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Fluorometric investigation of interaction of 3-acetyl-4-oxo-6,7-dihydro-12H indolo-[2,3-*a*] quinolizine with bovine serum albumin

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Abstract

Interaction of 3-acetyl-4-oxo-6,7-dihydro-12H indolo-[2,3-*a*] quinolizine (AODIQ) with a model transport protein, bovine serum albumin (BSA), has been studied using steady state fluorescence and fluorescence anisotropy experiments. Upon binding with BSA, the charge transfer (CT) fluorescence exhibits appreciable hypsochromic shift along with an enhancement in the fluorescence intensity. Gradual addition of BSA leads to the marked increase in the fluorescence anisotropy (r). From the high value of fluorescence anisotropy (r=0.30) it is argued that the probe molecule is located in motionally restricted environment of the protein. Addition of urea to the protein bound AODIQ leads to the decrease in fluorescence intensity as well as fluorescence anisotropy (r) indicating the release of AODIQ molecule to the aqueous buffer medium, thus supporting the idea that the protein, in its native form, binds with the probe. The binding constant and free energy change (ΔG^0) for the interaction of AODIQ with BSA have been evaluated from relevant fluorescence data. Polarity of the microenvironment has been determined from a comparison of the variation of fluorescence property of the probe in dioxane–water mixture with varying composition.

Keywords: Bovine serum albumin; Fluorescence anisotropy; Polarity; Microenvironment; Probe–protein interaction

1. Introduction

Serum albumins are the most widely studied abundant proteins in plasma. Many researchers have studied the structure and properties of serum albumins and their interaction with other proteins in order to understand how serum albumins affect the functionality of foods containing proteins. The three-dimensional structure of human serum albumin (HSA) has been resolved [1]. However, for bovine serum albumin (BSA), there are conflicting results so far as structural aspect is concerned. According to Brown [2], this protein has three domains, each consisting of a large double loop, a short connecting segment, a small double loop, a long connecting segment (Hinge), another large double loop and a connecting segment to the next domain. The exact 3D structure of BSA is not clearly known till date due to unavailability of suitable crystals for X-ray diffraction (XRD) studies [3]. It is noteworthy that the presence of more than one tryptophan residues in BSA makes the system more complex than HSA that contains only one tryptophan residue in it [4,5]. Serum albumins have conformational adaptability towards binding to a variety of biological molecules.

BSA is the principal carrier of fatty acids that are otherwise insoluble in circulatory plasma. As a multifunctional transport protein, albumin is the key carrier or reservoir of nitric oxide, which has been implicated in a number of physiological processes including neurotransmission. BSA has a great affinity for fatty acids, hematin, bilirubin, etc. It can form covalent adducts with pyridoxyl

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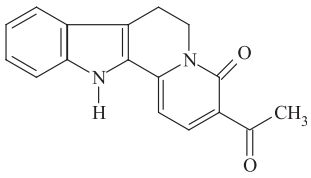
phosphate, cysteine, glutathione and a various metals such as Cu (II), Ni(II), Ag (II), and Au (I) [1]. The molecular interactions are often monitored using different optical techniques. Fluorescence spectroscopy is the most important technique to study the interactions of ligands with micro-heterogeneous environments including proteins because of its high sensitivity and relative ease of use [5–9]. The interactions of different fluorophores such as 3-hydroxy flavone (3-HF) [5], 1-anilino-8-naphthalene sulfonate (ANS) [7], Diethylamino coumarin [8], 2-[(2-bis-[carboxymethyl]amino-5-methylphenoxy)-methyl]-6-methoxy-8-bis[carboxymethyl] aminoquinoline (quin-2) [9] with BSA has been studied by different authors. Owing to the great importance in the determination of microscopic polarity of biological systems using fluorescent probes [10–12] attention has been drawn for the last few decades to this aspect. In a recent study [13] we have observed that the fluorometric behavior of AODIQ is very much dependent on solvent polarity. Taking advantage of this polarity sensitive fluorescence property of AODIQ, we have already determined the polarity of the microenvironment in micellar environments [14]. However, very few reports have appeared so far on the prospective use of fluorescence characteristics of such polarity sensitive fluorescent molecules for the determination of micropolarity in protein. Particular attention has been given to the probe AODIQ because of the fact that its structure resembles very much with those of the biologically relevant alkaloids, β-carbolines possessing an extensive pharmacological activity as central nervous system stimulant, hallucinogens, paralysants of cardiac muscle, etc. [15,16]. We have exploited the fluorometric technique to study the interaction of AODIQ with BSA. The present investigation reflects that there is a marked change in the fluorescence property of AODIQ upon binding with BSA, which has been utilized to characterize the interaction parameters and also to assess the micropolarity around the fluorophore within the protein.

Measurement of fluorescence anisotropy is claiming the role for its tremendous potential in bio-chemical research owing to the fact that any factor affecting size, shape or segmental flexibility of a molecule will affect this parameter [17]. Keeping this aspect in mind and also to exploit the technique as an independent evidence of AODIQ–BSA interaction, steady state fluorescence anisotropy has also been studied in the present work.

2. Experimental

3-Acetyl-4-oxo-6,7-dihydro-12H indolo-[2,3-*a*]quinolizine (AODIQ) (Scheme 1) was synthesized in the laboratory using the method mentioned elsewhere [18]. It was purified by column chromatography and the purity of the compound was checked by thin layer chromatography (TLC). Further, the compound was vacuum sublimed before use. Triply distilled water was used for making the experimental



Scheme 1. Structure of AODIQ.

solutions. BSA and urea were obtained from SRL, India. HEPES buffer was a sigma product; 50 mM HEPES buffer solution was prepared in water and the pH was adjusted to 7.0 and used in all studies of AODIQ in protein. The solvent *p*-dioxane used was of UV spectroscopic grade (Spectrochem India). Urea (Loba Chemie, India) was used to study the denaturation of the protein.

The absorption and steady state fluorescence measurements were performed using a Shimadzu MPS 2000 spectrophotometer and a Spex Fluorolog-2 spectrofluorimeter, respectively. The steady state fluorescence anisotropy measurements were performed with a Hitachi spectrofluorimeter (F-4010). For the anisotropy measurements the excitation and emission band widths were both 5 nm. Steady state anisotropy, r, is defined by:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

where I_{VV} and I_{VH} are the intensities obtained with the excitation polarizer oriented vertically and the emission polarizer oriented in vertical and horizontal orientation, respectively. The G factor is defined as:

$$G = I_{HV}/I_{HH}$$

I refers to the similar parameters as mentioned above for the horizontal position of the excitation polarizer. AODIQ concentration was 8 μM for all the experiments.

3. Results and discussion

3.1. Probe–protein binding interaction

The absorption spectrum of AODIQ in HEPES buffer at pH 7.0 shows a broad and unstructured lowest energy band with a maximum at around 420 nm. Addition of BSA to the aqueous buffer solution of AODIQ hardly changes the absorption spectrum (absorption spectrum not shown).

The room temperature emission spectrum of the AODIQ solution in HEPES buffer shows a single broad and unstructured band ascribed to the CT state [13]. Fig. 1 depicts the emission spectra of AODIQ (in buffer) as a function of BSA concentration. Gradual addition of BSA to the buffer solution leads to a hypsochromic shift of the emission maximum associated with an enhancement of the fluorescence intensity and a narrowing of the fluorescence band. The observations reflect that the microenvironment around the fluorophore in the protein solution is quite different from that of pure aqueous phase. Following the fluorometric work of

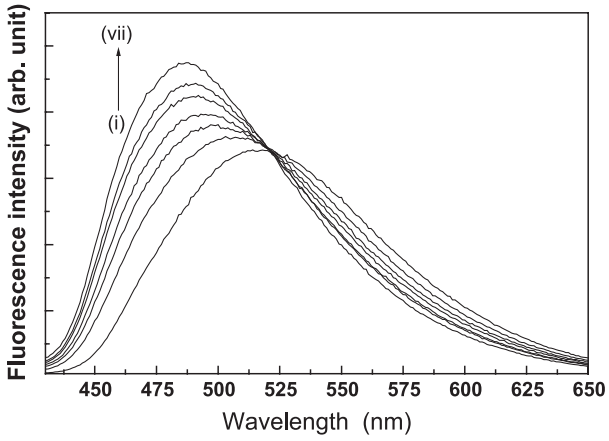


Fig. 1. Emission spectra of AODIQ as a function of BSA concentration (λ_{exc}=420 nm). Curves (i)→(vii) correspond to 0, 55, 100, 140, 200, 240, 320 μM BSA, respectively.

AODIQ in pure solvents [13], the blue shift in the fluorescence spectrum of the fluorophore with an addition of BSA is ascribed to a lowering in the polarity of the protein environment compared to that of the bulk aqueous phase.

The changes in the fluorometric behavior of the fluorophore with the addition of BSA in buffered aqueous solution can be rationalized in terms of binding of the probe with the protein leading to a less polar microenvironment around the fluorophore. A small increase in the fluorescence yield of AODIQ in BSA is also ascribed to a lowering in the polarity in the near vicinity of the probe and is explained from a comparison of the ease of formation of the CT state and an enhancement in the non-radiative decay of the same in more polar aqueous medium [14,19]. A blue shift in the fluorescence maximum also suggests a reduction in the polarity of the microenvironment [13].

Fig. 2 represents the variation of fluorescence anisotropy (r) of the emission of AODIQ as a function of BSA concentration.

The plot shows a marked increase in the fluorescence anisotropy of the fluorophore while moving from aqueous to BSA environment. The gradual increase in the anisotropy value indicates an interaction between AODIQ and BSA. It further suggests that the rotational diffusion of the probe is constrained significantly in the protein medium. Attainment of the plateau in Fig. 2 implies saturation in the binding interaction between the two partners. The high anisotropy value (r=0.30) suggests that the probe molecules are bound in a motionally restricted environment introduced by BSA.

In order to see the interaction between AODIQ and the protein, the binding constant value was determined from the fluorescence intensity considering the following equation as developed by Bhattacharya et al. [20].

$$1/\Delta F = 1/\Delta F_{max} + (1/K[\mathrm{L}])(1/\Delta F_{max}) \quad (1)$$

where $\Delta F=F_x-F_0$ and $\Delta F_{max}=F_\infty-F_0$ where F_0, F_x and F_∞ are the fluorescence intensities of AODIQ in the absence of BSA, at an intermediate concentration of BSA, and at the saturation of interaction, respectively; K being the binding constant and [L], the protein concentration. Rearranging the above equation we have the following form

$$(F_\infty - F_0)/(F_x - F_0) = 1 + (K[\mathrm{L}])^{-1} \quad (2)$$

The linearity in the plot of $(F_\infty-F_0)/(F_x-F_0)$ against $[\mathrm{L}]^{-1}$ confirms a one-to-one interaction between the two partners (Fig. 3).

The slope of the plot led to the determination of the binding constant between the two and the value obtained was 3.0×10^3 mol^{-1}. The estimated value (±15%) is in the range of the binding constant values for some other systems of the type [21,22]. From the K value, the free energy of the probe–protein binding interaction was calculated to be −20 kJ mol^{-1} at ambient temperature.

3.2. Effect of urea on protein bound AODIQ

More insight about AODIQ–BSA interaction comes from the fluorometric studies of the protein-bound probe in the presence of urea, a potent denaturing agent for

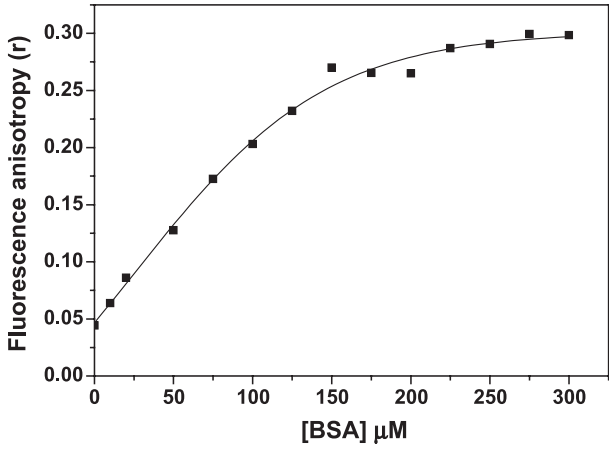


Fig. 2. Variation of fluorescence anisotropy (r) of AODIQ with increasing BSA concentration (λ_{em}=488 nm).

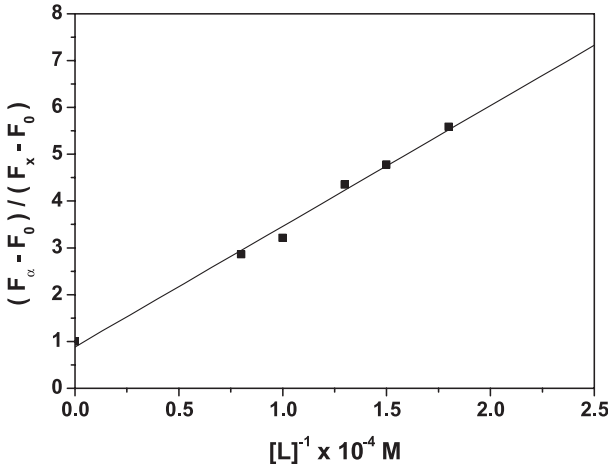


Fig. 3. Plot of $(F_\infty-F_0)/(F_x-F_0)$ against $[\mathrm{L}]^{-1}$.

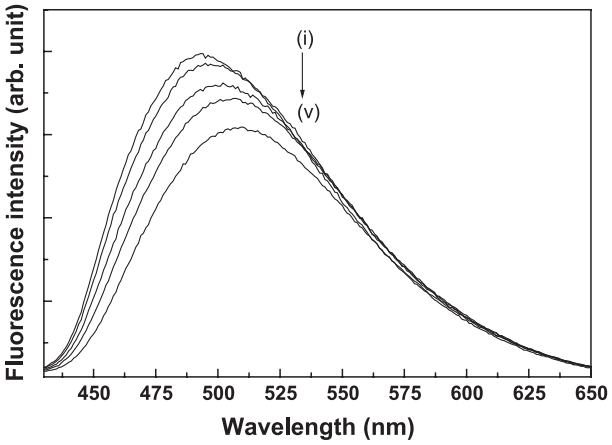


Fig. 4. Fluorescence spectra of BSA-bound AODIQ as a function of added urea. Curves (i)→(v) correspond to 0.0, 1.0, 3.0, 5.0 and 7.0 M urea, respectively (λ_{exc}=420 nm and [BSA]=250 μM).

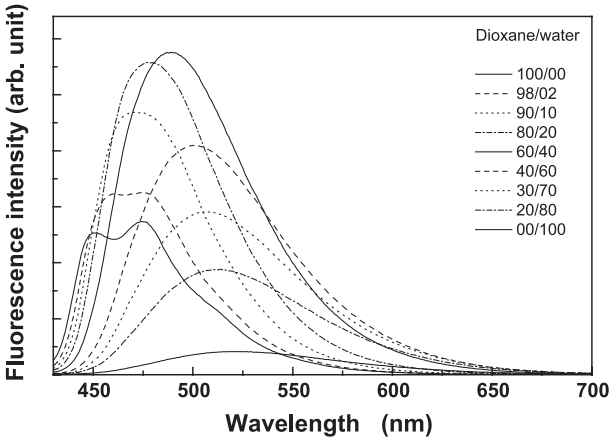


Fig. 6. Emission spectra of AODIQ in dioxane–water mixture under the same experimental condition (λ_{exc}=420 nm). Compositions of the solvent mixtures for different curves are given in the inset. The figure has been reproduced from Ref. [13].

proteins. Gradual addition of urea to the protein-bound AODIQ results in a gradual bathochromic shift in the fluorescence maximum with a concomitant lowering in the fluorescence intensity and a widening in the fluorescence band. Fig. 4 depicts these observations. Fig. 5 presents the variation of the fluorescence anisotropy as a function of urea concentration.

Fig. 5, having a pattern quite opposite to that of Fig. 2, suggests that addition of urea leads to weakening of the probe–protein interaction resulting in the release of the probe into the bulk aqueous phase. The red shift in the fluorescence band maximum (Fig. 4) with the addition of urea to the solution indicates that the urea leads to an increase in the micropolarity near the vicinity of the probe and thus suggests the release of AODIQ from the protein to the aqueous environment. Urea is known to be a good denaturing agent for different microheterogeneous environments including proteins [5,23]. We believe that urea displaces some water molecules adjacent to the probe in the protein environment. The resulting destabilization leads to the desolvation of the guest molecule and expulsion of it to the bulk aqueous phase [23]. It is important to mention here that in the presence of 8 M urea the emission maximum and the anisotropy value correspond to the values in aqueous environment. The observations thus suggest that BSA binds with the fluorophore, AODIQ, in its native form and denaturation of BSA leads to the release of the fluorophore from the protein environment to the bulk aqueous phase.

3.3. Polarity of the BSA microenvironment

Micropolarity of a biological system like protein can be estimated by comparison of the spectral properties of a fluorophore in that environment with those of the probe in solvents of known polarity [24]. To determine the micropolarity in BSA the fluorescence behavior of

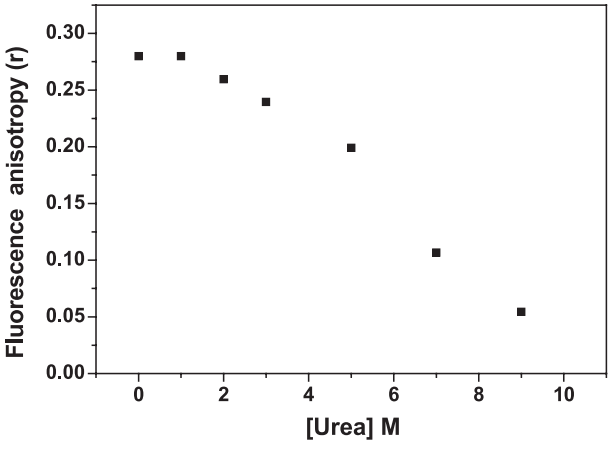


Fig. 5. Variation of fluorescence anisotropy (r) of BSA-bound AODIQ with increasing urea concentration (λ_{em}=488 nm, [BSA]=250 μM).

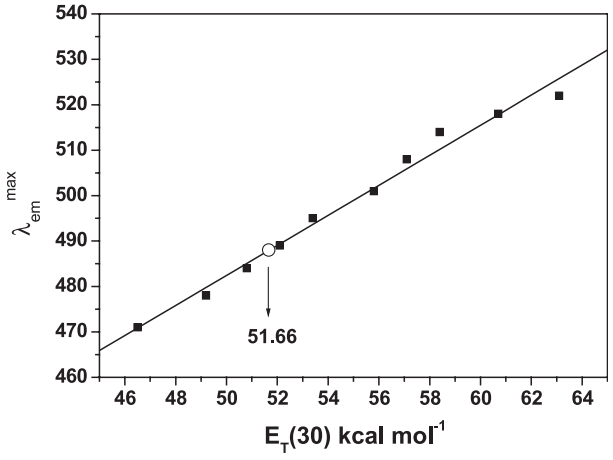


Fig. 7. Variation emission maximum (λ_{em}^{max}) of AODIQ in dioxane–water mixture against E_T(30). The open circle gives the λ_{em}^{max} value in BSA environment.

AODIQ in the protein environment was compared with that in water–dioxane mixture of varying composition (Fig. 6).

From Fig. 6 it is evident that the emission maximum of AODIQ is nearly 50 nm blue shifted on changing the solvent from pure water to 90/10 mixture of dioxane–water. Since the shift of the CT emission maximum in the protein environment is around 30 nm, one can safely assume that the micropolarity around the probe is intermediate between the polarities of the two aforesaid media [24]. In order to get a quantitative measure of the polarity at the protein–fluorophore binding site, empirical solvent polarity parameter, $E_T(30)$, based on the transition energy for the solvatochromic intramolecular charge transfer (CT) absorption of the betaine dye, 2,6-diphenyl-4(2,4,6 triphenyl-1-pyridono)phenolate, as developed by Reichardt, was used [14,25,26]. Representative plot monitoring the fluorescence maximum of AODIQ in water–dioxane mixture against $E_T(30)$, establishes a linear correlation between the two parameters (Fig. 7).

Interposing the emission maximum of AODIQ in the presence of BSA in Fig. 7 led to the determination of the micropolarity around the probe. In terms of $E_T(30)$ parameter, the polarity of the BSA environment was, thus, found to be 51.7. The $E_T(30)$ value suggests that the microenvironment around the probe in the proteneous medium is very similar to pure ethanol so far as polarity is concerned [27].

4. Conclusion

The present work reports the study of interaction of a polarity sensitive fluorophore with BSA. The photophysical behavior of AODIQ is modified remarkably in this environment compared to that in pure aqueous phase. This has been exploited to determine binding efficiency, nature of microenvironment around the probe and the micropolarity at the binding site. This work also demonstrates that addition of urea to the aqueous solution of protein (in buffer) changes the solvation of the latter leading to release of the fluorophore molecules bound to the protein. Extension of the present work in different serum albumins and allied enzymes should be of significant impact from the point of understanding of the environments in detail.

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