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ORIGINAL PAPER

Origin of Tryptophan Fluorescence Lifetimes. Part 2: Fluorescence Lifetimes Origin of Tryptophan in Proteins

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Abstract Fluorescence intensity decays of L-tryptophan in proteins dissolved in pH 7 buffer, in ethanol and in 6 M guanidine pH 7.8 and in lyophilized proteins were measured. In all protein conditions, three lifetimes were obtained along the emission spectrum (310–410 nm). The two shortest lifetimes are in the same range of those obtained for L-Trp in water or in ethanol. Thus, these two lifetimes originate from specific two sub-structures existing in the excited state and are inherent to the tryptophan structure independently of the surrounding environment (amino acids residues, solvent, etc.) In proteins, the third lifetime originates from the interactions that are occurring between tryptophan residues and neighboring amino acids. Populations of these lifetimes are independent of the excitation wavelength and thus originate from pre-defined sub structures existing in the excited state and put into evidence after tryptophan excitation. Fluorescence decay studies of different tripeptides having a tryptophan residue in second position show that the best analysis is obtained with two fluorescence lifetimes. Consequently, this result seems to exclude the possibility that peptide bond induces the third fluorescence lifetimes. Indole dissolved in water and/or in ethanol emits with two fluorescence lifetimes that are completely different from those observed for L-Trp. Absence of the third lifetime in ethanol demonstrates that indole behaves differently when compared to tryptophan. Thus, it seems not adequate to attribute fluorescence lifetime or fluorescence properties of tryptophan to indole ring and to compare tryptophan fluorescence properties in proteins to molecules having close structures such as NATA which fluoresces with one lifetime.

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Introduction

Fluorescence lifetimes detection and measurement of Trp residues in proteins has now been developed and applied since more than 50 years. Protein structure and dynamics, protein ligand interaction, protein denaturation, protein functions were among the different purposes for measuring protein tryptophan residues fluorescence lifetimes [1–38]. All these studies showed clearly that Tryptophan residue in proteins emits generally with a multiexponential decay. Although first spectrofluorometers built and used were not able to give exact numbers of lifetimes and their accurate values, results were obvious and showed that Trp residues in proteins emit with at least two lifetimes. In order to understand origin of fluorescence lifetimes of tryptophan in proteins, different and combined models were described. Rotamers model and charge transfer from indole ring to the carbonyl group of the peptide bond are considered as responsible for the bi or multiexponential decays. However, the real question is whether fluorescence properties of indole ring are the same as those of tryptophan? In general, researchers working on tryptophan fluorescence have always considered that all tryptophan fluorescence observables are or should be similar to those of indole ring. But is this the case? Nevertheless, none of these models was experimentally proven.

Modern instruments allow, by exciting tryptophan residue in a protein, to measure in general three fluorescence lifetimes, independently of the number of Trp residues present within the protein. Also, the values of these lifetimes are almost the same or very close, for all studied proteins.

In the present study, we are describing straighforwarded experiments that clearly demonstrate that two of the three

lifetimes are identical to those found for L-Trp in water and in ethanol and thus are inherent to the tryptophan structure itself independently of surrounding environment. Thus, these two lifetimes are generated by two sub-structures pre-existing in the excited state and revealed after tryptophan excitation. Experiments performed on tri-peptides containing a single tryptophan residue show that peptide bond is not responsible of the presence of the third lifetime in proteins. Nevertheless, our experiments indicate that this lifetime is the result of the permanent contact between tryptophan residue(s) and neighboring amino acids. Also, we present experiments showing clearly that indole ring dissolved in water and/or in ethanol has fluorescence lifetimes completely different from those observed for L-Trp in solution or Trp residues in proteins. Therefore, fluorescence properties of indole molecule differ from those of Trp residues and of free tryptophan in water or in ethanol. This suggests that tryptophan fluorescence observables are independent of indole ring.

Materials and Methods

Indole was from Sigma and was used as received. The fluorophore was dissolved in deionised water, or in ethanol (Sigma). Proteins were purchased from Sigma except Ubiquitine conjugated enzyme E2 which was prepared. Peptides used were prepared by Genescreen with 99 % purity.

Absorbance data were obtained with a Shimadzu MPS-2000 double-beam spectrophotometer (Shimadzu, Champs Sur Marne, France) using 1-cm pathlength cuvettes.

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. A minimal value of χ^2 indicates the best fit. A χ^2 value that approaches 1 indicates a good fit.

All experiments were performed in 10 mM Tris pH 7, 10 mM phosphate pH 7 or 6 M guanidine pH 7.8.

Results

Fluorescence Intensity Decay Parameters of Proteins in Native Solvents

Table 1 displays fluorescence lifetimes of tryptophan free in water and in ethanol and of proteins of different structures and containing one, two, three or eight tryptophan residues. Interpretation of the data does not need the presence of a protein or a peptide around the tryptophan. Our data characterize an internal reorganization of the tryptophan structure independently of its environment. These structures obtained in the excited state yield the two fluorescence lifetimes (0.4 and 3 ns) found for tryptophan whether free in solution or present within a protein. In most of the proteins, a third lifetime around 1 ns is in general recorded. In human and bovine serum albumins and in odorant binding protein, this third lifetime ranges from 6 to 9 ns. Values of the relative amplitudes of the 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. In fact, our results (Table 1) indicate that lifetimes relative amplitudes are not the same for all the proteins.

Data displayed in Table 1 show that proteins having one tryptophan residue or more emit with three fluorescence lifetimes. Thus, number of fluorescence lifetime is independent of that of tryptophan residues present within a protein, although exceptions could exist.

Table 1 also indicates that tryptophan residues in both BSA and HSA fluoresce with three identical lifetimes. Thus, one Trp residue emission can be described with three fluorescence lifetimes and it is not possible, in BSA, to assign a specific lifetime to a specific Trp residue.

Emission maximum of tryptophan residue in HSA, mutant Cyclophilin B and Odorant binding protein is located at 335, 325 and 340 nm, respectively. Thus, modification of the environment of a tryptophan affects fluorescence emission peak. However, in the three proteins, tryptophan residue emits with three lifetimes, two are almost identical to those found for tryptophan free in water and close to two lifetimes found for L-Trp in ethanol. Thus, two fluorescence lifetimes of tryptophan residue in proteins are independent of the fluorophore environment. This implies that spatial structure of a protein does not affect these two fluorescence lifetimes.

Figure 1 displays fluorescence lifetimes variation with emission wavelength of Trp residues of α_1-acid glycoprotein. One can observe an increase in the lifetimes (especially the two longest ones) with emission wavelength. Thus, these two lifetimes are sensitive to the polarity of the medium. The same results were obtained with two other lipocalin proteins, odorant binding protein [39] and β-lactoglobulin [40].

Figure 2 displays the value of the mean fluorescence lifetime τ_0 along emission spectrum, for L-tryptophan dissolved in 3 pH (2, 7 and 11) buffers and in ethanol and of Trp residues in α_1-acid glycoprotein and human serum albumin (HSA). τ_0 variation is not the same for tryptophan in the different conditions. This variation depends mainly on that of the longest fluorescence lifetime and of pre-exponential values.

Figure 3 displays pre-exponential values of tryptophan residues fluorescence lifetimes of native α_1-acid glycoprotein and of β-lactoglobulin. It is clear that only populations of the longest lifetimes are very close one to each other, the two other

Table 1 Fluorescence lifetimes and corresponding relative amplitudes in % of tryptophan free in solution (L-Trp) and present in different proteins

Sample	λ_{em} (nm)	τ_1	α_1	τ_2	α_2	τ_3	α_3	χ^2	Trp
L-Trp in water	350	0.43	4.71	3.06	95.29	–	–	1.008	
L-Trp in ethanol	338	0.152	18.56	1.78	70.07	4.873	11.37	1.004	
Porcine odorant binding protein	340	0.86	35.7	2.76	53.4	9.2	10.9	1.167	1
Human serum albumin	325	0.53	12.35	3.57	31.04	7.46	56.6	1.066	1
Mutant Cyclophilin B (CyPBw128A)	330	0.413	35	2.096	56	5.45	9	1.086	1
Cyclophilin B	330	0.684	21.4	1.784	60.3	4.376	18.3	1.067	2
Bovine serum albumin	355	0.265	4.73	3.055	25.7	6.58	69.6	1.006	2
Bovine β-lactoglobulin	330	0.56	14.40	1.306	50.2	4.475	35.4	1.156	2
Helicase	330	0.47	17.83	1.735	37.2	4.373	44.96	1.128	3
α_1-Acid glycoprotein	330	0.24	6.67	1.4	34.88	3.410	58.45	1.046	3
Chymotrypsin	330	0.69	27.26	1.472	45.03	3.82	27.71	1.154	8
Serotransferrin	330	0.31	25.65	1.25	37.69	3.784	36.66	1.137	8

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populations differ between the two proteins. This difference could be the result of the surrounding Trp residues environments. Differences also exist in the pre-exponential values obtained for fluorescence lifetimes of tryptophan residues in human serum albumin and β-lactoglobulin (Fig. 4). Thus, if in general, fluorescence lifetimes observed for tryptophan residues are very close in most proteins, populations emitting are not necessary equal.

Fluorescence Intensity Decay Parameters of Proteins in Denatured Solvent (6 M Guanidine)

In order to check whether the three lifetimes are dependent on the protein structure or not, fluorescence lifetimes of HSA and of β-lactoglobulin have been measured in 6 M guanidine pH 7.8. Data indicated that in both proteins, three fluorescence lifetimes still exist, although denaturation induces a decrease

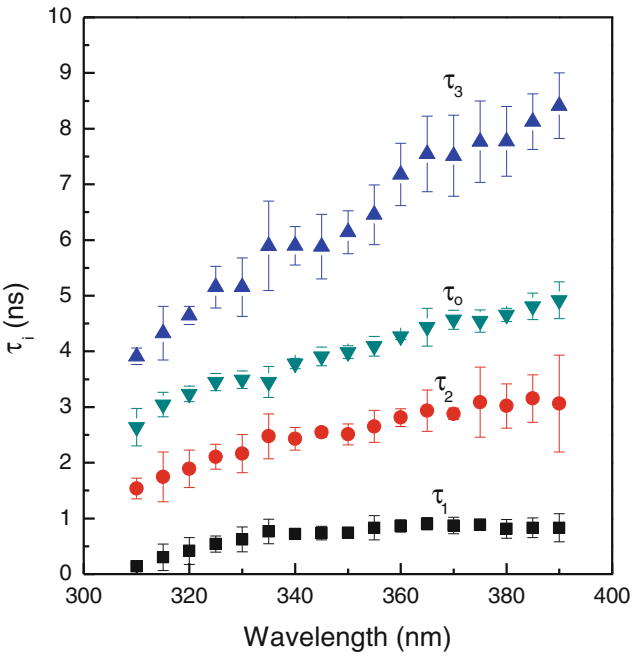

Fig. 1 Variation of fluorescence lifetimes of α_1-acid glycoprotein with emission wavelength. Results of 4 experiments from 2 protein preparations. λ_{ex}=296 nm

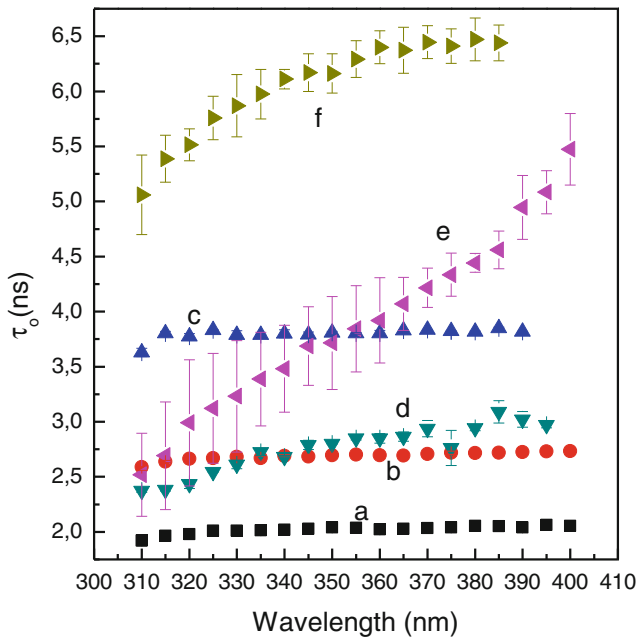

Fig. 2 Mean lifetime variation with emission wavelength of L-Trp dissolved in PBS buffer at pH 2 (**a**), pH 7 (**b**) and pH 12 (**c**) and in ethanol (**d**), of α_1-acid glycoprotein tryptophan residues (**e**) and of human serum albumin tryptophan residue (**f**). λ_{ex}=296 nm

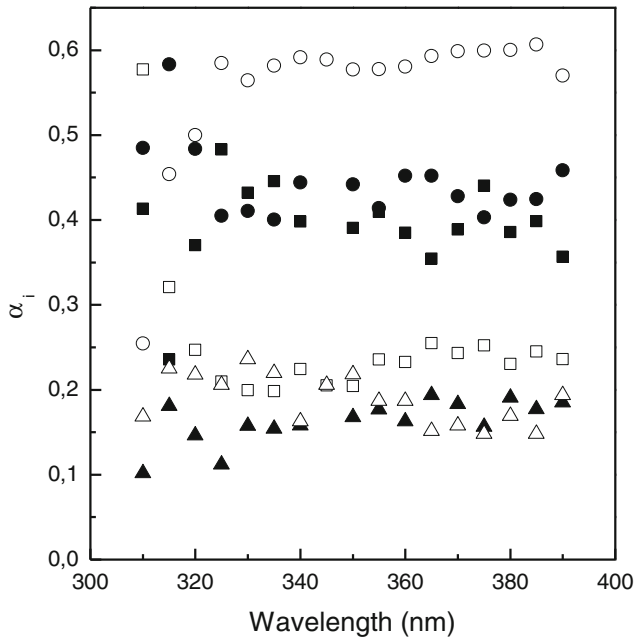

Fig. 3 Pre-exponential values of tryptophan residues fluorescence lifetimes of α_1-acid glycoprotein (*filled symbols*) and of β-lactoglobulin (*open symbols*), dissolved in 10 mM pH 7 Tris buffer and excited at 296 nm. *Squares*: α_1; *Circles*: α_2; *Triangles*: α_3

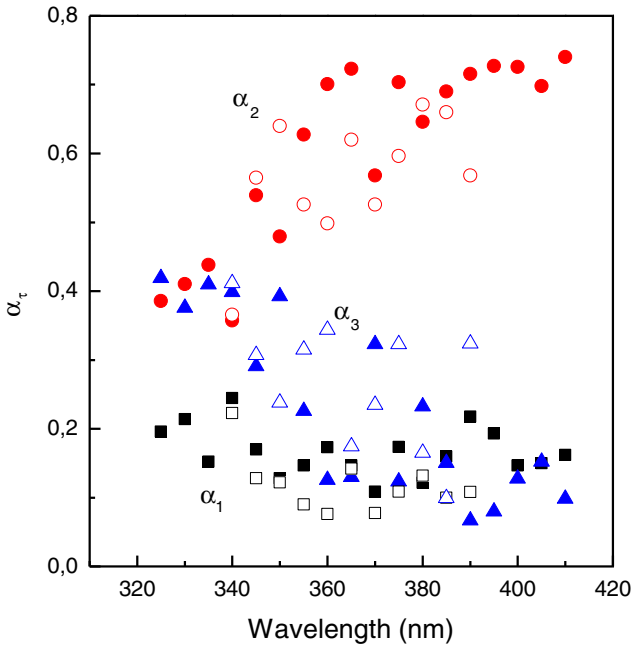

Fig. 5 Pre-exponential values of tryptophan residues fluorescence lifetimes of β-lactoglobulin (*filled symbols*) and of human serum albumin (*open symbols*), dissolved 6 M guanidine pH 7.8 buffer and excited at 296 nm. *Squares*: α_1; *Circles*: α_2; *Triangles*: α_3

in the fluorescence lifetimes values [40, 41]. Figure 5 displays values of lifetimes pre-exponentials with emission wavelength of both proteins in 6 M guanidine. One can notice that, for both proteins, each of the three pre-exponentials follow the same feature in both proteins. Thus, in 6 M guanidine, proteins tertiary structure is abolished. Denaturation with 6 M guanidine homogenizes lifetimes populations.

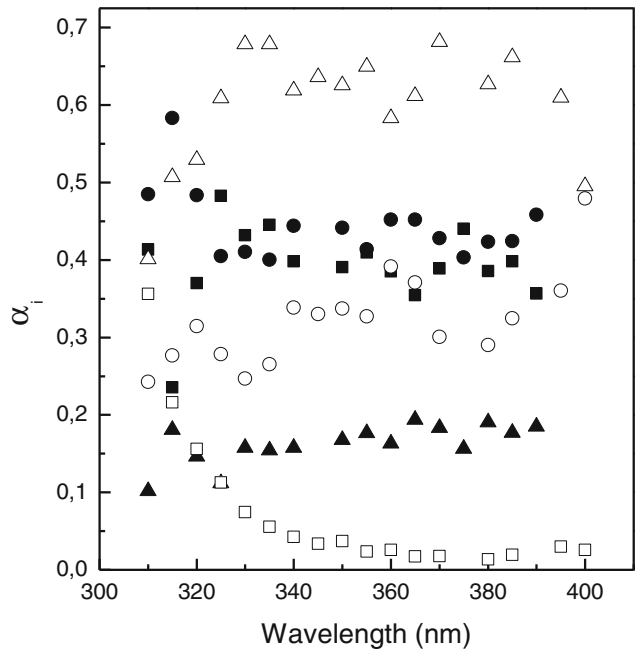

Fig. 4 Pre-exponential values of tryptophan residues fluorescence lifetimes of β-lactoglobulin (*filled symbols*) and of human serum albumin (*open symbols*), dissolved in 10 mM pH 7 Tris buffer and excited at 296 nm. *Squares*: α_1; *Circles*: α_2; *Triangles*: α_3

Nevertheless, one should question whether populations homogenization is the result of a new structure imposed by 6 M guanidine or it is simply the result of an interaction occurring between guanidine and tryptophan residue? In the latter case, fluorescence lifetimes measurements of L-Trp dissolved in 6 M guanidine should give identical result to that observed for proteins dissolved in guanidine.

Table 2 displays χ^2 values of L-Trp fluorescence intensity decay in 6 M guanidine. One can notice that there is no 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 6 M guanidine can be best described with two fluorescence lifetimes. Figure 6 displays values of both fluorescence lifetimes along emission wavelength. Lifetimes values (0.5 and 2.5 ns) are equal to those found for L-Trp in water (see first paper). Thus, guanidine does not affect the number or the values of fluorescence lifetimes of L-Trp observed in pure water.

Figure 7 displays lifetimes pre-exponentials variations of L-Trp in 6 M guanidine with emission wavelength. Measured values and their variations with emission wavelength are identical to those observed for L-Trp in water. Thus, even the populations responsible for the two lifetimes of L-Trp in hydrophilic medium are not affected by guanidine. Therefore, one can conclude that 6 M guanidine impose to all proteins identical structures.

Table 2 Values of χ^2obtained for L-Trp fluorescence intensity decay, dissolved in 6 M guanidine pH 7.8 and analyzed with one, two and three fluorescence lifetimes

Wavelength	χ^2 (1 τ)	χ^2 (2 τ)	χ^2 (3 τ)
315	6.8	1.18	1.13
320	4.8	1.137	1.13
325	4.5	1.009	1.03
330	4.8	1.17	1.14
335	3.8	0.905	0.916
340	3.8	1.25	1.24
345	2.7	1.38	1.36
350	2.4	1.26	1.25
355	2.26	1.24	1.22
360	2.25	1.25	1.18
365	2.05	1.31	1.24
370	1.96	1.21	1.21
375	1.49	1.01	1.1
380	1.72	1.05	1.05

Native and denatured proteins display three fluorescence lifetimes, two of them are equal to or close to the two lifetimes observed for L-Trp in water and to two of the three lifetimes of L-Trp in ethanol. Presence and values of these two lifetimes observed in proteins are independent of number of Trp residues and of their positions in the protein. Therefore, these two lifetimes are inherent to the tryptophan structure independently of its local environment. Only values of the pre-exponentials

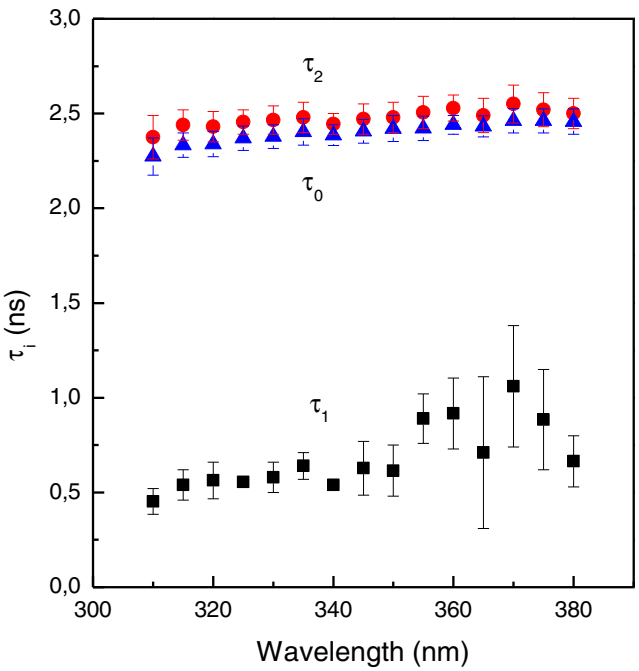

Fig. 6 Fluorescence lifetimes of L-Trp dissolved in 6 M guanidine pH 7.8 at 20 °C. λ_{ex}=296 nm. *Squares*: τ_1. *Circles*: τ_2. *Triangles*: τ_0

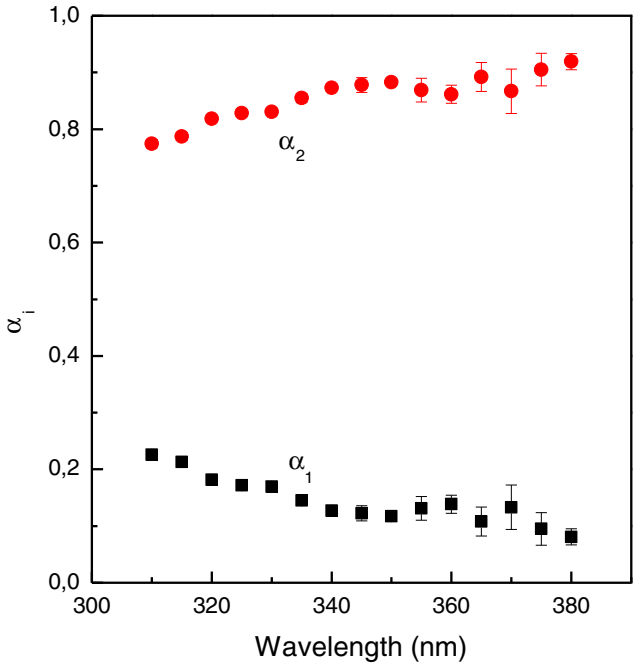

Fig. 7 Fluorescence lifetimes pre-exponential of L-Trp dissolved in 6 M guanidine pH 7 at 20 °C. λ_{ex}=296 nm

measured in native proteins are different from a protein to another, indicating that pre-exponentials characterize the local environment of tryptophan residues. In denatured proteins, pre-exponentials are homogenized in all proteins as the result of the loss of proteins tertiary structure.

We showed in the first paper that the third fluorescence lifetime observed in ethanol is the result of the hydrophobic/hydrophilic chemical functions interaction of ethanol with the tryptophan. Thus, it is important here to find out the origin of the third lifetime in proteins.

Origin of the Third Fluorescence Lifetime in Proteins

Does the Peptide Bond Induce the Third Fluorescence Lifetime?

Tryptophan in proteins is covalently linked to two neighboring amino acids, thus one can suppose that peptide bond generates the third fluorescence lifetime via different mechanisms such as charge transfer from indole ring to the carbonyl group of the peptide bond and/or the rotation of the fluorphore within peptide bonds. In order to check these two hypotheses, fluorescence lifetimes of several di and tri-peptides have been measured in water and ethanol at different emission wavelengths. Table 3 displays fluorescence lifetimes of tri-peptides in water and in ethanol. One can observe that in both solvents, two lifetimes were obtained for the peptides, whatever the nature of the amino acids surrounding the Trp residues was. In fact, in presence of hydrophilic and/or hydrophobic amino acids, only two lifetimes were measured in both water and ethanol. In ethanol, both

Table 3 Values of fluorescence lifetimes measured at 350 nm for different peptides in pure distilled water and in ethanol

Peptide	τ_1 Water/Ethanol	τ_2 Water/Ethanol	τ_3 Water/Ethanol
Ala-Trp-Ala	0.62/0.94	1.76/2.33	–
Arg-Trp-Lys	0.76/1.74	2.1/3.00	–
Arg-Trp-Pro	0.67/1.10	1.47/2.5	–
Leu-Trp-Leu	0.73/1.22	2.41/3.14	–
Phe-Trp-Ala	0.68/0.85	2.36/2.45	–
Phe-Trp-Phe	1.05/1.39	3.01/3.00	–

lifetimes were slightly higher than those measured in water although in both solvents, the values obtained were in the same range of those found for tryptophan in water and which are identical to two lifetimes found for L-tryptophan when dissolved in ethanol or for tryptophan residues in proteins. It results that peptide bonds are not responsible for the third fluorescence lifetime of tryptophan residues in proteins.

Tables 4 and 5 display values of χ^2 for Leu-Trp-Leu and Phe-Trp-Phe respectively, when fluorescence intensity decay analysis were performed with one, two and three fluorescence lifetimes. It is clear that best analysis is done with two fluorescence lifetimes, although from 310 to 320 nm, analysis is better with three fluorescence lifetimes. We do not have any explanation for the moment of this observation.

Figure 8 displays fluorescence lifetimes of tri-peptides Leu-Trp-Leu and Ala-Trp-Ala in ethanol. τ_1 are identical however τ_2 differs. It is higher when Leucine is present. Nevertheless, in both peptides, values of the two lifetimes are in the same range of that found for free L-Trp in water or in ethanol and for Trp residues in proteins. Thus, these two lifetimes are inherent to the tryptophan structure itself and their presence and values are independent of the fluorophore environment.

Figure 9 displays values of the two lifetimes pre-exponentials of tryptophan residue in both peptides. One can notice that values of each pre-exponential are identical in both peptides. Therefore, we have the same tryptophan populations in both peptides. Also, as we have observed for L-Trp in water and in ethanol, α_2 is much higher than α_1. However, values of pre-exponentials in peptides differ from those observed for free tryptophan in water or ethanol. Thus, tryptophan environment affects the populations emitting.

Experiments described in this paragraph show clearly that the third lifetime observed for tryptophan in proteins is not the result of the peptide bond.

Does Trp Residue-Neighboring Amino Acids Interactions Induce the Third Fluorescence Lifetime in Proteins?

In most of the studied proteins, tryptophan residue(s) emit with three lifetimes. This emission occurs independently of the

Table 4 Values of χ^2 obtained for Leu-Trp-Leu decay in water and analyzed with one, two and three fluorescence lifetimes

Wavelength	χ^2 (1 τ)	χ^2 (2 τ)	χ^2 (3 τ)
310	26.3	1.44	1.14
315	11	1.5	1.18
320	8.04	1.11	1.05
325	5.8	1.08	0.9
330	4.7	0.97	0.97
335	4.46	0.99	0.99
340	5	0.9	0.9
345	4.7	0.92	0.92
350	4.5	0.98	–
355	4.6	0.98	–
360	5.6	0.97	–
365	5.3	0.93	–
370	4.74	0.98	–
375	4.7	0.89	–
380	5.3	1.07	–
385	4.7	0.93	–
390	4.5	0.93	–
395	5.2	0.87	–
400	5.5	1.12	1.10
405	5.7	0.98	–
410	3.7	1.1	–

Table 5 Values of χ^2 obtained for Phe-Trp-Phe decay in ethanol and analyzed with one, two and three fluorescence lifetimes

Wavelength	χ^2 (1 τ)	χ^2 (2 τ)	χ^2 (3 τ)
310	6.82	1.25	1.08
315	5	1.04	1.04
320	4.35	1.07	0.98
325	3.77	0.98	0.90
330	3	1.04	0.95
335	2.84	1.11	1.02
340	3	1.04	0.98
345	2.31	0.99	0.90
350	2.3	1.19	1.15
355	2.21	0.96	0.9
360	2.34	0.96	0.90
365	2.24	0.95	0.85
370	2.16	0.96	0.9
375	2.21	1.05	1.04
380	2	1.1	1.1
385	2.32	0.99	0.85
390	2	0.99	0.90
395	2	1.07	1.1
400	2.15	0.98	0.90
405	2.1	1.31	1.26
410	2.3	1.10	1.15

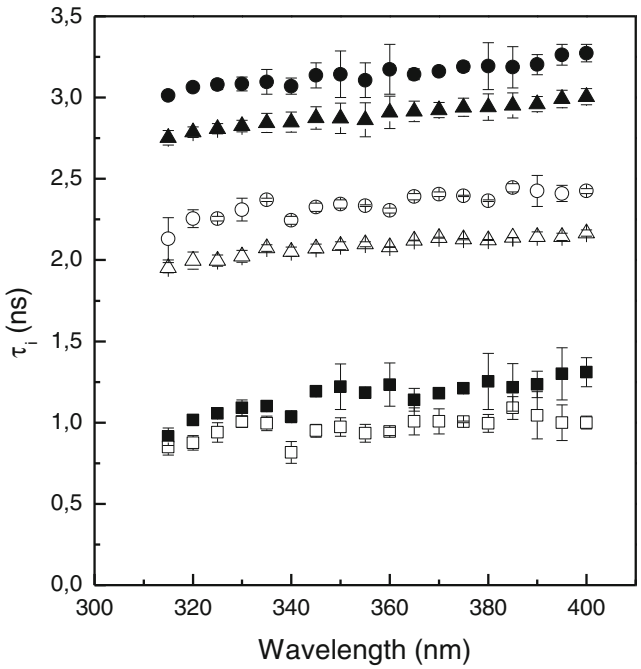

Fig. 8 Fluorescence lifetimes of Trp residue in the peptides Leu-Trp-Leu (*closed symbols*) Ala-Trp-Ala (*open symbols*) measured in ethanol at 20 °C. λ_{ex}=296 nm. *Squares*: τ_1. *Circles*: τ_2. *Triangles*: τ_0

number of tryptophan residues and of their positions in the protein (Table 1). Also, we found that two of these fluorescence lifetimes are identical or equal to those found for L-Trp in water or ethanol. Thus, presence of these two lifetimes is inherent to the tryptophan structure and is independent of the structure surrounding the fluorophore. Only, values of pre-exponentials vary from a protein to another and thus tryptophan residue environment affects emitting populations (Figs. 3 and 4). Thus, the only possible explanation of the presence of the third lifetime in proteins is the permanent contact existing between Trp residue and neighboring amino acids. This contact is the result of the protein folding within the proteins (secondary and tertiary structures in the case of a native protein and random folding in the case of a denatured protein). This contact is similar to that of free tryptophan in ethanol where the fluorophore interacts with both chemical hydrophobic and hydrophilic function of ethanol. In proteins, chemical functions of the amino acids radicals neighboring tryptophan residues replace the two chemical functions of ethanol. Therefore, if protein folding induces indirectly the third fluorescence lifetime of Trp residue, one should obtain close results when the protein is lyophilized or dissolved in solvents such as water and alcohol. In other terms, if the third fluorescence lifetime is generated by the contacts existing between tryptophan residue and neighboring amino acids, for a same protein, this lifetime would be independent of the nature of the solvent where the protein is dissolved. This could be true only when the solvent does not modify global structure of the protein and does not denature it, which is the case for 6 M guanidine.

Figure 10 displays fluorescence lifetimes with emission wavelength of Ubiquitine conjugated enzyme E2 in 10 mM phosphate buffer pH 7 and in lyophilized form. Fluorescence

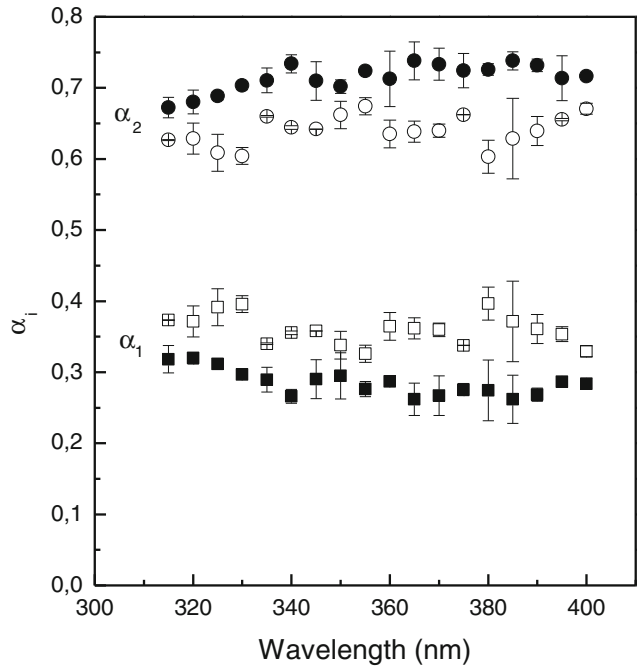

Fig. 9 Fluorescence lifetimes pre-exponentials of Trp residue in the peptides Leu-Trp-Leu (*closed symbols*) Ala-Trp-Ala (*open symbols*) measured in ethanol at 20 °C. λ_{ex}=296 nm. *Squares*: α_1. *Circles*: α_2

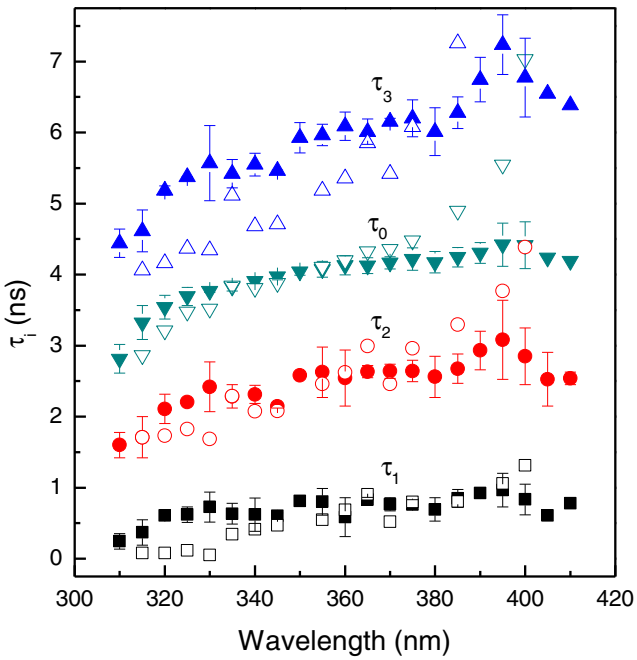

Fig. 10 Variation of fluorescence lifetimes of AP UbC (E2) dissolved in PBS buffer, pH 7 (*closed symbols*) and of lyophilized protein (*open symbols*) with emission wavelength. λ_{ex}=296 nm

lifetimes are superimposed indicating clearly that permanent interactions between Trp residues of the protein and neighboring amino acids are responsible of the third fluorescence lifetime. Buffer does not modify protein structure obtained upon lyophilisation. This result indicates clearly that none of the three fluorescence lifetimes of tryptophan is generated by the buffer. Therefore, solvent relaxation does not generate any of the three fluorescence lifetimes. Two measured lifetimes (0.7 and 2.5 ns) are inherent to the tryptophan structure itself, independently of its local environment. The third lifetime results from interactions between tryptophan residues with local environments as the result of protein folding. It is important here to remind that substructures generating these lifetimes are from the tryptophan excited state.

Figure 11 displays fluorescence lifetimes pre-exponential values of Ubiquitine conjugated enzyme E2 in 10 mM phosphate buffer pH 7 and in lyophilized form, at different emission wavelengths. In both conditions, pre-exponential values are superimposed at almost all wavelengths. This result indicates that tryptophan populations emitting in the excited state are the same.

Figure 12 displays fluorescence lifetimes variation with emission wavelength of lyophilized Ubiquitine conjugated enzyme E2 and dissolved in ethanol. Values are similar indicating that ethanol does not modify the global structure and folding of the protein. However, values of pre-exponentials are not the same in both conditions (Fig. 13). This means that interactions between tryptophan residues and neighboring amino acids have been modified when protein has been dissolved in ethanol.

These experiments show clearly that the third fluorescence lifetime observed in proteins results from interactions

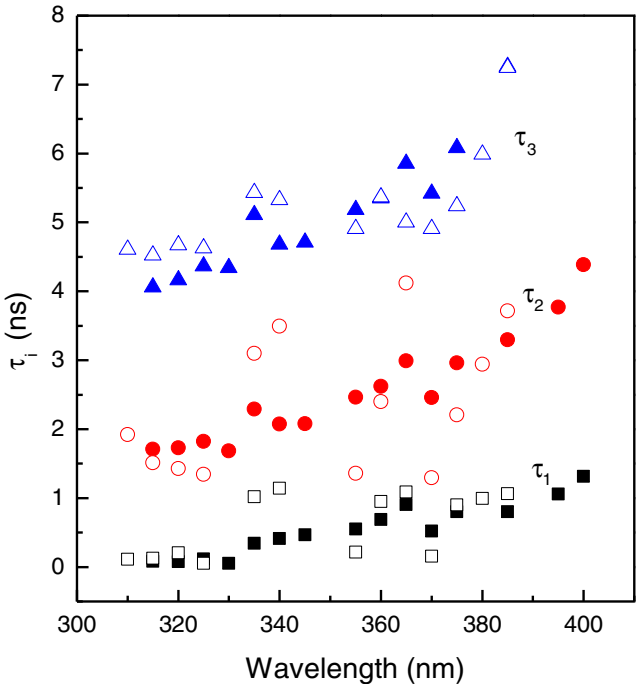

Fig. 12 Variation of fluorescence lifetimes of AP UbC (E2) dissolved in ethanol (*open symbols*), and of lyophilized protein (*closed symbols*) with emission wavelength. λ_{ex}=296 nm

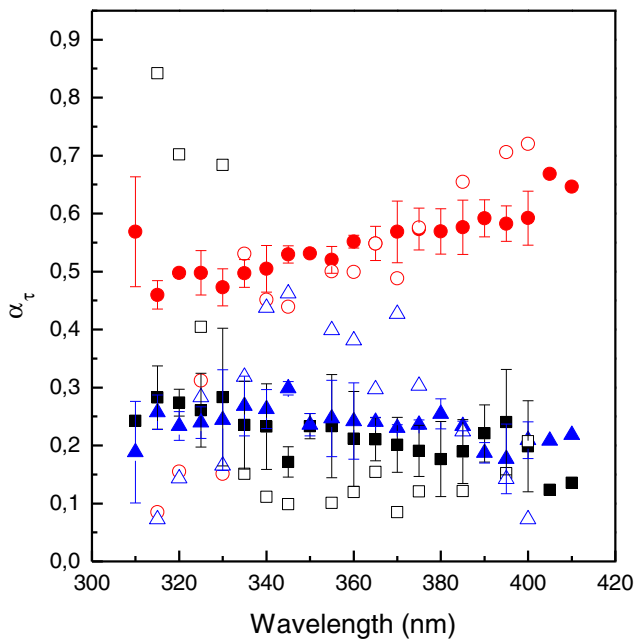

Fig. 11 Variation of fluorescence lifetimes pre-exponentials of AP UbC (E2) dissolved in PBS buffer, pH 7 (*closed symbols*) and of lyophilized protein (*open symbols*) with emission wavelength. λ_{ex}=296 nm. *Squares*: α_1. *Circles*: α_2. *Triangles*: α_3

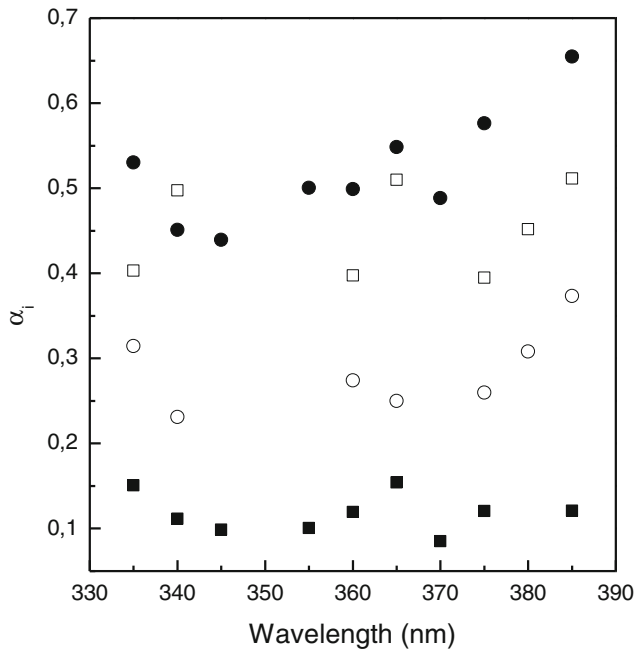

Fig. 13 Variation of fluorescence lifetimes pre-exponentials of AP UbC (E2) dissolved in ethanol (*open symbols*) and of lyophilized protein (*closed symbols*) with emission wavelength. λ_{ex}=296 nm. Only α_1 (*squares*) and α_2 (*circles*) are displayed for clarity

occurring between Trp residue(s) and neighboring amino acids. These contacts are facilitated by proteins folding.

Effect of Excitation Wavelength on Fluorescence Lifetimes Parameters

Fluorescence lifetimes of Trp residues of α_1-acid glycoprotein in tris buffer pH 7 were measured at λ_{ex} 266, 281 and 296 nm and analysed with one, two and three fluorescence lifetimes. Table 6 displays χ^2 values obtained at the three excitation wavelengths. The results obtained clearly indicate that at the three excitation wavelengths, best analysis is done with three fluorescence lifetimes.

Figures 14 and 15 display respectively fluorescence lifetimes and their pre-exponential values of α_1-acid glycoprotein measured along the emission spectrum and obtained at three excitation wavelengths, 266, 281 and 296 nm. Both fluorescence decay 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 three 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 emitting sub-structures or populations are not arbitrary but pre-existing, pre-defined ones and they are revealed after excitation (see also Discussion).

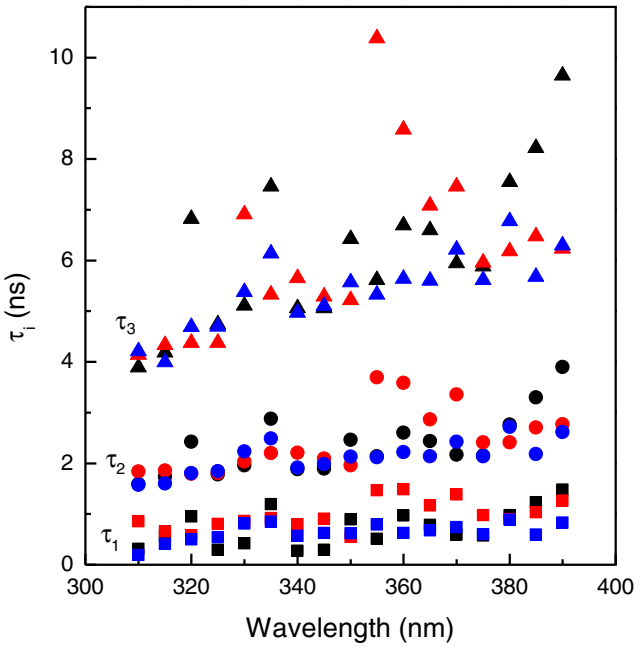

Fig. 14 Fluorescence lifetimes of α_1-acid glycoprotein in 100 mM PBS buffer, pH 7, are measured with three excitation wavelengths, 266 nm (*black*), 281 nm (*red*) and 296 nm (*blue*). τ_1 (*squares*), τ_2 (*circles*) and τ_3 (*triangles*), are equal and thus independent of the excitation wavelength. Thus, the recorded values correspond to those of Trp residues since at 296 nm, only Tryptophan is excited

Table 6 Values of χ^2 obtained for fluorescence intensity decay of α_1-acid glycoprotein in pH 7 tris buffer 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	χ^2 (1 τ)	χ^2 (2 τ)	χ^2 (3τ)	χ^2 (1 τ)	χ^2 (2 τ)	χ^2 (3τ)	χ^2 (1 τ)	χ^2 (2 τ)	χ^2 (3τ)
310	9.63	1.22	0.987	9.79	1.15	1.02	33.35	3.48	0.924
315	12.38	1.32	1.09	9.92	1.24	0.95	17.44	1.97	1.264
320	11.49	1.487	1.144	10.76	1.37	1.16	19.06	1.924	1.083
325	11.92	1.487	1.144	11.98	1.32	1.23	20.39	1.813	1.026
330	13.36	1.457	0.925	12.65	1.38	1.16	19.35	1.726	1.108
335	15.12	1.51	1.082	11.88	1.14	0.87	18.6	1.954	1.167
340	14.6	1.46	1.087	13.2	1.31	0.89	18.4	1.674	1.174
345	14.82	1.63	1.26	13.92	1.07	0.907	20.46	1.75	1.253
350	16.13	1.862	1.354	14.73	1.09	0.865	20.4	1.73	0.987
355	16.09	1.675	1.149	14.44	1.45	1.15	21.5	1.55	1.109
360	15.21	1.518	1.06	14.67	1.583	1.359	21	1.98	1.17
365	15.52	1.538	1.028	15.67	1.48	1.04	20.3	1.86	1.23
370	16.19	1.375	1.064	16.78	1.41	1.16	20.9	1.95	1.065
375	15.13	1.343	1.06	16.24	1.28	0.97	20.8	1.78	1.007
380	19	1.933	1.202	18	1.53	1.175	21.6	2.046	1.26
385	18	1.693	1.217	17.76	1.46	1.051	22.2	1.844	1.12
390	16.5	1.613	1.314	17.22	1.38	1.206	21.12	2.017	1.36

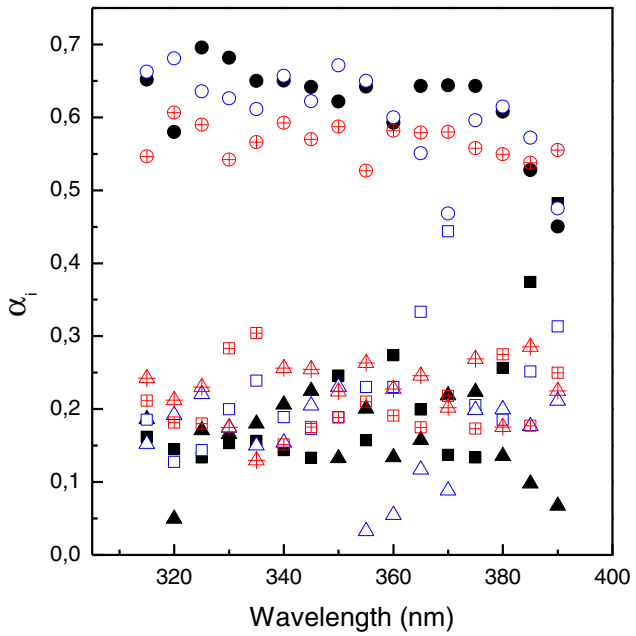

Fig. 15 Fluorescence lifetimes pre-exponentials of α_1-acid glycoprotein in 100 mM PBS buffer pH 7, measured at λ_{ex}=265 nm (*black*), 280 nm (*blues*) and 295 nm (*red*). α_1 are represented by the *squares*, α_2 by the *circles* and α_3 by the *triangles*

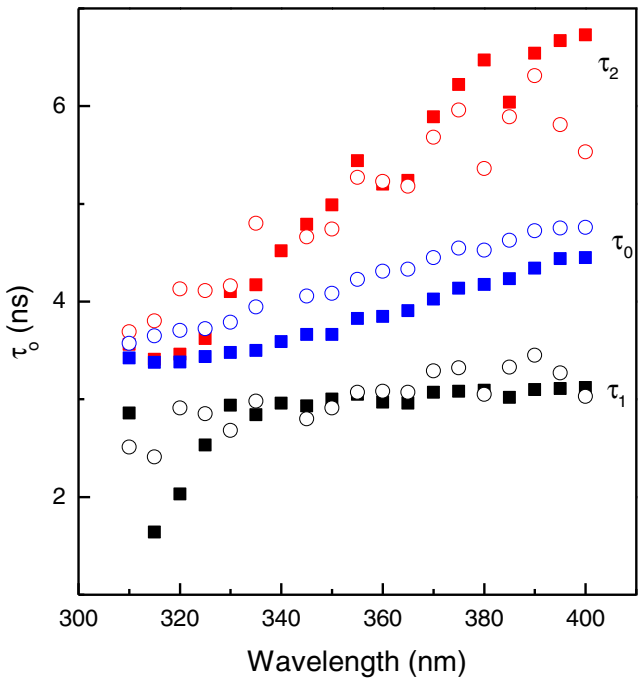

Fig. 16 Fluorescence lifetimes of indole in water (*squares*) and ethanol (*circles*) as a function of emission wavelength. λ_{ex}=296 nm

Fluorescence Intensity Decay Parameters of Indole in Water and Ethanol

Tryptophan structure is mainly formed by indole ring. Thus, it is normal in a first approximation to consider that indole ring is responsible for tryptophan fluorescence properties. However, if this is the case, then indole fluorescence lifetimes should be affected by the solvent and the environment in the same way as tryptophan is.

Figure 16 displays fluorescence lifetimes variation of indole dissolved in water and in ethanol at different emission wavelengths. Results show that intensity decays in both solvents occur with two lifetimes. Both lifetimes are identical in the two solvents. Values of these two lifetimes are different from those measured for L-Trp in water or ethanol or for tryptophan residue in proteins. One can notice also that in ethanol, indole emission does not occur with three lifetimes but only two. Thus, interactions between indole and hydrophobic/hydrophilic chemical properties of ethanol, if they exist, do not yield a third fluorescence lifetime. Therefore, indole cannot be used as a model to understand origin of fluorescence lifetimes of tryptophan, whether in solution or in proteins (see also Discussion).

Figure 17 displays fluorescence lifetimes pre-exponentials of indole in both water and ethanol at different emission wavelengths. In both solvents, pre-exponential variation is identical and is completely different from that obtained for L-Trp in water. Also, values of Tryptophan emitting populations differ completely from those of indole. Thus, in water, ethanol, or proteins, populations responsible of tryptophan emission are totally different from indole emitting populations.

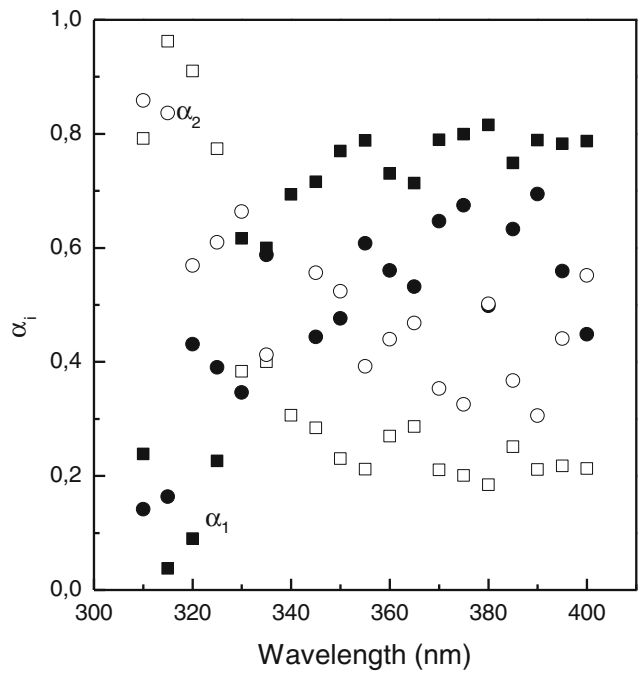

Fig. 17 Lifetime pre-exponentials variations with emission wavelength of indole in water (*squares*) and in ethanol (*circles*). α_1: *closed symbols*, α_2: *open symbols*

Discussion

The present paper displays data indicating that tryptophan in proteins emits in general with three lifetimes. Two lifetimes are identical or very close to those found for L-Trp in water and to two of the three lifetimes found for L-Trp in ethanol. Thus, these two lifetimes are inherent to the tryptophan structure and result from two sub-structures formed in the excited state. Each sub-structure is formed by tryptophan backbone along with a specific electronic distribution. The third lifetime is generated by interactions existing between Trp residue with neighboring amino acids as the result of protein folding. Also, since fluorescence lifetimes and populations are independent of excitation wavelengths and thus of excitation energy, then these sub-structures are pre-existing in the excited state and are put into evidence upon excitation. Also, it is always the same tryptophan populations that are emitting and not random ones. Therefore, in proteins as well as when tryptophan is free in a solvent, internal specific configurations do exist in the excited state and these configurations are the only ones which can be excited and/or able to emit. From a protein to another, in the native state, surrounding tryptophan residue environment affects organization of emitting populations.

Tryptophan absorbs with two transitions $S_o \rightarrow {}^1L_a$ and $S_o \rightarrow {}^1L_b$. Recently, we have measured fluorescence lifetimes and we have 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. 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 absence of progesterone, recorded fluorescence lifetimes were equal to 0.50386±0.05425 and 2.775±0.01158 ns ($\chi 2$=1.0056) and in presence of progesterone, recorded lifetimes were equal to 0.46024±0.0330 and 2.752±0.0111 ns ($\chi 2$=1.0107). Both short and long lifetimes do not vary significantly in presence of progesterone [42]. The same experiments performed on L-Trp in ethanol [42] or on proteins [40] showed that tryptophan emission occurs with the same three lifetimes in presence of both $S_o \rightarrow {}^1L_a$ and $S_o \rightarrow {}^1L_b$ transitions or in presence of $S_o \rightarrow {}^1L_b$ transition, only. These data are in good agreement with the fact that fluorescence lifetimes values and numbers are not correlated to the excitation energy. The latter excites the tryptophan, putting into evidence the sub-structures (each formed by the tryptophan backbone along with its specific electronic distribution) which are able to fluoresce.

Let us discuss our results in terms of rotamers model. The purpose of this discussion is to find out how far we can go in the application of this model. Three lifetimes are observed in most of the studied proteins. Thus, presence of these lifetimes is independent of the structure surrounding the tryptophan residue(s), of the proteins tertiary structure and of proteins dynamic properties. In other terms, presence of protein matrix around Trp residues does not play any fundamental role in the fluorescence lifetimes of Trp residues. In this case, protein backbone has no or non significant effect on the fluorescence lifetimes of proteins. In other terms, rotamers model cannot explain the presence of three lifetimes in proteins.

Also, attributing fluorescence lifetimes to conformers, means that, in proteins with more than one tryptophan residue, we should have a combination of conformers that yields three fluorescence lifetimes.

Rotamers model cannot explain the absence of significant differences in fluorescence lifetimes for example between Cyclophilin B wild type and the mutant $CyPBw_{128A}$. One can consider fluorescence lifetimes as the result of Trp residues interaction with their microenvironments. However, since fluorescence lifetimes do not vary significantly between wild type protein and the mutant, one should consider the possibility of having, around the Trp residues, a common identical protein structure responsible of the three measured fluorescence lifetimes.

Also, N-acetyl-L-tryptophanamide (NATA) and non-terminal tryptophan residues in proteins display the same structures in the ground state. Nevertheless, NATA emits with a single fluorescence lifetime while tryptophan residues emission is bi- or multiexponential. This clearly means that the structures generating fluorescence lifetimes are in the excited state and differ from a fluorophore to another. The same conclusion can be drawn from results obtained with indole. In fact, indole dissolved in water or ethanol emits with two lifetimes which are equal in both solvents and which completely differ from those measured for L-Trp in water or in ethanol. Also, populations of emitting species in indole are not equal to those observed for tryptophan. For all these reasons, although tryptophan, NATA and indole share a common structure, indole ring, fluorescence of tryptophan cannot be studied by making parallels with fluorescence of NATA and/or indole.

In conclusion, the present work allows, for the first time and by describing straight forwarded experiments, to prove that tryptophan emission lifetimes are inherent to the fluorophore itself and that the third fluorescence lifetime observed in proteins is generated by the interaction between Trp residue(s) and neighboring amino acids. Protein

and solvent relaxation do not generate the third lifetime and one should consider tryptophan structure and properties as different from those of NATA and indole. Finally, as it is the case for tryptophan free in solution, tryptophan in proteins cannot be described as a simple structure with one electronic distribution, but should be described as composed by three different substructures, each composed by the tryptophan backbone with its specific electronic distribution. This means that, in the excited states, a protein can also be described as a combination of three different substructures, each playing a specific role in the energy distribution within the protein matrix [43].

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