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# Interaction between hypocrellin A and some biological substrates with emphasis on an electron transfer mechanism

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**Abstract**

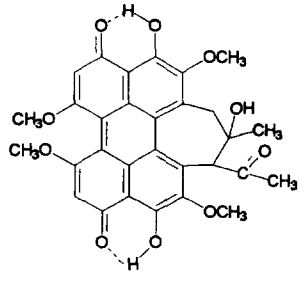
Hypocrellin A (HA) is an efficient phototherapeutic agent. Laser flash photolysis was used to produce and investigate the properties of the lowest excited triplet state ($T_1$) and the semiquinone radical anion of hypocrellin A ($HA^{\cdot -}$) at room temperature. In the presence of biological substrates, such as ascorbic acid and cysteine, the formation and decay of $HA^{\cdot -}$, attributed to electron transfer between HA and the substrates, were observed. Production of the superoxide radical anion ($O_2^{\cdot -}$) by photoactivated HA in the presence of biological substrates was examined by using the Nitro Blue Tetrazolium (NBT) trapping method in order to elucidate the mechanism of formation of superoxide and to quantify this formation. Specifically, production of $O_2^{\cdot -}$ was demonstrated unequivocally by reaction with superoxide dismutase. In addition, in weak acidic solution, $HA^{\cdot -}$ could also be produced and observed by laser flash photolysis, but it decays much faster than that in neutral or basic solution. The values of the free energy change for electron transfer between HA* and some biological substrates are less than zero, indicating that this process is permissible thermodynamically. Based on the experimental results, an electron transfer (Type I) mechanism may play a hitherto unrecognized role in the photodynamic interaction between HA and some biological substrates. © 1997 Elsevier Science S.A.

*Keywords:* Hypocrellin A; Photosensitization; Superoxide radical anion; Nitro Blue Tetrazolium

## 1. Introduction

Hypocrellin A (HA) (Fig. 1), a new photosensitizer, has been isolated from the natural fungus sacs of *Hypocrella bambusae* in China. This lipid-soluble perylenequinone derivative [1,2], in combination with phototherapy, has produced promising results as an antitumour agent [3–5]. It exhibits several advantages over the presently widely used haematoporphyrin derivatives (HPDs), i.e. ready preparation and easy purification relative to HPDs, low aggregation tendency, strong red-light absorptivity, and significantly reduced normal tissue photosensitivity because of its rapid metabolism in vivo [6]. As a result, HA has been successfully employed in the clinical photodynamic therapy (PDT) treatment of certain skin diseases, such as white lesion of vulva, keloid, vitiligo, psoriasis, tinea capitis and lichen amyloidosis [7–9], without observing the prolonged normal tissue photosensitivity that occurs with porphyrin photosensitizers [6].

Experiments have shown that the photodynamic reaction targets of HA are the cell membrane in accordance with its lipid affinity [10]. Most evidence suggests that singlet oxygen is responsible for most of these photodynamic activities




**Hypocrellin A**

Fig. 1. Structure of hypocrellin A.

[11,12]. Moreover, previous studies have shown that, on visible light irradiation, HA in organic solvents undergoes an energy transfer reaction, leading to the generation of singlet oxygen. However, very few studies have been reported on the photodestruction mechanism of various biological substrates by HA. Visible light illumination of oxygen-saturated DMSO solutions of HA containing biological substrates leads to damage of these substrates in the presence of singlet oxygen quenchers such as $NaN_3$ and DABCO. Although HA has been identified as a new efficient singlet oxygen generator [11], a Type II ($^1O_2$) mechanism might not be involved in the photodynamic action of biological substrates by HA

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according to the above experimental results. Usually $^1O_2$ is considered to play a key role in the photosensitization of tumours; however, recently, other reactive species (such as the photosensitizer radical, superoxide and hydroxy radical) have also been determined to be involved in the PDT of tumours. These considerations prompted our interest in the studies of the interaction mechanism between HA and biological substrates.

Although there are suggestions in the literature that $O_2^{\bullet-}$ should be generated via an electron transfer mechanism [13], complete quantitative data on the yield of superoxide have not been reported. In view of these considerations, the Nitro Blue Tetrazolium (NBT) trapping method appeared to be more applicable for the quantitative analysis of compared with DMPO spin trapping, although the spin trapping method is at least 15 times more sensitive than the NBT trapping method for the measurement of superoxide anions.

In addition, Hu et al. [13] considered that the semiquinone radical anion of HA ($HA^{\bullet-}$) could be produced and observed only in neutral or basic solution in the presence of ascorbic acid. In the present work, laser flash photolysis was used to investigate the properties of $HA^{\bullet-}$ in the presence of various biological substrates. Meanwhile, $O_2^{\bullet-}$ was observed and studied by ESR. The purpose of this study was to elucidate the mechanism of photodynamic interaction of HA with biological substrates, confirm the production of $HA^{\bullet-}$ in an acidic medium, and quantify the formation of $O_2^{\bullet-}$ using the NBT trapping method.

## 2. Materials and methods

### 2.1. Materials

HA (Fig. 1) was isolated from the fungus sacs of *Hypocrella bambusae* and recrystallized twice from acetone before use. Ascorbic acid, cysteine, kynurenine, indole, methionine, tryptophan, tyrosine, aspartic acid, 1,4-diazabicyclo-[2,2,2]octane (DABCO) and superoxide dismutase (SOD) were purchased from Biotech Technology Corporation, Chinese Academy of Sciences. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) Nitro Blue Tetrazolium (NBT) were purchased from Aldrich Chemical Company and stored at −20 °C under argon. Other reagents used, all of analytical grade, were purchased from Beijing Chemical Plant. The required organic solvents of high purity were prepared by further purification of the commercial products. Solutions were freshly prepared before use. The solutions were purged with argon, air or oxygen according to the experimental requirements.

### 2.2. Methods

The absorbance of NBT was monitored at 560 nm using a Shimadzu 160 UV–visible spectrophotometer and recorded at 4 min intervals. Irradiations were carried out by using a medium-pressure sodium lamp (500 W) on a "merry-go-round" apparatus. Light of wavelength below 470 nm was cut off by a long-pass filter, and the apparatus was immersed in running water in a thermostat at 20 °C. The solutions examined were put in long-necked glass cuvettes. Argon was bubbled through to remove oxygen and then the cuvettes were sealed with rubber stoppers. After illumination, their absorption spectra were measured immediately with a UV–visible spectrophotometer. Time-resolved transient absorption spectra were measured with a Q-switch Nd:YAG nanosecond laser apparatus (FWHM < 5 ns, 35 mJ per pulse, $\lambda_{exe} = 532$ nm). A xenon flash was used as an analysis flash for the detection of transients. The monitoring light passing through a grating monochromator was analysed by a detection system consisting of a photomultiplier tube and an oscilloscope. The samples in a 10 mm × 10 mm glass cell were bubbled with highly purified argon for 30 min before measurement. Measurements of the ESR spectra were carried out on a Bruker ER-300 EPR spectrometer operating at room temperature (X band: microwave frequency, 9.5 GHz). Samples (25 μl) were injected quantitatively into specially made quartz capillaries for ESR analysis and were illuminated with 450 W medium-pressure sodium lamp. A long-pass filter was employed to eliminate light of wavelength less than 470 nm.

## 3. Results and discussion

### 3.1. Generation of triplet states

Direct excitation of HA in $CHCl_3$ and $CH_3CN$ in the absence of biological substrates produced a detectable amount of triplet state. Fig. 2 shows the time-resolved absorption spectra of the transients produced after 532 nm laser excitation of a deaerated solution of HA in $CHCl_3$. The major transient with triplet character shows three bands with absorption maxima at around 530, 560 and 600 nm. The transient observed was assigned to the lowest triplet state ($T_1$) of HA because (1) it was produced during the laser pulse, (2) it was quenched by oxygen with a rate constant close to the

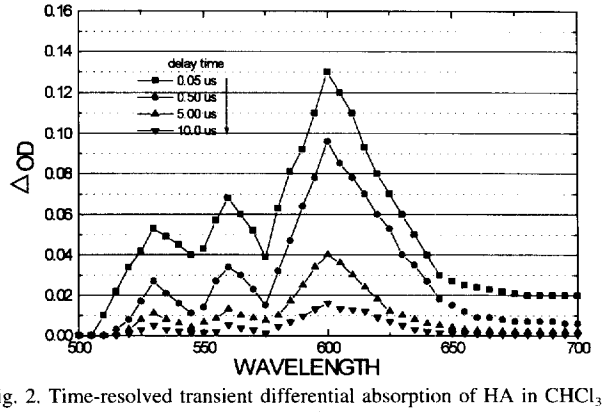


Fig. 2. Time-resolved transient differential absorption of HA in $CHCl_3$ at room temperature ($[HA] = 1.2 \times 10^{-4}$ mol $l^{-1}$, Ar saturated for 30 min, $\lambda_{ex} = 532$ nm). The delay times after the pulse are shown above the spectra.

diffusional limit, (3) its decay followed first-order kinetics, (4) its population increased in the presence of a heavy atom and (5) it was sensitized by benzophenone.

Using perylene or azulene as triplet scavengers, faster decays of the band maxima at 530, 560 and 600 nm are also observed, accompanied by the observation of the T–T absorption of perylene at 485 nm or azulene at 360 nm. These results further confirm the correct assignment of the three band maxima at 530, 560 and 600 nm to the T–T absorption of HA.

### 3.2. Formation of the semiquinone radical anion in the presence of biological substrates

Aromatic amines, such as *N,N*-diethylaniline (DEA), known to be an excellent electron donor, have often been used to study radical anions. The interaction between HA* and DEA was investigated by laser flash photolysis. The time-resolved absorption spectra obtained by laser irradiation of HA in acetonitrile ($\lambda_{exe}=532$ nm) in the presence of DEA is shown in Fig. 3. For HA in the presence of DEA there was no evidence of T–T absorption and the so transient observed might be $HA^{\bullet -}$.

$$HA^* + DEA \rightarrow HA^{\bullet -} + DEA^{\bullet +} \quad (1)$$

The time-resolved transient absorption spectra of HA and biological substrates in acetonitrile are shown in Figs. 4 and 5. These figures show that, concomitant with the rapid decay of bands I and II ($\lambda_{max}=530$, 560 nm respectively), band III ($\lambda_{max}=600$ nm) changed with some bathochromic shift following pulse excitation. The final maximum absorption of band III ($\lambda_{max}=620$ nm) is in agreement with the presence of $HA^{\bullet -}$ for the related system used here (Fig. 2). In the presence of a biological substrate (BS), $HA^{\bullet -}$ may be generated by

$$HA^* + BS \rightarrow HA^{\bullet -} + BS^{\bullet +} \quad (2)$$

In addition, the decay of the semiquinone radical anion in Fig. 4 is much faster than that in Fig. 3. It is well known that the semiquinone radical anion is quite basic and can be easily

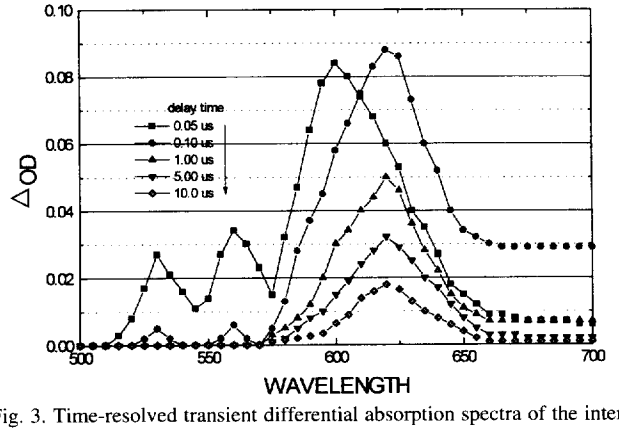


Fig. 3. Time-resolved transient differential absorption spectra of the interaction between HA and DEA in $CH_3CN$ at room temperature ([HA] = $1.2\times10^{-4}$ mol $l^{-1}$, [DEA] = $3\times10^{-3}$ mol $l^{-1}$, λex = 532 nm). The delay times after the pulses are shown above the spectra.

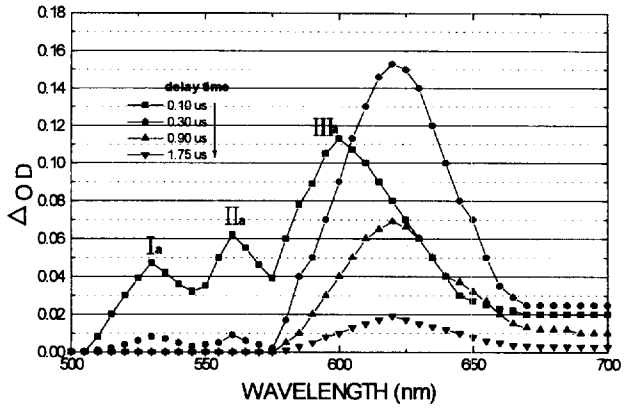


Fig. 4. Time-resolved transient differential absorption spectra of the interaction between HA and ascorbic acid in $CH_3CN$ at room temperature ([HA] = $1.2\times10^{-4}$ mol $l^{-1}$, [ascorbic acid] = $3.5\times10^{-3}$ mol $l^{-1}$, λex = 532 nm). The delay times after the pulses are shown above the spectra.

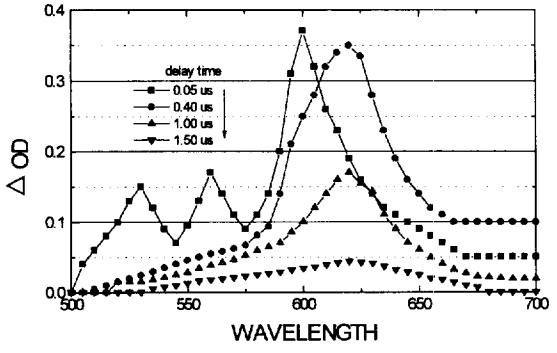


Fig. 5. Time-resolved transient differential absorption spectra of the interaction between HA and cysteine in $CH_3CN$ at room temperature ([HA] = $1.2\times10^{-4}$ mol $l^{-1}$, [cysteine] = $3.5\times10^{-3}$ mol $l^{-1}$, λex = 532 nm). The delay times after the pulses are shown above the spectra.

protonated to semiquinone radical in acidic condition. The protonation may catalyse the disproportionation of the radical anion and the transfer of the second electron to give the two-electron reduction product and the starting quinone. Under the experimental condition employed in Fig. 4, the HA radical anion might be easily protonated since ascorbic acid is a strong acid ($pK_1=4.04$). Hu et al. [13] considered that the HA radical anion could be produced only in medium of pH > 6.8; however, the process of electron transfer between HA and ascorbic acid was observed in acidic medium. The formation and decay of the HA radical anion was also observed under our experimental conditions, but it decays much faster than that in the medium of pH > 6.8, so the semiquinone radical anion of HA could not be detected, attributed to its very fast decay, by steady-state ESR in the presence of ascorbic acid at room temperature.

The decay of $HA^{\bullet -}$ is strongly influenced by oxygen; with a lifetime > 7.5 μs, it has larger quenching rate constants by oxygen than the triplet. Thus, in the presence of oxygen, $HA^{\bullet -}$ (lifetime < 2 μs) decays faster than the electron back-transfer. Reaction (3), leading to the $O_2^{\bullet -}$ radical anion is suggested as the quenching process.

$$HA^{\bullet -} + O_2 \rightarrow HA + O_2^{\bullet -} \quad (3)$$

In order to confirm that the above process is possible and detect the formation of $O_2^{\bullet-}$ quantitatively, the following experiments were carried out.

### 3.3. Formation and detection of the superoxide radical anion

One of the commonly used method for the detection of superoxide anions is the chemical trapping of NBT. $O_2^{\bullet-}$ can be characterized by its reaction with NBT leading to an absorbance change at 560 nm, and by the inhibition of this reaction by SOD. The increase in absorbance at 560 nm is characteristic of the reaction of NBT with $O_2^{\bullet-}$. In the presence of 10 μg $ml^{-1}$ of active SOD, under the same conditions of irradiation, no alteration in the initial spectrum was observed whereas, in the presence of the same concentration of boiled (denatured) SOD, the result was identical to that observed without SOD; this lack of effect of denatured SOD gives proof of the involvement of $O_2^{\bullet-}$ in the reaction with NBT. The reasons which dictated the choice of SOD as a suitable competitive inhibitor are the following: (1) the reaction of $O_2^{\bullet-}$ with NBT was inhibited by SOD specifically and efficiently; (2) SOD has negligible visible absorbance at the concentration used and thus does not interfere optically with the production of superoxide by our system; (3) its rate constant for reaction with superoxide has been directly demonstrated to be independent of pH [14]. Control experiments indicated that HA, oxygen and light are essential for the reaction of NBT and, in the absence of reductant biological substrates, a very slight absorbance change at 560 nm was observed when irradiating the oxygen-saturated HA solution containing NBT for a long time. In a previous paper, Hu et al. [13] demonstrated that the HA radical anion could be generated by self-electron transfer between the ground and excited species, and thus the above phenomenon observed in the absence of biological substrates may be due to the production of $O_2^{\bullet-}$ via electron transfer from $HA^{\bullet-}$ to oxygen. However, in the presence of various reductant biological substrates, such as ascorbic acid or cysteine, the change of absorption at 560 nm is greatly intensified (Fig. 6). From the results of laser flash photolysis, it could be found that the addition of biological substrate promotes the formation of the semiquinone radical anion, and thus the consistent environment effects of formation of $O_2^{\bullet-}$ with $HA^{\bullet-}$ suggest that $HA^{\bullet-}$ could be the precursor for the formation of $O_2^{\bullet-}$. Alternatively, direct electron transfer from substrate to singlet oxygen, yielding a substrate radical anion and the superoxide radical anion, should also be considered [15]. In order to test the possible contribution of singlet oxygen in the formation of $O_2^{\bullet-}$ in the presence of a biological substrate, we performed the absorbance change at 560 nm with a constant concentration of HA in the presence of singlet oxygen quenchers: sodium azide, DABCO and histidine. As shown in Fig. 6, the addition of these quenchers did not decrease the formation of $O_2^{\bullet-}$, indicating that the involvement of singlet oxygen in the $O_2^{\bullet-}$ photosensitization process was excluded. Along these lines and taking into account the above results, the probable mechanism of $O_2^{\bullet-}$ formation in the presence of a biological substrate is the reaction of the semiquinone radical anion $HA^{\bullet-}$ which is produced by electron transfer from the biological substrate to HA* with oxygen. The yields of formation of the superoxide radical anion in the absence and presence of biological substrates are listed in Table 1.

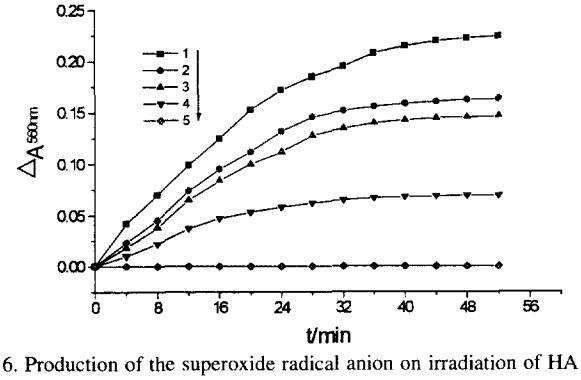


Fig. 6. Production of the superoxide radical anion on irradiation of HA in the presence of various biological substrates in $CH_3CN$ as solvent: 1, HA + ascorbic acid + NBT; 2, HA + cysteine + NBT; 3, HA + tryptophan + NBT; 4, HA + methionine + NBT; 5, HA + ascorbic acid + NBT + SOD (NBT = Nitro Blue Tetrazolium).

In addition, formation of $O_2^{\bullet-}$ during irradiation of oxygen-saturated HA solution (0.1 mM) containing DMPO (30 mM) and biological substrate was confirmed by the DMPO–spin trapping method. A multiplet ESR spectrum appeared; this ESR spectrum is characterized by three coupling con-

Table 1
Concentration of the superoxide radical anion on irradiation of HA in the presence of various biological substrates (20 °C)

| Number | Biological substrate | Oxidation potential of biological substrate | $[O_2^{\bullet-}]$/ppm (in 10 min) |
|---|---|---|---|
| 1 | Kynurenine | 0.95 | 0.5 |
| 2 | Indole | 0.98 | 0.8 |
| 3 | Methionine | 0.85 | 1.6 |
| 4 | Tryptophan | 0.80 | 4.6 |
| 5 | Tryptophan + $NaN_3$ | 0.80 | 5.2 |
| 6 | Tryptophan + DABCO | 0.80 | 5.8 |
| 7 | Cysteine | 0.72 | 5.5 |
| 8 | Ascorbic acid | 0.57 | 9.2 |
| 9 | Tyrosine | 0.94 | 0.6 |
| 10 | Aspartic acid | 1.24 | 0.3 |

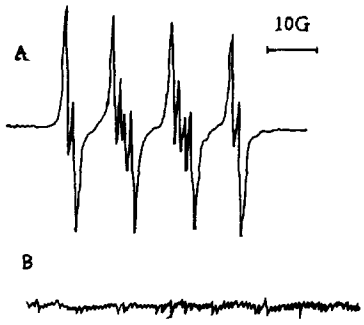


Fig. 7. (A) ESR spectrum of the DMPO–superoxide radical adduct produced from the irradiation of an oxygenated DMSO solution of HA (0.1 mM) and DMPO (30 mM). (B) Same as (A) but in the presence of SOD. Instrument settings: microwave power 5.05 mW; modulation amplitude 1.05 G; receiver gain $2 \times 10^4$.

stants, which are due to the presence of the nitrogen atom and two hydrogen atoms in the $\beta$ and $\gamma$ positions.

The hyperfine coupling constants ($a_N = 12.8$ G, $a_\beta^H = 10.4$ G, $a_\gamma^H = 1.5$ G) determined for this ESR spectrum are consistent with previous reported values for the DMPO–$O_2^{\bullet -}$ radical adduct [14]. Addition of superoxide dismutase (SOD), a specific and efficient scavenger for superoxide, could inhibit the oxygen-dependent DMPO–$O_2^{\bullet -}$ adduct formation (Fig. 7).

## 4. Conclusions

Using laser flash photolysis and the reaction of $O_2^{\bullet -}$ with NBT, the process of electron transfer from some biological substrates to HA* in acidic medium and the relative quantum yield of the superoxide anion photoproduced by HA and the biological substrates have been observed and determined for the first time. According to the results described here, it seems reasonable to conclude that an electron transfer (Type I) superoxide-mediated photodynamic mechanism may play a hitherto unrecognized role in the photodynamic interaction between HA and some biological substrates.

## Acknowledgements

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