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Spectroscopic studies of the interaction between hypocrellin B and human serum albumin

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Abstract—Previous work has proved that hypocrellin B (HB) binds to human serum albumin (HSA) at a specific site instead of distributed randomly on the surface of a protein. In the current work, further investigation by using bilirubin as a site I marker indicates that HB can compete for the same site with bilirubin, suggesting that the HB binding site is located at sub-domain IIA (site I) of HSA. Moreover, bound to HSA, the HB fluorescence was found to be pH sensitive in physiological range (pH 6.0–8.0). The increasing of binding constant of HB to HSA in the pH range 6–8 also indicates that the N ↔ B transition modulates the microenvironment changes of the binding site and influences considerably the binding between HB and HSA. Furthermore, picosecond time-resolved fluorescence spectra of HB–HSA complex in PBS indicate an additional short-lived component compared to that for HB in benzene, which may be assigned to the process of electron transfer from Trp-214 to HB.

1. Introduction

Hypocrellins, including hypocrellin A (HA) and hypocrellin B (HB) (Fig. 1), were well known for their photodynamic activities to tumors and viruses,[1–6] especially for the vascular-capillary diseases, such as, port-wine stain and age-related macular degeneration (AMD).[7] To have a full understanding of the modes of drug actions, their interaction with all possible biological targets, including nucleic acids, enzymes, and other proteins, is required. Human serum albumin (HSA) is the most abundant carrier protein in blood circulation that can bind many endogenous and exogenous drugs reversibly. The three-dimensional structure of human serum albumin has been determined through X-ray crystallographic measurements.[8] HSA is composed of three homologous domains (labeled I–III) and each of these is further divided into two sub-domains (A and B). The capability of binding aromatic and heterocyclic compounds depends largely on existence of two major regions, namely Sudlow's site I and site II, located within the special cavities in sub-domains IIA and IIIA.[9,10] HSA can bind and carry through the bloodstream many drugs which are poorly soluble in water. Therefore, studying on the interaction between drugs and HSA can also provide a good way for clinical application of the lipophilic hypocrellins. In our previous work, both the absorption spectra and fluorescence spectra suggest a strong binding of HB to HSA, proved by redshifts of the spectra and also by the increases in absorbance and fluorescence intensity. And based on the fluorescence responses of both HB and Trp-214 to the increases in concentration of HB and also the molecular configuration of HSA, it is suggested that HB molecules should be preferentially located on the

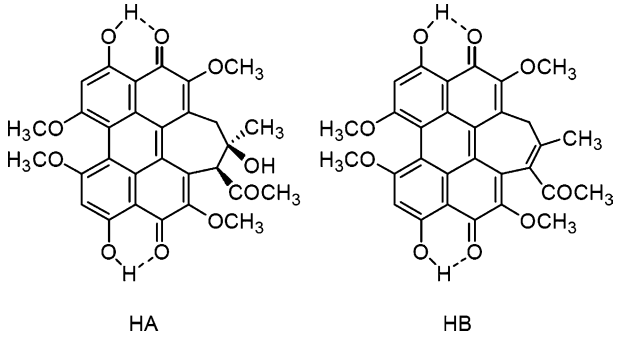

Figure 1. The molecular structures of HA and HB.

Abbreviations: HA, hypocrellin A; HB, hypocrellin B; HSA, human serum albumin; Trp, tryptophan; PBS, phosphate-buffered saline.

Keywords: Hypocrellins; Human serum albumin; Fluorescence; Ligand binding site; pH-sensitive fluorescence.

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sub-domain IIA, the area near to the Trp-214, rather than distributed randomly on the surface of a protein.[11] In the current paper, we have performed extended studies on the interaction of HSA with HB in aqueous solutions at different physiological pH values. And the competition between bilirubin and HB binding to HSA was investigated.

2. Results and discussion

2.1. pH-sensitive fluorescence for HB in HSA

It was proved that the fluorescence of hypocrellins were originated from the excited proton transfer[12] therefore, all factors that exert an electrostatic interaction on the hydrogen bonds must influence the HB fluorescence, such as pH value and ionic strength. It was reported that a lower pH value is an important characteristic differentiating tumor tissues from the normal ones. Therefore, it is interesting to evaluate pH values in biological tissues by monitoring fluorescence responses. Figure 2 shows the fluorescence emission spectra for HB in HSA with pH values varying from 5.8 to 8.2. From the figure, it can be seen that the fluorescence peak at 610 nm is strongly quenched, while that at 650 nm is strengthened with the increase in pH values. The normalized spectra (Fig. 2b) clearly show the relative fluctuation of the two peaks. The changes in the fluorescence intensities at 650 nm (normalized to 610 nm) at different pH values are shown in Figure 2c. Obviously, the absolute intensity of HB fluorescence at a certain wavelength is not only related to pH values but also the concentration of HB, so it is not a specific evaluation of the pH values, however, the ratio of the fluorescence intensities at the two wavelengths (F_{650}/F_{610}) (Fig. 2c) is a specific parameter, which may be used to monitor the pH values of the microenvironments in cells or tissues.

It was reported that the fluorescence intensity of free HB molecules changed quantitatively but the shape of the spectra kept invariable in PBS with various pH values.[13] Therefore, it may be deduced that the pH-induced fluctuation of HB fluorescence mentioned above should be attributed to interaction of microenvironment of HSA which is variable during the pH changes. It was reported that HSA underwent a conformational change known as N ↔ B transition in response to rises in pH value from 6.0 to 9.0,[14]; therefore, it may be argued that a loosening structure of the HSA molecules should have been responsible for the fluorescence fluctuation. To clarify this, the binding constants of HB to HSA at various pH values were determined. Figure 3 shows fluorescence spectra of HSA with a series of concentrations of HB at pH 6, 7, and 8, from which the Lineweaver–Burk curves of quenching of HSA fluorescence by HB were derived as shown in Figure 4. Thus, the binding constants were obtained from the slopes of the curves as listed in Table 1. The data show that the affinity of HB to HSA at pH 8 is about 6 times of that at pH 6. It can be deduced that the N ↔ B transition modulated changes on the microenvironment of the binding site influence the binding between HB and HSA, which in turn induces the fluctuation of the HB fluorescence. It was deduced by the previous work that there should be an electron transfer from tryptophan to HB in the HB–HSA complexes. With increase in pH value, the strengthened affinity of HB to HSA could promote the electron transfer, which quenched the fluorescence of HB at 610 nm. On the other hand, the appearance of the emission peak at 650 nm may be due to formation of the electron transfer complexes.

2.2. Influence of bilirubin on the HB binding to HSA

To provide further information about the HB binding site to HSA, the binding of bilirubin as a site I marker was investigated. It is well known that bilirubin possesses very high affinity to the site I, located in sub-domain IIA near Trp-214.[15–17] HB has a weak fluorescence at 620 nm in PBS but a much higher fluorescence in hydrophobic environments. For bilirubin, it did not fluoresce in PBS but its fluorescence was enhanced when bound to HSA (Fig. 5). Bilirubin–HSA solution gave a fluorescence

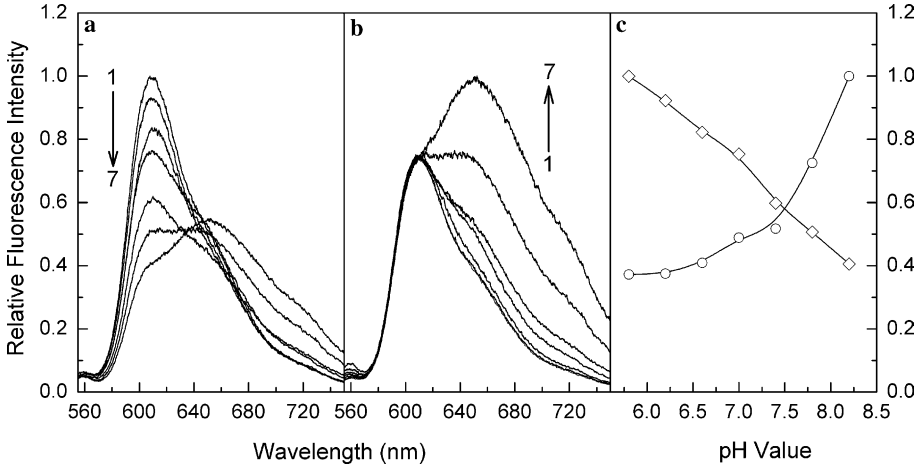

Figure 2. (a) Relative fluorescence emission spectra of HB in the complexes ([HB] = [HSA] = 5 μM) at different pH values with an excitation wavelength at 470 nm. Spectra 1–7 correspond to pH = 5.8, 6.2, 6.6, 7.0, 7.4, 7.8, and 8.2. (b) The same as shown in (a) but normalized to the 610 nm peak. (c) Plot of the fluorescence intensity at 610 nm (–◇–) and 650 nm (normalized to 610 nm, –○–) against the pH value.

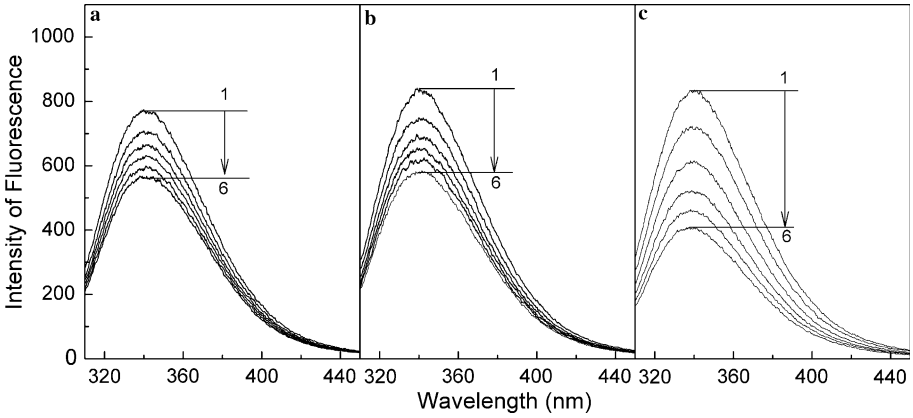

Figure 3. Fluorescence spectra of HSA (10 μM) with excitation at 295 nm at pH 6.0 (a), 7.0 (b), and 8.0 (c) at rt. 1–6 corresponding to [HB] = 0, 2, 4, 6, 8, and 10 μM.

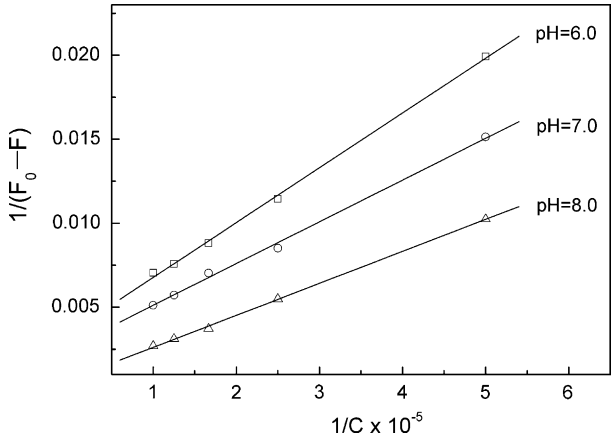

Figure 4. Lineweaver–Burk curve of fluorescence quenching of HSA with HB at various pH values. Conditions are the same as those in Figure 3.

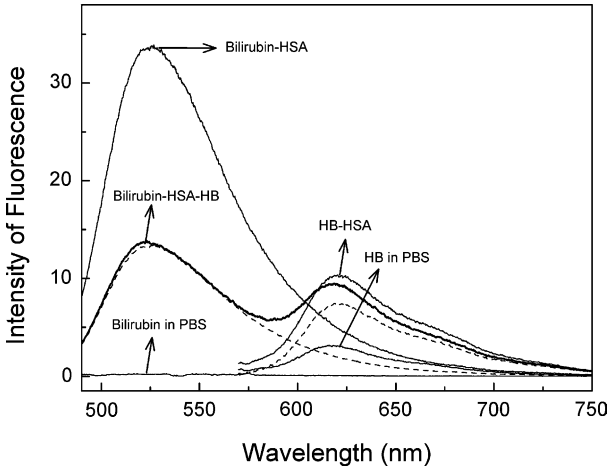

Figure 5. Fluorescence changes of HB and bilirubin in PBS in the absence and presence of HSA at rt, pH = 7.0. The overstriking line is the fluorescence spectra of bilirubin–HSA–HB in PBS. The two dashed lines are the deconvolution of the bilirubin–HSA–HB fluorescence spectra. The concentration of HSA, HB, and bilirubin is 5 μM in each case, λ_{ex} = 470 nm.

Table 1. The binding constants between HB with HSA at various pH values

pH value	K (L/mol)	R
6.0	1.1×10^4	0.9993
7.0	4.9×10^4	0.9986
8.0	6.3×10^4	0.9996

emission spectrum in the wavelength range 500–600 nm with emission maxima at 525 nm when excited at 470 nm. Figure 5 shows the relative fluorescence changes of HB and bilirubin in the absence and in the presence of HSA in PBS. Two dashed lines are the deconvoluted components of the bilirubin–HSA–HB fluorescence spectra. The fluorescence spectra suggest a competition of HB and bilirubin for binding to the same site in HSA, that is, the site I in sub-domain II A.

To obtain the influence of bilirubin on HB binding to HSA, fluorescence quenching for tryptophan in HSA–bilirubin complexes (the bilirubin–HSA molar ratio: 0; 0.5; 1.0) was detected with increase in the concentration of HB. Figure 6 shows the fluorescence spectra of HSA in the presence of different concentrations of HB at various bilirubin–HSA molar ratios. Figure 7 shows the Lineweaver–Burk curves of each quenching spectrum of HSA fluorescence by HB. The results of the binding constants are given in Table 2. It is shown that the affinity of HB to HSA was decreased with the increasing of the bilirubin–HSA molar ratios. It should be concluded that the binding site of HB to HSA is the same to that of bilirubin, the site I in the vicinity of Trp-214 located on the sub-domain IIA.

2.3. Transient fluorescence spectra measurement

Currently, time-resolved fluorescence analysis is a tool to probe the interaction between drug and protein. To investigate the interaction between HB and HSA further, picosecond time-resolved fluorescence spectra of HB in different systems were carried out. Figure 8 shows the fluorescence decays of HB in benzene and HB–HSA in PBS. The decay for HB in benzene was fitted to a single exponential function with a time constant of 955 ± 11 ps, which is roughly same as the reported τ = 1 ns in benzene.[18] On the other hand, the decay for

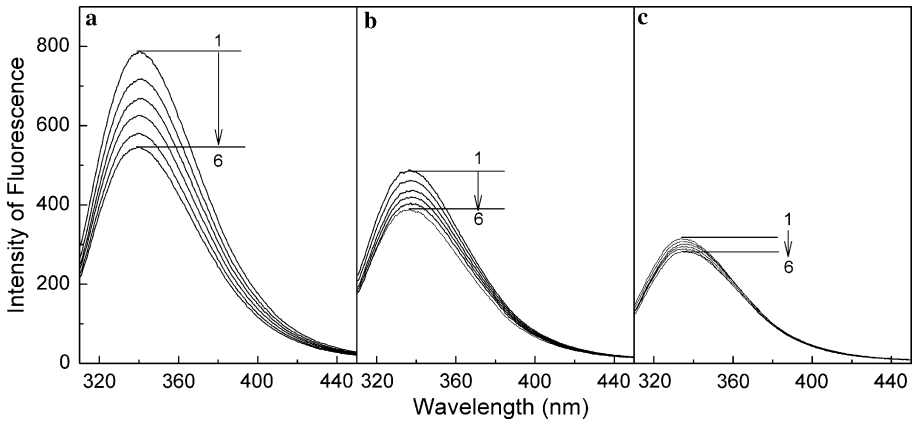

Figure 6. Fluorescence quenching spectra of HSA ([HSA] = 10 μM; spectra 1–6 correspond to [HB] = 0, 2, 4, 6, 8, and 10 μM) at rt, λ_{ex} = 295 nm, [bilirubin]/[HSA] = 0 (a), 0.5 (b), and 1.0 (c).

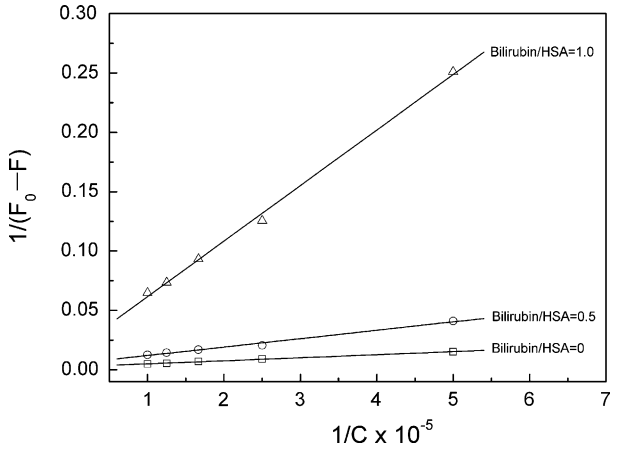

Figure 7. Lineweaver–Burk curve of fluorescence quenching of HSA with HB at various [bilirubin]/[HSA]. Conditions are the same as those in Figure 7.

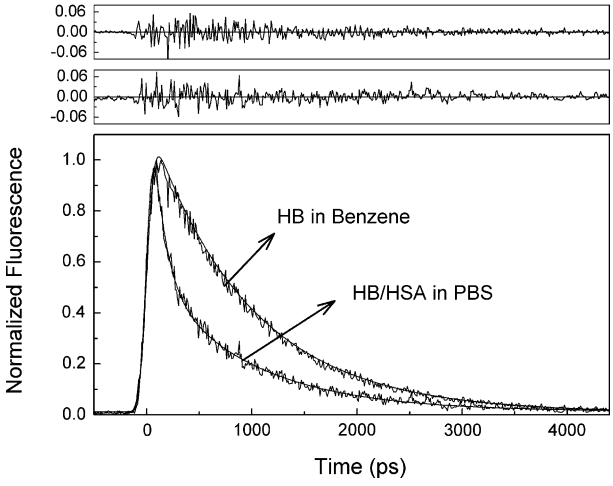

Figure 8. Fluorescence decays of HB in benzene and HB–HSA in PBS at pH 7.0 ([HSA] = [HB] = 10 μM; λ_{ex} = 465 nm, $\lambda_{em} \geqslant$ 610 nm). The decay for HB in benzene is fit to a single exponential function with a time constant of 955 ± 11 ps. The decay for HB–HSA in PBS is biexponential with time constants of 118 ± 12 and 918 ± 39 ps, with relative amplitudes of 0.63 and 0.37, respectively. The top set of residuals are those for HB in benzene; the bottom set, for HB–HSA in PBS.

Table 2. The binding constants between HB with HSA at various [bilirubin]/[HSA]

Bilirubin/HSA	*K* (L/mol)	*R*
0.0	4.9×10^4	0.9988
0.5	2.7×10^4	0.9951
1.0	0.61×10^4	0.9988

HB–HSA in PBS could be well fitted by two components: 118 ± 12 and 918 ± 39 ps, with relative amplitudes of 0.63 and 0.37, respectively. The time constant for the long-lived component is similar to that in benzene, which should be assigned to the process of $S_1^* \rightarrow S_0$. The additional short-lived component should be assigned to the intensive interactions between HB and HSA, which enhance the radiationless pathways of the excited state. As mentioned above, electron transfer from Trp-214 to HB may be a good explanation for this fast process. This aspect will be verified in further studies.

3. Conclusions

In this paper, the interaction of HB with HSA has been monitored by steady-state and transient fluorescence methods. The binding constants of HB with HSA at various pH values and in different bilirubin–HSA ratios were obtained. Bilirubin displacement experiments further confirm binding of HB to the site I of HSA. Moreover, it was found that the fluorescence for HB bound to HSA was sensitive to the pH changes in physiological range, and ratios of the fluorescence intensities at the two different wavelengths, 610 and 650 nm, may be taken as a specific parameter to probe pH values in biological environments.

4. Materials and methods

4.1. Materials

HA was extracted from the fungus of *Hypocrella bambusae* and crystallized twice from chloroform–petroleum ether before use. HB was quantitatively produced by

dehydration of HA. The purities of HA and HB were determined by thin layer chromatography (TLC). Bilirubin IX and HSA (Fraction V) were purchased from Porphyrin Products Inc. and Sigma Chemical Co., respectively. Phosphate-buffered saline (PBS) was prepared by the use of 10 mM KH_2PO_4 and 10 mM K_2HPO_4. The working solutions were prepared immediately before use.

4.2. Preparation of HB–HSA complexes

The HSA solution was prepared in 10 mM phosphate buffer. A concentrated solution of HB in dimethylsulfoxide (DMSO) (2 mg/ml) was added to the protein solution in microliter quantities and mixed quickly by shaking. After 30 min, the solution was stored in the dark for 12 h to allow for further interaction.

4.3. Spectroscopy measurements

Steady-state absorption and fluorescence spectra were recorded with a UV-1601 UV–Vis spectrophotometer (Hitachi, Japan) and F-4500 spectrofluorimeter (Hitachi, Japan), respectively. For the fluorescence measurements, HB was selectively excited at 470 nm and Trp-214 of HSA at 295 nm. Samples were dark-adapted at room temperature for 30 min prior to fluorescence measurement.

The time-resolved fluorescence measurements were carried out on a photo-counting streak camera (C2909, Hamamatsu). This machine uses a femtosecond laser source running at 1 kHz. The laser's output wavelength can be set to the desired excitation with OPA (OPA-800CF, Spectra Physics). To optimize the signal-to-noise ratio, 5000 photon counts were collected in the peak channel. This arrangement also prevents any prolonged exposure of the sample to the excitation beam thereby avoiding any possible optical damage of the fluorophore. The data detected by digital camera (C4742-95, Hamamatsu) were routinely transferred to a PC for analysis with HPDTA software. Intensity decay curves so obtained were fitted by a sum of exponential terms:

$$F(t) = \sum \alpha_i \exp(-t/\tau_i). \quad (1)$$

Here α_i is a pre-exponential factor representing the fractional contribution to the time-resolved decay of the component with lifetime τ_i. The decay parameters were recovered using a nonlinear least-squares iterative fitting procedure based on Matlab 5.3 (Mathworks). The program also includes statistical and plotting subroutine packages. The goodness of the fit of a given set of observed data and the chosen function were evaluated by the weighted residuals. A fit was considered acceptable when plots of the weighted residues showed a random deviation of about zero.

4.4. Calculation of the binding constant

The binding constant has been calculated by the Trp-214 fluorescence quenching of HSA and using the static quenching equation as analyzed on the previous paper.[11] This method is based on the general equation:

$$(F_0 - F)^{-1} = F_0^{-1} + K^{-1}F_0^{-1}[Q]^{-1}, \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and in the presence of quencher, respectively. [Q] is the concentration of quencher. K is the binding constant of drug and biomolecule, which can be determined by the inverse slope of the Lineweaver–Burk curve—$(1/(F_0 - F)$ vs $1/[Q])$.

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