J Fluoresc (2014) 24:93–104
DOI 10.1007/s10895-013-1277-8

ORIGINAL PAPER

Origin of Tryptophan Fluorescence Lifetimes Part 1. Fluorescence Lifetimes Origin of Tryptophan Free in Solution

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Received: 21 March 2013 / Accepted: 22 May 2013 / Published online: 4 August 2013

Abstract Fluorescence intensity decays of L-tryptophan free in polar, hydrophobic and mixture of polar-hydrophobic solvents were recorded along the emission spectrum (310–410 nm). Analysis of the data show that emission of tryptophan occurs with two lifetimes in 100 % polar and hydrophobic environments. The values of the two lifetimes are not the same in both environments while their populations (pre-exponentials values) are identical. Fluorescence lifetimes and pre-exponentials values do not change with the excitation wavelength and thus are independent of excitation energy. Our results indicate that tryptophan emission occurs from two specific sub-structures existing in the excited state. These sub-structures differ from those present in the ground states and characterize an internal property and/or organization of the tryptophan structure in the excited state. By sub-substructure, we mean here tryptophan backbone and its electronic cloud. In ethanol, three fluorescence lifetimes were measured; two lifetimes are very close to those observed in water (0.4–0.5 ns and 2–4 ns). Presence of a third lifetime for tryptophan in ethanol results from the interaction of both hydrophobic and hydrophilic dipoles or chemical functions of ethanol with the fluorophore.

Keywords Tryptophan · Fluorescence lifetimes · Lifetimes pre-exponentials · Hydrophilic solvent · Hydrophobic solvent · Sub-structures at the excitation state

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Introduction

Tryptophan possesses specific fluorescence properties, which are intrinsic. These properties (anisotropy, emission and excitation spectra, lifetimes) can be measured and characterized. Also, tryptophan fluorescence properties are modified depending on its environment. For example, when a tryptophan is buried inside the hydrophobic core of a protein, its fluorescence is blue-shifted compared to the fluorescence observed from a tryptophan present at the protein surface. Also, anisotropy is low when fluorophore motions are important and high when motions are slow. Still, one main problem exists and which is the origin of bi or multiexponential decay of tryptophan in solution and in peptides and proteins. A folded protein could have a set of different conformations, thus we have here a first definition of a protein structure: the global structure is a combination of sub-structures or conformations. The interconversion between them is not too fast. Each conformation is rigid and has a definite specific structure. This model is known as the rotamers model. Many authors attributed these bi and multiexponential decays to the presence of conformers or rotamers in equilibrium in the folded state. Each conformer exhibits one specific fluorescence lifetime. In polypeptides, lifetime of each rotamer is explained as the result of the quenching interactions between the indole and quenching groups in the fluorophore. Charge transfer from excited indole moiety to the carbonyl group of the peptide bond, excited state electron, proton transfer and solvent-quenching are described to play a role in the deexcitation process of tryptophan fluorescence [1–4]. However, no explanation is given why two lifetimes are always found the same (0.4–0.5 ns and 2–4 ns) in almost all studied proteins independently of the number of tryptophan residues and of their positions in the proteins. These two values are similar to those found for free tryptophan in solution [5–10].

We recently published results on fluorescence lifetimes measurements of tryptophan dissolved in ethanol [11]. We found that intensity decays can be analyzed with three fluorescence lifetimes. Two lifetimes are common to water and proteins (0.4–0.5 ns and 2–4 ns). However, pre-exponential factors of the three fluorescence lifetimes differ from those observed in proteins. Therefore, nature of interaction between tryptophan and its environment (ethanol and protein matrix) is not necessarily the same.

In the first part of this work, we present experimental data allowing to identify fluorescence lifetimes origin of free tryptophan in solution, while in the second part, the displayed data allow to explain origin of multiexponential fluorescence intensity decay in peptides and proteins.

Materials and Methods

L-Tryptophan was from Sigma and was used as received. The fluorophore was dissolved in deionised water, phosphate buffer or in ethanol (Sigma). CCl_4 and $CHCl_3$ were purchased from Sigma.

Absorbance data were obtained with a Shimadzu MPS-2000 double-beam spectrophotometer (Shimadzu, Champs Sur Marne, France) using 1-cm pathlength cuvettes. Steady state fluorescence spectra were recorded with a Perkin-Elmer LS-5B spectrofluorometer (PerkinElmer, Courtaboeuf, France). Bandwidths used for excitation and emission were 5 nm. The quartz cuvettes had optical pathlengths equal to 1 and 0.4 cm for emission and excitation wavelengths, respectively. Observed fluorescence intensities were corrected for absorption as described [12, 13]. Finally, fluorescence spectra were corrected for the background intensities of the buffer solution.

Fluorescence lifetime measurements were obtained with a Horiba Jobin Yvon FluoroMax-4P (Horiba Jobin Yvon, Longjumeau, France) using the time correlated single photon counting method. A Ludox solution was used as scatter. Excitation was performed at 296 nm with a nanoLED. Each fluorescence decay was analyzed with one, two and three lifetimes and then values of $\chi 2$ 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. Distribution of arrival times represents the decay curve called also 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), called also 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 [12, 14, 15]

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

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

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

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 $R_c(t)$ are 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 analysed with one, two or more lifetimes, values of χ^2 have to be compared. For example, we shall 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 [16].

Results

Fluorescence Observables of L-Tryptophan in Buffer

Figure 1 displays excitation and emission spectra of L-Trp dissolved in 10 mM phosphate buffer at pH 2, 7 and 12. One can notice that intensities of excitation spectra increase with

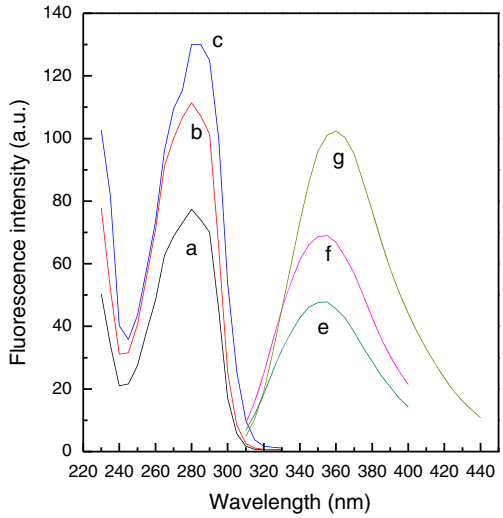

Fig. 1 Fluorescence excitation (λ_{em}=360 nm) and emission spectra (λ_{ex}=295 nm) of L-Trp in pH 2 (**a** and **e**), pH 7 (**b** and **f**) and pH 12 (**c** and **g**)

pH without any modification of the excitation peak position (280 nm). Thus, presence of hydrogen on the tryptophan seems to quench amino acid fluorescence excitation intensity. Tryptophan electronic distribution in the ground state differs between protonated (low pH) and non-protonated (high pH) states. Intensities of emission spectra are proportional to those of the excitation wavelength (here 295 nm) as we can observe in spectra e, f and g, and not necessarily on whether ground state is protonated or not. In fact, excitation at 260 nm yields emission spectra with the same peak intensities for pH 7 and 12 and higher than emission spectrum recorded at pH 2 (data not shown). This result is in good agreement with the fact that fluorescence emission intensity of a fluorophore is proportional to the intensity at the excitation wavelength [7].

Figure 2 exhibits fluorescence intensity decay of L-Trp dissolved in 10 mM phosphate buffer, pH 2 and recorded at 330 nm. Decay analysis shows that the fit can be best described with two lifetimes equal to 0.5 and 2 ns with amplitudes equal to 0.2 and 0.8, respectively.

Table 1 displays χ^2 values of L-Trp fluorescence intensity decay at pH 2, 7 and 12. One can notice that there is no

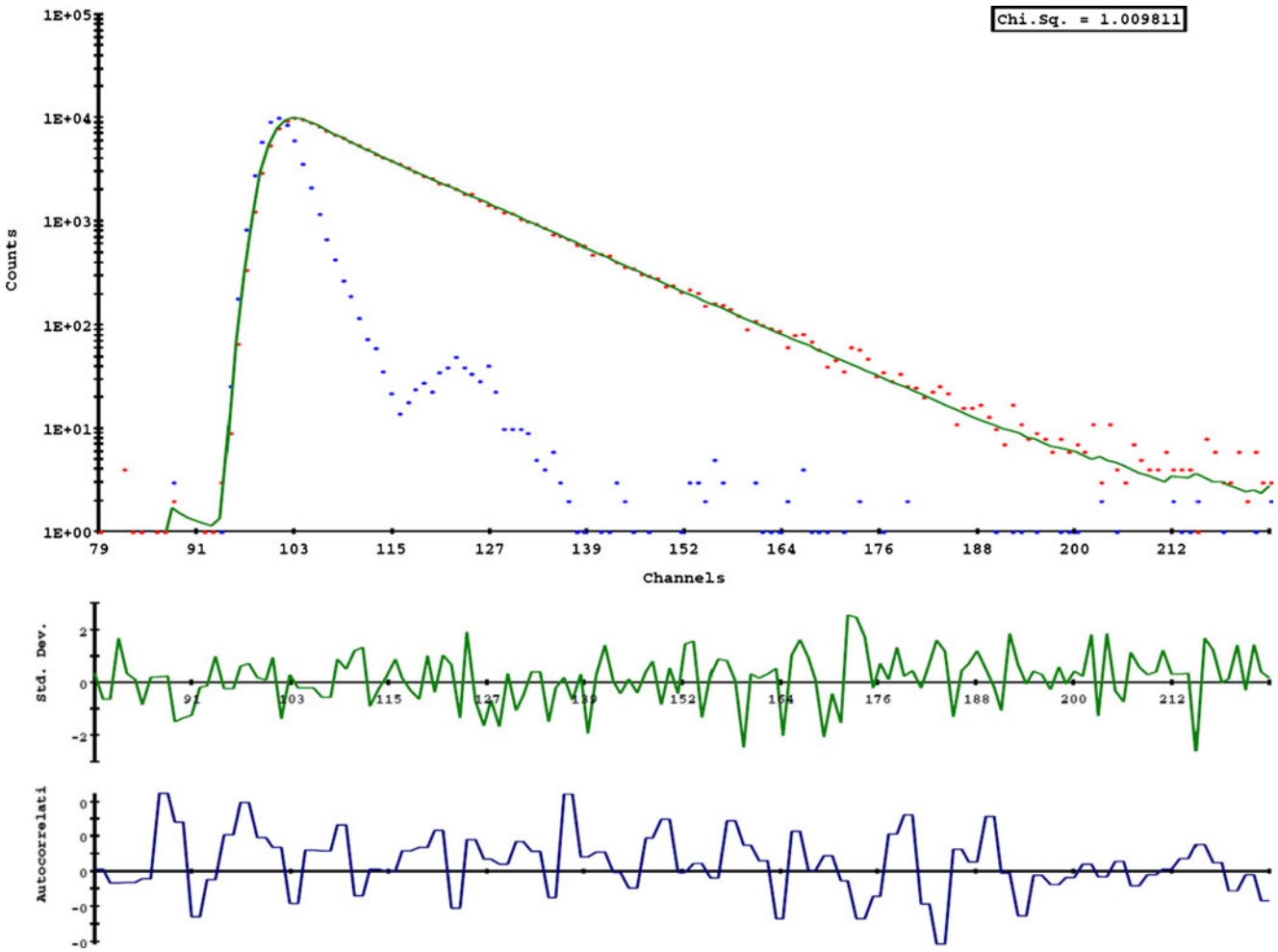

Fig. 2 Fluorescence intensity decay of L-Trp in pH 2, 10 mM phosphate buffer observed at 330 nm. λ_{ex}=296 nm

improvement in χ^2 values when analyses were done with three lifetimes, compared to those performed with two lifetimes. Therefore, fluorescence intensity decay of L-Trp in buffer at different pH can be best described with two fluorescence lifetimes. Our results are in good agreement with those already published [1, 7, 11, 17–22].

Figure 3 shows that value of the longest lifetime increases with pH, although it remains stable along emission wavelength. Thus, protonation of L-Trp decreases fluorescence lifetimes values, a phenomenon identical to that observed with fluorescence excitation intensities. Thus, we have a correlation between tryptophan protonation and fluorescence excitation intensity and lifetimes values. Figure 4 displays values of pre-exponentials of both fluorescence lifetimes. Population of longest fluorescence lifetime is much more important than that of shorter one and increases with wavelength. This increase is more important at pH 2 (protonated state) than at pH 7 or pH 12. Nevertheless, global shape of the increase is similar at pH 2 and 7. Only at pH 12, populations are stable and do not vary significantly with wavelength. At this pH, population of longest lifetime is almost equal to 100 %. Since populations of both fluorescence lifetimes are not reversed with pH, they characterized two sub-structures of L-tryptophan specific to the excited state. In fact, emission occurs from an excited state.

Table 1 Values of χ^2 obtained for L-tryptophan decay in 10 mM phosphate buffer pH 2, 7 and 12 and analyzed with two and three fluorescence lifetimes

	pH 2		pH 7		pH 12	
Wavelength	$\chi^2(2\tau)$	$\chi^2(3\tau)$	$\chi^2(2\tau)$	$\chi^2(3\tau)$	$\chi^2(2\tau)$	$\chi^2(3\tau)$
310	1.006	0.824	1.086	1.099		
315	1.096	1.111	0.959	0.969	1.23	1.126
320	0.996	1.011	1.163	1.158	1.13	1.147
325	1.043	1.032	1.005	1.02	0.877	0.877
330	1.194	1.19	1.264	1.277	1.061	1.058
335	1.147	1.109	1.029	1.011	1.051	1.022
340	1.069	1.033	0.845	0.856	1.056	1.048
345	1.061	1.071	1.048	1.048	0.929	0.936
350	1.028	1.03	1.074	1.091	1.153	1.163
355	0.827	0.834	0.792	0.782	1.13	1.099
360	1.002	1.004	1.02	0.996	1.274	1.297
365	0.95	0.966	1.074	1.087	0.913	0.894
370	1.014	0.654	0.951	0.957	0.967	0.927
375	1.02	1.028	1.151	1.157	1.012	1.013
380	1.149	1.169	1.21	1.218	0.982	0.988
385	0.916	0.926	0.973	0.984	0.994	1.007
390	1.1	1.11	1.09	1.106	1.168	1.174
395	1.012	0.988	1.12	1.133	0.752	0.758
400	0.948	0.962	1.076	1.094		

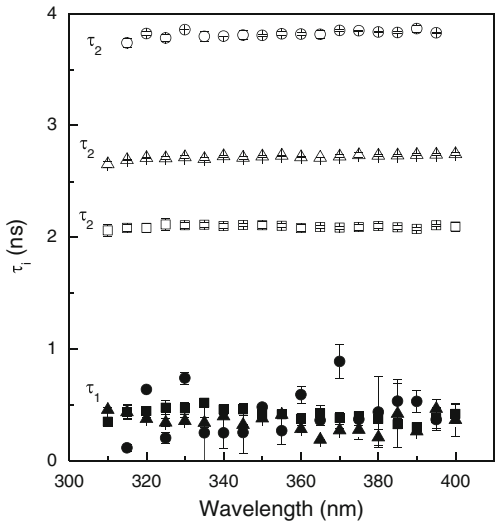

Fig. 3 Lifetimes values of L-Trp with emission wavelength recorded at pH 2 (*squares*), 7 (*triangles*) and 12 (*circles*). λ_{ex}=296 nm

Figure 5 displays mean fluorescence lifetime variation of L-tryptophan at the three pH. Deprotonation of fluorophore increases mean lifetime as the result of the increase of the longest fluorescence lifetime and its population. One can see from these results that mean lifetimes differ between the three pH.

Fluorescence Lifetimes of L-Tryptophan in Ethanol

Table 2 displays χ^2 values of L-tryptophan fluorescence intensity decay in pure ethanol, analysis of the decay was done with two and three fluorescence lifetimes. One can notice that analysis with three lifetimes yields a value of χ^2 closer to 1 than when analysis was performed with two lifetimes. Thus, fluorescence intensity decay of L-tryptophan in ethanol is best

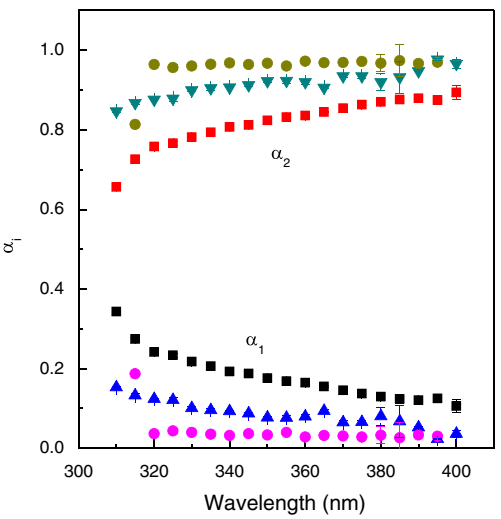

Fig. 4 Fluorescence lifetimes pre-exponential values of L-Trp in pH 2 (*circles*), 7 (*triangles*) and 12 (*circles*). λ_{ex}=296 nm

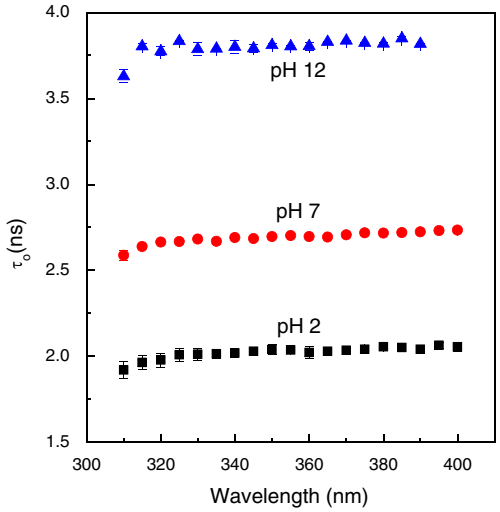

Fig. 5 Mean lifetime variation with emission wavelength of L-Trp in pH 2, 7 and 12. Results are from 2 experiments. λ_{ex}=296 nm

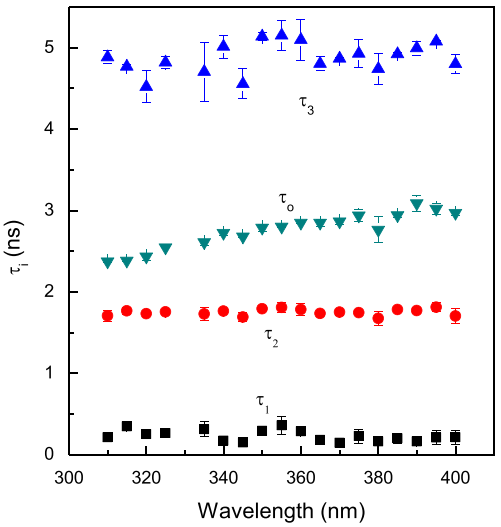

Fig. 6 Lifetimes variation of L-Trp in ethanol with emission wavelengths. λ_{ex}=296 nm. Set of two experiments.

analysed with three lifetimes. Three lifetimes were also obtained when tryptophan fluorescence was measured in methanol [17].

At 315 nm, fluorescence intensity decay can be adequately represented as

$$I\ [\lambda, t] = 0.124\ e^{-t/0.347} + 0.793\ e^{-t/1.768} + 0.083\ e^{-t/4.77}$$

where 0.124, 0.793 and 0.083 are the preexponential factors and 0.347, 1.768 and 4.77 ns the decay times (χ2=1.073).

Figure 6 displays values of the three fluorescence lifetimes and mean one along emission wavelengths. One can notice that lifetimes do not vary with emission wavelength. The two shorter lifetimes are very close to those found for L-Trp in water or buffer. Thus, origin of these two lifetimes is the same whether tryptophan is dissolved in pure polar solvent or in a mixture of hydrophobic/hydrophilic environment such as ethanol. Therefore, the 0.35–0.5 and 1.76–2.5 ns lifetimes originate from two substructures existing in the excited state. These substructures possess different electronic distributions. The third lifetime observed for L-Tryptophan in ethanol is the result of the hydrophobic/hydrophilic functions (CH_3 and OH) interactions with the fluorophore. These interactions

Table 2 Values of χ^2 obtained for L-tryptophan decay in ethanol and analyzed with two and three fluorescence lifetimes. (J. R. Albani, 2009, J. Fluoresc. 19: 1061–1071)

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

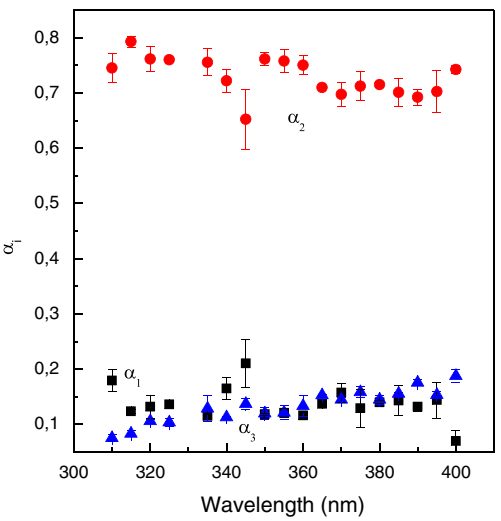

Fig. 7 Mean pre-exponential variation of Trp lifetimes in ethanol with emission wavelengths. λ_{ex}=296 nm. Set of two experiments

induce a third substructure with a specific electronic cloud generating an emission with a lifetime equal to about 4.7 ns.

Figure 7 shows lifetimes pre-exponential values at different emission wavelengths. The values are wavelengths independent, the second pre-exponential being the most important (α_2=0.7) while α_1 and α_3 are both equal to 0.15. Thus, hydrophilic/hydrophobic interactions of ethanol with L-Trp induce a new population in the excited state (τ_3=4.7 ns with α_3=0.15) and modify the populations of the two original L-Trp substructures (τ_1=0.35–0.5 ns and τ_2=1.76–2.5 ns with τ_1=0.15 and α_2=0.7).

Experimental Controls Proving Origin of the Third Fluorescence Lifetime in Ethanol

Fluorescence Lifetimes Measurements in Water/Ethanol Solutions

In order to check hypothesis that hydrophobic/hydrophilic interactions of ethanol with L-tryptophan induce the third lifetime (4.7 ns), fluorescence lifetime measurements of L-Trp were performed at different water/ethanol volume ratios. Table 3 displays χ^2 values of L-tryptophan fluorescence intensity decay in pure distilled water and in mixture of water/ethanol solutions and analyzed with one, two and three fluorescence lifetimes. In pure water, analysis with two and three lifetimes gives almost the same value of χ^2. Thus, fluorescence intensity decay of L-tryptophan in water is best analyzed with two lifetimes. However, in presence of ethanol, values of χ^2 approach 1 only when the fit was performed with three fluorescence lifetimes.

Figure 8 displays values of the different lifetimes as a function of different water/ethanol mixtures. In 100 % water, two lifetimes equal to 0.5 and 2.5 ns were calculated with pre-exponential values (populations) of 0.08 and 0.92, respectively. From the first water/ethanol ratio tested (90/10), fluorescence intensity decays were best fitted with three lifetimes

Table 3 Values of χ^2 obtained for L-tryptophan decay in pure distilled water and in mixture of water/ethanol solutions and analysed with one, two and three fluorescence lifetimes

% Ethanol	$\chi^2(1\tau)$	$\chi^2(2\tau)$	$\chi^2(3\tau)$
0	1.833	1.105	1.117
10	2.888	1.44	1.071
20	3.274	1.672	1.011
30	4.416	2.23	1.186
40	3.244	1.438	1.401
50	6.556	1.687	0.966
60	2.44	1.41	1.314
70	3.361	1.417	0.997
80	8.02	1.692	1.077
90	15.85	1.187	0.927
100	14.74	1.694	1.206

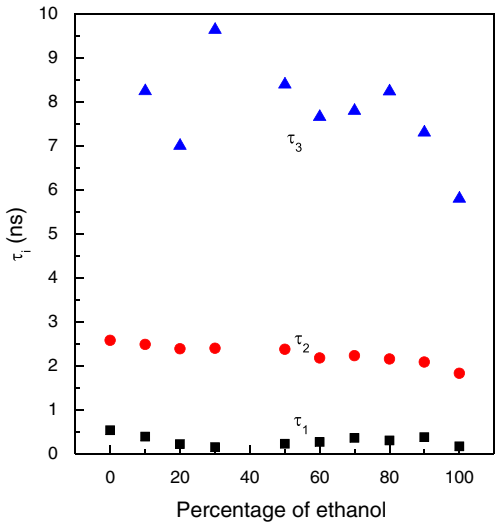

Fig. 8 Fluorescence lifetimes of L-Trp in water-ethanol mixtures. λ_{ex}=296 nm. λ_{em}=340 nm

instead of two observed in pure water. Two lifetimes are equal to or close to 0.5 and 2.5 ns, values identical to those measured when L-Trp was dissolved in pure water. Thus, these two lifetimes characterize two specific substructures of the fluorophore in the excited state (tryptophan backbone with specific electronic distribution). The third lifetime is clearly the result of the hydrophobic/hydrophilic chemical functions interaction of ethanol with the tryptophan.

Figure 9 displays lifetimes pre-exponential values measured at the different water/ethanol ratios. The most important population is that corresponding to the 2.5 ns fluorescence lifetime. Increasing ethanol percentage induces a decrease in the value of this population and in the same time it increases slightly the other two populations.

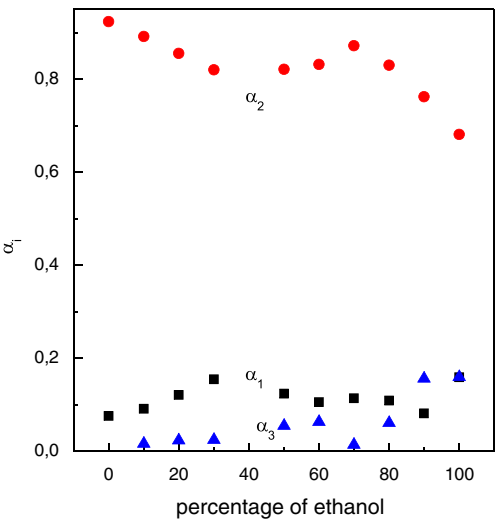

Fig. 9 Fluorescence lifetimes pre-exponentials of L-Trp in water-ethanol mixtures. λ_{ex}=296 nm. λ_{em}=340 nm. The dominant value is that coming from the second component, although in presence of ethanol its value decreases

Fluorescence Lifetimes Measurements in Hydrophobic Solvents (CCl_4 and $CHCl_3$)

Fluorescence lifetimes measurements performed in carbon tetrachloride or in chloroform show that two lifetimes are sufficient to describe intensity decays observed for L-tryptophan in both solvents. Table 4 gives the values of χ^2 obtained for one, two and three lifetimes for measurements performed in chloroform. It is clear from the values obtained that a fit with one exponential is not good and a fit with two exponentials is sufficient to describe intensities decays. Value of τ_1 was found unstable along emission wavelength varying between 0.05 and 3 ns, while value of second lifetime was found stable and equal to 3.4 ns, a value in the same range of that (2.5 ns) found for L-Trp in water.

Figure 10 displays pre-exponential values of both lifetimes along emission spectrum, for measurements performed in pure water (hydrophilic medium) and in chloroform (hydrophobic medium). In both solvents, longest and smallest populations are the same. Thus, in both solvents, L-Trp emits from two identical sub-structures. In ethanol, presence of hydrophobic and hydrophilic chemical properties modifies the solvent interaction with L-tryptophan inducing a third emitting population.

Figure 11 displays fluorescence excitation spectra of L-Trp dissolved in water and in chloroform. Shapes of the spectra and peak positions are not the same. Since fluorescence excitation spectrum characterizes electronic distribution within a fluorophore in the ground state, then this distribution is not the same whether L-Trp is dissolved in water or in chloroform. Thus, tryptophan substructure(s) population(s) in the ground state is/are not the same in water and in chloroform. However, since emitting populations in both water and chloroform are the same, they originate from same substructures induced at or existing in the excited state and not in the ground state. Therefore, third fluorescence lifetime induced in ethanol by hydrophobic/hydrophilic chemical functions interaction with tryptophan originates also from a substructure formed in the excited state.

Table 4 Values of χ^2 obtained for L-tryptophan decay in $CHCl_3$ and analyzed with one, two and three fluorescence lifetimes

Wavelength	$\chi^2(1\tau)$	$\chi^2(2\tau)$	$\chi^2(3\tau)$
310	4.27	0.9	0.86
315	1.31	0.99	1.01
320	1.55	1.15	1.14
325	1.24	1.16	1.17
330	1.28	1.12	1.13
335	1.06	0.95	0.96
340	1.18	1.05	1.05
345	1.04	0.95	0.92
350	1.11	0.84	0.84
355	1.1	1.02	1.02
360	1.2	1.04	1.01
365	1.18	1.04	1.03
370	1.44	1.18	1.17
375	1.02	0.96	0.97
380	1.2	1.08	1.13
385	1.29	1.27	1.26
390	1.23	1.19	1.2
395	1.08	1.01	0.97

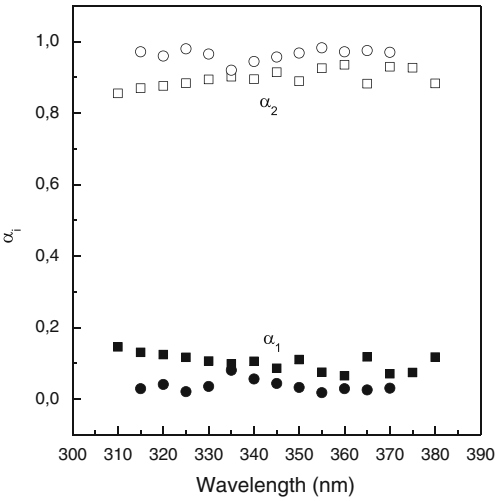

Fig. 10 Fluorescence lifetimes pre-exponential values of L-Trp in water (*squares*) and in $CHCl_3$ (*circles*). λ_{ex}=296 nm

Temperature Effect on Fluorescence Decay Parameters

Figure 12 displays fluorescence lifetimes of L-Trp dissolved in water at three temperatures, 13.6, 25.6 and 46 °C. It is difficult to observe a significant difference in the short fluorescence lifetime with temperature. However, longest and mean lifetimes decrease when temperature increases. This decrease is the result of high Brownian motions. Thus, in pure

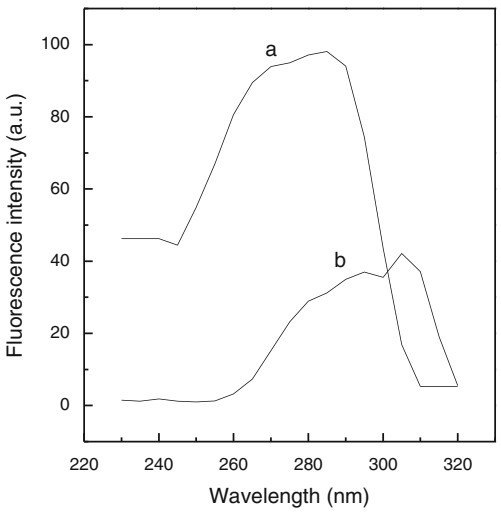

Fig. 11 Fluorescence excitation spectra of L-Trp in water (λ_{max}=280-nm) (**a**) and in $CHCl_3$ (λ_{max}=295 nm) (**b**). λ_{em}=360 nm

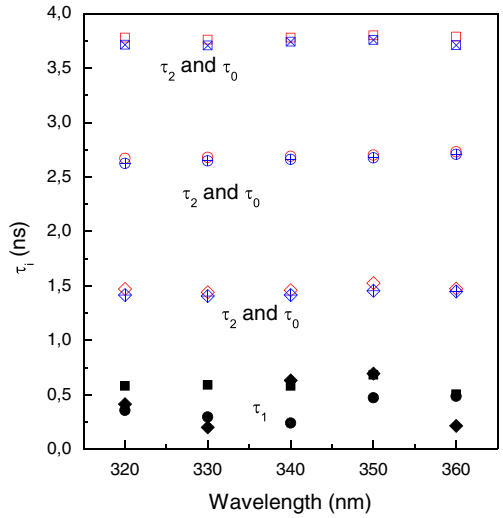

Fig. 12 Fluorescence lifetime variation of L-Trp in water with temperature. *Squares*: 13.6 °C. *Circles*: 25.6 °C. *Lozenges*: 46 °C

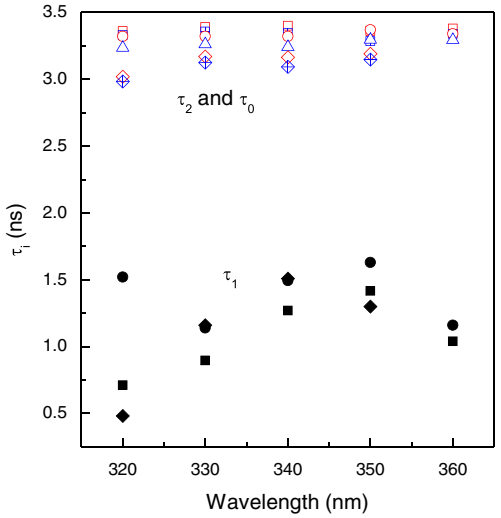

Fig. 14 Fluorescence lifetime variation of L-Trp in CCl_4 with temperature. *Squares*: 13.6 °C. *Circles*: 25.6 °C. *Lozenges*: 46 °C

hydrophilic solvent (water), interaction between solvent and L-Trp molecules is very important, facilitating fluorophore solubility in water.

Figure 13 displays fluorescence lifetimes pre-exponentials of L-Trp in water with temperature. The results clearly show that pre-exponential values of both lifetimes do not change with temperature. Thus, temperature has no effect on emitting populations. Figure 13 indicates presence of two specific L-Trp populations emitting in the excited states, these two populations are pre-existing within the fluorophore structure (backbone and electronic cloud). These two populations emit each with one lifetime which value is affected by Brownian motions.

Figure 14 displays fluorescence lifetimes of L-Trp dissolved in CCl_4 measured at 13.6, 25.6 and 46 °C. Unlike the results obtained in water, temperature does not modify lifetimes values. Thus, interaction of hydrophobic medium (CCl_4) with L-Trp is weak, which explains the difficulty of dissolving very quickly the fluorophore in CCl_4 and in $CHCl_3$. Pre-exponential values are not affected by temperature variation (Fig. 15) indicating that emission occurs from specific pre-existing populations and which are put into evidence after excitation.

Effect of Excitation Wavelength on Fluorescence Lifetimes Parameters: Fluorescence Lifetimes Occur from Pre-existing, Pre-defined Populations in the Excited State

Fluorescence lifetimes of L-Trp in water were measured at three excitation wavelengths, 266, 281 and 296 nm. Table 5 displays χ^2 values obtained at each excitation wavelength, for

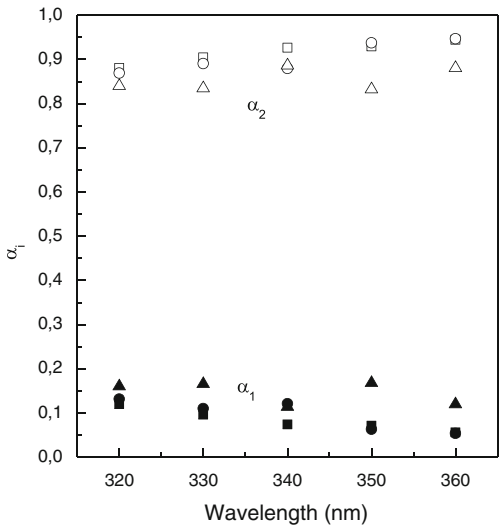

Fig. 13 Fluorescence lifetime pre-exponential variation of L-Trp in water with temperature. *Squares*: 13.6 °C. *Circles*: 25.6 °C. *Lozenges*: 46 °C

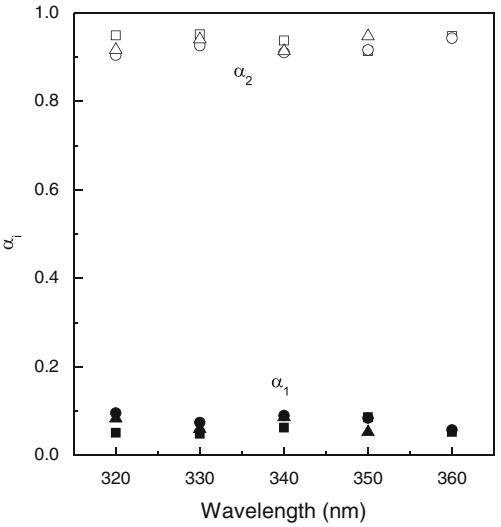

Fig. 15 Fluorescence lifetime pre-exponential variation of L-Trp in CCl_4 with temperature. *Squares*: 13.6 °C. *Circles*: 25.6 °C. *Lozenges*: 46 °C

one, two and three lifetimes. Data obtained clearly indicate that at each excitation wavelength, the best analysis is done with two fluorescence lifetimes.

Figures 16 and 17 display respectively fluorescence lifetimes and their pre-exponential values of L-Trp dissolved in pure water measured along the emission spectrum and obtained at three excitation wavelengths, 266, 281 and 296 nm. One can see that both fluorescence parameters do not change with excitation wavelength and are constant along emission spectrum. Thus, excitation energy does not play any role in the attribution of fluorescence lifetimes values. Upon excitation, new electronic distributions, different from that or those existing in the ground state, appear, favouring two sub-structures having each one specific fluorescence lifetime. Each sub-structure is formed by tryptophan backbone along with a specific electronic distribution. The fact that lifetimes and their pre-exponential values are independent of the excitation wavelength indicates that the two emitting sub-structures or populations are not arbitrary but pre-existing, pre-defined ones and they are revealed after excitation occurs.

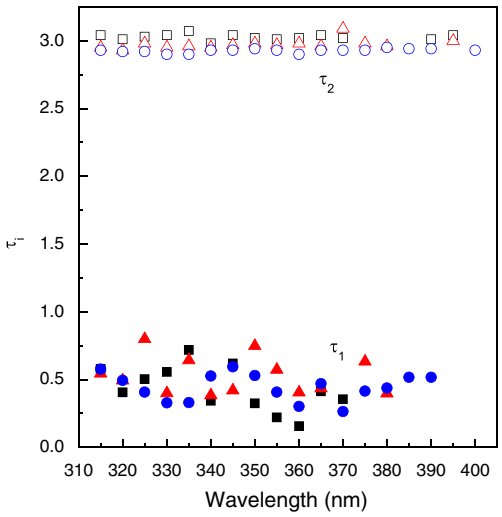

Fig. 16 Fluorescence lifetimes of L-Trp in pure water as a function of emission wavelength, recorded at three excitation wavelength, 266 (*squares*), 281 (*triangles*) and 296 nm (*circles*). Lifetimes values are independent of excitation wavelength

Discussion

The present work describes different experiments dealing with origin of fluorescence lifetimes of L-tryptophan free in solution (pure hydrophilic solvent (water), pure hydrophobic solvent (chloroform and carbon tetrachloride) and solvent possessing both hydrophobic and hydrophilic chemical functions (ethanol)). Understanding origin of tryptophan lifetimes is studied since more than 30 years by many authors [23–27]. Whether tryptophan was free in solution, in peptides or in proteins, explanations given on the origin of measured fluorescence lifetimes were always the same: emission is occurring from rotamers and charge transfer from excited indole moiety to the carbonyl group of the peptide bond. Excited state electron and proton transfer and solvent-quenching are described to play a role in

Table 5 Values of χ^2 obtained for L-tryptophan decay in twice distilled water at λ_{ex} 266, 281 and 296 nm and analyzed with one, two and three fluorescence lifetimes

	λ_{ex}=266 nm			λ_{ex}=281 nm			λ_{ex}=296 nm		
Wavelength	$\chi^2(1\tau)$	$\chi^2(2\tau)$	$\chi^2(3\tau)$	$\chi^2(1\tau)$	$\chi^2(2\tau)$	$\chi^2(3\tau)$	$\chi^2(1\tau)$	$\chi^2(2\tau)$	$\chi^2(3\tau)$
315	3.81	1.17	1.15	3.49	1.23	1.21	3.68	1.07	0.97
320	2.65	0.98	0.97	2.65	1.24	1.25	4.14	1.02	0.99
325	2.29	1.13	1.10	2.39	1.11	0.988	2.53	1.02	1.02
330	2.80	0.90	0.83	2.21	1.07	1.02	2.17	1.2	1.19
335	2.24	1.008	0.96	2.07	1.2	1.2	2.5	1.22	1.19
340	1.7	0.98	0.95	1.75	1.07	1.17	2.16	1.13	1.05
345	1.78	1.05	1.02	1.82	1.16	1.1	1.98	1.07	1.05
350	1.39	0.94	0.91	1.52	1.08	1.05	1.96	0.96	0.91
355	1.75	1.16	1.14	1.32	1.11	1.07	1.81	1.11	1.09
360	1.44	1.06	1.04	1.25	0.993	0.999	1.68	1.05	1.01
365	1.41	1.07	1.13	1.37	1.08	1.07	1.74	1.08	1.05
370	1.36	1.12	1.11	1.33	1.15	1.15	1.63	1.08	1.06
375	1.09	0.99	0.99	1.11	0.98	0.95	1.48	1.13	1.07
380	1.09	1.01	1.002	1.04	0.94	0.98	1.54	1.16	1.09
385	1.07	1.008	0.98	1.19	0.98	0.94	1.31	1.07	1.08
390	1.13	0.91	0.89	1.29	1.22	1.22	1.28	1.07	1.11
395	1.13	1.09	1.08	1.03	0.92	0.87	1.25	1.13	1.03
400	1.21	1.09	1.1	1.08	1.01	1.02	1.25	1.04	1.04

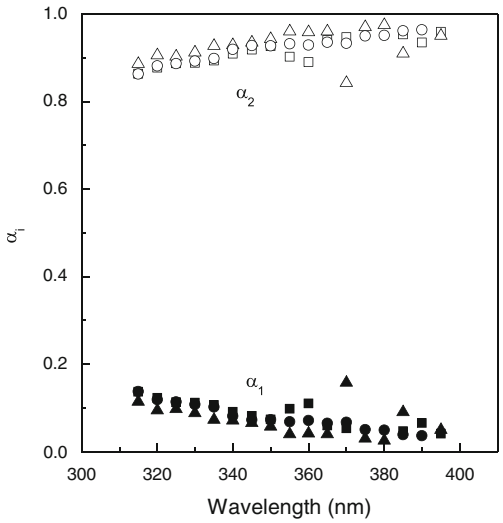

Fig. 17 Fluorescence pre-exponential lifetimes of L-Trp in pure water as a function of emission wavelength, recorded at three excitation wavelength, 266 (*squares*), 281 (*triangles*) and 296 nm (*circles*). Pre-exponential lifetimes values are independent of excitation wavelength

the deexcitation process of tryptophan fluorescence. However, experiments proving clearly these different possibilities were not shown. In our present work, we performed clear and straightforward experiments that prove that the two fluorescence lifetimes of tryptophan free in water originate from two specific structures formed each by the tryptophan backbone with its electronic distribution in the excited state. Also, our experiments show that the third lifetime observed for tryptophan in ethanol is the result of interactions occurring between the fluorophore and hydrophobic/hydrophilic chemical functions of ethanol.

Figure 1 shows that protonation of tryptophan modifies intensity of its excitation spectrum, thus electronic distribution of tryptophan in the ground state is not the same at different pHs. Fluorescence emission spectrum of tryptophan in water is proportional to the intensity at the excitation wavelength (here 295 nm) and not to the intensity of the excitation maximum (280 nm). Therefore, fluorescence emission cannot be regarded as the result of electronic distribution within the ground state but to the new electronic distribution occurring within the excited state. A logical conclusion since emission occurs from the excited state where electronic distribution is not necessarily identical to that of the ground state. This new excited state occurs independently of the surrounding environment of the fluorophore [7].

Also, one can consider that protonation affects mainly and only excitation spectrum while emission spectrum intensity is simply proportional to the intensity at the excitation wavelength.

Fluorescence emission decay of L-Trp whether in water or in buffer occurs with two lifetimes (0.5 and 2–3.5 ns). It is clear from our data that state of protonation of L-Trp modifies value of the longest one. Also, values of the populations of both lifetimes are not reversed with pH, the most important population is that of longest lifetime. Thus, these lifetimes occur from two substructures inherent to L-Trp itself independently of its state of protonation. The latter affects lifetimes values, mainly longest one, such as it affects fluorescence excitation spectra intensities.

When dissolved in ethanol (CH_3CHOH), L-Trp emits with three lifetimes. Interaction of L-Trp with the dual chemical nature of ethanol (hydrophobic with CH_3 and hydrophilic with OH) is responsible of presence of the third lifetime. Two lifetimes (0.5 and 1.7 ns) are very close to those found for L-Trp in water (0.5 and 2.5 ns) and thus characterize the two substructures observed in water and in buffers of different pHs. We can notice that population of the second lifetime is equal to 0.75 and, although lower than that (0.95) obtained for the second lifetime in buffer, is still the main fluorescent component. The third lifetime appears at low percentage of ethanol (10 %) indicating clearly that presence of hydrophobic chemical function, even at low concentration compared to that of hydrophilic one, is sufficient to induce a new lifetime (the third one).

Fluorescence intensity decay of L-Trp in pure hydrophobic medium (CCl_4 or $CHCl_3$) is best described with two lifetimes. Although the value of the shortest one fluctuates as the result of the bad and weak interactions between L-Trp and solvent molecules, populations of both lifetimes are identical to those of the two lifetimes measured when L-Trp is dissolved in water (pure hydrophilic medium). This result confirms the fact that in pure solvent (hydrophobic or hydrophilic), emission of L-Trp occurs from two populations generated in the excited state and which are inherent to the fluorophore itself independently of the surrounding environment. The latest will play a role, if any, in the values of the two lifetimes by modifying them, although this modification is rather weak. When solvent contains both hydrophilic and hydrophobic chemical functions such as in ethanol, where both hydrophobic (CH_3) and hydrophilic (OH) structures interact together with L-Trp, a third lifetime is generated.

Fluorescence excitation spectra of L-Trp in water and in chloroform are not the same (Fig. 11), however emitting populations are equal. Excitation spectrum characterizes electronic distribution in the ground state while emission occurs from the excited state. Thus, the two tryptophan populations characterize two excited states of the fluorophore and which are the same in pure hydrophilic and hydrophobic environments. This means that we cannot attribute lifetimes to conformers in the ground state since in this state, L-Trp dissolved in pure water should possess conformers that differ from those present when L-Trp is dissolved in pure chloroform or carbon tetrachloride.

In pure water, the longest fluorescence lifetime decreases when temperature increases as the result of Brownian motions increase (Fig. 12), while populations of both lifetimes are not modified with the temperature change (Fig. 13). This also means that these two populations and the two lifetimes are inherent to the L-Trp structure itself.

When L-Trp is dissolved in CCl_4, temperature increase does not affect any of the fluorescence lifetimes (Fig. 14), i.e. interaction between fluorophore and solvent molecules is not the same as that of fluorophore with water molecules.

Most interesting is the fact that in water, fluorescence lifetimes values and their populations are independent of the excitation energy. In fact, at three excitation wavelengths, 260, 280 and 295 nm, we measured identical fluorescence lifetimes (0.5 and 2.5 ns) and we obtained for each lifetime the same pre-exponential value (0.1 and 0.9, respectively). The lower the excitation wavelength is, the higher the excitation energy gained by the fluorophore will be. In principle, one would expect to measure fluorescence lifetimes that are dependent on the excitation wavelength and to obtain populations that vary from an excitation wavelength to another. However, this was not the case, which means that whatever excitation energy is, the same populations are emitting. Therefore, populations that are emitting from the excited states are independent of excitation energy and thus are pre-selected populations. In other terms, in pure hydrophilic or hydrophobic medium, L-Trp possesses in the excited state, two and only two specific sub-structures which can emit. Excitation of L-Trp allows reorganization of these two sub-structures formed each by L-Trp backbone with a specific electronic distribution.

Results described in the present work are from straight forwarded experiments showing clearly that L-Trp emits with two lifetimes when it is dissolved in pure chemical solvent (pure hydrophobic or pure hydrophilic one). These two lifetimes are generated in the excited state and are inherent to the L-Trp itself. The most interesting is the fact that these two excited states are always the same, whatever excitation energy is, i.e. it is always the same L-Trp populations that are emitting and not random ones. Therefore, it appears that internal specific configurations of L-Trp do exist in the excited state and these configurations are the only ones which can be excited and/or able to emit. In other words, description of molecules with one structure, as it is done up today, is oversimplified.

Interaction of L-Trp in solution with both hydrophobic and hydrophilic chemical structures, such as it is the case in ethanol, generates a third fluorescence lifetime.

In the second part of this paper, we display results obtained on proteins in different conditions, in order to find out origin of measured lifetimes in proteins.

Acknowledgements The author wishes to thank professors Bernard Alpert and Daniel Kmiecik for their helpful discussion in this work.

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