Spectroscopic Investigation on the Interaction of ICT Probe 3-Acetyl-4-oxo-6,7-dihydro-12H Indolo-[2,3-a] Quinolizine with Serum Albumins

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Interaction of 3-acetyl-4-oxo-6,7-dihydro-12H indolo-[2,3-a] quinolizine (AODIQ), a biologically active molecule, with model transport proteins, bovine serum albumin (BSA) and human serum albumin (HSA) have been studied using steady state and picosecond time-resolved fluorescence and fluorescence anisotropy. The polarity dependent intramolecular charge transfer (ICT) process is responsible for the remarkable sensitivity of this biological fluorophore to the protein environments. The CT fluorescence exhibits appreciable hypsochromic shift along with an enhancement in the fluorescence yield, fluorescence anisotropy (r) and fluorescence lifetime upon binding with the proteins. The reduction in the rate of ICT within the hydrophobic interior of albumins leads to an increase in the fluorescence yield and lifetime. Marked increase in the fluorescence anisotropy indicates that the probe molecule is located in a motionally constrained environment within the proteins. Micropolarities in the two proteinous environments have been determined following the polarity sensitivity of the CT emission. Addition of urea to the protein-bound systems leads to a reduction in the fluorescence anisotropy indicating the denaturation of the proteins. Polarity measurements and fluorescence resonance energy transfer (FRET) studies throw light in assessing the location of the fluorophore within the two proteinous media.

Introduction

Serum albumins are the most widely studied proteins abundant in plasma. Many researchers have studied the structure and properties of serum albumins and their interactions 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 the structural aspect is concerned. As a matter of fact, both BSA and HSA are similar in sequence and conformation but differ in the number of tryptophan (Trp) residues. BSA (583 aa) and HSA (585 aa) are characterized by a high homology (80%) and similar conformation, containing 17 disulfide bridges and a series of nine loops, assembled in three domains (I, II, III), each containing two subdomains, A and B.[2] The principal binding sites are located in subdomain IIA and IIIA, and it is generally assumed that in BSA and HSA these sites are homologous, although they may differ in affinities.[2] From the determined crystallographic structure of HSA, it was proposed that in this protein the single tryptophan residue (Trp-214) is located in IIA binding site, while Lys-199 and His-242 are involved in the protein ligand interaction. In case of BSA, there are two tryptophan residues, Trp-212 and Trp-134. Trp-212 is located in a similar hydrophobic microenvironment as the single Trp-214 in HSA (subdomain IIA),[2] whereas Trp-134 is more exposed to solvent and it is localized in the subdomain IA. It has been demonstrated that, in serum albumins the domains are assembled to form heart shaped molecules.[3] However this assembling of the domains can be modified depending on the condition.[2] Furthermore, from a comparison of the decay of the fluorescence anisotropy for Trp-214 in HSA it was concluded that the protein can easily adopt many conformations, ranging from more compact to a more relaxed form.[3]

Albumins are identified as major transport proteins in blood plasma for many compounds such as fatty acids, which are otherwise insoluble in plasma, hormones, bilirubin and many drugs. As a multifunctional transport protein albumin is the key carrier of nitric oxide that has been implicated in a number of physiological processes including neurotransmission. Moreover, albumins are involved in the maintenance of colloid-blood pressure and are implicated in the facilitated transfer of many ligands across organ-circulatory interfaces such as in the liver, intestine, kidney and brain.[4] They form covalent adducts with pyridoxyl sulfate, cysteine, glutathione and various metals such as Cu (II), Ni (II), etc.[1]

Fluorescence-probe spectroscopy of proteins as a methodology yielding structural and dynamical information concerning the fluorophore environment has a long history of the application of excited-state molecular interactions between a probe and its environment.[5−8] This interaction is effective for description of both dipole-reorientation dynamics of molecules surrounding the chromophore and their dielectric properties. Thus, a fluorescent molecular probe plays an important role in demonstrating as well as characterizing the microenvironment.[9,10] The fluorophore 3-acetyl-4-oxo-6,7-dihydro-12H indolo-[2,3-a] quinolizine (AODIQ), a neutral molecules, used in the present experiment has recently been shown to be an excellent probe for biological systems.[11,12] It belongs to the group of bioactive indole family. It is well-known that reactive oxygen species (ROS) participate in a number of pathological processes in the nervous system. Compounds able to interfere with the action of ROS might be useful in prevention and treatment of these pathologies. Recently attention has been focused toward designing and synthesizing

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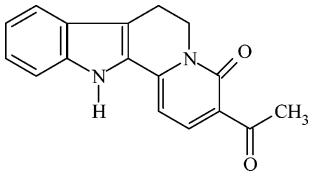
SCHEME 1 : Structure of AODIQ



compounds with a suitable spectrum of pharmacological and pharmacokinetic properties, among which indole derivatives are distinct group with great potential. The indole nucleus seems to be a promising basis for designing, synthesizing of new derivatives able to protect the nervous system.[13] Molecules containing indole nucleus such as β-carbolines, carbazoles, etc., are by now well established as bioactive molecules.[14−18] The single step synthesis of AODIQ from 1-methyl-3,4-dihydro-β-carboline[19] also leads to the presumption that the molecule should have biological activity. The spectroscopic and photophysical data of this molecular system in homogeneous and microheterogeneous media is most welcome for a better understanding of the biodistribution of this dye inside the living cell. The basic intention of the present spectroscopic study on AODIQ is to explore the potential usefulness of its fluorescence properties for understanding its interaction with relevant biological targets such as proteins, membranes etc. In our continuing effort we have already studied the photophysics of this molecular system in micellar and reverse micellar environments.[11,12,20] From the viewpoint of future biophysical applications, it seems interesting to study the photophysical processes of AODIQ in different serum albumins. Among most of the important parameters for the understanding of such an interaction are the determination of the binding affinity, location of the probe molecule in the microenvironment and the micropolarity therein. Although it is a challenge to identify the exact location of a small probe molecule in a complex protein environment, the binding parameters, micropolarity of the environment and fluorescence resonance energy transfer (FRET) studies have thrown some light on this aspect. The work has also addressed to the effect of addition of denaturing agents such as urea on the photophysics of the probe.

Experimental Section

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.[19] It was purified by column chromatography and the purity of the compound was checked by thin-layer chromatography (TLC). The compound was further vacuum sublimed before use. Triply distilled water was used for making the experimental solutions. BSA and urea were obtained from SRL, India. HSA and HEPES buffer were Sigma products; 50 mM HEPES buffer solution was prepared in water, the pH was adjusted to 7.0, and the solution was used in all studies of AODIQ in protein. Solvent p-dioxane used was of UV spectroscopic grade (Spectrochem India).

Absorption and steady-state fluorescence measurements were performed using a Shimadzu MPS 2000 spectrophotometer and a Spex Fluorolog II spectrofluorimeter, respectively. The steady-state fluorescence anisotropy was performed with a Hitachi spectrofluorimeter F-4010 model. Excitation and emission bandwidths were 5 nm. Steady-state anisotropy, r, was defined by

$$r = (I_{VV} - G\cdot I_{VH})/(I_{VV} + 2G\cdot I_{VH}) \quad \text{(i)}$$

where I_{VV} and I_{VH} are the intensities obtained with the excitation polarizer oriented vertically and the emission polarizer oriented vertically and horizontally, respectively. The G factor was defined as

$$G = I_{HV}/I_{HH} \quad \text{(ii)}$$

I terms refer to parameters similar to those mentioned above for the horizontal position of the excitation polarizer. Quantum yields were determined using quinine sulfate in 0.1 N H_2SO_4 ($\varphi_f = 0.54$).[21] Fluorescence lifetimes were determined from time-resolved intensity decay by the method of time-correlated single-photon counting using a picosecond diode laser at 408 nm (IBH, U.K., nanoLED-07) as light source. The typical response time of this laser system was 70 ps. The decays were analyzed using IBH DAS-6 decay analysis software. The same software was also used for anisotropy analysis. For all the lifetime measurements the fluorescence decay curves were analyzed by biexponential iterative fitting program provided by IBH. Mean (average) fluorescence lifetime (τ) for biexponential iterative fitting were calculated from the decay times and the preexponential factors using the following relation

$$\langle\tau\rangle = a_1\tau_1 + a_2\tau_2 \quad \text{(iii)}$$

Results and Discussion

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 and HSA to the aqueous buffer solution of AODIQ hardly changes the absorption spectrum.

The room-temperature emission spectrum of the AODIQ solution in buffer shows a single broad and unstructured band with a maximum at around 520 nm, ascribed to the CT state.[22] It is pertinent to mention here that AODIQ has dual emission, one locally excited (LE) and the other charge transfer (CT), depending upon the polarity of the medium; the former obtained predominantly in less polar and the latter in high polar environments.[22] The same report has further shown that the quantum yield (φ_f) of AODIQ fluorescence depends very much on the polarity of the environment in which the probe is located. The variation of φ_f with the polarity function forms a bell shaped curve. Thus, in aqueous (buffered) solution the quantum yield is rather low; it increases with a lowering in the polarity function, attains a maximum and then decreases again with further decrease in solvent polarity. Figure 1 depicts the emission spectra of AODIQ (in aqueous buffer solution) as a function of BSA and HSA concentrations.

The figure reveals that gradual addition of BSA and HSA is associated with a hypsochromic shift of the emission maximum along with an enhancement in the fluorescence yield. The effects reflect that the microenvironments around the fluorophore in the protein solutions are quite different from that in pure aqueous phase. The blue shift in the fluorescence spectrum suggests that the polarities of the protein environments are less than the polarity of the bulk aqueous phase since similar hypsochromic shift is observed in less polar solvents.[22,23] Apart from the hypsochromic shift the emission yield of the CT band increases appreciably as and when the fluorophore is included in the protein environments. This can be rationalized in terms of binding of the probe in a less polar site in the protein environment. As mentioned above the fluorescence quantum yield of AODIQ in a highly polar solvent like water is low compared to that in a somewhat less polar medium. Although the formation of CT state is favored in a polar solvent like water,

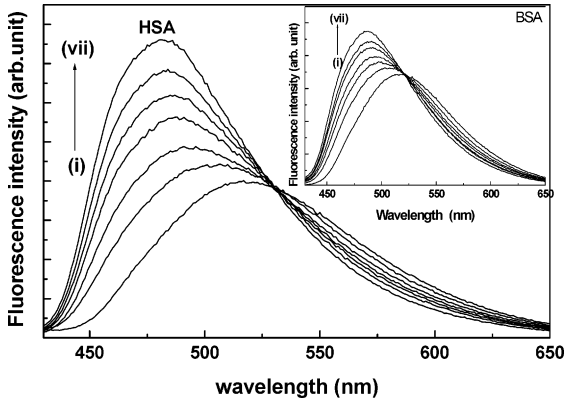


Figure 1. Emission spectra of AODIQ (9.0×10^{-6} M) as a function of HSA concentration ($\lambda_{exc} = 420$ nm). Curves i → vii correspond to 0, 10, 25, 35, 50, 70 and 100 μM HSA, respectively. [Inset represents the emission spectra of AODIQ (9.0×10^{-6} M) as a function of BSA concentration ($\lambda_{exc} = 420$ nm). Curves i → vii correspond to 0, 55, 100, 140, 200, 240, and 320 μM BSA, respectively.]

the stabilization of the state is so great that, owing to the proximity of the stabilized CT state and the low lying triplet/ground states, the nonradiative decay is facilitated markedly, resulting in a reduction in the net fluorescence yield of the CT emission.[23,24] Within the protein environment, the reduced polarity destabilizes the CT state, resulting in an increase in energy gap between the CT state and the triplet/ground states. According to the energy gap law this would lead to a reduction in the nonradiative decay and, hence, enhance the CT emission yield. Thus the blue shift as well as enhancement can be attributed to the reduced polarity of the microenvironment.

Probe−Protein Binding

To see the binding interaction between AODIQ and albumin proteins, the binding constant values have been determined from the fluorescence intensity data following the modified Benesi-Hildebrand equation:[16,25]

$$1/\Delta F = 1/\Delta F_{max} + (1/K\cdot[L])\,(1/\Delta F_{max}) \qquad \text{(iv)}$$

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 intensity of AODIQ considered in the absence of protein, at an intermediate protein concentration, and at a concentration of complete interaction, respectively, K being the binding constant and [L] is the protein concentration. Rearranging the above eq iv, we have the following form

$$(F_\infty - F_0)/(F_x - F_0) = 1 + (K\,[L])^{-1} \qquad \text{(v)}$$

Plots of $(F_\infty - F_0)/(F_x - F_0)$ against $[L]^{-1}$ for both BSA and HSA show linear variations (Figure 2), justifying the validity of the above equation and hence confirm one-to-one interaction between the fluorophore and the proteins. The binding constant values have been determined from the slope of the individual plots. From the determined K value, the free energy changes (ΔG) for the probe−protein binding have been calculated at ambient temperature. The data for the binding phenomena are tabulated in Table 1.

The determined K values (± 15%) fall in the normal range reported earlier for such type of complexations.[26,27] From a look at the relative values of K (Table 1), it is evident that the probe binds in a stronger way with HSA as compared to BSA. This greater degree of association of the probe with HSA is reflected

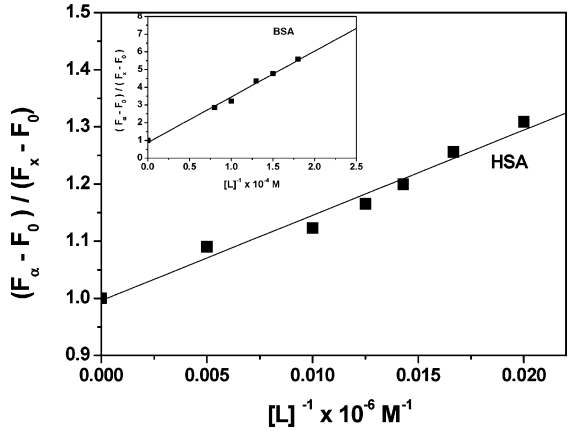


Figure 2. Plot of $(F_\infty - F_0)/(F_x - F_0)$ against $[L]^{-1}$ for HSA [Inset shows similar variation for BSA].

TABLE 1: Binding Constants and Free Energy Changes for AODIQ−Protein Interaction at 300K

environment	binding constant *(K)* mol^{-1}	free energy change (ΔG) kJ mol^{-1}
BSA	3.0×10^3	−20.0
HSA	7.0×10^4	−27.8

on the larger fluorescence enhancement of the fluorophore in case of HSA with the addition of a definite amount of BSA or HSA.

Steady-State Fluorescence Anisotropy Study. Measurement of fluorescence anisotropy has a great impact for its tremendous potential in biochemical research because any factor which affect size, shape, or segmental flexibility of a molecule will affect the observed anisotropy.[28] An increase in the rigidity of the surrounding environment of a fluorophore results in an increase in the fluorescence anisotropy, i.e., fluorescence anisotropy reflects the extent of restriction imposed by the microenvironment on the dynamic properties of the probe, thus it can be exploited for finding out the probable location of the probe in the microheterogeneous environments including proteins.[11,28] Figure 3 represents the variation of fluorescence anisotropy (r) of the emission of AODIQ as a function of protein concentration for both BSA and HSA.

The plots show marked increase in anisotropy of AODIQ with increasing concentration of both the proteins. The gradual increase in anisotropy of the emission of AODIQ with an increasing protein concentration implies an imposed motional restriction on the fluorophore in the proteinous environments. Figure 3 reveals that with increasing protein concentrations fluorescence anisotropy (r) increases rapidly at the beginning (upto [BSA] = 125 μM and [HSA] = 30 μM) and then levels off gradually. Similar results have been reported by Sengupta et al.[29,30] with other fluorophore systems. The differences in the amounts of proteins needed for the leveling effect is possibly due to differences in their affinity toward the probe molecule. Although the maximum anisotropy values are nearly same for the two proteins (r is 0.34 in HSA and 0.30 in BSA), attainment of the value at a much lower concentration of HSA compared to that of BSA suggests a higher motional restriction on the fluorophore in the former, a corroboration of the greater binding interaction between the probe and the HSA than probe-BSA interaction as reported above (Table 1).

Since fluorescence anisotropy is intimately connected with the viscosity of the microenvironment around the fluorophore, microviscosity is often estimated from a comparison of the fluorescence anisotropy of a fluorophore in an environment with

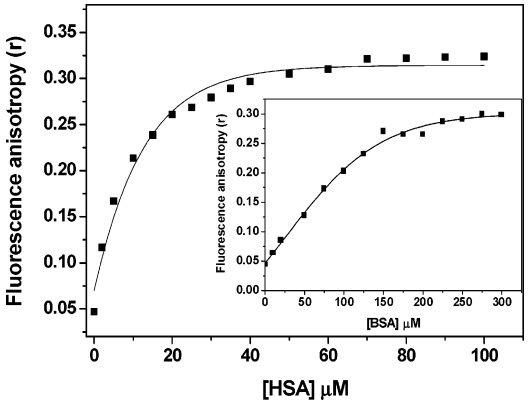


Figure 3. Variation of fluorescence anisotropy (r) of AODIQ with increasing concentrations of HSA and BSA (in inset). λ_{em} is 483 nm for HSA and 488 nm for BSA.

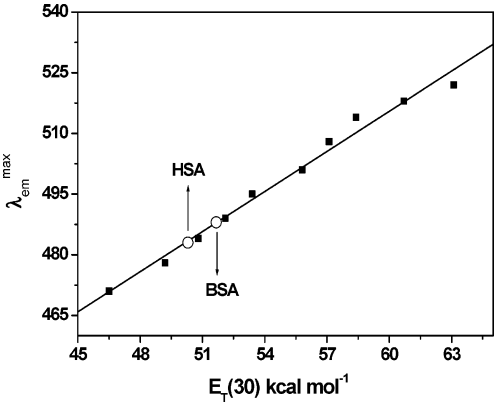


Figure 4. Variation of emission maximum (λ_{em}^{max}) of AODIQ in dioxane-water mixture against $E_T(30)$. The open circles give the interpolated λ_{em}^{max} values in BSA and HSA environments.

those of the probe in environments of known viscosities.[11,16,20,31] With a similar intention, we have extended the anisotropy measurements of AODIQ in glycerol-water mixture of different composition and compared the values with the anisotropy values of the protein bound situations. Surprisingly, we have noticed that the anisotropy value of AODIQ in 90/10 glycerol/water mixture is still remarkably lower than the anisotropy in the protein environment at the saturation level. However, it is evident that the viscosity in the BSA and HSA environment cannot be high enough to exceed the viscosity of a 90/10 glycerol/water mixture.[32] This observation thus indicates the dominance of the rotational correlation time for proteins over the normal viscosity effect. Following our previous work,[16] this interesting observation can be rationalized invoking some specific interactions such as hydrogen bonding centered around the protein and the heteroatoms present in the probe molecule, leading to some additional restriction imposed on the motion of the overall molecule or trapping of the probe in some motionally contrained site (such as crevice) of the protein. Some steric constrain may also play a role in fixing the spatial orientation of the probe molecule lowering the mobility of it.

Polarity of the Microenvironment Around the Fluorophore. Owing to the great importance of the determination of microscopic polarity of biological systems using fluorescent probes,[33−36] attention has been drawn for the last few decades to this direction. In an earlier study,[22] it was observed that the CT fluorescence of AODIQ is very much dependent on the solvent polarity. Taking advantage of this polarity sensitive fluorescence property of AODIQ, we have already studied the polarities of microenvironments such as micelles and reverse micelles.[11,20] In the present report, we have attempted to have an estimate of the micropolarity of the proteinous environments around the fluorophore considering the fact that there are very few reports so far on the prospective use of polarity sensitive fluorophore molecule for the determination of micropolarity in proteins. 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 polarities.[11,16,36−38] Because of the incomplete solvent relaxation around the fluorophore bound to motionally constrained environments, it is questionable whether the polarity of homogeneous environments (pure solvents or solvent mixtures) can simulate the polarity of protein media. However, to have a qualitative estimate, the micropolarities in microheterogeneous environments such as micelles, reverse micelles, proteins and lipids are often determined and expressed in equivalent $E_T(30)$ scale comparing the fluorescence behavior of the probe in microheterogeneous environments to that in a mixture of homogeneous solvents of varying composition.[11,16,36−38] This polarity has been referred to the static polarity by Kasha.[39]

We have studied the fluorescence behavior of AODIQ in water-dioxane mixture of varying composition.[22] The CT 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 shifts of the emission maxima in the protein environments are 32−35 nm, one can logically assume that the micropolarity around the probe is intermediate between the polarities of the two aforesaid media, viz., dioxane and water. To get a quantitative measure of the polarity of the local environments of AODIQ in HSA and BSA an empirical solvent polarity parameter, $E_T(30)$, based on the transition energy for the solvatochromic intramolecualar charge-transfer absorption of the betaine dye 2,6-diphenyl-4(2,4,6 triphenyl-1-pyridono) phenolate as developed by Reichardt have been used.[40,41] Representative plot monitoring the CT fluorescence maximum of AODIQ in water-dioxane mixture against $E_T(30)$ as represented in Figure 4, establishes a linear correlation between the two.

Interpolating the values of the CT emission maxima of AODIQ bound to BSA and HSA with the above correlation, we have determined the micropolarities around the probe to be 51.7 and 50.3, respectively, in the two environments. The micropolarity values suggest that the probe is located in a more hydrophobic region in HSA compared to that in BSA environment.

Effect of Urea. Steady-state fluorescence measurements, detecting changes in the tertiary structure, are complementary tools to investigate the conformational stability of globular proteins.[28] The unfolding process of serum albumins on increasing concentration of urea (1−8 M) has been well studied.[42−45] The studies suggest that the denaturation of BSA and HSA in the presence of increasing urea concentration takes place at a single, two state transition through intermediate state at 4−6 M urea.[42,43] It is further concluded that the formation of the intermediate in the unfolding-refolding transition of serum albumin involves (i) unfolding of domain III, (ii) minor structural transformation of domain II, and/or (iii) the separation of the subdomains of domain III from each other.[42−45] After finding the binding interaction between AODIQ and BSA/HSA, we intended to see the denaturing effect of the protein on its binding activity and on the overall photophysics of the probe. In the present work, urea induced modification of the protein bound probe have been determined by means of steady-state fluorescence measurements (Figure 5).

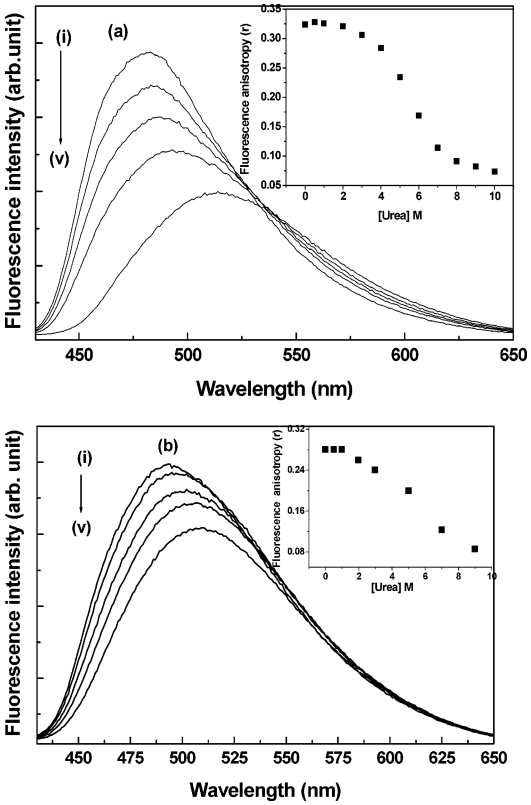


Figure 5. Fluorescence spectra of HSA-bound (a) and BSA-bound (b) AODIQ as a function of added urea. Curves i → v correspond to 0.0, 3.0, 4.0, 5.0, and 9.0 M urea in panel a and 0.0, 1.0, 3.0, 5.0, and 7.0 M urea in b. (λ_{exc} = 420 nm, [HSA] = 100 μM and [BSA] = 250 μM). Insets show the variation of fluorescence anisotropy (r) of protein-bound AODIQ with increasing urea concentration.

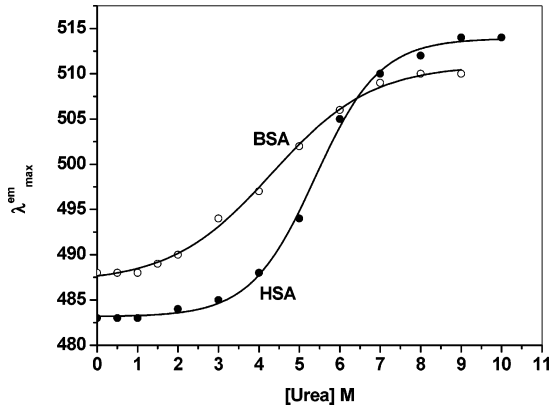


Figure 6. Plot of emission maximum of protein-bound AODIQ as a function of urea concentration.

The fluorescence spectrum of protein bound AODIQ shows a maximum at 488 nm in case of BSA and 483 nm in case of HSA under normal condition (when the tertiary structure is folded). On gradual addition of urea to the protein bound AODIQ the emission maximum undergoes a progressive red shift along with the decrease in fluorescence intensity and fluorescence anisotropy (Figure 5). Figure 6 depicts the plot of emission maximum of AODIQ in the proteinous environments with the addition of urea. The figure reveals transitions for both the serum albumins at urea concentrations 3−7 M, consistent with the literature.[42,43] The red shift indicates that the addition of urea increases the polarity of the microenvironment. The shift in the emission maximum and decrease in fluorescence intensity and anisotropy as reflected from Figure 5 remain unexplained, unless and until we assume urea causes a decrease in the number of fluorophores bound to the protein. Figure 5 with a pattern opposite to that of Figure 1 suggests that addition of urea leads to weakening of the probe-protein binding resulting in the release of probe molecules into the bulk aqueous phase. Consistent with our earlier work, we believe that urea displaces some water molecules adjacent to the probe in the protein environment with the denaturation of the latter.[11,16,46] The resulting destabilization leads to the desolvation of the guest molecule and expulsion of it to the bulk aqueous phase. It is pertinent to mention here that in the presence of sufficient amount of urea (9 M) the emission maxima and the anisotropy values closely correspond to the values in aqueous environment. These observations thus suggest that the proteins bind with the probe, in their native forms and denaturation of BSA and HSA leads to the release of the fluorophore from the protein environment to the bulk aqueous phase.

Fluorescence Resonance Energy Transfer Studies. Fluorescence resonance energy transfer (FRET)[28,47] is a distance dependent interaction between the different electronic excited states of dye molecules in which excitation energy is transferred from one molecule (donor) to another molecule (acceptor) without emission of a photon from the former molecular system. According to Förster's theory, the efficiency of FRET depends mainly on the following factors: (i) the extent of overlap between the donor emission and the acceptor absorption, (ii) the orientation of the transition dipole of donor and acceptor, and (iii) the distance between the donor and the acceptor.[28,47] Fluorescence (Förster's) resonance energy transfer (FRET) is an important technique for investigating a variety of biological phenomena including energy transfer processes.[28,48,49] One important consequence of energy transfer is photosensitization, a classic example of which is photosynthesis.[47] Moreover, FRET plays a key role in photodynamic therapy (PDT) of cancer[16,28] and is extensively used to study the structure, conformation, spatial distribution and assembly of complex proteins.[49] Since FRET causes a number of important biological phenomena,[48] much interest lies in FRET studies between AODIQ and protein in vitro. Furthermore, in a proteinous environment the proximity of a guest molecule to the tryptophan moiety is often determined through FRET study. Given the broad range biological activities of the probe molecule, AODIQ, and to assess the probable location of the probe in the protein environment, we became interested in the FRET studies between BSA/HSA and AODIQ. For this purpose the donors (BSA and HSA) were excited at 280 nm (tryptophan absorption) where the absorbance of the acceptor (AODIQ) is negligible. On gradual addition of AODIQ, fluorescence intensity of the tryptophan residue present in BSA and HSA decreased with a concomitant increase in the fluorescence intensity of AODIQ through an isoemissive point at 438 nm (Figure 7). This indicates an efficient energy transfer from the tryptophan residue present in BSA and HSA to AODIQ.

The excitation profile monitoring the emission of AODIQ (at 488 nm) shows that in the presence of BSA and HSA, besides the $S_0 \rightarrow S_1$ transition of AODIQ ($\lambda_{exc}^{max} \sim 420$ nm), a band with $\lambda_{exc}^{max} \sim 280$ nm (corresponding to the tryptophan) appears. This provides strong evidence for the occurrence of Förster's type resonance energy transfer (FRET)[29,50−53] from the tryptophan moiety (donor) present in BSA and HSA to the AODIQ molecule (acceptor).

Efficient FRETs in both HSA and BSA suggest that the probe guest molecule resides closer to the tryptophan moiety of the proteins. Following Sengupta et al.[29,52,53] and other workers,[54,55]

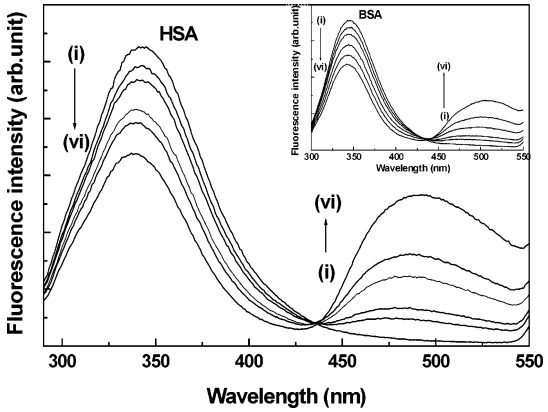


Figure 7. Fluorescence spectra of HSA (10μM) as a function of AODIQ concentrations (λ_{exc} = 280 nm). Curves i → vi correspond to 0, 4.36, 6.54, 13.08, 17.44, and 21.8 μM AODIQ concentrations. Inset shows the case of BSA (10μM) for the same concentrations of acceptor.

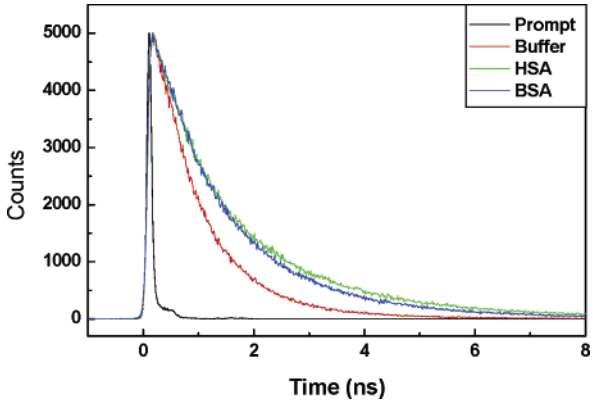


Figure 8. Time-resolved fluorescence decays of AODIQ in aqueous buffer, HSA and BSA media (λ_{exc} = 408 nm). The sharp profile on the left is the lamp profile.

TABLE 2: Lifetimes of AODIQin BSA and HSA Environment

environment	a_1	τ_1 (ps)	a_2	τ_2 (ps)	χ^2
buffer	0.780	654	0.220	1350	1.126
BSA (200 μM)	0.604	804	0.396	1947	1.368
HSA(100 μM)	0.583	719	0.417	2236	1.185

TABLE 3: Radiative and Nonradiative Rate Constants of AODIQ in Aqueous Buffer and Protein Environments

environments	φ_f	$\langle\tau_f\rangle$ ps	$\langle\tau_c\rangle$ ps	$k_r \times 10^{-9}$ (s^{-1})	$k_{nr} \times 10^{-9}$ (s^{-1})
buffer	0.08	807	180	0.099	1.139
BSA (200 μM)	0.12	1257	471	0.095	0.663
HSA (100 μM)	0.18	1351	1149	0.133	0.607

we thus conclude that the probe is located in the inter domain cleft region of HSA (near Trp-214). But for BSA, it is not so straightforward because there are two Trp residues namely Trp-212 and Trp-132. Trp-212 is thought to be located in a similar hydrophobic microenvironment as the single Trp-214 in HSA (subdomain of IIA)[2] whereas Trp-132 is more exposed to the aqueous environment. The residues, Trp-214 (present in HSA) and Trp-212 (present in BSA), show similar fluorescence behavior.[56] In our present investigation from the similarity in fluorescence behavior, and from the proximity in the micropolarity values around the probe in BSA and HSA environments, it is reasonable to assume that the probe is located near Trp-212 rather than Trp-132 in case of BSA environment. To determine the relative proximity of the probe molecule to Trp residue in BSA and HSA environments, we have determined the energy transfer efficiency which is mainly dependent upon the extent of overlap between the donor emission and the acceptor absorption and the distance between the donor and acceptor molecules maintaining 1:1 situation of donor:acceptor concentrations defined by the following equation

$$E = 1 - F/F_0 \qquad \text{(vi)}$$

here, E is the energy transfer efficiency, F and F_0 are the fluorescence intensities in the presence and absence of the acceptor molecule.

Due to the presence of two Trp residues in BSA, the fluorescence quantum yield of BSA is greater than that of HSA. Despite this fact it was shown that the energy transfer efficiency is greater in HSA environment (0.15) than that of in BSA environment (0.14), which clearly indicates that the probe molecule is relatively closer to Trp residue in HSA environment compared to the situation in BSA. Measured polarity and anisotropy values also support this conjecture.

Time Resolved Studies

Fluorescence lifetime serves as a sensitive parameter for exploring the local environment around a fluorophore,[57,58] and it is sensitive to excited state interactions. Differential extents of solvent relaxation around a fluorophore could also be expected to give rise to a difference in its lifetime. In proteins, the decay of AODIQ becomes biexponential and the lifetime values are observed to be longer than those measured in aqueous buffered solution. Extraction of meaningful rate constants in such heterogeneous systems is really difficult. Such behavior may be attributed to the possible existence of different hydrogen bonding species formed with surrounding solvent (water) molecules.[59] Typical biexponential decay profiles of AODIQ in protein environments are shown in Figure 8. The fluorescence lifetimes of AODIQ in protein environments are represented in Table 2.

Instead of placing too much emphasis on the magnitude of individual decay constants in such biexponential decays, we chose to use the mean fluorescence lifetime defined by eq iii as an important parameter for exploiting the behavior of AODIQ molecule bound to proteins. The average lifetime values of AODIQ in protein environments calculated using eq iii are tabulated in Table 3. Interestingly, the decay parameters are affected by the protein environment, with the average lifetime $\langle\tau_f\rangle$ changing from 807 ps in buffer to 1257 and 1351 ps in BSA and HSA, respectively.

The decreased polarity around the probe in the proteinous environments is reflected in the increase in fluorescence lifetime. From the observed φ_f and τ_f, we can calculate the radiative and nonradiative rate constants for the ICT processes using the following relations (vii) & (viii), which is more important in supporting and for explaining the above observation

$$k_r = \varphi_f/\tau_f \qquad \text{(vii)}$$

$$1/\tau_f = k_r + k_{nr} \qquad \text{(viii)}$$

where φ_f, τ_f, k_r, and k_{nr} are the fluorescence quantum yield, mean fluorescence lifetime, radiative rate constant and nonradiative rate constant, respectively. All these photophysical parameters are tabulated in Table 3.

It is apparent from the Table 3 that in the protein environments the nonradiative rate constants k_{nr} are decreased from

that in the aqueous phase. So the enhanced lifetime is attributable to a reduction in the nonradiative rates in the protein environments.

Fluorescence anisotropy is a property that is dependent upon rotational diffusion of the fluorophore as well as the fluorescence lifetime. To ensure that the observed change in the steady-state anisotropy of AODIQ in the protein environments is not due to any change in the lifetime, the apparent (average) rotational correlation times were calculated using Perrin's equation[28] for AODIQ in BSA and HSA environments at their saturation level

$$\tau_c = (\langle\tau_f\rangle\, r)/(r_0 - r) \qquad \text{(ix)}$$

where r_0, r, and $\langle\tau_f\rangle$ are the limiting anisotropy, steady-state anisotropy, and fluorescence lifetime of AODIQ, respectively. Although Perrin's equation is not strictly valid in this case yet using the mean fluorescence lifetime we assume the equation to be valid. Using eq ix we have determined the rotational correlation times in protein environments taking $r_0 = 0.38$ for BSA and HSA and 0.18 for buffer medium (determined through time resoled anisotropy study not represented here). The rotational correlation times in BSA and HSA environments are very much increased upon binding with the proteins and the values are tabulated in Table 3. Significant increase in the rotational correlation times in BSA and HSA environments clearly establishes that the observed change in the anisotropy values (Figure 3) were not due to lifetime-induced phenomena and reinforces our earlier prediction that there is an increase in rotational restriction experienced by the probe molecule.

Conclusion

The present work reports the study of interaction of a polarity sensitive fluorophore with HSA and BSA proteins. The photophysical behaviors of AODIQ are modified remarkably in these environments compared to those in aqueous phase. This has been exploited to determine the binding efficiency, nature of microenvironment around the probe and the micropolarity at the binding site. The studies suggest that AODIQ binds with both to BSA and HSA, the binding is however to a greater extent with HSA than with BSA. This work further demonstrates that addition of urea to the aqueous solution of the proteins changes the solvation of the latter in such a way that the fluorophore molecules bound to protein are released to the bulk phase. AODIQ is thus proved to a potential indicator to follow different aspects of the bio and organized assemblies. FRET study throws some light about the location of the fluorophore in the two protein environments. It suggests that the probe molecule is relatively closer to the tryptophan residue in HSA environment than in BSA.

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