Trade Science Inc.

December 2009 Volume 8 Issue 4

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 8(4) 2009 [621-626]

Study on the interaction between bilirubin and human serum albumin by fluorescence spectroscopy

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Received: 5th August, 2009 ; Accepted: 15th August, 2009

ABSTRACT

The interactions between bilirubin and human serum albumin (HSA) have been studied by fluorescence spectroscopy. The fluorescence quenching spectrum was studied at different temperature by Stern-Volmer and Lineweaver-Burk equations. The main binding force of bilirubin to HSA was judged according to the thermodynamic parameters. The binding distance between bilirubin and protein was also obtained. Furthermore, the binding constant was measured by fluorescence quenching and enhancement spectrum, respectively. The observed spectral results revealed that there were strong interactions between bilirubin and protein, the mechanism of quenching belonged to static quenching and the main binding force was van der Waals interactions and hydrogen bonds.

KEYWORDS

Human serum albumin;
Bilirubin;
Fluorescence spectrum;
Binding constant;
Energy transfer.

INTRODUCTION

As the major soluble protein constituents of the circulatory system, serum albumins have many physiological functions. They affect pharmacokinetics of many drugs and account for most of the antioxidant capacity of serum. They often increase the apparent solubility of hydrophobic drugs in plasma and modulate their delivery to cells in vivo and in vitro[1]. They play a dominant role in the transport and deposition of endogenous and exogenous compounds. They bind different classes of ligands at multiple sites, and the principal regions of ligand binding sites of albumin are located in hydrophobic cavities in subdomains IIA and IIIA[1,2].

Among various endogenous ligands that bind to serum albumin, more important are bilirubin (BR) which is well known because of its toxicity under conditions of hyperbilirubinemia[3]. The development of hyperbilirubinemia is associated with the risk of neurological dysfunctions due to deposition of bilirubin in brain and its enhanced toxic effects on cellular functions in this tissue[4]. Studies on the binding of BR to albumin become important for the development of various preventive measures against kernicterus[5].

Otherwise, bilirubin is an efficient antioxidant against free radical species such as superoxide anion and peroxide radicals which induced oxidation in vitro[6,7]. It is the basis of Bezoar which is contained in various traditional Chinese medicine. The investigation of albumin–drug binding can provide information of the structural features which determine the therapeutic effectivity of drugs, and is of critical for understanding the drug toxicity and its distribution in the organism[8].

At present, there have been some reports that in-

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vestigate the binding of proteins to ligands by various techniques[9-15]. It has been known that bilirubin binds tightly to the primary binding site (at or near loop 4 in subdomain IIA) in serum albumin[1,16]. The binding of BR to albumin is stabilized by various non-covalent forces such as salt linkages, hydrogen bonds, van der Waals attractions and hydrophobic interactions[17].

In this paper, the binding reactions of bilirubin and HSA were studied in detail by the technique of fluorescence. The binding constant, binding mode, binding distant as well as the quenching mode for the first binding site between HSA and BR was measured by fluorescence quenching method. The binding constant was also obtained by fluorescence enhancement method.

EXPERIMENTAL

Apparatus

The fluorescence spectrum and the intensity of fluorescence were measured with a Shimadzu RF-540 spectrofluorometer (Kyoto, Japan). The absorption spectra were performed on a Perkin-Elmer lambda 17 UV/VIS spectrophotometer (P-E Co., America). A WH-2 vortex mixer (Huxi Instrumental Co., China) was used to blend the solution.

Reagents

All reagents for synthesis were of analytical-reagent grade. Bilirubin stock solution was prepared in ultrasonic bath by dissolving power (Fluka Chemika) in a small quantity of 5.0 mmol L^{-1} NaOH solution, and was ultimately diluted to 0.2 mmol L^{-1}. The stock solution of HSA was prepared by dissolving commercially purchased HSA (Sigma) in doubly distilled water at 0-4°C. The working solution of human serum albumin was 1.0 g L^{-1}. Tris (hydroxymethyl) aminomethane -HCl solution (pH 7.0) was used to control the acidity. Doubly distilled water was used throughout.

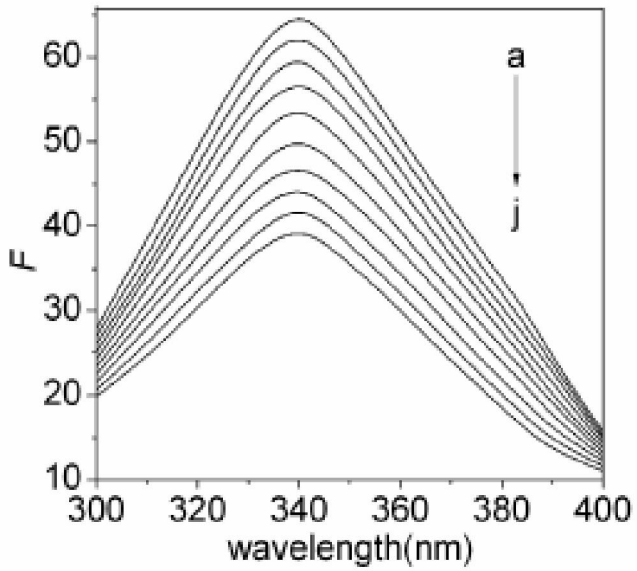
Fluorescence spectrum

Appropriate working solution of human serum albumin and bilirubin solution were added to a 25 mL volumetric flask. The mixture was diluted to 12.5 mL with doubly distilled water and vortexes. The fluorescence quenching spectra were measured by exciting BR-HSA at 288 nm, and scanning through the wavelength range 300-400 nm and the maximum wavelength was at 340 nm. The emission spectra of the fluorescence enhancement titration were recorded in the wavelength range 500-600 nm by exciting the BR-albumin complex at 446 nm. The excitation and emission slits were 5 and 10 nm, respectively.

RESULTS AND DISCUSSION

Fluorescence quenching studies

The fluorescence spectra of HSA and its decreased fluorescence intensity quenching by BR were shown in Figure 1. The maximum excitation and emission wavelengths were at 288 and 340 nm, respectively. In the present of BR, the fluorescence intensity of HSA could be quenched. As the concentrations of bilirubin increased, the quenching degree increased. This result suggested that the HSA conformation was changed by BR, HSA and bilirubin might form compound, and intermolecular energy transfer occurred between BR and HSA.



C_{HSA}: 3.00 μmol L^{-1}, C_{BR}: (a → j): 0.000, 0.300, 0.600, 0.900, 1.20, 1.50, 1.80, 2.10, 2.40, 3.00 μmol L^{-1}

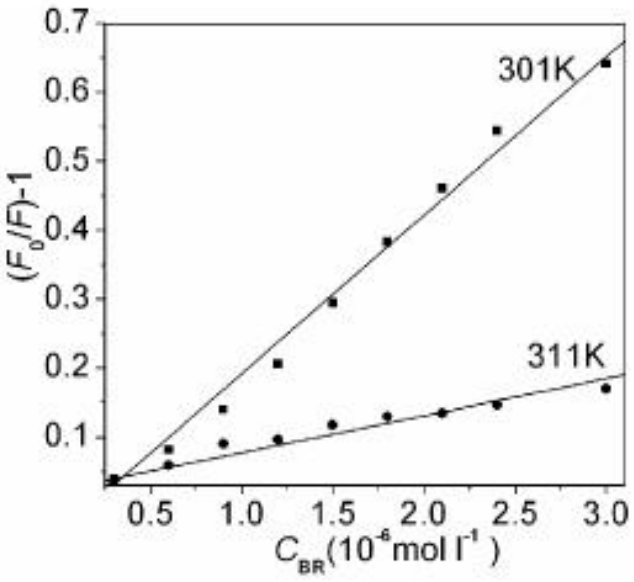
Figure 1 : Fluorescence spectra of HSA with different concentration of BR.

Fluorescence quenching includes dynamic and static quenching. They were differentiated by the fluorescence results at different temperatures. In order to judge the type of quenching, the procedure was firstly assumed

to be dynamic quenching[18] which can be described by the Stern-Volmer equation:

$$F_0/F = 1 + K_q \tau_0 [Q] = 1 + K_{sv}[Q] \quad (1)$$

where, F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively. τ_0 is the average lifetime of molecule without quencher and K_q is quenching rate constant of bimolecule. K_{sv} is the Stern-Volmer quenching constant, [Q] is the concentration of the quencher. The Stern-Volmer graph was shown in Figure 2, under the condition of various temperatures, and K_q, K_{SV} were shown in TABLE 1.



C_{HSA}: 3.00 μmol L^{-1}

Figure 2 : Stern-Volmer curves.

TABLE 1 : Binding parameters of HSA with BR at different temperature

Protein	T /K	K_{sv} /(L mol^{-1})	K_q /(L mol^{-1} s^{-1})	K /(L mol^{-1})
HSA	301	2.30×10^{5}	2.30×10^{13}	1.28×10^{6}
HSA	311	5.38×10^{4}	5.38×10^{12}	1.52×10^{6}

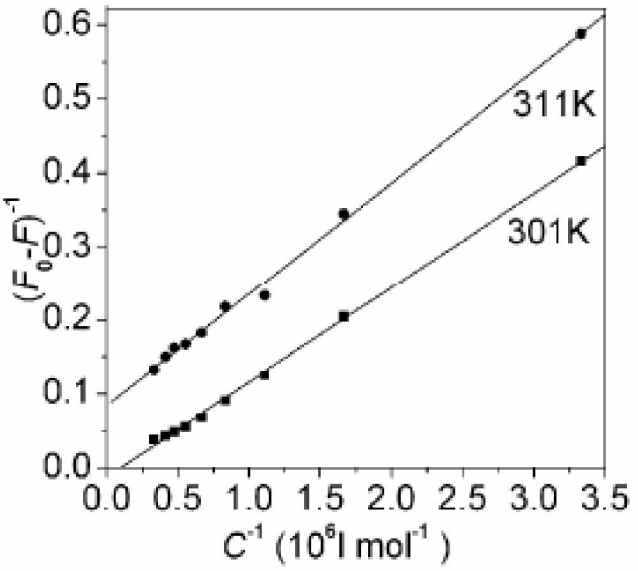
As shown in Figure 2, the term, F_0/F, linearly increased with increasing the concentration of quencher. The quenching efficient of HSA fluorescence by BR underwent an intense decrease with increasing temperature. This indicated the static quenching interaction between BR and HSA, because the quenching rate constants decrease with increasing temperature for the static quenching and the reversed effect is observed for the dynamic quenching[18]. Since the fluorescence lifetime of biomacromolecule is 10^{-8} s, the quenching rate constant can be obtained by the slope ($K_q = K_{sv}/\tau_0$). Maximum scatter collision quenching constant of quencher to biomacromolecule is 2.0×10^{10} L mol^{-1} s^{-1}. Obviously, the rate constant of protein quenching procedure initiated by BR was greater than *K*q of scatter procedure. It suggested that the static quenching was attributable to the formation of a non-fluorescence ground state complex between BR and HSA.

The static quenching follows the Lineweaver-Burk relationship:

$$(F_0-F)^{-1} = F_0^{-1} + K^{-1}F_0^{-1}[Q]^{-1} \quad (2)$$

where F_0, F and [Q] are the same as formerly. K is formation constant.

Figure 3 and the formation constant K (shown in TABLE 1) showed that there was a strong interaction between BR and HSA. The interaction was weakened when the temperature rose.



C_{HSA}: 3.00 μmol L^{-1}

Figure 3 : Lineweaver-Burk curves.

Thermodynamic analysis

According to Ross' view, the main sorts of binding force can be judged by thermodynamic parameters (ΔH and ΔS). From the thermodynamic standpoint, $\Delta H > 0$ and $\Delta S > 0$ implies a hydrophobic interaction, $\Delta H < 0$ and $\Delta S < 0$ reflects the van der Waals force or hydrogen bond formation, with $\Delta H < 0$, and $\Delta S > 0$ suggesting an electrostatic force[19,20]. If the temperature changes little, the reaction enthalpy change (ΔH) is regarded as

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a constant. Therefore, from the following equations:

$$\ln (k_2/k_1) = (1/T_1 - 1/T_2)\Delta H/R \quad (3)$$

$$\Delta G = -RT \ln K \quad (4)$$

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

ΔH, ΔG and ΔS which are enthalpy change, free energy change and entropy change, respectively, and can be obtained. In the binding reaction between BR and HSA, ΔH, ΔG and ΔS were calculated to be -113 KJ mol^{-1}, -30.9 KJ mol^{-1} and -273 J mol^{-1} K^{-1}. The negative values of free energy (ΔG) supported the assertion that the binding process was spontaneous. The negative enthalpy (ΔH) and entropy (ΔS) values of the interaction of BR and HSA indicated that the binding was mainly enthalpy-driven and the entropy was unfavorable for it. It suggested that van der Waals interactions and hydrogen bonds played major role in the reaction[20], which consistent with Jacobsen J. *et al*.'s earlier report[17].

The energy transfer between BR and HSA

According to the theory of Förster non-radiative energy transfer[21,22], a transfer of energy could take place through direct electro-dynamic interaction between the primarily excited molecule and its neighbors and the energy transfer will happen under the following conditions: (i) the donor can produce fluorescence light (ii) fluorescence emission spectrum of the donor and UV absorption spectrum of the acceptor have more overlap and (iii) the distance between the donor and the acceptor is lower than 8 nm. The energy transfer effect is related not only to the distance between the acceptor and the donor, but also to the critical energy transfer distance. The relation between these factors is:

$$E = \frac{R_0^6}{R_0^6 + r_0^6} \quad (6)$$

where E is the energy transfer efficiency, R_0 is the critical distance when the transfer efficiency is 50%,

$$R_0^6 = 8.8\times10^{-25} K^2 N^{-4} \Phi J \quad (7)$$

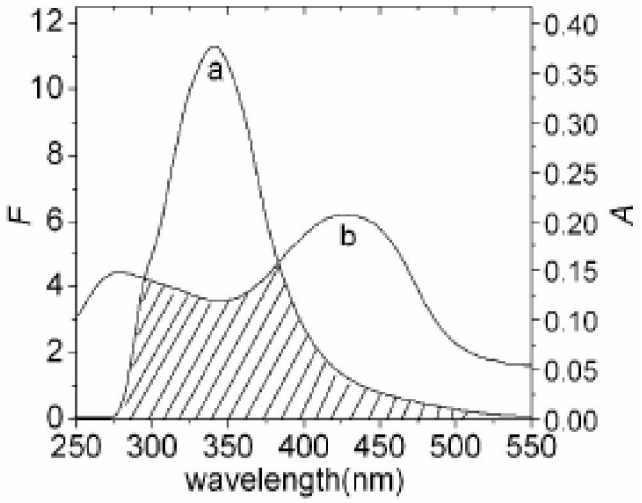
In Eq. (7), K^2 is the spatial orientation factor of the dipole, N is the index of refraction of the medium, Φ is the fluorescence quantum yield of the donor, J is the overlap integral of the fluorescence emission spectra of the donor and the absorption spectra of the acceptor. Therefore:

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

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor of wavelength λ, then the energy transfer efficiency is:

$$E = 1 - \frac{F}{F_0} \quad (9)$$

The overlapping spectra of the absorption spectra of BR and the fluorescence spectra of HSA (BR: HSA =1:1) were shown in Figure 4. So J can be evaluated by integrating the spectra in Figure 4 for λ= 250~550 nm. Under these experimental conditions, we found R_0 = 5.04 nm from Eq. (7) using K^2 = 2/3, N = 1.336, Φ = 0.15[23]. At last, the distance between BR and the amino-acid residue in HSA can be evaluated from Eq. (6) where r = 6.42 nm. The average distance r < 8 nm, indicated that the energy transfer from HSA to BR occurred with high probability.



$C_{HSA} = C_{BR} = 9.60$ μmol L^{-1}

Figure 4 : Overlap of the fluorescence emission spectra of HAS (a) with the absorption spectra (b) of BR.

Analysis of binding equilibrium

The composition of the binary complex can be deduced from the following formula[24,25].

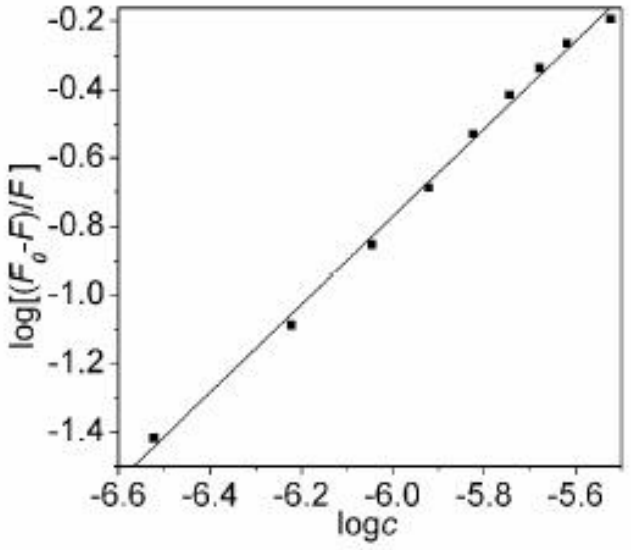
$$M + nL = MLn \quad (10)$$

$$\log [(F_0 - F)/F] = \log Ka + n \log [M] \quad (11)$$

where M is the biological molecule with fluorophores, L is the quencher, MLn is the binary complex whose constant is Ka, F_0

is the fluorescence of the overall amount of biological molecules (bound and unbound), F is the fluorescence of unbound biological molecules. A plot of log $[(F_0-F)/F]$ versus log [M] will give straight line with a slope of n and y-axis intercept of log Ka.

Figure 5 was obtained by keeping the HSA concentration (3.00 μmol L^{-1}) constant and changing the concentration of BR. The data were well fitted to Eq. (5) and the slope was 1.28 and the calculated resultant binding constant was 8.57×10^6 L mol^{-1}. The coefficient was 0.998. The results also indicated that the HSA could form a stable 1: 1.28 complex with BR.



C_{HSA}: 3.00 μmol L^{-1}

Figure 5 : Estimation of the composition of BR-HSA complex.

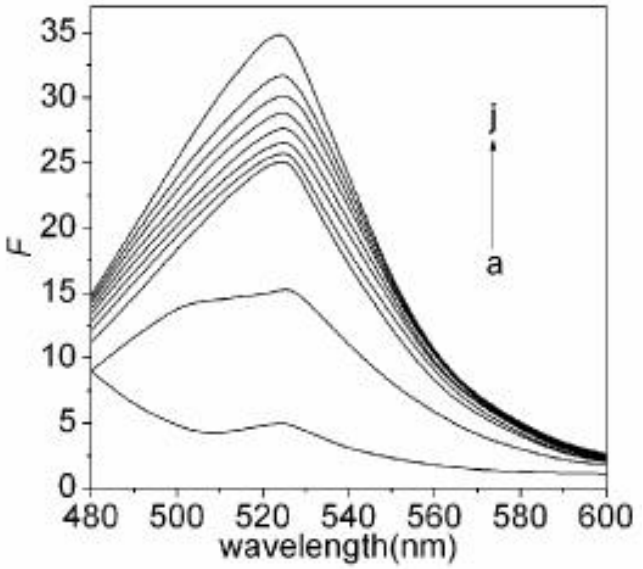
Fluorescence enhancement

Binding of BR to albumin was also studied by fluorescence enhancing spectroscopy. Fluorescence emission spectra were recorded in the wavelength range 500-600 nm by exciting the BR-albumin complex at 446 nm and the maximum emission wavelength was 525 nm. Increasing concentrations of BR were added to a fixed concentration of albumin (3.00 μmol L^{-1}), which produced an enhancement of fluorescence intensity. The BR/albumin molar ratios were obtained between 0 and 0.9. The data were analyzed by fitting them in Scatchard[26,27] plot using the following equation:

$$nK_A - K_AE = \frac{E}{[BR]} = \frac{E}{(R-E)[HSA]_T} \qquad (12)$$

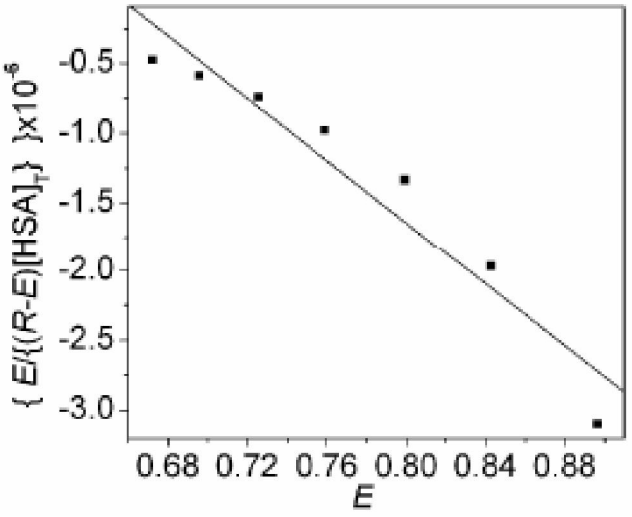
where n is the binding capacity, K_A is the association constant, E is the fractional enhancement, [BR] is the free BR concentration, $[HSA]_T$ is the total albumin concentration and R is the BR/albumin molar ratio. The value of K_A was obtained from the slope of a straight line plot between E/[BR] versus E.

The data of fluorescence enhancenment and the Scatchard plots were showed in Figure 6 and Figure 7, respectively. Obtained from the slope and intercept of the plots, the values of association constant and binding capacity for the first binding site were found to be 1.12×10^7 L mol^{-1} and 0.963, which agreed well with the results in fluorescence quenching experiments.



C_{HSA}: 3.00 μmol L^{-1}, C_{BR}: (a→j): 0.000, 0.300, 0.600, 0.900, 1.20, 1.50, 1.80, 2.10, 2.40, 3.00 μmol L^{-1}

Figure 6 : Fluorescence spectra of the BR-HSA complexes in the presence of different concentrations of BR.



C_{HSA}: 3.00 μmol L^{-1}

Figure 7 : Scatchard plots for the interaction of BR with HSA.

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CONCLUSIONS

The binding characteristic of BR and HSA was identified by measuring the fluorescence spectra. The results showed that HSA fluorescence was quenched by BR through static quenching mechanism. The distance r was noticed to be 6.42 nm, which was calculated in the present study between the bound BR and the tryptophan residues in HSA. The thermodynamic results indicated that the BR interacted with HSA through van der Waals interactions and hydrogen bonds. It was found that the association and binding constant were 1.12×10^{7} L mol^{-1} and 8.57×10^{6} L mol^{-1} by fluorescence enhancement and quenching method, respectively.

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