Studies on the Interaction of Cinnamic Acid with Bovine Serum Albumin

Hedong BIAN, Hairong ZHANG, Qing YU, Zhenfeng CHEN, and Hong LIANG*

College of Chemistry and Chemical Engineering, Guangxi Normal University; Guilin, Guangxi 541004, People's Republic of China. Received December 31, 2006; accepted March 7, 2007

The interaction between cinnamic acid and bovine serum albumin (BSA) have been studied at three temperatures, 296, 303 and 310 K. Fluorescence quenching spectra in combination with Fourier transform infrared (FT-IR) spectroscopy and circular dichroism (CD) spectroscopy was used to investigate the drug-binding mode, the binding constant and the protein structure changes in the presence of cinnamic acid in aqueous solution at pH 7.40. The fluorescence quenching constant K_q, K_{sv} and the binding constant K were calculated according to Stern–Volmer equation based on the quenching of the fluorescence of BSA in the presence of cinnamic acid. The thermodynamic parameters, the enthalpy (ΔH) and the entropy change (ΔS) were estimated to be -16.457 kJ mol^{-1} and 38.028 J mol^{-1} K^{-1} according to the van't Hoff equation. The displacement experiment shows that cinnamic acid can bind to the subdomain IIA (corresponding to Sudlow's drug binding site I). The distance between the tryptophan residues in BSA and cinnamic acid bound to site I was estimated to be 1.63 nm using Föster's equation on the basis of fluorescence energy transfer. The decreased binding constant in the presence of common ions indicates that common ions have effect on drug–BSA system.

Key words cinnamic acid; bovine serum albumin (BSA); binding; fluorescence quenching; FT-IR spectra; circular dichroism

Cinnamic acid (3-phenyl-2-propenoic acid, structure shown in Fig. 1) is a compound which can be found in nature. It mainly occurs in flavor compositions and products containing cinnamon oil.[1] It has a broad possible therapeutic activities, including antimicrobial activity and antifungal activity.[2,3] It was shown that cinnamic acid has antitumor activity against human malignant tumors, such as melanoma, glioblastoma and adenocarcinoma of the prostate and lung.[4] It was also approved its use in cancer prevention and therapy because it has low toxicity in rats and rabbits.[1,5]

Serum albumin, one of the most available and extensively studied of all proteins, is the most abundant protein in plasma, accounting for about 60% of its total protein content and providing about 80% of the blood osmotic pressure. It plays an important role in drug transport and storage in vertebrates.[6,7] In the current work, bovine serum albumin (BSA) is selected as our protein model because it is well suited to these initial studies and has been extensively characterized.[8] BSA consists of 583 amino acids in a single polypeptide chain. It posses a wide range of physiological functions involving the binding, transport and delivery of fatty acids, porphyrins, bilirubin, tryptophan, thyroxin and steroids. It contains three homologous α-helices domains (I, II and III), and each domain is further divided into two subdomains (IA, IB, *etc.*).[9] It posses two tryptophans embedded in two different domains, one of them is located in the proximity of the protein surface, but buried in a hydrophobic pocket of domain I (Trp-134), whereas the other is located in an internal part of domain II (Trp-214).[10] Interactions between cinnamic acid and human serum albumin have been reported using Fourier transformed infrared (FT-IR) spectroscopy.[11] But BSA has more widely application than human serum albumin because it is not only suitable for humans but also suitable for other animals, and there is lack of information on the cinnamic acid–BSA binding mode, the binding constant, the effects of cinnamic acid complexation on the protein secondary structure, and the effect of common ions.

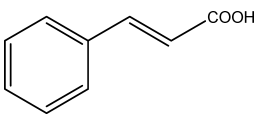

Fig. 1. The Chemical Structure of Cinnamic Acid

So in this work, the interaction of cinnamic acid and BSA was studied at physiological pH by fluorescence, CD spectroscopy and FT-IR spectroscopy, and the effect of common ions on drug–BSA system in aqueous solutions at physiological pH have also been investigated. Spectroscopic evidence regarding the drug binding mode, the association constant, and the change of protein secondary structure are provided here.

Experimental

Materials BSA was purchased from Sino-American Biotechnology Company and used without further purification and its molecular weight was 66210. BSA (1.0×10^{-4} mol/l) solution was prepared in pH 7.40 Tris–HCl buffer solution and kept in the dark at 4 °C. Cinnamic acid (analytical grade) was obtained from the National Institute for Control Pharmaceutical and Products, China. Cinnamic acid stock solution (1.0×10^{-3} mol/l) was prepared in ethanol. NaCl (analytical grade, 0.1 mol/l) solution was used to maintain the ionic strength at 0.1 mol/l Tris–HCl buffer (pH 7.40). The pH was checked with Sartorius PP-20 standardized pH meter (Germany). The solution of four displacement probes such as ibuprofen, chlorphenamine maleate, bilirubin, ketoprofen which (obtained from the National Institute for Control Pharmaceutical and Products, China) was obtained by dissolving it in ethanol, respectively. All starting materials were analytical reagent grade and doubly deioned distilled water was used throughout.

Apparatus and Methods Fluorescence emission spectra were measured with RF-5310PC spectrofluorophotometer (Shimadzu). UV–Vis absorbance spectra were measured with a Cary-100 UV–Visible spectrophotometer (Varian). CD spectra were measured with a Jasco-810 automatic recording spectropolarimeter (Japan) and a 0.1 cm pathlength cell was used. CD spectra of cinnamic acid and the cinnamic acid–BSA complex were recorded from 200 to 350 nm. Corresponding absorbance contributions of buffer and free cinnamic acid solutions were recorded and subtracted with the same parameters. The results are expressed as mean residue ellipticity (MRE) in deg cm$^2\cdot$dmol^{-1}, which is defined as $\mathrm{MRE}=\theta_{obs}(\mathrm{m\,deg})/(10\times n\times l\times C_p)$, where θ_{obs} is the CD in millidegree, n is the number of amino acid residues (583), l is the path length of the cell and C_p is mole fraction. The α-helical content of BSA was calculated from the MRE value at 208 nm using the equation $\alpha\%\mathrm{helix}=[(\mathrm{MRE}_{208}-4000)/(33000-4000)]\times100$.[12] FI-

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

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 resolution of 4 cm^{-1} and 60 scans. Spectra processing procedures: spectra of buffer solution were collected under the same conditions. Then, the absorbance of buffer solution from the spectra of sample solution was subtracted to get the FT-IR spectra of proteins. The subtraction criterion was that the original spectrum of protein solution between 2200 and 1800 cm^{-1} was featureless.[13)]

For the quantitative analysis of the potential interaction between cinnamic acid and BSA, the fluorometric titration was used. Quenching measurements were taken in 3.0 ml solution containing 1.5×10^{-6} mol/l BSA, which was titrated by successive additions of cinnamic acid solution (to get a final concentration of 1.95×10^{-5} mol/l), the fluorescence intensity was recorded (excitation at 280 nm using slit widths 10/10 nm and emission at 344 nm). All experiments were measured at three temperatures (296, 303, 310 K). The data were analyzed by the Stern–Volmer equation to calculate the binding constants.

Molecular Probe Experiments: A 3 ml solution of BSA with an appropriate cinnamic acid concentration was titrated by successive additions of ibuprofen, chlorphenamine maleate, bilirubin and ketoprofen solutions, respectively. The fluorescence intensity was recorded (excitation at 280 nm using slit widths 10/10 nm and emission at 344 nm) at 296 K. The data were analyzed according to the method of Sudlow *et al.*

Results and Discussion

Interaction of Cinnamic Acid with BSA In order to ensure cinnamic acid binds to BSA, fluorescence quenching spectra of BSA without and with cinnamic acid in pH 7.40 Tris–HCl buffer were carried out. The intrinsic fluorescence intensity of BSA measured before and after addition of cinnamic acid provides information about conformational changes of BSA. The effect of the cinnamic acid on BSA and the molecular environment in a vicinity of the chromophore molecules can be embodied by fluorescence measurements. Figure 2 shows the fluorescence emission spectra of BSA with the addition of different concentrations of cinnamic acid. It can be found that the fluorescence intensity of BSA decreased regularly and the maximum fluorescence emission wavelength had a slight blue-shift (from 344 to 341 nm) with the increasing of cinnamic acid concentration. The results showed that the binding of cinnamic acid is associated with changes in the dielectric environment of at least one of the two indole rings in BSA, suggesting after adding the solution of cinnamic acid the chromophore was placed in a more hydrophobic environment.[13)]

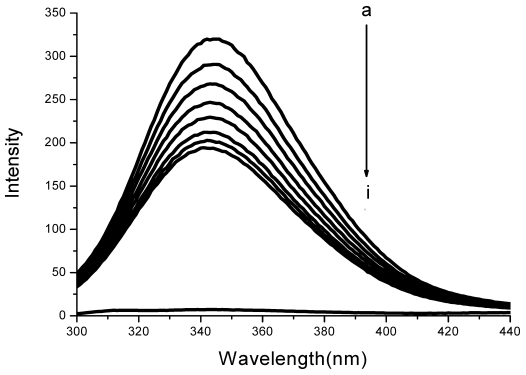

Fig. 2. Emission Spectra Excited at 280 nm (pH=7.40)

(a) 1.5×10^{-6} mol/l BSA; (b—h) 1.5×10^{-6} mol/l BSA in the presence of 1.5×10^{-6} mol/l, 4.5×10^{-6} mol/l, 7.5×10^{-6} mol/l, 10.5×10^{-6} mol/l, 13.5×10^{-6} mol/l, 15.5×10^{-6} mol/l, 18.5×10^{-6} mol/l cinnamic acid, respectively; (i) 7.5×10^{-6} mol/l cinnamic acid.

The static quenching and dynamic quenching can be distinguished by the results at different temperatures. The quenching rate constants are expected to decrease with increasing temperature for the static quenching. In contrast, the reversed effect was observed for the dynamic quenching.[14)] The Stern–Volmer graphs at various temperatures are shown in Fig. 3. It can be found that plots are linear and the slopes decrease with increasing temperature. The results indicate that the probable quenching mechanism of fluorescence of BSA by cinnamic acid is a static quenching procedure, because K_{sv} is decreased with increase in temperature. In order to confirm this view, we assumed the procedure was a dynamic quenching procedure. The quenching equation is:

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

Where F and F_0 are the fluorescence intensity in the absence and presence quencher, K_q, K_{sv}, τ_0 and $[Q]$ are the quenching rate constant of the biomolecule, the dynamic quenching constant, the average lifetime of molecule without quencher and concentration of quencher, respectively. And K_{sv} is also can be written as $K_{sv}=K_q\tau_0$ and $K_q=K_{sv}/\tau_0$. So the quenching constant and the dynamic quenching constant can be obtained from the slope because the fluorescence lifetime of the biopolymer is 10^{-8} s.[15)] The data and the correlation coefficients are listed in Table 1. The maximum scatter collision quenching constant, K_q of various quenchers with the biopolymer is 2.0×10^{10} l/mol/s.[16)] Thus, the rate constant of protein quenching procedure initiated by cinnamic acid is greater than the K_q of the scatter procedure. So, this shows that the quenching is not initiated by dynamic collision but from compound formation.[17)]

Further experiments were carried out with CD and FT-IR technique to verify the binding of cinnamic acid to BSA. Figure 4 shows the CD spectra of BSA in the absence and pres-

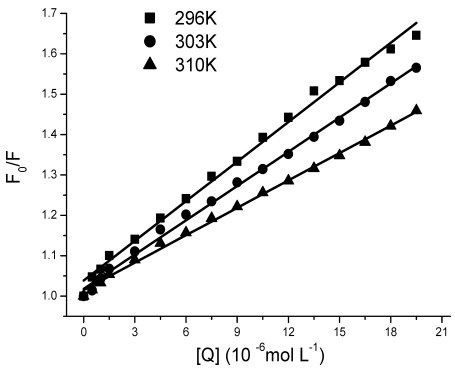

Fig. 3. The Stern–Volmer Curves for Quenching of Cinnamic Acid with BSA

λ_{ex}=280 nm, λ_{em}=344 nm.

Table 1. The Dynamic Quenching Constants (l/mol) and Quenching Rate Constants (l/mol/s) between Cinnamic Acid and BSA

Temperature (K)	Dynamic quenching constants ($\times10^4$ l/mol)	Quenching rate constants ($\times10^{12}$ l/mol/s)
296	3.270	3.270
303	2.827	2.827
310	2.262	2.262

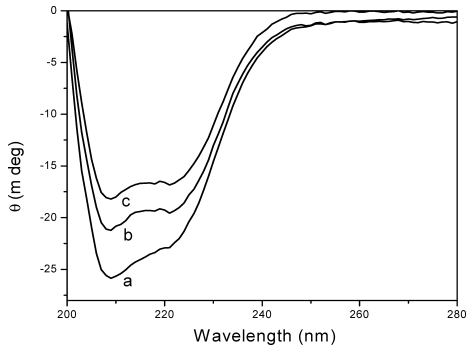

Fig. 4. CD Spectra of the BSA–Cinnamic Acid System

(a) 1.5×10^{-6} mol/l BSA; (b) 1.5×10^{-6} mol/l BSA+1.5×10^{-6} mol/l cinnamic acid; (c) 1.5×10^{-6} mol/l BSA+3.0×10^{-6} mol/l cinnamic acid. T=303 K, pH=7.40.

ence cinnamic acid. It can be seen that the CD spectra of BSA exhibited two negative bands in the ultraviolet region at 208 and 220 nm, which is characteristic of α-helical structure of protein. The binding intensity of cinnamic acid to BSA decreases both of these negative bands after adding cinnamic acid solution, suggesting the helix structure content of BSA has been changed. Otherwise, the CD spectra of BSA without and with cinnamic acid are similar in shape, which indicated that the structure of BSA after cinnamic acid binding to BSA is predominantly α-helical. The α-helical content of the protein changes from 67% content in native BSA to 55% content (1 : 1) and 47% content (1 : 2) after cinnamic acid binding to BSA, which is evidence that the binding of cinnamic acid to BSA may cause some conformational changes. That is, there is an interaction between the BSA and cinnamic acid. That may be because the drug–BSA combination caused the rearrangement of the protein polypeptide chain and changes of the protein's secondary structure.[11)]

Figure 5 was the FT-IR spectra of BSA in the absence and presence of cinnamic acid. The FT-IR spectrum of free BSA is shown in Fig. 5a, which was obtained by subtracting the absorption of Tris–HCl buffer from the spectrum of protein solution. Figure 5b shows the spectrum of BSA bound with cinnamic acid, which was obtained by subtracting the spectrum of the cinnamic acid-free form from that of the cinnamic acid-bound form. Hydrogen bonding and the coupling between transition dipoles are the key factors in the most important factors governing conformational sensitivity of the amide bands. The protein amide I band *ca.* 1653 cm^{-1} (mainly C=O stretch) and amide II band *ca.* 1548 cm^{-1} (C–N stretch coupled with N–H bending mode) both have a relationship with the secondary structure of protein.[18)] As shown in Fig. 5, there is the evident peak shift of amide II from 1559.2 to 1573.4 cm^{-1} and the peak position of amide I hasn't changed, which indicate that the secondary structure of BSA is perturbed after cinnamic acid was added. That is, cinnamic acid had bound to BSA.

Binding Parameters For static quenching, fluorescence quenching can be analyzed using modified Stern–Volmer equation.[19)]

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

where F and F_0 are the fluorescence intensities of BSA in the

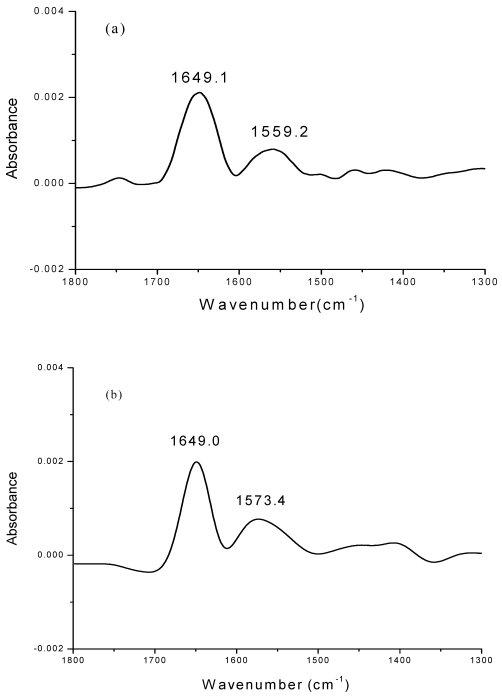

Fig. 5. FT-IR Spectra and Difference Spectra [(BSA Solution+Cinnamic Acid Solution)−(BSA Solution)] of Free BSA (a) and Its Cinnamic Acid Complexes (b) in Buffer Solution in the Region of 1800—1300 cm^{-1}

[cinnamic acid]=1.2×10^{-4} mol/l, [BSA]=3.0×10^{-5} mol/l.

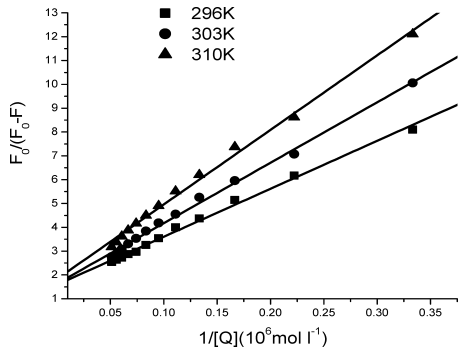

Fig. 6. The Binding Constant, K, of the BSA–Cinnamic Acid

λ_{ex}=280 nm, λ_{em}=342 nm, pH=7.40, $C_{BSA}=1.5\times10^{-6}$ mol/l, $C_{\text{cinnamic acid}}=5\times10^{-7}$ to 1.95×10^{-5} mol/l) at 296 K (■); 303 K (●); 310 K (▲).

presence and absence of quencher, respectively; K is the Stern–Volmer quenching constant and [Q] is quencher concentration; f is the fraction of the initial fluorescence accessible to quencher. The plots of $F_0/(F_0-F)$ *versus* 1/[Q] (Fig. 6) yields 1/f as the intercept, and 1/(fK) as the slope. Table 2 shows the results of the quenching constant (K). It was found that the quenching constant (K) decreased with the temperatures increasing, which indicates the static quenching interaction between cinnamic acid and BSA. The results can be interpreted as the binding constant of the complexation reaction because static quenching arises from the formation of a dark complex between fluorophore and quencher.[20)]

Binding Mode and Binding Site In order to further characterize the acting forces between cinnamic acid and BSA, the thermodynamic parameters calculated from the van't Hoff equation were analyzed. The acting forces between a drug and a biomolecule may include hydrogen bond,

Table 2. Binding Constants and Thermodynamic Parameters for the Binding of Cinnamic Acid to BSA at pH=7.40

Temperature (K)	Binding parameters ($\times10^4$ l/mol)	ΔG (kJ/mol)	ΔS (J/mol/K)	ΔH (kJ/mol)
296	7.901	−27.713		
303	6.443	−27.979	38.028	−16.457
310	5.846	−28.246		

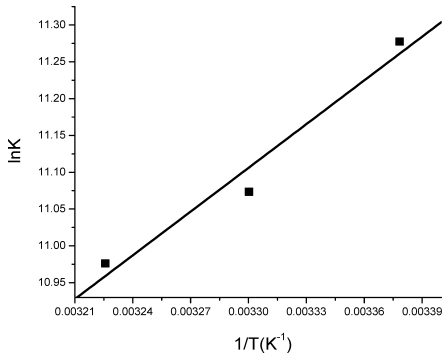

Fig. 7. Van't Hoff Plot for the Interaction of BSA and Cinnamic Acid
pH=7.40, [BSA]=1.5×10^{-6} mol/l.

van der Waals force, electrostatic force and hydrophobic interaction force and so on.[21] The thermodynamic parameters, enthalpy ($\Delta H°$), entropy ($\Delta S°$) and free energy change ($\Delta G°$), play important roles in estimating the binding mode. The reaction enthalpy change is regarded as a constant if the temperature changes little. These data can be determined from the van't Hoff equation:

$$\ln K = -\Delta H°/RT + \Delta S°/R \quad (3)$$

According to static quenching equation, the binding constants (K) at three different temperatures (T) were obtained, and the results were listed in Table 2. The values of $\Delta H°$ and $\Delta S°$ can be calculated from the slope and intercept, and the value of $\Delta G°$ can be obtained by the following relation:

$$\Delta G° = \Delta H° - T\Delta S° \quad (4)$$

Figure 7 is the Van't Hoff plot for the interaction of BSA and cinnamic acid and the values of $\Delta H°$, $\Delta S°$ and $\Delta G°$ were listed in Table 2. From it, it can be seen that the reaction of BSA and cinnamic acid was an exothermic reaction accompanied with negative enthalpy ($\Delta H°$) and positive entropy ($\Delta S°$) changes. And the binding process is spontaneous because of the negative free energy ($\Delta G°$). For drug–protein interaction, positive entropy is frequently taken as the evidence for hydrophobic interaction, but it may also be a manifestation of electrostatic interaction.[22] Furthermore cinnamic acid is a negative ion, it is possible that the drug–protein complex is form by electrostatic interactions between the ionic species.

Trp-214 is located in subdomain IIA in BSA, being known to bind a variety of ligands in its hydrophobic cavity.[7,23] To check the specificity of the drug binding, the displacement of fluorescent probes measured by fluorescence titration was also investigated in the presence of four binding site probes for subdomains IIA (corresponding to Sudlow's drug binding site I) and IIIA (corresponding to Sudlow's drug binding site II).[24] The displacement probes were bilirubin[23] and ketoprofen[25] for site I, ibuprofen[23] and chlorphenamine maleate[26] for site II. The percentage of displacement of the probe was determined according to the method of Sudlow *et al.*[27]

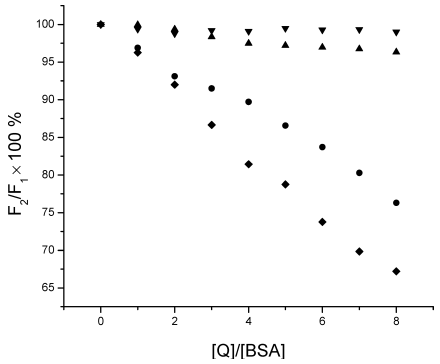

Fig. 8. Effect of Site Maker Probe on the Fluorescence of Cinnamic Acid–BSA

The concentration of BSA and cinnamic acid were 1.5×10^{-6} mol/l and 7.5×10^{-6} mol/l; respectively. [Q]: ▼, chlorphenamine maleate; ▲, ibuprofen; ●, bilirubin; ■, ketoprofen; pH=7.40; λ_{ex}=280 nm, λ_{em}=344 nm.

$$\text{probe displacement (\%)} = F_2/F_1 \times 100 \quad (5)$$

where F_1 and F_2 represent the fluorescence of cinnamic acid plus BSA in the absence and presence of probe, respectively.

Figure 8 shows the changes in fluorescence of cinnamic acid bound to BSA on the addition of several probes. The relative fluorescence intensity significantly decreased after the addition of Bilirubin and Ketoprofen, but when added Ibuprofen and Chlorphenamine Maleate the fluorescence has no obviously change, which indicates that Bilirubin and Ketoprofen can displace the cinnamic acid but Ibuprofen and Chlorphenamine Maleate have little effect on the binding of cinnamic acid to BSA. This means that cinnamic acid binds to the site I of BSA.

The Energy Transfer between Cinnamic Acid and BSA Fluorescence quenching studies proved that BSA could bind with cinnamic acid and the energy transfer between cinnamic acid and BSA occurred. The efficiency of energy transfer can be used to evaluate the distance between the ligand and the tryptophan residues in the protein. In this work, the efficiency of energy transfer was investigated according to the Förster energy transfer theory to calculate the distance between donor and accepter.[28] The efficiency of energy transfer, E, is described by the following equation:

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

where r is the distance between donor and acceptor and R_0 is the distance at 50% transfer efficiency.

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

where K^2 is the spatial orientation factor of the dipole and $K^2=2/3$ for random orientation as in fluid solution, n is the refraction index for the medium, Φ is the fluorescence quantum yield of the donor in the absence of the acceptor and J is the overlap integral between the donor fluorescence emission spectrum and the acceptor absorption spectrum. J can be given by

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda / \sum F(\lambda)\Delta\lambda \quad (8)$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescence

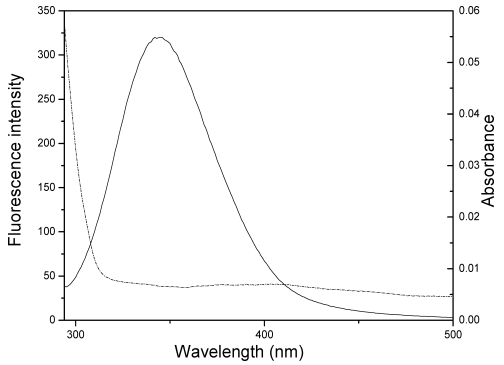

Fig. 9. The Fluorescence Spectra and the Absorption Spectra of BSA–Cinnamic Acid

The straight line: the fluorescence spectra of BSA; the point segment line: the absorbance spectra of cinnamic acid, [BSA]=1.5×10^{-6} mol/l; [cinnamic acid]=6.0×10^{-6} mol/l, pH=7.40, T=296 K.

reagent of wavelength λ, $\varepsilon(\lambda)$ is the molar absorbance coefficient of the acceptor at the wavelength of λ. From these relationships, J, E and R_0 can be calculated; so r also can be calculated.

Figure 9 showed the overlap of the UV absorption spectrum of cinnamic acid with the fluorescence emission spectra of BSA. From Fig. 9, the overlap integral of the UV absorption spectrum of cinnamic acid and the fluorescence emission spectrum of BSA calculated according to the above relationship is 4.5087×10^{-16} cm^3 l/mol. Using these present data $K^2=2/3$, $n=1.36$, $\Phi=0.15$.[29] So the value of R_0 is 1.36 nm and the value of r is 1.63 nm. The average distance r is less than 8 nm, which indicates that the energy transfer from BSA to cinnamic acid occurs with high probability.

The Effect of Common Ions on the Binding Constants of Cinnamic Acid and BSA The effect of common ions on the binding constants was investigated at 23 °C and the adopted common ions concentration are all 10 times to the concentration of BSA in order to keep enough common ions. The results are summarized in Table 3. It is shown that the binding constant between protein and drug was decreased because of the competition between common ions and drug, indicating that the binding force between protein and drug also decreased. Thus, the common ions shortening the storage time of drug in blood plasma and enhancing the maximum effectiveness of the drug.[30]

Conclusion

In this paper, the interaction of cinnamic acid with BSA under physiological condition has been studied by fluorescence methods in combination with FT-IR and CD techniques. The results suggest that cinnamic acid can interact with BSA strongly in site I mainly through the hydrophobic interaction, which induce the changes of secondary structures of BSA.

Acknowledgements This project was supported by the National Natural Science Foundation of China (No. 20671023, 20361002, 30460153), the key project of Chinese Ministry of Education (No. 03101, 204111), the Teaching and Research Award Programme for Outstanding Young Teachers in Higher Education Institutions of MOE, China, and the foundation of Key Laboratory of Medicinal Chemical Resources and Molecular Engineering, Guangxi Normal University, China.

Table 3. The Effect of Common Ions on the Quenching Constants and the Binding Parameters between Cinnamic Acid and BSA at 296 K

Common ions	Quenching rate constants ($\times10^{12}$ l/mol/s)	Binding parameters ($\times10^4$ l/mol)
Co^{2+}	1.378	1.054
Mg^{2+}	1.587	1.885
Zn^{2+}	1.721	1.016
Fe^{3+}	1.240	1.136
Cu^{2+}	1.797	3.790
Al^{3+}	1.733	1.072
NH_4^+	1.477	2.584
K^+	1.488	5.117
Br^-	1.651	1.858
I^-	1.620	4.884

References

1) Hoskins J. A., *J. Appl. Toxicol.*, **4**, 283—292 (1984).
2) Ramanan P. N., Rao M. N., *Ind. J. Exp. Biol.*, **25**, 42—43 (1987).
3) Tawata S., Taira S., Kobamoto N., Zhu J., Ishihara M., Toyama S., *Biosci. Biotechnol. Biochem.*, **60**, 909—910 (1996).
4) Liu L., Hudgins W. R., Shack S., Yin M. Q., Samid D., *Int. J. Cancer*, **62**, 345—350 (1995).
5) Opdyke D. L. J., *Food Cosmet. Toxicol.*, **13**, 687—690 (1975).
6) Carter D., Ho J. X., *Adv. Protein Chem.*, **45**, 153—203 (1994).
7) Peters T., "All About Albumin. Biochemistry, Genetics and Medical Applications," Academic Press, San Diego, CA, 1996.
8) Guo M., Zou J. W., Yi P. G., Shang Z. C., Hu G. X., Yu Q. S., *Anal. Sci.*, **20**, 465—470 (2004).
9) Peters T., "All About Albumin, Biochemistry, Genetics and Medical Applications," Academic Press, San Diego, 1995.
10) Liu J. Q., Tian J. N., Zhang J. Y., Hu Z. D., Chen X. G., *Anal. Bioanal. Chem.*, **376**, 864—867 (2003).
11) Jiang M., Xie M. X., Zheng D., Liu Y., Li X. Y., Chen X., *J. Mol. Struct.*, **692**, 71—80 (2004).
12) Khan A. M., Muzammil S., Musarrat J., *Int. J. Biol. Macrcomol.*, **30**, 243—249 (2002).
13) Yuan T., Weljie A. M., Vogel H. J., *Biochemistry*, **37**, 3187—3195 (1998).
14) Chen G. Z., Huang X. Z., Xu J. G., Zheng Z. Z., Wang Z. B., "The Methods of Fluorescence Analysis," 2nd ed., Science Press, Beijing, 1990, p. 112.
15) Lakowica J. R., Weber G., *Biochemistry*, **12**, 4161—4170 (1973).
16) Ware W. R. J., *Phys. Chem.*, **66**, 445—458 (1962).
17) Deepa S., Mishra A. K., *J. Pharm. Biomed. Anal.*, **38**, 556—563 (2005).
18) Tian J. N., Liu J. Q., Tian X., Hu Z. D., Chen X. G., *J. Mol. Struct.*, **691**, 197—202 (2004).
19) Lakowica J. R., "Principles of Fluorescence Spectroscopy," 2nd ed., Kluwer Academic Publishers/Plenum Press, New York, 1999.
20) Eftink M. R., Ghiron C. A., *J. Phys. Chem.*, **80**, 486—493 (1976).
21) Nie L. H., Zhao H. C., Wang X. B., Wang X. J., *Beijing Normal University (Nat. Sci.)*, **37**, 87—91 (2001).
22) Maruyama A., Lin C. C., Yamasaki K., Miyoshi T., Imai T., Yamasaki M., Otagiri M., *Biochem. Pharm.*, **45**, 1017—1026 (1993).
23) He X. M., Carter D. C., *Nature* (London), **358**, 209—215 (1992).
24) Artali R., Bombieri G., Calabi L., Pra A. D., *Il Farmaco*, **60**, 485—495 (2005).
25) Mignot I., Presle N., Lapicque F., Monot C., Dropsy R., Netter P., *Chirality*, **8**, 271—278 (1996).
26) Gonzàlez-Jimènez J., *Chem-Biol. Interact.*, **91**, 65—74 (1994).
27) Sudlow G., Birkett D. J., Wade D. N., *Mol. Pharmacol.*, **12**, 1052—1061 (1976).
28) Förster T., Sinanoglu O. (eds.), "Modern Quantum Chemistry," Vol. 3, Academic Press, New York, 1966, p. 93.
29) Hu Y. J., Liu Y., Zhao R. M., Dong J. X., Qu S. S., *J. Photochem. Photobiol. A, Chem.*, **179**, 324—329 (2006).
30) Ying L., Wenying H., Jiaqin L., Fenling S., Hu Z., Xingguo C., *Biochim. Biophys. Acta*, **1722**, 15—21 (2005).