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ORIGINAL CONTRIBUTION

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Fluorescence of 1,3-Di(1-pyrenyl)propane probe incorporated into human serum albumin protein enforced conformations of the probe

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Abstract Steady-state and time-resolved fluorescence spectra of 1,3-di(1-pyrenyl)propane (1Py-(3)-1Py) incorporated into macromolecules of human serum albumin (HSA), into micelles of dodecyltrimethylammonium chloride (DTAC), and dissolved in 1,4-dioxane were compared. The steady-state fluorescence spectra indicated that in all the mentioned environments, upon excitation of 1Py-(3)-1Py, light was emitted from the single pyrene chromophores (1Py*) and from the 1Py, 1Py* excimers. The time-resolved fluorescence emission registered at 480 nm (excimer emission) for 1Py-(3)-1Py in the DTAC micelles and dissolved in 1,4-dioxane allowed to monitor formation of excimer with time constant $\tau_1 = 40.0$ ns and 9.6 ns, for 1Py-(3)-1Py in the DTAC micelles and in 1,4-dioxane, respectively. However, when the 1Py-(3)-1Py probe was located inside of the macromolecules of HSA, only the decay of emission was observed for excimer with our set-up ($t > 2$ ns after excitation). The instantaneous formation of excimer, unrelated to the decay of monomer excitation, indicates that the considerable fraction of 1Py-(3)-1Py in the hydrophobic pockets of HSA is present as the ground state dimer. The red shift ($\Delta\lambda = 8$ nm) and broadening of UV absorption for 1Py-(3)-1Py in HSA (when compared with absorption 1Py-(3)-1Py in 1,4-dioxane) and comparison of excitation spectra of 1Py-(3)-1Py in HSA and in 1,4-dioxane also indicate that label molecules bound to some sites of HSA are in the ground state in the dimer conformation. Moreover, the close values of the ratios of intensities of monomer emission to excimer emission, registered 2 ns (5 ns gate) after excitation pulse with duration 300 ps and at the steady-state conditions, indicate that the interconversion between conformers of 1Py-(3)-1Py inside of the macromolecules of HSA is slow in comparison with the decay time of Py chromophore in the excited state in HSA (two-exponential decay with decay times $\tau_1 = 2.41$ ns, $\tau_2 = 69.0$ ns). Thus, ratios of the intensities of monomer and excimer emissions of 1Py-(3)-1Py in HSA do not allow to obtain any information on the local microfluidity inside of the protein macromolecules but could be used for discrimination between different conformations of the probe, possibly located in different protein pockets.

Key words 1,3-di(1-pyrenyl)propane – human serum albumin – steady state fluorescence emission spectroscopy – time-resolved fluorescence emission spectroscopy

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Introduction

Sensitivity of the fluorescent emission to the nearest environment of fluorophores is often used as a basis for experiments allowing to characterize local properties of matrices into which these fluorophores were embedded. Thus, compounds with appropriate fluorophores were used for probing local polarity, pH, and concentrations of particular ions [1]. Many fluorophore bearing compounds were used for determination of the local fluidity of the matrices. To this group belong compounds with planar fluorophores with condensed aromatic rings (e.g. dansyl) for which it is possible to characterize the rotational mobility by measuring the depolarization of emission [2] and compounds bearing more than one fluorophore which can live in an excited state for a time long enough to allow the conformational changes bringing the chromophores of the probe (one in the excited state) together. This results in formation of an excimer emitting light at wavelengths different from those of the single excited fluorophore [3]. Probes with pyrene substituents are very convenient for this purpose.

In the absence of quenchers (e.g., oxygen) the single pyrene moiety is notable for its long life in the excited state (ca 200–400 ns depending on the medium [4, 5]) and provides a long time span for formation of an excimer. Thus, fluorescent spectroscopy of macromolecules labeled at the ends with pyrene labels was used for studies of molecular dynamics [4, 6, 7] and the short linear molecules with pyrene groups at the ends were found to be suitable for characterization of the microfluidity of their environment [8–10].

In every case, when the fluorescence spectra of any probe with fluorophores capable to form excimer are analyzed, one has to address the questions related to the conformations of the probe in the ground state. For example, it has been found that 1,*n*-bis(1-pyrene-Pycarboxy)alkanes and 1,*n*-bis(2-pyrenecarboxy)alkanes form intramolecular Py, Py ground state dimers, respectively for $n = 3$–16, $n = 22$ and for $n = 3$–16, $n = 32$ [11]. It has been disputed whether in the case of 1Py-(3)-1Py the time resolved spectra could be used as a basis for elucidation of information on the distribution of various conformers of this compound in the ground state [12, 13]. In spite of some disagreements on this subject, data gathered until now indicate that in solutions, prior to excitation, fractions of molecules of 1Py-(3)-1Py in the conformations with an intramolecular sandwich-like 1Py, 1Py dimer prearranged in the ground state are negligible and that the time-resolved emission spectroscopy of this compound provides information on its molecular dynamics [12–14].

We were interested in using 1Py-(3)-1Py as a probe for studies of human serum albumin (HSA). Human serum albumin is a protein most abundant in the blood serum, being responsible, among others, for transportation of hydrophobic compounds [15–17]. X-ray studies of HSA crystals revealed that this single chain protein is composed of three domains, with binding sites for hydrophobic compounds located in domain III and in the part of domain II close to domain III [15]. HSA is known also for its tendency to form aggregated structures composed of the two or more HSA macromolecules, i.e. HSA dimers and oligomers, respectively [18, 19]. We expected that the steady state and the time resolved fluorescent spectra of 1Py-(3)-1Py, located inside of the HSA binding centers, will provide information on the properties of the HSA segments surrounding the probe.

In a recent review, F.M. Winnik collected and analyzed experimental data indicating that in the various kinds of organized media (e.g., Langmuir–Blodgett films and micelles) the pyrene chromophores can become prearranged in the ground state into dimers [20]. Thus, we wanted to explain whether location of 1Py-(3)-1Py in the binding sites of HSA will result in any matrix enforced formation of the ground state pyrene dimer of this bichromophoric compound.

Experimental

Human serum albumin (HSA) (Sigma, Cohn fraction V) and dodecyltrimethylammonium chloride were used as received. 1,3-Bis(1-pyrenyl)propane (1Py-(3)-1Py) (Molecular Probes) was used without further purification.

HPLC traces of HSA before and after labeling with 1Py-(3)-1Py were obtained using a setup of a 2150 HPLC pump (LKB), a Superose column (Pharmacia), and a SEM 4000 UV detector (LDC). Phosphate buffer was used as an eluent. Flow rate was 0.5 ml/min.

Human serum albumin was labeled in a similar manner as was described earlier [18] by slow addition of 500 μl of 1Py-(3)-1Py solution in 1,4-dioxane ([1Py-(3)-1Py] = $5.0 \cdot 10^{-4}$ mol/l) to 49.5 ml of HSA solution in PBS ([HSA] = 1.0 g/l). Finally, the solution was filtered through a 0.2 μm filter with the purpose to remove any possible protein agglomerates. HPLC traces of HSA before and after labeling with 1Py-(3)-1Py are given in Fig. 1. It was found, by HPLC method, that in solutions of HSA labeled with 1Py-(3)-1Py not less than 98% of the label was bound to protein macromolecules. HSA used in our experiments contains monomeric macromolecules as well as dimeric and oligomeric protein aggregates in proportion 1:0.209:0.087, respectively. In our earlier studies, we found that 1Py-(3)-1Py labels predominantly oligomeric HSA [18]. Results presented in Fig. 1 conform to these findings.

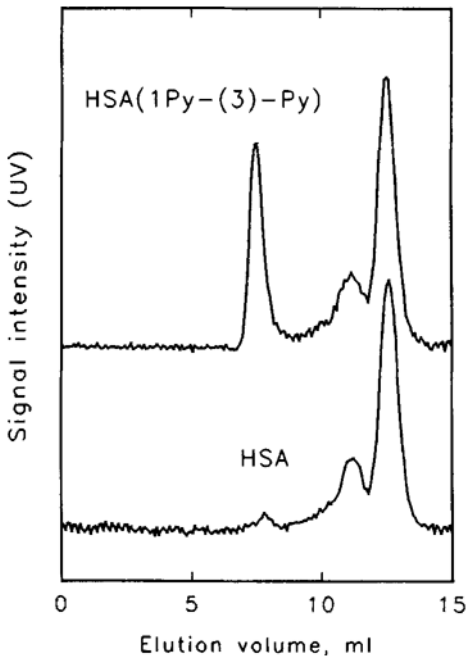

Fig. 1 HPLC traces of HSA before and after labeling with 1Py-(3)-1Py. UV detection at 280 nm and 345 nm for HSA and HSA(1Py-(3)-1Py), respectively

Absorption spectra were monitored using a Hewlett Packard 8452A diode array spectrophotometer. Emission and excitation spectra were recorded using a Perkin Elmer LS 50 spectrometer. For monitoring the emission decays the samples were excited at $\lambda = 337.1$ nm with a nitrogen laser (Laser Photonics, USA, model LN 120C; energy up to 78 μJ in the 0.3 ns pulse). Orientation of samples kept in a 1 cm quartz cell was adjusted in order to reduce scattered-light interference from the laser pulse. Appropriate optical filters were used to avoid reflections of the laser light directly onto the monochromator slit. The emission after passing through the monochromator (Bausch & Lomb, slit setting 0.1) was detected with a Hamatsu R928 or R3890 photomultipliers or a biplanar vacuum photodiode type F-4014 manufactured by ITT (Fort Wayne, USA) with 60 ps response time. Then the signal was recorded on a digitizing oscilloscope (Hewlett-Packard 54510 A), transferred via an interface (HP-IB) to an IBM compatible computer for storage and analysis. The Optical Spectrometric Multichannel Analyzer (OSMA) (Princeton Instruments Inc., USA) based on proximity focuses MCP (multichannel plate) image intensified diode array detector (equipped with controller ST 120, high voltage gate generator PG 200, and spectrograph Acton Research Spectra Pro 275) was used to collect emission transient spectra. The fiber-optic adapter has been applied to permit optimum coupling of an emission light collected via a fiber-optic cable to the OSMA system. The emission spectra were recorded approximately 30–40 times per delay setting to improve the signal-to-noise ratio. Emission spectra and decays of HSA(1Py-(3)-1Py) were recorded for protein solutions in PBS in the presence of oxygen from air. We did not remove oxygen from these samples because the freeze-thaw procedure as well as purging with Ar resulted in foaming, due to the low surface tension of the solutions of HSA. It has been found that foaming results in an extensive denaturation of bovine serum albumin [21], a protein with structure and properties very similar to HSA. We expected that foaming would affect binding of 1Py-(3)-1Py. For comparison, we registered (also in presence of oxygen from air) the decays of excitation of 1Py-(3)-1Py in 1,4-dioxane solution and placed inside of the dodecyltrimethylammonium chloride (DTAC) micelles.

Results and discussion

Figure 2 shows the steady state emission spectrum of HSA(1Py-(3)-1Py) (spectrum (a)). In this spectrum signals in the region from 360 nm to 430 nm were assigned to emission from single 1Py* fluorophores and the broad signal with maximum at 480 nm was assigned to the 1Py, 1Py* excimer. In Fig. 2 (spectrum (b)) there is shown the emission spectrum of HSA(1Py-(3)-1Py) registered with the diode array detector 2 ns (gate 5 ns) after the N_2 laser pulse (337.1 nm) with duration 300 ps. Ratio of the intensity of monomeric 1Py* emission to intensity of emission by 1Py, 1Py* excimer (I_M/I_E) was 0.187 and 0.152, for spectra (a) and (b) respectively. Close values of I_M/I_E found from these spectra indicated that proportions of 1Py-(3)-1Py molecules with conformations with only one pyrene in the excited state and in conformations with two pyrene fluorophores in an excimer were similar 2 ns after excitation and at the steady-state conditions. This observation suggested that either the 1Py-(3)-1Py probes undergo extremely fast conformational changes inside of the macromolecules of HSA (this seems to be rather improbable) or that prior to excitation the considerable fraction of 1Py-(3)-1Py bound to the binding sites of HSA was in the dimeric state. With purpose to discriminate between these possibilities we analyzed the absorption and excitation spectra of HSA(1Py-(3)-1Py) and of 1Py-(3)-1Py in 1,4-dioxane solutions. We monitored the time-resolved emissions of monomeric 1Py* (at 400 nm) and of 1Py, 1Py* excimers (at 480 nm) for HSA(1Py-(3)-1Py) and we compared results of these experiments with time-resolved emissions registered for 1Py-(3)-1Py in the 1,4-dioxane solution and in the dodecyltrimethylammonium chloride (DTAC) micelles.

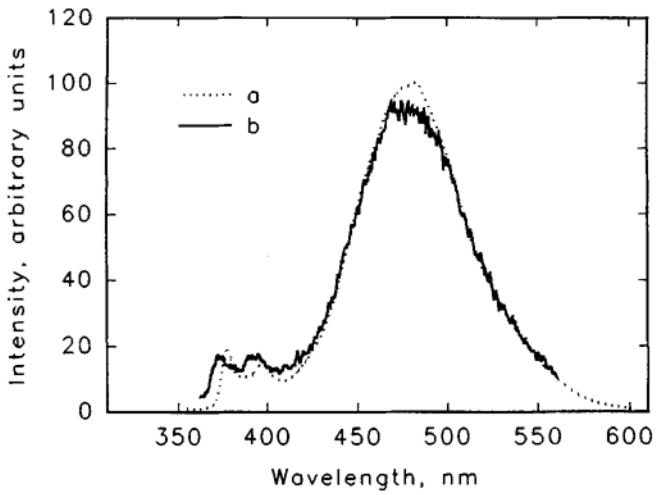

Fig. 2 Steady-state emission spectrum of HSA(1Py-(3)-1Py) in 1,4-dioxane (a) and emission spectrum of HSA(1Py-(3)-1Py) in PBS registered 2 ns (5 ns gate) after the N_2 laser pulse (b)

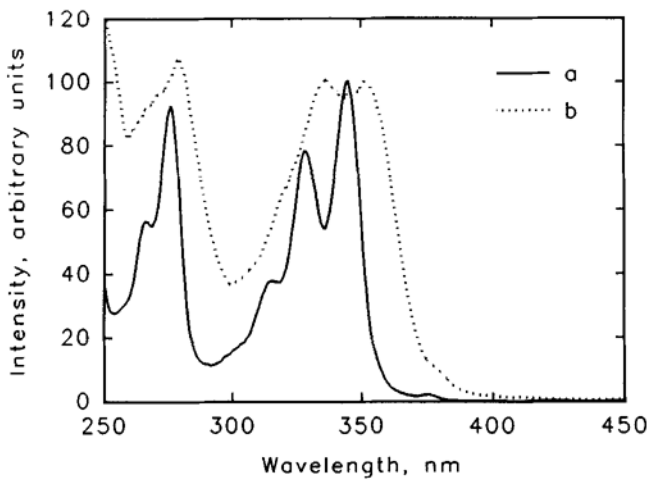

Fig. 4 Excitation spectra of 1Py-(3)-1Py in 1,4-dioxane (a) and of HSA(1Py-(3)-1Py) in PBS (b). Emission monitored at $\lambda = 480$ nm (excimer emission)

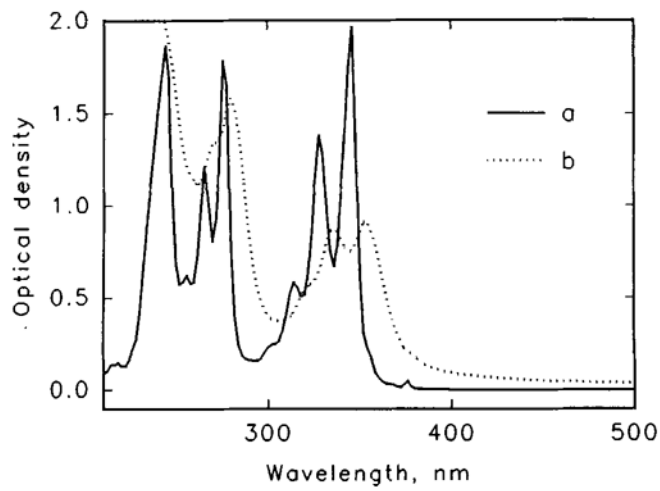

Fig. 3 Absorption spectra of 1Py-(3)-1Py in 1,4-dioxane (a) and of HSA(1Py-(3)-1Py) in PBS (b). Concentrations: [HSA(1Py-(3)-1Py)] $= 1.14 \cdot 10^{-5}$ mol/l, [1Py-(3)-1Py] $= 2.46 \cdot 10^{-5}$ mol/l

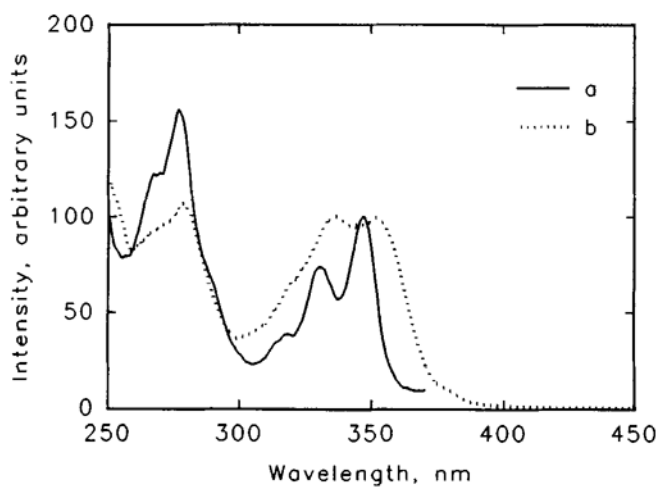

Fig. 5 Excitation spectra of HSA(1Py-(3)-1Py) in PBS monitored at 378 nm monomer emission (a) and at 480 nm excimer emission (b)

The absorption spectra of HSA(1Py-(3)-1Py) and of 1Py-(3)-1Py in 1,4-dioxane solution are given in Fig. 3. In these spectra absorptions in the region 300–400 nm correspond to the ^1L_a and ^1L_b states of pyrene molecule, whereas absorptions in the region 200–300 nm results from the overlapping signals due to tryptophan and tyrosine of HSA and ^1B_b states of pyrene [13, 22]. Absorptions corresponding to 1Py-(3)-1Py in HSA are considerably broader and shifted to the red ($\Delta\lambda = 8$ nm for pyrene in ^1L_a state) when compared with the absorption of 1Py-(3)-1Py in 1,4-dioxane. Such relation occurs between spectra of compounds with two pyrene moieties preassociated into the ground state dimer and with free pyrene groups [20, 23]. Important information can be obtained also from comparison of excitation spectra of HSA(1Py-(3)-1Py) and for 1Py-(3)-1Py in 1,4-dioxane monitored at 480 nm (excimer emission). These spectra are shown in Fig. 4. Significant red shift, broadening, and changes of the fine structure indicate that different species contribute to formation of excimers of 1Py-(3)-1Py in HSA and in 1,4-dioxane. With purpose to distinguish between influence of the HSA matrix on single pyrene chromophores and an effect resulting from the ground state dimer formation, we compared also the excitation spectra of 1Py-(3)-1Py in HSA monitored at 378 nm and at 480 nm, i.e. at the wavelengths corresponding to the 1Py* and 1Py, 1Py* emissions (c.f. Fig. 5). These spectra indicate that indeed,

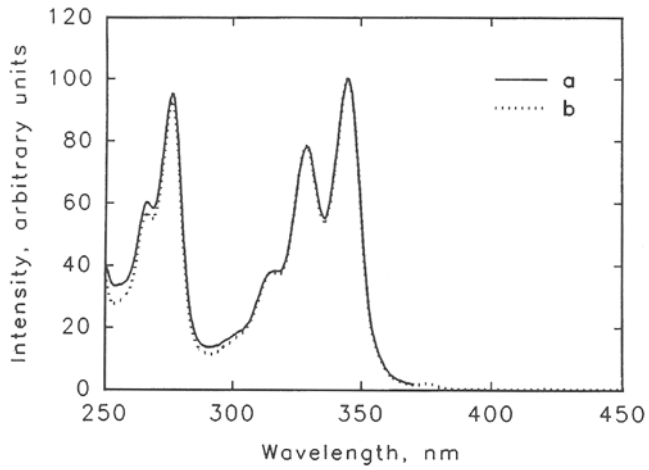

Fig. 6 Excitation spectra of 1Py-(3)-1Py in 1,4-dioxane monitored at 378 nm monomer emission (a) and at 480 nm excimer emission (b)

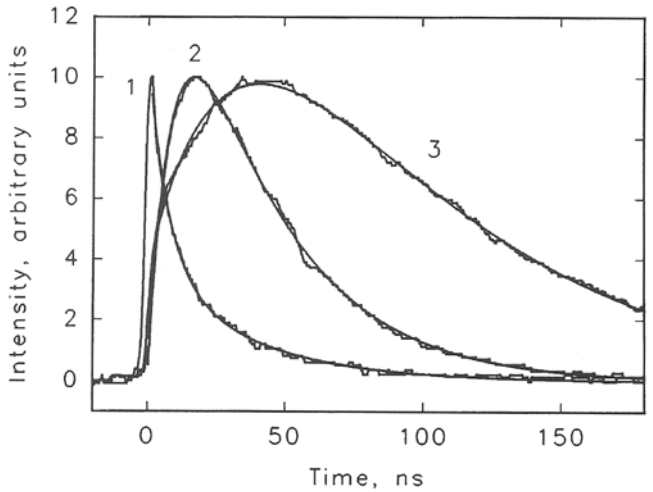

Fig. 7 Time-resolved emission of 1Py, 1Py* excimers of 1Py-(3)-1Py in HSA (1), 1,4-dioxane (2), and DTAC (3)

the species (or their mixtures) which, after excitation, form excimers are not identical. This again supports the hypothesis that at least a part of 1Py-(3)-1Py chromophores in HSA is in the form of the ground state dimer. Similar relation between the excitation spectra with monitored monomer and excimer emissions were observed for pyrene labeled hydroxypropyl cellulose in water when pyrene substituents form the ground state dimers [24]. It is worth to note that for 1Py-(3)-1Py in 1,4-dioxane solutions the excitation spectra monitored at 378 nm and at 480 are identical (c.f. Fig. 6) indicating absence of the ground state dimers in this system.

The time-resolved emissions of 1Py, 1Py* for 1Py-(3)-1Py in 1,4-dioxane solution and in the DTAC micelles shown in Fig. 7 were typical for this bichromophoric compound, i.e., conformed to Scheme 1, indicating formation of excimer with time constant τ_1 followed with decay with time constant τ_2.* In Scheme 1 k_1 and k_2 denote rate constants of the radiative and nonradiative decay of 1Py*, k_3 and k_4 denote the rate constants of excimer formation and dissociation, k_5 and k_6 correspond to the radiative and nonradiative decay of 1Py, 1Py*. The time-resolved emissions of 1Py* (I_M) and 1Py, 1Py* (I_E) in 1,4-dioxane and in DTAC were fitted with two-exponentials.

$$I_M = A_{M1} \cdot \exp(-t/\tau_1) + A_{M2} \cdot \exp(-t/\tau_2) \quad (1)$$

$$I_E = A_E \cdot [-\exp(-t/\tau_1) + \exp(-t/\tau_2)] \quad (2)$$

* In some papers there is proposed for 1Py-(3)-1Py a more complex scheme involving formation of two conformationally different excimers with different lifetimes [13]. However, for the purpose of this study, we do not introduce such detailed discrimination between various kinds of excimers.

according to Scheme 1:

$$\tau_{1,2} = (1/2)\{k_1 + k_2 + k_3 + k_4 + k_5 + k_6 \mp [(k_1 + k_2 + k_3 - k_4 - k_5 - k_6)^2 + 4k_3k_4]^{1/2}\} . \quad (3)$$

In the case of 1Py-(3)-1Py in HSA the resonable fit was obtained for two-exponentials with decay times which were different for 1Py* monomer and for 1Py, 1Py* dimer:

$$I_M = A_{M1} \cdot \exp(-t/\tau_{M1}) + A_{M2} \cdot \exp(-t/\tau_{M2}) \quad (4)$$

$$I_E = A_{E1} \cdot \exp(-t/\tau_{E1}) + A_{E2} \cdot \exp(-t/\tau_{E2}) . \quad (5)$$

Apparently, inside of the macromolecules of HSA the probe was located in various environments.

Values of the decay times for 1Py* and for 1Py, 1Py* produced by excitation of 1Py-(3)-1Py in 1,4-dioxane and inside of DTAC and HSA are collected in Table 1. The measurements we carried out intentionally for the non-deaerated samples (to avoid foaming of HSA and to compare, in similar conditions, formation of 1Py, 1Py* in HSA with formation of excimer in 1,4-dioxane and in DTAC micelles) and thus, we were fully aware that especially the long time decays would be affected by quenching. The longest decay times of 1Py* registered in our studies did not exceed 69.0 ns and were significantly shorter than the longest time constant determined in deoxygenated systems indicating the significant quenching of 1Py* emission with oxygen. For example, for 1Py-(3)-1Py in *n*-heptane Zachariasse et al. observed the three exponential decay of monomer emission with half-lifetimes $\tau_M = 2.5$ ns, 36 ns and 140 ns [13]. The first two lifetimes correspond to

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Scheme 1

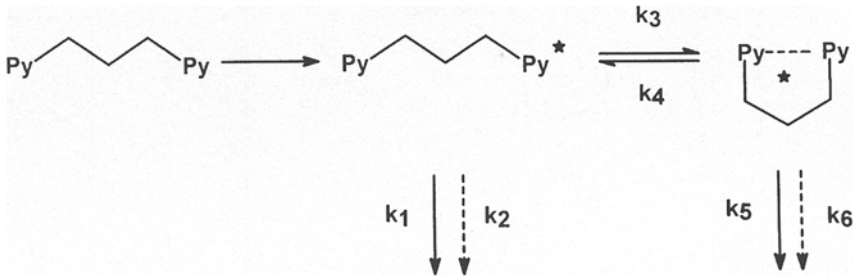

Table 1 Decay times of excitation of 1Py-(3)-1Py probe in HSA, DTAC, and dissolved 1,4-dioxane. Excitation at 337.1 nm with 300 ps pulse

1Py-(3)-1Py in HSA			
τ_{M1} ns	τ_{M2} ns	A_{M1}	A_{M2}
2.41	69.0	13.225	1.775
τ_{E1} ns	τ_{E2} ns	A_{E1}	A_{E2}
7.33	35.5	7.015	3.845
1Py-(3)-1Py in DTAC			
τ_1 ns		τ_2 ns	
40.0		60.9	
1Py-(3)-1Py in 1,4-dioxane			
τ_1 ns		τ_2 ns	
9.6		36.6	

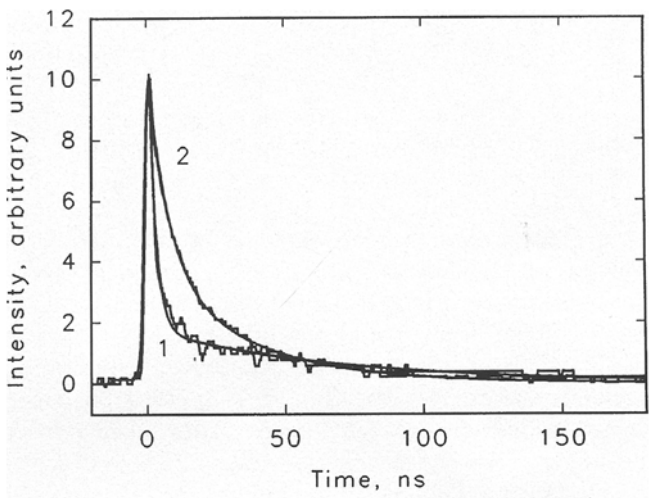

Fig. 8 Time-resolved emission of 1Py, 1Py* (1) and 1Py* (2) of 1Py-(3)-1Py label in HSA

formation of two different excimers, the third one illustrates the effect of the radiative and nonradiative decays.

Nevertheless, for 1Py-(3)-1Py in 1,4-dioxane and in DTAC micelles, in spite of quenching, it was possible to observe formation of 1Py, 1Py* excimer. Formation of excimer was faster for 1Py-(3)-1Py in the 1,4-dioxane solution ($\tau_1 = 9.6$ ns) than in the case when this probe was inside of the apparently more viscous DTAC micelles ($\tau_1 = 40.0$ ns). On the other hand, the time dependent emission of 1Py, 1Py* in HSA(1Py-(3)-1Py) was characterized by formation of excimer in time shorter than could be determined with our setup (i.e., shorter than 2 ns).

Traces of the decays of 1Py* emission and time dependence of 1Py, 1Py* emission for HSA 1Py-(3)-1Py) are shown in Fig. 8. It is worth noting that for HSA(1Py-(3)-1Py) the decay times are different for monomeric 1Py* and for 1Py, 1Py* excimer indicating that emissions of these species are kinetically decoupled. Therefore, the absorption, excitation, and steady-state state emission spectra, as well as the time-resolved emissions indicate without any doubt that from the mentioned earlier two possibilities that for 1Py-(3)-1Py in binding sites of HSA: i) the 1Py, 1Py* excimer is formed very fast and ii) 1Py-(3)-1Py is in conformations including also the prearranged 1Py, 1Py ground state dimer, the second alternative has to be chosen. This conclusion was confirmed also by the emission spectra of HSA(1Py-(3)-1Py) registered at 77 K (in the ethylene glycol matrix). Also at this low temperature in the glassy state of the matrix the 1Py, 1Py* excimer emission was observed immediately (at time shorter than 2 ns) after the excitation pulse.

In earlier studies it was found that in macromolecules of HSA the binding sites for fatty acids, L-tryptophan, tyroxine, diazepam, bilirubin, digitoxin, ibuprofen, and 2,3,5-triiodobenzoic acid are in domain III and in the part of domain II adjacent to domain III [15, 16]. At the moment, we cannot specify locations of the 1Py-(3)-1Py probe inside of the macromolecules of HSA. However, the two-exponential decays observed in our studies for emissions of 1Py-(3)-1Py in HSA suggests that

the probe was located in different binding sites. We can conclude also that the changes of the emission of the 1Py-(3)-1Py probe in HSA do not reflect changes in the local fluidity inside of the HSA macromolecule, as we supposed earlier [18], but should be related either to redistribution of the probe between the various binding sites or to the conformational changes of protein enforcing conformational changes in the incorporated 1Py-(3)-1Py probe.

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