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A study of the binding of C.I. Mordant Red 3 with bovine serum albumin using fluorescence spectroscopy

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ABSTRACT

The interaction of C.I. Mordant Red 3 and bovine serum albumin was investigated using fluorescence and UV–vis absorption spectroscopy. Fluorescence quenching, from which binding parameters were evaluated, revealed that the quenching of the serum by C.I. Mordant Red 3 resulted from the formation of a dye–serum complex. The enthalpy and entropy changes for the reaction were −50.49 kJ mol^{-1} and −50.88 J mol^{-1} K^{-1} respectively. van der Waals forces and hydrogen bonds were the dominant intermolecular forces that stabilize the complex; the distance between donor and acceptor was 2.79 nm, according to Förster's non-radiative energy transfer theory. The effect of the dye upon the conformation of bovine serum albumin was analyzed using synchronous fluorescence spectrum.

1. Introduction

Bovine serum albumin (BSA), a major soluble protein, has many physiological functions, including regulation of colloid osmotic pressure and transporting of a variety of endogenous and exogenous ligands, such as fatty acids, drugs, metabolites and dyes throughout the circulation [1–3]. It has been used as model protein in several biophysical, biochemical and physicochemical studies for many years. BSA is made up of three homologous domains (I–III), which are divided into nine loops by 17 disulfide bridges. Each domain is composed of two sub-domains (A and B). Aromatic and heterocyclic ligands were found to bind within two hydrophobic pockets in sub-domain IIA and IIIA, site I and site II [4,5]. BSA has two tryptophans, Trp-134 and Trp-212, embedded in the first sub-domain IB and sub-domain IIA, respectively. There is evidence of conformation changes of BSA induced by its interaction with dyes and low molecular weight ligands. And these changes appear to affect the secondary and tertiary structure of albumin. So, it is important to study the interaction of dyes with BSA, and hence become an important research field in chemistry, life sciences and clinical medicine.

Nowadays, many researches on the binding of drugs to BSA have been carried out, but seldom report concentrates on the binding of BSA with toxic anthraquinone dyes. C.I. Mordant Red 3, 1,2-dihydroxy-9,10-anthraquinonesulfonic acid sodium salt (alias *Alizarin Red S*; Fig. 1) is a water-soluble, anthraquininoid dye obtained from the sulfonation of *alizarin*, a natural dye obtained from madder and known since third millennium B.C. [6]. C.I. Mordant Red 3 is used mostly in the textile industry, but also enjoys other applications such as biological histochemical staining [6,7]. However, C.I. Mordant Red 3 is a recalcitrant dye that is both mutagenic and carcinogenic [8]. The high incidence of cancer among workers in the dye and chemical industries has been associated with exposure to anthraquinone dyes [9,10] and there is no experimental evidence about the molecular mechanisms involved in these processes [11]. In this context, a fuller understanding of the disposition and transportation of anthraquinone dyes in blood would benefit from the investigation of the interaction of this dye with serum albumin. Although both an electrochemical method as well as a resonance light scattering technique have been developed for the quantitative determination of protein [12,13], other parameters such as the mode of interaction, association constant and number of binding sites involved are important, as these may provide important theoretical information about metabolism and distribution of C.I. Mordant Red 3 [14]. Furthermore, to the best of our knowledge, the detailed investigation of C.I. Mordant Red 3–BSA association using fluorescence spectrum has not been reported in the literature.

Fluorescence quenching is a powerful method to study the molecular interactions involving proteins because it is sensitive,

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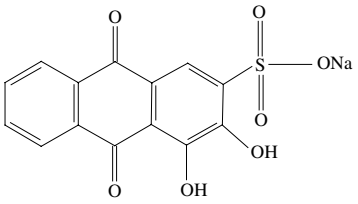

Fig. 1. Molecular structure of C.I. Mordant Red 3.

rapid and relatively easy to use [15]. Fluorescence spectroscopy is essentially a probe technique sensing changes in the local environment of the fluorophore, help understand albumin's binding mechanisms to compounds and provide clues to the nature of the binding phenomenon. It has advantages over conventional methods such as equilibrium dialysis, ultrafiltration and ultracentrifugation. These methods suffer from lack of sensitivity or long analysis time or both and use of protein concentrations far exceeding the dissociation constant for the ligand–protein complex under investigation [16]. In the present paper, the binding of C.I. Mordant Red 3 to BSA was studied by spectroscopy including fluorescence and UV–vis spectrum. The binding mechanism between C.I. Mordant Red 3 and BSA regarding the binding parameters, the thermodynamic functions, the binding distances and the effect of C.I. Mordant Red 3 on the protein conformation were investigated in our work.

2. Materials and method

2.1. Materials

Bovine serum albumin (fatty acid free <0.05%) and C.I. Mordant Red 3 (CAS number: 130-22-3) were obtained from Sigma–Aldrich Company (USA) and used without further purification. BSA was dissolved in the pH 7.25 buffer solution (6.7×10^{-6} M) and BSA stock solution was kept in the dark at 277 K. C.I. Mordant Red 3 was prepared into (6.7×10^{-6} M) stock solution by dissolving appropriate amount of the compound in Milli-Q ultrapure water. NaCl (0.5 M) solution was used to keep the ionic strength at 0.1 M. Buffer consists of Tris (0.2 M) and HCl (0.1 M), and the pH was adjusted to 7.25. All other chemicals were of analytical reagent grade and Milli-Q ultrapure water was used throughout the experiment.

2.2. Apparatus

Fluorescence measurements were performed with an RF-5301PC spectrofluorimeter (Shimadzu, Japan) equipped with 1.0 cm quartz cuvette and a 150 W xenon lamp, the temperature was controlled by F12-ED refrigerated/heating circulator bath (Julabo, Germany). The UV–vis spectrum was recorded at room temperature on a Cary-100 UV–vis spectrophotometer (Varian, USA) equipped with 1.0 cm quartz cell. pH measurement was carried out with an Orion-868 digital pH-meter (Orion, USA).

2.3. Procedures

A 2.0 mL solution, containing appropriate concentration of BSA, was titrated by successive additions of a 6.7×10^{-6} M stock solution of C.I. Mordant Red 3. Titrations were done manually by using trace syringes. The fluorescence spectrum was recorded at three temperatures (297, 303 and 309 K) in the range of 250–500 nm with exciting wavelength at 280 nm. The width of the excitation and emission slit was set to 5 nm and 5 nm, respectively. The UV–vis spectrum was performed at 297 K.

3. Results and discussion

3.1. Interactions between C.I. Mordant Red 3 and BSA

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule, including exciting-state reactions, molecular rearrangements, energy transfer, ground-state complex formation and collisional quenching processes [17]. Quenching can be classified as either dynamic or static quenching by different mechanisms. Dynamic quenching results from collision between fluorophore and quencher, and static quenching is due to the formation of ground-state complex between fluorophore and quencher. In general, dynamic and static quenching can be distinguished by their different dependence on temperature and viscosity [18]. The quenching rate constants decrease with increasing temperature for state quenching, but the reverse effect is observed for dynamic quenching. The fluorescence spectrum of BSA in the presence of different amounts of C.I. Mordant Red 3 was recorded in the range of 250–500 nm upon excitation at 280 nm (Fig. 2). As can be seen from Fig. 2, the fluorescence intensity of BSA gradually decreased upon increasing the concentration of C.I. Mordant Red 3, indicating the binding of C.I. Mordant Red 3 to BSA. The maximum wavelength of BSA shifted from 348 to 342 nm after the addition of C.I. Mordant Red 3. Under the same condition, no fluorescence of C.I. Mordant Red 3 was observed. Therefore, the observed spectral shift due to the polarities of the protein environments is less than the polarity of the bulk aqueous phase since similar blue shift is observed in less polar solvents [19].

In order to confirm the quenching mechanism, the fluorescence quenching data are analyzed by the Stern–Volmer equation [20]:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the steady-state fluorescence intensities in the absence and presence of quencher, respectively. k_q is the quenching rate constant, τ_0 is the average lifetime of the biomolecule without quencher ($\tau_0 = 10^{-8}$ s [21]), K_{SV} is the Stern–Volmer quenching constant and [Q] is the concentration of quencher. Fig. 3 displays the Stern–Volmer plots of the quenching of BSA fluorescence by C.I. Mordant Red 3 at different temperatures. The plot shows that within the investigated concentrations, the results agree with the

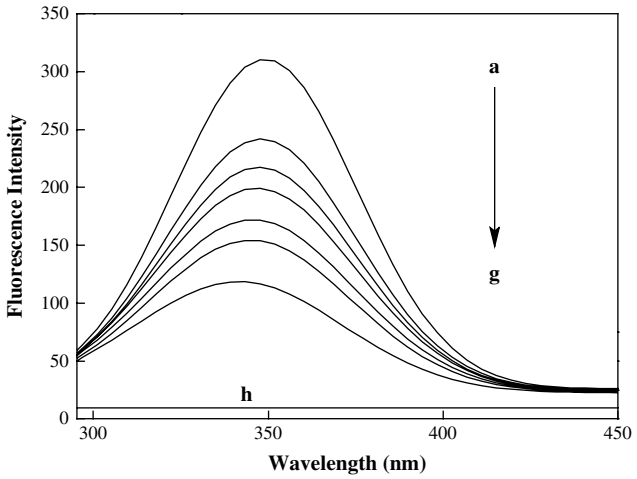

Fig. 2. The fluorescence spectra of C.I. Mordant Red 3–BSA system. The concentration of C.I. Mordant Red 3 corresponding to 0, 1.34, 2.68, 4.02, 5.36, 6.70, 9.38 $\times 10^{-7}$ M from (a) to (g); c(BSA) = 6.70×10^{-7} M; (h) 1.34×10^{-7} M C.I. Mordant Red 3. λ_{ex} = 280 nm; pH = 7.25; T = 297 K.

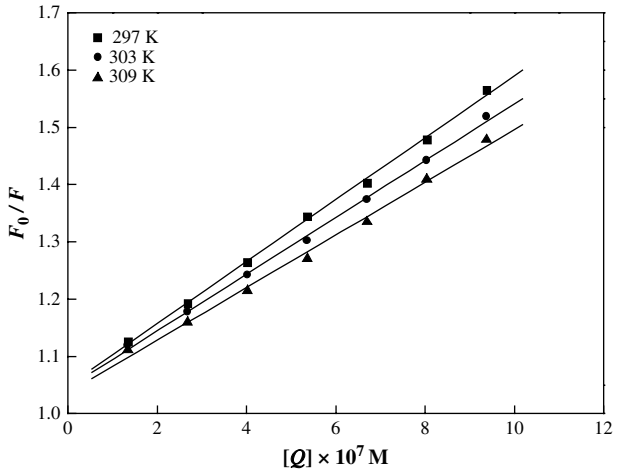

Fig. 3. Stern–Volmer plots for the C.I. Mordant Red 3–BSA system at three different temperatures. c(BSA) = 6.70 × 10^{-7} M; λ_{ex} = 280 nm; pH = 7.25.

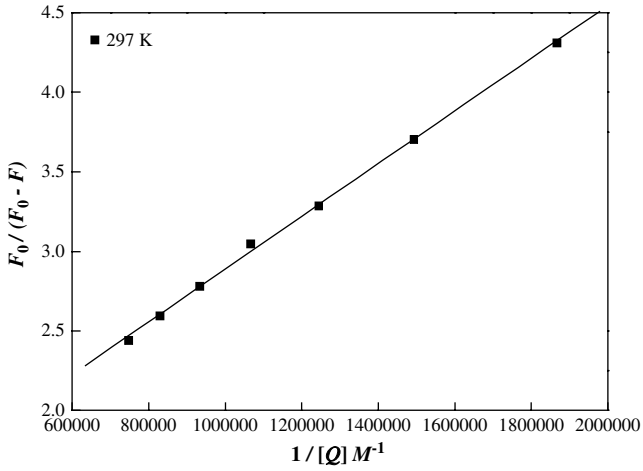

Fig. 4. Modified Stern–Volmer plots for the quenching of BSA by C.I. Mordant Red 3 at 297 K. c(BSA) = 6.70 × 10^{-7} M; λ_{ex} = 280 nm; pH = 7.25.

Stern–Volmer equation (1). Table 1 summarizes the calculated K_{SV} and k_q at each temperature studied. The results showed that the values of Stern–Volmer quenching constants K_{SV} and k_q decreased with increasing temperature and the values of k_q were much greater than 2.0 × 10^{10} M^{-1} s^{-1} [22], which indicated that the probable quenching mechanism of C.I. Mordant Red 3–BSA interaction was initiated by complex formation rather than by dynamic collision. In other words, the fluorescence quenching of BSA resulting from complex formation is predominant, while from dynamic collision could be negligible.

The fluorescence data obtained at room temperature (297 K) was further examined using modified Stern–Volmer equation [23]:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{K_a f_a [Q]} \quad (2)$$

where f_a is the fraction of the initial fluorescence which is accessible to quencher and K_a is the effective quenching constant for the accessible fluorophore. Fig. 4 displays the modified Stern–Volmer plot of $F_0/(F_0 - F)$ versus $1/[Q]$, the value of f_a was found to be 1.23 indicating that 81.16% of the total fluorescence of BSA is accessible to quencher [16].

UV–vis absorption spectrum is a very simple method and applicable to explore the structural change and to know the complex formation [24]. In the present study, the UV–vis spectrum of BSA, C.I. Mordant Red 3, C.I. Mordant Red 3–BSA system in aqueous solution was measured (Fig. 5). The absorbance of C.I. Mordant Red 3 decreased with the addition of BSA, and a slight red shift of maximum peak position (from 257 to 260 nm) was observed. A reasonable explanation for the two evidences may come from the complex formation between C.I. Mordant Red 3 and BSA [25].

3.2. Analysis of binding equilibria

When small molecules bind independently to a set of equivalent sites on a macromolecule, the binding constant (K_b) and the numbers of binding sites (n) can be determined by the following equation [26]:

$$\log \frac{F_0 - F}{F} = \log K_b + n \log[Q] \quad (3)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, [Q] is the quenching concentration. Fig. 6 shows the plots of $\log[(F_0 - F)/F]$ versus $\log[Q]$ for the C.I. Mordant Red 3–BSA system at different temperatures, the calculated results are presented in Table 2. As can be seen from Table 2 the binding constants K_b decreased with the temperature, which may indicate forming an unstable compound. The unstable compound would be partly decomposed with the rising temperature, therefore, the values of K_b decreased. The values of n at the experimental temperatures were approximately equal to 1, which indicated that there was one class of binding sites to C.I. Mordant Red 3 in BSA. In BSA, the tryptophan residues involved in binding could be either Trp-134 or Trp-212. Trp-134 was embedded in the first sub-domain IB and was more exposed to a hydrophilic environment, whereas Trp-212 was embedded in sub-domain IIA and deeply buried in the hydrophobic loop [27]. So, from the value of n, it may be inferred that C.I. Mordant Red 3 most likely binds to the hydrophobic pocket located in sub-domain IIA [28].

Table 1
Stern–Volmer quenching constants for the interaction of C.I. Mordant Red 3 with BSA at three different temperatures.

T (K)	K_{SV} (×10^{-5} M^{-1})	k_q (×10^{-13} M^{-1} s^{-1})	R[a]	SD[b]
297	5.408	5.408	0.9992	0.0068
303	4.952	4.952	0.9991	0.0069
309	4.593	4.593	0.9973	0.011

[a] R is the correlation coefficient.
[b] SD is the standard deviation for the K_{SV} values.

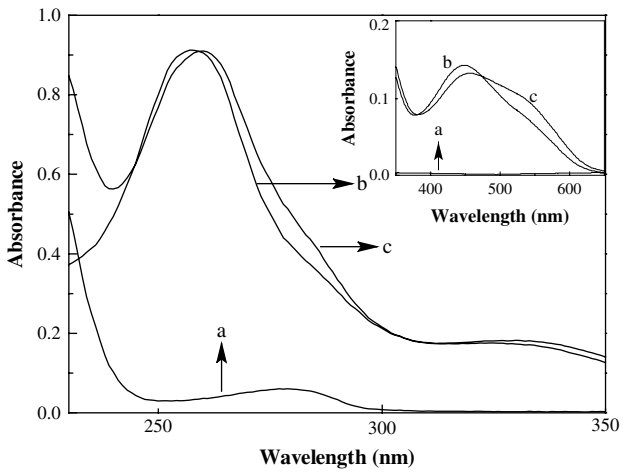

Fig. 5. The UV–vis absorption spectra of C.I. Mordant Red 3, BSA and C.I. Mordant Red 3–BSA system. (a) The absorption spectrum of BSA only; (b) the absorption spectrum of C.I. Mordant Red 3 only; (c) the absorption spectra of C.I. Mordant Red 3–BSA; c(C.I. Mordant Red 3) = 3.0 × 10^{-5} M, c(BSA) = 1.34 × 10^{-6} M; pH = 7.25; T = 297 K. The inset shows the UV–vis spectrum ranging from 350 to 650 nm.

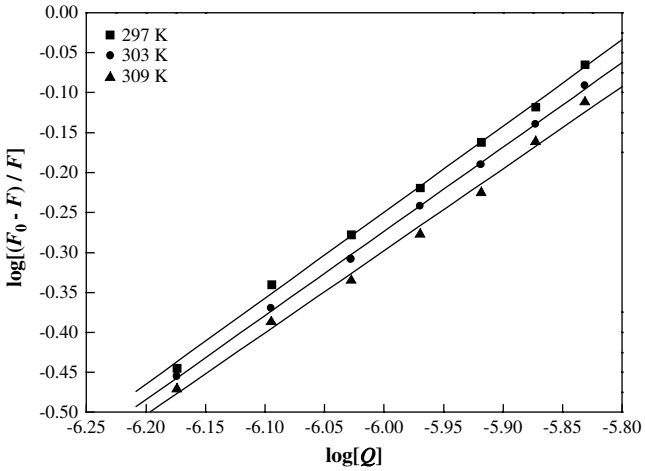

Fig. 6. The plots of $\log[(F_0 - F)/F]$ versus $\log[Q]$. c(BSA) = 6.70×10^{-7} M; λ_{ex} = 280 nm; pH = 7.25.

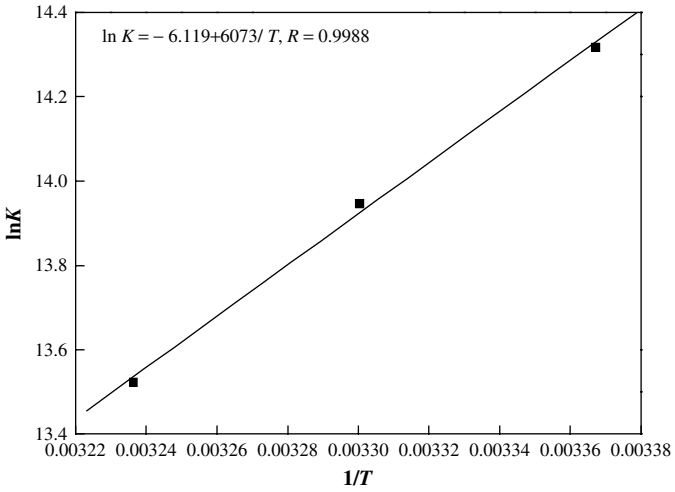

Fig. 7. van't Hoff plot for the interaction of BSA and C.I. Mordant Red 3 in Tris buffer. pH = 7.25.

3.3. Binding mode

There are essentially four types of non-covalent interactions that could play a role in ligand binding to proteins. There are hydrogen bonds, van der Waals interaction, electrostatic and hydrophobic interactions [29]. The thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) of reaction are important for confirming the acting force. For this reason, the temperature dependence of the binding constant was studied. The temperatures chosen were 297, 303 and 309 K so that BSA does not undergo any structural degradation. The thermodynamic parameters can be determined from the van't Hoff equation:

$$\ln K = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \tag{4}$$

where K is the binding constant at temperature T and R is the gas constant. The values of ΔH and ΔS were obtained from linear van't Hoff plot (Fig. 7) and are presented in Table 2. The value of ΔG is calculated from the relation

$$\Delta G = \Delta H - T\Delta S \tag{5}$$

From Table 2, it can be seen that ΔH and ΔS for the binding reaction between C.I. Mordant Red 3 and BSA are found to be -50.49 kJ mol^{-1} and -50.88 J mol^{-1} K^{-1}, which indicated that the binding processes were an exothermic reaction. The negative sign for ΔG means that the spontaneity of the binding of C.I. Mordant Red 3 with BSA. Ross and Subramanian [30] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes. From the point of view of water structure, a positive ΔS value is frequently taken as typical evidence for hydrophobic interaction, because the water molecules that are arranged in an orderly fashion around the ligand and protein acquire a more random configuration as a result of hydrophobic reaction. Further, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS and a negative ΔH value, while negative ΔS and ΔH arise from van der Waals forces and hydrogen bond formation. From the negative values of ΔH and ΔS observed in the present study, it can be concluded that the acting forces are mainly hydrogen bonding and van der Waals forces.

Table 2
Binding parameters and thermodynamic parameters of C.I. Mordant Red 3–BSA.

T (K)	K_b ($\times 10^{-6}$ M^{-1})	n	R[a]	SD[b]	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
297	1.653	1.08	0.9989	0.0069	−50.49	−35.36	−50.88
303	1.141	1.06	0.9996	0.0039		−35.14	
309	0.7468	1.03	0.9969	0.011		−34.74	

[a] R is the correlation coefficient.
[b] SD is the standard deviation for the K_b values.

3.4. Energy transfer between C.I. Mordant Red 3 and BSA

According to the Förster non-radiative resonance energy transfer theory [31], the rate of energy transfer depends on (i) the relative orientation of the donor and the 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. Here the donor and acceptor were BSA and C.I. Mordant Red 3, respectively. The absorption spectrum of C.I. Mordant Red 3 was recorded in the range of 300–500 nm in the pH 7.25 Tris buffer. The emission spectrum of BSA was also recorded under the same condition. There was a spectral overlap between the fluorescence emission spectrum of BSA and UV–vis absorption spectrum of C.I. Mordant Red 3 (Fig. 8). The efficiency of energy transfer between the donor and acceptor, E, could be calculated using the equation:

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

where F_0 and F are the fluorescence intensities without and with C.I. Mordant Red 3, respectively. r is the distance between acceptor and donor, and R_0 is the critical distance when the transfer efficiency is 50%,

$$R_0^6 = 8.8 \times 10^{-25} k^2 n^{-4} \varphi J \tag{7}$$

where k^2 is the spatial orientation factor between the emission dipole of the donor and the absorption dipole of the acceptor, n is the average 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 receptor. J is given by

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{8}$$

where $F(\lambda)$ is the fluorescence intensity of the donor in the wavelength range λ to $\lambda + \Delta\lambda$, $\varepsilon(\lambda)$ is the molar absorption coefficient of

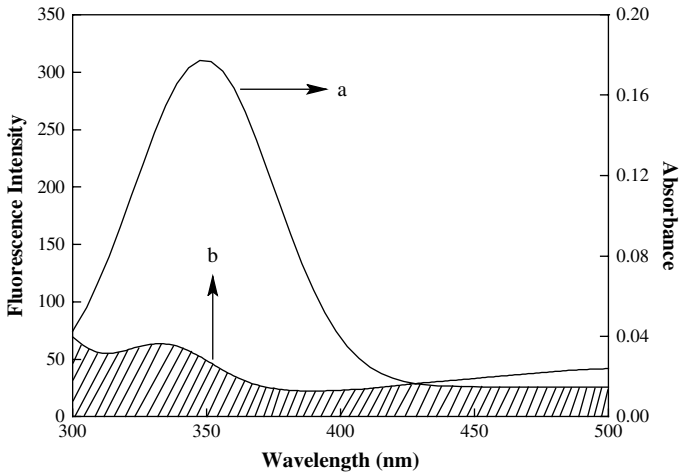

Fig. 8. Spectral overlap between the fluorescence emission spectrum of BSA (a) and UV–vis absorption spectrum of C.I. Mordant Red 3 (b). c(C.I. Mordant Red 3) = c(BSA) = 6.70×10^{-7} M; pH = 7.25; T = 297 K.

the acceptor at λ. In the present case, $k^2 = 2/3$, $n = 1.336$, $\varphi = 0.15$ [32]. According to Eqs. (6)–(8), the values of the parameters were found to be $J = 5.552 \times 10^{-14}$ cm^3 L M^{-1}, $R_0 = 3.39$ nm, $E = 0.759$ and $r = 2.79$ nm. The donor-to-acceptor distance r is less than 8 nm [33], which implied that the energy transfer from BSA to C.I. Mordant Red 3 occurred with high probability. This accorded with conditions of Förster non-radiative resonance energy transfer theory and indicated again a static quenching between C.I. Mordant Red 3 and BSA.

3.5. Conformation investigation

Synchronous fluorescence spectroscopy introduced by Lloyd and Evett [34] has been used to characterize complex mixtures as it can provide fingerprints of complex samples [35]. It gives information about the molecular environment in the vicinity of the chromospheres molecules and has several advantages, such as sensitivity, spectral simplification and avoiding different perturbing effects [25]. Yuan et al. [36] suggested a useful method to study the environment of amino acid residues by measuring the possible shift in wavelength emission maximum λ_{max}, the shift in position of emission maximum corresponding to the changes of the polarity around the chromospheres molecule. According to the theory of Miller [37], when $\Delta\lambda$ between excitation and emission wavelength is stabilized at 15 nm or 60 nm, the spectrum characteristic of the protein tyrosine or tryptophan residues was observed. The effect of C.I. Mordant Red 3 on the synchronous fluorescence spectrum of BSA is shown in Fig. 9. It can be seen from Fig. 9 that the maximum emission wavelength of tyrosine and tryptophan has a slight blue shift (tyrosine: from 322 to 320 nm; tryptophan: from 361 to 358 nm), which indicated that the conformation of BSA was changed and the polarity around the tyrosine or tryptophan residues was decreased [38,39]. Moreover, the fluorescence intensity decreased regularly with the addition of C.I. Mordant Red 3, which further demonstrated the occurrence of fluorescence quenching in the binding process. It has been also shown in Fig. 10 that the slope was higher when $\Delta\lambda$ was 60 nm indicating that a significant contribution of tryptophan residues in the fluorescence of BSA, C.I. Mordant Red 3 was closer to tryptophan residues compared to tyrosine residues [14].

3.6. Further considerations

In multimeric protein, the individual polypeptide chains are termed protomers or subunits (monomer). Proteins composed of two subunits are termed dimeric proteins. Hydrogen bonds and electrostatic bonds formed between surface residues of adjacent subunits stabilize the association of subunits [40]. There is evidence that BSA can form non-covalent dimers [41,42]. In this study, the negative ΔH and ΔS values indicate that van der Waals force and

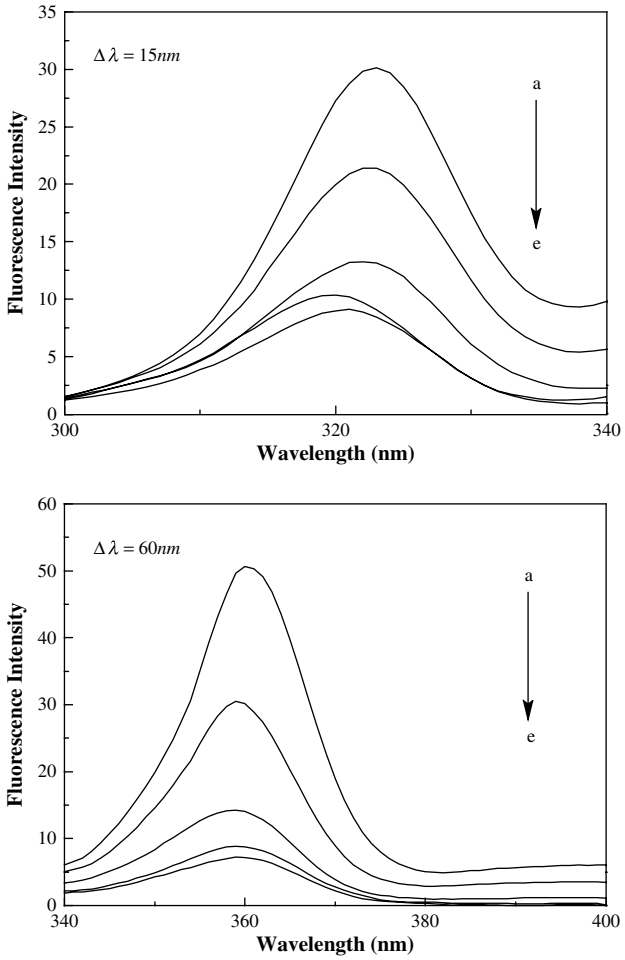

Fig. 9. Synchronous fluorescence spectrum of BSA. From (a) to (e): c(BSA) = 6.70×10^{-7} M, c(C.I. Mordant Red 3) = 0, 4.02, 9.38, 14.74, 20.01 $\times 10^{-7}$ M; pH = 7.25; T = 297 K.

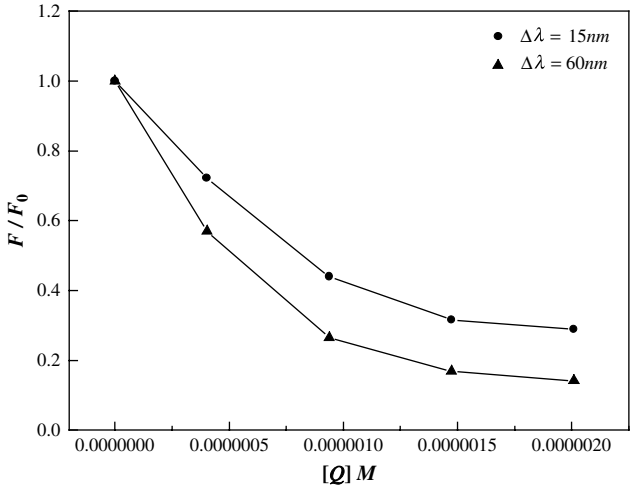

Fig. 10. The quenching of BSA synchronous fluorescence by C.I. Mordant Red 3. c(BSA) = 6.70×10^{-7} M; pH = 7.25; T = 297 K.

hydrogen bond played major roles in the C.I. Mordant Red 3–BSA binding reaction and contributed to the stability of the complex. So, it can be concluded that C.I. Mordant Red 3 affects the monomer–dimer equilibrium of the protein. This result is a novelty in the field of BSA binding studies and should be taken into account because they affect binding results.

4. Conclusions

In this paper, the interaction between C.I. Mordant Red 3 and BSA was studied by fluorescence spectroscopy combined with UV–vis absorption spectrum under simulative physiological conditions. Fluorescence experiment results revealed that the intrinsic fluorescence of BSA was quenched through static quenching process. The enthalpy change (ΔH) and entropy change (ΔS) for the reaction were calculated to be -50.49 kJ mol^{-1} and -50.88 J mol^{-1} K^{-1}, which indicated that van der Waals force and hydrogen bond played major roles in stabilizing the complex. The distance between C.I. Mordant Red 3 and BSA was close enough ($r = 2.79$ nm) to arose non-radiative energy transfer from BSA to C.I. Mordant Red 3. In the conformational investigation, the synchronous fluorescence spectrum revealed that the conformation and microenvironment of BSA molecular were changed in the presence of C.I. Mordant Red 3. The binding study of C.I. Mordant Red 3 with BSA has toxicological importance. This report is expected to provide important insight into the interaction mechanism of BSA with cancerogenic anthraquinone dyes, which may be a useful guideline for further toxicology investigation.

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