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Characterization of human serum albumin forms with pH. Fluorescence lifetime studies

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ABSTRACT

Fluorescence lifetimes of human serum albumin (HSA) tryptophan 214 residue were measured in solution at different pH (from 2 to 12). The results indicate that tryptophan emission occurs with three lifetimes at all pH. However, lifetimes and pre-exponential values are dependent on the pH and thus on the protein form. Three different protein populations have been differentiated: one population for pH 2 and 3 (extended form), the second one from pH 4 to 9 containing HSA migrating (F), normal (N) and basic (B) forms. Another type of population is obvious for pH higher than 9, characterizing the aged (A) form of HSA.

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1. Introduction

Human serum albumin (HSA) is the most abundant protein in human blood plasma. It has a molecular mass of 67 kDa and is composed of 585 amino acid residues. Three-dimensional structure of human serum albumin has been determined crystallographically at a resolution of 2.8 Å. It comprises three homologous domains that form a heart-shaped molecule and contains a total of 17 disulfide bonds. Each domain is a product of two subdomains that possess common structural motifs. The principal regions of ligand binding to human serum albumin are located in hydrophobic cavities in subdomains IIA and IIIA, which exhibit similar chemistry [1].

It is known that HSA undergoes reversible conformational transformation with change in pH. At pH 7, HSA assumes the normal form (N) which abruptly changes to highly charged fast migrating form (F) at pH values less than 4.3, as this form moves "fast" upon gel electrophoresis. At pH less than 2.7 the F-form changes to the fully extended form (E). On the basic side of the normal pH above pH 8, the N-form changes to basic form (B) and above pH 10, the structure changes to the aged form (A) [2] (Scheme 1).

HSA contains a single tryptophan residue at position 214 in domain II. Emission of this tryptophan residue occurs with three fluorescence lifetimes [3]. Fluorescence of the tryptophan residue in HSA provides information on the local environment of the protein. In fact, tryptophan residues in proteins are largely used to study dynamics and structure of proteins by measuring fluorescence intensity and anisotropy decays [4–7].

Since tryptophan fluorescence is sensitive to modifications occurring within proteins and in the surrounding environment of the fluorophore and since HSA structure is modified at different pH, we tried in the present work to find out how fluorescence emission decay parameters (lifetimes and pre-exponentials) of Trp-214 residue are affected by the pH modification.

2. Materials and methods

Human serum albumin (purity >98%) was from Sigma–Aldrich (Saint Quentin Fallavier, France). Protein concentrations were determined at 278 nm with the following extinction coefficient $3.5219 \times 10^4\ M^{-1}\ cm^{-1}$ [8]. Protein concentration in all the experiments was equal to 8 μM.

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

Steady state fluorescence spectra were recorded with a PerkinElmer LS-5B spectrofluorometer (PerkinElmer, Courtaboeuf, France). 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 [9,10],

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Scheme 1. Conformational changes of HSA induced by pH modification.

although optical densities at the excitation and emission wavelengths were low (Fig. 1).

Fluorescence lifetime measurements were performed with a Horiba Jobin Yvon FluoroMax-4-P (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 nano-LED. Each fluorescence decay was analysed with one, two and three lifetimes and then values of χ^2 were compared in order to determine the best fit. Since lifetimes data are based on the value of χ^2, it is important to make a description of the meaning and importance of this value. In the single photon counting method, the detection system measures the time between the excited pulse and the arrival of the first photon. The distribution of arrival times represents the decay curve called also the impulse response function. In order to obtain a decay curve that characterizes the sample only, excitation pulse should be infinitely sharp or should have a pulse much shorter than the decay time of the sample. Nevertheless, even if these two conditions are met, this is not always the case; the presence of the light pulse does not allow identifying the zero time point of the fluorescence decay. This will induce errors in number of lifetimes determination and calculation. The time distribution of the lamp pulse $L(t)$, called also the instrument response function, is measured in a separate experiment using scatter solution. The observed fluorescence decay is called $R(t)$ and the final response function is called $F(t)$.

$R(t)$ is given by the convolution of the lamp pulse with the impulse response of the sample [9,11,12]

$$R(t) = \int_0^t L(t')\,F(t-t')\,dt' \qquad (1)$$

A method applied to estimate the impulse response function $F(t)$ is the least-squares analysis. The method calculates the expected value of $R(t)$ given assumed values of α_i and τ_i and the calculated value $[R_c(t)]$ is compared with the observed value $R(t)$. The α_i and τ_i values are varied until the best fit is obtained. The goodness of fit χ^2 is calculated from

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

where

$$w = \frac{1}{R(t)} \qquad (3)$$

is a statistical weighting factor to account for the expected error in each value of $R(t)$. A minimal value of χ^2 indicates the best fit. A χ^2 value that approaches 1 indicates a good fit. Differences between $R(t)$ and $R_c(t)$ is described by a function called the autocorrelation function of the differences and can be displayed by experimental curves. Close values of $R(t)$ and $R_c(t)$ yield experimental autocorrelation curves that are randomly distributed around zero, indicating by that a good fit. Finally, to find out whether a decay curve should be best analysed with one, two or more lifetimes, values of χ^2 have to be compared. For example, let us consider a value of χ^2 equal to 1.054, 1.06 and 1.1 when analysis is done with 1, 2 and 3 lifetimes,

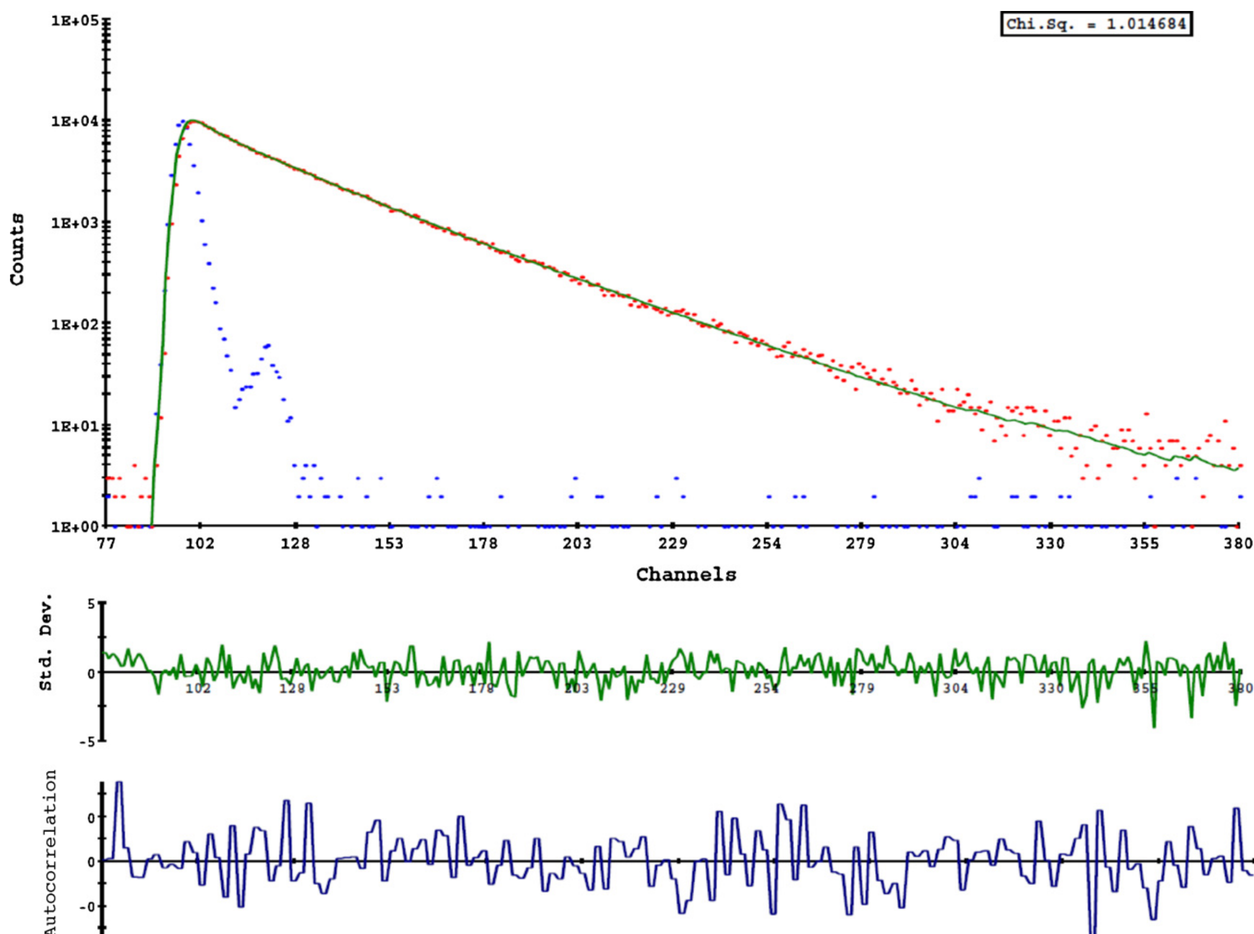

Fig. 1. Fluorescence intensity decay of Trp residue in human serum albumin at pH 6. λ_{ex} = 296 nm and λ_{em} = 340 nm.

respectively. One lifetime should be considered as the best description of the decay curve since there was no real improvement in χ^2 value when the experimental decay was fitted with two or three lifetimes [13].

The mean fluorescence lifetime is the second order mean [9]

$$\tau_0 = \sum f_i \tau_i \quad (4)$$

and

$$f_i = \frac{\alpha_i \cdot \tau_i}{\sum \alpha_i \tau_i} \quad (5)$$

where α_i are the pre-exponential terms, τ_i are the fluorescence lifetimes and f_i the fractional intensities.

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

3. Results and discussion

Fluorescence intensity decay of HSA Trp-214 residue observed at 340 nm and measured at pH 6 can be adequately described by a sum of three exponentials (Fig. 1)

$$I(\lambda, t) = 0.0579e^{-t/0.862} + 0.3415e^{-t/4.357} + 0.6006e^{-t/7.617}$$

where 0.0579, 0.3415 and 0.6006 are the pre-exponential factors and 0.862, 4.357 and 7.617 ns the decay times ($\chi^2 = 1.015$). Analysing the decay curve with two lifetimes yield a χ^2 equal to 1.29. Fluorescence emission decay of HSA Trp-214 residue measured along the emission spectrum can be described by a sum of three exponentials at all studied pH. Table 1 shows the data obtained at 340 nm from pH 2 to 12. Thus, presence of three lifetimes is independent of the HSA global form or structure whether the extended form (E) observed at pH 2 and 3, basic form (B) at pH 8 and aged form (A) at pH 12. Recent studies on tryptophan have indicated that fluorescence emission lifetimes of tryptophan in solution and in proteins occur from substructures generated in the excited state upon fluorophore excitation [3,14,15]. The same conclusion was obtained for the extrinsic fluorophore 6,*p*-toluidinylnaphthalane-2-sulfonate (TNS), dissolved in ethanol or bound to proteins [13]. The pre-exponentials factors would characterize the population of each of these substructures [3,13–15].

Fig. 2 displays the variation of the three fluorescence lifetimes at pH 2, 8 and 12 along the emission spectrum. The shape of the three lifetimes is identical at the three pH. This indicates the presence of three substructures, each having a specific fluorescence lifetime. Although it is difficult to observe a real difference between the three shortest lifetimes τ_1 measured at the three pH, it is very clear that values of τ_2 and τ_3 measured at pH 2 are lower than those obtained at pH 8 and 12. This allows us to conclude that the extended form (E) of HSA at pH 2 has a structure that differs significantly from those of basic (B) or aged forms (A). This conclusion is confirmed by the variation of the mean fluorescence lifetime τ_0 with emission wavelength at different pH (Fig. 3). In fact, two different populations can be separated from the plot obtained. A population containing protein form at pH 2 and 3 (extended form E) and a second population containing the other protein forms (from pH 4 to 12). Also, at all pH, value of τ_0 increases with the emission wavelengths (Fig. 3). This is the result of the increase of the longest fluorescence lifetime τ_3 value at high emission wavelengths (Fig. 2). At these emission wavelengths, there will be a more important contribution of the tryptophan substructures populations that are exposed to a polar environment.

In order to find out whether it is possible to separate the different forms present at pH higher than 4, τ_0 as function of the pH was plotted. Fig. 4 displays the results obtained at three wavelengths, 320 (squares), 360 (circles) and 400 nm (triangles). Two

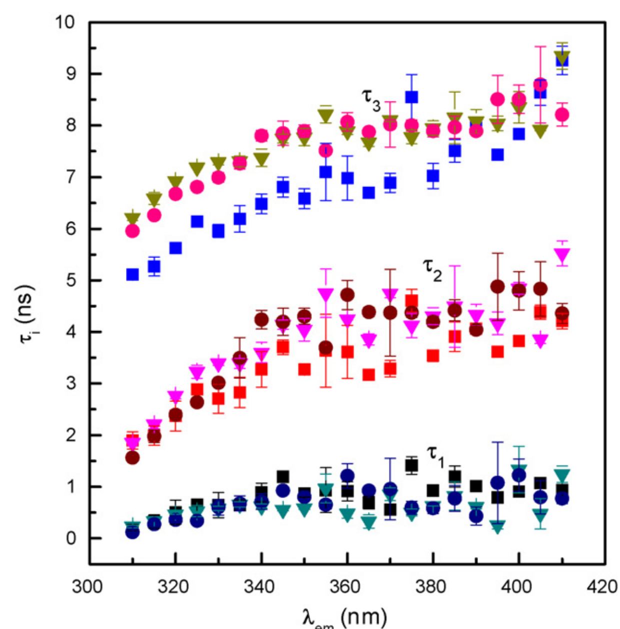

Fig. 2. Variation of the three fluorescence emission lifetimes of HSA Trp-214 residue with emission wavelength measured at pH 2 (squares), 8 (triangles) and 12 (circles).

main observations can be gained from the plot, we notice first that the data can be displayed in three different populations: one population for pH 2 and 3 (extended form), the second one from pH 4 to 8 containing HSA migrating (F) and normal (N) forms. Another type of population is obvious for pH higher than 8, characterizing the basic (B) and aged (A) forms of human serum albumin. Thus, mean fluorescence lifetime τ_0 calculated at different pH allows to separate three different populations, each containing one or two protein forms. Since it was not possible to differentiate the entire forms one from each other, this means that fluorescence technique used is not sensitive to the structural differences between the forms within each population or this could also mean that structural dif-

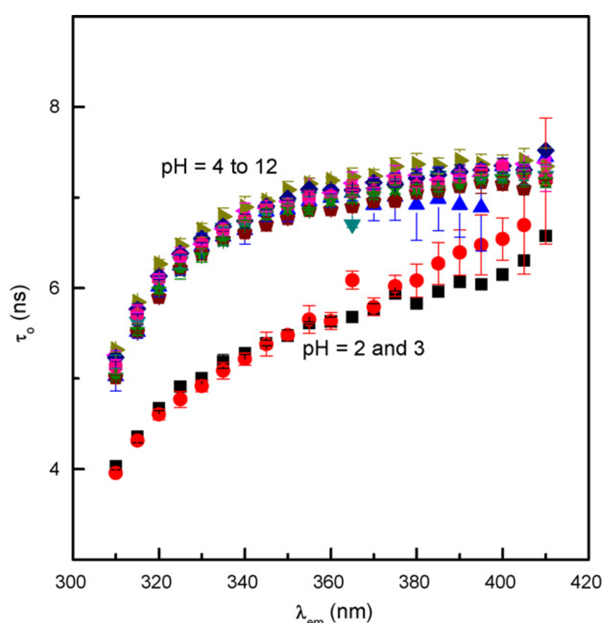

Fig. 3. Variation of mean fluorescence lifetime τ_0 of HSA Trp-214 residue with the emission wavelength, measured from pH 2 to 12.

Table 1
Fluorescence lifetime parameters of human serum albumin Trp-214 residue at different pH observed at 340 nm.

	pH										
	2	3	4	5	6	7.5	8	9	10	11	12
τ_1 (ns)	0.889	0.621	0.450	0.278	0.859	0.559	0.623	0.412	0.755	0.595	0.712
Error τ_1	0.179	0.009	0.179	0.105	0.003	0.147	0.037	0.099	0.081	0.001	0.151
τ_2 (ns)	2.827	3.016	3.218	3.199	4.428	3.570	3.600	3.412	4.219	3.791	4.240
Error τ_2	0.296	0.038	0.261	0.064	0.071	0.386	0.197	0.322	0.179	0.124	0.183
τ_3 (ns)	6.478	6.434	7.214	7.326	7.876	7.510	7.373	7.331	7.635	7.103	7.802
Error τ_3	0.190	0.116	0.225	0.113	0.258	0.148	0.170	0.202	0.118	0.063	0.102
τ_0 (ns)	5.280	5.215	6.639	6.699	6.853	6.896	6.741	6.613	6.760	6.278	6.716
Error τ_0	0.027	0.066	0.085	0.011	0.077	0.116	0.031	0.014	0.033	0.014	0.034
α_1	0.099	0.071	0.046	0.052	0.062	0.041	0.046	0.048	0.055	0.051	0.056
Error α_1	0.019	0.003	0.007	0.015	0.004	0.007	0.002	0.001	0.002	0.005	0.007
α_2	0.460	0.489	0.253	0.252	0.289	0.259	0.266	0.302	0.344	0.352	0.303
Error α_2	0.035	0.010	0.021	0.014	0.052	0.043	0.029	0.024	0.023	0.025	0.049
α_3	0.441	0.440	0.701	0.696	0.649	0.700	0.687	0.302	0.590	0.597	0.661
Error α_3	0.054	0.007	0.013	0.002	0.047	0.050	0.031	0.024	0.035	0.031	0.007

ferences between the forms present within each population are not important, even absent.

Second observation obtained from Fig. 4 is that the mean fluorescence lifetime measured at all the pH is lower at 320 nm than at 360 or 400 nm. At 320 nm, observed fluorescence occurs mainly from an environment that is more hydrophobic than at 360 or 400 nm. Since τ_0 value depends on those of the three lifetimes and of their populations characterized by the pre-exponential values, Fig. 4 clearly indicates that the values of τ_2 and τ_3 are much more important at long wavelengths than at lower ones, a conclusion in good agreement with the results obtained in Fig. 3. Also, the populations, characterized by the pre-exponential terms, of these two lifetimes may also be more important at high than at low wavelengths.

Fig. 5 compares lifetimes pre-exponentials with emission wavelengths obtained at pH 6 and 8. The data show that from 320 nm, the first pre-exponential α_1 decreases along the emission spectrum, α_2 does not change while α_3 increases with the emission wavelengths. These variations can explain why τ_0 is lower at 320 than at 360 or 400 nm (Fig. 4). Also, results obtained in Fig. 5 mean that tryptophan population characterized by the first pre-exponential α_1 is more sensitive to hydrophobic than hydrophilic area, whereas the population characterized by α_3 is much more in a contact with hydrophilic area. The second pre-exponential α_2 is sensitive equally to both areas.

Thus, the population α_1 of the shorter lifetime (τ_1) is characteristic of a hydrophobic area since it decreases with the emission wavelength. At high wavelength, its value approaches even zero. α_2 does not vary with the emission wavelength, it is stable at all wavelengths. α_3 which characterizes the population of the longest fluorescence lifetime (τ_3) is characteristic of both hydrophilic and hydrophobic areas although its sensitivity to hydrophilic area is clearly established since it increases with emission wavelengths. 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 HSA domain IIA, 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 indica-

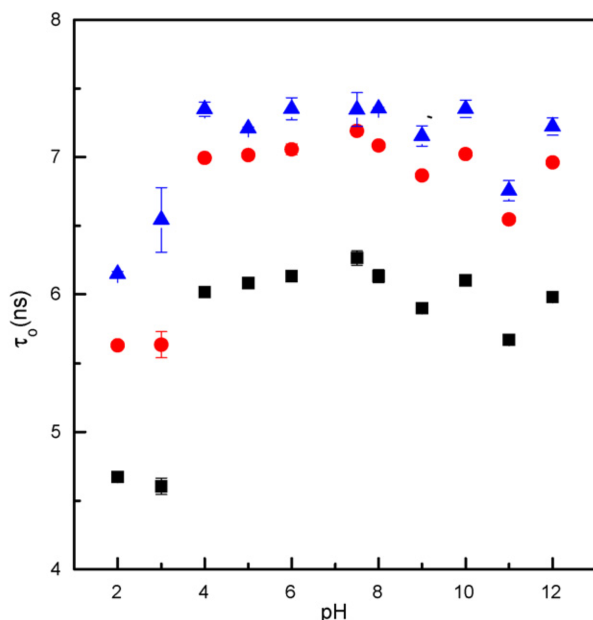

Fig. 4. Mean fluorescence lifetime τ_0 variation of HSA Trp-214 residue with pH, measured at 320 (squares), 360 (circles) and 400 nm (triangles).

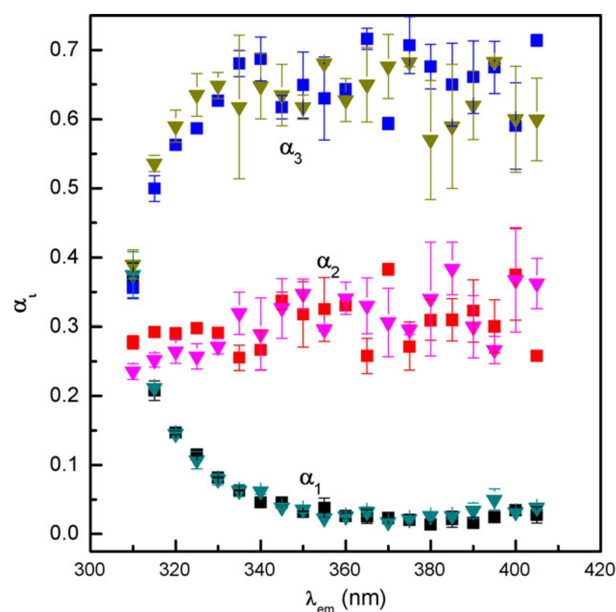

Fig. 5. Pre-exponentials values of HSA Trp-214 residue fluorescence lifetimes with emission wavelengths at pH 6 (squares) and pH 8 (triangles).

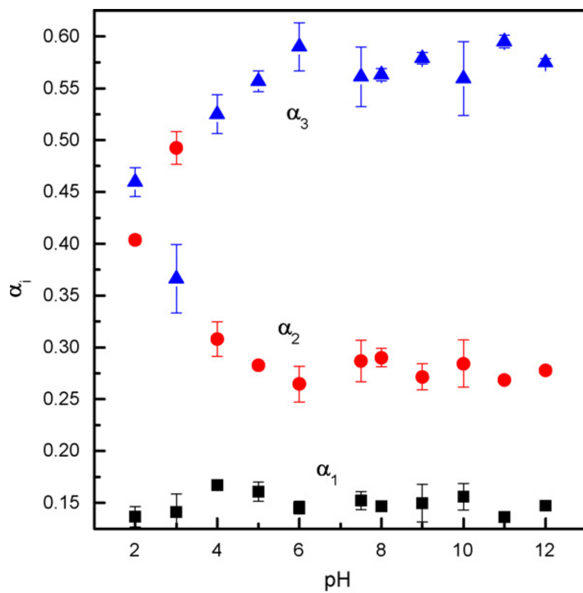

Fig. 6. Lifetimes pre-exponentials variation of HSA Trp-214 residue with pH at 320 nm.

tion on the interaction that exists between each substructure and its environment.

In order to differentiate the different forms of HSA at different pH, the three lifetimes pre-exponentials values were plotted at different pH. Figs. 6–8 display population variation of the three substructures of Trp-214 at 320, 360 and 400 nm at the different pH. At the three wavelengths, three different populations of HSA forms can be observed: one population is clearly observed at pH 2 and 3, corresponding to the extended form (E) of HSA, a second one is observed from pH 4 to 9 and which corresponds to the fast migrating (F), normal (N) and basic (B) forms. The third population observed at pH higher than 10 contains aged (A) HSA form. These results are in good agreement with those observed with fluorescence lifetimes (Fig. 5), although the third protein populations contain forms B and A when lifetimes values are plotted as a function of the pH. Nevertheless, data displayed in Figs. 5–8,

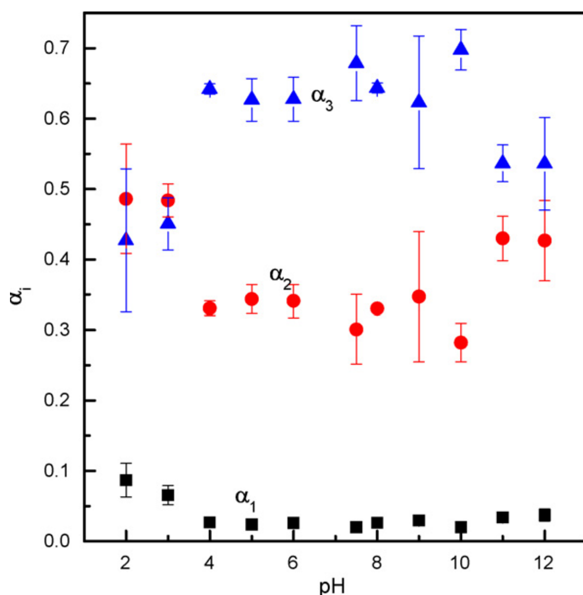

Fig. 7. Lifetimes pre-exponential variation of HSA Trp-214 residue with pH at 360 nm.

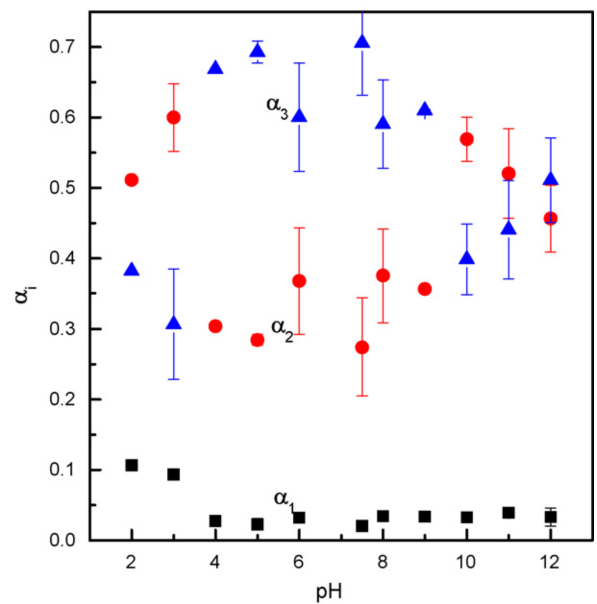

Fig. 8. Lifetimes pre-exponential variation of HSA Trp-214 residue with pH at 400 nm.

indicate that fluorescence lifetime measurements (lifetime and pre-exponentials values) allow differentiating the protein forms observed at different pH. Also, the results allow us to suggest that structural differences between certain forms such as F, N, or B are not so important, and thus this small difference in the structures, if it exists, cannot be detected by fluorescence lifetimes measurements. Pre-exponentials variation shape with pH is not identical at the three wavelengths (Figs. 6–8). This means that the tryptophan substructures populations differ between a hydrophobic area, observed at 320 nm, and more hydrophilic areas observed at 360 and 400 nm.

The data clearly indicate that protein structure at pH 2 and 3 (Extended form) is different from the other forms observed at other pH. HSA in acid medium is in a structure that is completely different from the protein when dissolved in pH higher than or equal to 4. Also, the data show that the aged form of HSA could have a different structure than those observed in the other pHs. Finally, let us remind that human serum albumin undergoes several transitions in dependence of pH, the N–F transition between pH 5 and 3.5, the F–E transition or acid expansion below pH 3.5, and the N–B transition between pH 7 and 9. Human serum albumin secondary and tertiary structures have been studied by circular dichroism spectroscopy at different pH. The results have shown a reduction of helical structure and an increase of sheet structure content of the N–F transition (pH 5.0–3.5) [16,19,20]. This secondary structural modification, very important between pH 3.5 and 2.5 [16,19,21], is accompanied by tertiary structural alterations in the N–F and F–E transitions. At pH 2, the authors considered from the observed signals the existence of tertiary packing, at least around aromatic residues and disulfide bridges [16]. In the alkaline pH range between pH 7.4 and 9.0 (N–B transition), HSA secondary structure displays a slight reduction in helical content and a small increase in sheet structure, [16,22] accompanied by modifications in the tertiary structure [16].

In order to find out whether HSA is unfolded or not at pH 2 and 3, fluorescence spectra of the protein were recorded at pH going from 2 to 12. From pH of 4 to 12, emission peak found is equal to 339 ± 1 nm and thus the structure surrounding Trp residue in HSA is stable at all pH. On the contrary, at pH 2 and 3, tryptophan residue is surrounded by a more hydrophobic area than at pH equal

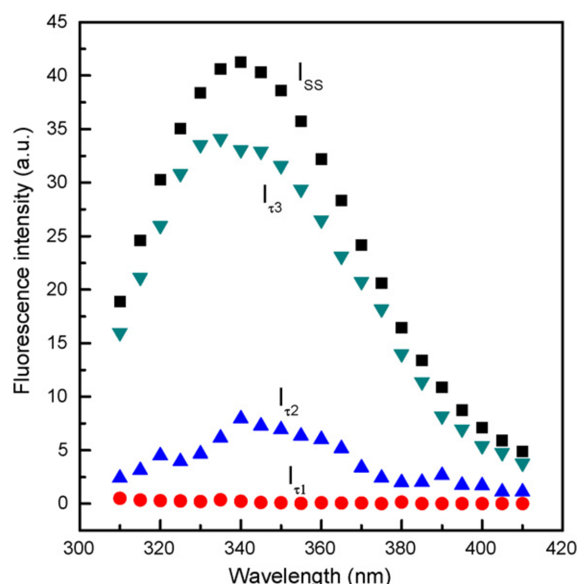

Fig. 9. Time-resolved spectra of HSA Trp residue at pH 7.5. Emission peaks are equal to 340 nm (first and second lifetimes) and 335 nm for the third lifetime.

to or higher than 4 since the observed peak is at 329 ± 1 nm (data not shown). Thus, at pH 2 and 3, no HSA denaturation occurs but a structural reorganization. Tryptophan 214 residue at pH 2 and 3 is more embedded in the protein core than at higher pH. Complete protein denaturation leads to an emission peak for the tryptophan around 350–355 nm. This result is in good agreement with those published in [16] where the authors studied HSA fluorescence from pH of 6 to 12 and in [17] where the authors recorded the emission spectra of HSA from pH of 1.5 to 9.

The structure observed at pH 2 and 3 (extended form) corresponds to a molten globule form [18] which is an intermediate state of the protein before reaching denaturation. In this state, the protein could be partly unfolded; importance of this partial unfolding depends on the protein studied.

Contribution of each lifetime to global fluorescence intensity was calculated and plotted with time-resolved emission spectroscopy at all the pHs. Fig. 9 displays steady-state emission spectrum (I_{ss}) of HSA Trp-214 residue and those of the three lifetimes at pH 7.5. The shortest lifetime τ_1 contributes the less to overall fluorescence intensity. The main contribution to fluorescence intensity is provided by the third fluorescence lifetime τ_3. Also, the observed peaks intensities were at 340 nm (for τ_1 and τ_2) and 335 nm for the third lifetime. These results were observed at all studied pH. One should mention here that we plotted the emission spectra every 5 nm instead of 1 nm, and therefore the peaks of the calculated spectra could be slightly different and located between 335 and 340 nm.

In conclusion, the present work shows that fluorescence lifetimes of tryptophan are generated by sub-structures formed in the excited state. Two of the three lifetimes were observed for tryptophan free in solution. In most of the proteins, a third lifetime is recorded. This lifetime is the result of the interaction between the tryptophan residue and the surrounding amino acids. Structure or the global form of the protein has no influence on the presence of the three lifetimes. However, lifetimes and pre-exponentials values are dependent on the number of emitting tryptophan residues [14] and on the type of interaction that is occurring between Trp residues and the surrounding environment ([14] and present work). Fluorescence of human serum albumin gives three lifetimes, each lifetime refers to a specific orientation of tryptophan residue within the protein.

Also, the present work clearly set up the possibility of observing the different forms of HSA obtained at different pH and to confirm that fluorescence lifetimes measurements allow to differentiate three protein populations: extended form (E) at pH 2 and 3. For pH values in a range 4–10, three protein forms: migrating (F), normal (N) and basic forms (B). The third type of population is obvious for pH values 11 and 12 characterizing the aged form (A) of human serum albumin.

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