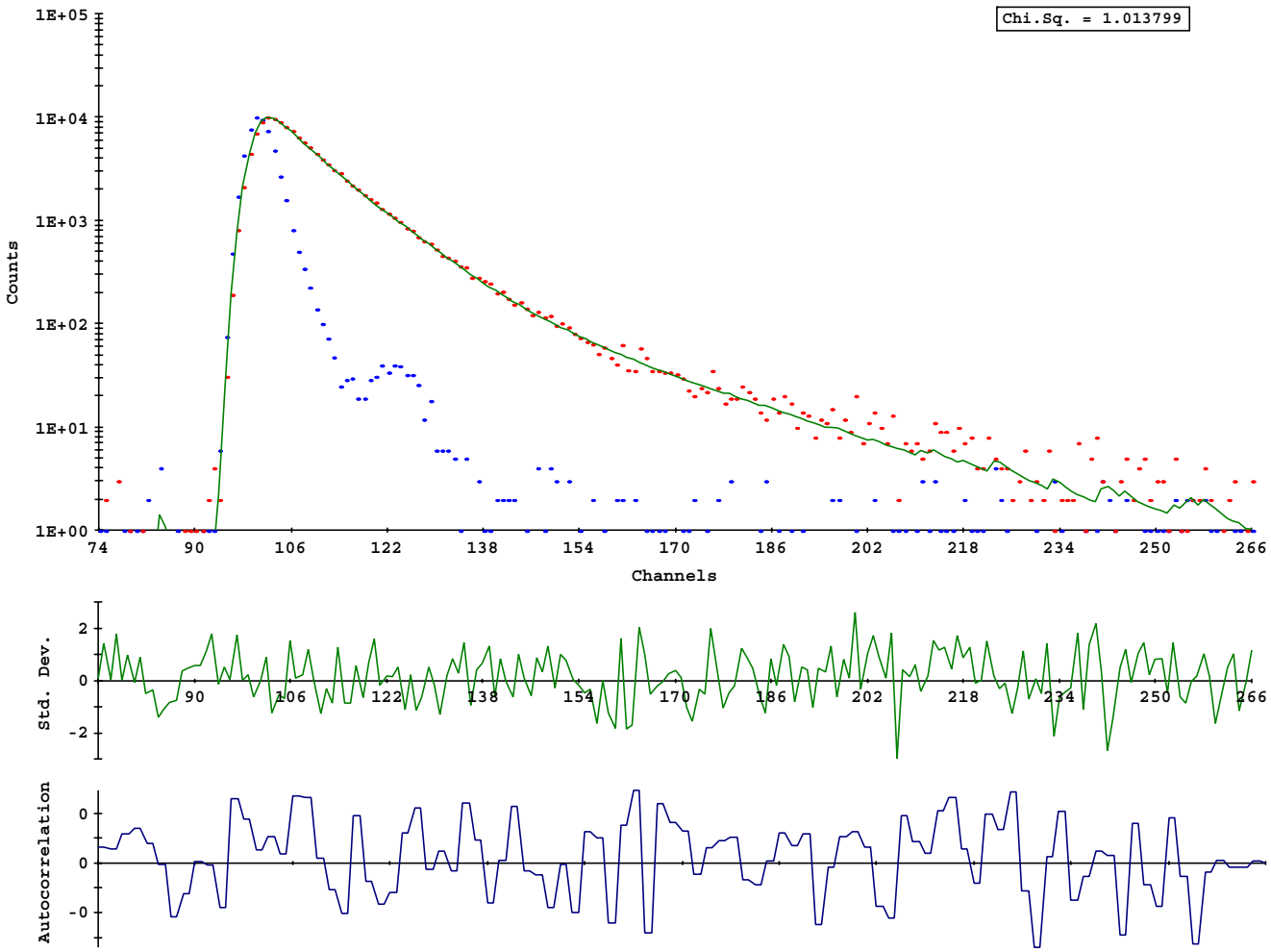

Fig. 12 Fluorescence intensity decay of L-tryptophan in distilled ethanol in presence of 240 μM progesterone. λ_{ex}=296 nm and λ_{em}=315 nm

excited state electron and proton transfer and solvent-quenching are described to play a role in the deexcitation process of tryptophan fluorescence [10–13]. But we do not see how and why the rotamers model, if it is correct, can be applied to free tryptophan in solution.

What is intriguing is the fact that the two lifetimes measured for free tryptophan in water (≈ 0.5 and 2.5 ns) are found in almost all proteins [14]. Thus, these two values are in a certain way independent of the surrounding environment of tryptophan, revealing that they characterize intrinsic properties of the fluorophore, i.e. specific substructures reached formed upon excitation. These substructures differ slightly with the environment of the tryptophan modifying the values of the two lifetimes and of their corresponding amplitudes. In ethanol, the three lifetimes are generated by the presence of three substructures induced by an environment (ethanol) which chemical and physical properties differ from those of water. The same analysis can be drawn for Trp residues in proteins where the fluorophore is, in the excited state, within substructures identical or very close to those observed in ethanol and affected by their surrounding environment. The major problem with the rotamers model is the fact that one considers that the tryptophan residue displays the same conformation in both, ground and excited states, a postulate that is not correct. Also, another problem with this model is its extrapolation to free tryptophan in solution, something non-coherent since in solution tryptophan does not have the same structural environment as in polypeptides (absence of peptide backbone,etc…) This is why it is more logical in a first step to correlate fluorescence lifetime of tryptophan with its main structure or substructures in the excited state, independently of the surrounding environment. Then, in a second step, modifications of the fluorescence decay parameters (lifetimes and pre-exponentials) should be analyzed as the effect of the environment on the sub-structures of the fluorophore in the excited state.

This latest suggestion is in good agreement with the fact that although N-acetyl-L-tryptophanamide (NATA) and nonterminal tryptophan residues in proteins display the

Table 1 Value of χ^2 obtained for L-tryptophan decay in ethanol and analysed with two and three fluorescence lifetimes

Wavelength	$\chi^2(3\ \tau)$	$\chi^2(2\ \tau)$
310	1.008	2.638
315	1.129	2
320	1.096	1.645
325	1.036	1.509
330	1.070	1.303
335	0.952	1.421
340	1.166	1.484
345	0.94	1.317
350	0.896	1.448
355	0.91	1.324
360	1.29	1.785
365	0.972	1.32
370	0.968	1.45
375	1.02	1.29
380	1.112	1.65
385	1.152	1.55
390	1.076	1.612
395	1.12	1.54

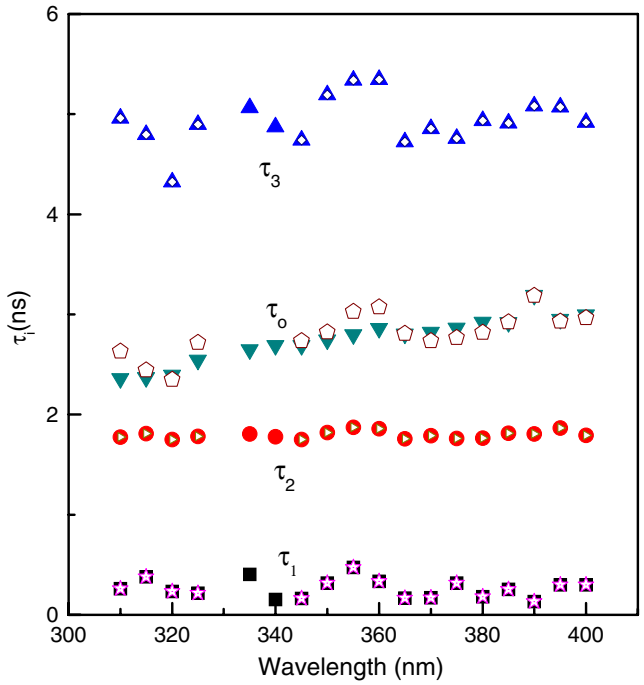

Fig. 13 Lifetimes variation of L-Trp in ethanol with emission wavelengths in absence (closed symbols) and in presence of 240 μM progesterone (open symbols) λ_{ex}=296 nm

same structures in the ground state, NATA emits with a single fluorescence lifetime while tryptophan residues emission is bi or multiexponential. This clearly means that the structures that are responsible of these lifetimes differ. Therefore, lifetimes characterize substructures formed in the excited state and not in the ground state, otherwise both molecules, NATA and tryptophan residues, should yield same lifetime values and numbers. Also, structures of NATA and tryptophan differ and if tryptophan residues in polypeptides have two lifetimes very close to those

Table 2 Value of χ^2 obtained for L-tryptophan decay in ethanol in presence of 240 μM progesterone and analysed with two and three fluorescence lifetimes

Wavelength	$\chi^2(3\ \tau)$	$\chi^2(2\ \tau)$
310	1.065	2.54
315	1.073	2.02
320	1.184	1.88
325	1.058	1.703
345	1.173	1.621
350	0.886	1.33
355	1.009	1.356
360	0.86	1.338
365	1.124	1.581
370	1.019	1.398
375	1.161	1.427
380	1.083	1.619
385	1.159	1.58
390	1.009	1.457
395	0.945	1.313
400	1.06	1.254

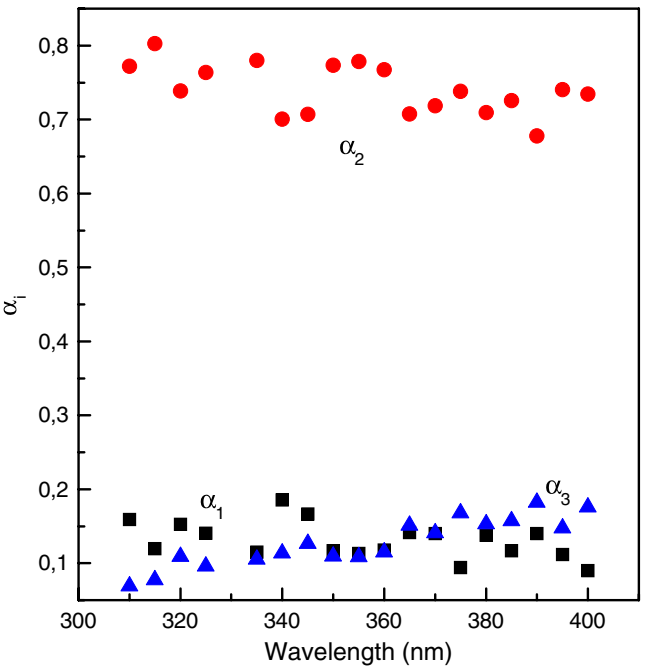

Fig. 14 Pre-exponential variation of Trp lifetimes in ethanol with emission wavelengths. λ_{ex}=296 nm

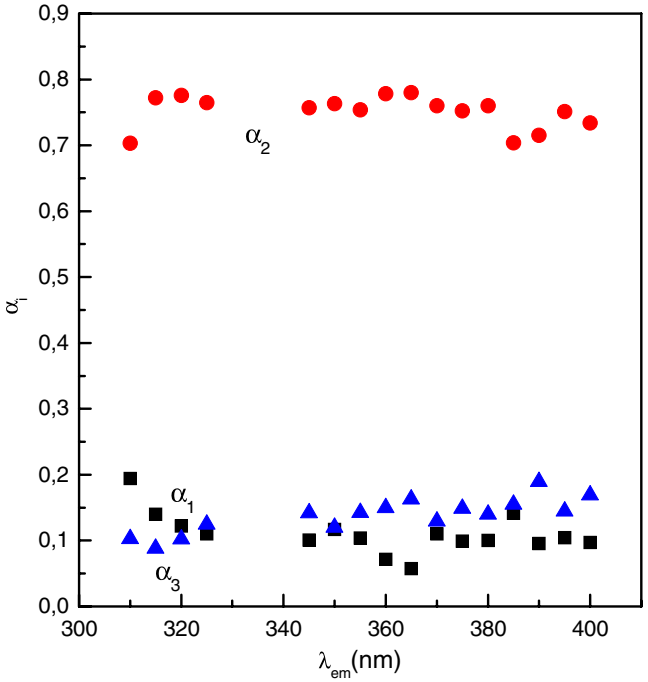

Fig. 15 Pre-exponential variation of Trp lifetimes in ethanol with emission wavelengths, in presence of progesterone. λ_{ex}=296 nm

observed for free tryptophan in solution, this can be explained by the fact that these lifetimes are generated by specific molecule within specific sub-structures.

In this case, what is the purpose of comparing NATA and tryptophan residues fluorescence? In a recent work, Broos et al [15] compared fluorescence excitation anisotropy spectra of NATA and azurin in propyleneglycol. The authors found similar spectra with a trough at 291 nm and a peak at 285 nm. Since NATA in a polar solvent emits from the ^1L_a transition, the authors concluded that emission of azurin occurs also from the same transition. However, when we look to the excitation polarization spectrum of indole already published [8, 16]. We found that ^1L_b transition displays two peaks at 289 and 281 nm and a trough at 285 nm. Thus, spectrum shape of indole does not match with that found by Broos et al. and with that of NATA. Finally, it is important to indicate that azurin emission is multiexponential [17] and thus we do not see how it can be compared to NATA. This is a second example showing that NATA cannot be used as a reference to understand origin of tryptophan fluorescence in proteins. Comparison should be made only with free tryptophan in solution (water, ethanol, etc…).

In a recent work performed on 6,p-toluidinyl-naphthalene-2-sulfonate (TNS), we showed that its fluorescence decay observables (lifetime and pre-exponentials) and excitation and emission spectra are specific to (1) sub-structures of the fluorophore within the studied environment, (2) interactions that exist between the fluorophore molecules themselves and (3) their interactions with the environvenment [18]. In fact, by recording the fluorescence spectra and measuring fluorescence lifetimes of TNS dissolved in ethanol, aggregated at high concentration in water and bound to BSA, we fluorescence lifetimes of TNS do not yield the same information as fluorescence spectra. Excitation spectrum characterizes the state of the fluorophore in the medium (aggregated, free in solution or bound to the protein), while fluorescence lifetimes depend on the interaction that exists between the fluorophore molecules themselves and of the different substructures of the fluorophore in the excited state. Therefore, when dealing with fluorescence measurements, at least two parameters should be taken into account, the choice of the best fluorophore to be used as a reference and the meaning of the measured observables. Our data on TNS [18] and tryptophan (this work) show that lifetimes and spectra do not yield the same information.

The results described in the present work clearly reveals that tryptophan lifetimes do not depend solely and necessary on both $S_o \rightarrow {}^1L_a$ and / or $S_o \rightarrow {}^1L_b$ transitions but they can be observed even in presence of $S_o \rightarrow {}^1L_b$ transition only. Therefore, emission lifetimes are not necessarily correlated to specific tryptophan structures observed in the ground state.

In conclusion, this work describes for the first time a method that allows separating the $S_o \rightarrow {}^1L_a$ and the $S_o \rightarrow {}^1L_b$ transitions. Also, we found that $S_o \rightarrow {}^1L_b$ transition alone allows exciting the tryptophan molecules whether in

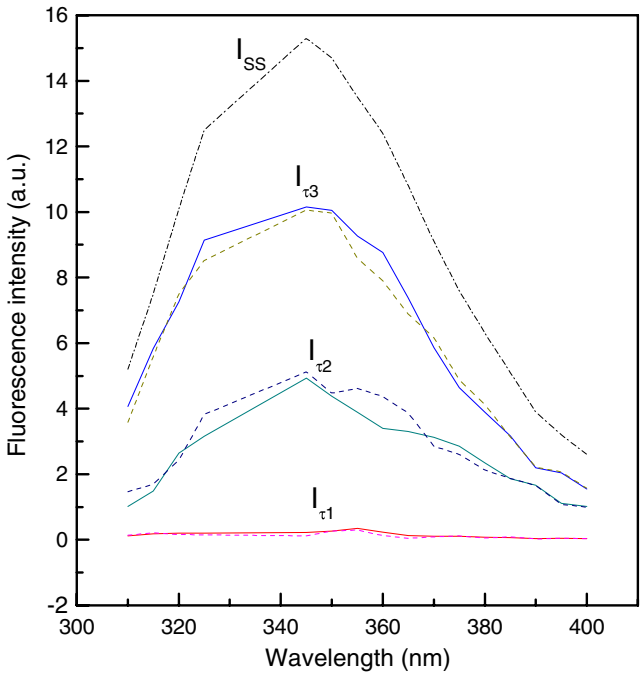

Fig. 16 Emission spectra of lifetimes of L-Trp in ethanol in absence (*full lines*) and presence (*dashed lines*) of progesterone. λ_{ex}=296 nm. Addition of ethanol does not affect the steady-state spectrum

water or ethanol inducing fluorescence lifetimes equal to those measured in presence of the two transitions. Experiments are now undertaken so that we find out whether there is any correlation between tryptophan ground state and fluorescence lifetimes.

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