# Study of the Interaction of Indirubin with Bovine Serum Albumin

Hedong BIAN, Mei LI, Qing YU, Zhenfeng CHEN, Jianniao TIAN, and Hong LIANG*

*College of Chemistry and Chemical Engineering, Guangxi Normal University; Guilin, Guangxi 541004, People's Republic of China.* Received December 19, 2005; accepted March 8, 2006

**This study examined the interaction of indirubin with bovine serum albumin (BSA) at three temperatures (286, 297, 308 K) at pH 7.40. In the presence of indirubin, the drug–BSA binding mode, binding constant and the protein structure changes in aqueous solution were determined by fluorescence quenching methods including Fourier transform infrared (FT-IR) spectroscopy and UV–Vis spectroscopy. The FT-IR change indicates that indirubin binds to BSA. The change in protein secondary structure accompanying ligand binding has been proved by fluorescence spectra data. The thermodynamic parameters, the enthalpy change ($\Delta H$), and the entropy change ($\Delta S$) calculated by the van't Hoff equation possess small negative ($-2.744\ \mathrm{kJ\cdot mol^{-1}}$) and positive values ($112.756\ \mathrm{J\cdot mol^{-1}\cdot K^{-1}}$), respectively, which indicated that hydrophobic interactions play the main role in the binding of indirubin to BSA. Furthermore, the displacement experiment shows that indirubin can bind to the subdomain IIA and the distance between the tryptophan residues in BSA and indirubin bound to site I was estimated to be 2.24 nm according to Föster's equation on the basis of fluorescence energy transfer.**

**Key words** indirubin; bovine serum albumin (BSA); binding; fluorescence quenching; UV–Vis spectra; FT-IR spectra

Indirubin (2*H*-indol-2-one, 3-(1,3-dihydro-3-oxo-2*H*-indol-2-ylidene)-1,3-dilydro) constitutes the main active ingredient of a traditional Chinese medicinal recipe, Danggui Longhui Wan, used to treat various diseases including chronic myelocytic leukemia.[1,2] Indirubin is an inhibitor of cyclin dependent kinases and glycogen synthase kinase.[3—5] Indirubin has been recently discovered to be a potent ligand of the aryl hydrocarbon receptor (AhR), also known as the "dioxin receptor".[6] Recent studies also showed that indirubin inhibits 2,4,6-trinitro-1-chlorobenzene (TNBC)-induced inflammatory reactions in mice.[7]

Bovine serum albumin (BSA) is a single-chain 582 amino acid globular nonglycoprotein cross-linked with 17 cystine residues (8 disulfide bonds and 1 free thiol). As one of the most abundant proteins, BSA plays an important role in the transport and deposition of a variety of endogenous and exogenous ligands in blood. BSA is divided into three linearly arranged, structurally distinct, and evolutionarily related domains (I—III)[8—11]; each domain is composed of two subdomains (A, B). BSA has two tryptophans embedded in two different domains, one is Trp-134, located in the proximity of the protein surface, but buried in a hydrophobic pocket of domain I, the other is Trp-214, located in an internal part of domain II.[12] BSA has a wide range of physiological functions involving the binding, transport, and delivery of fatty acids, bilirubin, porphyrins, thyroxine, tryptophan and steroids.[8—11] It is home to specific binding sites for metals, pharmaceuticals, and dyes. The binding ability on the interaction of drug with protein will significantly affect the apparent distribution volume of the drugs and also affect the elimination rate of drugs in most cases; so the studies on this aspect become an important research field in life sciences, chemistry and clinical medicine because it can provide important information on the structural features which determine the therapeutic effectiveness of drugs.

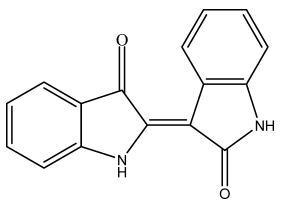


Chart 1. The Chemical Structure of Indirubin

Serial study methods concerning the interaction between drugs and protein include NMR,[13] CD,[14] ROD,[15] Raman[16] attenuated total reflectance-Fourier transform infrared (ATR-FTIR), UV–Vis absorbance and fluorescence spectroscopy. Fluorescence quenching techniques are great aids in the study of binding of drugs to plasma proteins and serum albumin in particular because of their high sensitivity, rapidity and ease of implementation. FT-IR spectroscopy has recently become very popular for the structural characterization of proteins. The most important advantage of FT-IR spectroscopy for biological studies is that the spectra of almost any biological system can be obtained in a wide variety of environments. For secondary-structure analysis of proteins, so far, many studies have been carried out to investigate the interaction of proteins with drugs. However, the information on the indirubin–BSA binding mode, the binding constant, and the effects of indirubin complexation on the protein secondary structure is obscure.[17]

In this work, we report the fluorescence, UV–Vis and FT-IR spectroscopic results on the binding of indirubin to BSA in aqueous solutions at physiological pH. Spectroscopic evidence regarding the drug binding mode, binding constant, and the change of protein secondary structure is described.

## Experimental

**Materials** BSA (molecular weight, 66210) was obtained from Sino-American Biotechnology Company and used without further purification. Indirubin was extracted from indigo without further purification. Indigo is first treated with 18% chlorhydric acid, then with ethyl acetate, dissolved in 95% ethanol, and finally precipitated with 0.1% NaOH. Indirubin stock solution was prepared in ethanol.[18] NaCl (analytical grade, 0.1 M) solution was used to keep the ion strength at 0.1 M Tris–HCl buffer (pH 7.40). A 16 $\mu$M solution of BSA was prepared in pH 7.40 Tris–HCl buffer solution. The pH was checked with a Sartorius PP-20 standardized pH meter (Germany). All other chemicals were of analytical reagent grade.

**Apparatus and Methods** Fluorescence emission spectra were measured with a RF-5310PC spectrofluorophotometer (Shimadzu), using slit widths of 10/10 nm, recorded at an excitation wavelength of 280 nm and an

* To whom correspondence should be addressed. e-mail: bianhd@mailbox.gxnu.edu.cn 

emission wavelength of 300—500 nm. UV–Vis absorbance spectra were measured with a Cary-100 UV-Visible spectrofluorophotometer (Varian). FI-IR measurements were carried out at room temperature on Perkin-Elmer FT-IR spectrometer (America) equipped with a germanium-attenuated total reflection (ATR) accessory, a DTGS KBr detector, and a KBr beam splitter. All spectra were taken *via* the attenuated total reflection (ATR) method with a resolution of 4 $cm^{-1}$ and 60 scans. Spectra processing procedures: spectra of buffer solution were collected under the same conditions. The absorbance of buffer solution from the spectra of sample solution was then subtracted to obtain the FT-IR spectra of the proteins. The subtraction criterion was that the original spectrum of the protein solution between 2200 and 1800 $cm^{-1}$ was featureless.[19)]

Fluorometric Titration Experiments: A 3.0 ml solution containing an appropriate concentration of BSA was titrated by successive additions of a $4.0\times10^{-5}$ M ethanol stock solution of indirubin to final concentrations of 0 to 1.6 $\mu$M. Titrations were done manually using trace syringes, and the fluorescence intensity was measured at an excitation of 280 nm and an emission of 344 nm. All experiments were measured at three temperatures (286, 297, 308 K). The temperature of samples was kept constant. The data thus obtained were analyzed by the Stern–Volmer equation to calculate the binding constants.

## Results and Discussion

Fluorescence quenching spectra of BSA in the absence and presence of indirubin in pH 7.40 Tris buffer were carried out. Tyrosine, a common amino acid in proteins shows high fluorescence intensity in pure solution, but it presents weak emission when part of a protein chain,[20)] especially at the wavelength actually used (290 nm). When excited around 280 nm, the emission spectra of intrinsic fluorescence is mainly due to the presence of trytophan residues. The intrinsic fluorescence intensity of BSA measured provides information on conformational changes of BSA before and after addition of indirubin. Figure 1 shows typical fluorescence emission spectra of BSA in a vicinity of the chromophore molecules. BSA has a strong fluorescence emission with a peak at 344 nm when excitated at 280 nm. Its fluorescence intensity decreases and the maximum emission wavelength around 344 nm had a slight blue shift when the solution of indirubin was added. The result suggested that the binding of indirubin is associated with changes in the local dielectric environment of at least one of the two indole rings in BSA, which indicates the chromophore was placed in a more hydrophobic environment after the addition of indirubin.[19)] The quantitative analysis of the binding of indirubin–BSA was carried out using the relative fluorescence quenching at 344 nm according to indirubin concentration, [*Q*], at various temperatures as shown in Fig. 2. When the titration was carried out at a low concentration of protein, indirubin was only partially bound. The plateau in each titration curve obtained indicates saturation of the BSA binding site.

Figure 3 shows the FT-IR spectra of the indirubin-free and indirubin-bound form of BSA with their different absorption spectra. The protein amide I in the region 1600—1700 $cm^{-1}$ (mainly C=O stretch) and amide II band ≈1548 $cm^{-1}$ (C–N stretch coupled with N–H bending mode) both have a relationship with the secondary structure of protein, and amide I band is more sensitive to the change of protein secondary structure than amide II.[21,22)] As shown in Fig. 3, the peak position of the amide I band moved from 1644.4 to 1654.3 $cm^{-1}$ and the amide II band moved from 1552.7 to 1583.6 $cm^{-1}$, which indicate that the protein secondary structure has been changed because of the interaction of indirubin

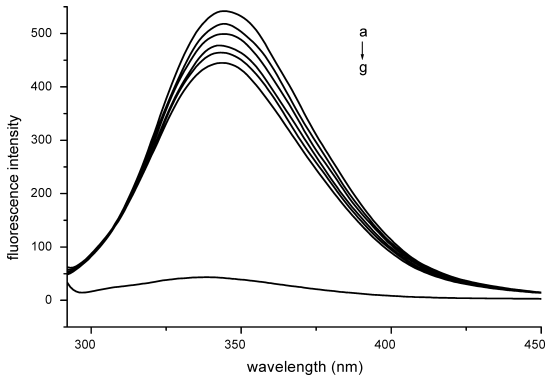


Fig. 1. Emission Spectra Excited at 280 nm (pH=7.40)

(a) 1.6 $\mu$M BSA; (b)—(g) 1.6 $\mu$M BSA in the presence of 0.12, 0.32, 0.48, 0.88, 1.6 $\mu$M indirubin, respectively; (g) 1.28 $\mu$M indirubin.

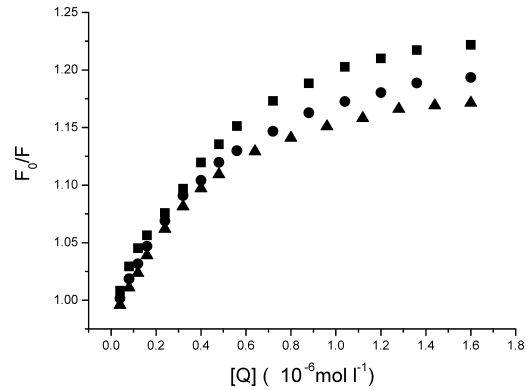


Fig. 2. Relative Fluorescence Intensity for the Indirubin–BSA Interaction Obtained by Titration with Indirubin

$C_{BSA}$=1.6 $\mu$M; pH 7.40; $\lambda_{ex}$=280 nm, $\lambda_{em}$=344 nm at 286 K (■); 297 K (●); 308 K (▲).

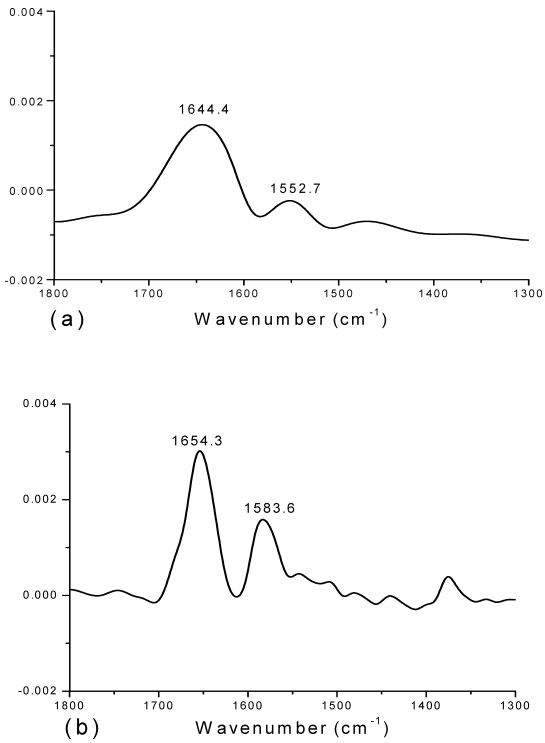


Fig. 3. FT-IR Spectra and Difference Spectra [(BSA Solution+Indirubin Solution)−(Indirubin Solution)] of Free BSA (a) and Its Indirubin Complexes (b) in Buffer Solution in the Region of 1800—1300 $cm^{-1}$

[indirubin]=2 $\mu$M, [BSA]=30 $\mu$M.

Table 1. Thermodynamic Parameters of Indirubin–BSA Interaction at pH 7.40

| Temperature (K) | Binding parameters ($10^6$ M$^{-1}$) | $f^{-1}$ | $\Delta G^0$ (kJ mol$^{-1}$) | $\Delta H^0$ (kJ mol$^{-1}$) | $\Delta S^0$ (kJ mol$^{-1}$ K$^{-1}$) |
|---|---|---|---|---|---|
| 286 | 2.47 | 1.1 | −35.001 | | |
| 297 | 2.34 | 1.2 | −36.216 | 2.744 | 112.756 |
| 308 | 2.28 | 1.3 | −37.482 | | |

with BSA.

**Binding Parameters** Quenching data were also analyzed according to the Stern–Volmer equation[23]:

$$\frac{RF_0}{\Delta RF}=\frac{1}{[Q]}\frac{1}{fK}+\frac{1}{f}$$

where $[Q]$ is the concentration of quencher (indirubin), $RF_0$ and $\Delta RF$ are the relative fluorescence intensities of protein in the absence and presence of quencher, respectively; $f$ is the fractional maximum fluorescence intensity of the protein summed up, and $K$ is a constant. The dependence of $RF_0/\Delta RF$ on the value of $1/[Q]$ is linear with a slope equal to the value of $(fK)^{-1}$. The value $1/f$ is fixed on the ordinate. The association constant $K$ is a quotient of an ordinate $1/f$ and the slope of $(fK)^{-1}$.

Figure 4 shows the Stern–Volmer plot for the indirubin–BSA system under various temperatures. The binding constant $K$ calculated according to the Stern–Volmer equation is shown in Table 1. The intercept on the $y$-axis ($f^{-1}$) is 1.1, 1.2, 1.3 varying with the temperatures, indicating that almost the total fluorescence of BSA is accessible to the quencher. All of the tryptophan residues are accessible to the quencher if $f=1$. In Table 1, it can be seen that the quenching constants decrease with the increasing temperature, which suggests that the quenching mechanism of indirubin to BSA is a static type. Static quenching arises from the formation of a dark complex between the fluorophore and the quencher, so the association constant or binding constant of the complexation reaction can be employed for interpreting the quenching constant.[24]

**Binding Mode and Binding Site** In order to estimate the interaction of indirubin with BSA, the thermodynamic parameters were calculated from the van't Hoff plots. The acting forces between a pharmaceutical and biomolecules include hydrogen bonds, van der Waals forces, electrostatic forces, hydrophobic interaction forces, and so on. The thermodynamic parameters enthalpy ($\Delta H^0$) and entropy ($\Delta S^0$) of a reaction play important roles in confirming the binding mode.

If the enthalpy change ($\Delta H^0$) does not vary significantly over the temperature range studied, then its value and that of $\Delta S^0$ can be determined from the van't Hoff equation:

$$\ln K=-\Delta H^0/RT+\Delta S^0/R \quad (1)$$

In Eq. 1, $K$ is the binding constant at the corresponding temperature and $R$ is the gas constant. The temperatures used were 286, 297 and 308 K. The enthalpy change ($\Delta H^0$) is calculated from the slope of the van't Hoff relationship. The free energy change is estimated from the following relationship:

$$\Delta G^0=\Delta H^0-T\Delta S^0 \quad (2)$$

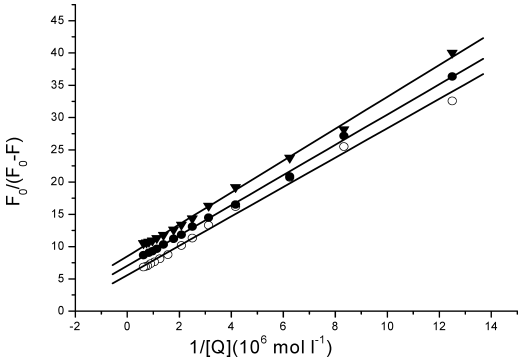


Fig. 4. The Ster–Volmer for the Indirubin–BSA at pH 7.40

BSA concentration: 1.6 $\mu$M; ▼ 286 K; ● 297 K; ○ 308 K; $\lambda_{ex}$=280 nm, $\lambda_{em}$=344 nm.

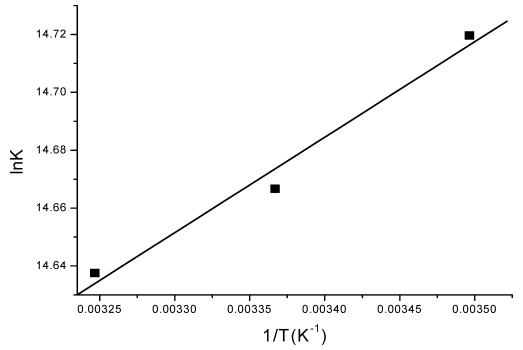


Fig. 5. Van't Hoff Plot

pH 7.40, [BSA]=1.6 $\mu$M.

Figure 5, by fiting our data (Table 1), shows that the assumption of a near constant $\Delta H^0$ is justified. Table 1 shows the values of $\Delta H^0$ and $\Delta S^0$ obtained for the binding site from the slopes and ordinates at the origin of the fitted lines and lists the corresponding value of $\Delta S^0$ at 297 K.

In the indirubin–BSA system, the data were best fitted to obtain $\Delta H^0$ and $\Delta S^0$ values with small negative (−2.744 kJ · mol$^{-1}$) and positive values (112.756 J · mol$^{-1}$ K$^{-1}$), respectively. The binding process was always spontaneous as evidenced by the negative sign of the $\Delta G^0$ values. The positive $\Delta S^0$ value indicates that the acting force is mainly a hydrophobic interaction force, but it has been pointed out that positive entropy and negative enthalpy may also be a manifestation of electrostatic interaction.[25] Furthermore, the main source of the $\Delta G^0$ value is derived from a large contribution of the $\Delta S^0$ term with little contribution from the $\Delta H^0$ factor, so hydrophobic interaction is the main force, but the electrostatic interaction cannot be excluded. These are similar to the interactions of other drugs with serum albumin.[26—28]

Trp 214 in BSA is part of the so-called subdomain IIA, which is known to bind a variety of ligands in its hydropho-

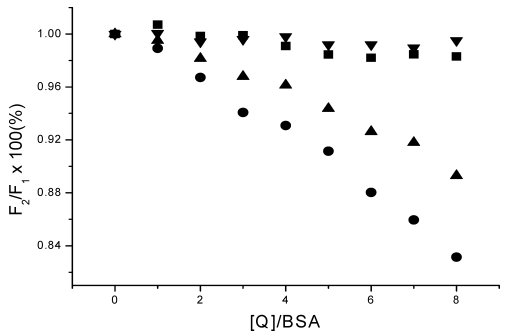


Fig. 6. Effect of Site Maker Probe on the Fluorescence of Indirubin–BSA

BSA and indirubin concentration both were 2 $\mu$M. ■ Ibuprofen; ▼ Chlorphenamine Maleate; ▲ Bilirubin; ● Ketoprofen; pH 7.40; $\lambda_{ex}$=280 nm, $\lambda_{em}$=344 nm.

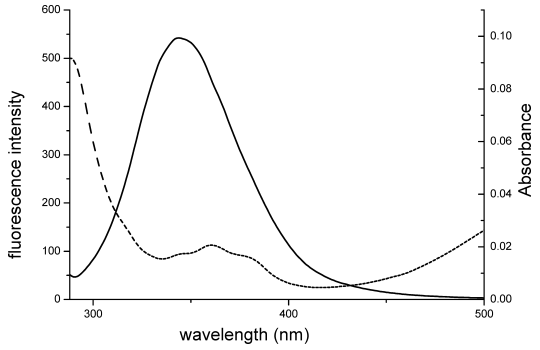


Fig. 7. The Fluorescence Spectra and the Absorption Spectra of BSA–Indirubin

The straight line: the fluorescence spectra of BSA; the point segment line: the absorbance spectra of indirubin, [BSA]=1.6 $\mu$M; [indirubin]=1.28 $\mu$M, pH=7.40, 294 K.

bic cavity.[29,30] Quenching of the Trp fluorescence was also investigated in the presence of four binding site probes for subdomains IIA and IIIA. The binding cavities associated with subdomains IIA and IIIA are also referred to as site I and site II according to the terminology proposed by Sudlow *et al.*[31] The displacement of indirubin bound to BSA by several drugs was measured by fluorescence titration. A 3 ml solution of BSA was titrated by the displacement probe (Bilirubin[30] and Ketoprofen[32] for site I, Ibuprofen[30] and Chlorphenamine Maleate[33] for site II) with an appropriate indirubin concentration. The percentage of displacement of the probe was determined according to the method of Sudlow *et al.*[31]

$$F_2/F_1\times 100\%$$

where $F_1$ and $F_2$ represent the fluorescence of indirubin plus BSA without the probe and with the probe, respectively.

The displacement experiments to determine the specificity of the binding site of indirubin to BSA is shown in Fig. 6. The relative fluorescence intensity significantly decreased after the addition of Bilirubin and Ketoprofen while the addition of Ibuprofen and Chlorphenamine Maleate caused no obviously change, which indicates that Bilirubin and Ketoprofen can displace the indirubin but Ibuprofen and Chlorphenamine Maleate have little effect on the binding of indirubin to BSA. In all, the displacement experiments implied that indirubin binds to the site I of BSA.

**Energy Transfer between Indirubin and BSA** The distance from the tryptophan residue (donor) to the bound drug (acceptor) in BSA can be calculated according to Förster's theory.[34] The efficiency of energy transfer, $E$, is given by

$$E=1-F/F_0=R_0^6/(R_0^6+r^6)$$

Where $r$ is the distance between donor and accepter and $R_0$ is the distance at 50% transfer efficiency.

$$R_0^6=8.8\times 10^{-25}K^2n^{-4}\Phi J$$

Where $K^2$ is the orientation factor related to the geometry of the donor-accepter of the dipole and $K^2=2/3$ for random orientation as in fluid solution, $n$ is the refractive index of the medium, $\Phi$ is the fluorescence quantum yield of the donor, $J$ is the spectra overlap of the donor emission and the acceptor absorption. $J$ is given by

$$J=\sum F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda \Big/ \sum F(\lambda)\Delta\lambda$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescence donor in wavelength $\lambda$, and $\varepsilon(\lambda)$ is the molar absorbance coefficient of the acceptor in wavelength $\lambda$. From these relationships, $J$, $E$ and $R_0$ can be calculated; so the value of $r$ also can be calculated.

The overlap spectra of the absorption spectra of indirubin and the fluorescence emission spectra of BSA are shown in Fig. 7. The fluorescence emission spectrum of the tryptophan residue in BSA was quenched. Energy transfer in the spectra may depend on the distance between the tryptophan residue and indirubin bound to BSA. From Fig. 7, the overlap integral calculated according to the above relationship is $1.754\times 10^{-15}\,\mathrm{cm^3 1\cdot mol^{-1}}$. Thus, the value of $R_0$ is 1.74 nm and the value of $r$ is 2.24 nm.

## Conclusion

In this work, an investigation of the interaction between indirubin and BSA was undertaken using many spectroscopic methods. The experimental results indicate that indirubin can interact with BSA in site I mainly through a hydrophobic interaction, which induces the changes in the secondary structures of BSA.

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