Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Photochemistry and Photobiology A: Chemistry 167 (2004) 213–221
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

The study on the interaction between human serum albumin and a new reagent with antitumour activity by spectrophotometric methods

Hong Gao [a], Liandi Lei [a], Jiaqin Liu [a,b], Qin Kong [a], Xingguo Chen [a,*], Zhide Hu [a]

[a] *Department of Chemistry, Lanzhou University, Lanzhou 730000, China*
[b] *Mianyang Teacher's College, Mianyang 621000, China*

Received 30 January 2004; received in revised form 9 May 2004; accepted 17 May 2004

Available online 22 July 2004

Abstract

In this work, the binding of 2-hydroxy-3-nitro-9-fluorenone (HNF; a new reagent with antitumour activity) to human serum albumin (HSA) was investigated by fluorescence spectroscopy combined with UV-Vis absorption, circular dichroism (CD), and Fourier transform infrared (FT-IR) spectrophotometric techniques under simulative physiological conditions for the first time. A strong fluorescence quenching reaction of HNF to HSA was observed and the quenching mechanism was suggested as static quenching according to the Stern–Volmer (S–V) equation. The binding constants of HNF with HSA at 300, 310 and 320 K were calculated as 6.08×10^5, 3.80×10^5 and 2.79×10^5 M^{-1}, respectively, and corresponding numbers of binding sites (n) were 1.1, 1.0 and 1.0. Experimental results observed showed that the binding of HNF to HSA induced conformational change of HSA. The quantitative analysis data of CD spectra from that of the α-helix 60.3% in free HSA to 56.5% in the HNF–HSA complex further confirmed that the secondary structure of the protein was modified by HNF. The thermodynamic parameters, standard enthalpy change (ΔH°) and the standard entropy change (ΔS°), were obtained to be -31.10 kJ mol^{-1} and 6.87 J mol^{-1} K^{-1}, respectively, which indicated that a hydrophobic force played a major role in the interaction of HNF with HSA. All these experimental results and theoretical data clarified that HNF could bind to HSA and be effectively transported and eliminated in body, which could be a useful guideline for further drug design.
© 2004 Elsevier B.V. All rights reserved.

Keywords: 2-Hydroxy-3-nitro-9-fluorenone; Human serum albumin; Binding study; Fluorescence quenching; UV-Vis spectroscopy; CD spectroscopy; FT-IR spectroscopy

1. Introduction

Polycyclic aromatic compounds (PACs) as basic structural unit are widely distributed in nature and fossil fuels. In organic synthesis, the use of PACs and their derivatives as anticancer agents has been explored [1,2]. These studies mainly focused on the synthesis of the polycyclic ring systems and the examination of their metabolic activation within target cells. In addition, the structure–activity relationships of PACs as anticancer agents were studied, because the anticancer agents revealed two common structural features: they have a planar ring system and a basic side chain group [3]. This would then put PACs in a class of potential anticancer agents that have been called generically membrane-stabilizing agents (MSA) [4].

Human serum albumin (HSA) is the most abundant protein in plasma. As the major soluble protein constituent of the circulatory system, it has many physiological and pharmacological functions. It contributes to colloid osmotic blood pressure and is chiefly responsible for the maintenance of blood pH. Moreover, it also plays an important role in the transport and disposition of endogenous and exogenous ligands present in blood [5]. Its surprising capacity to bind a large variety of drugs results in its prevailing role in drug pharmacokinetics and pharmacodynamics. Its primary pharmacokinetics function is participating in absorption,

Abbreviations: HNF, 2-hydroxy-3-nitro-9-fluorenone; HAS, human serum albumin; PACs, polycyclic aromatic compounds; MRE, mean residue ellipticity

* Corresponding author. Tel.: +86 9318912540; fax: +86 9318912582.
E-mail addresses: gaohong01@st.lzu.edu.cn (H. Gao), chenxg@lzu.edu.cn (X. Chen).

1010-6030/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotochem.2004.05.017

distribution, metabolism and excretion of drug, of which the drug distribution is the one that HSA controls, because most drugs travel in plasma and reach the target tissues by binding to HSA [6]. So plasma protein after binding to drug has a significant influence on the pharmacokinetics of many drugs. Strong binding can decrease the concentrations of free drug in plasma, whereas weak binding can lead to a short lifetime or poor distribution. This is why pharmaceutical companies have developed and standardized screens for HSA binding in the first step of drug design. Therefore, it is an important guideline to investigate the interaction between a new compound with biological activity and HSA in early stages of drug discovery. The small molecule substances, such as drugs and other chemicals that can bind to protein, have been studied for decade years [7–12]. However, seldom studies are available in the binding of new synthesis reagent with bioactivity to protein [13]. Up to now, several spectrophotometric methods mainly including fluorescence, UV-Vis, CD, FT-IR, nuclear magnetic resonance (NMR) have been used to study the interaction of small molecule substances and protein and clarify the conformational change of protein [14–18]. Some techniques such as electrochemical technique [19], capillary electrophoresis [20], high-performance liquid chromatography [21], have also been utilized for the evaluation of binding mode and binding constants. Among them, fluorescence spectroscopy has been widely used owing to its exceptional sensitivity, selectivity, convenience and abundant theoretical foundation.

2-Hydroxy-3-nitro-9-fluorenone (HNF) was a new reagent, whose structure was shown in Fig. 1 [22]. Its antitumour activity was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay using human leukemia HL-60 cells for the first time, and the data are still unpublished. In this work, the binding of HNF to HSA was investigated by fluorescence spectroscopy combined with some other spectrophotometric techniques including UV-Vis, CD and FT-IR, under simulative physiological conditions of human blood (pH 7.4, ionic strength 0.1). Experimental results observed in this work indicated that the binding of HNF to HSA led to the conformational change of HSA. The difference of α-helical contents of HSA in the presence and absence of HNF also confirmed that the secondary structure of protein was modified by HNF. The binding constants and the numbers of binding sites were determined. Additionally, binding mechanism and binding mode were suggested according to experimental results.

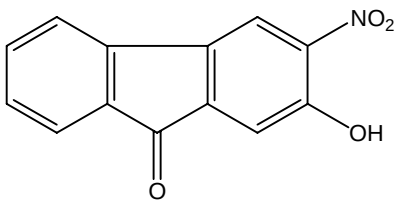

Fig. 1. The chemical structure of 2-hydroxy-3-nitro-9-fluorenone.

2. Materials and methods

2.1. Materials

The HSA (fatty acid-free <0.05%) was purchased from Xian Rui Ke Biological Products Co. Ltd. (Xian, China), was used without further purification and its molecular weight was assumed to be 66 500. HNF was provided by The State Key Laboratory of Applied Organic Chemistry, Lanzhou University. The dimethyl sulphoxide (DMSO) purchased from The Second Tianjin Chemical Reagent Factory (Tianjin, China) was of analytical grade and was redistilled prior to use. Tris (hydroxymethylaminomethane) was of biochemical grade and was purchased from The Shanghai Chemical Reagent Head Factory (Shanghai, China). Ultra-pure water was prepared with a Milli-Q water purification system (Millipore, USA). All other reagents were of analytical grade and ultra-pure water was used throughout all the experiments.

2.2. Characterization of HNF

HNF was obtained by reacting 2-hydroxy-9-fluorenone with nitric oxide containing trace O_2 in CH_2Cl_2 at room temperature. The solvent was evaporated off after completion of the reaction and the crude product was separated and purified by silica gel (200–300 mesh) column with a total length of 50 cm, using a mobile phase of petroleum ether and acetone (8:1, v/v).

The structure of HNF was identified by IR, ^{1}H NMR and HRMS as follows: IR (KBr, ν_{max} (cm^{-1})): 3048 (OH), 1709 (CO), 1541, 1329 (NO_2); ^{1}H NMR (300 MHz, CD_3COCD_3): 7.41 (1H, s, H1), 7.44 (1H, dd, $J = 7.5$, 0.9 Hz, H5), 7.69 (2H, m, H6, H7), 7.89 (1H, dd, $J = 7.5$, 0.9 Hz, H8), 8.34 (1H, s, H4). HRMS: Cald., 240.0302 $(M - H)^+$; Found, 240.0301.

According to above data, the structure of HNF was confirmed as Fig. 1.

2.3. Preparation of solutions

HNF was dissolved in redistilled DMSO as the stock solution (1.0×10^{-3} M). The 1.0×10^{-4} M HSA stock solution was prepared by directly dissolving HSA in ultra-pure water. Both of above stock solutions were kept in a refrigerator and diluted as required. A 0.05 M Tris–HCl buffer solution was prepared and its pH was adjusted to 7.4 using 0.1 M of either hydrochloric acid or sodium hydroxide solution. In addition, A 1 M NaCl stock solution was prepared by directly dissolving NaCl in ultra-pure water. The 0.1 M NaCl working solution used in this work was obtained by further dilution of stock solution to keep the ionic strength of simulative physiological condition at 0.1.

2.4. Apparatus and operation conditions

All fluorescence spectra and intensities were recorded using an RF-5301PC spectrofluorimeter (Shimadzu) equipped

with a xenon lamp source and 1.0 cm quartz cells, excitation and emission wavelengths were 285 and 342 nm, respectively, and excitation and emission bandwidths were both 5 nm. A Tu-1901 UV-Vis spectrophotometer (Beijing, China) was used for the measurement of UV-Vis absorption spectra in a given wavelength range, using 1 cm cells. A Jasco-20C automatic recording spectropolarimeter (Japan) was employed for the measurement of CD spectra, and a 0.2 cm pathlength cell was used. CD spectra of HSA and the HNF–HSA complex (the molar ratio of HNF to HSA was 2:1) were recorded from 200 to 350 nm. Corresponding absorbance contributions of buffer and free HNF solutions were recorded and subtracted with the same instrumental parameters. The infrared spectra were acquired on a Nicolet Nexus 670 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 ATR method with resolution of $4\,cm^{-1}$ and 60 scans. The infrared spectra of HSA and the HNF–HSA complex (the molar ratio of HNF to HSA was 3:1) were obtained in the featured region of $1800–1400\,cm^{-1}$. Corresponding absorbance contributions of buffer and free HNF solutions were recorded and digitally subtracted with the same instrumental parameters.

2.5. Methods

In a 10 mL standard volumetric flask with a stopper, appropriate volume of HNF standard solution (1.0×10^{-3} M) was added, then definite volume of 1.0×10^{-4} M HSA stock solution, 2.0 mL of 0.05 M Tris–HCl buffer and 1.0 mL of 1.0 M NaCl solution were added; then diluted to 10.0 mL with ultra-pure water and mixed well. Two reagent blanks were prepared in a similar way but without HNF or HSA. Both the mixtures and the reagent blanks mentioned above were scanned by various spectrophotometers, and all experiments were performed at room temperature.

Measurements of the HAS–NHF binding at different temperatures were carried out by fluorimetric titration of HSA according to following titration procedure (sample temperatures were maintained by a thermostat bath with recycling water throughout all the experiments): a cuvette of 1 cm was filled with 3 mL 3.0×10^{-6} M HSA solution. After recording the fluorescence intensities of HSA, 10 μL HNF stock solution (1.0×10^{-3} M) was added consecutively. The fluorescence intensity data in the concentration range of HNF from 6.7×10^{-6} to 2.7×10^{-5} M were collected to calculate the various parameters of the interaction between HNF with HSA.

3. Results and discussion

3.1. The interaction between HNF and HSA

It is well known that HSA is a monomeric protein comprising 585 amino acids. And its secondary structure is mainly α-helix and 17 disulfide bridges. The initial crystal structure analyses have revealed that the principal regions of ligand binding sites of albumin are located in hydrophobic cavities in subdomains IIA and IIIA, and the sole tryptophan residue (Trp-214) is in subdomain IIA [23]. Numerous studies confirmed that the binding of small molecule substances to HSA could induce the conformational change of HSA, because the intramolecular forces involved to maintain the secondary structure could be altered, which results in the conformational change of protein [7–18].

For macromolecules, fluorescence measurements can give some information of the binding of small molecule substances to protein, such as the binding mechanism, binding mode, binding constants, binding sites, intermolecular distances, etc. For HSA, there are only three intrinsic fluors-tryptophan, tyrosine and phenylalanine. Actually, the intrinsic fluorescence of HSA is almost contributed by tryptophan alone, because phenylalanine has a very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is ionized, or near an amino group, a carboxyl group, or a tryptophan. This viewpoint was well supported by the experimental observation coming from Sulkowska [24]. That is, the change of intrinsic fluorescence intensity of HSA is that of fluorescence intensity of tryptophan residue when small molecule substances are added to HSA. The fluorescence quenching spectra of HSA at various concentrations of HNF are shown in Fig. 2. Obviously, HSA had a strong fluorescence emission band at 342 nm by fixing the excitation wavelength at 285 nm, while HNF had no intrinsic fluorescence. The fluorescence intensity of HSA decreased regularly, and slight blue shift was observed for the emission wavelength with increasing HNF concentration, indicating that a HNF–HSA complex, which could quench the

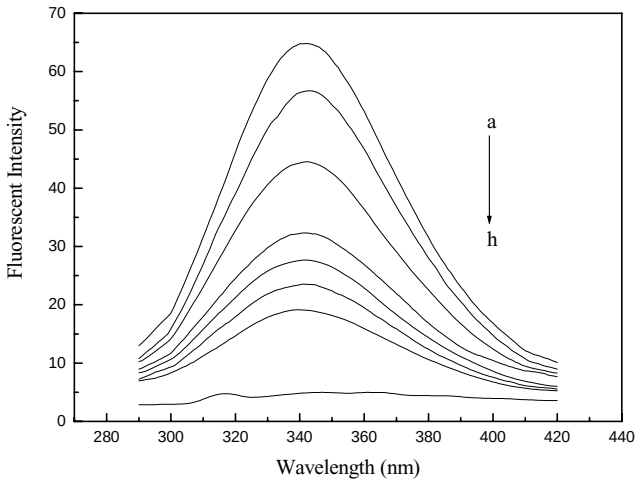

Fig. 2. Fluorescence emission spectra of the HAS–HNF system obtained in 0.05 M Tris–HCl buffer, pH 7.4, ionic strength 0.1, at room temperature. Excitation wavelength was 285 nm. The concentration of HSA was fixed as 8.0×10^{-7} M: (a) HSA, 8.0×10^{-7} M; (b–g) HNF–HSA, the HNF concentrations were 1.0×10^{-6}, 2.0×10^{-6}, 4.0×10^{-6}, 6.0×10^{-6}, 8.0×10^{-6} and 1.0×10^{-5} M, respectively; (h) HNF, 3.0×10^{-6} M.

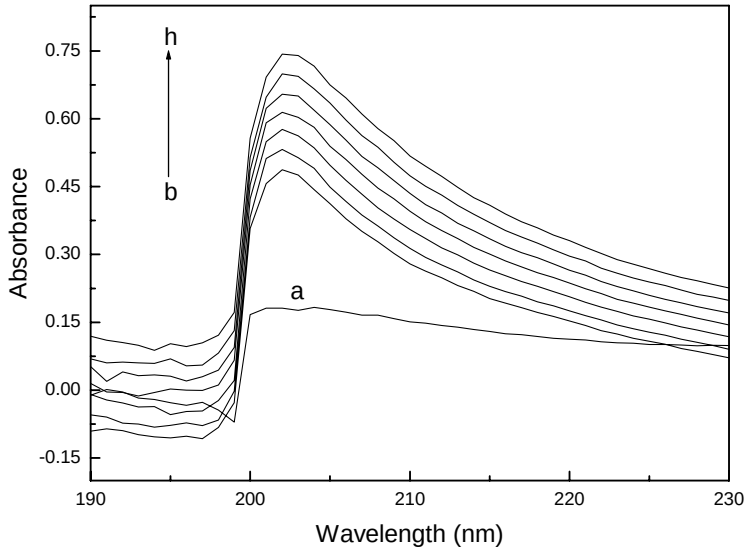

Fig. 3. UV absorption spectra of the HAS–HNF system obtained in 0.05 M Tris–HCl buffer, pH 7.4, ionic strength 0.1, at room temperature: (a) HNF, 1.0×10^{-5} M; (b) HSA, 2.0×10^{-7} M; (c–h) HNF–HSA, the HNF concentrations were 2.0×10^{-6}, 5.0×10^{-6}, 8.0×10^{-6}, 1.1×10^{-5}, 1.4×10^{-5} and 1.7×10^{-5} M, respectively.

fluorescence of HSA and change the microenvironment of tryptophan residue, was formed. Therefore, we inferred that the binding took place near Trp-214 and led to a conformational change with a local perturbation of the IIA binding site in HSA.

UV-Vis absorption measurement is a very simple method and applicable to the binding study of small molecule substances to protein. In this work, we investigated the UV absorption spectra of HNF and the HNF–HSA complex (see Fig. 3). Clearly, the UV absorption intensity of HSA increased regularly with the variation of HNF concentration, but its maximum peak position and shape almost kept the same. In order to obtain more information on the binding of HNF to HSA, CD and FT-IR were employed to study the conformational change of HSA.

CD is an extraordinarily sensitive technique to monitor the conformational change in the protein. From the change of CD spectra of HSA, we may deduce that there must be a conformational change in HSA. In this work, the 2:1 molar ratio of drug to HSA was used for the CD measurement. The CD spectra of HSA in the absence (line a) and presence (line b) of HNF were shown in Fig. 4, which indicated that the binding of HNF to HSA induced a significant conformational change in HSA. From line a of Fig. 4, it can be seen that free HSA exhibited two significative negative bands located at 208 and 216 nm, which was in accord with the CD

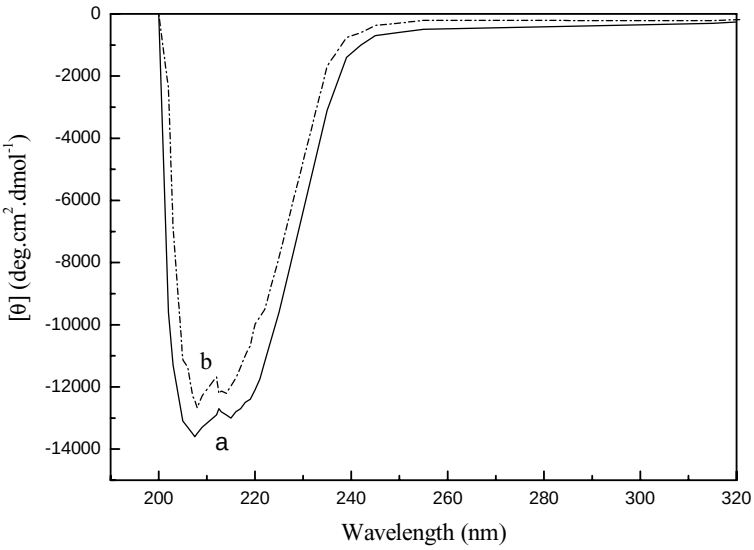

Fig. 4. CD spectra of the HAS–HNF system obtained in 0.05 M Tris–HCl buffer, pH 7.4, ionic strength 0.1, at room temperature; the 2:1 molar ratio of HNF to HSA was used. (a) HSA, 1.0×10^{-6} M; (b) HNF–HSA, the HNF concentration was 2.0×10^{-6} M and the HSA concentration was 1.0×10^{-6} M.

spectrum of HSA containing three conformations (α-helix, β-form, and random coil) [25]. Clearly, the addition of HNF to HSA led to a significant intensity decrease for both negative bands, as shown in line b of Fig. 4. In other words, the binding of HNF to HSA induced the conformational change of HSA. Furthermore, the α-helical contents of HSA in the absence and presence of HNF were calculated by quantitative analysis of the CD spectra data. The CD results were expressed in terms of mean residue ellipticity (MRE) in deg cm^2 dmol^{-1} according to the following equation:

$$\text{MRE} = \frac{\text{observed CD (mdeg)}}{C_\text{p} n l \times 10}$$

where C_p is the molar concentration of the protein, n the number of amino acid residues (585) and l the pathlength (0.2 cm). The α-helical contents of free and combined HSA were calculated from the MRE value at 208 nm using the following equation as described by Lu et al. [26]:

$$\alpha\text{-Helix (\%)} = \left[\frac{-\text{MRE}_{208} - 4000}{33\,000 - 4000}\right] \times 100$$

where MRE_{208} is the observed MRE value at 208 nm, 4000 is the MRE of the β-form and random coil conformation cross at 208 nm, and 33 000 is the MRE value of a pure α-helix at 208 nm.

From above equation, the quantitative analysis results of the α-helix in the secondary structure of HSA were obtained. They differed from that of 60.3% in free HSA to 56.5% in the HNF–HSA complex, which was indicative of the loss of α-helical content. Under physiological conditions, fatty acids are present and normally can stabilize the conformation of HSA; furthermore they occupy various binding sites of HSA [27]. However, under the simulative physiological conditions of this study, the contents of fatty acids are very low and HSA is rather flexible. After added to HSA solution, HNF can bind to HSA and the binding of HNF with HSA was hardly interfered by existing fatty acids. So we concluded that the decrease of α-helical content of HSA was the results of HNF bound to HSA.

To further prove the conformational change of HSA induced by the binding of HNF to HSA, we investigated the FT-IR spectra of HSA and the HNF–HSA complex (see Fig. 5a and b). The FT-IR spectrum of free HSA was acquired by subtracting the absorption of Tris buffer from the spectrum of protein solution, which exhibited the characteristic amide I and amide II absorption bands at 1647 and 1555 cm^{-1}, respectively, as shown in Fig. 5a. The difference spectrum of HSA was obtained by subtracting the spectrum of HNF-free form from that of HNF-bound form, as shown in Fig. 5b. It was clear that amide I and amide II bands shifted from 1647 to 1652 cm^{-1} and 1555 to 1566 cm^{-1}, respectively. In addition, a new peak appeared at 1541 cm^{-1}, indicating that the change of the secondary structure of HSA was attributed to the addition of HNF.

3.2. Binding mechanism, binding constants and the number of binding sites

In order to speculate the fluorescence quenching mechanism, the fluorescence quenching data at different temperatures (300, 310 and 320 K) were firstly analyzed using the classical Stern–Volmer equation [28]:

$$\frac{F_0}{F} = 1 + K_\text{SV}[Q]$$

where F and F_0 are the fluorescence intensities with and without quencher (HNF), respectively, K_SV the Stern–Volmer quenching constant, and $[Q]$ the concentration of quencher (HNF). The K_SV values at 300, 310 and 320 K were 3.3647×10^5, 3.0308×10^5 and 2.6777×10^5 M^{-1}, and correlation coefficients of equation were 0.9926, 0.9949 and 0.9974, respectively. From above results, we can see that the Stern–Volmer quenching constants decreased with increasing temperature, which was consistent with the static type of quenching mechanism. So the possible quenching mechanism between HNF and HSA was suggested as static quenching but not dynamic quenching. That is, HNF bound to HSA and an HNF–HSA complex formed, which resulted in a fluorescence quenching of the fluorophore.

For static quenching, the following equation was employed to calculate the binding constant and the number of binding sites [9]:

$$\lg\left[\frac{F_0 - F}{F}\right] = \lg K + n \lg[Q]$$

where F_0 and F are the fluorescence intensities of HSA in the absence and presence of quencher (HNF), and K and n are the binding constant and the number of binding sites, respectively. Thus, a plot of $\lg[(F_0 - F)/F]$ versus $\lg[Q]$ can be used to determine K as well as n. The binding data (K and n) at different temperatures were presented in Fig. 6 and Table 1. It was found that the binding constant (K) decreased with the increasing of temperature, resulting in a reduction of the stability of the HNF–HSA complex. Meanwhile, from the data of n, we may infer that there was one independent class of binding sites on HSA for HNF. HNF most likely binds to the hydrophobic pocket located in subdomain IIA; that is to say, Trp 214 is near or within the binding site.

3.3. Binding mode

Considering the dependence of binding constant on temperature, a thermodynamic process was considered to be responsible for the formation of the complex. Therefore, the thermodynamic parameters dependent on temperatures were analyzed in order to further characterize the acting forces between HNF and HSA. The acting forces between a small molecule substance and macromolecule mainly include hydrogen bond, van der Waals force, electrostatic force, hydrophobic interaction force, and so on. The thermodynamic

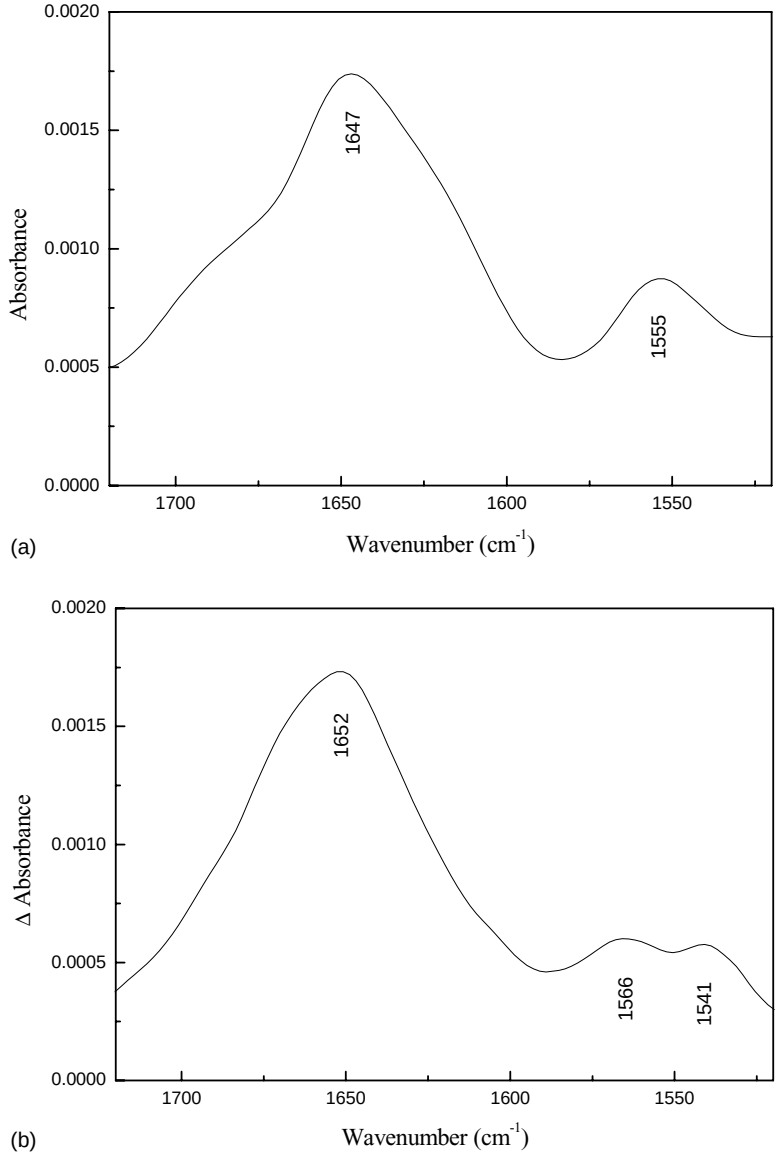

Fig. 5. FT-IR spectra of the HAS–HNF system obtained in 0.05 M Tris–HCl buffer, pH 7.4, ionic strength 0.1, at room temperature; the 3:1 molar ratio of HNF to HSA was used. (a) The FT-IR spectrum of free HSA was acquired by subtracting the absorption of Tris–HCl buffer from the spectrum of protein solution; (b) FT-IR difference spectrum of HSA obtained by subtracting the spectrum of the HNF-free form from that of the HNF-bound form in the region of 1720–1520 cm^{-1} (HSA, 3.0×10^{-5} M; HNF, 9.0×10^{-5} M).

parameters, enthalpy (ΔH°), entropy (ΔS°) and free energy change (ΔG°), are the mainly evidence to estimate the binding mode. According to static quenching equation [9], the binding constants (K_T) at three different temperatures (T) were obtained, and the results were listed in Table 1. These data were also used to calculate the thermodynamic parameters by using the Gibbs–Helmholtz equation [8]:

$$\ln K_T = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}$$

It was seen that there was a good linear relationship between $\ln K_T$ and $1/T$ (see Fig. 7), and the correlation coefficient

Table 1
Binding constants[a] (K), the numbers of the binding sites[a] (n) and thermodynamic parameters for the binding of HNF to HSA

Temperature (K)	K ($\times 10^5$ M^{-1})	n	ΔG° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})	ΔH° (kJ mol^{-1})
300	6.08	1.1	−33.16	6.87	−31.10
310	3.80	1.0	−33.23		
320	2.79	1.0	−33.30		

[a] The values were the mean value of three measurements.

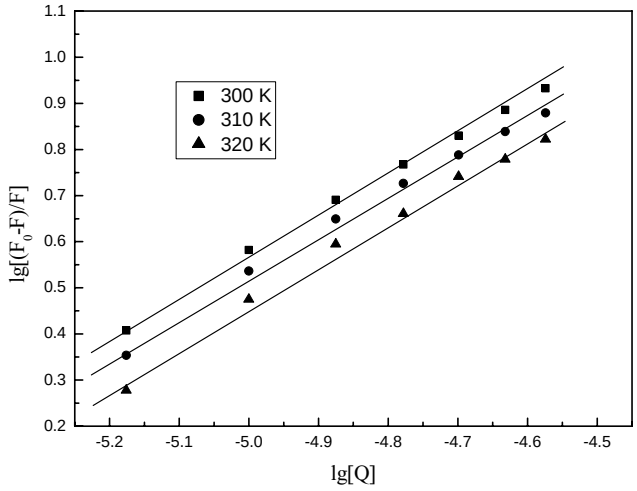

Fig. 6. The Stern–Volmer curves for quenching of HNF to HSA at different temperatures (λ_{ex} = 285 nm; λ_{em} = 342 nm; HNF, from 6.7×10^{-6} to 2.7×10^{-5} M; HAS, 3.0×10^{-6} M).

was 0.9948. Here R is the gas constant, so the values of ΔH° and ΔS° can obtained from the slope and intercept, then the value of ΔG° also can be calculated by the following relation:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

The values of ΔH°, ΔS° and ΔG° were also listed in Table 1. From it, it was seen that the formation of the HNF–HSA complex was an exothermic reaction accompanied with negative enthalpy (ΔH°) and positive entropy (ΔS°) changes. From the point of view of water structure, a positive entropy (ΔS°) value is usually considered as the evidence for hydrophobic interaction. In addition, specific electrostatic interaction between ionic species in aqueous solution is also characterized by a positive ΔS° and a negative ΔH°. In this work, the dissociation constant value of HNF (K_a) was determined as 6.91×10^{-7} (pK_a, 6.16) by using the spectrophotometric method [29]. Additionally, the negative charge density (δ) of HNF was calculated to be 0.95 under the simulative physiological condition (pH 7.4) according to the following equation [30]:

$$\delta = \frac{10^{\mathrm{pH}-\mathrm{p}K_a}}{1 + 10^{\mathrm{pH}-\mathrm{p}K_a}}$$

That is, HNF at pH 7.4 was nearly all ionized, and electrostatic interaction could not be excluded because of the high negative charge density on HNF. So we inferred that the hydrogen bridges between the OH group and Keto group with the protein might be formed due to the ionization of HNF. Generally, ΔG° mainly comes from the large contribution of ΔS° with little contribution from ΔH° for electrostatic interaction [9]. However, in this work, ΔG° was mainly from the contribution of ΔH° but not from ΔS°. Therefore, we inferred that hydrophobic interaction might play a major role in the interaction of HNF with HSA. Meanwhile, two other acting forces, electrostatic force and hydrogen bond, were also involved in the binding process.

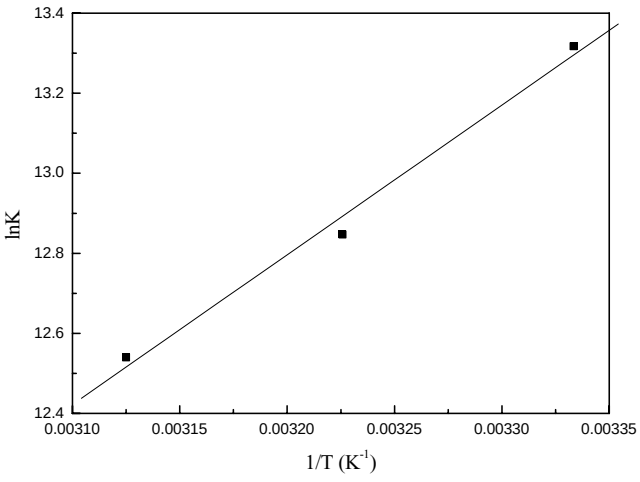

Fig. 7. Temperature dependence of K.

3.4. Energy transfer between HNF and HSA

The fluorescence quenching of HSA after binding with HNF indicated that the energy transfer between HNF and HSA occurred. The importance of the energy transfer in biochemistry is that the efficiency of transfer can be used to evaluate the distance between the ligand and the tryptophan residues in the protein. In this work, the efficiency of energy transfer was studied according to the Förster energy transfer theory [31]. The efficiency of energy transfer, E, is described by the following equation:

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

where F and F_0 are the fluorescence intensities of HSA in the presence and absence of HNF, r the distance between acceptor and donor, and R_0 the critical distance when the transfer efficiency is 50%:

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

where K^2 is the spatial orientation factor of the dipole, N the refractive index of the medium, Φ the fluorescence quantum yield of the donor, J the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. J is given by

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

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. From the above relationships, J and E can be easily obtained; therefore, R_0 and r can be further calculated.

Fig. 8 showed the overlap of the fluorescence spectrum of HSA and the absorption spectrum of HNF when the

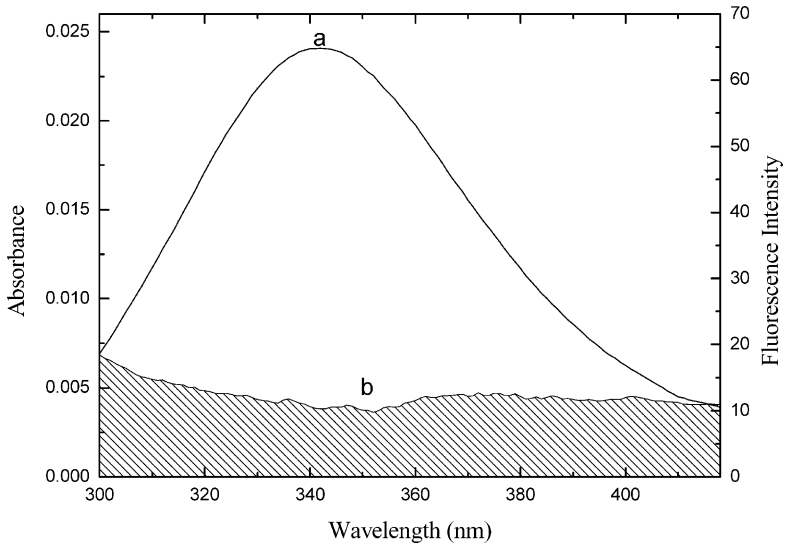

Fig. 8. The overlap of the fluorescence emission spectrum of HSA (a) with the UV absorption spectrum of HNF (b) obtained in 0.05 M Tris–HCl buffer, pH 7.4, ionic strength 0.1, at room temperature. The 1:1 molar ratio of HNF to HSA was used. HSA, 1.0×10^{-6} M; HNF, 1.0×10^{-6} M.

molar ratio of HNF to HSA is 1:1. J was obtained to be $5.3368 \times 10^{-15}\,\mathrm{cm^3\,M^{-1}}$ by integrating the overlap of the UV absorption spectrum of HNF and the fluorescence emission spectrum of HAS, and E was calculated to be 0.2386. It was reported earlier that $K^2 = 2/3$, $\Phi = 0.118$, $N = 1.336$ for HSA [13]. Based on these data, we found $R_0 = 2.21$ nm and $r = 2.68$ nm. So the distance between HNF and tryptophan residue in HSA is 2.68 nm, which is far lower than 7 nm. These accord with conditions of Förster energy transfer theory.

4. Conclusions

The interaction between HNF and HSA was studied by fluorescence spectroscopy combined with UV-Vis, CD, and FT-IR spectrophotometric techniques under simulative physiological conditions for the first time. This study showed that the intrinsic fluorescence of HSA was quenched through static quenching mechanism and HNF most likely bound to the hydrophobic pocket located in subdomain IIA. Experimental results also showed that the binding of HNF to HSA induced a conformational change of HSA, which was further proved by the quantitative analysis data of CD spectra. In addition, we inferred that the binding force was mainly hydrophobic interaction; two other interactions, electrostatic attraction and hydrogen bond were also involved in the binding process. We hope this work will be used as a useful guideline for efficient drug design.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 20275014).

References

[1] R.V. Smalley, D. Goldstein, D. Bullowski, C. Hannon, D. Buchler, C. Knudsen, R.L. Tuttle, Invest. New Drugs 10 (1992) 107–112.
[2] F.F. Becker, C. Mukhopadhyay, L. Hackfeld, I. Banik, B.K. Banik, Bioorgan. Med. Chem. 8 (2000) 2693–2699.
[3] B. K Banik, F.F. Becker, Bioorgan. Med. Chem. 9 (2001) 593–605.
[4] F.F. Becker, B.K. Banik, Bioorgan. Med. Chem. Lett. 8 (1998) 2877–2880.
[5] D.C. Carter, J.X. Ho, Adv. Protein Chem. 45 (1994) 153–203.
[6] F. Herve, S. Urien, E. Albengres, J.C. Duche, J. Tillement, Clin. Pharmacokinet. 26 (1994) 44–58.
[7] V. Henrik, Danish Med. Bull. 46 (1999) 379–399.
[8] J.Q. Liu, J.N. Tian, J.Y. Zhang, Z.D. Hu, X.G. Chen, Anal. Bioanal. Chem. 376 (2003) 864–867.
[9] J.N. Tian, J.Q. Liu, J.Y. Zhang, Z.D. Hu, X.G. Chen, Chem. Pharm. Bull. 51 (2003) 579–582.
[10] R.E. Royer, L. David, V. Jagt, FEBS Lett. 157 (1983) 28–30.
[11] E.B. Thomas, H. Bertil, L. Björn, FEBS Lett. 86 (1978) 25–28.
[12] E.O. Bishop, S.J. Kimber, B.E. Smith, P.J. Beynon, FEBS Lett. 101 (1979) 27–30.
[13] F.L. Cui, J. Fan, D.L. Ma, M.C. Liu, X.G. Chen, Z.D. Hu, Anal. Lett. 26 (2003) 2151–2166.
[14] S. Nahar, R. Carpentier, H.A. Tajmir-Riahi, J. Inorg. Biochem. 65 (1997) 245–250.
[15] A.I. Filyasova, I.A. Kudelina, A.V. Feofanov, J. Mol. Struct. 173 (2001) 565–566.
[16] C. Bertucci, G. Ascoli, G.U. Barretta, L.D. Bari, P. Salvadori, J. Pharmaceut. Biomed. Anal. 13 (1995) 1087–1093.
[17] F. Zsila, Z. Bikádi, M. Simonyi, Biochem. Pharmacol. 65 (2003) 447–456.
[18] C.Q. Jiang, M.X. Gao, X.Z. Meng, Spectrochim. Acta A 59 (2003) 1605–1610.
[19] Y. Yu, Q.L. Li, Anal. Chim. Acta 436 (2001) 147–152.
[20] S. Hu, L. Zhang, J.N. Dovichi, J. Chromatogr. A 924 (2001) 369–375.
[21] S.F. Sun, S.W. Kuo, R.A. Nash, J. Chromatogr. A 288 (1984) 377–388.
[22] L.D. Lei, H.R. Zhang, D.S. Yang, L.M. Wu, Chem. J. Chinese U., in press.

[23] G. Colmenarejo, Med. Res. Rev. 23 (2003) 275–301.
[24] A. Sulkowska, J. Mol. Struct. 614 (2002) 227–232.
[25] J.Q. Liu, J.N. Tian, X. Tian, Z.D. Hu, X.G. Chen, Bioorgan. Med. Chem. 12 (2004) 469–474.
[26] Z.X. Lu, T. Cui, Q.L. Shi, Applications of Circular Dichroism and Optical Rotatory Dispersion in Molecular Biology, 1st ed., Science Press, 1987, pp. 79–82.
[27] S. Curry, P. Brick, N.P. Franks, Biochim. Biophys. Acta 1441 (1999) 131–140.
[28] M.R. Eftink, Fluorescence Quenching Reaction: Probing Biological Macromolecular Structures, Biophysical and Biochemical Aspects of Fluorescence Spectroscopy, Plenum Press, New York, 1991.
[29] X.Y. Zhang, et al., Theory of Chemical Analysis, 1st ed., Science Press, 1991, pp. 407–411.
[30] Wuhan University, Analytical Chemistry, 1st ed., People Press, 1978, pp. 131–132.
[31] T. Förster, O. Sinanoglu (Eds.), Modern Quantum Chemistry, vol. 3, Academic Press, New York, 1966.