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Spectroscopic investigations on the mechanism of interaction of bioactive dye with bovine serum albumin

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Abstract

The mechanism of binding of rose bengal (RB) with bovine serum albumin (BSA) was investigated by spectroscopic methods. The analysis of fluorescence data indicated the presence of both dynamic and static quenching mechanisms in the binding. Various binding parameters have been evaluated. The thermodynamic parameters, ΔH^0 and ΔS^0 were observed to be $-79.61\ \mathrm{kJ\,mol^{-1}}$ and $-143.37\ \mathrm{J\,mol^{-1}\,K^{-1}}$, respectively. The quantitative analysis of CD results revealed that the α-helicity of BSA decreased from 66.4% (in free BSA) to 48.64% (in bound BSA). The binding average distance, r between the BSA (donor) and RB (acceptor) was determined based on Förster's theory and it was found to be 2.75 nm. The effects of common ions on the binding constant of RB–BSA were also examined.

Keywords: Bovine serum albumin; Rose bengal; 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 destroy pathogenic organisms without causing appreciable harm to the host. Due to this, some azo, thiazine, triphenyl methane and acridine dyes came into use as antiseptic trypanocides and for other medicinal purposes [2]. RB, chemically, 4,5,6,7-tetrachloro 2′,4′,5′,7′-tetraiodofluorescein di sodium salt (Fig. 1), has been used as a dye, biological stain and diagnostic aid. *In vivo*, the ophthalmic dye, RB displays profound antiviral effects against herpes simplex virus (HSV)-1 [3]. It is also known to inactivate the Friend leukemia virus inoculum at low doses [4]. RB is a clinically significant dye as it finds application in detecting the organic anions in liver plasma.

Serum albumins are the most abundant proteins in the circulatory system of a wide variety of organisms. Being the major macromolecules in contributing to osmotic blood pressure [5], they can play a dominant role in drug disposition and efficacy. Many drugs and other bioactive small molecules bind reversibly to albumin and other serum components, which then function as carriers. Serum albumin often increases the apparent solubility of hydrophobic drugs in plasma and modulates their delivery to cell *in vivo* and *in vitro*. Consequently, it is important to understand the mechanism of interaction of a bioactive compound with protein. This kind of study may provide salient information on the structural features that determine the therapeutic effectiveness of drugs/dyes, and hence become an important research field in chemistry, life sciences and clinical medicine [5–8].

Quenching measurement of albumin fluorescence is an important method to study the interactions of compounds with protein [9]. It can reveal the accessibility of quenchers to albumins (fluorophores), help to understand albumin's binding mechanisms to compounds with the aid of time resolved fluorescence technique and provide clues about the nature of the binding phenomenon. The application of dyes in protein determination is well established [10,11]. However, other parameters such as mode of interaction, association constant and

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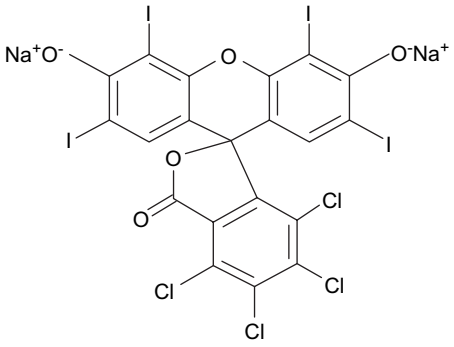

Fig. 1. Structure of rose bengal.

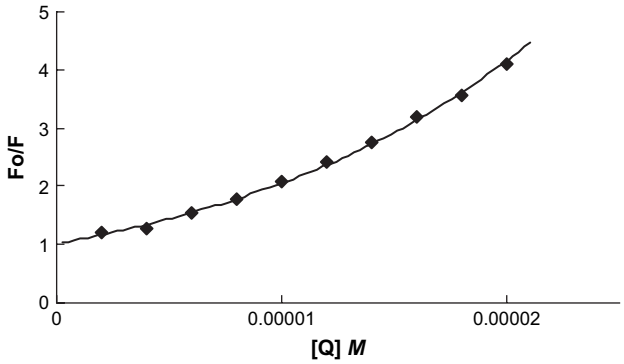

Fig. 3. Stern–Volmer plot for the binding of RB to BSA.

number of binding sites are important, when dyes are used as drugs. The binding of dyes to proteins has seldom been investigated [12,13]. The literature survey reveals that attempts have not been made so far to investigate the binding mechanism of RB with BSA by fluorescence, UV–vis absorption, circular dichroism and lifetime measurements. The energy transfer between RB and protein (BSA) is also reported in the present study 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 RB to BSA, the fluorescence spectra were recorded in the range of 300–500 nm upon excitation at 296 nm. RB causes a concentration dependent quenching of the intrinsic fluorescence of BSA (Fig. 2) without changing the emission maximum and shape of the peaks. These results indicated that there were interactions between RB and BSA. The interaction of RB with BSA was further confirmed by absorption and circular dichroism techniques. The fluorescence quenching data are analyzed by the Stern–Volmer equation,

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

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 (RB). 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 [14] by the same fluorophore (Fig. 3).

Fluorescence lifetimes were estimated for BSA from the fluorescence decays (Fig. 4). A biexponential decay was observed with two nanosecond lifetimes, 6.08 and 2.08 ns [15]. Hence, due to the presence of two tryptophan residues of the protein, the fluorescence decays are characterized by two nanosecond lifetimes. Interestingly, the effect of addition of drug to protein showed the quenching of both the lifetimes as a function of the increase in the drug concentration. The lifetimes (τ_1 and τ_2), relative amplitudes (A_1 and A_2) and χ^2

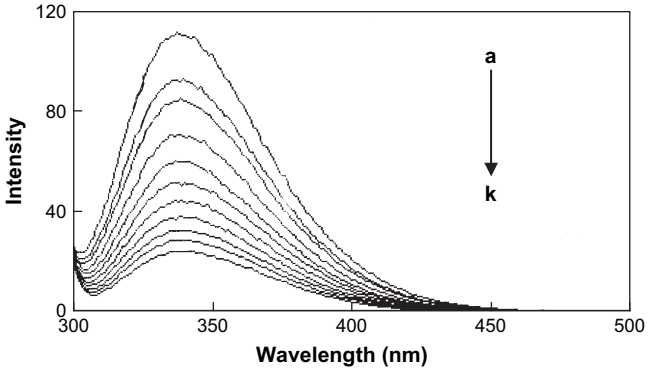

Fig. 2. Fluorescence spectra of BSA in the presence of RB. BSA concentration was fixed at 12 μM (a). RB concentrations were 2 (b), 4 (c), 6 (d), 8 (e), 10 (f), 12 (g), 14 (h), 16 (i), 18 (j), and 20 μM (k).

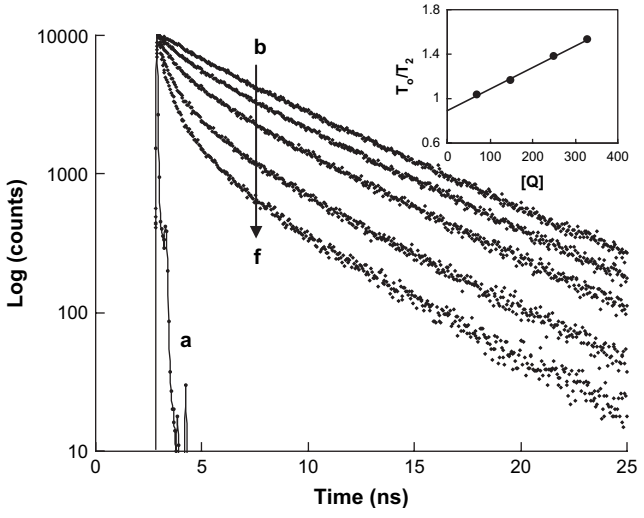

Fig. 4. Fluorescence decay profiles of BSA in the absence and presence of RB in 0.1 M phosphate buffer of pH 7.4, $\lambda_{ex} = 296$ nm and $\lambda_{em} = 344$ nm, (a) Laser profile, BSA concentration was fixed at 30 μM (b). In RB–BSA, RB concentrations were 70(c), 150 (d), 250 (e) and 300 μM (f).

of the various decay analysis of the BSA–RB system are listed in Table 1. The dynamic portion of observed quenching was determined by lifetime measurements using the equation,

$$\tau_0/\tau = 1 + K_D[Q] \quad (2)$$

where τ_0 (6.08 ns) and τ (equal to τ_2 in the present study) are the fluorescence lifetimes of BSA in the absence and presence of RB, respectively, and K_D is the dynamic quenching constant. 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_2 *versus* [Q]. The value of static quenching constant, K_S was obtained using the equation,

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

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 $4.76 \pm 0.023 \times 10^4\ M^{-1}$. The fluorescence data obtained at room temperature were further examined using the modified Stern–Volmer equation,

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

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. The value of f_a refers to the fraction of fluorescence accessible to quenching, which need not be the same as the fraction of tryptophan residues i.e. accessible to quenching [14]. 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 to be 1.18 ± 0.007 at 298 K indicating that $84 \pm 0.39\%$ of the total fluorescence of BSA is accessible to the quencher. The K_{SV} was found to be $7.43 \pm 0.051 \times 10^4\ M^{-1}$.

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 [16],

$$\log\ (F_0 - F)/F = \log\ K + n\ \log[Q] \quad (5)$$

where K and n are the binding constants 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 of $28.05 \pm 0.027 \times 10^{-5}\ M^{-1}$ and 1.1 ± 0.016, respectively. The value of n is approximately equal to 1 indicating that there is one class of binding site to RB in BSA.

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

Number	RB (μM)	Analysis	Lifetime (ns)		Amplitude		χ^2
			τ_1	τ_2	A_1	A_2	
BSA (30 μM)	–	Biexp	2.08	6.08	6.98	93.02	1.18
1	70	Biexp	1.25	5.66	14.40	85.60	1.13
2	150	Biexp	1.02	5.33	25.42	74.58	1.22
3	250	Biexp	7.56	4.68	36.77	63.23	1.39
4	330	Biexp	5.94	3.85	61.65	38.35	1.46

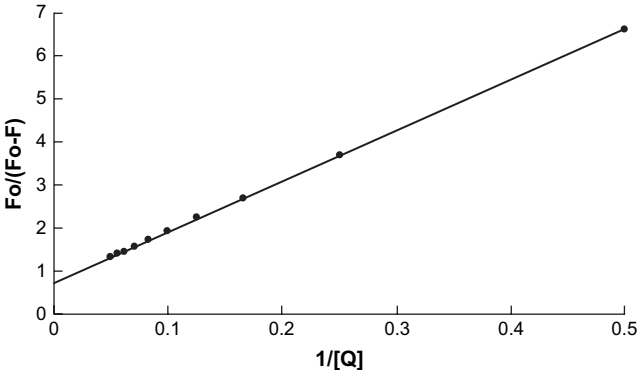

Fig. 5. Modified Stern–Volmer plot for the binding of RB to BSA.

2.3. Type of interaction force between RB and BSA

The binding studies were carried out at 288, 298, 303 and 308 K. At these temperatures the BSA does not undergo any structural degradation. The interaction between a ligand and protein may involve hydrogen bonds, van der Waals forces, electrostatic forces, and/or hydrophobic interactions. The thermodynamic parameters were determined using the following equations,

$$\log\ K = -\Delta H^0/2.303RT + \Delta S^0/2.303R \quad (6)$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (7)$$

The log K *versus* 1/T plot (Fig. 6) enabled the determination of ΔH^0 and ΔS^0 for the binding process. ΔH^0, ΔG^0 and ΔS^0 are standard enthalpy change, standard free energy change and standard entropy change, respectively. The values of K, ΔH^0, ΔS^0 and ΔG^0 are summarized in Table 2. The negative value of ΔG^0 reveals that the interaction process is spontaneous. An important source of negative contribution to ΔH^0 and ΔS^0 will arise if a hydrogen bond is formed [17]. The negative ΔH^0 and ΔS^0 values for the interaction of RB and BSA indicate that the binding is mainly enthalpy driven and entropy

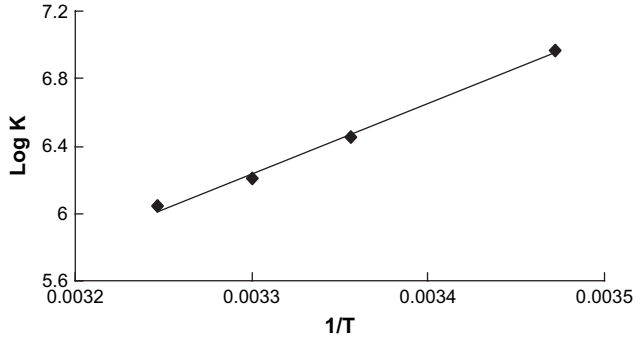

Fig. 6. Log K *versus* 1/T for RB–BSA system.

Table 2
Thermodynamic parameters of BSA–RB system

Drug	Temperature (K)	Binding constant ($K \times 10^{-5}$, M^{-1})	r	ΔG^0 (kJ mol^{-1})	ΔH^0 (kJ mol^{-1})	ΔS^0 (J mol^{-1} K^{-1})
RB	288	(92.17 ± 0.031)	0.9914	−38.38	−79.61	−143.37
	298	(28.05 ± 0.027)	0.9982	−37.40		
	303	(16.20 ± 0.036)	0.9922	−35.96		
	308	(10.99 ± 0.038)	0.9896	−35.61		

is unfavorable for it, and that the hydrogen bonding and van der Waals forces played major role in the interaction [17].

2.4. Energy transfer between RB and BSA

The overlap of the UV absorption spectra of RB with the fluorescence emission spectra of BSA is shown in Fig. 7. The energy transfer has been used as a ''spectroscopic ruler'' for the measurement of distance between the sites on proteins [14]. The importance of 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 [18], 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

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{8}$$

where F and F_0 are the fluorescence intensities of BSA in the presence and absence of RB, 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,

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \tag{9}$$

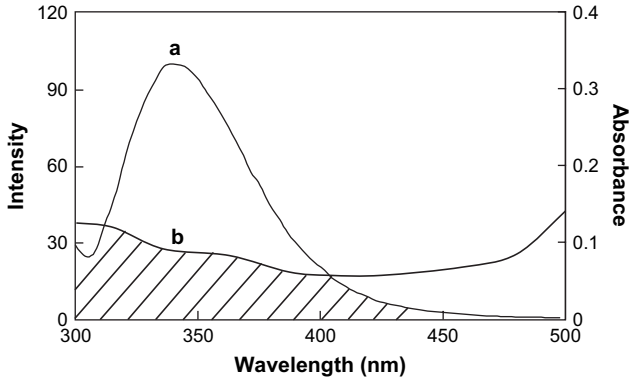

Fig. 7. The overlap of the fluorescence spectrum of BSA (a) and the absorbance spectrum of RB (b) {$\lambda_{ex} = 296$ nm, $\lambda_{em} = 344$ nm, [BSA]:[RB] = 1:1}.

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

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{10}$$

where $F(\lambda)$ is the fluorescence intensity of fluorescent donor of wavelength, λ, and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength, λ. In the present case, $k^2 = 2/3$, $N = 1.336$ and $\Phi = 0.15$ [19,20]. From Eqs. (8)–(10), we were able to calculate that $J = 1.52 \times 10^{-14}$ cm^3 L M^{-1}, $R_0 = 1.86$ nm, $E = 0.45$ and $r = 2.75$ nm. The donor-to-acceptor distance, $r < 7$ nm [21,22] indicated that the energy transfer from BSA to RB occurs with high possibility. Larger BSA–RB distance, r compared to that of R_0 value observed in the present study also reveals the presence of static type quenching mechanism to a larger extent [23,24].

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

Since the common ions which are distributed in human and animals (in ionized or non-ionized or complex form) do influence the binding of drugs to proteins, such studies have been reported in the literature. Hence, we have examined the effects of common ions *viz*., Fe^{2+}, Co^{2+}, K^+, Ni^{2+}, V^{5+} and Cu^{2+} on the binding constant of RB–BSA at 298 K. The solutions of cations were prepared from chlorides of respective cations while that of V^{5+} solution was prepared from ammonium metavanadate. Under experimental conditions, no cation gave precipitate in phosphate buffer. The binding constant for RB–BSA increased in the presence of ions *viz*., Co^{2+}, K^+, Ni^{2+} and Cu^{2+} (Table 3) implying stronger binding of

Table 3
Effects of common ions on binding constants of BSA–RB system

System	Binding constant (M^{-1})
BSA + RB	$(28.05 \pm 0.027) \times 10^5$
BSA + RB + Fe^{2+}	$(14.21 \pm 0.041) \times 10^5$
BSA + RB + Co^{2+}	$(32.74 \pm 0.038) \times 10^5$
BSA + RB + K^+	$(81.11 \pm 0.047) \times 10^5$
BSA + RB + V^{5+}	$(10.13 \pm 0.026) \times 10^5$
BSA + RB + Ni^{2+}	$(87.09 \pm 0.019) \times 10^5$
BSA + RB + Cu^{2+}	$(84.02 \pm 0.035) \times 10^5$

RB to BSA. This prolongs the storage time of RB in blood plasma (if RB is administered to a subject in presence of Co^{2+}, K^{+}, Ni^{2+} and Cu^{2+}) and enhances the maximum effectiveness of the dye [24]. Hence, in the presence of above ions, RB can be stored and removed better by BSA [24]. The decrease in binding constant of RB–BSA in the presence of Fe^{2+} and V^{5+} (Table 3) revealed the availability of higher concentration of free RB in plasma which could be quickly cleared from the blood through urine [25].

2.6. Conformation investigations

UV–vis absorption measurement is a very simple method and applicable to explore the structural change [22] and to know the complex formation [9]. In the present study, we have recorded the UV absorption spectra of RB, BSA and RB–BSA system (Fig. 8). It is evident that the UV absorption intensity of BSA increased regularly with the variation of RB concentration. The maximum peak position of RB–BSA was shifted slightly towards lower wavelength region possibly due to complex formation between RB and BSA [24]. The slight shift of the peaks also indicated that with addition of RB, the peptide strands of BSA molecules extended more and the hydrophobicity was decreased [22].

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 RB are shown in Fig. 9. The CD spectra of BSA exhibited two negative bands in the UV region at 209 and 222 nm, characteristic of an α-helical structure of protein [26]. The CD results were expressed in terms of mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ according to the following equation [8],

$$\mathrm{MRE} = \frac{\text{Observed CD(mdeg)}}{C_p n l \times 10} \tag{11}$$

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

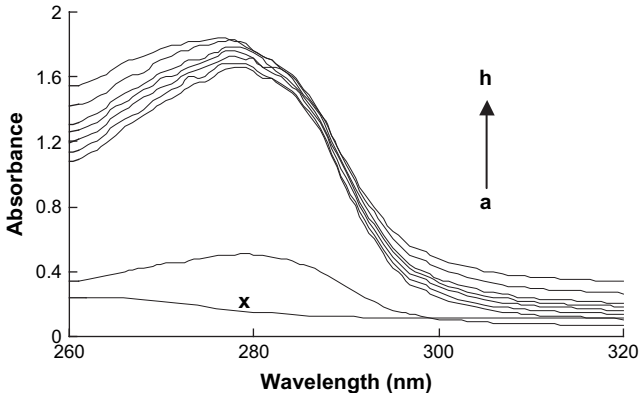

Fig. 8. Absorbance spectra of RB, BSA and BSA–RB system. BSA concentration was kept fixed at 12 μM (a). RB concentrations in RB–BSA system were 7 (b), 14 (c), 21 (d) 28 (e), 35 (f), 49 (g) and 63 μM (h). A concentration of 12 μM was used for RB only (**x**).

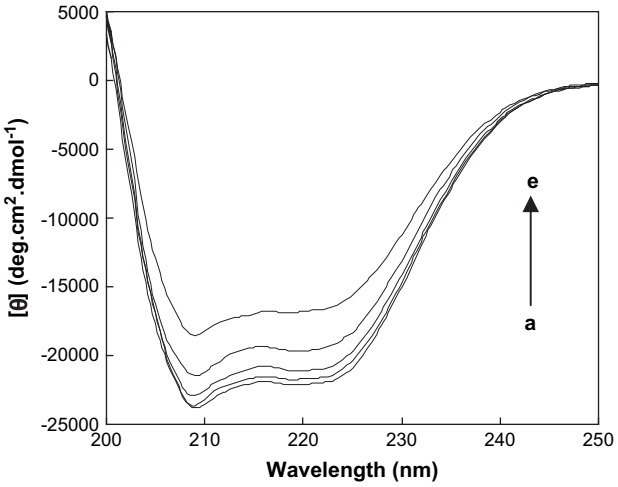

Fig. 9. CD spectra of the BSA–RB system obtained in 0.1 M phosphate buffer of pH 7.4 at room temperature. BSA concentration was kept fixed at 12 μM (a). In RB–BSA system, RB concentrations were 6 (b), 18 (c), 42 (d) and 90 μM (e).

α-helical contents of free and bound BSA [5] were calculated from MRE value at 208 nm using the equation,

$$\alpha\text{-Helix}(\%) = \frac{-\mathrm{MRE}_{208} - 4000}{33{,}000 - 4000} \times 100 \tag{12}$$

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 α-helicity differed from that of 66.41% in free BSA to 48.64% in the BSA–RB system, which was indicative of the loss of α-helical. The percentage of α-helical structure of protein decreased indicating that RB bound with the amino acid residues of the main polypeptide chain of protein and destroyed their hydrogen bonding networks [24]. The CD spectra of BSA in presence and absence of RB are similar in shape, indicating that the structure of BSA after RB binding to BSA is also predominantly α-helical [27]. So, we concluded that the binding of RB to BSA induced some conformational changes in BSA.

3. Conclusions

This paper provided an approach for studying the binding of protein to RB by employing absorption, fluorescence, circular dichroism and lifetime measurements. The results showed that the BSA fluorescence was quenched by RB through both dynamic and static quenching. The spectral data revealed the conformational changes in BSA upon interaction with RB. The binding constant of RB–BSA was affected in presence of common ions. This work also reports the distance between

the donor (BSA) and the acceptor (RB) for the first time based on Förster's energy transfer theory.

4. Experimental

4.1. Materials

Bovine serum albumin (BSA, Fraction V) was obtained from Sigma Chemical Company, St Louis, USA. AnalaR grade rose bengal was used in the study. The solutions of RB 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 5 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 5 nm.

Fluorescence decays were recorded using standard time-correlated single photon counting (TCSPC) method using the following set up: a diode pumped millena continuous wave (CW) laser (Spectra Physics) 532 nm was used to pump the titanium:sapphire rod in Tsunami picoseconds mode locked laser system (Spectra Physics). The 750 nm (80 MHz) was taken from the titanium: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 a microchannel plate (MCP) photo multiplier tube (PMT) [Hamamatsu R 3809] and processed through CFD, TAC and microchannel analyzer (MCA). The instrument response function for this system is ~52 ps. Fluorescence decay was analyzed by using the software provided by IBH (Decay Associated emission Spectra-6) and PTI Global Analysis Software. A 1.0 cm quartz cell was used throughout the study.

4.3. RB–BSA interactions

Based on preliminary experiments, BSA concentration was kept fixed at 12 μM and drug concentration was varied from 2 to 20 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 RB (1:0.5, 1:1.5, 1:3.5 and 1:7.5) 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 RB 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 RB concentration was varied from 10 to 30 μM.

4.6. Effects of some common ions

The fluorescence spectra of RB (2–20 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 of 300–500 nm upon excitation at 296 nm.

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