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ORIGINAL PAPER

Transport of a Cancer Chemopreventive Polyphenol, Resveratrol: Interaction with Serum Albumin and Hemoglobin

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Abstract Resveratrol is a natural phytoalexin with pharmacologic effects on several human diseases: carcinogenesis, coronary heart disease and neurodegenerative disease. Due to its poor water solubility, resveratrol must be bound to proteins to keep it at a high concentration in serum. In our work, the bindings of resveratrol to plasma proteins, human serum albumin (HSA) and hemoglobin (Hb), have been investigated systematically by fluorescence quenching technique, synchronous fluorescence, UV–vis absorption spectroscopy, circular dichroism (CD) spectroscopy and molecular modeling method. The fluorescence data show that the binding of resveratrol to HSA or Hb is a static quenching procedure and each protein has only one binding site for the drug. The binding constant of resveratrol to HSA is larger than that of resveratrol to Hb at corresponding temperature, which indicates that the affinity of HSA toward the drug is higher than that of Hb. The CD spectroscopy indicates that the secondary structures of the proteins are changed in the presence of resveratrol with the reduction of α-helices, which decreased about 18.75% for HSA and 9.43% for Hb at the drug to proteins molar ratio of 2. Thermodynamic analysis and molecular modeling suggest that hydrophobic interaction plays a major role in the binding of resveratrol to HSA, and hydrogen bonding is the mainly binding force in the binding of resveratrol to Hb. The study of molecular modeling shows that resveratrol is located in the hydrophobic cavity between subdomain IB and IIA of HSA (the entrance of site I), or located in the central cavity of Hb (partial to the subunit A).

Keywords Resveratrol · Human serum albumin · Hemoglobin · Fluorescence quenching · Circular dichroism · Molecule modeling

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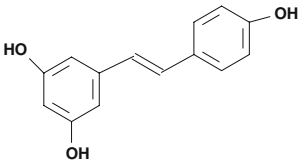
Introduction

Resveratrol (3, 4′, 5-trihydroxystilbene) (Fig. 1) is a naturally occurring phytoalexin synthesized in response to injury or fungal attack [1]. It has been found in at least 72 plant species, a number of which are dietary components, such as mulberries, peanuts, and grapes [1, 2]. Resveratrol exists in two isomeric forms as biologically active *trans*-resveratrol and inactive *cis*-resveratrol. Recently resveratrol attracted great interest in the science due to the "French paradox" (despite the fat-rich diets, mortality from coronary heart disease is lower in French than in the other countries due to the moderate consumption of red wine) [3]. Epidemiological studies have suggested that resveratrol is one of red wine's active ingredients responsible for the decreased coronary heart disease mortality [4]. Resveratrol has been found to display many pharmacologic effects, including modulator lipoprotein metabolism inhibiting the oxidation of low-density lipoproteins [5, 6] and inhibition either platelet aggregation or proatherogenic eicosanoids production by human platelets and neutrophils [7]. Jang et al. [8] first reported that resveratrol has strong anti-carcinogenesis effects and block the carcinogenesis stages of initiation, promotion, or progression. Since then, a lot of research results have confirmed that resveratrol indeed has potent

Fig. 1 Structure of *trans*-resveratrol



anti-carcinogenesis effects on a variety of cancer types. Although the biological positive effects of resveratrol are largely admitted, little is known about its transport and the distribution in the body. Resveratrol has poor water solubility [9], so it must be bound to proteins to keep it at a high concentration in plasma. The efficiency of a therapeutic substance is related to its affinity to bind protein transporters [10]. In the transport of resveratrol, it can bind to serum proteins [11] and can passively diffuse through the plasma membrane [12]. Fiorani et al. [13] reported that quercetin could penetrate the plasma membrane by passive diffusion and bind to hemoglobin, so red blood cells could accumulate large amounts of the drug and act as natural flavonoid reservoir. In order to gain a better understanding of resveratrol transport, the interaction of resveratrol with serum albumin and hemoglobin was investigated in this work.

Experimental

Materials

Resveratrol, HSA and bovine hemoglobin (Hb) were purchased from Sigma (St. Louis, MO, USA). The purity of resveratrol was 99% according to the manufacture. The stock solution of resveratrol was prepared in 50% DMSO and kept in the dark at 277 K, the molar concentration was based on its molecular weight of 228. HSA and Hb solution were prepared immediately in phosphate buffer solution (pH 7.4) before use and the molar concentrations were based on their molecular weights of 66,500 Da and 67,000 Da, respectively. All reagents were of analytical reagent grade. Water from a Milli Q system apparatus (Millipore, USA) was used throughout the experiments.

Apparatus

UV–vis absorption spectra were recorded on Cary-100 UV–vis spectrophotometer (Varian, USA) with 1.0 cm quartz cell. Fluorescence spectroscopy measurements were performed on F-4500 fluorescence spectrophotometer (Hitachi, Japan) equipped with a thermostatically controlled cell holder and a 1.0 cm quartz cell. Excitation and emission slit widths were 5 nm for HSA and 10 nm for Hb. The scan speed was 240 nm min^{-1}. CD spectra were recorded on JASCO J-820 spectropolarimeter (Japan Spectroscopic, Japan) using 0.1 cm cell at 0.2 nm intervals, the bandwidth was 5.0 nm and the scan speed was 60 nm min^{-1}.

Procedures

UV–vis measurements

The UV–vis measurements of HSA and Hb in the presence or absence of resveratrol were made in the range of 190–300 nm at 298 K. Protein concentration was fixed at 2.0×10^{-6} mol l^{-1} while the drug concentration was varied from 0.8×10^{-6} mol l^{-1} to 4.0×10^{-6} mol l^{-1}. As a control, the UV–vis spectra of buffer titrated with resveratrol, were also measured and subtracted from the spectra of the samples.

Fluorescence quenching measurements

The excitation wavelength of 280 nm was used, and the emission spectra were recorded from 290 to 410 nm at three temperatures (288, 298 and 308 K). Titration quenching experiments were carried out as follows: A 3 ml protein sample (2.0×10^{-6} mol l^{-1}) was placed in a quartz cell and was titrated by successive additions of resveratrol (concentration from 1.0 to 7.0×10^{-6} mol l^{-1}). The overall dilution did not exceed 3.5%. All solutions were mixed thoroughly and kept 10 min before measurements.

CD measurements

The CD measurements of HSA and Hb in the presence or absence of resveratrol were made in the range of 200–260 nm with three scans averaged for each CD spectrum. HSA or Hb concentration was fixed at 2.0×10^{-6} mol l^{-1} while the molar ratio of proteins to drug concentration was 1:1 and 1:2.

Molecular docking

Molecular docking simulations of HSA–resveratrol and Hb–resveratrol were performed with AutoDock 3.05 program [14]. The crystal structure of proteins and resveratrol were from the Brookhaven Protein Data Bank (entry codes: 1h9z for HSA, 1g09 for bovine Hb and 1dvs for resveratrol). The structure of HSA and bovine Hb were assigned with Kollman charges. The geometries of these compounds were optimized using the software AutoDockTools [15] with Gasteiger charges. The Lamarckian genetic algorithm was used to calculate the interactions. Modified docking parameters were set as follow [14]: the number of individuals in the population is 150 and the number of energy evaluations is 10 million. One hundred calculating

cycles were performed. The docked complexes were selected according to the criteria of interacting energy combined with geometrical matching quality.

Results and discussion

UV–vis absorption spectra

Figure 2 shows the UV–vis absorption spectra of HSA and Hb in the absence or presence of resveratrol. HSA and Hb have strong absorbance with a peak at about 208 nm and it represents the content of α-helix structure of proteins [16]. The absorbance of HSA and Hb decreased with the addition of resveratrol. Furthermore, the addition of resveratrol results in the distinct red shifts of maximum peak position (from 208 to 219 nm). These two results indicate that there are interactions between resveratrol with HSA or Hb.

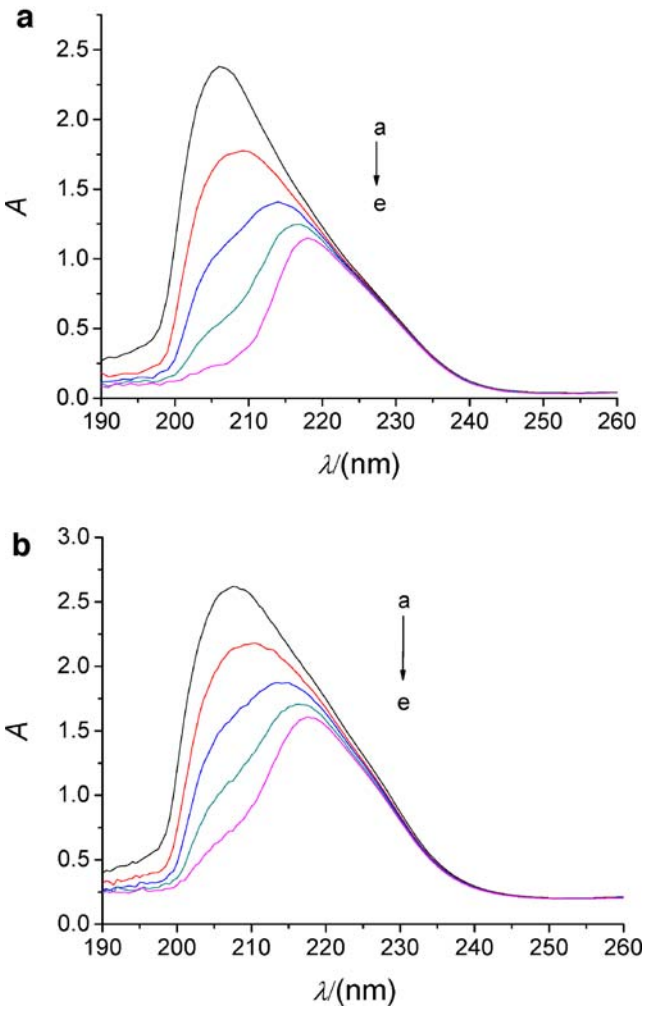


Fig. 2 UV–vis absorption spectra of HSA (**a**) and Hb (**b**) in the presence of resveratrol at 298 K. Concentration of resveratrol: (*a*) 0; (*b*) 0.8×10^{-6}; (*c*) 1.6×10^{-6}; (*d*) 2.4×10^{-6}; (*e*) 3.2×10^{-6} mol l^{-1}. The concentration of HSA or Hb is 2.0×10^{-6} mol l^{-1}

Fluorescence quenching mechanism of HSA and Hb induced by resveratrol

Figure 3 shows the fluorescence spectra of HSA and Hb in the presence of resveratrol at different concentrations. HSA has an emission peak at 337 nm and Hb at 330 nm with the excitation wavelength at 280 nm. Resveratrol caused concentration dependent quenching of the intrinsic fluorescence of HSA and Hb without changing the emission maximum peaks. These results further indicate that there are interactions between resveratrol with HSA or Hb.

The fluorescence quenching data are analyzed by the Stern–Volmer equation [17]:

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

where F_0 and F are the steady-state fluorescence intensities in the absence or presence of quencher (resveratrol), respec-

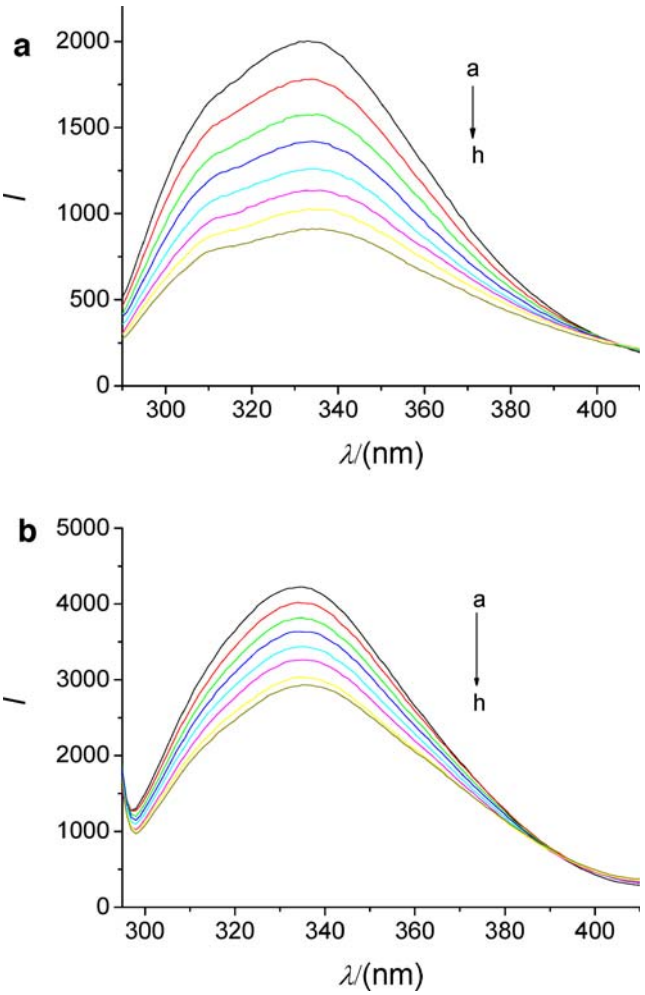


Fig. 3 Fluorescence spectra of HSA (**a**) and Hb (**b**) in the presence of resveratrol at 298 K. Concentration of resveratrol: (*a*) 0; (*b*) 1.0×10^{-6}; (*c*) 2.0×10^{-6}; (*d*) 3.0×10^{-6}; (*e*) 4.0×10^{-6}; (*f*) 5.0×10^{-6}; (*g*) 6.0×10^{-6}; (*h*) 7.0×10^{-6} mol L^{-1}. The concentration of HSA or Hb is 2.0×10^{-6} mol l^{-1}

tively, K_{SV} is the Stern–Volmer quenching constant and [Q] is the concentration of quencher. The values of K_{SV} at different temperatures are shown in Table 1. The linearity of the F_0/F versus [Q] plots is shown in Fig. 4. As shown in Table 1, the quenching constant K_{SV} decreases with increasing temperature which indicates that the probable quenching mechanism of HSA or Hb is a static quenching procedure and complex between resveratrol with HSA or Hb may be formed.

The formation of complex is further confirmed from the values of quenching rate constant, K_q, which are evaluated using the equation:

$$K_q = \frac{K_{SV}}{\tau_0}, \tag{2}$$

where τ_0 is the average lifetime of the protein without the quencher. The value of τ_0 of the biopolymer is 10^{-8} s^{-1} and the values of K_q are at the order of 10^{13} l mol^{-1} s^{-1} for HSA, and 10^{12} l mol^{-1} s^{-1} for Hb (in Table 1). The maximum scatter collision quenching constant K_q of various quenchers with the biopolymer is 2×10^{10} l mol^{-1} s^{-1} [18]. Obviously, the K_q values of the proteins quenching procedure initiated by resveratrol are greater than that of the scattered procedure. This indicates that the quenching of HSA or Hb by resveratrol is not initiated by dynamic collision but from the formation of complex.

Binding parameters

The binding constant (K) and the number of binding sites (n) between resveratrol with HSA or Hb can be calculated using the equation for the static quenching process [17]:

$$\log\left[\frac{F_0 - F}{F}\right] = \log K + n\log[Q] \tag{3}$$

A plot of log $[(F0-F)/F]$ versus log[Q] gives a straight line, whose slope equals to n and the intercept on Y-axis equals to log$K$. The values of K and n at 288, 298 and 308 K are listed in Table 1. The fact that the binding constant between resveratrol with HSA or Hb decreases with increasing temperature indicates that the stability of the HSA–resveratrol and Hb–resveratrol complexes is weakened with increasing temperature. It is also shown in Table 1 that there is one resveratrol binding site in HSA or Hb. Furthermore, the binding constant of HSA–resveratrol is larger than that of Hb-resveratrol, which indicates that the affinity of HSA toward resveratrol is higher than that of Hb.

Types of interaction force between resveratrol with HSA or Hb

There are four major noncovalent interaction forces between small molecules and proteins: hydrogen bonding, van der Waals force, hydrophobic interaction and electrostatic interaction [19]. The signs and magnitudes of thermodynamic parameters for protein reactions account for the main forces contributing to protein stability. If the enthalpy change (ΔH) does not vary significantly over the temperature range studied, then the thermodynamic parameters of ΔH, entropy change (ΔS) and free energy (ΔG) can be determined from the Van't Hoff equation:

$$\ln K = -\frac{\Delta H}{\mathrm{RT}} + \frac{\Delta S}{R}, \tag{4}$$

where K is the binding constant at the corresponding temperature and R is the gas constant. The ΔH and ΔS are determined from the linear Van't Hoff plots. The ΔG is estimated from the following equation:

$$\Delta G = \Delta H - T\Delta S \tag{5}$$

The results of ΔH, ΔS and ΔG are presented in Table 2. The negative ΔG and the negative ΔH indicate that the binding process of resveratrol to HSA or Hb is spontaneous and the formation of resveratrol–HSA or resveratrol–Hb complex is exothermic reactions. Ross and Subramanian [20] have characterized the sign and magnitude of the thermodynamic parameter associated with various individ-

Table 1 Binding parameters of HSA–resveratrol and Hb–resveratrol interactions

T (K)	KSV ($\times10^5$ l mol^{-1})	R	K_q ($\times10^{13}$ l mol^{-1})	K ($\times10^5$ l mol^{-1})	n
HSA					
288	1.81	0.9967	1.81	10.21	1.05
298	1.76	0.9943	1.76	7.43	1.15
308	1.67	0.9962	1.67	5.81	1.14
Hb					
288	0.58	0.9971	0.58	1.25	0.94
298	0.54	0.9969	0.54	0.74	1.04
308	0.48	0.9986	0.48	0.39	1.11

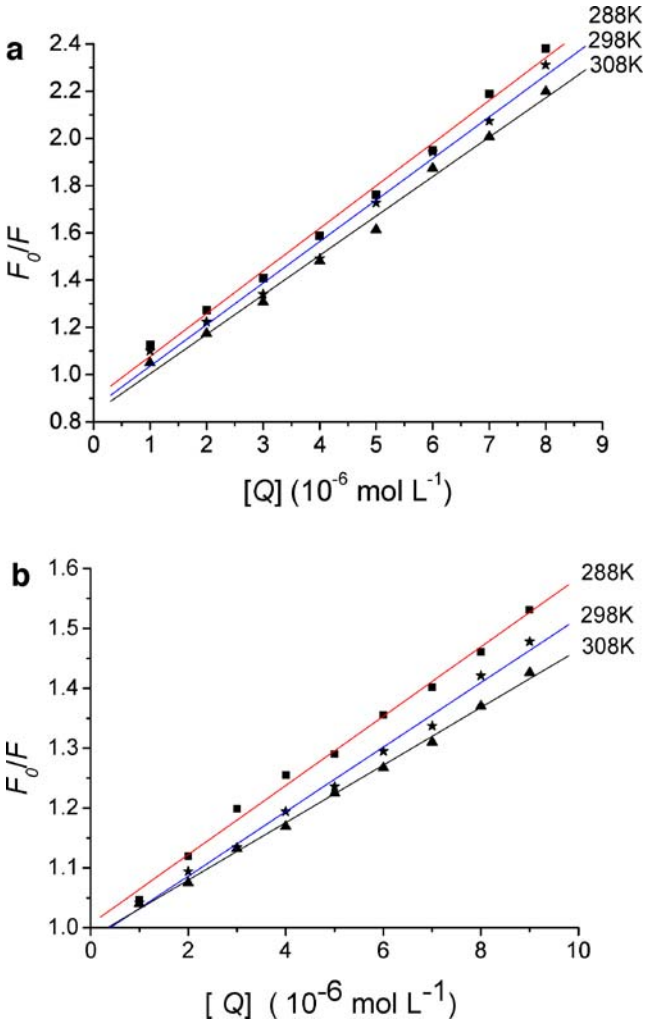


Fig. 4 Stern–Volmer curves for the binding of resveratrol with HSA (**a**) or Hb (**b**) at 288, 298 and 308 K. The concentration of HSA or Hb is 2.0×10^{-6} mol l^{-1}

ual kinds of interaction that may take place in protein association processes. From the viewpoint of water structure, the binding of resveratrol to HSA had negative ΔH and positive ΔS values, which suggests that both hydrophobic interaction and hydrogen bonding play roles in the binding process [17, 20]. Meanwhile, the major contribution to ΔG is from ΔH term (74.8%, 298 K) rather than from ΔS, which shows that the binding process is enthalpy and hydrophobic interaction is the major interaction force in the binding of resveratrol to HSA. The binding of resveratrol to Hb had negative ΔH and negative ΔS values, both of which are considered as the typical evidence for hydrogen bonding [20, 21]. So hydrogen bonding might play a major role in the binding process of resveratrol to Hb. In addition, resveratrol is known to have three pK_a values, 6.4, 9.4 and 10.5 [22]. In present study, resveratrol is ionized partly under the experimental conditions (pH 7.4), so electrostatic interaction can be included both in the binding processes of resveratrol to HSA or Hb. The additional evidences for the binding processes of resveratrol–HSA and resveratrol–Hb came from the results of synchronous fluorescence and molecular modeling.

Table 2 Thermodynamic parameters of HSA–resveratrol and Hb–resveratrol interactions

T (K)	*K* ($\times10^5$ l mol^{-1})	*G* (kJ mol^{-1})	*H* (kJ mol^{-1})	*S* (J mol^{-1} K^{-1})
HSA				
288	10.21	−27.42		
298	7.43	−27.65	−20.69	23.37
308	5.81	−27.89		
Hb				
288	1.25	−27.98		
298	0.74	−27.47	−42.61	−50.79
308	0.39	−26.97		

Conformation investigation

The synchronous fluorescence spectra give information about the molecular environment in the vicinity of the chromophore molecules. According to Yuan et al. [23], the environment of amino acid residues can be explored by measuring the possible shift in maximum emission wavelength λ_{max}, and it is corresponding to the changes of the polarity around the chromophore molecule. When the $\Delta\lambda$ between excitation wavelength and emission wavelength are stabilized at 30 or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine residues or tryptophan residues [23]. To explore the structural change of HSA and Hb induced by addition of resveratrol, synchronous fluorescence spectra of HSA (Fig. 5) and Hb (data not shown) with various amounts of resveratrol were measured.

It is shown in Fig. 5 that the addition of the drug resulted in the strong fluorescence quenching of HSA. The position of the maximum emission wavelength had a little blue shift (from 283 to 279 nm) when $\Delta\lambda$ was 30 nm, which indicates that the polarity around the tyrosine residues decreased and the hydrophobicity increased. The position of the maximum emission wavelength did not change when $\Delta\lambda$ was 60 nm. It is reported that the maximum emission wavelength at 330–332 nm showed that tryptophan residues are located in the nonpolar region, which means, they are buried in a hydrophobic cavity in HSA; the maximum emission wavelength at 350–352 nm showed that tryptophan residues are exposed to water, namely, the hydrophobic cavity in HSA is disagglomerated and the structure of HSA is looser [16]. Figure 5b shows that resveratrol is mainly bound to the hydrophobic cavity of HSA, which is in accordance with the results from

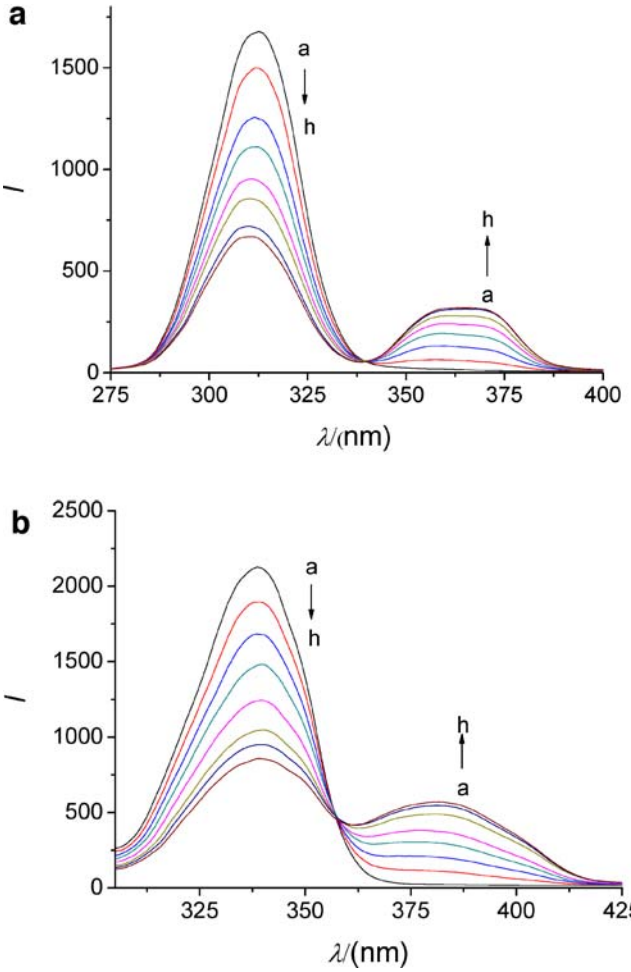


Fig. 5 Synchronous fluorescence of HSA–resveratrol system at 298 K. **a** Δλ=30 nm; **b** Δλ=60 nm. Concentration of resveratrol: (*a*) 0; (*b*) 1.0×10^{-6}; (*c*) 2.0×10^{-6}; (*d*) 3.0×10^{-6}; (*e*) 4.0×10^{-6}; (*f*) 5.0×10^{-6}; (*g*) 6.0×10^{-6}; (*h*) 7.0×10^{-6} mol l^{-1}. The concentration of HSA is 2.0×10^{-6} mol l^{-1}

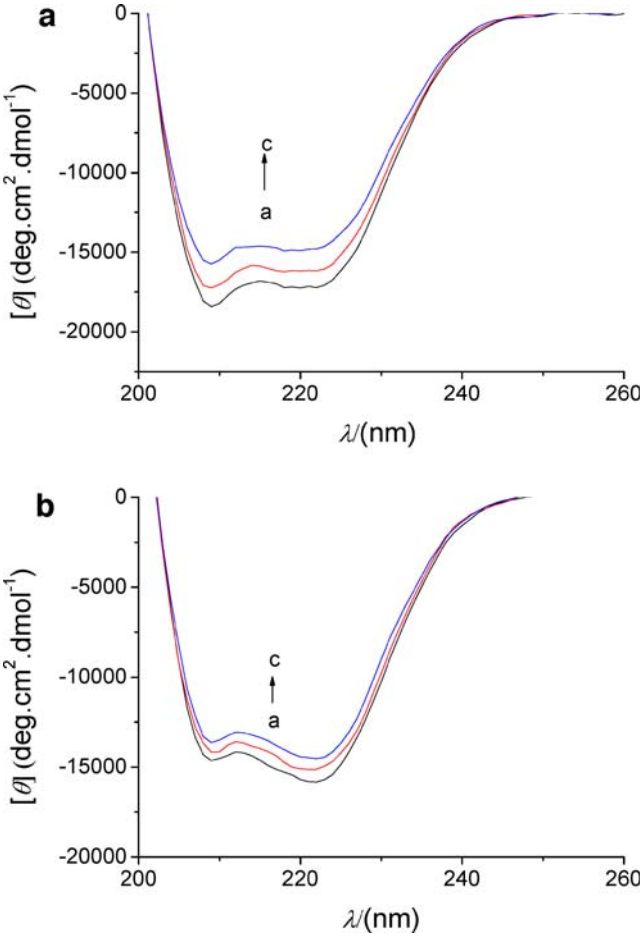


Fig. 6 CD spectra of HSA (**a**) and Hb (**b**) in the presence of resveratrol at 298 K. Concentration of resveratrol: (*a*) 0; (*b*) 2.0×10^{-6}; (*c*) 4.0×10^{-6} mol L^{-1}. The concentration of HSA or Hb is 2.0×10^{-6} mol L^{-1}

thermodynamic analysis and molecular modeling. On the other hand, the isoactinic points at 310 for 30 nm synchronous fluorescence spectra and 297 for 60 nm spectra indicate the existence of bound and free resveratrol in equilibrium, respectively.

The position of the maximum emission wavelength of either tyrosine residues or tryptophan residues did not change in the binding of resveratrol to Hb.

To ascertain the possible influence of resveratrol binding on the secondary structure of HSA and Hb, CD measurements were performed in the presence of resveratrol at different concentrations (Fig. 6). CD spectra of HSA and Hb exhibit two negative bands in the ultraviolet region at 209 and 222 nm, which are characteristic for α-helical structure of proteins [17]. The binding of resveratrol to HSA or Hb caused decrease in both two bands, indicating the decrease of the α-helical content in proteins. The CD results were repressed in terms of mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ according to the following equation:

$$\mathrm{MRE} = \frac{\text{observed CD } (m\,\mathrm{deg})}{C_p n l \times 10}, \tag{6}$$

where C_p is the molar concentration of the protein, n is the number of amino acid residues of the protein and l is the path length. The α-helical content of HSA and Hb are calculated from MRE values at 209 nm using the following equation [24]:

$$\alpha-\mathrm{helix}(\%) = \frac{-\mathrm{MRE}_{209} - 4,000}{33,000 - 4,000} \times 100, \tag{7}$$

where MRE_{209} is the observed MRE value at 209 nm, 4,000 is the MRE of the β-form and random coil conformation cross at 209 nm.

From the above equation, the α-helical content of HSA and Hb were determined. The α-helical structure of HSA is

reduced from 49.65 to 45.51 and 40.34% at the molar ratio of HSA/resveratrol is 1:1 and 1:2, respectively, and that of Hb is reduced from 36.55 to 34.82 and 33.10% at the molar ratio of Hb/resveratrol is 1:1 and 1:2. However, the CD spectra of HSA (and Hb) in the presence or absence of resveratrol are similar in shape, indicating that the structure of HSA (and Hb) is also predominantly α-helical. These results are agreement with the UV–vis absorption spectra analysis. The decrease of α-helical content is 18.75% for HSA and 9.43% for Hb at the molar ratio of drug/protein is 2. It is inferred that the conformational change of HSA is larger than that of Hb in the presence of resveratrol.

Molecular modeling

Although the solution experiments show that resveratrol has one binding site in HSA or Hb, it is difficult to determine the location of the binding site. The Autodock program was used to examine the binding mode of resveratrol in HSA or Hb. The best docking energy result is shown in Fig. 7. In resveratrol-HSA system, resveratrol possibly positions in the hydrophobic cavity between subdomain IB and IIA. Within 4 Å around resveratrol, six hydrophobic residues, Pro147, Tyr148, Phe149, Tyr150, Ala151 and Leu250, make hydrophobic interactions with the benzene rings of resveratrol. This fact suggests that hydrophobic force is the main interaction force in the binding of resveratrol to HSA, which supported by thermodynamic analysis. It is also worthy of to noted that hydrophobic interactions of Tyr148 and Tyr150 with the benzene rings of resveratrol cause increasing hydrophobicity around tyrosine residues, which is in agreement with the result of synchronous fluorescence. In addition, there are three amino acids residues with positive charge, Lys106, Lys199 and His242, within 4 Å around resveratrol, electrostatic interactions are also involved in the binding of the drug to HSA.

In resveratrol–Hb system, the docking result shows that resveratrol is located in the central cavity of Hb, partial to the subunit A. Within 4 Å around resveratrol, the atoms of Pro95, Asp126 and Thr134 are in close proximity to form hydrogen bonding with 5-OH, 4′-OH and 3-OH of the drug, which is supported by the thermodynamic analysis. Electrostatic interaction is also involved in the binding of resveratrol to Hb considering that two amino acids residues with positive charge, Lys99 and His103, are in the proximity of the binding drug.

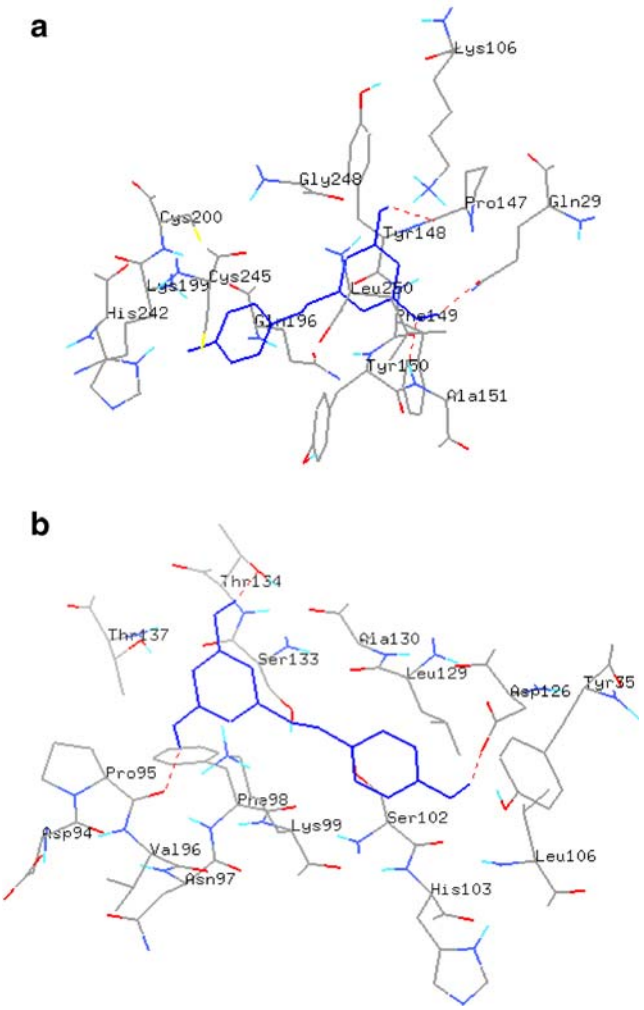


Fig. 7 The binding mode of resveratrol–HSA (**a**) and resveratrol–Hb (**b**). Resveratrol is shown with *line model* and *colored* by *blue*. The residues of HSA and Hb are shown with *line model* and *colored by atom type* (carbon, *gray*; oxygen, *red*; nitrogen, *blue*; hydrogen, *cyan*)

Conclusions

In the transport of resveratrol, it must be bound to proteins to keep it at a high concentration in serum due to its poor water solubility. Previous work showed that human red blood cells accumulate large amounts of quercetin and act as a natural flavonoid reservoir. In this work, the binding properties of resveratrol to plasma proteins, HSA and Hb have been investigated by spectroscopy and molecular modeling method. The binding constant of resveratrol–HSA complex is larger than that of resveratrol-Hb, which indicates that the affinity of HSA toward resveratrol is larger than that of Hb. Considering the concentration of HSA at about 40 g l^{-1} and Hb at about 140 g l^{-1} in plasma, both of them play important roles in the drug transport. Furthermore, Hb can accumulate some resveratrol when drug concentration is high in serum. The binding parameters and thermodynamic parameters for resveratrol binding to HSA or Hb were obtained. Thermodynamic analysis suggests that hydrophobic interaction plays a major role in the binding of resveratrol to HSA, and hydrogen bonding is

the main force in the binding of resveratrol to Hb. The secondary structures of proteins are changed in the presence of resveratrol with reduction of α-helices, and the change of HSA is larger than that of Hb. Molecular modeling result shows that resveratrol is located in the hydrophobic cavity between subdomain IB and IIA of HSA and in the central cavity of Hb. This work might give more comprehensive understanding about the transport of resveratrol in vivo.

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