Novel two-color fluorescence probe with extreme specificity to bovine serum albumin

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Received 25 November 2002; revised 13 January 2003; accepted 27 January 2003

First published online 7 Februari 2003

Edited by Michael R. Bubb

Abstract **We report on strong, highly specific and stochiometric binding to bovine serum albumin of novel fluorescence probe FA, 2-(6-diethylaminobenzo[*b*]furan-2-yl)-3-hydroxychromone, that exhibits a very characteristic two-band fluorescence spectrum. Both absorption band and two fluorescence bands of FA are very sensitive to non-covalent interactions in the immediate environment of the probe. Multiparametric analysis of this spectroscopic information allows us to conclude that the binding site is characterized by very low polarity, high extent of screening from aqueous environment and unusually high electronic polarizability. The latter suggests the proximal location of probe FA to the aromatic amino acid residues in the binding site. The new probe can be proposed for the study of interaction of ligands and drugs of different nature with serum albumins.**

Key words: Fluorescence probe; Ligand binding; Bovine serum albumin; Polarity sensing; Two-color ratiometric dye; 3-Hydroxychromone

1. Introduction

Serum albumin is one of the most available and extensively studied of all proteins. It is the major constituent of blood plasma of many species, accounting for about 60% of its total protein content and providing about 80% of the blood osmotic pressure. It is a carrier in the blood of many low-polar metabolites and drugs [1,2]. Initially it was considered as a universal carrier of compounds with poor solubility in water. A discrete number of binding sites of different specificity was described later. The lack of absolute specificity towards particular ligands, multiple binding at different sites, the allosteric effects, and overlap between these sites are the major complications in the studies of albumin binding sites [1–5].

Fluorescence probes are often aromatic heterocyclic compounds with poor solubility in water and high affinity to hydrophobic binding sites. Their role in the studies of ligand binding properties of serum albumins is very important. The probes can be the analogs of natural ligands and drugs [5,6] or serve as the binding site markers in competition experiments with various ligands [7,8]. They can also be important tools for albumin determination in the presence of other proteins and also in clinically important media, such as blood plasma and urine [9]. The studies of fluorescent probe binding to serum albumin started already half of the century ago [10], and since then this protein has become the test object for various probes [11–18]. 3-Hydroxyflavone derivatives were among these probes [15–18]. Meantime, the highly specific and stochiometric binding have been demonstrated in none of these studies unequivocally. There was no attempt to explore in full the unique spectroscopic properties of these probes, which involve the presence of well-separated two bands in fluorescence emission that are sensitive strongly and differently to a variety of environment perturbations [19].

In this study we present the data showing that the fluorescence probe FA, 2-(6-diethylaminobenzo[*b*]furan-2-yl)-3-hydroxychromone, which is a 3-hydroxychromone derivative synthesized in our group [20], binds to bovine serum albumin (BSA) with a very high binding constant without detectable binding to the other proteins in the blood serum. The binding occurs at a single binding site in the equimolar quantities, and low-specific or multiple binding is not observed. Unique photophysical properties of this probe allow providing multiparametric analysis of the interactions within the albumin ligand binding site.

2. Materials and methods

BSA, of 'essentially fatty acid free' grade was the product of Sigma. The probe FA was synthesized and purified as described elsewhere [20]. Fluorescence emission spectra were obtained on Quanta Master spectrofluorometer PTI (Photon Technology International, Canada) operating in quanta counting mode. Excitation and emission slits for titration experiments were 1 and 10 nm, respectively, and the excitation wavelength was 450 nm. All the measurements were made in thermostated cell holders at 24°C. Precise wavelength positions of the band maxima were obtained from the first derivatives of spectra. All the light absorption measurements were made on Cary 3 Bio spectrophotometer (Varian, Australia). Fluorescence quantum yield was calculated by the reference method [21] using the solution of 4′-diethylamino-3-hydroxyflavone in ethanol as a reference with quantum yield $Q = 0.52$ [22], as described elsewhere [23]. In these measurements the excitation wavelength was 430 nm, the probe FA concentration was $5 \cdot 10^{-7}$ M, the molar ratio BSA:FA was 2:1.

Titration experiments were performed in 50 mM phosphate buffer, pH 7.0, with the addition of microliter amounts of FA solution in *N*,*N*-dimethylformamide to the final volume of 3 ml. Concentration of BSA was $5 \cdot 10^{-7}$ M and the range of variation of FA concentrations was $5 \cdot 10^{-8}$–$1 \cdot 10^{-6}$ M. All titration points were obtained with new preparations, and the mixtures were incubated for 7.5 min in a dark

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Abbreviations: BSA, bovine serum albumin; ESIPT, excited-state intramolecular proton transfer; FA, 2-(6-diethylaminobenzo[*b*]furan-2-yl)-3-hydroxychromone

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00116-9

cell compartment of the instrument before the measurements. Titration of FA with BSA was done by using the same procedure. Both titrations were used for calculation of the binding constant and the number of binding sites along the common Scatchard procedure. Errors of all the estimated values were calculated from the corresponding linear fits.

3. Results and discussion

Upon electronic excitation the probe FA exhibits the excited-state intramolecular proton transfer (ESIPT) reaction (Fig. 1), which results in both blue–green emission of the excited state normal form (N*) and orange–red emission of the phototautomer (T*) form. This property is known for many 3-hydroxychromone and 3-hydroxyflavone derivatives that exhibit ESIPT [15,19,22], and the distinguished feature of FA is the ability to observe both bands in emission with high intensity in the range of low-polar solvents [23,24].

This important feature of displaying two highly intensive well-separated emission bands is clearly observed for the FA–BSA complex (Fig. 2). Fluorescence spectra of the probe FA bound to BSA demonstrate two bands of comparable intensities, whereas the fluorescence intensity of this probe dissolved in aqueous medium is negligibly small, even in comparison with the Raman scattering band of water. Therefore there is no necessity for accounting the fluorescence emission of unbound probe, which simplifies greatly the studies of probe binding. The N* band maximum is observed at 537 nm, while the T* band maximum is at 617 nm. Fig. 2 presents also the comparison of fluorescence spectrum in the FA–BSA complex with the spectra obtained for FA in the solvents with similar positions of the N* band and the I_{N*}/I_{T*} ratios. We observe a remarkable variation of spectra even between very similar low-polar solvents. The spectrum obtained for FA in complex with BSA is characteristic, it is similar to that in studied solvents, but exhibits some deviations.

Light absorption and fluorescence spectroscopic parameters for FA in bovine serum which is diluted to the same concentration of BSA of $5\cdot10^{-7}$ M are identical to that obtained in the solution of purified BSA. This means that the probe does not bind to other serum proteins than BSA and suggests FA as an albumin-specific probe. The Scatchard analysis of titration curves of $5\cdot10^{-7}$ M albumin by variable concentrations of FA and of $5\cdot10^{-7}$ M FA with variable concentrations of BSA resulted in affinity constant $1.7\cdot10^{7}\pm0.4\cdot10^{7}$ M^{-1} and the number of binding sites $n=1.1\pm0.2$ (Fig. 3A). This indicates a very strong binding in comparison with many other albumin ligands [25]. The identical binding parameters were obtained by plotting the intensity of either N* or T* band, and the I_{N*}/I_{T*} ratio does not change as a function of relative BSA:FA

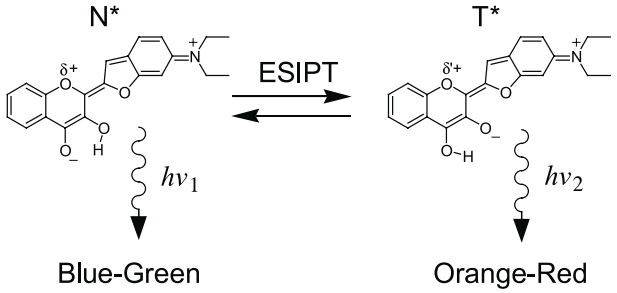

Fig. 1. The excited-state transformation of FA that gives rise to two emission bands in different wavelength ranges.

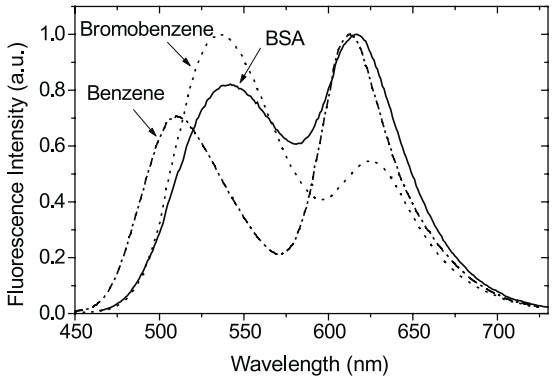

Fig. 2. Fluorescence emission spectrum of FA ($5\cdot10^{-7}$ M) in complex with BSA ($1\cdot10^{-6}$ M), and its comparison with correspondent spectra in benzene and bromobenzene.

concentration (Fig. 3B). The latter indicates that at low and at high ratios of protein–probe concentrations identical binding sites are occupied. In general, this observation may serve as a very sensitive test for the single-site binding.

For FA, the probe of 3-hydroxychromone family in contrast to common fluorescence probes that do not undergo ESIPT, a number of site-sensitive spectroscopic parameters can be derived. They are the position of absorption band maximum (ν_{abs}) and the positions of two emission band maxima (ν_{N*} and ν_{T*}) expressed in wavenumbers, $\nu(cm^{-1})=10^{7}/\lambda(nm)$, the ratio of intensities between them, I_{N*}/I_{T*}, and the fluorescence quantum yield (Q) [26]. Therefore, the spectroscopic data that can be obtained with FA must be very characteristic for the protein and its binding site. In contrast to common description by one parameter (e.g. by binding site polarity) this approach allows providing multiparametric description of this site [26].

As it was previously shown with the 3-hydroxyflavone analog of FA (4′-diethylamino-3-hydroxyflavone, probe FE), the

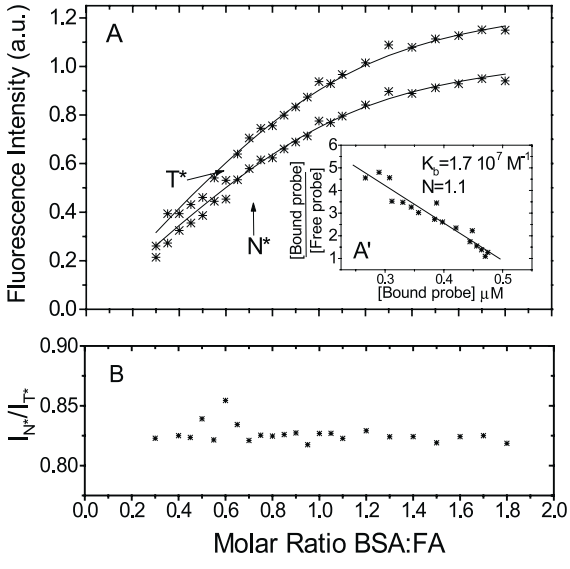

Fig. 3. The results of typical titration experiment with variation of BSA concentration at constant FA concentration $5\cdot10^{-7}$ M. A: Fluorescence intensity at N* (at 537 nm) and T* (at 617 nm) band maxima. The fits were obtained from the data based on the calculated binding constant and single binding site. A′ is the correspondent Scatchard plot. B: The ratio of N* and T* band intensities.

position of absorption maximum, ν_{abs}, is a linear function of electronic polarizability of solvent, $f(n) = (n^2-1)/(2n^2+1)$ [26]. In the case of probe FA we observe a similar correlation based on the results obtained in 15 representative solvents [23] (Fig. 4A). Electronic polarizability can be a useful measure of dispersive van der Waals interactions in the binding site. From the linear fit of absorption maximum the value of electronic polarizability of the FA environment in the BSA binding site can be estimated as $f(n) = 0.24 \pm 0.01$, and the effective refraction index can be derived as $n = 1.54 \pm 0.06$. This is a relatively high value that corresponds to that of aromatic molecules in liquid or solid states. Thus, for benzene $n_{20}{}^{D} = 1.5011$, toluene – 1.4969 and phenol – 1.5425 [27]. The first two solvents may simulate electronic polarization produced by Phe residues, and the latter by Tyr residues. The aromatic amino acid residues of Trp, Phe and Tyr were frequently reported as constituents of ligand binding sites of serum albumins of different species [1,28–31], and thus the direct interaction of FA with one of them or their clusters may be suggested. This may be one of important factors that determine the strength and specificity of binding. Together with high estimated electronic polarizability of the FA binding site this may witness for the formation of π–π stacking interactions [32] between the FA molecule and aromatic residues of the binding site.

It is known that the position of the N* band maximum of probe FA displays classical positive solvatochromy [23], similar to a variety of other polarity-sensitive fluorescence dyes, such as PRODAN [33] or Nile Red [34]. This allows using this parameter for the determination of the binding site polarity. The previous studies performed for FA in the broad range of low-polar solvents [23,24] demonstrated that the position of the N* band correlates with the solvent polarity index E_T^N linearly (the E_T^N is the most popular empirical solvent polarity normalized scale that is based on the shifts of absorption spectra of the betaine dye in a great number of solvents [35]). From the linear fit of the N* band position vs. E_T^N [23] presented in Fig. 4B, it can be found that the E_T^N value for the BSA binding site is 0.21 ± 0.03. This corresponds to the range of polarity between bromobenzene (0.182) and ethyl acetate (0.228). Thus, the binding site of probe FA can be considered as low polar.

Since the probe FA is essentially non-fluorescent in water and highly fluorescent in the low-polar media, the fluorescence quantum yield, Q, can be a measure of probe screening from the solvent. In complex with BSA the Q value of the probe is high (0.28), and close to that in solvents of similar polarity (ethyl acetate – 0.26) [23]. This witnesses for the substantial screening of the bound probe from aqueous environment.

An interesting problem appeared with our attempt to esti-

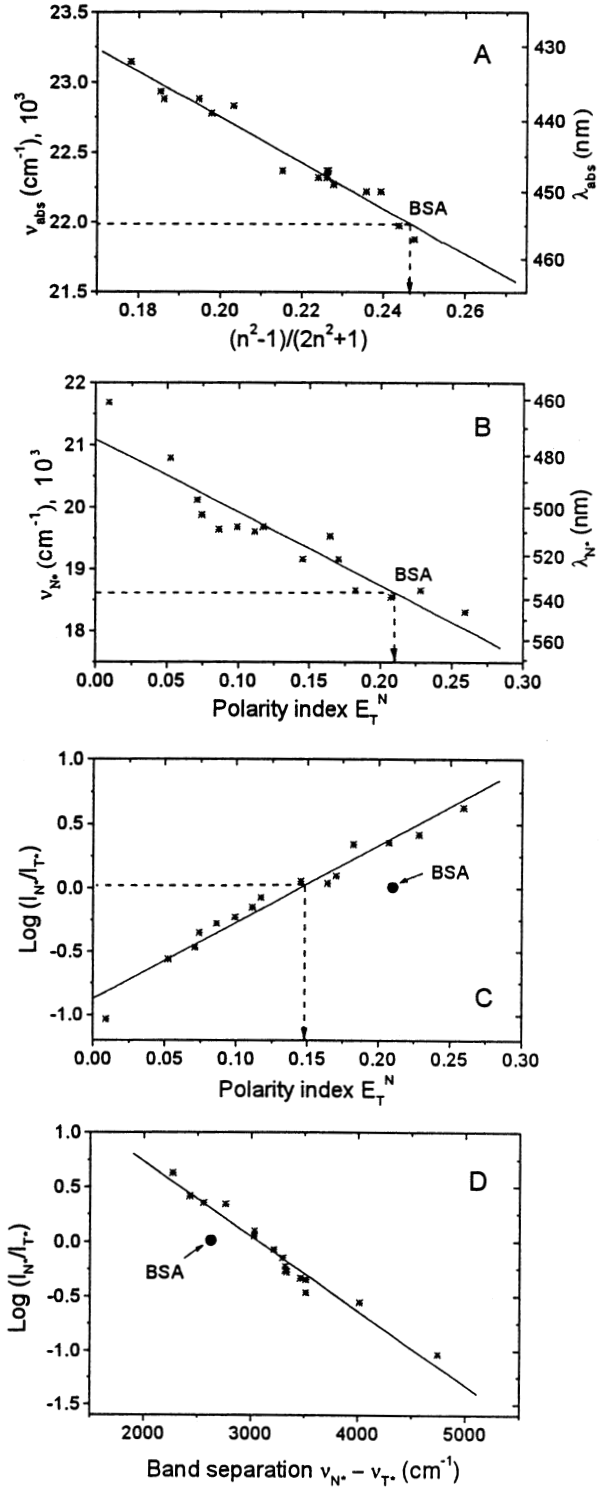

Fig. 4. Multiparametric analysis of spectroscopic information for probe FA in complex with BSA based on the data for this probe obtained in neat solvents [23]. A: Position of absorption maximum vs. electronic polarizability of solvents. Correlation equation: $\nu_{abs} = 26010 - 16330\ f(n)$, correlation coefficient $r = -0.979$, standard deviation SD = 80. Electronic polarizability of the FA binding site of BSA was obtained according to position of absorption maximum, 21980 cm^{-1} (455 nm). B: Position of N* band in emission spectrum vs. polarity index E_T^N. Correlation equation: $\nu_{N^*} = 21100 - 11830E_T^N$, $r = -0.933$, SD = 330. E_T^N value of the FA binding site of BSA was obtained according to the position of N* band maximum at 18622 cm^{-1} (537 nm). C: Logarithm of the I_{N^*}/I_{T^*} ratio vs. E_T^N. Correlation equation: $\log(I_{N^*}/I_{T^*}) = -0.875 + 6.04E_T^N$, $r = 0.979$, SD = 0.092. According to the linear fit of $\log(I_{N^*}/I_{T^*})$ versus E_T^N, the E_T^N value of BSA binding site was derived as 0.15 ± 0.02. The point correlating the $\log(I_{N^*}/I_{T^*})$ value for BSA binding site with the E_T^N value 0.21 obtained from the shift of the N* band is also shown. It deviates strongly from this regularity. D: Logarithm of the I_{N^*}/I_{T^*} ratio vs. band separation $\nu_{N^*} - \nu_{T^*}$. Correlation equation: $\log(I_{N^*}/I_{T^*}) = 2.10 - 6.85 \times 10^{-4}(\nu_{N^*} - \nu_{T^*})$, $r = -0.980$, SD = 0.087. The correspondent point for BSA binding site deviates downward.

mate the binding site polarity based on the ratio of band intensities, I_{N*}/I_{T*}. According to previous data on FA [23] in low-polar aprotic solvents, I_{N*}/I_{T*} strongly correlates with E_T^N, and its logarithm, log (I_{N*}/I_{T*}), is a linear function of E_T^N. In the meantime this relation can be influenced by other interactions that can modulate the ESIPT reaction [26]. An attempt to determine E_T^N for the FA–BSA complex based on correlation of log (I_{N*}/I_{T*}) with E_T^N (see Fig. 4C) leads to E_T^N value 0.15 ± 0.02, which corresponds to much lower polarity than that determined from the N* band position. In order to resolve this problem we used the correlation between log (I_{N*}/I_{T*}) and band separation on wavenumber scale, $\nu_{N*} - \nu_{T*}$. This function is linear, independently of the type of solvent (Fig. 4C) [24,26]. In the meantime the point corresponding to FA bound to BSA deviates from this function downwards. This suggests the existence of some specific interaction of the probe within the binding site, which increases substantially the apparent efficiency of ESIPT reaction [26]. This effect can not be modeled with the aid of liquid solvents, and it may be connected with anisotropic rigid environment of FA molecule in the protein binding site.

The experiments on serum albumins from different species, including human, are currently in progress. According preliminary data, probe FA exhibits specific binding and a characteristic fluorescence profile for each of the species. These data will be reported in due course.

4. Conclusions

The first two-band ratiometric site-sensitive fluorescence probe, FA, is introduced for the studies of ligand binding to serum albumin molecule. We demonstrate that FA, and probably other heterocyclic compounds of similar structure, can exhibit strong and specific binding at a single site of the BSA molecule. The binding site is characterized by very low polarity, high extent of screening from aqueous environment and unusually high electronic polarizability, which suggests the participation of aromatic amino acid residues. We have also detected anomalous photochemical behavior (ESIPT perturbation) of the bound probe indicating its specific surrounding in the binding site. We expect that this albumin-specific probe will find an important application in the studies of topology of binding sites on serum albumin molecule of different species and of its interactions with ligands and drugs of different nature.

Acknowledgements: This work was supported by TUBITAK research grants. The authors thank to TUBITAK Research Institute for Genetic Engineering and Biotechnology (Turkey) for providing instrumentation and financial support. The authors are grateful to Drs. Y. Mely and G. Duportail for reading the manuscript and helpful discussions.

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