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Probing the binding of morin to human serum albumin by optical spectroscopy

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Abstract

Morin [2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one], a member of flavonols, is an important bioactive compound by interacting with nucleic acids, enzymes and protein. Its binding to human serum albumin was investigated by fluorescence quenching, fluorescence anisotropy, and UV–vis absorbance under the simulative physiological condition. Fluorescence quenching data show that the interaction of morin with HSA forms a non-fluorescent complex with the binding constants of 1.394×10^5, 1.489×10^5, 1.609×10^5 and 1.717×10^5 M^{-1} at 292, 298, 303 and 310 K, respectively. The thermodynamics parameters, enthalpy change (ΔH) and entropy change (ΔS) were calculated to be 8.97 kJ mol^{-1} and 129.15 J mol^{-1} K^{-1} via van't Hoff equation. From the spectroscopic results and thermodynamics parameters, it is observed that van der Waals and hydrogen bonds are predominant intermolecular forces when forming the complex. The distance $r = 4.25$ nm between donor (Trp214) and accepter (morin) was estimated based on the Förster theory of non-radiative energy transfer. The red shift of UV–vis absorbance shows that morin is bound to several amino acids on the hydrophobic pocket of HSA. Moreover, the competitive probes, such as warfarin and ibuprofen (site I and II probes, respectively), reveal that the binding location of morin to HSA in the site I of the hydrophobic pocket, which corresponds to the results of UV–vis absorbance, while morin also binds other lower affinity binding sites on HSA from the fluorescence anisotropy spectroscopy.

Keywords: Morin; Human serum albumin; Fluorescence quenching; Fluorescence anisotropy; Competitive probe

1. Introduction

Serum albumin, an abundant protein constituent of blood plasma [1], facilitates the disposition and transportation of various exogenous and endogenous ligands. They are capable of binding an extraordinarily broad range of pharmaceuticals, including fatty acids, amino acids, steroids, metal ions, etc. [2–4]. Much of the clinical and pharmaceutical interest in serum albumin derives from its effects on the drug of pharmacokinetics when bound to serum albumin reversibly. X-ray measurements have revealed that ligands binding to human serum albumin (HSA) are located in hydrophobic cavities in subdomains IIA and IIIA referred to as Sudlow site I and II [5,6]. Identify the binding site location can be aided by examining the competitive binding of probes to HSA. One of the thoroughly characterized ligands of HSA is warfarin (see Fig. 1), whose site is located in the region of subdomain IIA (Sudlow site I). Another known probe, ibuprofen, a non-steroidal anti-inflammatory substance, is bound to HSA in the subdomain IIIA (Sudlow site II).

The polyphenolic compounds of flavonoids occur ubiquitously in foods of plant origin, including flavonols, flavones, flavanones, and isoflavones [7], which have received much attention because of their broad-spectrum pharmacological activities and extensive biological effects [8]. The basic structure of flavonoids is usually characterized by two aromatic rings, ring A and ring B, joined by a three-carbon linked *c*-pyrone ring (ring C), forming a C_6–C_3–C_6 skeleton unity (see Fig. 1) where polar groups, usually hydroxyl, methoxyl, or glycosyl, are appended at various positions [9]. Morin [2-(2,4-dihydroxyphenyl)-3,5,7 trihydroxy-4H-1-benzopyran-4-one], a member of flavonols, is a yellowish pigment found in the old fustic (*Chlorophora tinctoria*) and many Chinese herbs. Based on *in vitro* studies, morin has

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been shown various pharmacological activities, including anti-inflammatory [10], chemoprotective, antimutagenic, antibacterial and antiviral activity [11], antioxidant to against oxyradicals damage [12], and anti-tumor promotion [13,14]. Recently, reporters [15] have demonstrated that morin can be a fairly potent P-gp inhibitor. In addition, morin has also the ability to modulate the activities of metabolic enzymes such as cytochrome P450 (CYPs) [16]. However, there is growing evidence that conjugative metabolism is central to the biological fate of flavonoids. A previous study reported that the serum level of conjugated metabolites of morin was much higher than the parent form after oral administration of morin to rabbits [17]. Conjugated metabolites of flavonoids supposedly possess profoundly different physicochemical properties from their parent forms and therefore might show differences regarding the solubility, transport across cell membrane or intrinsic activity [18]. The plasma concentrations of flavonoids with the best bioavailability profiles range from 1 to 5 mM including monomeric flavonols, flavones and flavanols [19]. However, it is possible that some phenolic compounds accumulate inside specific target tissues causing higher local concentration compared to their plasma concentrations. Because the elimination of polyphenols from the body is generally quite fast, they are being consumed daily in order to maintain high concentrations [20]. Morin exerts its functions usually by regulating the activation or modifying the structure of enzymes, which emphasizes the need to understand the detail of mechanism of morin interacting with macromolecules. High-affinity binding of morin to serum albumin, the most abundant plasma protein, has been reported by Xie et al. [21], who have characterized that the binding of morin to serum albumin was a specific binding site on HSA with the binding constant $1.13 \pm 0.11 \times 10^5\ M^{-1}$ and the protein secondary structures of the reduction of α-helix and β-sheet. However, no experimental information on the localization and nature of morin binding site(s) has been reported. For the various pharmacological activities as a drug in pharmacology, its interaction with human serum albumin deserves a detail investigation. And an insight into binding location of HSA is important to the drugs' pharmacology and pharmacodynamics. The crystallographic HSA interaction with fatty acids, warfarin and so on, creates a solid basis for understanding its interaction with other substance, so it is necessary to study the specific interaction between morin and HSA.

In this work, the fluorescence quenching of the intrinsic tryptophan fluorescence of HSA has been used as a tool to study the interaction of morin with HSA in an attempt to characterize the chemical associations taken place in the simulative physiological condition. Meanwhile, several measurements including fluorescence quenching, fluorescence anisotropy, UV–vis absorption, serve as aids to better understand the mechanism of the drug binding to HSA. The conformational changes of HSA were discussed on the basis of fluorescence spectra and UV–vis absorbance. The binding site of morin to HSA was done by employing the known probe of warfarin and ibuprofen which bind specifically at Sudlow site I and site II, respectively. The results reveal that morin mainly binds to the site I, but interacts with other lower affinity binding sites at high concentration.

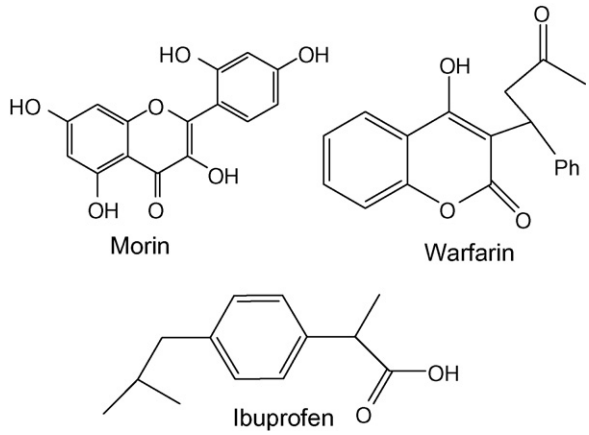

Fig. 1. Structure of morin, warfarin, and ibuprofen.

2. Materials and methods

2.1. Materials

Human serum albumin (HSA, fatty acid free) and morin (≥99%), purchased from Sigma. Chemical Co. Ltd., was used without further purification. Tris–Base had a purity of no less than 99.5%, and NaCl, etc., were all of analytical purity. HSA was dissolved in the buffer of Tris–HCl solution (50 mM Tris–Base, 100 mM NaCl, pH 7.4 ± 0.1). The pH of buffer was adjusted by the solution of HCl or NaOH when the temperature was changed. The concentration of the protein was determined spectrophotometrically using an extinction coefficient ($\varepsilon = 280$) of 36,600 $M^{-1}\ cm^{-1}$. Morin was prepared by absolute dimethyl formamide (DMF) to form 5 mM as the stock solution. Warfarin, obtained from Medicine Co. Ltd. of Jiangshu in China, was prepared by doubly distilled water to form 5 mM solution. Ibuprofen, presented by the company of Hubei Biocause Heilen Pharmaceutical Co. Ltd. of China, was prepared by DMF. All other solutions were prepared with doubly distilled water.

To evaluate the effect of DMF on the quenching of HSA, DMF was considered as a quencher to study the fluorescence quenching of HSA. A 2.0 mL of 10 μM HSA was titrated by DMF which was in the range of concentration 0.1–1.5% (v/v), and the spectra of UV–vis absorbance and fluorescence quenching was studied. There was no change observed from the spectral profiles (data not shown), which suggested no change in HSA conformation and can be considered negligible in the amount used.

2.2. Fluorescence measurements

Fluorescence spectra were measured on an F-2500 Spectrofluorimeter (Hitachi, Japan) equipped with a 1.0 cm quartz cell and a thermostat bath. The excitation wavelength was 295 nm, and the emission spectra was read at 300–450 nm, using 2.5 nm/2.5 nm slit widths. To quantify the binding constants of morin to HSA, a 2.0 mL solution of 10 μM HSA was titrated by successively additions of morin solution using trace syringes (to give a concentration from 0 to 50 μM). Fluorescence quenching

experiments were studied at four different temperature (292, 298, 304, and 310 K) with recycle water keeping the temperature constant. The appropriate blanks corresponding to the buffer were subtracted to correct background of fluorescence. The results obtained were analyzed by using Stern-Volmer equation or modified Stern-Volmer equation to calculate the binding constants.

2.3. UV absorbance measurement

UV–vis absorption spectra of 10 μM of free morin in buffer solution (pH 7.4 ± 0.1, 0.2% of DMF) as well as the UV–vis absorption spectra of morin/HSA varieties of molar ratio complexes were recorded on a TU-1901 UV–Vis spectrometer (Puxi Analytic Instrument Ltd. of Beijing, China) from 200 to 500 nm. To accomplish this, a 2.0 mL solution of 10 μM HSA was titrated by successively additions of morin solution.

2.4. Fluorescence anisotropy measurement

The anisotropy (r) is defined as the difference between the fluorescence intensity emitted parallel and perpendicular ($I_{||}$ and $I_{\perp}$) divided by the total intensity. Fluorescence anisotropy was calculated from fluorescence intensity measurement employing a vertical excitation polarizer and a vertical horizontal emission polarizer according to Eq. (1) [22]:

$$r = \frac{I_{||} - GI_{\perp}}{I_{||} + 2GI_{\perp}} \quad (1)$$

where $I_{||}$ is the intensity of emitted light measured in the direction parallel to excitation, $I_{\perp}$ is the intensity of emitted light measured in the direction perpendicular to excitation and $G = I_{\perp}/I_{||}$ is the instrument grating correction factor. Fluorescence polarization measurement was measured by using the automatic polarization device of a LS50B spectrofluorometer (Perkin-Elmer) equipped with a polarization at room temperature. Excitation and emission bandwidths were all adjusted to 10 nm. Each titration point of the sample equilibration at least four times with an integration time of 1 min was collected.

The fluorescence anisotropy of the interaction between probes and the complexes of morin/HSA was followed at the excitation and emission wavelengths: 390 and 520 nm (the wavelengths of maxima absorption and emission of morin), respectively.

3. Results and discussion

3.1. Interaction of morin with HSA and fluorescence quenching mechanism

Fluorescence quenching is the decrease of quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecules. Fluorescence quenching can be quenched both by collision and by complexes formation with the quencher [23]. Collision (dynamic) and complexes formation (static) can be distinguished by their differing dependence on temperature and viscosity, or preferably by lifetime measurements. In order to illustrate the mechanism of the interaction between morin and HSA, the effect of morin on the fluorescence spectra of HSA was explored and the spectral changes were shown in Fig. 2. Upon additions of morin, the fluorescence intensity of HSA at around 340 nm decreased along with the emission peak of a slight blue shift (from 342 to 337 nm), which indicated that the amino acid residue Trp214 in the binding pocket has been brought to a more hydrophobic environment.

For clarifying the fluorescence quenching induced by morin can be described by the well-known Stern-Volmer Eq. (2):

$$\frac{F_0}{F} = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, k_q is the biomolecular quenching constant, τ_0 is the life time of the fluorescence in absence of quencher, $[Q]$ is the concentration of quencher, and K_{SV} is the Stern-Volmer quenching constant. Herein, Eq. (1) was applied to determine K_{SV} by linear regression of a plot of F_0/F against $[Q]$. In this case, both dynamic and static quenching was involved, which was demonstrated by the fact that the Stern-Volmer plots deviated from linearity toward y-axis at high morin concentrations (Fig. 2). As we known, quenching can also be occurred by a formation between the two compounds that does not fluorescence after returning from excited state and that is due to specific interaction [24]. To verify that quenching was a formation between morin and HSA, it is important to calculate the apparent biomolecular quenching constant. The bimolecular quenching constant was obtained from the plot of Stern-Volmer (Fig. 3a) $K_{SV} = 8.66 \pm 0.03 \times 10^4\ M^{-1}$, which corresponds with the results obtained by Xie [21]. The fluorescence lifetime for HSA was approximately about 5 ns [25], so we can get $k_{dif}^{app} = (1.73 \pm 0.006) \times 10^{13}\ M^{-1}\ s^{-1}$. It can be seen that k_{dif}^{app} is largely higher than the limiting diffusion constant K_{dif} of the biomolecule ($K_{dif} = 2.0 \times 10^{10}\ M^{-1}\ s^{-1}$) [26], which suggest that is a specific interaction. To accurately quantify the

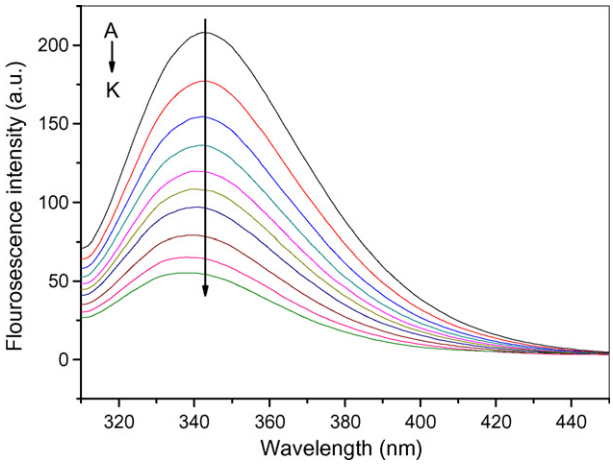

Fig. 2. Effect of morin on fluorescence spectrum of HSA ($T = 298$ K, $\lambda_{ex} = 295$ nm). c(HSA) = 10 μM; c(morin)/(10 μM), (A–K) 0; 0.25; 0.5; 0.75; 1.0; 1.5; 2.0; 2.5; 3.0.

Table 1
Stern-Volmer quenching constants (K_{SV}) of the interaction of morin with HSA at different temperatures

pH	T (K)	$10^{-4}K_{SV}$ (M^{-1})	R[a]	S.D.[b]
7.4	292	9.468 ± 0.037	0.9942	0.050
	298	9.053 ± 0.026	0.9966	0.107
	304	8.325 ± 0.054	0.9953	0.084
	310	7.880 ± 0.041	0.9964	0.065

[a] R is the linear correlated coefficient.
[b] S.D. is standard deviation.

mechanism, the linear range (C_{morin}/C_{HSA} = 0–3.0) of the Stern-Volmer quenching constants was calculated from the slope of the regression curve at different temperature. The result was shown in Table 1. It was observed that the quenching constants are inversely with the temperature, which together with the binding constant (k_{dif}^{app}) revealed that the fluorescence quenching caused by morin was a specific interaction between HSA and morin, and the quenching was mainly arisen from static quenching by the complex formation. At higher concentration of morin, the deviation from the linearity of the Stern-Volmer plot indicated that both dynamic and static quenching was involved as discussed in literature [27]. As discussed above, the quenching mechanism was a specific interaction rather than dynamic collision. The data must be analyzed via modified Stern-Volmer Eq. (3) [28].

$$\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F} = \frac{1}{f_a K_a}\frac{1}{[Q]} + \frac{1}{f_a} \quad (3)$$

where ΔF is the difference of fluorescence in the absence and presence of the quencher at concentration $[Q]$, f_a is the fraction of accessible fluorescence and K_a is the binding constant of morin to HSA. The plots of $F_0/\Delta F$ versus $1/[Q]$ (Fig. 3b) yield the f_a^{-1} as the intercept, and $(f_a K_a)^{-1}$ as the slope. The corresponding results at different temperatures are shown in Table 2. As Table 2 showed, the binding constant decreases with the increasing temperature, a characteristic that coincides with the static type of quenching mechanism.

3.2. Steady-state fluorescence anisotropy: motional information of morin

Fluorescence polarization (anisotropy) is a technique specially applied to study molecular interactions. It gives a direct, nearly instantaneous measure of a tracer's bound/free ratio in solution. Fluorescence polarization is based on the observation of the molecular movement of fluorescent molecules in solution and does not require physical separation of excess ligands or acceptors [29]. Coupling of the chromophore to a larger molecule reduces its mobility resulting in an increase of anisotropy [30]. This characteristic can be used to study the microenvironment on the dynamic properties of the molecules when accessed to biomolecules, and hence it can be exploited to assess the motional information in the surface cavity of the biomolecules.

We have monitored the fluorescence anisotropy of morin saturation on binding to HSA as a function of protein concentration. Fig. 4 presents the variation of the fluorescence anisotropy (r) of the morin (510 nm emission and 390 nm excitation) as a function of protein concentration for HSA. The plot shows a marked increase in the anisotropy value with the increasing concentration of HSA until the molar ratio 1.2 (morin/HSA), which suggests an imposed motional restriction on the fluorophore in the microenvironments of HSA. The overall dimension of the protein-bound probe is logically much larger than that of the unbound species itself. This leads to a marked reduction in the tumbling motion for the former, resulting in an increase in the anisotropy value [31]. The result further reveals that the high anisotropy value (r = 0.29) suggests that morin is bound to a motional restricted site on HSA [32], whose binding site will discuss subsequently.

3.3. The binding interaction force between morin and HSA

Ligands integrating with biomolecules are usually determined by several binding interactions, including hydrophobic and electrostatic interactions, van der Waals forces, hydrogen bonds and covalent bonds between ligands and biomolecules. The thermodynamic parameters, the enthalpy (ΔH) and entropy (ΔS) of binding reaction, are important to confirm the force of interactions of ligands with biomolecules. In order to investigate the mode of the drug binding to HSA, the temperature-dependence of binding constants was studied at four different temperatures (292, 298, 304, and 310 K). The slope of a plot of the biomolecular binding constant versus $1/T$ (T, absolute temperature) are linear within experimental error (Fig. 5), which allows one to calculate the energy change for the binding process.

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

$$\Delta G = \Delta H - T\,\Delta S = -RT\,\ln K \quad (5)$$

where constants K are K_a at the corresponding temperature (the temperatures used were 292, 298, 304 and 310 K). The enthalpy

Table 2
Modified Stern-Volmer association constant K_a and relative thermodynamic parameters at pH 7.40

T (K)	$10^{-5}K_a$ (M^{-1})	R[a]	S.D.[b]	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
292	1.394	0.9999	0.026	8.97	−28.74	129.15
298	1.489	0.9998	0.012		−29.52	
304	1.609	0.9997	0.049		−30.29	
310	1.717	0.9998	0.034		−31.07	

[a] R is the linear correlated coefficient.
[b] S.D. is standard deviation.

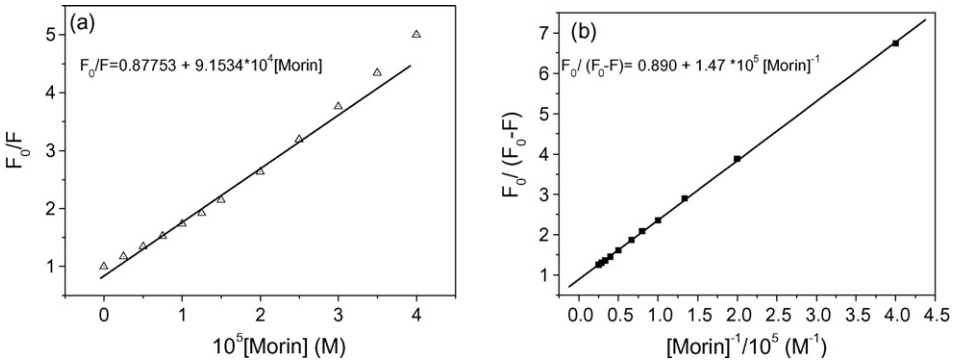

Fig. 3. (a) Stern-Volmer curve and (b) modified Stern-Volmer curve of protein fluorescence quenching treated with different concentrations of morin in the drug to protein molar ratios ranged from 0 to 4 (a) and from 0 to 3 (b), respectively; c(HSA) = 10 μM, λ_{ex} = 295 nm, at 298 K.

is considered as a constant when the temperature of system is changed a little. According to the Eqs. (4) and (5), we can obtain the value of ΔH, ΔG and ΔS shown in the Table 2. From the results, it can be observed that the interaction process is spontaneous due to the negative free energy change. The exothermic reaction of the morin with HSA has both a positive of enthalpy (ΔH) and entropy (ΔS) values. Ross and Subramanian [33] have characterized the sign and magnitude of the thermodynamic parameter various individual interactions that may take place in the protein of association processes based only upon calorimetrically determined enthalpy changes. From the point of view, the positive ΔH and ΔS value is frequently taken as mainly entropy driven and the enthalpy is unfavorable for it, the hydrophobic forces played major role in the reaction. From the structure of morin (Fig. 1), aromatic hydrocarbon was easily integrating into the hydrophobic pocket of HSA.

3.4. Energy transfer between HSA and morin

The main binding regions of HSA are located in subdomains IIA and IIIA as elucidated by the crystallography study of X-ray diffraction for several ligands [5,6,34]. HSA has one tryptophan (Trp214) in subdomain IIA, we can estimate the drug binding site of distance between the site and the fluorophore (Trp214) according to Förster theory of molecular resonance energy transfer [35].

In addition to radiation and reabsorption, the Förster theory of molecular resonance energy transfer points out that a transfer of energy could also take place through direct electrodynamic interaction between the primarily excited molecule and its neighbors. According to this theory, the efficiency of energy transfer between the donor and acceptor, E, is given by Eqs. (6) and (7) in Ref. [36] and $K^2 = 2/3$ was employed for the random orientation as in fluid solution, which provide a reasonably good estimate for this system because the tryptophan chromophore rotates very quickly in protein [37]. The overlap of the absorption spectra of the morin with the fluorescence emission spectra of HSA was shown in Fig. 6. In the present case, $N = 1.36$, $\varphi = 0.074$ [38], we could calculate that $R_0 = 3.5$ nm, and $r = 4.25$ nm, which is consistent with the previous report [39].

The donor-to-acceptor distance $r < 8$ nm, and $0.5R_0 < r < 1.5R_0$, indicated that the energy transfer from HSA to morin occurred with high possibility, which also can explain the efficient quenching of the tryptophan fluorescence. The distance of the flavonoids derivatives as probes binding to human serum albumin had been reported previously. Sytnik and Litvinyuk [40] have been calculated the distance of

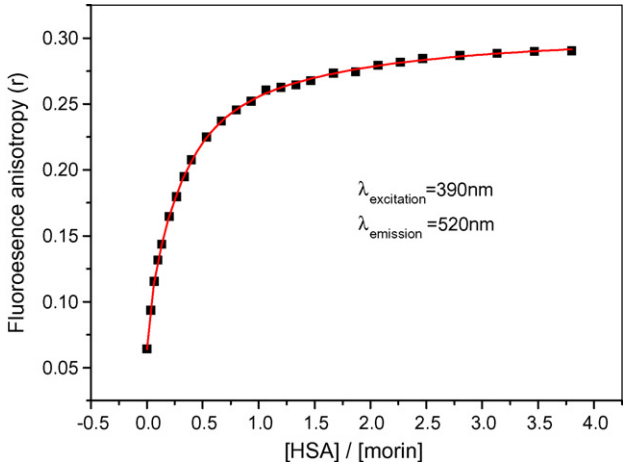

Fig. 4. Fluorescence anisotropy (r) measurements of morin binding to HAS ([morin] = 10 μM; λ_{ex} = 390 nm, λ_{em} = 520 nm; pH 7.4; at room temperature). Each data point indicates the average of three determinations.

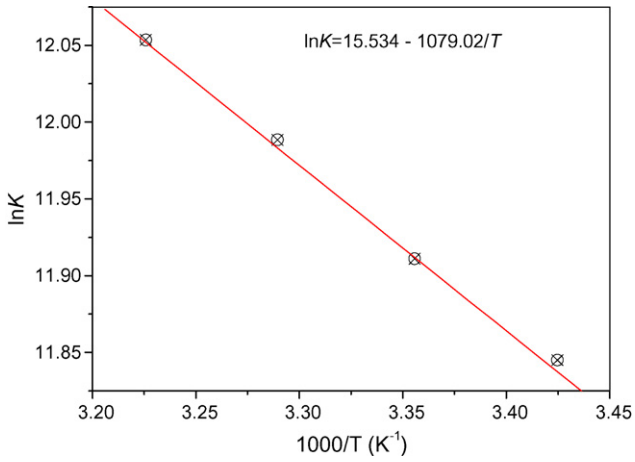

Fig. 5. van't Hoff plot, pH 7.40, c(HSA) = 10 μM.

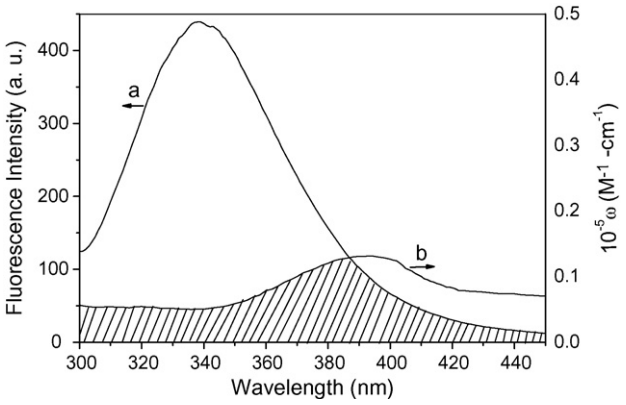

Fig. 6. Spectral overlap of HSA fluorescence (a) with morin absorption (b). c(HSA) = c(morin) = 10 μM.

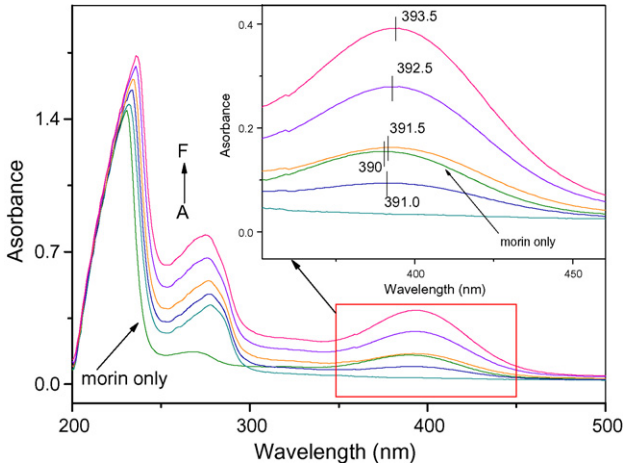

Fig. 7. UV–vis absorbance spectra of HSA in the absence and presence of morin. (A) Morin only c(morin) = 10 μM; (B–F) c(HSA) = 10 μM, c(morin)/(10 μM): 0, 0.5, 1, 1.5, 2.0. The inset shows the enlarger of the chosen area.

Trp214-3-hydroxyflavone based on Förster theory by means of the quantum yield of human serum albumin and its binding to 3-hydroxyflavone complexes. The value of Förster radius for the Trp214–3-HF anion donor–acceptor pair was determined to be 2.54 nm, indicating the 2.55 nm separation between Trp-214 and 3-HF anion. However, Xie and co-workers [41] have been calculated the flavonoids derivatives of myricetin according to the molecular modeling method, who have been obtained three rings binding to the amino acid residues and the binding distance of Trp214 to the drug was only 0.640 nm. Therefore, the calculated distance of drug-Trp214 may just tell us a range of distances between probes and serum albumin, which was affected by many factors when calculated according to Förster theory including the method.

3.5. Conformation investigation

When drugs interacted with a globular protein, the intramolecular forces that are responsible for maintaining the secondary or tertiary structures can be altered, and resulted in a protein conformational change. A valuable feature of intrinsic fluorescence of protein is the high sensitivity of the chromophore to its local environment. The intrinsic fluorescence of protein can provide information about protein conformational transitions, subunit association, substrate binding or denaturation. Brustein et al. [42] considered that maximum emission wavelength (λ_{max}) of the tryptophan residues is relative to the polarity of microenvironment and the values of λ_{max} are 331, 334 and 341 nm when the tryptophan residues located in hydrophobic medium, exposure in water partly and completely. The changes of maximum emission wavelength of the tryptophan residues will reflect the conformation changes of HSA. From Fig. 2, it was observed that the emission of HSA has a little blue shift of up to 8.5 nm (from 342 to 334 nm) with increasing morin concentration, which a blue shift indicates a less polar (or more hydrophobic) environment of tryptophan residue [43].

To further verify the structural change of HSA when exposed to morin, we measured the UV/vis absorbance spectra (Fig. 7) of HSA with various amounts of morin. The absorption spectra of the HSA of the tryptophan (Trp214) are sensitive to the microenvironment surrounding the chromophore. It can be seen from Fig. 7 that the UV absorption band I (300–450 nm) of morin had an obvious red shift upon binding to HSA owing to the microenvironment of morin changed, which suggested a specific interaction together with a complex formation between the drug and protein [44]. The absorption peaks in the visible region showed a red shift (from 391.0 to 393.5 nm, see the inset of Fig. 7) indicating the increase of the conjugation of phenyl ring with the residues of HSA [45]. A small red shift of nearly 2.5 nm observed in the position of the absorption maxima suggests that the energy gap between the ground and excited states of the chromophore was reduced by a small amount [46]. Therefore, the observed spectral changes with morin provide strong evidence for the increased hydrophobicity of the drug microenvironment [47], consistent with the fluorescence quenching observed. According to the absorption of the reports, the OH group on the cinnamoyl part of morin molecule was dissociated nearly completely in the physiological condition (pH 7.4) existed as an anionic species. However, the –OH groups of morin had not be disassociated when it interacts with protein [21]. As a result, the hydrophobic forces played major role in the binding force between morin and HSA, but not the electrostatic interactions.

3.6. Identification of the binding sites

The topology of human serum albumin is heart-shaped, with three of the repeating helical domains I, II and III, and each domain was quantified to consist of two subdomains A and B. Most of ligands binding sites on HSA were shown to be located in subdomains IIA and IIIA, although numerous other low-affinity sites also exist. In the subdomains IIA and IIIA regions are characterized by a hydrophobic surface on one side, and a positively charged surface on the other, which allow them to specifically bind negatively charged heterocyclic ligands of average size and small aromatic carboxylic acids, respectively. Drug site I, where warfarin binds, has been characterized as a conformationally adaptable region subdomain

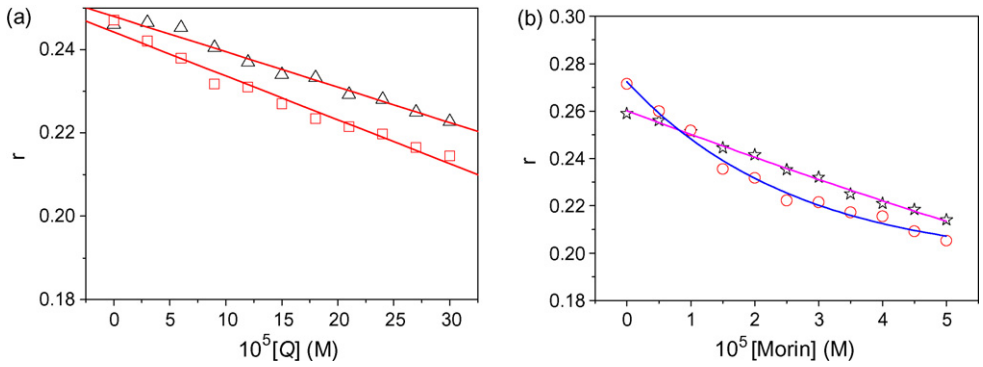

Fig. 8. (a) Effect of competitive probes ([*Q*] represents the ibuprofen or warfarin) on the fluorescence anisotropy values of the morin–HSA complex. (b) Effect of morin on the fluorescence anisotropy values of the competitive probes (ibuprofen or warfarin)–HSA complexes (the molar ratio of protein to ligand 1:1). (triangle △, ibuprofen was titrated into the morin–HSA complex; square □, warfarin was titrated into the morin–HSA complex; round ◯, morin was titrated into the warfarin–HSA complex; pentacle ☆, morin was titrated into the ibuprofen–HSA complex; [*Q*] represents the concentration of ibuprofen or warfarin; [HSA] = 10 μM; λ_{ex} = 390 nm, λ_{em} = 520 nm; pH 7.4; at the room temperature). Each data point indicates the average of three determinations.

IIA (site I) which have been studied by X-ray crystallography [48].

Competitive binding of probes to serum albumin, such as warfarin (site I) or ibuprofen (site II) is often used to probe an unknown ligand of the binding region on serum albumin. The binding constants of the high-affinity site are in the range 8.9×10^4–$3.4 \times 10^5\ M^{-1}$ for warfarin binding to HSA, 3.0×10^5–$3.6 \times 10^6\ M^{-1}$ for racemic ibuprofen [49]. Most reports observed that both warfarin and ibuprofen possesses one high-affinity sites and several low-affinity binding sites on the domains of HSA [50]. Moreover, the high-affinity sites and low-affinity binding sites are both located in site I for warfarin, but the high-affinity sites and low-affinity binding sites for ibuprofen lie in site II and site I regions, respectively. In general, the molar ratio of ligand/protein not larger than 1, the ligand is mainly bound to its HAS but otherwise, it begins to bind to its low-affinity binding sites [51]. The probes of warfarin and ibuprofen which bind to the site I and site II of HSA, respectively, competing with morin in binding to HSA were investigated by the fluorescence anisotropy [52,53]. The fluorescence anisotropy can get the movement of morin in protein solution in absence and presence of probes [54].

We first examined the change of the fluorescence anisotropy when probes (warfarin or ibuprofen) were added to the binary mixture of 10 μM morin with 10 μM HSA. As we can see from Fig. 8a, the anisotropy value of equimolar concentrations (10 μM) of morin and human serum albumin (the excitation and emission wavelengths: 390 and 520 nm), was dependent on the concentration of morin, which indicates that the morin binding site on HSA is not altered or replaced by two known probes. From the binding constant of morin (1.489×10^5 M, T = 298 K), we can put forward that the high affinity of drugs binding to the known region of human serum albumin is not easy to be displaced by competitive probes. To further clarify these differences, we considered morin as a probe to compete with the binary mixture of warfarin–HSA or ibuprofen–HSA. It is observed that, the anisotropy value of equimolar concentrations (10 μM) of warfarin–HSA complex was quickly decreased approximately 17% (the anisotropy value (r) decrease from 0.27 to 0.23) in the molar ratio (morin/warfarin) 1.5 (Fig. 8b) when morin was added into the warfarin–HSA complex, while the change of the anisotropy value of ibuprofen–HSA complex was caused by a concentration-dependence. Putting the facts that the interaction is slightly exothermic [55], and different enantioselectivities [56], it seems that morin does bind at the region of site I (subdomain IIA). As discussed in the Section 3.5, morin binding to HSA enter into a more hydrophobicity of microenvironment and the site I was also a hydrophobic microenvironment, so we can come to a conclusion that morin bound to the site I of HSA, which consistent with other reports of the flavonoids derivatives of quercetin [57], myricetin [40,41] binding region (subdomain IIA). However, morin affected warfarin–HSA complex may reason that the similarly aromatic structures and the interaction force of the hydrophobic forces. It would therefore appear that the binding of such compounds occurs at several sites on the protein, but the structural constraints on favorable accommodation of morin within the binding site I. Considering the results discussed presented above, we can put forward that morin, under the condition used in this work, only binds to one, or predominately to one site on the HSA and the hydrophobic forces play an important role in the binding energy.

4. Conclusions

Fluorescence method is an important tool for the investigation of the interaction between small probe molecule and protein. The fluorescence of HSA is mainly originated from the tryptophan residues, which can be quenched by morin, and the results showed that a non-fluorescent complex was a major part in the fluorescence quenching, but both static and dynamic occur together with complex formation at high concentration. The binding process was exothermic, enthalpy driven and spontaneous, as indicated by the thermodynamic parameters analyzed, and the major part of the binding force is van der Waals interaction and H-bonds. The competitive probes revealed that morin may be located in the region of site I (subdomain IIA). Also, the possibility of the competitive probes in determination of the binding on the HSA using the fluorescence anisotropy should be pursued based on more comprehensive and in-depth investigation of the basic mechanisms, on which

a precise method for determining the sites of HAS should be further studied.

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References

[1] C.V. Kumar, A. Buranaprapuk, Angew. Chem. Int. Ed. Engl. 36 (1997) 2085–2087.
[2] G. Colmenarejo, Med. Res. Rev. 23 (2003) 275–301.
[3] S. Sugio1, A. Kashima, S. Mochizuki, M. Noda, K. Kobayashi, Protein Eng. 12 (1999) 439–446.
[4] A. Molla, S. Vasavanonda, G. Kumar, H.L. Sham, M. Johnson, B. Grabowski, J.F. Denissen, W. Kohlbrenner, J.J. Plattner, J.M. Leonard, D.W. Norbeck, D.J. Kempf, Virology 250 (1998) 255–262.
[5] G. Sudlow, D.J. Birkett, D.N. Wade, Mol. Pharmacol. 11 (1975) 824–832.
[6] S. Curry, H. Mandelkow, P. Brick, N. Franks, Nat. Struct. Biol. 5 (1998) 827–835.
[7] D.K. Das, Methods Enzymol. 234 (1994) 410–420.
[8] G.G. Duthic, S.J. Duthie, J.A.M. Kyle, Nutr. Res. Rev. 13 (2000) 79–106.
[9] J.B. Harborne, C.A. Williams, Phytochemistry 55 (2000) 481–504.
[10] S.H. Fang, Y.C. Hou, W.C. Chang, S.L. Hsiu, P.D. Chao, B.L. Chiang, Life Sci. 74 (2003) 743–756.
[11] H.K. Kim, B.S. Cheon, Y.H. Kim, S.Y. Kim, H.P. Kim, Biochem. Pharmacol. 58 (1999) 759–765.
[12] D.P. Makris, J.T. Rossiter, J. Food Composit. Anal. 15 (2002) 103–113.
[13] Y. Iwase, Y. Takemura, M. Ju-ichi, T. Mukainaka, E. Ichiishi, C. Ito, H. Furukawa, M. Yano, H. Tokuda, H. Nishino, Cancer Lett. 173 (2001) 105–109.
[14] K. Kawabata, T. Tanaka, S. Honjo, M. Kakumoto, A. Hara, H. Makita, N. Tatematsu, J. Ushida, H. Tsuda, H. Mori, Int. J. Cancer 83 (1999) 381–386.
[15] S. Zhang, M.E. Morris, J. Pharmacol. Exp. Ther. 304 (2003) 1258–1267.
[16] P. Hodek, P. Trefil, M. Stiborova, Chem. Biol. Interact. 139 (2002) 1–21.
[17] S.L. Hsiu, T.Y. Huang, Y.C. Hou, D.H. Chin, P.D.L. Chao, Life Sci. 70 (2002) 1481–1489.
[18] C.Y. Hsiang, S.L. Wu, T.Y. Ho, Biochem. Pharmacol. 69 (2005) 1603–1611.
[19] C. Manach, A. Scalbert, C. Morand, C. Remesy, L. Jimenez, Am. J. Clin. Nutr. 79 (2004) 727–747.
[20] J. Alvesalo, H. Vuorela, P. Tammela, M. Leinonen, P. Saikku, P. Vuorela, Biochem. Pharmacol. 71 (2006) 735–741.
[21] M.X. Xie, M. Long, Y. Liu, C. Qin, Y.D. Wang, Biochim. Biophys. Acta 1760 (2006) 1184–1191.
[22] K.L. Bentley, L.K. Thompson, R.J. Klebe, P.M. Horowitz, BioTechniques 3 (1985) 356–366.
[23] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Press, New York, 1999, pp. 695–698.
[24] A. Papadopoulou, R.J. Green, R.A. Frazier, J. Agric. Food Chem. 53 (2005) 158–163.
[25] E.L. Gelamo, C.H.T.P. Silva, H. Imasato, M. Tabak, Biochim. Biophys. Acta 1594 (2002) 84–99.
[26] W.R. Ware, J. Phys. Chem. 66 (1962) 455–458.
[27] Y.L. Wei, J.Q. Li, C. Dong, S.M. Shuang, D.S. Liu, C.W. Huie, Talanta 70 (2006) 377–382.
[28] S. Lehrer, Biochemistry 10 (1971) 3254–3263.
[29] W.Y. He, Y. Li, H.Z. Si, Y.M. Dong, F.L. Sheng, X.J. Yao, Z.D. Hu, J. Photochem. Photobiol. A: Chem. 182 (2006) 158–167.
[30] J. Kusba, J.R. Lakowicz, J. Chem. Phys. 111 (1999) 89–99.
[31] A. Chakrabarty, A. Mallick, B. Haldar, P. Das, N. Chattopadhyay, Biomacromolecules 8 (2007) 920–927.
[32] Y. Li, W.Y. He, H.X. Liu, X.J. Yao, Z.D. Hu, J. Mol. Struct. 831 (2007) 144–150.
[33] D.P. Ross, S. Subramanian, Biochemistry 20 (1981) 3096–3102.
[34] D.C. Carter, J.X. Ho, Adv. Protein Chem. 45 (1994) 153–203.
[35] T. Förster, Ann. Phys. 2 (1948) 55–75.
[36] Z.D. Qi, B. Zhou, X. Qi, C. Shi, Y. Liu, J. Dai, J. Photochem. Photobiol. A: Chem. 193 (2007) 81–88.
[37] Y.V. Il'ichev, J.L. Perry, J.D. Simon, J. Phys. Chem. 106 (2002) 452–459.
[38] Y.J. Hu, Y. Liu, Z.B. Pi, S.S. Qu, Bioorg. Med. Chem. 13 (2005) 6609–6614.
[39] W. He, Y. Li, C. Xue, Z. Hu, X. Chen, F. Sheng, Bioorg. Med. Chem. 13 (2005) 1837–1845.
[40] A.I. Sytnik, I. Litvinyuk, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 12959–12963.
[41] C. Qin, M.X. Xie, Y. Liu, Biomacromolecules 8 (2007) 2182–2189.
[42] E.A. Brustein, N.S. Vedenkina, M.N. Irkova, Photochem. Photobiol. 18 (1973) 263–279.
[43] X.M. He, D.C. Carter, Nature 358 (1992) 209–215.
[44] F. Zsila, Z. Bikadi, M. Simonyi, Biochem. Pharmacol. 65 (2003) 447–456.
[45] Y. Chen, T. Xu, X. Shen, H. Gao, J. Photochem. Photobiol. A: Chem. 169 (2005) 123–129.
[46] C.V. Kumar, L.M. Tolosa, J. Phys. Chem. 97 (1993) 13914–13919.
[47] C.V. Kumar, A. Buranaprapuk, J. Am. Chem. Soc. 121 (1999) 4262–4270.
[48] I. Petitpas, A.A. Bhattacharya, S. Twine, M. East, S. Curry, J. Biol. Chem. 276 (2001) 22804–22809.
[49] D.E. Epps, T.J. Raub, F.J. Kézdy, Anal. Biochem. 227 (1995) 342–350.
[50] S. baroni, M. Mattu, A. Vannini, R. Cipollone, S. Aime, P. Ascenzi, M. Fasano, Eur. J. Biochem. 268 (2001) 6214–6220.
[51] Y. Ni, S. Su, S. Kokot, Anal. Chim. Acta 580 (2006) 206–215.
[52] R. Dagher, C. Pigault, D. Bonnet, D. Boeglin, C. Pourbaix, M.C. Kilhoffer, P. Villa, C.G. Wermuth, M. Hibert, Biochim. Biophys. Acta 1763 (2006) 1250–1255.
[53] J.C. Owicki, J. Biomol. Screen. 5 (2000) 297–306.
[54] C. Dufour, O. Dangles, Biochim. Biophys. Acta 1721 (2005) 164–173.
[55] V. Maes, Y. Engelborghs, J. Hoebeke, Y. Maras, A. Vercruyysse, Mol. Pharmacol. 21 (1981) 100–107.
[56] T.A.G. Noctor, C.D. Pham, R. Kaliszan, I.W. Wainer, Mol. Pharmacol. 42 (1992) 506–511.
[57] B. Sengupta, P.K. Sengupta, Biochem. Biophys. Res. Commun. 299 (2002) 400–403.