

# Affinity Interaction Between Lysozyme and Hypocrellin A: Electron Transfer and Fluorescence Quench

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**Abstract** Based on the known binding interaction between lysozyme and hypocrellin A, a photosensitizing drug used in photodynamic therapy, and the fluorescence quenching mechanism of lysozyme by hypocrellin A was studied. The results indicated that the photoinduced electron transfer between the tryptophan and tyrosine residues of lysozyme and hypocrellin A plays an important role in this fluorescence quenching process.

**Key words** hypocrellin A, lysozyme, binding interaction, photoinduced electron transfer

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## 竹红菌甲素和溶菌酶之间的电子转移与荧光猝灭作用

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**[摘要]** 在光动力学疗法中, 竹红菌甲素作为敏剂与溶菌酶发生了相互作用。本文对竹红菌甲素同溶菌酶作用的荧光猝灭机理进行了研究。结果表明, 竹红菌甲素与溶菌酶结构中的酪氨酸和色氨酸之间的光诱导电子转移机制, 在荧光猝灭过程中起了重要作用。

**[关键词]** 竹红菌甲素, 溶菌酶, 结合作用, 光诱导电子转移

Photodynamic therapy (PDT) is a treatment that involves injection of photosensitizing and tumor-localizing dyes followed by exposure of the tumor region to high fluence rates of light usually from a laser<sup>[1]</sup>. 4,9-dihydroxyperylene-3,10-quinone (HA, Fig. 1), extracted from *Hypocrella bambusae*, has been used as a phototherapeutic agent to cure various skin diseases, and taken orally as a folk medicine for several centuries in China<sup>[2]</sup>. Recently, studies show that this natural perylenequinonoid compound also has antitumor and antiviral activities, including human immunodeficiency virus<sup>[3]</sup>. It possesses several advantages over the presently used hematoporphyrin derivatives (HPD), i.e. ready preparation, easy purification relative to HPD, small aggregation tendency, strong red light absorptivity and high quantum yields of singlet oxygen<sup>[4]</sup>. In the photodynamic ther-

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peutic procedure, HA or its analogues will be injected intravenously into the patients, then incubation step is needed before the treatment with light. Previous studies show that during this incubation step, HA will bind with various serum proteins such as albumin, globulins, and lipoproteins to form protein-HA complex. Finally the binding will be gradually released in either vascular stroma or intercellular bed, such as lysosomes, golgi, endoplasmic reticulum, and cellular membranes<sup>[5]</sup>. This binding affinity of HA to proteins influence on transport, uptake, and the distribution of HA in vivo, as well as the photophysical and photochemical characters of HA. It's possible determining the photodynamic properties of HA. Such as HAS-C-PC<sup>[6]</sup>. These studies indicated that HA can interact with these proteins and quench the fluorescence of these proteins.

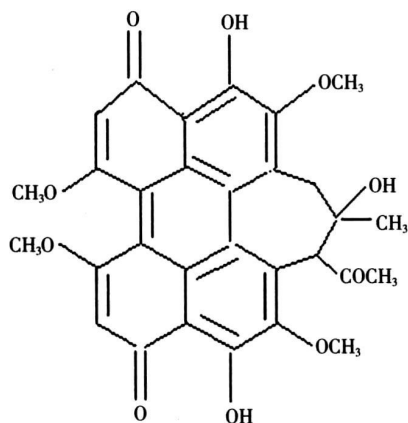


Fig.1 Molecular structure of hypocrellin A

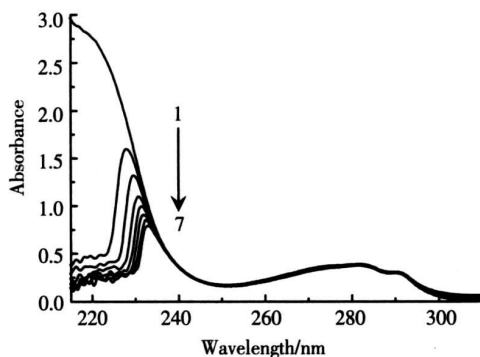


Fig.2 UV-vis absorption spectra of  $1.1 \times 10^{-5}$  M lysozyme solution with (1) 0, (2) 6.67, (3) 13.3, (4) 20.0, (5) 26.7, (6) 33.3 and (7)  $40.0 \times 10^{-6}$  M HA at 25°C

In this paper, lysozyme was chosen as the target of HA due to its well-known structure and lower molecular weight. We found that HA can effectively bind with lysozyme and significantly vary the conformation of lysozyme. In addition, the fluorescence of lysozyme was quenched. Based on these results, the fluorescence quenching mechanism was systematically discussed.

## 1 Materials and Methods

### 1.1 Materials

Lysozyme was the product of Sigma Chemical Company (St Louis, MO, USA) and dissolved in 0.04 mol/L pH 7.4 PBS containing 0.10 mol/L NaCl and the final concentration of Lysozyme was  $1.6 \times 10^{-5}$  mol/L. HA was obtained according to the procedure described in literature, and the purity was confirmed as > 97% by HPLC. Dimethylsulfoxide (DMSO) was dried by distillation over KOH prior to use. 2 mmol/L HA aqueous solution was prepared by adding 10 mL DMSO solutions of HA to 50 mL double distilled water.

### 1.2 Spectroscopic measurements

UV-visible absorption spectroscopy was performed with Lambda 17 UV-Vis spectrophotometer (Perkin-Elmer, USA), and absorption spectrum from 210 to 310 nm was recorded. All the fluorescence measurements were done with a LS 50 B spectrofluorimeter (Perkin-Elmer, USA), and the excitation was at 286 nm. Unless otherwise stated, incubated for 40 minutes, and all experiments were carried out at room temperature 25°C.

### 1.3 Electron paramagnetic resonance (EPR) measurements

EPR spectra were obtained using a Bruker ESP-300E spectrometer operating at room temperature, and the operating conditions were as follows: microwave bridge, X-band with 100 Hz field modulation, sweep width 100 G, modulation amplitude 1.0 G, modulation frequency, 100 kHz, receiver gain  $1 \times 10^5$ , microwave power 5 mW. Samples were injected into the specially made quartz capillaries for EPR analyses and purged with argon, air or oxygen for 40 minutes in the dark orderly according to the experimental requirements and illuminated directly in the cavity of the EPR spectrometer with a Nd:YAG laser (355 nm, 5–6 ns of pulse width, repetition frequency 10 Hz, 10 mJ/pulse). The kinetics of spin adduct were studied by recording peak heights of EPR

spectra every 30 s

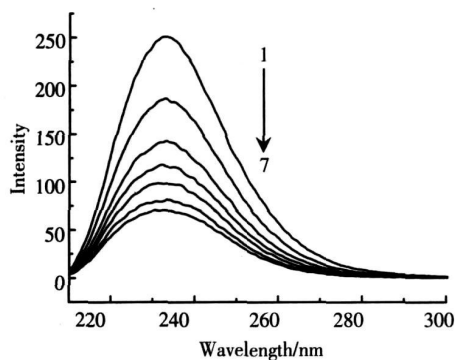
## 2 Results and Discussion

### 2.1 Binding interaction between lysozyme and HA

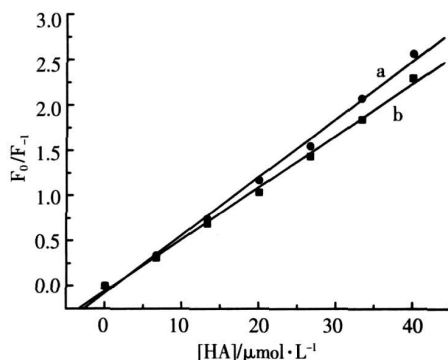
The experiments of the UV-visible absorption spectroscopy and the fluorescence spectroscopy were carried out to study the interaction between lysozyme and HA.

In the region of 210–310 nm, lysozyme and HA have two characteristic absorption bands with absorption maxima at 219 nm and 280 nm, respectively (Fig 2). After addition of different concentration of HA to a series of lysozyme aqueous solution, notable changes in the absorption spectrum of lysozyme were observed. The absorption peaks at 210 nm shifted to longer wavelength and the intensities decreased greatly, while the other absorption peaks at 280 nm showed no obvious changes. When the concentration of HA reached 40  $\mu\text{M}$ , the absorption peak at 219 nm shifted to 233 nm, and the intensity decreased by 72% (Fig 2). According to the previous reports<sup>[7]</sup>, the absorption band at 280 nm can be attributed to the amidic acid residues in lysozyme, and the absorption band at 219 nm depends strongly on the conformation that lysozyme adopts, e.g. the helix of lysozyme. Therefore, these results suggest that HA can bind onto lysozyme, and this binding interaction causes the conformation of lysozyme to change remarkably.

Furthermore, the controlled experiments show that DMSO present in the above-mentioned solutions did not affect the absorption spectrum of lysozyme, which is consistent well with the reference [8].



**Fig.3** Fluorescence emission spectra of  $1.6 \times 10^{-5}$  M Hb solution with (1) 0, (2) 6.67, (3) 13.3, (4) 20.0, (5) 26.7, (6) 33.3 and (7)  $40.0 \times 10^{-6}$  M HA at 25°C.  $\lambda_{\text{ex}}$ : 286 nm



**Fig.4** Stern-Volmer plots for the fluorescence quenching of lysozyme by HA at 25°C (line a) and 42°C (line b)

The fluorescence spectra of lysozyme with varying concentrations of HA are shown in Fig 3. It is clear that HA can quench the fluorescence of lysozyme effectively. Fig 4 gives the Stern-Volmer plots for the fluorescence quenching of lysozyme by HA at 25°C and 42°C, respectively. Both the straight lines have good linear correlations of 0.998 (25°C) and 0.999 (42°C) (Fig 4). On the basis of Stern-Volmer equation<sup>[9]</sup> (equation 1):

$$F_0/F = 1 + K_q \tau_0 [Q] \quad (1)$$

In equation 1,  $F_0$  and  $F$  are the fluorescence intensities of the fluorophore in the absence or presence of the quencher  $Q$ ,  $\tau_0$  is the singlet excited state lifetime of the fluorophore in the absence of the quencher. For biopolymers,  $\tau_0$  of  $10^{-8}$  s can be used in calculation. So the bimolecular quenching constants ( $K_q$ ) are calculated to be  $6.43 \times 10^{12} \text{ mol}^{-1} \text{ L} \cdot \text{s}^{-1}$  (25°C) and  $5.75 \times 10^{12} \text{ mol}^{-1} \text{ L} \cdot \text{s}^{-1}$  (42°C), respectively. The two  $K_q$  are nearly three orders of magnitude higher than the diffusion constant of water ( $7 \times 10^9 \text{ mol}^{-1} \text{ L} \cdot \text{s}^{-1}$  at 25°C estimated from its viscosity<sup>[10]</sup>), indicating that the fluorescence quenching may result from the static interaction due to the binding of HA onto lysozyme. The static quenching mechanism is in line with the observed smaller quenching constant at elevated temperature (42°C vs 25°C). Generally, bimolecular quenching constant will increase with the increase of the temperature when quenching is dynamic, i.e., relying on the diffusion collision between excited fluorophore and quencher. In contrast, elevated temperature favors the dissociation of the complex formed

non-covalently, e.g. by way of hydrogen bond, electrostatic attraction, or hydrophobic force, thus alleviates the fluorescence quenching originated from the complex formation between fluorophore and quencher. The conclusion is consistent with the results of UV-visible absorption spectroscopy.

## 2.2 Fluorescence quenching mechanism

Fluorescence emissions of proteins mainly originate from tryptophan residues and tyrosine residues, and their emission bands overlap closely in the normal fluorescence spectrum, thus to know the fluorescence quenching object of HA in this fluorescence quenching process, the synchronous fluorescence experiments were carried out. In synchronous fluorescence spectrum, when the wavelength interval ( $\Delta\lambda$ ) between the excitation and emission wavelengths ( $\lambda_{ex}$  and  $\lambda_{em}$ ) is set to be 20 nm, the obtained emission peak can be ascribed to tyrosine residues. In contrast, when the value of  $\Delta\lambda$  is 80 nm, the obtained emission peak can be attributed to tryptophan residues<sup>[11]</sup>.

Fig. 5 shows the synchronous fluorescence spectra of lysozyme in the presence of varied concentrations of HA with  $\Delta\lambda = 20$  nm (A) or 80 nm (B). In the absence of HA, the emission maximum of tyrosine residues locates at 314 nm, while that of tryptophan residues is at 349 nm. Upon addition of HA, both the emission bands decreased markedly in intensity, and the peak positions red shifted slightly. It is well known that the emission maxima of tyrosine residues or tryptophan residues in proteins depend greatly on the microenvironment surrounding them. The red shift of the emission maxima suggests a change of microenvironment from hydrophobic to hydrophilic, as the result of the conformation variation of proteins. In our experimental conditions, the emission peak of tryptophan residues shifted by about 6 nm upon addition of HA, from 349 nm to 355 nm, very close to the value in the tryptophan aqueous solution, indicating that the interactions between HA and lysozyme made the tryptophan residues exposed almost completely to the aqueous medium. These findings are in good agreement with the remarkable changes of the shortwavelength absorption band of lysozyme after binding by HA (Fig. 2), as the result of conformation change of lysozyme.

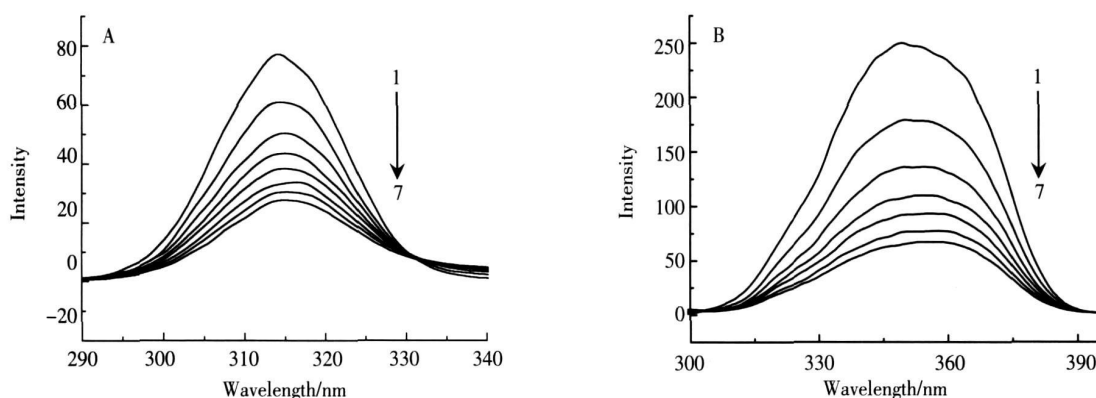


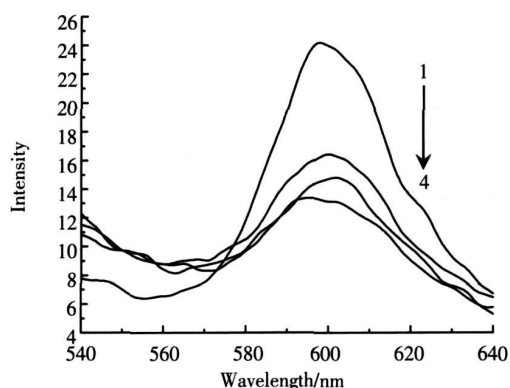
Fig. 5 Synchronous fluorescence spectra of  $1.6 \times 10^{-6}$  mol/L lysozyme solutions with (1) 0, (2) 6.67, (3) 13.3, (4) 20.0, (5) 26.7, (6) 33.3 and (7)  $40 \times 10^{-6}$  mol/L HA.  $\Delta\lambda$ : (A) 20 nm, (B) 80 nm

Both the normal and synchronous fluorescence spectra show that the binding of HA quenched the fluorescence emission of lysozyme efficiently through forming the complex between lysozyme and HA, but the fluorescence quenching may either via energy transfer mechanism or electron transfer mechanism or both of them, so further experiments are necessary.

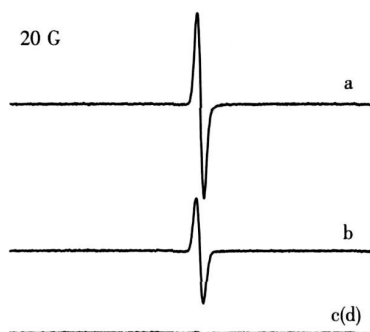
To examine the possibility of energy transfer, we measured the fluorescence spectra of HA upon addition of lysozyme, in which the excitation wavelength was set at 286 nm. It was found that the fluorescence intensity of HA decreased with the increase of the concentration of lysozyme (Fig. 6). This indicates that the energy transfer from the excited lysozyme to HA, if any, did not play an important role in the interactions between HA and lysozyme.

In contrast, the photoinduced electron transfer between HA and lysozyme is evident. Fig. 7 shows the EPR

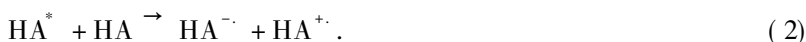
spectra of the irradiated HA solutions with or without the presence of lysozyme. Irradiation of the deaerated aqueous solution of HA ( $20\text{ }\mu\text{M}$ ) by a Nd:YAG laser at 355 nm for 1 min gave an EPR signal (spectrum b, Fig 7), which can be reasonably attributed to the semiquinone anion radical of HA ( $\text{HA}^{\cdot-}$ ) by comparison with our previous study<sup>[12]</sup>. The semiquinone anion radical of HA is believed to be generated by the electron transfer between an excited HA with a ground state HA (equation 2).



**Fig.6** Fluorescence emission spectra of  $6.67\times 10^{-6}$  M HA with (1) 0, (2)  $0.5$ , (3)  $1.0$  and (4)  $1.5\times 10^{-6}$  M lysozyme.  $\lambda_{\text{ex}} = 286\text{ nm}$



**Fig.7** (a) EPR spectrum of deaerated aqueous solution of HA ( $20\times 10^{-4}$  M) in the presence of lysozyme ( $40\times 10^{-6}$  M) after irradiation at 355 nm for 1 min. (b) Same as a but without lysozyme. (c) Same as b but oxygen saturated. (d) Same as b but HA or irradiation was omitted



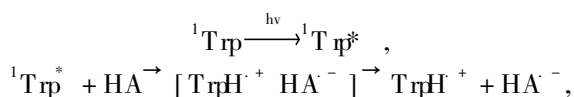
When lysozyme was added into the deoxygenated aqueous solution of HA, the intensity of the  $\text{HA}^{\cdot-}$  signal was enhanced significantly (spectrum a Fig 7). This implies lysozyme may serve as electron donor to favor the formation of  $\text{HA}^{\cdot-}$ .

The oxidation potentials of tryptophan and tyrosine are 0.88 V and 0.93 V vs NHE, respectively<sup>[13]</sup>, and the reduction potential of HA is  $-0.37\text{ V}$  vs NHE<sup>[14]</sup>. Thus, based on their absorption edges (313 nm for tryptophan, 290 nm for tyrosine and 640 nm for HA)<sup>[15]</sup>, the oxidation potentials of the excited tryptophan and tyrosine are estimated to be  $-3.08\text{ V}$  and  $-3.35\text{ V}$  vs NHE respectively, and the reduction potential of the excited HA is  $1.57\text{ V}$  vs NHE. According to Rehm-Weller equation<sup>[16]</sup>,

$$\Delta G = E_{\text{ox}}(\text{donor}) - E_{\text{red}}(\text{acceptor}) - E_{\text{q0}}(\text{excited state energy}) \quad (3)$$

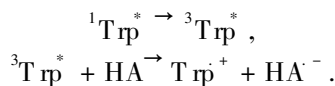
the free energy change of the photoinduced electron transfer process can be estimated. When the photoinduced electron transfer occurred between the excited HA and lysozyme,  $G(\text{Trp})$  is  $-15.9\text{ kcal/mol}$ ,  $G(\text{Tyr})$  is  $-14.8\text{ kcal/mol}$  respectively. On the other hand, when the photoinduced electron transfer occurred between the excited lysozyme and HA,  $G(\text{Trp})$  is  $-62.5\text{ kcal/mol}$ ,  $G(\text{Tyr})$  is  $-68.7\text{ kcal/mol}$  respectively. From these values, it can be known that the photoinduced electron transfer from tryptophan or tyrosine to HA will be a thermodynamic favorable process no matter whether HA or lysozyme is excited (Fig 8). In addition, the participation of tryptophan residues and tyrosine residues in the photoinduced electron transfer between lysozyme and HA agree very well with the fact that their fluorescence emissions were quenched effectively by HA (Fig 5).

According to the above analysis, the photoinduced electron transfer between lysozyme and HA can be illustrated as following process



	$\text{HA}/\text{HA}^{\cdot-}(-0.37\text{ V})$	
$\text{Trp}^*/\text{Trp}^*(-3.08\text{ V})$		$\text{Tyr}^*/\text{Tyr}^*(-3.35\text{ V})$
$\text{Trp}^*/\text{Trp}(0.88\text{ V})$		$\text{Tyr}^*/\text{Tyr}(0.93\text{ V})$
	$\text{HA}^*/\text{HA}^{\cdot-}(1.57\text{ V})$	

**Fig.8** Reduction potentials of HA and oxidation potentials of tryptophan and tyrosine in their ground and excited states. All potentials are vs NHE



The  $\text{HA}^{\cdot-}$  is formed due to the photoinduced electron transfer from tryptophan residues of protein to  $\text{HA}$  either in singlet or in triplet state. The  $\text{Trp}^{\cdot+}$  are generated predominantly through electron transfer rather than energy transfer.

### 3 Conclusions

In summary, the experimental results indicate that  $\text{HA}$  can effectively bind on lysozyme, which is driven mainly by hydrophobic interactions, and cause the fluorescence of lysozyme to be quenched. In addition, Based on the above discussions, it is very clear that the fluorescence quenching mechanism is the photoinduced electron transfer from tryptophan or tyrosine residues of lysozyme to  $\text{HA}$ , no matter whether  $\text{HA}$  or lysozyme is excited.

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