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ORIGINAL PAPER

New Insights in the Interpretation of Tryptophan Fluorescence

Origin of the Fluorescence Lifetime and Characterization of a New Fluorescence Parameter in Proteins: The Emission to Excitation Ratio

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Abstract Origin of tryptophan fluorescence is still up to these days a quiz which is not completely solved. Fluorescence emission properties of tryptophan within proteins are in general considered as the result of fluorophore interaction within its environment. For example, a low fluorescence quantum yield is supposed to be the consequence of an important fluorophore–environment interaction. However, are we sure that the fluorophore has been excited upon light absorption? What if fluorophore excitation did not occur as the result of internal conformation specific to the fluorophore environment? Are we sure that all absorbed energy is used for the excitation process? Fluorescence lifetimes of Trp residues are considered to originate from rotamers or conformers resulting from the rotation of the indole ring within the peptide bonds. However, how can we explain the fact that in most of the proteins, the two lifetimes 0.5 and 3 ns, attributed to the conformers, are also observed for free tryptophan in solution? The present work, performed on free tryptophan and tyrosine in solution and on different proteins, shows that absorption and excitation spectra overlap but their intensities at the different excitation wavelengths are not necessarily equal. Also, we found that fluorescence emission intensities recorded at different excitation wavelengths depend on the intensities at these excitation wavelengths and not on the optical densities. Thus, excitation is not equal to absorption. In our interpretation of the data, we consider that absorbed photons are not necessary used only for the excitation, part of them are used to reorganize fluorophore molecules in a new state (excited structure) and another part is used for the excitation process. A new parameter that characterizes the ratio of the number of emitted photons over the real number of photons used to excite the fluorophore can be defined. We call this parameter, the emission to excitation ratio. Since our results were observed for fluorophores free in solution and present within proteins, structural reorganization does not depend on the protein backbone. Thus, fluorescence lifetimes (0.5 and 3 ns) observed for tryptophan molecules result from the new structures obtained in the excited state. Our theory allows opening a new way in the understanding of the origin of protein fluorescence and fluorescence of aromatic amino acids.

Keywords Tryptophan · Emission spectrum · Excitation spectrum · Emission to excitation ratio

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Introduction

Fluorescence is characterized by different parameters such as emission and excitation spectra, intensity, lifetime, anisotropy and quantum yield. Since only part of the energy absorbed by the fluorophore is reemitted as photons (fluorescence), fluorescence quantum yield is defined as:

$$\Phi_F = \frac{\text{number of photons emitted by the fluorophore}}{\text{number of photons absorbed by the fluorophore}} \quad (1)$$

In order to explain the meaning of the emission to excitation ratio, we need to focus on (1) the relation that exists between the fluorescence intensity and the optical density at the excitation wavelength, (2) the relation that exists between absorption and excitation spectra of the fluorophore and (3) the relation that exists between the fluorescence intensity and the excitation spectrum. At dilute

solutions, i. e., at low fluorophore concentrations, fluorescence emission intensity I_f is related to the quantum yield Φ_F and to the optical density by the equation

$$I_f = 2.3 I_o \varepsilon_{(\lambda_{ex})} cl \Phi_F \quad (2)$$

where I_o is the lamp intensity and the product $\varepsilon_{(\lambda_{ex})} cl$ is the optical density at the excitation wavelength. Thus, according to Eq. 2, since ε is wavelength dependent, fluorescence intensity will be also wavelength dependent. Fluorescence intensity is proportional to the molar extinction coefficient or/and to the optical density. Thus, in this case, normalizing fluorescence spectra for the same optical density at the excitation wavelength should yield identical spectra. We are considering here the case where corrections for the inner filter effect have been already performed or when they are not necessary as the result of the low optical densities at the excitation and emission wavelengths.

The second parameter that interests us here is the fluorescence excitation spectrum. It is stated that excitation is equivalent to absorption since upon absorption the molecule reaches an excited state S_n. For a pure product and in the absence of any interference with other molecules in the solution, excitation and absorption spectra of a fluorophore should, in theory, be identical. In the present case, we are dealing with excitation spectra that are corrected for technical and instrumental problems.

In the work described in this manuscript, we have compared the fluorescence excitation and absorption spectra of free tryptophan and tyrosine in solution and of ten different proteins. For all the analyzed samples, we have found that absorption and excitation spectra do not match. In fact, the spectra look very close but at many wavelengths, they do not display identical intensities.

Also, we have recorded the fluorescence emission spectra of all the products at different excitation wavelengths, then, we have plotted the spectra after normalization to a same optical density. We have found that there is no correlation between emission intensities and optical densities at the excitation wavelengths. In other terms, we did not obtain superimposable emission spectra after normalizing the intensities for the same optical density. However, we have found that emission spectra are proportional to the excitation spectrum intensity at the corresponding excitation wavelength. Our results are interpreted in terms of emission to excitation ratio, a parameter that characterizes the ratio of the number of emitted photons over the real number of photons used to excite the fluorophore. In our interpretation of the data, we consider that absorbed photons are not necessary used for the excitation, part of them are used to reorganize the fluorophore molecules in a new state (excited structure) and another part is used for the excitation process.

In the present work, we show the results obtained for one (Bovine serum albumin) of the ten proteins we have studied and for L-Tryptophan and L-Tyrosine free in solution. Our theory does not concern one or two proteins but can be generalized to all proteins. Also, our theory allows opening a new way in the understanding of the origin of protein fluorescence and fluorescence of aromatic amino acids.

Materials and methods

L-tryptophan, L-tyrosine, bovine β-lactoglobulin, deoxyribonuclease I, bovine serum albumin, human serum albumin, trypsin and creatine phosphokinase were from Sigma.

Helicase was from Pharindustrie, Clichy, France and rat albumin fraction V was from Cappel Laboratories, USA.

Serotransferrin and human α_1-acid glycoprotein were prepared by Professor H. Debray (actually retired) of University of Lille 1.

Absorbance data were obtained with a Shimadzu MPS-2000 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 5 nm. The quartz cuvettes had optical pathlengths equal to 1 and 0.4 cm for the emission and excitation wavelengths, respectively. Fluorescence spectra were corrected for the background intensities of the buffer solution. Observed fluorescence

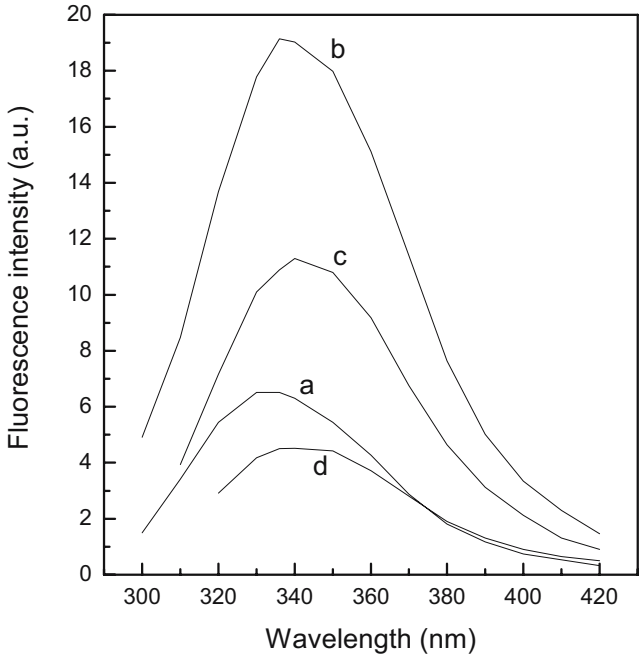

Fig. 1 Fluorescence emission spectra of bovine serum albumin at four excitation wavelengths. **a** λ_{ex}=260 nm. **b** λ_{ex}=280 nm. **c** λ_{ex}=295 nm. **d** λ_{ex}=300 nm. The optical densities for 0.4 cm pathlength are respectively 0.0236, 0.0388, 0.008 and 0.0028

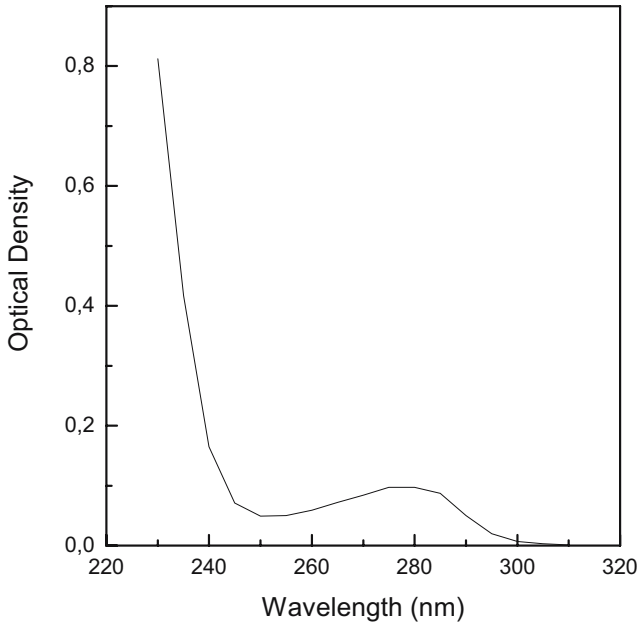

Fig. 2 Absorption spectrum of Bovine serum albumin

intensities were first corrected for the dilution, then corrections were made for the inner filter effect, although weak, as described [1, 2].

Fluorescence lifetime measurements were obtained with a Horiba Jobin Yvon FluoroMax-3-P, using the time correlated single photon counting method. Excitation was performed at 295 nm with a nanoLED and emission was observed at 330 nm for the proteins and at 350 nm for free L-Trp in twice distilled water.

All experiments on proteins were performed at 20°C in 10 mM phosphate buffer and 0.143 M NaCl buffer (PBS buffer), pH 7, while experiments on free L-Trp and L-Tyr were performed in twice distilled water.

Results

Figure 1 displays the fluorescence emission spectra of bovine serum albumin (BSA) obtained at four excitation wavelengths, 260 (a), 280 (b), 295 (c) and 300 nm (d). Emission peak is not identical for the four spectra, it shifts to higher wavelengths with the excitation wavelength. This red-edge excitation spectra shift can be explained by the absence of any residual motions, in the nanosecond time scale, around Trp residues of BSA [3–5]. The presence or the absence of a red-shift in the emission peak with the excitation wavelength is not the subject of this paper and thus will not be discussed.

The spectra obtained at the four excitation wavelengths display different intensities at the emission peaks (Fig. 1). The weakest intensity corresponds to the excitation wavelength of 300 nm and the highest intensity to 280 nm excitation wavelength. Since the spectra displayed are corrected for the inner filter effect, they are interpreted by the classical fluorescence theory as the result of the absorption at the excitation wavelength. Therefore, on the basis of this theory, one should conclude that the highest optical density is that at 280 nm followed by 295, 260 and 300 nm.

Figure 2 displays absorption spectrum of BSA. The optical density values are equal to 0.097 (280 nm), 0.020 (295 nm) 0.059 (260 nm) and 0.007 (300 nm). Thus, optical density at 260 nm is three times higher than that recorded at 295 nm. Therefore, if emission intensity is proportional to optical density at the excitation wavelength, it should be higher when excitation was performed at 260 nm than at 295 nm. Normalizing the emission spectra obtained in Fig. 1 for the optical density at 280 nm yields spectra displayed in Fig. 3. We can notice that the four spectra do not have identical intensities and that there is no direct correlation between intensity value at the emission peak with the optical density of the excitation wavelength.

Figure 4 displays the fluorescence excitation spectrum of BSA ($\lambda_{em} = 360$ nm). Although the general feature of this spectrum looks like the absorption spectrum (Fig. 2), there is an important difference between them: the intensity of the excitation spectrum does not vary with the wavelengths in the same way than the optical density along the absorption spectrum. An example, the optical density at 260 nm is higher at 295 nm than at 260 nm while the excitation intensity is higher at 295 nm than at 260 nm.

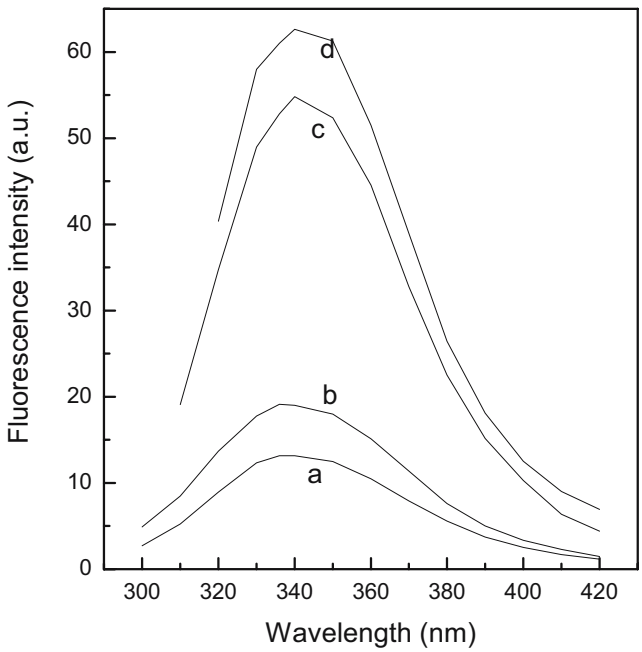

Fig. 3 Fluorescence emission spectra of bovine serum albumin normalized at the optical density at 280 nm. **a** $\lambda_{ex} = 260$ nm. **b** $\lambda_{ex} = 280$ nm. **c** $\lambda_{ex} = 295$ nm. **d** $\lambda_{ex} = 300$ nm

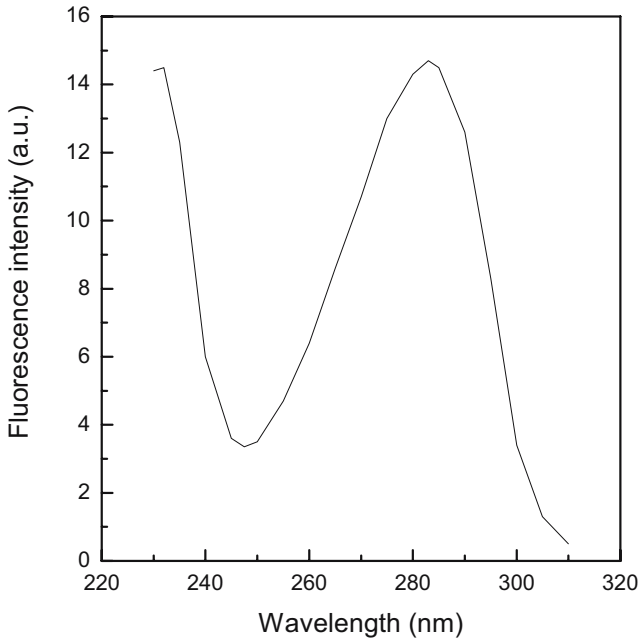

Fig. 4 Fluorescence excitation spectrum of bovine serum albumin. $\lambda_{em} = 360$ nm

Normalizing absorption and excitation spectra at 280 nm allows us to see the differences between them (Fig. 5). Excitation peak is shifted to the red compared to the absorption peak. It is clear that the optical density at 260 nm is higher than that at 295 nm, while in the excitation spectrum, we have the opposite, the intensity at 295 nm is higher than that at 260 nm. Normalizing the emission spectra obtained in Fig. 1 for the excitation intensity at 280 nm yields the spectra displayed in Fig. 6. The four normalized spectra display very close or equal emission intensities. Thus, fluorescence emission intensities obtained at different excitation wavelengths (Fig. 1) are directly proportional to the intensity of the excitation wavelength of the excitation spectrum.

The same results (spectra not shown) were obtained for human serum albumin, serotransferrin, β-lactoglobulin, human α_1-acid glycoprotein, creatine phosphokinase, deoxyribonuclease I, rat albumin, helicase and trypsin. For all the studied proteins, the results indicate that fluorescence emission intensities are not proportional to the optical densities of the excitation wavelengths but to the excitation intensities of the excitation spectrum. In proteins, although we were observing the emission from Trp residues, some excitation wavelengths such as 260 and 280 nm allow to excite also the other two aromatic amino acids, phenylalanine and tyrosine residues. Therefore, in order to check whether our data result from the interference between the different amino acids and the protein backbone or if they originate from the amino acids themselves, we performed the same type of experiments on L-tryptophan and L-tyrosine free in solution.

Figure 7 displays absorption spectrum of L-Trp in twice distilled water. Optical densities measured with pathlength of 1 cm at 260, 280, 295 and 300 nm are equal to 0.09, 0.134, 0.025 and 0.003, respectively. Thus, if fluorescence intensity is proportional to the optical density at the excitation wavelength, one should expect to obtain an

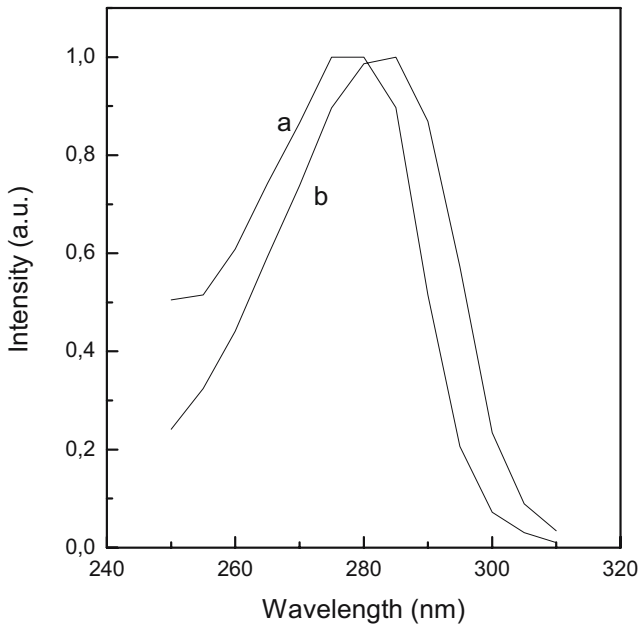

Fig. 5 Normalized absorption (**a**) and excitation (**b**) spectra of bovine serum albumin

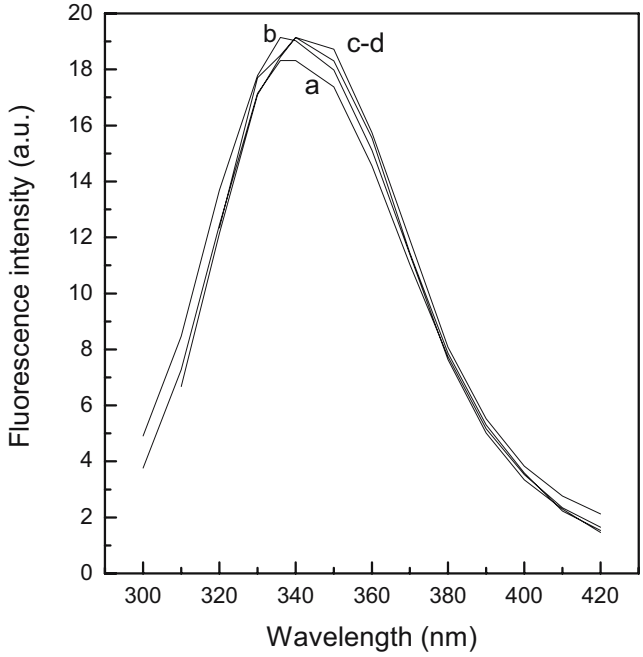

Fig. 6 Fluorescence emission spectra of bovine serum albumin normalized for the excitation intensity at 280 nm. **a** $\lambda_{ex} = 260$ nm. **b** $\lambda_{ex} = 280$ nm. **c** $\lambda_{ex} = 295$ nm. **d** $\lambda_{ex} = 300$ nm

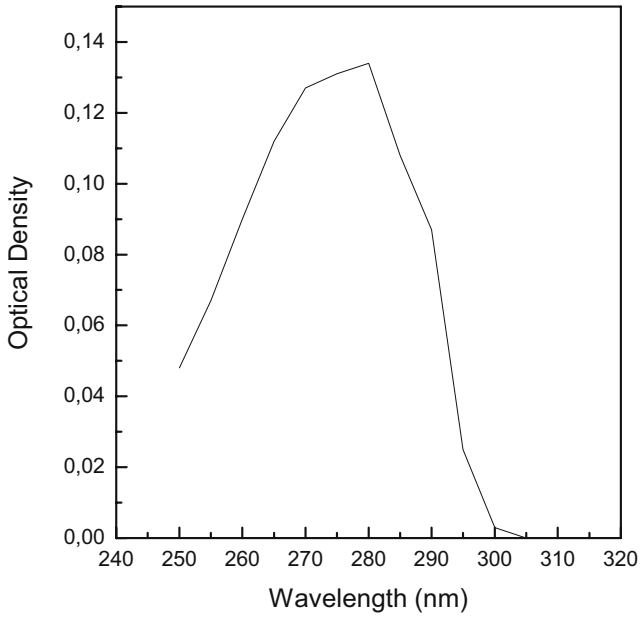

Fig. 7 Absorption spectrum of L-tryptophan in water buffer

emission spectrum intensity with $\lambda_{ex} = 260$ nm, three times that obtained with $\lambda_{ex} = 295$ nm. Also, one would not expect to observe any significant fluorescence with $\lambda_{ex} = 300$ nm as the result of the weak optical density, close to zero.

Figure 8 displays fluorescence emission spectra of L-Trp recorded at four excitation wavelengths, 260 (a), 280 (b), 295 (c) and 300 nm (d). The results clearly indicate that the

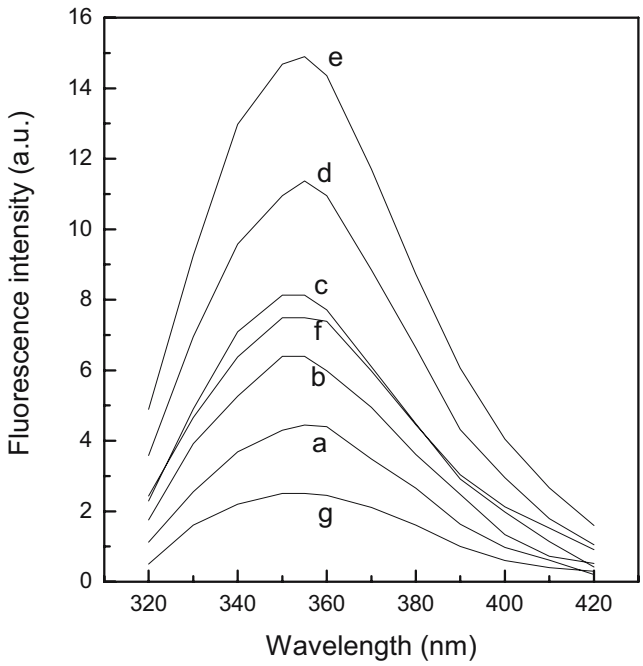

Fig. 8 Fluorescence emission spectra of L-tryptophan in water buffer as function of λ_{ex} 250 nm (**a**), 255 nm (**b**), 260 nm (**c**), 265 nm (**d**), 280 nm (**e**), 295 nm (**f**) and 300 nm (**g**)

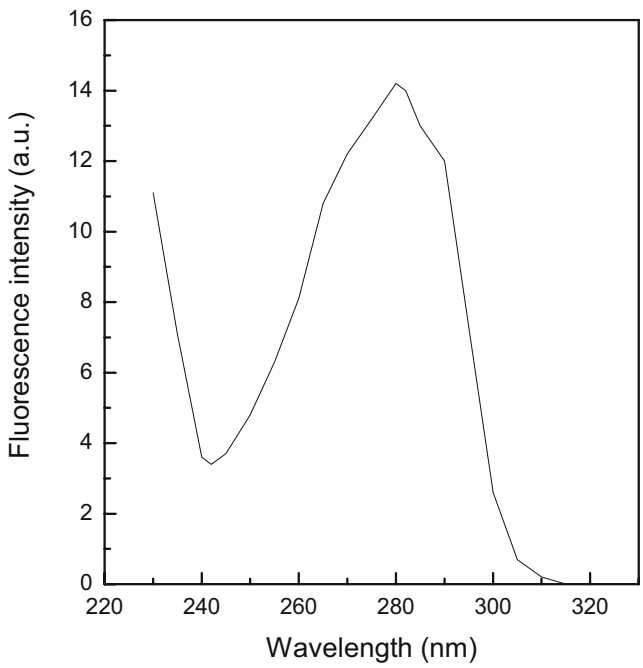

Fig. 9 Fluorescence excitation spectrum of L-tryptophan in water buffer. $\lambda_{em} = 360$ nm

fluorescence intensities are not proportional to the optical densities at the excitation wavelengths. The most significant result is the fluorescence spectrum recorded at $\lambda_{ex} = 300$ nm. It is clear that a very low optical density such as 0.003 should not yield such a significant fluorescence spectrum. Thus, the spectrum recorded with $\lambda_{ex} = 300$ nm does not reflect the tryptophan absorption at this wavelength. Figure 9 displays the fluorescence excitation

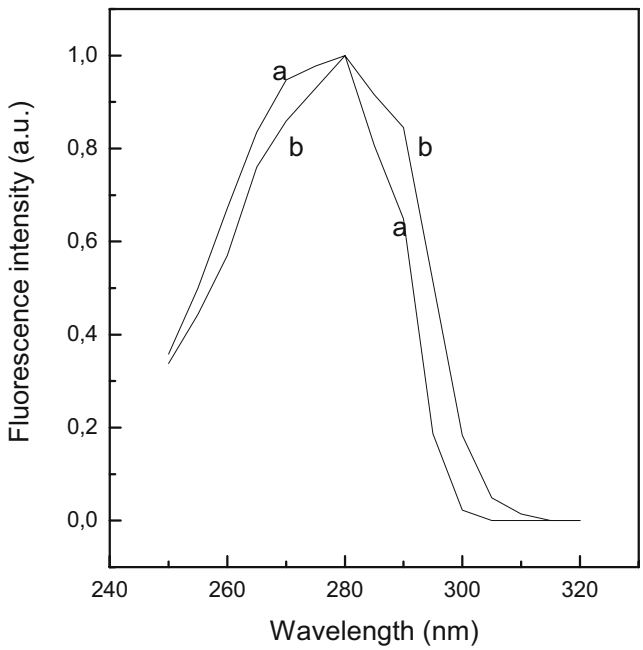

Fig. 10 Normalized absorption (**a**) and excitation (**b**) spectra of L-Trp in water. $\lambda_{em} = 360$ nm

spectrum of L-Trp ($\lambda_{em} = 360$ nm). One can notice that excitation intensity at 295 nm is higher than that at 260 nm and the intensity at 300 nm is not equal to zero but is 5.5 times lower than the intensity measured at 280 nm. The normalized absorption and excitation spectra of L-Trp are displayed in Fig. 10. Although the two spectra show similar shape, they do not superimpose. Figure 11 displays the normalized emission spectra of L-Trp for the optical density at 280 nm. The results clearly indicate that emission spectra are not proportional to the optical densities at the excitation wavelengths. However, normalization for the excitation intensity at 280 nm yields fluorescence emission spectra of equal or very close intensities (Fig. 12). The fluorescence emission spectra are proportional to the excitation intensity of the excitation wavelength.

Figure 13 displays the emission spectra of L-tyrosine in PBS buffer obtained at 250 (a), 255 (b), 260 (c), 265 (d), 270 (e) and 280 nm (f). Optical densities measured with pathlength of 1 cm are 0.02, 0.023, 0.027, 0.033, 0.039 and 0.038, respectively. Thus, one should, in principle, expect to observe fluorescence emission spectra that are proportional to the optical densities at the excitation wavelength. Since excitation was performed with 0.4 cm pathlength, the real optical densities are even weaker. Figure 13 indicates that fluorescence intensity is not proportional to the optical density at the excitation wavelength. Even when the optical densities are identical or close such as 0.02 and 0.023 or 0.039 and 0.038, the emission intensities are not equal to each other.

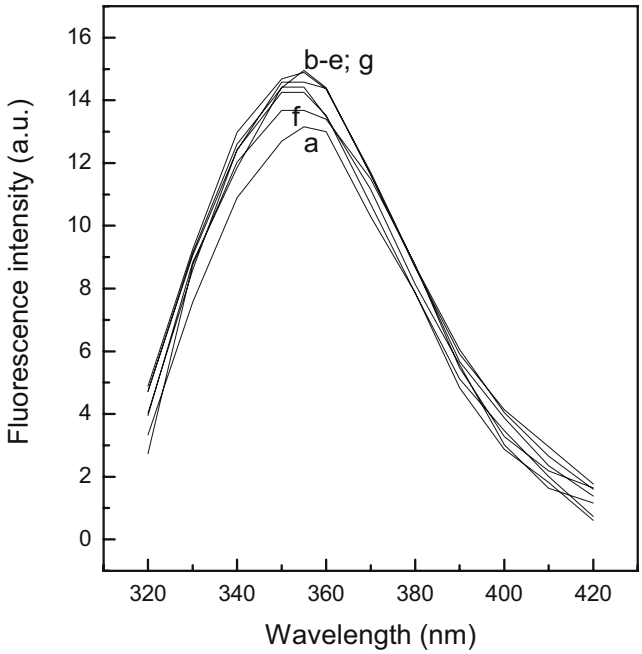

Fig. 12 Fluorescence spectra of L-tryptophan in water as function of λ_{ex}. 250 nm (**a**), 255 nm (**b**), 260 nm (**c**), 265 nm (**d**), 280 nm (**e**), 295 nm (**f**) and 300 nm (**g**). The spectra are normalized for the intensity at 280 nm of the excitation spectrum

Normalization of the fluorescence emission spectra for the optical densities at 280 nm does not yield fluorescence spectra of equal intensities (Fig. 14) confirming the fact that the emission spectra are not proportional to the optical

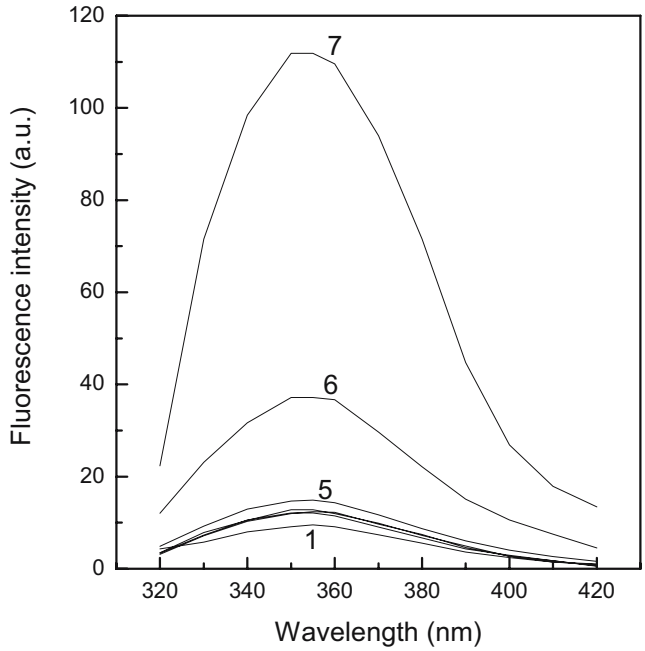

Fig. 11 Fluorescence emission spectra of L-tryptophan in water as function of λ_{ex}. (**1**) 250 nm, (**2**) 255 nm, (**3**) 260 nm, (**4**) 265 nm, (**5**) 280 nm, (**6**) 295 nm et (**7**) 300 nm. The spectra are normalized for the optical density at 280 nm. Spectra 2–4 have not equal intensities

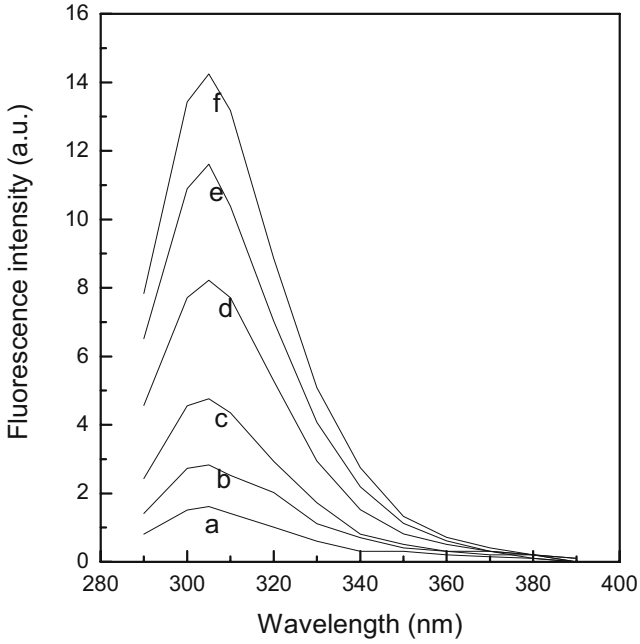

Fig. 13 Fluorescence emission spectra of L-tyrosine in water at different excitation wavelengths. λ_{ex}. 250 nm (**a**), 255 nm (**b**), 260 nm (**c**), 265 nm (**d**), 270 nm (**e**) and 280 nm (**f**). Optical densities measured with pathlength of 0.4 cm are 0.008, 0.0092, 0.027, 0.0108, 0.0156 and 0.0152, respectively

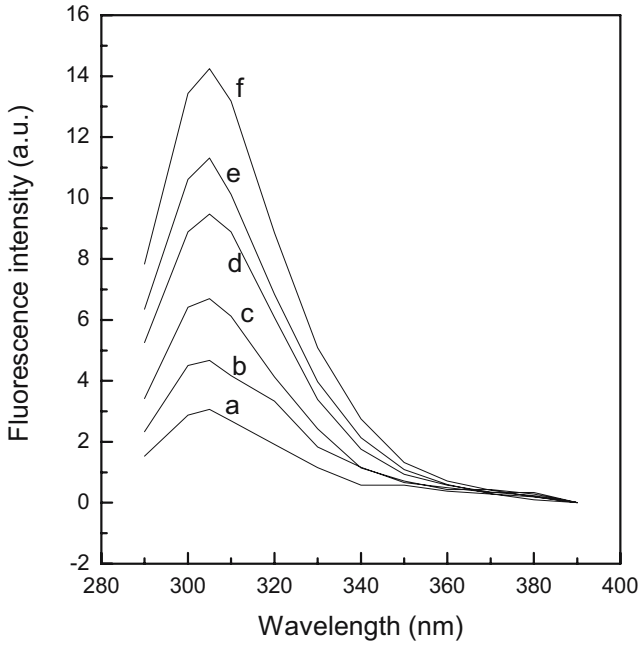

Fig. 14 Fluorescence spectra of L-tyrosine in water buffer as function of λ_{ex}. (**a**) 250 nm, (**b**) 255 nm, (**c**) 260 nm, (**d**) 265 nm, (**e**) 270 nm and (**f**) 280 nm. The spectra are normalized for the optical density at 280 nm. The result shows that the emission spectra vary with the optical density at λ_{ex}

densities at the excitation wavelength. Figure 15 displays the fluorescence excitation spectrum of L-tyrosine (λ_{em} = 310 nm). The fluorescence intensities are equal to 1.5, 2.8, 4.6, 7.4, 10.4 and 13.6 at 250, 255, 260, 265, 270 and 280 nm, respectively. We notice that the fluorescence intensities at the six wavelengths are not equal and thus

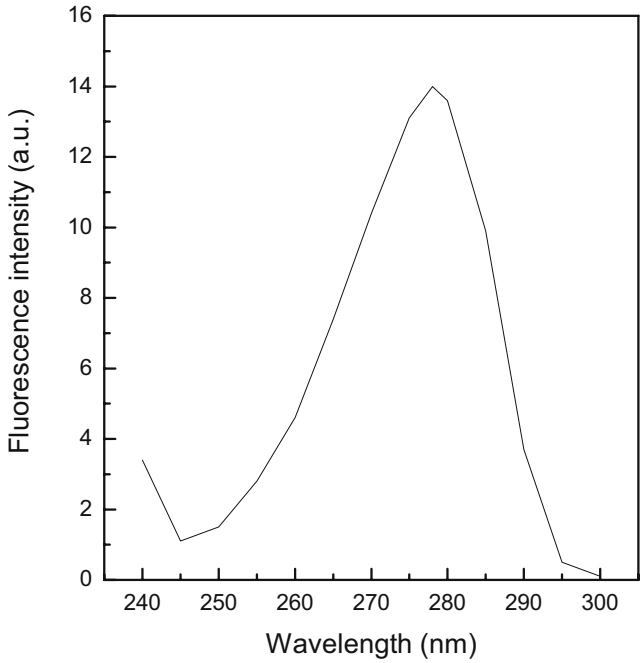

Fig. 15 Fluorescence excitation spectrum of L-tyrosine in water

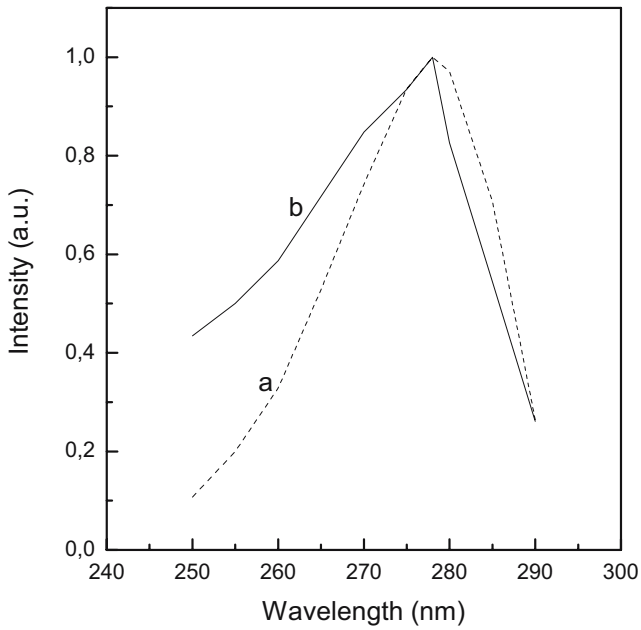

Fig. 16 Excitation (**b**) and absorption (**a**) spectra of L-tyrosine in water buffer

absorption and excitation spectra of L-tyrosine are not identical (Fig. 16). Normalization of the spectra obtained in Fig. 13 for the excitation intensity at 280 nm yields emission spectra of equal or very close intensities (Fig. 17). Therefore, fluorescence emission spectra of L-tyrosine are proportional to the intensity of the excitation spectrum at the excitation wavelength. This result is identical to that observed for L-tryptophan in solution and

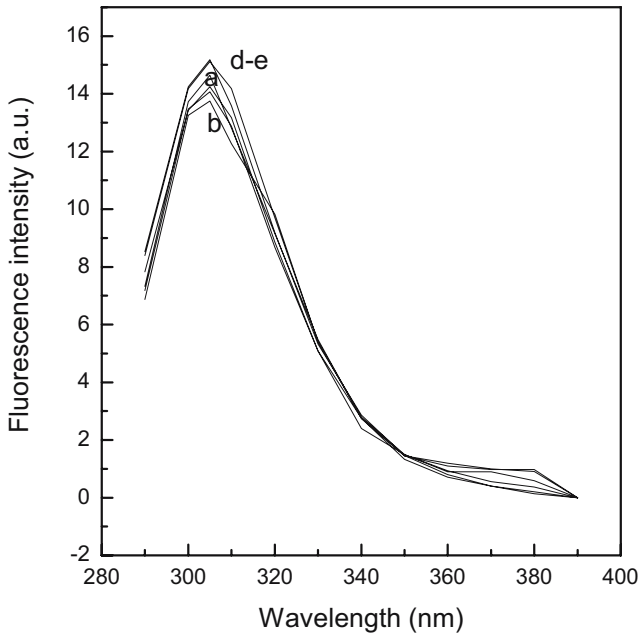

Fig. 17 Fluorescence spectra of L-tyrosine in water as function of the excitation wavelength: 250 nm (**a**), 255 nm (**b**), 260 nm (**c**), 265 nm (**d**), 270 nm (**e**) and 280 nm (**f**). The spectra are normalized for the intensity at 280 nm of the excitation spectrum

for the analyzed proteins, confirming the fact that in proteins the fluorescence emission spectra of aromatic amino acids is proportional to the intensity of the excitation spectrum at the excitation wavelength. The protein matrix around the fluorophores is not responsible of this dependence.

Discussion

It is common to read in the literature that absorption is equal to excitation. However, the results described in this paper show for the first time that absorption is not necessarily equivalent to excitation. Also, one can say that excitation spectrum yields information and details that can be hidden in the absorption spectrum especially at wavelengths where optical densities are very small.

The results described in this manuscript are dependent on whether the emission spectra are corrected for the output of the lamp, i.e., for the excitation sources intensities at the different excitation wavelengths. The LS5B provides corrected excitation spectra for the output of the lamp. Nevertheless, we compared fluorescence excitation spectrum of L-tryptophan dissolved in twice distilled water and recorded with the LS5B fluorometer, with the standard excitation spectrum of tryptophan dissolved in tri-distilled water [6]. We found that the two excitation spectra overlap (not shown).

If the spectra are not corrected for the output of the lamp, the relative emission peak intensities should approximate the excitation intensities at the four wavelengths. These intensities are predominately a function of the xenon arc lamp. For such a lamp, the excitation intensity as a function of wavelength increases when going from 260 to 300 nm. For the output spectrum of the xenon arc lamp, we found that intensities, given in relative values, are 0.417, 1, 1.46 and 1.917 at 260, 280, 295 and 300 nm, respectively. In this case, if the spectra are not corrected for the output of the lamp, we should have emission spectra proportional to the lamp intensities and thus one should record emission intensity at λ_{ex} equal to 300 nm higher than that recorded with the other three excitation wavelengths. We notice that this is not the case. In fact, in the excitation spectrum, the intensity at 300 nm is much lower than that at 295 nm and the emission spectra obtained are proportional to the intensities of the excitation wavelengths. Therefore, all the fluorescence spectra displayed in the present work are corrected for the output of the lamp.

The data displayed in the present work show that fluorescence emission intensities of aromatic amino acids free in solution or present in proteins are proportional to the excitation spectrum at the excitation wavelength but not to the optical density at the excitation wavelength. Thus, Eq. 2, when applied to aromatic amino acids fluorescence studies, is not correct. Similarity between the results obtained with free tryptophan in solution and proteins is clear evidence that within proteins, these results are not the effects of cysteine or peptide bonds absorption. Therefore, energy absorbed by the fluorophore, characterized by the optical density parameter at the excitation wavelength, does not reflect necessarily the effective energy used to excite the fluorophore. Absorbed energy helps to reorient fluorophore structure within the excited state, is dissipated in the medium (solvent and/or protein matrix) and is used to excite the fluorophore molecules. Therefore, excitation is not equal to absorption. This is put into evidence by the fact that a difference does exist between excitation and absorption spectra and mainly to the fact that emission spectra recorded at different excitation wavelengths can be normalized for the intensities of the excitation spectrum and not for the optical densities.

Difference between absorption and excitation spectra can be detected in many spectra already published in the literature, but it has been never mentioned. The reason for that is the fact that absorption is considered equal to excitation. For example, Teale published in 1960 [7] absorption and excitation spectra of different proteins. We compared the spectra together and we found that the intensities differ at many wavelengths and thus excitation and absorption spectra do not superimpose although their shape is similar. An example, the ratio $\frac{I_{280nm}}{I_{295nm}}$ for the absorption and the excitation spectra are equal to 1.88 and 2.83, respectively. Also, we found that this ratio is not the same for all the excitation spectra.

Another example, excitation and absorption spectra of fluorescein were displayed by Lakowicz [1]. One can notice that if the global shapes of the two spectra are similar, the intensities at many wavelengths are not equal.

We published absorption and excitation spectra of quinine present in Tonic Soda. Although the two spectra are very similar, their intensities at many wavelengths are not equal [2].

Thus, since absorption has always been considered equivalent to excitation, the differences between absorption and excitation spectra have been minimized. This fact is evident when we are working with dilute solutions, although necessary for fluorescence experiments. For example, is it possible to accept the idea that optical density (0.007) observed at 300 nm for BSA has generated the emission spectrum observed in Fig. 1? Does an optical density equal to 0.007 mean something? However, in the excitation spectrum, the intensity recorded at $\lambda_{ex} = 300$ nm is not equal to zero (Fig. 3).

Excitation spectrum reflects the real energy transitions allowing the fluorophore to reach the excited state. Thus, only excitation spectrum indicates the transition energies,

which excite a fluorophore. Since energy used for excitation is not necessarily reemitted as photons, a quantum yield does exist. This parameter, that we are going to baptise the emission to excitation ratio and symbolize as R_{ex}^{em} is equal to the energy of the emitted light over the energy used to excite the fluorophore:

$$R_{ex}^{em} = \frac{\text{number of emitted photons}}{\text{number of photons used to excite the fluorophore}} \tag{3}$$

Determining the value of R_{ex}^{em} for a fluorophore can be obtained by comparison with another fluorophore of known R_{ex}^{em}

$$I_{F(sample)} = I_{ex(sample)} \times R_{ex^{sample}}^{em} \tag{4}$$

$$I_{F(reference)} = I_{ex(reference)} \times R_{ex^{reference}}^{em} \tag{5}$$

$$\frac{I_{F(sample)}}{I_{F(reference)}} = \frac{I_{ex(sample)} \quad R_{ex^{sample}}^{em}}{I_{ex(reference)} \quad R_{ex^{reference}}^{em}} \tag{6}$$

$$\Rightarrow$$

$$R_{ex^{sample}}^{em} = \frac{I_{F(sample)} \times I_{ex(reference)} \quad R_{ex^{reference}}^{em}}{I_{F(reference)} \times I_{ex(sample)}} \tag{7}$$

Integrating the fluorescence emission intensities along the emission spectrum yields:

$$R_{ex^{sample}}^{em} = \frac{\sum I_{F(sample)} \times I_{ex(reference)} \quad R_{ex^{reference}}^{em}}{\sum I_{F(reference)} \times I_{ex(sample)}} \tag{8}$$

where I_{ex} is the fluorescence intensity of the excitation spectrum at the excitation wavelength.

For simplicity, taking free L-Trp as reference with a value of R_{ex}^{em} equal to 1, the values of R_{ex}^{em} for the different studied proteins are shown in Table 1. The result is in clear opposition with that found when the classical quantum yield Φ is applied. In fact, in general, the value of Φ found for proteins is lower than that of Trp residue free in solution. This is explained as the result of energy loss of the excited state via different ways: dissipation within the medium, energy transfer by collisions with other amino acids, excited state proton transfer and excited state electron transfer from the indole ring to a neighboring electrophilic amino acid. However, in all these studies, absorption is considered as the phenomenon that excites the fluorophore and indeed in this case, the low value of Φ is considered as the result of the different deexcitation processes. A conclusion that is not necessarily correct since Φ is not the correct parameter describing the relation between fluorophore excitation and emission.

Fluorescence quantum yield Φ compares the emitted photons to the absorbed ones. However, there is no indication up to now that all the absorbed photons participate in the excitation process. Also, another question should be asked: are we sure that probability of excitation is always 100% effective each time a fluorophore absorbs light energy? Up to now, one is used to consider the absence of fluorescence from a fluorophore as the result of excited state energy dissipation into the medium via different processes that compete with fluorescence. However, can't we consider the case where fluorophore does not emit simply because it was not excited? The lack of excitation would be the result of structural rearrangement within the fluorophore microenvironment. The emission to excitation ratio R_{ex}^{em}, to the difference of Φ, yields direct information on whether a fluorophore is excited or not. In fact, in proteins, when the value of R_{ex}^{em} at 295 is lower than that at 280 nm, this means that excitation of tyrosine occurs at 280 nm although tyrosine contribution to the protein fluorescence could be very low or absent because of many processes such as for example energy transfer to neighboring amino acids or to tryptophan residues.

When the values of R_{ex}^{em} at 295 and 280 nm are identical or very close, this means that excitation of tyrosine residues within the protein is not occurring.

Table 1 Value of the emission to excitation ratio of different proteins with different amount of Trp residues, determined in comparison with free tryptophan in solution. The value of the emission to excitation ratio for L-Trp in solution is taken as equal to 1

Protein	R_{ex}^{em} at 280 nm	R_{ex}^{em} at 295 nm	Trp	Tyr
Bovine serum albumin	1.295	1.35	2	19
Human serum albumin	1.525	1.2	1	18
Serotransferrin	1.845	1.62	8	9
Bovine β- lactoglobulin	1.315	1.414	2	4
Human α_1-acid glycoprotein	1.7	1.57	3	11
Creatine Phosphokinase	1.465	1.493	4	10
Deoxyribonuclease I	1.68	1.486	3	12
Rat albumin Fraction V	1.425	1.093	1	_
Helicase (low optical densities)	1.35	1.171	3	6
Helicase (high optical densities)	1.335	1.286	3	6
Trypsin	1.46	1.286	4	10

Our results show that the emission to excitation ratio R_{ex}^{em} calculated via the excitation spectrum is higher than that of free L-Trp in solution. This means that protein structure does not quench fluorescence emission of amino acids but plays a role of gap in retaining and organizing the energy of this emission. It is a fact that energy emitted as photons is much lower than that absorbed inducing a low quantum yield Φ, however since absorbed energy is not used necessarily only to excite the fluorophore, one should determine this "quantum yield" only from the energy used to excite the fluorophore. In this case, Φ has different significance than R_{ex}^{em}. Φ characterizes the photon emission of the fluorophore compared to the absorbed photons, while R_{ex}^{em} characterizes the photon emission by the fluorophore compared to the real energy used for the excitation process only. Φ allows comparing photon emission importance to all the processes intervening in the absorption (energy dissipation in the medium, structural reorganization of the excited fluorophore, excitation of the fluorophore, etc..). However, the emission to excitation ratio R_{ex}^{em} allows having a direct relation between the number of emitted photons and that of the photons really used for the excitation. The ratios $\frac{R_{ex\ 295nm}^{em}}{R_{ex\ 280nm}^{em}}$ and $\frac{\Phi_{295nm}}{\Phi_{280nm}}$ are not equal for most of the proteins (Table 2). This is clear evidence that these two parameters have not the same significance. In proteins such as rat serum albumin, bovine serum albumin and human serum albumin, where the ratios values of R_{ex}^{em} and of Φ are equal or close, energy transfer from tyrosine residues to other amino acid residues is very high. Table 2 shows that the ratio of the quantum yields is much more dependent on the optical densities at the excitation wavelength than that of R_{ex}^{em} (data shown for helicase). This reveals that determination of R_{ex}^{em} is much more accurate than Φ and one can determine the value of R_{ex}^{em} independently of the optical density of the studied sample.

In proteins, the fact of exciting at wavelengths where only aromatic amino acids absorb and of obtaining a fluorescence emission intensity that is not proportional to the optical density, clearly indicates that absorption energy is not used only or entirely to excite the fluorophore. Part of this absorbed energy helps to reorganize the fluorophore molecules in the excited state inducing a structure different from that of the ground state. The low value of Φ in proteins compared to that of free tryptophan in solution is not the result of the interaction between the Trp residues with the neighboring amino acids but of the loss of a part of the absorbed energy within the system in order to reorganize it. Since similar results and identical conclusions can be drawn from our experiments whether performed on free Trp in solution or Trp present within a protein and of free tyrosine in solution, structural organization of the fluorophore in the excited state is independent of the surrounding environment.

A fluorophore is characterized by different parameters such as emission maximum position, anisotropy and fluorescence lifetime. Emission maximum is dependent on the polarity of Trp residue environment and anisotropy characterizes the motion of the Trp residue within its environment. Thus, these two parameters are dependent on the position of the Trp residue within the protein. Fluorescence lifetime is considered to originate from rotamers. In fact, tryptophan in peptides and proteins exhibits a bi or multi-exponential fluorescence decay. An explanation of this decay is the presence of conformers in equilibrium in the folded state. Each conformer exhibits one specific fluorescence lifetime. The origin of the conformers arises from the rotation of the indole ring within the Cα–Cβ bond and/or the Cβ–Cγ bond, the interconversion between the rotamers being slow relative to the fluorescence time scale. The rotamers are considered also as rigid entities. The rotamers model is applied to explain origin of the biexponential decay of tryptophan free in solution. In polypeptides, lifetime of each rotamer is explained as the result of the quenching interactions between indole and quenching groups in the fluorophore. Although the rotamers model approach could correspond to our interpretation of the

Table 2 Ratios of the emission to excitation ratio R_{ex}^{em} and the quantum yield Φ of different proteins calculated at 280 and 295 nm

Protein	$\frac{R_{ex}^{em}\text{ at }295nm}{R_{ex}^{em}\text{ at }280nm}$	$\frac{\Phi\text{ at }295nm}{\Phi\text{ at }280nm}$	Trp	Tyr
Bovine serum albumin	1.042	1.074	2	19
Human serum albumin	0.786	0.674	1	18
Serotransferrin	0.878	0.463	8	9
Bovine β- lactoglobulin	1.075	0.584	2	4
Human α_1-acid glycoprotein	0.923	0.424	3	11
Creatine Phosphokinase	1.019	0.577	4	10
Deoxyribonuclease I	0.885	0.467	3	12
Rat albumin Fraction V	0.767	0.75	1	_
Helicase (low optical densities)	0.867	0.391	3	6
Helicase (high optical densities)	0.963	0.674	3	6
Trypsin	0.88	0.62	4	10

Table 3 Fluorescence lifetimes and corresponding relative amplitudes in % of tryptophan free in solution (L-Trp) and present in different proteins

Sample	τ_1	α_1	τ_2	α_2	τ_3	α_3	χ^2
L-Trp	0.43	4.71	3.06	95.29	–	–	1.008
Serotransferrin	0.31	25.65	1.25	37.69	3.784	36.66	1.137
Helicase	0.47	17.83	1.735	37.2	4.373	44.96	1.128
Bovine β- lactoglobulin	0.56	14.40	1.306	50.2	4.475	35.4	1.156
α_1-acid glycoprotein	0.24	6.67	1.4	34.88	3.410	58.45	1.046

data obtained in this work, definition of this model is not correct. In fact, our results are obtained on free tryptophan in solution and on tryptophan present in proteins of different structures. Therefore, our 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. Table 3 shows the different lifetimes values we measured for some of the proteins studied in this work. In most of the proteins, a third lifetime around 1 ns is in general recorded. This lifetime could be the result of the interaction between the Trp residue (s) and the surrounding amino acids. Also, the values of the relative amplitudes of the three fluorescence lifetimes can be dependent on the number of emitting Trp residues or / and on the type of interaction that is occurring between Trp residues and the surrounding environment. In fact, our results (Table 3) indicate that the fluorescence lifetimes relative amplitudes are not the same for the measured proteins.

It is important to mention that our model concerning a possible structural reorganization of the tryptophan in the excited state with specific fluorescence lifetimes can be applied to the fluorescent probe 2,p-toluidinylnaphthalene-6-sulfonate (TNS). In fact, this extrinsic probe is almost non-fluorescing when free in solution with a lifetime equal to 0.4 ns. When bound to proteins, TNS emission is characterized with two lifetimes around 4.3 and 11 ns. These values have always been measured for TNS bound to different proteins such as apomyoglobin [8], mellitin [9], troponin c [10] α_1-acid glycoprotein [11] and *Lens culinaris* agglutinin [12]. In all these proteins, TNS was found to have restricted motions with a binding site that is hydrophobic. Therefore, the two lifetimes (4 and 10–11 ns) observed for the TNS-protein complexes are common features of TNS bound to a protein. Thus, these values are more specific to the TNS structure itself within the protein, rather to the protein itself, although they are observed only when TNS is bound to a protein. Therefore, structure of free TNS in solution is different from that of TNS bound to a protein. The values of the fluorescence lifetimes do not seem to be dependent on the nature of the macromolecule, i.e., specific and same type of interactions occur between TNS and its binding site. However, one should add the possibility of having local structure of TNS that varies slightly with the nature of the interacting macromolecule. In this case, lifetime measurements are not sufficiently sensitive to these small structural differences.

Nevertheless, it is important to indicate that what is true for TNS, Tryptophan and Tyrosine may not be applied to other fluorophores. Therefore, generalizing a theory is not necessary the right thing to do in exploring and understanding proteins or macromolecules structures and dynamics with fluorescence.

In conclusion, the present work puts into evidence for the first time that absorption and excitation spectra of tryptophan and tyrosine residues are not the same. Moreover, we have found that the fluorescence emission intensities of the two fluorophores are proportional to the intensity of the excitation spectrum at the excitation wavelength and not proportional to the absorption at the excitation wavelength. Our conclusion is that absorption spectrum of a fluorophore does not reflect the exact energy used to excite the fluorophore molecules, and only excitation spectrum gives the real energy transitions of the fluorophore excitation. These results were also observed for ten proteins having different numbers of Trp residues. Thus, our data are explained as the result of a structural reorganization of the tryptophan in the excited state independently of the protein matrix. We introduce a new parameter called the emission to excitation ratio R_{ex}^{em} in order to define the ratio of the emitted photons over the photons used for the excitation. These results allow us to look to the meaning and origin of protein fluorescence differently from the interpretations already applied up to now.

References

1. Lakowicz JR (1999) Principles of fluorescence spectroscopy. Plenum, New York, 1983
2. Albani JR (2004) Structure and dynamics of macromolecules: absorption and fluorescence studies. Elsevier, Amsterdam
3. Demchenko AP (2002) The red-edge effects: 30 years of exploration. Luminescence 17:19–42
4. Granjon T, Vacheron MJ, Vial C, Buchet R (2002) Mitochondrial creatine kinase binding to phospholipids decreases fluidity of membranes and promotes new lipid-induced beta structures as

monitored by red edge excitation shift, laurdan fluorescence, and FTIR. Biochemistry 40:6016–6026
5. Albani JR (2006) Progesterone binding to the tryptophan residues of human α_1-acid glycoprotein. Carbohydr Res 341:2557–2564
6. Miller JN (1961) Standards in fluorescence spectrometry: techniques in visible and ultraviolet spectrometry, vol 2. Chapman and Hall, New York
7. Teale FWJ (1960) The ultraviolet fluorescence of proteins in neutral solution. Biochem J 76:381–388
8. Lakowicz JR, Gratton E, Cherek H, Maliwal BP, Laczko G (1984) Determination of time-resolved fluorescence emission spectra and anisotropies of a fluorophore-protein complex using frequency-domain phase-modulation fluorometry. J Biol Chem 259:10967–10972
9. Demchenko AP (1985) Fluorescence molecular relaxation studies of protein dynamics: the probe binding site of melittin is rigid on the nanosecond time scale. FEBS Lett 182:99–102
10. Steiner RF, Norris L (1987) Fluorescence dynamics studies of troponin C. Biopolymers 26:1189–1204
11. Albani J (1992) Motions studies of the human α_1-acid glycoprotein (orosomucoid) followed by red-edge excitation spectra and polarization of 2-p-toluidinylnaphthalene-6-sulfonate (TNS) and of tryptophan residues. Biophys Chemist 44: 129–137
12. Albani JR (1996) Dynamics of *Lens culinaris* agglutinin studied by red-edge excitation spectra and anisotropy measurements of 2-p-toluidinylnaphthaline-6-sulfonate (TNS) and of tryptophan residues. J Fluoresc 6:199–208