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A study of the interaction between bromopyrogallol red and bovine serum albumin by spectroscopic methods

S.M.T. Shaikh, J. Seetharamappa*, S. Ashoka, P.B. Kandagal

Department of Chemistry, Karnatak University, Dharwad 580 003, India

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Abstract

The mechanism of binding of bromopyrogallol red (BPR) with bovine serum albumin (BSA) was investigated by fluorescence, absorption, circular dichroism (CD) and lifetime measurements. The analysis of fluorescence data indicated the presence of both dynamic and static quenching mechanism in the binding. Various binding parameters have been evaluated. The CD spectral data revealed the decrease in α-helical content of BSA from 70.7% (in free BSA) to 53.89% (in bound form) thereby indicating the conformational change in BSA upon binding. The thermodynamic parameters have also been evaluated. The binding average distance, *r* between the donor (BSA) and acceptor (BPR) was determined based on the Förster's theory and it was found to be 3.84 nm. The association constant of BPR–BSA decreased in the presence of common ions.

Keywords: Bovine serum albumin; Bromopyrogallol red; Spectroscopic studies

1. Introduction

Dyes are being increasingly used for clinical and medicinal purposes [1]. The discovery that some dyes would stain certain tissues and not others led to the idea that dyes might be found that would selectively stain, combine with and destroy pathogenic organisms without causing appreciable harm to the host. As a result some azo, thiazine, triphenyl methane and acridine dyes came into use as antiseptic trypanocides and for other medicinal purposes [2]. It is also known that certain dyes viz, fluorescein and rose bengal are preferentially adsorbed by cancerous cells [1].

Serum albumin is the major transport protein for unesterified fatty acids, but is also capable of binding an extraordinarily diverse range of metabolites, drugs, dyes and organic compounds [3]. Since the overall distribution, metabolism and efficacy of many drugs in the body are correlated with their affinities towards serum albumin [3], the investigation of compounds with respect to albumin–compound binding becomes important. These results provide salient information of the structural features that determine the therapeutic effectiveness of drugs, and hence become an important research field in chemistry, life sciences and clinical medicine [4–7]. There is evidence of conformational changes in BSA induced by its interaction with low molecular weight ligands. These changes appear to affect the secondary and tertiary structure of albumin [8]. These molecular interactions are often monitored by spectroscopic techniques. Quenching measurements of albumin fluorescence is an important method to study the interactions of compounds with protein [5,9]. It can reveal accessibility of quenchers to albumin's fluorophores, help understand albumin's binding mechanisms to compounds and provide clues to the nature of the binding phenomenon. However, the binding of dyes to proteins has seldom been investigated [10,11].

Dye–protein interaction governs the duration and intensity of pharmacological effect [12,13]. The use of dyes for protein determination is well established [14,15]. However, other parameters such as mode of interaction, association constant and number of binding sites are important, when dyes are used as drugs. Critical literature survey reveals that attempts have not been made so far to investigate the mechanism of

* Corresponding author. Tel.: +91 836 221 5286; fax: +91 836 274 7884.
E-mail address: jseetharam@yahoo.com (J. Seetharamappa).

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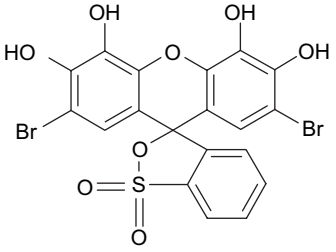

Fig. 1. Structure of bromopyrogallol red.

interaction of BPR, chemically, alpha-(5-bromo-2,3-dihydroxy-4-oxo-2,5-cyclohexadienylidene)-alpha-(5-bromo-2,3,4-trihydroxyphenyl) toluene sulphonic acid (Fig. 1.) with BSA. The present paper deals with the mechanism of binding of BPR with BSA by fluorescence, UV–vis absorption, circular dichroism and fluorescence lifetime measurements. The energy transfer between the BPR and protein (BSA) is also reported for the first time.

2. Results and discussion

2.1. Fluorescence measurements

The fluorescence intensity of a compound can be decreased by a variety of molecular interactions viz, excited-state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching. Such decrease in intensity is called quenching. In order to investigate the binding of BPR to BSA, the fluorescence spectra were recorded at 344 nm upon excitation at 296 nm. BPR causes a concentration dependent quenching of the intrinsic fluorescence of BSA (Fig. 2) without changing the emission maximum indicating that there is no change in the local dielectric environment. The interaction of BPR with BSA was further confirmed by absorption and circular dichroism techniques.

The fluorescence quenching data are analyzed by the Stern–Volmer equation,

$$F_0/F = 1 + K_{SV}[Q] \tag{1}$$

where F_0 and F are the steady state fluorescence intensities in the absence and presence of quencher, respectively, K_{SV} is the Stern–Volmer quenching constant and $[Q]$ is the concentration of quencher (BPR). The plot of F_0/F versus $[Q]$ showed positive deviation (concave towards the y axis) indicating the presence of both static and dynamic quenching [16] by the same fluorophore (Fig. 3). The dynamic portion of the observed quenching was determined by lifetime measurements using the equation,

$$\tau_0/\tau = 1 + K_D[Q] \tag{2}$$

where τ_0 and τ are the fluorescence lifetimes of BSA in absence and presence of BPR, respectively, and K_D is the dynamic quenching constant. The fluorescence decay of BSA in presence of different concentrations of the quencher was found to be biexponential (Fig. 4). The lifetime (τ), relative amplitudes (A) and χ^2 of the various decay analysis of the BSA–BPR system are listed in Table 1. The value of K_D was found to be $(2.12 \pm 0.021) \times 10^3\ M^{-1}$ from the plot of τ_0/τ versus $[Q]$. The value of static quenching constant, K_S was obtained using the equation,

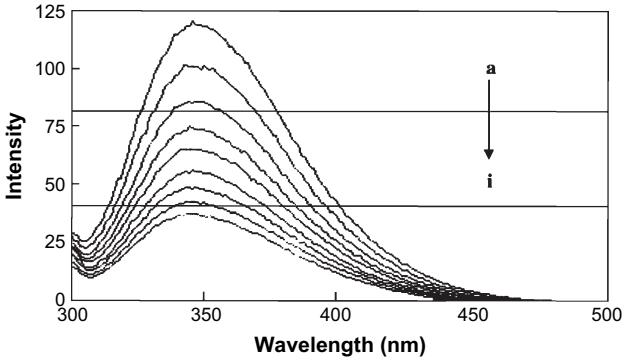

Fig. 2. Fluorescence spectra of BSA in the presence of BPR. BSA concentration was fixed at 12 μM (a). BPR concentrations were 2 (b), 4 (c), 6 (d), 8 (e), 10 (f), 12 (g), 14 (h), and 16 μM (i).

$$\frac{(F_0 - F)/F}{[Q]} = (K_S + K_D) + K_S K_D[Q] \tag{3}$$

by plotting the graph of $\{(F_0 - F)/F\}/[Q]$ versus $[Q]$ and using the value of K_D obtained from lifetime measurements. It was found to be $(8.12 \pm 0.044) \times 10^4\ M^{-1}$. The fluorescence data obtained at room temperature were further examined using modified Stern–Volmer equation,

$$F_0/(F_0 - F) = 1/f_a + 1/([Q]f_a K_{SV}) \tag{4}$$

where f_a is the fraction of the initial fluorescence which is accessible to the quencher and K_{SV} is the Stern–Volmer quenching constant. From the plot of $F_0/(F_0 - F)$ versus $1/[Q]$, the values of f_a and K_{SV} were obtained from the values of intercept and slope, respectively (Fig. 5). The value of f_a was observed

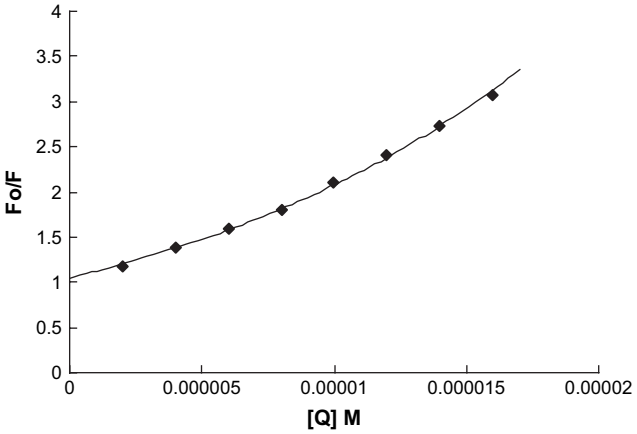

Fig. 3. Stern–Volmer plot for the binding of BPR to BSA.

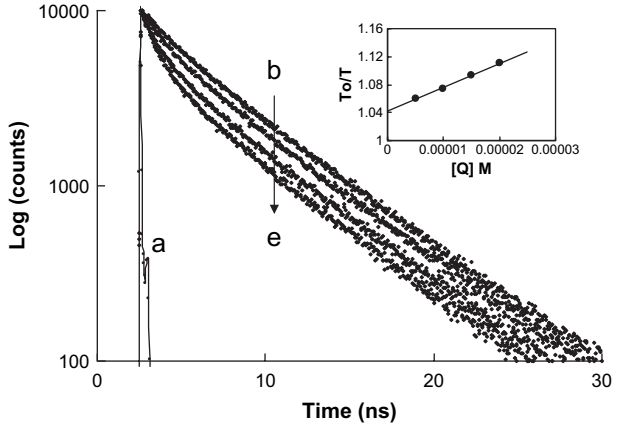

Fig. 4. Fluorescence decay profiles of BSA in the absence and presence of BPR in 0.1 M phosphate buffer of pH 7.4, $\lambda_{ex} = 296$ nm and $\lambda_{em} = 344$ nm (a). Laser profile, BSA concentration was fixed at 30 μM (b). In BPR–BSA, BPR concentrations were 10 (c), 20 (d), and 30 μM (e).

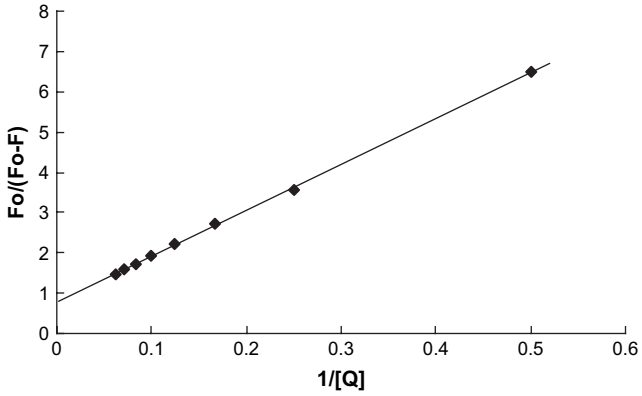

Fig. 5. Modified Stern–Volmer plot for the binding of BPR to BSA.

to be (1.29 ± 0.009) at 298 K indicating that $(77.5 \pm 0.54)\%$ of the total fluorescence of BSA is accessible to quencher. The K_{SV} was found to be $(6.78 \pm 0.032) \times 10^4\ M^{-1}$. The rate constant for the bimolecular quenching process, k_q was evaluated using the equation,

$$k_q = K_{SV}/\tau_0 \quad (5)$$

where τ_0 is the average lifetime of the protein without the quencher. The τ_0 value for BSA in the present study was found to be 6.46 ns and so the value of k_q was observed to be $(12.46 \pm 0.041) \times 10^{12}\ L\ M^{-1}\ S^{-1}$.

Parachor, which is a measure of molar volume of drug, was calculated for BPR from the atomic parachors and other structural features [17]. The value was found to be $(1613.83 \pm 0.85)\ (N\ m^{-1})^{1/4}\ m^3$. The large value of parachor indicates that the larger size of drug molecule probably has larger hydrophobic area, which can interact with hydrophobic surface on the protein molecule.

2.2. Analysis of binding equilibria

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation [18],

$$\log(F_0 - F)/F = \log K + n \log[Q] \quad (6)$$

Table 1
Lifetimes of fluorescence decay of BSA in phosphate buffer of pH 7.4 at different concentrations of BPR

Number	BPR (μM)	Analysis	Lifetime (ns)		Amplitude		χ^2
			τ_1	τ_2	A_1	A_2	
BSA	30	Biexp.	2.86	6.46	21.80	78.16	1.17
1	10	Biexp.	1.87	6.04	16.44	83.56	1.21
2	20	Biexp.	1.56	6.02	16.27	83.74	1.26
3	30	Biexp.	1.25	5.85	15.71	84.29	1.27

where K and n are the binding constant and the number of binding sites, respectively. Thus, a plot of $\log (F_0 - F)/F$ versus $\log [Q]$ yielded the K and n values to be $(13.49 \pm 0.038) \times 10^5\ L\ M^{-1}$ and (1.06 ± 0.011), respectively. The value of n is approximately equal to 1 indicating that there is one class of binding site to BPR in BSA. In BSA, the tryptophan residues involved in binding could be either Trp 135 or Trp 214. Of both tryptophans in BSA, Trp 135 is more exposed to a hydrophilic environment, whereas Trp 214 is deeply buried in the hydrophobic loop [19]. So, from the value of n it is proposed that BPR most likely binds to the hydrophobic pocket located in subdomain II A; that is to say Trp 214 is near or within the binding site [20].

2.3. Type of interaction force between BPR and BSA

Considering the dependence of binding constant on temperature, a thermodynamic process was considered to be responsible for the formation of the complex. Hence, the thermodynamic parameters depend on the temperatures and were analyzed to characterize the acting forces between BPR and BSA. The acting forces between a small molecule and macromolecule include hydrogen bond, van der-Waals force, electrostatic force, hydrophobic interaction force, and so on. The thermodynamic parameters were determined using the following equations:

$$\log K = -\Delta H^o/2.303RT + \Delta S^o/2.303R \quad (7)$$

$$\Delta G^o = \Delta H^o - T\Delta S^o \quad (8)$$

where ΔH^o, ΔG^o and ΔS^o are, respectively, enthalpy change, free energy change and entropy change. The binding studies were carried out at 288, 298, 303 and 308 K. At these temperatures the BSA doesn't undergo any structural degradation. The values of K, ΔH^o, ΔS^o and ΔG^o are summarized in Table 2. The negative value of ΔG^o reveals that the interaction process is spontaneous. The positive entropy change occurs because the water molecules that are arranged in an orderly fashion around the drug and protein acquire the more random configuration as a result of hydrophobic interactions. Thus, the

Table 2
Thermodynamic parameters of BSA–BPR system

Temperature, (K)	Binding constant ($K \times 10^{-5}$, M^{-1})	R^2	ΔG^o (kJ mol^{-1})	ΔH^o (kJ mol^{-1})	ΔS^o (J mol^{-1} K^{-1})
288	4.24 ± 0.026	0.9970	−30.99		
298	13.49 ± 0.038	0.9984	−33.55	93.13	430.6
303	11.12 ± 0.029	0.9968	−35.04		
308	55.89 ± 0.041	0.9953	−39.74		

positive ΔH^o and ΔS^o values showed that hydrophobic interactions play a role in the binding of BPR to BSA [21].

2.4. Energy transfer between BPR and BSA

The overlap of the UV absorption spectra of BPR with the fluorescence emission spectra of BSA is shown in Fig. 6. The importance of the energy transfer in biochemistry is that, the efficiency of transfer can be used to evaluate the distance between the ligand and the tryptophan residues in the protein. According to Förster's non-radiative energy transfer theory [22], the rate of energy transfer depends on: (i) the relative orientation of the donor and acceptor dipoles, (ii) the extent of overlap of fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor, and (iii) the distance between the donor and the acceptor. The energy transfer effect is related not only to the distance between the acceptor and donor, but also to the critical energy transfer distance, R_0, that is

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{9}$$

where F and F_0 are the fluorescence intensities of BSA in the presence and absence of BPR, r is the distance between acceptor and donor and R_0 is the critical distance when the transfer efficiency is 50%. R_0^6 is calculated using the equation,

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \tag{10}$$

where k^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor and J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. J is given by

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{11}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength, λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength, λ. In the present case, $K^2 = 2/3$, $N = 1.336$ and $\Phi = 0.15$ [23,24]. From Eqs. (9) to (11), we would be able to calculate that $J = 1.61 \times 10^{-15}$ cm^3 L M^{-1}, $R_0 = 2.28$ nm, $E = 0.45$ and $r = 3.84$ nm. The donor-to-acceptor distance, $r < 7$ nm [25,26] indicated that the energy transfer from BSA to BPR occurs with high possibility. Further the value of r obtained this way agrees very well with literature value of substrate binding to serum albumin at site II A [19].

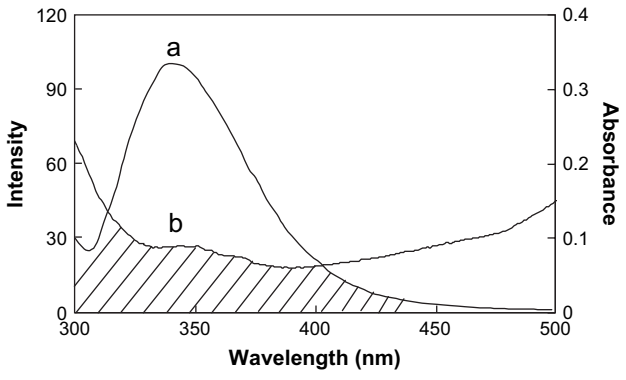

Fig. 6. The overlap of the fluorescence spectrum of BSA (a) and the absorbance spectrum of BPR (b) {$\lambda_{ex} = 296$ nm, $\lambda_{em} = 344$ nm, [BSA]:[BPR] = 1:1}.

2.5. The effect of ions on the binding constant of BPR–BSA

The fluorescence intensity was changed before and after the addition of some common ions such as Fe^{2+}, Co^{2+}, K^+, Ni^{2+}, V^{5+} and Cu^{2+}. The effect of these ions on the binding constant of BPR–BSA system was investigated at 298 K. The association constants of BPR–BSA were determined in presence of the above common ions and are shown in Table 3. The BPR–BSA binding constant was decreased by 50.06–83.39% in the presence of common ions.

2.6. Conformation investigations

UV–vis absorption measurement is a very simple method and applicable to explore the structural change [26] and to know the complex formation [27]. In the present study, we have recorded the UV absorption spectra of BPR, BSA and BPR–BSA system (Fig. 7). It is evident that the UV absorption intensity of BSA increased regularly with the variation of BPR concentration. The maximum peak position of BPR–BSA was shifted slightly towards higher wavelength region possibly due to complex formation between BPR and BSA.

CD is a sensitive technique to monitor the conformational changes in the protein upon interaction with the ligand. The CD spectra of BSA in the absence and presence of BPR are shown in Fig. 8. The CD spectra of BSA exhibited two

Table 3
Effects of common ions and other drugs on binding constants of BSA–BPR system

System	Binding constant ($\times 10^5$, M^{-1})
BSA + BPR	7.71 ± 0.043
BSA + BPR + Fe^{2+}	1.28 ± 0.052
BSA + BPR + Co^{2+}	1.30 ± 0.026
BSA + BPR + K^+	1.71 ± 0.038
BSA + BPR + V^{5+}	1.46 ± 0.047
BSA + BPR + Ni^{2+}	3.85 ± 0.029
BSA + BPR + Cu^{2+}	1.38 ± 0.061

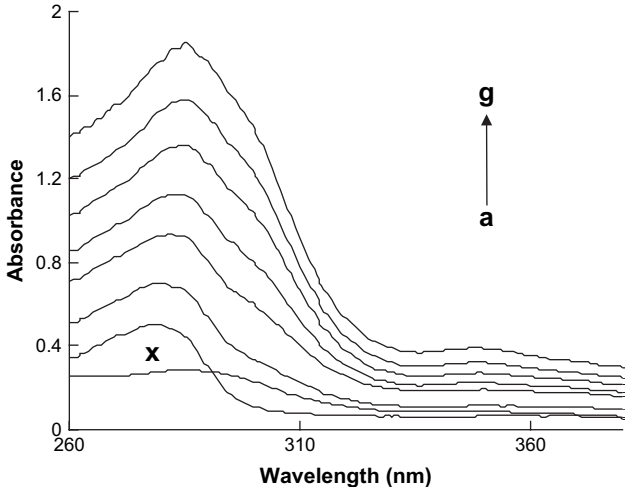

Fig. 7. Absorbance spectrum of BSA, BPR and BSA–BPR system. BSA concentration was kept fixed at 12 μM (a). BPR concentrations in BPR–BSA system were 6 (b), 12 (c), 18 (d) 24 (e), 30 (f), and 36 μM (g). Concentration of 12 μM (x) was used for BPR only.

negative bands in the UV region at 209 and 222 nm, characteristic of an α-helical structure of protein [28]. The CD results were expressed in terms of mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ according to the following equation [6]:

$$\text{MRE} = \frac{\text{Observed CD (mdeg)}}{C_\text{P} nl \times 10} \tag{12}$$

where C_P is the molar concentration of the protein, n is the number of amino acid residues and l is the path length. The α-helical contents of free and combined BSA [6] were calculated from MRE value at 208 nm using the equation

$$\alpha\text{-Helix}\ (\%) = \frac{[-\text{MRE}_{208} - 4000]}{[33,000 - 4000]} \times 100 \tag{13}$$

where MRE_{208} is the observed MRE value at 208 nm, 4000 is the MRE of the β-form and random coil conformation cross at 208 nm, and 33,000 is the MRE value of a pure α-helix at 208 nm. From the above equation, the quantitative analysis results of the α-helix in the secondary structure of BSA were obtained. They differed from that of 70.7% in free BSA to 53.89% in the BSA–BPR system, which was indicative of the loss of α-helical. The percentage of protein α-helix structure decreased indicated that the dye, BPR bound with the amino acid residue of the main polypeptide chain of protein and destroyed their hydrogen bonding networks [29]. The CD spectra of BSA in the presence and absence of BPR are similar in shape, indicating that the structure of BSA after BPR binding to BSA is also predominantly α-helical [30]. So, we concluded that the binding of BPR to BSA induced some conformational changes.

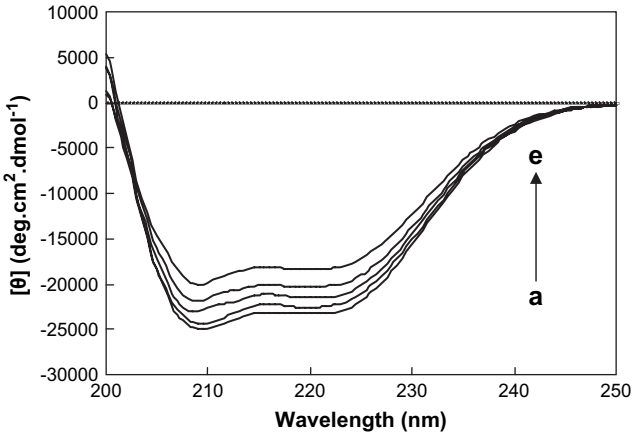

Fig. 8. CD spectra of the BSA–BPR system obtained in 0.1 M phosphate buffer of pH 7.4 at room temperature. BSA concentration was kept fixed at 12 μM (a). In BPR–BSA system, BPR concentrations were 5 (b), 15 (c), 30 (d), and 60 μM (e).

3. Conclusions

This paper provided an approach for studying the binding of protein with BPR using absorption, fluorescence, CD and lifetime measurements. The results showed that BSA fluorescence was quenched by BPR through both dynamic and static quenching. Since, a large hydrophobic cavity is present in the subdomain II A of BSA, it becomes the target of BPR binding. The spectral data revealed the conformational changes of BSA upon interaction with BPR. The decrease in the binding constant of BPR–BSA was observed in the presence of common ions. This work also reports the distance between tryptophan and bound BPR for the first time based on Förster's energy transfer theory.

4. Experimental

4.1. Materials

Bovine Serum Albumin (BSA, Fraction V, approximately 99%; protease free and essentially γ-globulin free) was obtained from Sigma Chemical Company, St. Louis, USA. AnalaR grade bromopyrogallol red was in the study. The solutions of BPR and BSA were prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. BSA solution was prepared based on its molecular weight of 65,000. All other materials were of analytical reagent grade and double distilled water was used throughout.

4.2. Spectral measurements

Fluorescence measurements were performed on a Hitachi spectrofluorimeter Model F-2000 equipped with a 150 W Xenon lamp and slit width of 10 nm. The CD measurements were made on a JASCO-J-715 spectropolarimeter using a 0.1 cm cell at 0.2 nm intervals, with three scans averaged for each CD spectrum in the range of 200–250 nm.

The absorption spectra were recorded on a double beam CARY 50-BIO UV–vis spectrophotometer equipped with a 150 W Xenon lamp and a slit width of 10 nm.

Fluorescence decays were recorded using TCSPC method using the following setup. A diode pumped millena CW laser

(Spectra Physics) 532 nm was used to pump the Ti–sapphire rod in Tsunami picoseconds mode locked laser system (Spectra Physics). The 750 nm (80 MHz) was taken from the Ti–sapphire laser and passed through pulse picker (Spectra Physics, 3980 2S) to generate 4 MHz pulses. The third harmonic output (296 nm) was generated by flexible harmonic generator (Spectra Physics, GWU 23 PS). The vertically polarized 296 nm laser was used to excite the sample. The fluorescence emission at magic angle (54.7°) was dispersed in a monochromator (f/3 aperture), counted by an MCP PMT (Hamamatsu R 3809) and processed through CFD, TAC and MCA. The instrument response function for this system is ~52 ps. The fluorescence decay was analyzed by using the software provided by IBH (DAS-6) and PTI Global Analysis Software. A 1.0 cm quartz cell was used throughout the study.

4.3. BPR–BSA interactions

Based on preliminary experiments, BSA concentration was kept fixed at 12 μM and drug concentration was varied from 2 to 16 μM. Fluorescence spectra were recorded at 288, 298, 303 and 308 K in the range of 300–500 nm upon excitation at 296 nm in each case.

4.4. Circular dichroism measurements

The CD measurements of BSA in the absence and presence of BPR (1:0.5, 1:1.5, 1:3 and 1:6) were made in the range of 200–250 nm. A stock solution of 150 μM BSA was prepared.

4.5. Lifetime measurements

The fluorescence lifetime measurements of BSA in the presence and absence of BPR were recorded by fixing 296 nm as the excitation wavelength and 344 nm as the emission wavelength. A stock solution of 200 μM BSA was prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. The BSA concentration was kept fixed at 30 μM and BPR concentration was varied from 10 to 30 μM.

4.6. Effects of some common ions

The fluorescence spectra of BPR (2–16 μM)–BSA (12 μM) were recorded in the absence and presence of various common ions (12 μM) viz, Fe^{2+}, Co^{2+}, K^{+}, Ni^{2+}, V^{5+} and Cu^{2+} in the range 300–500 nm upon excitation at 296 nm.

Acknowledgements

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