PICOSECOND TRYPTOPHAN FLUORESCENCE OF HUMAN BLOOD SERUM OROSOMUCOID

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The state of three tryptophyl residues in human serum orosomucoid was estimated by prediction methods based on parameters characterizing their hydrophobicity either directly, or in terms of buried surfaces of the individual amino acid residues. It is shown that tryptophan 25 is the most buried, while Trp 160 is the most exposed to the solvent. Trp 122 is in this respect in an intermediate state. The fluorescence decay behaviour was determined using a picosecond single photon counting system. The multiwavelength data were analyzed using a global analysis as well as a distribution of lifetimes program. Both procedures yielded the existence of four wavelength independent lifetimes (0.22 ns, 1.0 ns, 2.5 ns, and 8.4 ns). A tentative assignment of the decay associated spectra of the four components to the three individual tryptophans is presented.

Key words: Orosomucoid; Time-resolved fluorescence; Distribution analysis.

Human blood serum orosomucoid (acid α_1-glycoprotein; ORS throughout this paper) is a medium-size protein of the molecular weight 41 000. In this molecule 181 amino acids are arranged into one chain with two disulfide bridges joining Cys 5 and 147, and Cys 72 and 164. Five carbohydrate units representing together 42% of the total molecular weight are attached to asparagine residues; these units are located in the first half of the primary sequence. Typical for this protein is a high number of possible amino acid substitutions at 21 positions[1]. The high content of acidic components leads to isoelectric point varying between 1.8 and 2.7 depending on the buffer (for physicochemical properties of ORS see reviews by Schulze and Heremans[2], and Jeanloz[3]). The ORS is extraordinary stable: It acquires reversibly the original conformation after treatment with 2-chloroethanol; its denaturation introduced by 6 M urea is reversible, as well.

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Thermal stability of this protein is also unusual: It is denatured in aqueous solution upon heating at 100 °C after 24 h (ref.[4]). There are, however, signs that thermal changes follow rather complicated pattern. Differential scanning microcalorimetry of isoionic ORS was not fully reversible, and temperature perturbation difference spectra (TPDS) showed hysteresis of $\Delta\varepsilon_\lambda$ vs ΔT curves for some wavelengths even at pH 7 (ref.[5]).

Circular dichroic spectra in aqueous solution at pH 7 revealed an untypical structure: 10% α-helix, 60% β-sheet, and 10% β-turns type II. In the presence of methanol (volume fraction 70%) the α-helix content increased slightly compared to aqueous solution. After heating up to 80 °C and subsequent cooling down to 25 °C the α-helix content was found to be higher than in the native molecule[6]. Thus, it seems that in the environment with higher hydrophobicity ORS assumes an even more regular structure than in the native state. The native state is characterized by a high number of masked groups. In the pH region 2.5–12 the titration curve of this protein is reversible, but only 63 out of 76–78 potentially titratable groups (including 16 sialic acid residues) are titrated. There are 10 carboxylic groups buried[7,8], out of the total 47, and 3 to 5 tyrosines[9,10] out of the total 12. As found by TPDS, there is only 1 phenylalanine out of 12 accessible to the solvent in the ORS molecule in its native state[11]. These data indicate a compact structure with a very hydrophobic core.

Taking into consideration the relative low number of only three tryptophan residues in ORS, time-resolved fluorescence spectroscopy[12] appears to be a powerful tool to get more information about the structure and function of ORS. In order to apply this method in various functional investigations, two main requirements have to be fulfilled: First, the fluorescence decay behaviour of the three tryptophans has to be characterized. Though there has been already made such an attempt[13], the limited time resolution of these experiments together with the small amount of data gathered, led to a superficial picture of the tryptophan fluorescence in ORS. This fact motivated us to perform a picosecond multi-wavelength study to characterize the ORS fluorescence. Second, information about the microenvironments of the individual tryptophans has to be available. Although it is known from the steady-state fluorescence studies[14], chemical modification[9], and TPDS (ref.[5]) that only one residue is exposed to the solvent, we try in this study to get more detailed information about the tryptophan microenvironment using several prediction techniques.

EXPERIMENTAL

Materials

Human blood serum orosomucoid molecular mass = 41 kDa (a gift from Dr H. Haupt, Behringwerke, Marburg) was homogeneous on polyacrylamide gel electrophoresis; the content of impurities was below the level of detection. Concentrations were determined by ultraviolet spectrophotometry at 280 nm (ε = 29.7 mM^{-1} cm^{-1}). Protein solutions were stored in Tris(hydroxymethyl)aminomethane (Tris)

buffered saline (0.05 M Tris, 0.1 M NaCl, pH 7.4) at –70 °C. Experiments were carried out at a concentration of 4 μM ORS in Tris-buffered saline (0.01 M Tris, 0.1 M NaCl, pH 7.4) at 25 °C.

Prediction Techniques

The content of secondary structures in the ORS molecule and details of their potential accessibility to the solvent were estimated by prediction methods. In all cases examined, a segment of 25 amino acids was chosen with the tryptophyl in question in the central position. Following approaches were used:

1. The type and the content of secondary structures was predicted by the method of Chou, Fasman[15] using more precise parameters[16], and by the GOR method[15] with the parameters for the secondary structure content $\alpha < 20\%$, $\beta > 20\%$.

2. The approximate hydrophobicity was estimated by hydrophobic profile[17] using binary code (hydrophobic 1, hydrophilic 0). In this case sliding window 5 was calculated.

3. These data were compared with the hydrophobicity profile calculated using the scale of Fauchére and Pliska, normalized by Cornette et al.[18] based on octanol/water transfer.

4. The average fraction of buried surface was estimated using the data of Rose et al.[19].

For methods *3* and *4,* average values of studied parameters were calculated as both, sliding average (SA) and weighted sliding average (WSA). The second type of calculation takes in consideration decreasing influence of more distant residues on the central one[11]. Since it includes three residues to the left and to right, the window for WSA is 7 and the same was used for SA to make the results comparable.

Fluorescence Measurements

Corrected fluorescence spectra were recorded with a SLM 8000 spectrofluorometer (Urbana, IL) using a "magic angle" configuration. Bandwidths of 2 nm were set for the excitation as well as for the emission.

The fluorescence lifetime measurements were performed using a single photon counting technique as described elsewhere[20,21]. The excitation at 295 nm was vertically polarized. The emission was detected after passing through a "magic angle" polarizer and a J-Y H10 (Instrument SA Inc., Metochen, NJ) monochromator with a 2 nm band pass, on a Hamamatsu R1645U (Bridgewater, NJ) microchannel plate photomultiplier. Fluorescence decays were collected with 10 000 to 40 000 counts in the peak. The time scales were 14.3, 25.5 and 62.4 ps per channel, 512 channels collected. The instrument response functions (55 ps full width at half maximum) were measured using a light scattering solution of a non-diary coffee creamer. The fluorescence decays of ORS were collected from 300 to 420 nm in 10 nm steps. Blank experiments using Tris-buffered saline were performed to exclude distortions by scattered light. The intensity of the scattered light at 300 nm, as well as at the H_2O Raman band, was negligible.

Data Analysis

For the basic multiexponential analysis a non-linear least-squares iterative reconvolution procedure was used. The program is based on the Marquardt Levenberg algorithm[22] and the optimization includes stray light and time shift[23]. To account for the possibility of a continuous distribution of lifetimes, the data were fit using the symmetric Lorentzian distribution and the asymmetric Kohlrausch–Williams–Watts distribution[23].

A modified version of the commercially available Edinburgh Analytical Instruments (Edinburgh, U.K.) global analysis program was employed to simultaneously reconvolve the decays collected at different

emission wavelengths. The program uses the Marquardt Levenberg algorithm. The wavelength dependent sets of fluorescence decays were analyzed by linking the lifetime values.

To characterize the number of classes of fluorescence lifetimes the Edinburgh Analytical Instruments distribution program was used. The program is based on the minimum energy method where the lifetimes are equally spaced on a logarithmic scale.

All three programs use reduced χ^2 as a criterion for the goodness of fit evaluation. Furthermore, the quality of the fit was judged by weighted residuals and the Durbin–Watson parameter. In the case of the global analysis the mean reduced χ^2-value ($\chi^{2(\mathrm{Global})}$) was used to evaluate the goodness of the fit.

Decay-associated spectra (DAS) were obtained by combining the time-resolved data with the steady-state emission spectra, as described elsewhere[12]. To determine the emission maxima, the DAS were fit to log-normal distribution functions[24].

RESULTS

Prediction Methods

The combination of two methods for secondary structure prediction led to results slightly different from the output of previous attempts[25,26]. Thus, a more detailed analysis of the application of different prediction methods on glycoproteins is prepared. For the present study prediction was limited on shorter segments of the primary sequence. According to the present calculation only Trp 25 is located within a β-sheet formed between Ile 21 and Ile 28, while the other two tryptophyls are in the aperiodic regions of the ORS molecule. As shown before[26], the distribution of hydrophobic amino acids in ORS molecule is uneven; they prevail in the first half of the primary structure. The other half is more hydrophilic, particularly the last 20 amino acids. Trp 25 is located in one of strongly hydrophobic segments, as apparent from a high value of hydrophobic profile which at Tyr 27 reaches 5, the highest possible value for the chosen window. Two other parameters, hydrophobicity in octanol/water scale (Fig. 1), and the fraction of buried surface (Fig. 2), reflect the same property more precisely. These data show that Trp 25 is located in a strong hydrophobic region between Gly 23 and Ala 29.

TABLE I
Predicted parameters of tryptophyl residues in ORS

Trp	Predicted structure	Hydrophobic profile	Hydrophobicity	Fraction of buried surface
25	β-sheet	4	2.380	0.758
122	aperiodic	3	0.661	0.692
160	aperiodic	1	–0.018	0.655

Compared with these data, Trp 160 is located in a minimum of the hydrophobic profile and has the lowest average value of buried surface. This tryptophyl is the first amino acid of extremely hydrophilic C-end of ORS molecule.

The values of these parameters for Trp 122 are intermediate between the data for Trp 27 and Trp 160, close to the average for the whole ORS. The numerical values for all three tryptophyls are summarized in Table I.

Characterization of the Fluorescence Decay of ORS

To describe the fluorescence decays of tryptophans in proteins at a single emission wavelength, two possibilities have to be considered. Either the intensity decay is interpreted by a set of separate classes of lifetimes or, as in recent publications[27–30], by a (broad) continuous lifetimes distribution model. Although the second case seemed to be unlikely for a three tryptophans containing protein, the fluorescence decays of ORS were fit using the symmetric Lorentzian distribution profile and the asymmetric

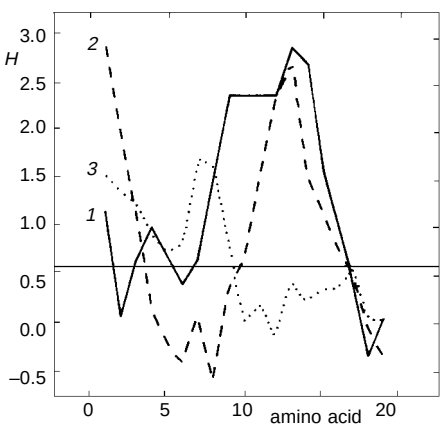

FIG. 1

Hydrophobicity, *H*, in octanol/water scale[18] calculated as the sliding average for a segment of ORS with tryptophyl at the position 10 in this graph. The curves correspond to individual tryptophan residues: *1* Trp 25, *2* Trp 122, *3* Trp 160. The horizontal line shows the average hydrophobicity per residue of the whole ORS molecule

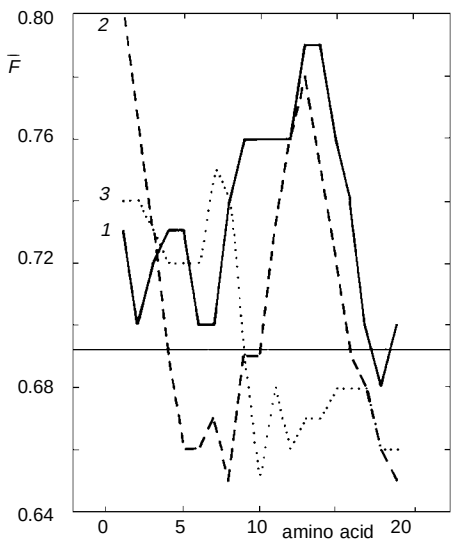

FIG. 2

The average buried area, $\bar{F}$, of amino acids along the segment of the primary chain of ORS with the tryptophan at the position 10 in this graph. The values are sliding averages based on the data of Rose et al.[19]. The curves correspond to individual tryptophan residues: *1* Trp 25, *2* Trp 122, *3* Trp 160. The horizontal line is the average value of the buried surface per residue of the whole ORS molecule

Kohlrausch–Williams–Watts distribution. Unacceptable high χ^2-values (> 2.0) led to the conclusion that excitation at 295 nm creates more than one class of tryptophan excited-states.

To characterize the number of separate classes of lifetimes, the Edinburgh Analytical Instruments distribution program was applied. In contradiction to the continuous distribution models used above, there is no "a priori" assumption about the distribution shape. The fluorescence decay is fit to a sum of one hundred lifetime values. The best fit is determined by the minimum energy method. It has to be stressed, that no application of a distribution of lifetimes program based on the minimum energy method has been published so far. Thus, it is necessary for a valid interpretation of the ORS results to characterize the significance and accuracy of obtained lifetime profiles. For this purpose the fluorescence decays of four standards (1,4-diphenyl-1,3-butadiene, 2,5-diphenyloxazole, anthracene, and pyrene, all in cyclohexane) with different lifetime values (0.56 ± 0.01 ns, 1.31 ± 0.01 ns, 5.09 ± 0.01 ns, and 19.5 ±00.01 ns, respectively) were accumulated to simulate a defined 4-exponential decay. The signal-to-noise ratio was comparable to the ORS experiments. Figure 3 demonstrates that the existence of four different relevant lifetime classes can be resolved. However, the width of the found four lifetime distributions is substantially larger than the four standard deviations that result from the conventional 4-exponential fit.

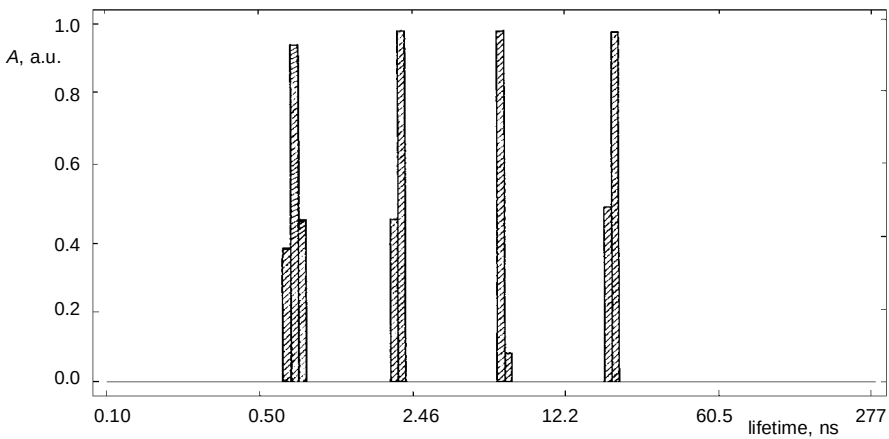

FIG. 3

Amplitude profile, *A*, of the fluorescence lifetimes distribution of a defined 4-exponential decay, produced by the Edinburgh Analytical Instruments distribution program. In order to create a defined 4-exponential decay, the fluorescence decays of four standard fluorophores with monoexponential decays were superimposed computationally. The standards (1,4-diphenyl-1,3-butadiene, 2,5-diphenyloxazole, anthracene, and pyrene, all in cyclohexane) have been selected to span for tryptophan relevant lifetime range (0.56 ± 0.01 ns, 1.31 ± 0.01 ns, 5.09 ± 0.01 ns, and 19.50 ±0.01 ns, respectively). The mean lifetime values from the distribution fits are 0.72 ns, 2.1 ns, 6.2 ns, and 20.0 ns

When applied to the fluorescence decays of ORS, the analysis of the decays detected at 300 to 420 nm yielded 4 lifetime classes. The values of the lifetime centers are identical for all emission wavelengths within the experimental error, while the fractional amplitudes vary. The overall wavelength averaged values are 0.22 ns (component A), 1.1 ns (B), 2.5 ns (C), and 8.6 ns (D). Comparison of the distribution profiles of ORS (Fig. 4) with those of a defined 4-exponential decay (Fig. 3) shows that the four components are subjected to a broadening arising from ground state heterogeneity. However, the resulting widths are small in comparison to those of proteins for which continuous distributions were discussed[27–30].

The conventional multiexponential analysis of the wavelength dependent set of the decay curves includes judging the fits by the above mentioned criteria and leads to the same conclusion, i.e. the existence of four components with different emission spectra. Again, the lifetime values were wavelength independent within the experimental error, whereas the amplitudes changed with the wavelength. In conclusion, the existence of four lifetime classes was concluded from the results of two different iteration methods, the Marquardt Levenberg algorithm for multiexponential analysis and the minimum energy method for a distribution of lifetimes.

Although the comparison of Figs 3 and 4 indicates that the four lifetime classes in the fluorescence decay of ORS exhibit significant distribution widths, a global multiexponential analysis of the fluorescence decays has been performed as well. Since the multiexponential analysis yielded constant lifetime values over the emission spectrum,

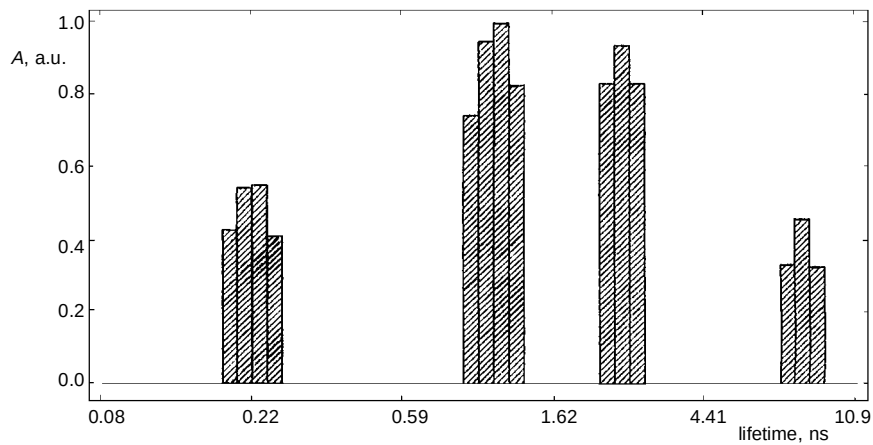

FIG. 4

Amplitude profile, *A*, of the fluorescence lifetimes distribution of ORS fluorescence decay (λ_{ex} = 295 nm; λ_{em} = 330 nm; 4 μM in Tris-buffer) resulting from Edinburgh Analytical Instruments distribution analysis. The resulting mean lifetimes for this measurement are 0.22 ns (component A), 1.1 ns (B), 2.5 ns (C), and 8.6 ns (D)

the fluorescence lifetimes were linked. Again, four lifetime components were necessary to reach an acceptable $\chi^{2(\text{Global})}$-value of 1.20. The fluorescence decay time values obtained this way are 0.22 ± 0.02 ns (component A), 1.0 ± 0.2 ns (B), 2.5 ± 0.2 ns (C), and 8.4 ± 0.5 ns (D). The standard deviations resulting from the global analysis were significantly smaller in comparison with the unlinked multiexponential analysis of individual decays. The decay-associated spectra of ORS, which were calculated from the steady-state intensities and the fractional intensities obtained by the global analysis, are shown in Fig. 5. The emission maxima of the log-normal fits of the five components are 334 nm (A), 334 nm (B), 337 nm (C), and 352 nm (D).

DISCUSSION

The values of the hydrophobicity parameters (Table I) should be discussed in the framework of possible limiting values of these parameters, as well as, of the properties of the entire ORS molecule. The extreme values of hydrophobicity in the scale by Cornette et al.[18] are +6.19 for tryptophan and –2.78 for arginine. The average value of this parameter for the entire ORS molecule is 0.870 and, thus, not far from 0.661, the value calculated for Trp 122. Moreover, the fraction of buried surface calculated as the average for the whole ORS molecule is 0.715. This value is again close to the corresponding value calculated for Trp 122 (0.692). Both results support the idea that this residue is just in an intermediate state with respect to hydrophobicity of the environment and the accessibility to the solvent. Already the data shown in Table I bring evidence that the two remaining tryptophyls are at both extremes: Trp 25, which is located in extreme hydrophobic surroundings, should be expected to be the least accessible, Trp 160, exposed to the solvent due to its strongly hydrophilic environment, should be the best accessible one.

The question which has not been answered satisfactorily is the effect of carbohydrate part on both, the stability of ORS molecule and the accessibility of its peptide part. Five

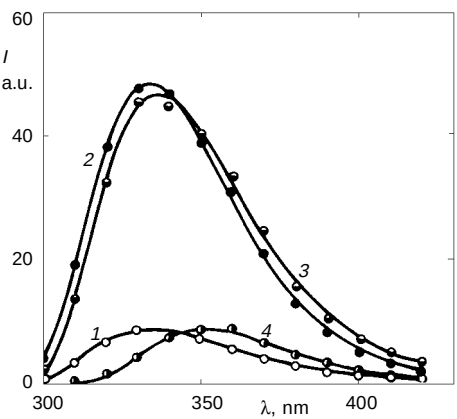

FIG. 5
Decay-associated emission spectra of ORS (λ_{ex} = 295 nm). The fluorescence decay time values are 0.22 ± 0.02 ns (component A, spectrum *1*), 1.0 ± 0.2 ns (B, *2*), 2.5 ± 0.2 ns (C, *3*), and 8.4 ± 0.5 ns (D, *4*). The emission maxima of the log-normal distribution fits are 334 nm (A, *1*), 334 nm (B, *2*), 337 nm (C, *3*), and 352 nm (D, *4*). Shown are the experimental points and the corresponding log-normal distribution fits

branched chains are attached to the polypeptide chain at the positions 15, 38, 54, 75, and 85 (ref.[1]). They are located in the first half of the ORS molecule, which has prevailing hydrophobic character. Along with the hydrophobic environment these sugar residues might contribute further to the masking of the first tryptophyl (Trp 25). The second one might be influenced by some branches of the fifth saccharide unit, but not to such extent like the previous one. Trp 160 appears from this point of view the more accessible one, due to the prevailing hydrophilic environment and the absence of carbohydrate parts in its vicinity.

These observations were supported by TPDS in aqueous and in mixed solutions[5]. While in the aqueous solution the number of accessible tryptophyls is always ≤ 1, in the presence of methanol (55 vol.%, pH 5) it increases to 2, obviously due to the increase in the hydrophobicity of the solvent[26]. The third tryptophyl remained masked in methanol solution even at pH 12. It was a further proof of the high stability of the hydrophobic core of ORS.

The description of the fluorescence decays of ORS by a decay model with four wavelength independent lifetimes results from the distribution of lifetimes analysis (minimum energy method) and the global analysis (Marquardt Levenberg algorithm). Both methods yield essentially identical mean lifetime values. Thus, the fluorescence of the three tryptophans is characterized by at least four different tryptophan excited-states. Contributions of excited-state reactions, as Trp-Trp energy transfer[31–33] or solvent relaxation during the excited-state lifetimes[34–36] are unlikely, since a reconvolution fit does not have any negative fractional amplitudes at the red edge of the emission spectrum and the steady-state spectra do not change with red edge excitation (data not shown).

The fluorescence decay of the basic model compound for tryptophan in proteins, *N*-acetyl-L-tryptophanamide, shows a monoexponential decay behaviour[12]. Since we found four lifetime components for the three tryptophan protein ORS, we conclude that at least one of tryptophans experiences two energetically favoured environments with significantly different tryptophan quenching properties. Moreover, a comparison of the distribution widths of the defined 4-exponential decay (Fig. 3) with those of ORS (Fig. 4) show that each of the four tryptophan excited states in their specific environments are subjected to a broadening arising from ground state heterogeneity. This conclusion finds support in the fact that the thermal movement of the peripheral residues of ORS is not sufficiently suppressed, so that the X-ray diffraction could not be evaluated in order to obtain the three-dimensional structure of this protein[4].

A fluorescence decay time is generally determined by the radiative fluorescence lifetime and the rate of nonradiative deactivation processes. The radiative lifetime for tryptophan can vary from 10 to 70 ns depending on the environment[36]. Though there are contradictory results in the literature[37], a qualitative rule indicates that buried, more blue emitting tryptophans will exhibit shorter radiative lifetimes than those exposed,

the more red emitting ones[36,38–40]. Thus, it is likely that components with shorter lifetimes (A and B) are due to the fluorescence of the more buried Trp 25 and/or Trp 122. This assumption is supported by the relatively blue emission of this components (334 nm). The relatively long lifetime value of component D (8.4 ns) together with the red shifted emission (352 nm) indicates that Trp160 is responsible for component D. From the lifetime value (2.5 ns) as well as from the emission maximum (337 nm) of component C no tentative assignments can be concluded. In order to unambiguously correlate the four observed lifetimes with the predicted state of the tryptophan residues further picosecond fluorescence data collected at different pH-values[11], as well as, studies including the use of low-molecular mass quenchers[12] will be necessary.

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