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Fluorescence Decay Time Distribution Analysis Reveals Two Types of Binding Sites for 1,8-Anilinonaphthalene Sulfonate in Native Human Oxyhemoglobin

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Abstract—Binding of 1,8-anilinonaphthalene sulfonate (1,8-ANS) with native human oxyhemoglobin (Hb) in 50 mM potassium phosphate buffer (pH 7.4) was studied by steady-state fluorescence spectroscopy and by laser spectrofluorimetry with subnanosecond time resolution. The distribution of fluorescence decay times and parameters of two- and three-exponential deconvolution of the fluorescence kinetics of 1,8-ANS in Hb solution demonstrate that the emission at wavelengths λ_{em} of 455-600 nm is not single-exponential and has components with mean decay times <0.5, 3.1-5.5, and 12.4-15.1 nsec with the amplitudes depending on the emission wavelength. Analysis of time-resolved fluorescence spectra shows that the shortest-lived component should be assigned to 1,8-ANS molecules in the aqueous medium, whereas the two longer-lived components are assigned to two types of binding sites for 1,8-ANS in the Hb molecule characterized by different polarity and accessibility to water molecules.

Key words: oxyhemoglobin, 1,8-anilinonaphthalene sulfonate, fluorescence

Fluorescent probes are extremely informative in studies of the conformational states of proteins [1, 2]. Anilinonaphthalene probes including 1,8-anilinonaphthalene sulfonate (1,8-ANS) are the best known and most often used fluorescent probes, and their fluorescence parameters are mainly determined by the structural and dynamic properties of protein molecules [1-5]. 1,8-ANS is most widely used in studies of various heme-free proteins [6-9]. In the case of heme proteins, this probe is, as a rule, used in studies of their apoforms [1, 10-12].

The first studies with 1,8-ANS showed that the interaction of this probe with apomyoglobin and apohemoglobin markedly enhanced the intensity of its fluorescence [10]. These changes were accompanied by a displacement of the maximum of the probe fluorescence spectrum to shorter wavelengths: from 515 nm for aqueous solutions of the probe to 454-457 nm for 1,8-ANS complexes with apomyoglobin and apohemoglobin. Specific binding sites for 1,8-ANS were found in the heme cavities of these proteins [10]. The lifetime of 1,8-ANS inside these complexes varies from 15.3 to 18.2 nsec [11, 12].

Although the use of 1,8-ANS and other probes in studies of the properties of myoglobin and hemoglobin apoforms was very successful [10-12], we suggest that studies of structural and dynamic properties of native hemoglobin molecules should be more interesting from the standpoint of functional significance. Thus, the use of 8-hydroxy-1,3,6-pyrenetrisulfonate (a fluorescent analog of 2,3-diphosphoglycerate (DPG)) provided the interpretation of changes in the DPG-binding site during liganding and deliganding and of rearrangements in the tertiary and quaternary structures of functionally active hemoglobin [13]. However, the use of 1,8-ANS for studies of the properties of oxyhemoglobin and of the native myoglobin was considered unpromising because of the possible strong quenching of its fluorescence by the heme group and because of the absence of binding sites other than that in the heme cavity [2, 12,

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14]. However, the binding of a certain amount of 1,8-ANS with oxyhemoglobin molecules was shown by equilibrium dialysis [10].

In the present work, we used 1,8-ANS in studies of native human hemoglobin by steady-state and time-resolved fluorescence spectroscopy. It is shown that analysis of fluorescence decay time distributions and time-resolved emission spectra allows to discriminate emission of oxyhemoglobin-bound 1,8-ANS on the background of its fluorescence in the buffer solution. Our results show that the 1,8-ANS probe, due to its unique features and simplicity in use, is very promising for studies of specific features of such a very physiologically important protein as hemoglobin and of other heme-containing proteins.

MATERIALS AND METHODS

Reagents. 1,8-ANS was from Merck (Germany). For isolation of the main hemoglobin fraction (HbA_1), DEAE-cellulose DE-52 (Whatman, England) was used. Tris from Serva (Germany) and KH_2PO_4 and KOH from Fluka (Switzerland) were used as constituents of buffer solutions.

Preparation of oxyhemoglobin. Blood samples from pregnant women with normal pregnancies were kindly provided by the Research Institute of Maternity and Child Protection, Ministry of Health of the Republic of Belarus. After lysis of erythrocytes [15], hemoglobin was isolated and purified to homogeneity by ion-exchange chromatography on a column with DEAE-cellulose pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.5) [16]. Prior to placing onto the column, the hemoglobin preparation was transferred by dialysis from potassium phosphate buffer into 50 mM Tris-HCl buffer (pH 8.6). The column was eluted with a linear reverse pH gradient of Tris-HCl buffer (pH 8.5-7.0). This method results in the homogenous main form of adult human hemoglobin (HbA_1) and the minor form HbA_2, and additionally eliminates organic phosphates [16]. Homogeneity of the resulting fractions was monitored by electrophoresis in polyacrylamide and agarose gels. The gels were stained with Coomassie Brilliant Blue R250 or with a Silver Stain Kit (Sigma, USA). With the Silver Stain Kit, protein admixtures ranging from 0.0025 to 0.0071% could be detected in the studied sample [17]. The resulting HbA_1 and HbA_2 preparations free of detectable apoglobin admixtures were concentrated by ultracentrifugation using Amicon YM-05 membranes (USA). The working solution of hemoglobin was prepared by dilution of the stock solution in 50 mM potassium phosphate buffer (pH 7.4) to 0.02 mM hemoglobin.

Spectroscopic determinations and data analysis. Hemoglobin concentration at all stages of isolation and purification was determined spectrophotometrically with a Cary 500 UV-VIS spectrophotometer (Varian, USA) using molar extinction coefficient 13.8 $mM^{-1}\cdot cm^{-1}$ at wavelength 541 nm [18].

Steady-state spectra of intrinsic fluorescence of the proteins and of the fluorescence of 1.8-ANS were recorded with an SFL-1211A spectrofluorimeter (SOLAR, Belarus) at 20°C in a thermostabilized cuvette. For titration of hemoglobin solution with increasing concentrations of 1,8-ANS, the spectral width of slits for the exciting and emitted light was set to 7.5 nm.

The dissociation constant of 1,8-ANS-hemoglobin complex was calculated from titration curves at excitation wavelength λ_{ex} = 296 nm and emission wavelength λ_{em} = 486 nm using the ENZFITTER program (Sigma).

Time-resolved fluorescence was recorded with an automated laser spectrofluorimeter [19]. In this setup, the sample is excited by emission from an atmospheric pressure nitrogen laser (λ = 337.1 nm, FWHM pulse duration 1.5 nsec, pulse repetition frequency up to 50 Hz) or by a dye laser pumped by it. The fluorescence emission wavelength is selected using a stepper motor-controlled double grating monochromator with dispersion of 6 Å/mm . The fluorescence signal is detected by a FEU-164 photomultiplier tube with a time response of 2 nsec. The signal from the photomultiplier tube is fed into a V4-24 boxcar integrator (Kalibr, Belarus) (bandwidth 1 GHz), and from its output the digitized signal enters a computer. To reduce the influence of amplitude fluctuations in the exciting light pulse on the results of measurements, the intensity of the signal recorded by the spectrofluorimeter is normalized by the integral intensity of Rhodamine 6G dye in the reference channel. The polarizer and analyzer (Glan prisms) in the optical scheme of the fluorimeter allow measurements of the emission anisotropy. All electronic and optical units are controlled by a personal computer. The spectrofluorimeter allows recording of time-resolved spectra of the fluorescence intensity and emission anisotropy (time resolution 200 psec) and fluorescence decay kinetics for a wide range of excitation wavelengths (337.1, 360-750 nm).

Because of the finite duration of the excitation pulse and of the limited time resolution of the spectrofluorimeter, the fluorescence pulse measured $I(t)$ is a convolution of the "true" fluorescence kinetics $F(t)$ (the fluorescence response of the system under investigation to a δ-pulse excitation) and of the apparatus response function $R(t)$, its particular shape being determined by those of the excitation pulse and response of the detection system [20]:

$$I(t) = \int_0^t R(t-t')F(t')dt'. \quad (1)$$

The fluorescence decay curves in the present work were analyzed by two approaches. The first was a standard approach of iterative deconvolution when kinetic data are

analyzed based on the assumption that the "true" fluorescence kinetics are described by either the single-exponential decay law or a sum of two or three decaying exponentials. The second approach recovers the fluorescence decay time distribution [21] based on the Tikhonov regularization [22]. In both approaches the "true" fluorescence decay is presented as a sum of decaying exponentials:

$$F(t) = \Sigma_{i=1}^{N} f_i \exp(-t/\tau_i). \quad (2)$$

However, these two approaches are significantly different. In the first approach, the number N of exponentials can be set equal to 1 to 3 depending on the shape of the kinetic curve and the quality of its fitting by the corresponding decay law and both the decay times τ_i and pre-exponential factors f_i are varied in the fitting process to provide the best fit of the calculated kinetics and experimental data. In the second approach, the number of exponentials N is fixed and in the present work it was set to 100; the fixed fluorescence decay times τ_i are uniformly spaced on the logarithmic scale ranging from 0.05 to 200 nsec, and the fluorescence kinetics are analyzed by numerical solution of a finite-dimensional regularized analog of Eqs. (1) and (2) relative to the set of pre-exponential factors $\{f_i\}_{i=1}^{N}$. The use of the generalized cross-validation procedure [23] to determine the optimal value of the regularization parameter provides a stable solution with the minimum *a priori* assumptions on the decay law. Therefore, this approach to the analysis can be used for the independent testing of results of the standard analysis of kinetics by one-, two-, or three-exponential deconvolution. In both approaches, convolution (1) was calculated using a local quadratic approximation of the apparatus response function [24]. The quality of data fitting was judged by values of the reduced χ^2 and Durbin–Watson parameter and also by the appearance of plots of weighted residuals and of their autocorrelation function [20].

RESULTS

Steady-state fluorescence. The tetrameric hemoglobin molecule contains six tryptophan and twelve tyrosine residues [25]. The intrinsic fluorescence of hemoglobin is determined by Trp-14α and Trp-15β [26].

Increasing the probe concentration in oxyhemoglobin solution in 50 mM potassium phosphate buffer (pH 7.4) reduced the intensity of the protein fluorescence and shifted the luminescence maximum of 1,8-ANS from 550 nm (which is specific for an aqueous solution of the probe) to ~486 nm in the presence of the protein. There was a corresponding increase in the intensity of the probe fluorescence in the range 470-486 nm. The increased intensity of the 1,8-ANS fluorescence and the observed shift of the maximum towards shorter wavelengths are ascribed to the complexation of the probe with the protein [10].

We found that the dependence of quenching of the protein fluorescence on the concentration of 1,8-ANS expressed in the Stern–Volmer coordinates was characterized by a concavity relatively to the F_0/F axis. This behavior of the curve is usually associated with the inner filter effect and with the protein complexation with a fluorophore [2]. The curve replotted taking into account the inner filter effect on the excitation and emission wavelengths was also characterized by a concavity relatively to the F_0/F axis, which proves that oxyhemoglobin complexes with 1,8-ANS.

Because fluorescence in the region of 470-486 nm is not detected in the absence of hemoglobin at the 1,8-ANS concentrations used, this effect should be ascribed to binding of the probe, and this allowed us to quantitatively characterize the interaction of 1,8-ANS with Hb. The binding of 1,8-ANS is quantitatively characterized by dissociation constants calculated from dependencies of the fluorescence intensity of 1,8-ANS on its concentration [11]. Processing of the dependencies obtained has shown the presence of two dissociation constants: 0.4 ± 0.1 μM (K_s^1) and 1.90 ± 0.09 μM (K_s^2).

Time-resolved fluorescence spectroscopy. Subnanosecond time-resolved fluorescence spectroscopy is one of the most informative methods for studying protein interactions with fluorophores. Time-resolved fluores-

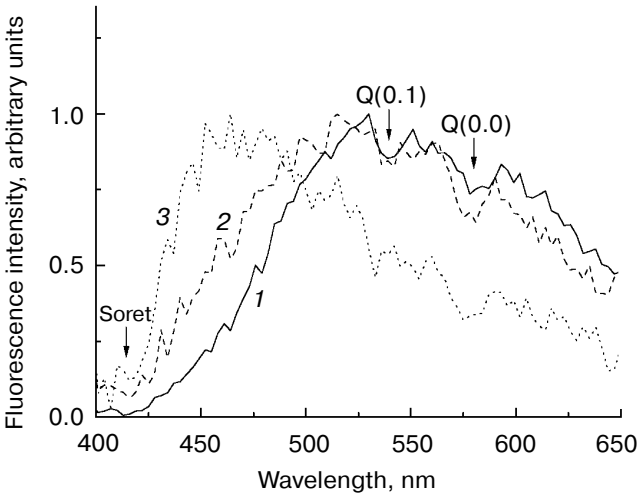

Fig. 1. Time-resolved emission spectra of 1,8-ANS fluorescence in oxyhemoglobin solution in 0.05 M potassium phosphate buffer (pH 7.4) recorded at the instant t = 0 (*1*), 4 (*2*), and 6 (*3*) nsec. [HbO_2] = 20 μM, [1,8-ANS] = 40 μM. Excitation wavelength, λ_{ex} = 337.1 nm; temperature, 20°C. Arrows show the Soret and visible absorption bands of oxyhemoglobin.

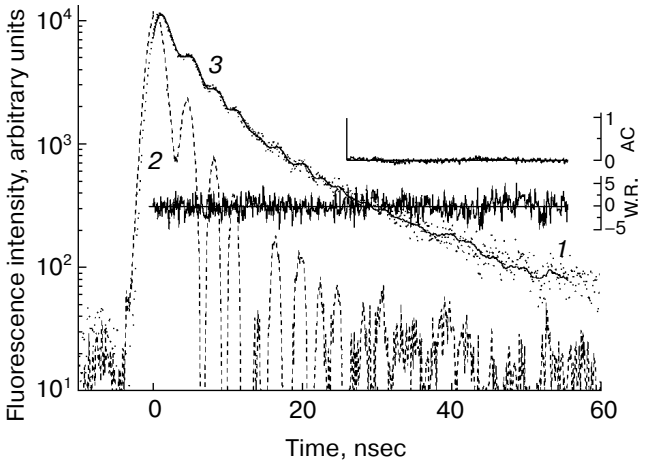

Fig. 2. Fluorescence decay kinetics of 1,8-ANS in human oxyhemoglobin solution in 0.05 M potassium phosphate buffer, pH 7.4 (*1*), apparatus response function of the spectrofluorimeter (*2*), and fluorescence decay kinetics recovered by the decay time distribution analysis (*3*). The horizontal inserts present curves of weighted residuals (W.R.) and their autocorrelation function (AC). Excitation and emission wavelengths: λ_{ex} = 337.1 nm; λ_{em} = 455 nm. Concentrations: [HbO_2] = 15 μM; [1,8-ANS] = 10 μM.

cence spectra of 1,8-ANS in oxyhemoglobin solution are presented in Fig. 1. The position of the spectrum recorded at the instant corresponding to the maximum of the excitation pulse (t = 0 nsec) indicates that it is related to the fluorescence of 1,8-ANS in the buffer. An increase in the delay time (t = 4 and 6 nsec) results in a significant shift of the time-resolved spectra towards shorter wavelengths.

The fluorescence decay kinetics in the range of 455-600 nm were nonexponential (the data for 455 nm are presented in Fig. 2). By analysis of the fluorescence decay curves, three components were revealed with significantly different decay times (Fig. 3, table). For convenience (Fig. 3), the decay time distributions are presented as plots of steady-state intensities ($f_i\tau_i$) versus decay times (τ_i). Insignificant difference in values of the fluorescence decay times and relative contributions of the decay components obtained by two different approaches that were used to analyze the fluorescence decay kinetics can serve as a measure of the error in evaluation of these parameters from the experimental data.

DISCUSSION

Optical excitation of 1,8-ANS results in an increase of the magnitude and change in the orientation of its dipole moment [27]. This leads to a strong dependence of the position of the fluorescence spectrum on the polarity of the environment. Thus, in strongly polar aqueous solutions the fluorescence of 1,8-ANS has a maximum at about 550 nm; in media of lower polarity the emission spectrum is significantly (~50 nm and more) shifted towards shorter wavelengths. The quantum yield of the fluorescence and the excited-state lifetime of 1,8-ANS are also very solvent-sensitive [28]. Thus, the decay time of the probe fluorescence changes from ~200 psec in aqueous media to 7 nsec in methanol and 11 nsec in ethanol [28], whereas in nonpolar solvents these values are ~3.5 nsec in ethylbenzene [29] and 12-14 nsec in dioxane [27, 29]. The quantum yield of 1,8-ANS fluores-

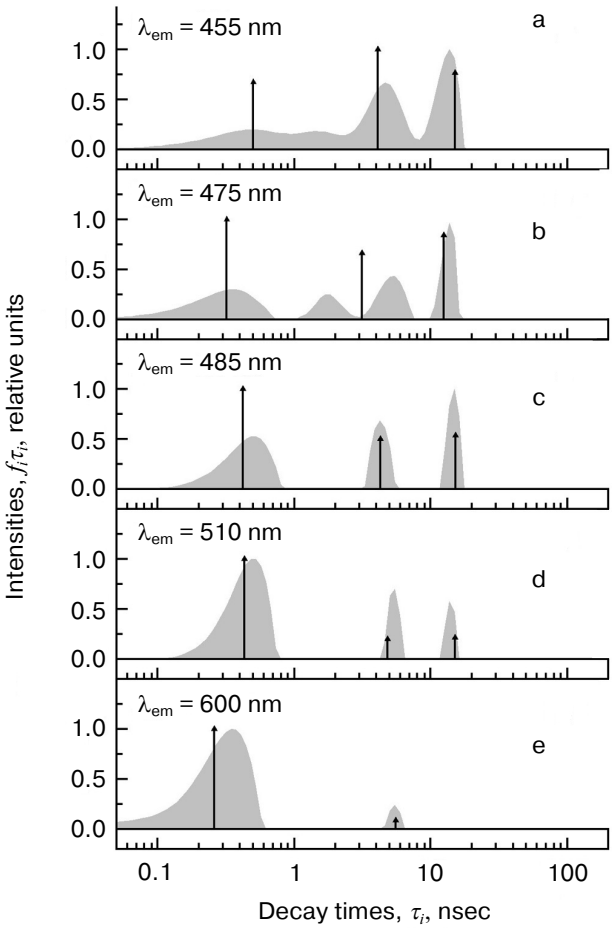

Fig. 3. Fluorescence decay time distributions recovered from the fluorescence kinetics of 1,8-ANS in human oxyhemoglobin solution in 0.05 M potassium phosphate buffer (pH 7.4). For comparison, δ-functions corresponding to results of the two- and three-exponential analysis of the fluorescence decay kinetics are schematically shown by the arrows. The emission wavelengths are as follows: λ_{em} = 455 (a), 475 (b), 485 (c), 510 (d), and 610 nm (e). Excitation wavelength, λ_{ex} = 337.1 nm. [HbO_2] = 15 μM, [1,8-ANS] = 10 μM.

Parameters of fluorescence decay of 1,8-ANS in solution of human oxyhemoglobin in 0.05 M potassium phosphate buffer (pH 7.4) for a set of emission wavelengths. The excitation wavelength λ_{ex} = 337.1 nm. [HbO_2] = 15 μM, [1,8-ANS] = 10 μM, temperature 20°C

Emission wavelength λ_{em}, nm	Method of analysis of kinetics data[a]	Parameters of fluorescence decay[b]					
		f_1	τ_1, nsec	f_2	τ_2, nsec	f_3	τ_3, nsec
455	3-exp	0.820	0.50	0.149	4.09	0.031	15.1
	DTD[c]	—	—	—	—	—	—
475	3-exp	0.918	0.32	0.062	3.13	0.020	12.4
	DTD[d]	0.928	0.30	0.062	3.88	0.010	13.4
485	3-exp	0.940	0.42	0.046	4.28	0.014	15.2
	DTD	0.949	0.43	0.039	4.29	0.012	14.6
510	3-exp	0.978	0.43	0.017	4.82	0.006	15.0
	DTD	0.982	0.44	0.015	5.33	0.004	13.9
600	2-exp	0.996	0.26	0.004	5.54		
	DTD	0.998	0.29	0.002	5.40		

[a] Three (3-exp) and two (2-exp) exponential deconvolution; DTD) analysis of decay time distribution.

[b] In the case of analysis of the fluorescence kinetics by recovery of decay time distributions, the relative amplitude contributions of each component f_i and the corresponding values of the decay time τ_i are presented.

[c] For λ_{em} = 455 nm the lifetime distribution is uninterrupted (Fig. 3a) and the unique determination of the distribution peaks is difficult.

[d] For λ_{em} = 475 nm the analysis of the decay time distribution reveals the presence of two peaks in limits of the range of 1.1-7.0 nsec (Fig. 3b) with pre-exponential multipliers and lifetimes as follows: f_{21} = 0.039, τ_{21} = 1.80 nsec; f_{22} = 0.023, τ_{22} = 5.10 nsec. In the corresponding column of the table, the total amplitude contribution and the mean decay time are presented for the components in the range of 1.1-7.0 nsec.

cence decreases by two orders of magnitude on changing from dioxane or ethanol to aqueous solutions [30-32] and is about 0.004 in aqueous media. Because the characteristics of 1,8-ANS fluorescence strongly depend on the microenvironment, they can be used to determine the locations of this probe in biopolymers.

In particular, 1,8-ANS was used in studies of the properties of apohemoglobin (apoHb) [10]. The binding of apoHb with 1,8-ANS resulted in about 200-fold increase in the fluorescence intensity and shift of the maximum of the spectrum from ~515 nm to ~450 nm. This effect is a consequence of the probe location in the heme cavities of the protein. The existence in apoHb of one type of binding sites for 1,8-ANS has been confirmed by various methods including equilibrium dialysis and steady-state and time-resolved fluorescence measurements [10, 11]. Fluorescence of 1,8-ANS bound to apoHb was shown to decay exponentially with lifetime ~18 nsec [11].

The data of the present work on kinetic parameters of 1,8-ANS fluorescence when complexed with oxyhemoglobin suggest that the oxyhemoglobin molecule has additional binding sites for the probe that are different from the heme cavities. The complexation of Hb with 1,8-ANS is confirmed by the presence of two slow components in the fluorescence decay kinetics (Fig. 3, table) and by the shift to shorter wavelengths of the maximum of time-resolved emission spectra of the probe (Fig. 1). The calculated values of K_s show a high specificity of the 1,8-ANS binding with oxyhemoglobin because they are even lower than 0.4 ± 0.1 μM (K_s^1) and 1.9 ± 0.09 μM (K_s^2) in the case of the same probe binding with the heme cavities of apoHb [10, 11]. However, it should be taken into account that the K_s values we have calculated are effective values because the 1,8-ANS fluorescence is quenched by the heme group. In fact, it is well known that heme is a strong quencher of the intrinsic fluorescence of the protein and also significantly affects the fluorescence of the probes that have been used [13, 33]. These findings indicate that the interaction of 1,8-ANS with oxyhemoglobin molecules is characterized by two K_s values, and this is in a good agreement with the presence of two slow components in the 1,8-ANS fluorescence decay in the presence of hemoglobin (Fig. 3, table).

Based on the findings, we can classify the three observed processes of quenching of the probe fluores-

cence as follows. The lifetime of the short-lived component of the fluorescence is about several hundreds of picoseconds, and its relative contribution increases with increasing emission wavelength. This component of the fluorescence most likely corresponds to probe molecules in the bulk buffer because, as discussed earlier, 1,8-ANS in aqueous media has a low quantum yield and a short lifetime and its fluorescence spectrum is significantly shifted toward longer wavelengths.

The longer-lived components of the fluorescence should be ascribed to 1,8-ANS molecules bound to the protein. Obviously, the component with the decay time of 12-15 nsec belongs to probe molecules in a hydrophobic environment [28, 30]. This is confirmed by the finding that the maximum of the time-resolved emission spectrum corresponding to the delay of 6 nsec was shifted towards shorter wavelengths compared to the aqueous solution. The contribution of the component with decay time of 3.1-5.5 nsec in the long wavelength region of the spectrum is higher than that of the longer-lived component (12-15 nsec). Therefore, the binding site for 1,8-ANS with the lifetime of 3.1-5.5 nsec is suggested to be more accessible to water molecules, and their presence reduces the quantum yield and the lifetime of the fluorescence.

Note that the nonexponential decay of the 1,8-ANS fluorescence with characteristic lifetimes in the ranges of 3-7 and 11-15 nsec has been observed earlier in studies of the interaction of this probe with various heme-free proteins and lipid dispersions [8, 9, 30, 34-39]. The presence of two components in the decay kinetics in the above-mentioned works was also explained by the existence of two types of binding sites of the probe with macromolecules. The presence of two types of binding sites for 1,8-ANS in biological macromolecular systems different in both the composition and structure is likely to be caused by the dual nature of 1,8-ANS, which can exist as a neutral hydrophobic molecule and as an ion [5]. These properties of the probe manifest themselves by the presence of both hydrophobic and electrostatic interactions of 1,8-ANS with protein molecules [40, 41].

Thus, since the isolation procedure of hemoglobin results in a protein preparation free from organic phosphates [16], the central cavity of the hemoglobin molecule is potentially accessible for binding of 1,8-ANS, as was shown for the interaction of 8-hydroxy-1,3,6-pyrenetrisulfonate with the DPG-binding site [13].

The results of the present work unambiguously show the presence of rather specific binding sites in the oxyhemoglobin molecule that are different from the heme cavity, which can be used in studies of specific features behavior of native hemoglobin molecules. It is quite possible that the highly specific binding sites found for 1,8-ANS can play a role in the regulation of physiological functions of this transport protein.

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