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ORIGINAL PAPER

Origin of Fluorescence Lifetimes in Human Serum Albumin. Studies on Native and Denatured Protein

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Abstract Human serum albumin consists of a single polypeptide of 585 amino acid residues with 1 Trp residue. In the present work, we measured fluorescence lifetimes of the protein in both native and denatured states. The results indicate that Trp emission occurs with three lifetimes in both states. Lifetimes values and contribution to the global emission decay differ between the two states. Data are interpreted as the results of an emission occurring from three substructures of the tryptophan formed in the excited state. Two of these substructures are already present for the tryptophan free in solution. The third lifetime is the result of the interaction between the tryptophan residue and surrounding microenvironment. The populations of these substructures characterized by the pre-exponential parameters of the fluorescence lifetimes are dependent on the fluorophore microenvironment and on the global protein structure.

Keywords Human serum albumin · Tryptophan fluorescence lifetime · Emission spectra · Guanidine · Native protein · Denatured protein

Introduction

Origin of tryptophan fluorescence lifetimes has been investigated since now more than 30 years. The thousands of experiments performed on proteins or / and free tryptophan in solution, allowed suggestion of different origins for tryptophan fluorescence lifetimes. Dynamics of the tryptophan residue within its microenvironment, different solvent relaxation state [1, 2], emission from rotamers [3, 4], presence of protein isoforms [5], and energy transfer within the protein matrix or to the heme in case of hemoproteins [6, 7], are some suggested origins of tryptophan fluorescence lifetimes.

In a recent work, by comparing fluorescence lifetimes of free tryptophan in solution and of tryptophan in proteins, we have suggested the following origin of tryptophan fluorescence lifetimes in proteins: The two lifetimes (0.5 and 3 ns) found for tryptophan free in water and in almost all measured proteins originate from the tryptophan structure itself. Each lifetime characterizes a substructure of the fluorophore reached in the excited state. The third lifetime found in the proteins, would be the result of the tryptophan-surrounding environment interaction [8]. Also, we found that values of lifetimes pre-exponentials and in many cases those of lifetimes depend on the type of protein-tryptophan interaction. The results published in [8] are in good agreements with those we obtained recently on tryptophan in solution (water or ethanol) [9] and which indicate that tryptophan lifetimes do not depend solely and necessary on both $S_o \rightarrow {}^1L_b$ and / or $S_o \rightarrow {}^1L_a$ transitions but they can be observed even in presence of $S_o \rightarrow {}^1L_b$ transition only. Therefore, emission lifetimes are not necessarily correlated to specific tryptophan structures observed in the ground state but to substructures formed in the excited state.

Tryptophan 214 residue of human serum albumin emits with three lifetimes [10, 11]. In the present work, in order to check whether these lifetimes are dependent on the protein structure or not, we measured fluorescence lifetimes of HSA Trp residue in two protein states, native and denatured. Experiments conducted along the emission spec-

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trum show that presence of three lifetimes is independent of the protein state, while, lifetimes and pre-exponential values are not the same whether HSA is native or denatured.

Materials and methods

Human serum albumins (purity >98%) was from Sigma. Protein concentrations were determined at 278 nm with the following extinction coefficient 3.5219×10^4 M^{-1} cm^{-1} [12]. Protein concentration in all the experiments was equal to 8 μM.

Guanidine hydrochloride was from Sigma. Solution of 6 M guanidine pH 7.8 was used in order to denature HSA.

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

Steady state 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 intensities were corrected for the inner filter effect as described [13, 14], although optical densities at the excitation and emission wavelengths were low.

Fluorescence lifetime measurements were performed with a Horiba Jobin Yvon FluoroMax-4-P, using the time correlated single photon counting method. A ludox solution was used as scatter. Excitation was performed at 296 nm with a nanoLED. Each fluorescence decay was analyzed with one, two and three lifetimes and then values of $\chi2$ were compared in order to determine the best fit. A χ^2 value that approaches 1 indicates a good fit.

The mean fluorescence lifetime is the second order mean [13]:

$$\tau_o = \sum f_j \tau_j \quad (1)$$

and

$$f_i = \alpha_i \tau_i / \sum \alpha_i \tau_i \quad (2)$$

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

All experiments were performed at 20°C in 10 mM bis Tris buffer.

Results and discussion

Emission spectrum of native HSA displays a peak at 340 nm at pH 7.5. When dissolved in 6 M guanidine solution, the emission peak is shifted to 352 nm accompanied with an increase of the fluorescence intensity (spectra not shown), indicating protein denaturation and thus an increase of the protein tryptophan residue exposure to the solvent. Identical results have been reported earlier [15, 16]. Figure 1 displays fluorescence intensity decay of native human serum albumin recorded at 350 nm. Data can be adequately represented by a sum of three exponentials

$$I(\lambda,t) = 0.0277\ e^{-t/0.492} + 0.2928\ e^{-t/3.961} + 0.6796\ e^{-t/7.777}$$

where 0.0277, 0.2928 and 0.6796 are the preexponential factors and 0.492, 3.961 and 7.777 ns the decay times ($\chi2$= 1.101). The mean fluorescence lifetime τ_o calculated from two experiments was found equal to 7.085±0.094 ns.

When human serum albumin is dissolved in 6 M guanidine, its fluorescence intensity decay can still be characterized by a sum of three exponentials (Fig. 2)

$$I(\lambda,t) = 0.1222\ e^{-t/0.380} + 0.6399\ e^{-t/2.350} + 0.2379\ e^{-t/4.725}$$

where 0.1222, 0.6399 and 0.2379 are the preexponential factors and 0.380, 2.350 and 4.725 ns the decay times ($\chi2$= 1.204). The mean fluorescence lifetime calculated from two experiments was found equal to 3.314±0.05 ns. Thus, denaturation of human serum albumin with guanidine decreases the three fluorescence lifetimes values and does not yield a single fluorescence lifetime. This means that presence of three lifetimes for tryptophan-214 residue in HSA does not depend on the protein structure only, but also on the tryptophan itself. It has been suggested that fluorescence lifetimes of Trp residue(s) within random coil polypeptides (denatured proteins) are very close, the differences would come from the position of the tryptophan within the protein primary structure (end of the chain or in its middle) and / or presence of quencher group near the Trp residue(s) [16–18]. Nevertheless, the data described in our work as those we have recently published [8–10] indicate that fluorescence lifetimes characterize intrinsic properties of the fluorophore. In fact, the two shortest lifetimes around 0.4–0.5 ns and 2–4 ns were measured for tryptophan free in solution and present within proteins [8–10, 16]. Thus, the presence of these two lifetimes is independent of any structure around tryptophan and characterize an internal property or / and organization of the tryptophan structure in the excited state [8]. The data displayed in the present work show that the two shortest lifetimes, whether the protein is in native or denatured states, are very close to those obtained for other proteins [8]. The third lifetime recorded in proteins could be attributed to interaction between the Trp residue (s) and the surrounding amino acids and to

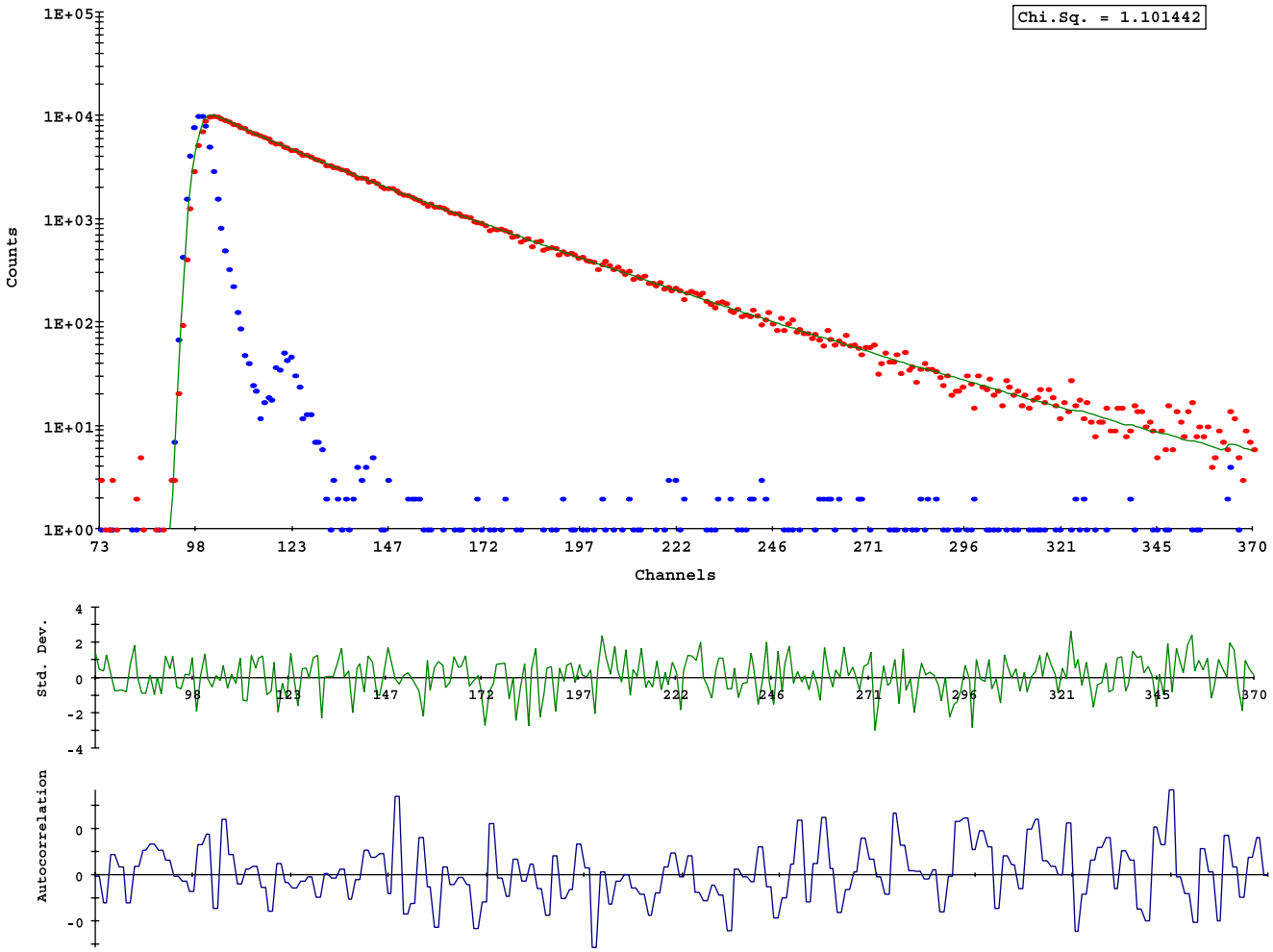

Fig. 1 Fluorescence intensity decay of human serum albumin dissolved in Tris-buffer, pH 7.5. λ_{ex}=296 nm and λ_{em}=350 nm

possible specific properties of the protein. Present and previous works [8] show that values of the three fluorescence lifetimes and of their pre-exponentials are dependent on the structure around the tryptophan residues and thus on the interaction between Trp residues and their environments. The 7.7 ns observed in the native state is the result of the interaction of Trp-214 residue with its surrounding environment, the amino acids of the hydrophobic part of the ligand binding pocket. This long lifetime indicates that corresponding radiative (k_r) and non-radiative (k_i) constants are low which means that sub-structure yielding this long lifetime displays important interaction within the neighboring amino-acids. The pre-exponential value (0.68) of this lifetime is high, which means that the population of the substructure emitting with lifetime equal to 7.7 ns is the most important between the three populations. This is in a complete opposition with the shorter lifetime (0.492 ns) which pre-exponential value is equal to 0.0277. Despite the presence of Trp-214 residue within a highly hydrophobic domain of HSA, this does not exclude contacts with the solvent molecules that are diffusing within the protein matrix. This diffusion is the result of local motions within HSA matrix.

Protein denaturation decreases the contact between Trp-214 residue and its microenvironment and thus induces an increase of the fluorescence intensity and of the radiative constant (k_r) value. Populations of substructures obtained in the denatured states are not necessarily identical to those observed for the native protein.

Figure 3 displays fluorescence lifetime variation of native and denatured HSA along emission wavelengths. While the shape of this lifetimes variation is identical for both proteins states, lifetimes values of denatured protein are almost half those of the native one. This decrease in the fluorescence lifetime, already observed at one emission wavelength [15–17] can be explained by the fact that in the native state, interaction between HSA Trp residue and neighboring amino acids is important compared to that observed when the protein is totally denatured. Thus, in the native state, radiative rate constant decreases as the result of the important interaction between fluorophore and neighboring amino-acids. In the unfolded state, interaction

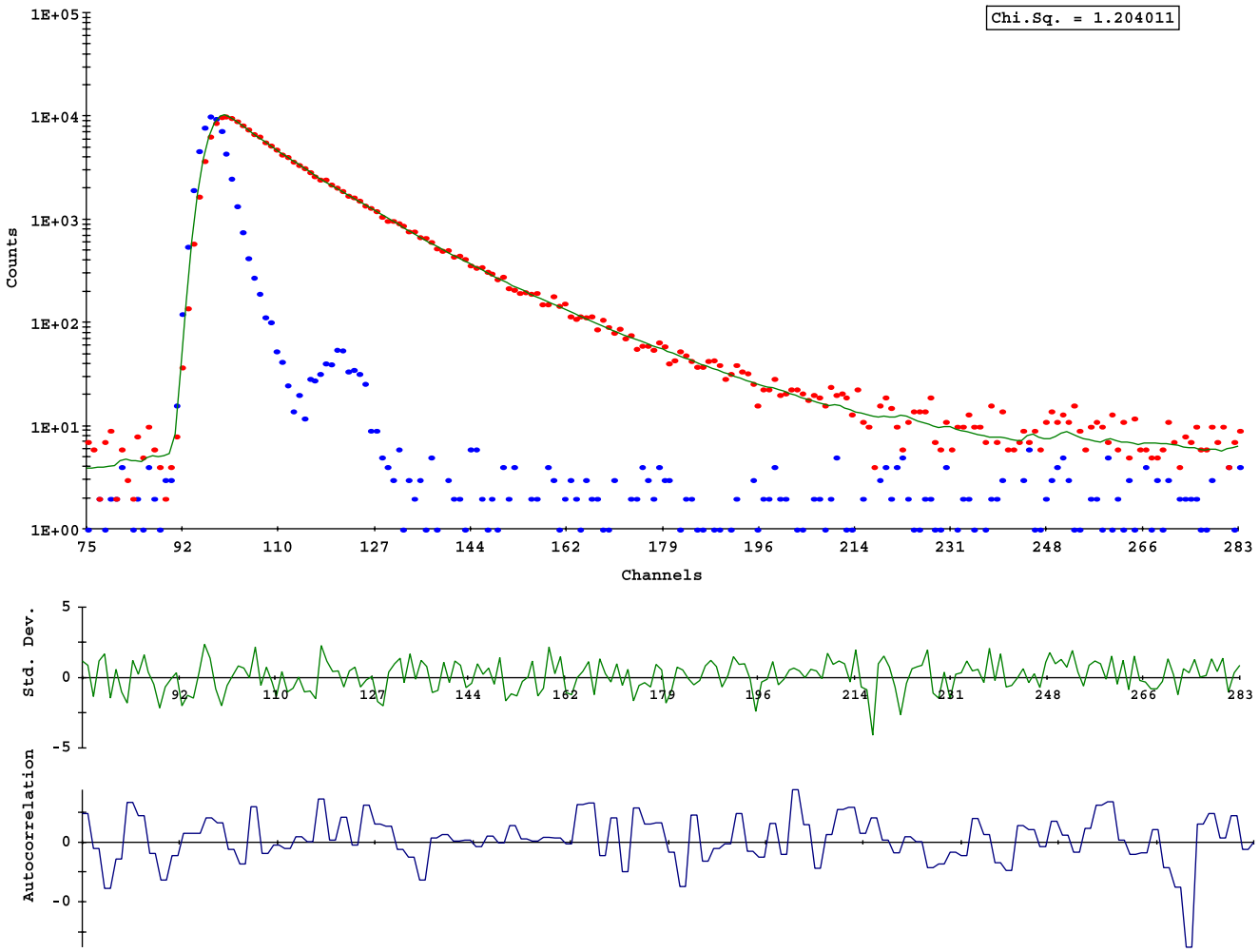

Fig. 2 Fluorescence intensity decay of human serum albumin dissolved in 6 M guanidine, pH 7.8. λ_{ex}=296 nm and λ_{em}=350 nm

between Trp residue and amino acids decreases, inducing by that an increase in the radiate rate constant and thus a decrease of the measured fluorescence lifetimes compared to that recorded in the native state.

Fluorescence lifetime decrease can also be explained by the difference in the local motions of the tryptophan residue between native and denatured states. In fact, in the native state, HSA tryptophan-214 residue displays local motions [14, 19, 20], which increase in the denatured state inducing an increase in the non-radiative rate constant k_i. This will decrease the value of fluorescence lifetime. Therefore, HSA structure plays a role of gap in retaining and organizing photon emission energy. Quenching efficiency characterized by the protein unfolding (passage from the native to the denatured state) is equal to

$$E = 1 - \frac{\tau_{o(D)}}{\tau_{o(N)}} \tag{3}$$

where $\tau_{o(D)}$ and $\tau_{o(N)}$ are the mean fluorescence lifetimes in the denatured and native states, respectively.

E was found equal to 0.5±0.05 along the emission spectrum from 340 to 390 nm. The value found for E reveals the importance of the interactions between HSA Trp-124 residue and surrounding amino-acids in the native state compared to the unfolded one. Denaturation induces structural modifications within the IIA subdomain where Trp-214 residue is located, affecting the different interactions that exist between IIA and IIIA subdomains and thus protein conformation and its structural stability.

Figure 4 displays lifetime pre-exponentials variation along the emission spectra of both denatured and native HSA. First of all, the data show that we have three different fluorophore populations independent of the surrounding environment. HSA denaturation induces an important modification in the pre-exponential values recorded in the native state. This means that denaturation has modified the population of each of the three tryptophan substructures. This result is clear evidence that pre-exponential values characterize the type and nature of interaction existing between Trp-214 residue and its environment, while the

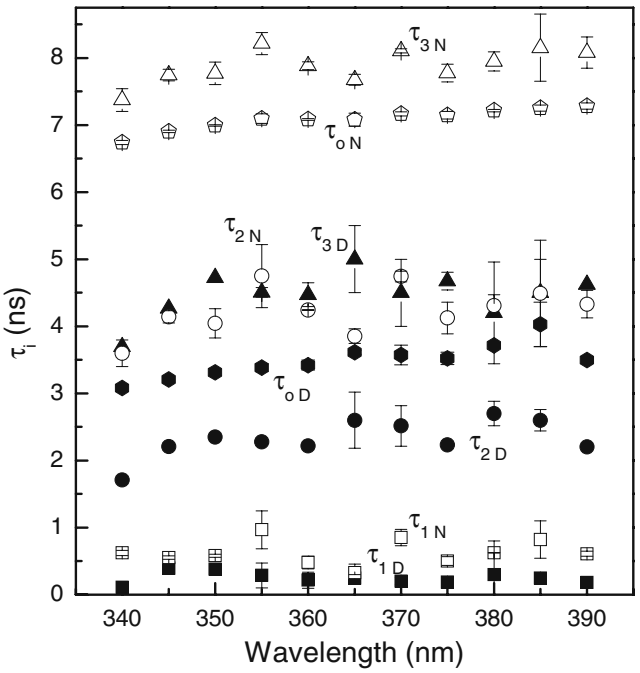

Fig. 3 Lifetimes values of HSA Trp residue with emission wavelength of both denatured (*closed symbols*) and native (*open symbols*) proteins. τ_1, τ_2 and τ_3 are symbolized by *squares*, *circles*, *triangles* and *pentagons*, respectively

number of lifetimes is an indication of the substructures that exist for the fluorophores in the excited state. Figure 4 indicates that in the denatured state, the population characterizing the two shortest lifetimes are much more important than those observed in the native state which is not the case for the population with the longest fluorescence lifetime. Since mean fluorescence lifetime value depends on those of the three lifetimes and of their pre-exponentials, important decrease in the fluorescence lifetimes (Fig. 3) and of the longest lifetime pre-exponential (Fig. 4) as the result of HSA denaturation, induces the decrease of the mean fluorescence lifetime.

HSA denaturation with guanidine follows a multistep process; Studies at different guanidine concentrations show that HSA unfolding begins with the destabilization of domain III, followed at guanidine concentration higher than 1.8 M with the unfolding of domain II, and finally denaturation of domain I occurs [21]. Around 1.8 M guanidine concentration, folding intermediate of molten globule type occurs. Although we did not measure fluorescence lifetimes at different guanidine concentrations, the results we obtained in the native state on other proteins of different structures [8] allow us to suggest that presence of 0.492 and 3.961 ns fluorescence lifetimes observed for Trp-214 residue of HSA is independent of the protein conformation. Nevertheless, values of the two lifetimes and those of their pre-exponentials are influenced by the protein global state and by the structure near the tryptophan residue.

The three substructures of tryptophan-214 residue, each with a specific lifetime, should have different orientations in space within HSA and thus should be surrounded by environments of different polarities. One should be careful here in the interpretation of meaning of "different polarities". By this we do not intend to pretend that one substructure is located in a hydrophobic area while the other is in polar one. In fact, the three substructures are located in the same hydrophobic location of domain IIA of HSA, but they could have different slight contact with the solvent molecules which are in the vicinity of the fluorophore, rendering one of the sub-structures more "hydrophilic" than the others. Lifetimes values give an indication on the interaction that exists between each substructure and its environment. The three lifetimes decrease after HSA denaturation shows that the substructures have weaker contact with the surrounding environment in the denatured state. Also, this means that solvent relaxation process differs in both states around the substructures.

In conclusion, our data reveal that HSA Trp-214 residue emits with three lifetimes whether the protein is in the native or in the denatured states. Although the values of these lifetimes differ from a state to another, the two shortest ones (0.5 and 4 ns) are within the range of the two

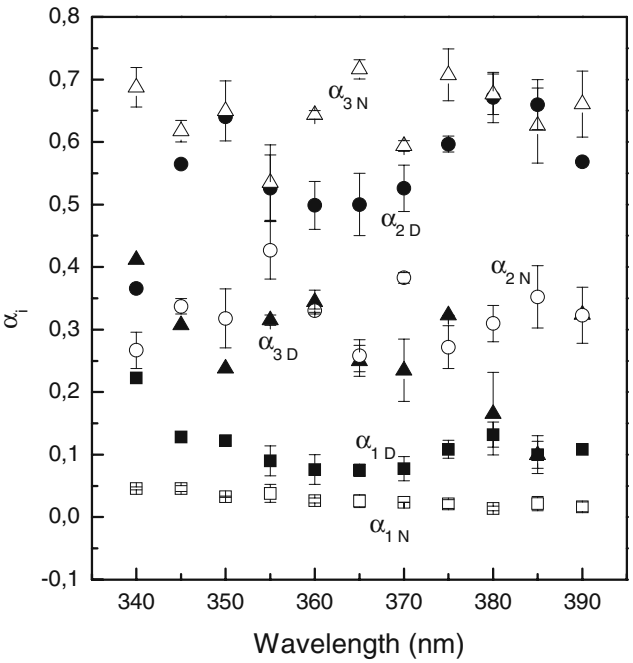

Fig. 4 Pre-exponential variation of fluorescence lifetimes of native (*open symbols*) and denatured (*closed symbols*) HSA with emission wavelengths. α_1, α_2 and α_3 are symbolized by *squares*, *circles* and *triangles*, respectively

fluorescence lifetimes observed for free tryptophan in solution and for tryptophan residues in proteins. Thus, these data show clearly that presence of these fluorescence lifetimes is independent of the protein structure and characterize intrinsic properties of the tryptophan, in the excited state, whether free in solution or part of a protein. These data are consistent with those already published [8–11] indicating that tryptophan fluorescence lifetimes originate from substructures formed in the excited state. Further works on other proteins and in different physiological conditions should confirm our model.

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