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UV–visible and electron spin resonance study of mercapto-substituted hypocrellin B: photogeneration of semiquinone radical anion and superoxide

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Abstract

Mercapto-substituted hypocrellin B derivatives (MHBDs), display, in the absence of electron donors, a characteristic absorbance peak and electron spin resonance (ESR) signal, which can be attributed to the semiquinone radical anion, formed by intermolecular electron transfer. In the presence of electron donors, the amplitude of the ESR signal increases significantly. Irradiation of MHBDs in the presence of oxygen leads to the generation of superoxide radicals, which may be registered by the spin trapping technique; the formation mechanism of superoxide in the absence or presence of electron donors is discussed. The free radicals and superoxide radicals formed on photoirradiation of MHBDs may play a hitherto unrecognized role in biological processes. © 1997 Elsevier Science S.A.

Keywords: Electron spin resonance; Mercapto-substituted hypocrellin B; Photogeneration; Semiquinone radical anion; Superoxide; UV–visible absorbance

1. Introduction

Hypocrellin A and B (HA and HB) (Fig. 1) have recently been isolated from the natural fungus sacs of *Hypocrella bambusae* in China. These lipid-soluble 4,9-dihydroxy-3,10-perylenequinone derivatives, employed in pioneering photodynamic therapy (PDT) applications of perylenequinonoid pigments (PQPS) [1,2], exhibit several advantages over the presently used haematoporphyrin derivative (HPD), i.e. ease of preparation and purification relative to HPD, small aggregation tendency (which decreases the efficiency of HPD), strong red light absorptivity and significantly reduced normal tissue photosensitivity because of the fast metabolism in vivo [3]. As a result, hypocrellins have been successfully employed in the clinical PDT treatment of a number of skin diseases, such as white lesions of vulva, keloid, vitiligo, psoriasis, tinea capitis and lichen amyloidosis [4–6] without observing the prolonged normal tissue photosensitivity that occurs with HPD [3]. It has also been shown that hypocrellins are efficient singlet oxygen generators [7], and demonstrate advantages over the classic $^1O_2$ sensitizers (such as porphyrins, rose bengal, methylene blue, etc.), including high molar extinction coefficients, wide UV–visible absorption, high quantum yields of singlet oxygen generation, high stability, good solubility and small solvent and concentration effects [8].

Recent studies have indicated that the target of photodynamic action of hypocrellin is the cell membrane; this photodynamic action causes a reduction in the quantity of mercapto groups in membrane proteins [9–11]. Until recently, relatively little effort had been devoted to the investigation of the reaction of HB with mercapto compounds and the mechanism of photodynamic activity of these HB derivatives.

In this work, we describe the detection of electron spin resonance (ESR) signals of two kinds of mercapto-substituted HB derivative (5-RS-HB, 5,8-RS-HB) in dimethylsulphoxide (DMSO) in the absence of electron donors on irradiation by visible light. In addition, an increase in the concentration of free radicals was detected in the presence of reducing agents. Finally, superoxide radicals were detected using the spin trapping technique, and the generation mechanism of $O_2^{\cdot -}$ was studied in detail.

2. Experimental section

HA and HB were extracted from the fungus sacs of *Hypocrella bambusae* and purified by recrystallization from acetone; 5-RS-HB and 5,8-RS-HB (Fig. 1) were prepared

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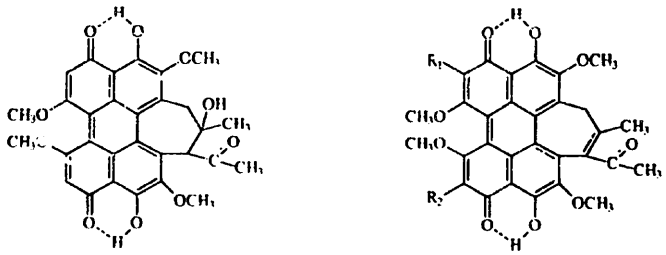

HA

HB (R_1=R_2=H)
5-RS-HB (R_1=$CH_3(CH_2)_{11}S$,R_2=H)
5,8-RS-HB (R_1=R_2=$CH_3(CH_2)_{11}S$)

Fig. 1. Chemical structures of HA, HB, 5-RS-HB and 5,8-RS-HB.

according to the method described previously [12] 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) and superoxide dismutase (SOD) were purchased from Aldrich Chemical Company; DMPO was purified as described previously [13]; the degree of DMPO purity was determined by the ESR method. Cysteine was purchased from Biotech Technology Corporation, Chinese Academy of Sciences. Diethylaniline, *N*-ethylaniline, aniline, ascorbate, ethylenediaminetetraacetic acid (EDTA), reduced nicotinamide adenine dinucleotide (NADH), dodecyl mercaptan, DMSO and other solvents, all of analytical grade, were purchased from Beijing Chemical Plant. Water was freshly distilled before use. The solutions were purged with oxygen, air or argon according to the experimental requirements. The required high-purity solvents were prepared by further purification of the commercial products, and no impurities were detected by absorption and/or fluorescence spectroscopy.

ESR spectra were recorded using a Bruker ER-300 EPR spectrometer with 100 kHz field modulation operating at 9.5 GHz in quartz capillaries. Photoinduced ESR spectra of directly illuminated samples were recorded inside a microwave cavity. A 450 W medium-pressure sodium lamp was used as light source. A long-pass filter was employed to eliminate light at wavelengths less than 470 nm. Samples were deoxygenated by bubbling with argon gas for 30 min and were then injected quantitatively (25 μl) into deoxygenated quartz capillaries. The concentration of the radicals was estimated by the double integration method. The radical 2,2,6,6-tetramethylpiperidine oxide (TEMPO), at a given concentration, was employed as reference; the accuracy of determination of the radical concentrations was 20%. UV–visible absorption difference spectra were recorded with a Shimadzu UV-160A UV–visible spectrophotometer; the samples were bubbled with highly purified argon for 30 min, irradiated with a 450 W medium-pressure sodium lamp and then measured immediately.

3. Results

Illumination of 5-RS-HB (1 mM) in an argon-gassed DMSO solution for 2 min leads to the generation of a strong ESR signal as shown in Fig. 2. The intensity of the ESR signal increases rapidly with increasing photoirradiation time, and decreases very slowly in the dark. The same spectrum is obtained when dimethylformamide (DMF) is used as solvent instead of DMSO. Irradiation is required for the generation of the 5-RS-HB radical. The kinetics reflect a fast increase in radical concentration, a rapid approach to a plateau and a slow decrease after the light is extinguished. When the sample is exposed to oxygen, the ESR signal is quenched rapidly. The ESR signal intensity of the 5-RS-HB radical

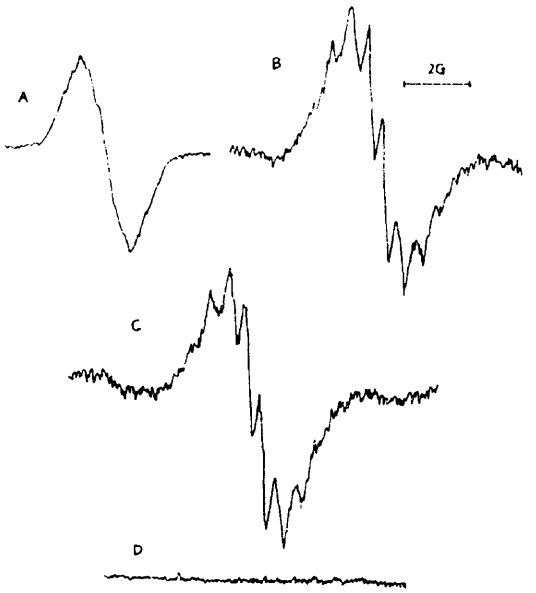

Fig. 2. Spectrum A: photoinduced ESR spectrum from a deaerated DMSO solution of 5,8-RS-HB (1 mM) on irradiation above 470 nm for 2 min. Spectrum B: same as spectrum A, but the photosensitizer was 5-RS-HB (1 mM). Spectrum C: same as spectrum A, but in the presence of DEA (5 mM) and irradiation for 30 s. Spectrum D: same as spectrum A, but oxygen was bubbled through the solution after illumination. Instrumental settings (spectra A, B and D): microwave power, 1.01 mW; modulation amplitude, 0.166 G; scan range, 20 G; receiver gain, 2×10^5. Instrumental settings (spectrum C): microwave power, 1.01 mW; modulation amplitude, 0.166 G; scan range, 20 G; receiver gain, 2×10^4.

depends on the concentration of 5-RS-HB, the presence of oxygen and the irradiation time and intensity. The 5-RS-HB concentration exerts such a strong effect on the generation of the 5-RS-HB radical that the signal of the 5-RS-HB radical can be observed even in aerated DMSO at high concentration. In order to identify the character of the ESR signal shown in Fig. 2, the following experiment was carried out. 5-RS-HB (1 mM) in DMSO was illuminated in the presence of *N,N*-diethylaniline (DEA), an excellent electron donor, for 30 s. The ESR spectrum obtained is very similar to that recorded in the absence of DEA. From Fig. 2, it can be seen that the addition of DEA intensifies the ESR signal significantly. Moreover a series of other electron donors, including *N*-ethylaniline, aniline, cysteine, ascorbate, EDTA and NADH, produce analogous results. This indicates the anionic character of the 5-RS-HB radical. As in the case of 5-RS-HB, 5,8-RS-HB also produces a semiquinone radical on illumination (Fig. 2). Its properties are similar to those of the 5-RS-HB radical.

The ESR signal of the 5-RS-HB radical is quenched significantly by oxygen; the signal disappears completely when oxygen is bubbled through a solution of the 5-RS-HB radical. Furthermore, visible light irradiation of an oxygenated solution of 5-RS-HB in the presence of a spin trap, DMPO, generates an ESR spectrum of the DMPO–superoxide radical adduct, immediately accompanied by the disappearance of the ESR signal of the 5-RS-HB radical (Fig. 3). This multiplet ESR spectrum is characterized by three coupling constants due to the presence of a nitrogen atom and two hydrogen atoms in the β and γ positions. The determined coupling constants ($a^N = 12.7$ G, $a_\beta^H = 10.3$ G and $a_\gamma^H = 1.5$ G) for this ESR spectrum are consistent with previously reported values for the DMPO–$O_2^{\cdot-}$ radical adduct in DMSO [14]. The addition of SOD, a specific and efficient scavenger for superoxide, inhibits the O_2-dependent DMPO–$O_2^{\cdot-}$ adduct formation (Fig. 3). SOD inhibits the ESR signal intensity in a dose-dependent manner. Analogous results are obtained when 5-RS-HB is replaced by 5,8-RS-HB.

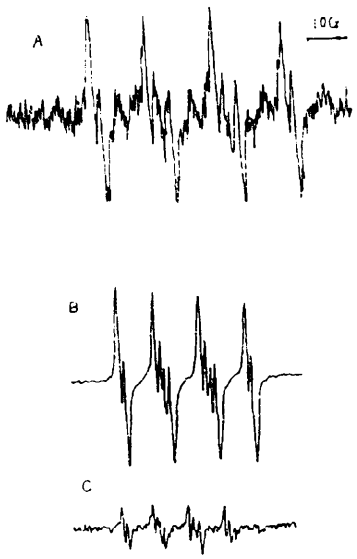

Fig. 3. Spectrum A: ESR spectrum of DMPO–superoxide radical adduct produced on irradiation of an oxygenated DMSO solution of 5,8-RS-HB or 5-RS-HB (1 mM) and DMPO (30 mM). Spectrum B: same as spectrum A, but in the presence of DEA. Spectrum C: same as spectrum A, but in the presence of SOD. Instrumental settings: microwave power, 5.05 mW; modulation amplitude, 1.05 G; receiver gain, 2×10^5 (spectra A and C); 2×10^4 (spectrum B).

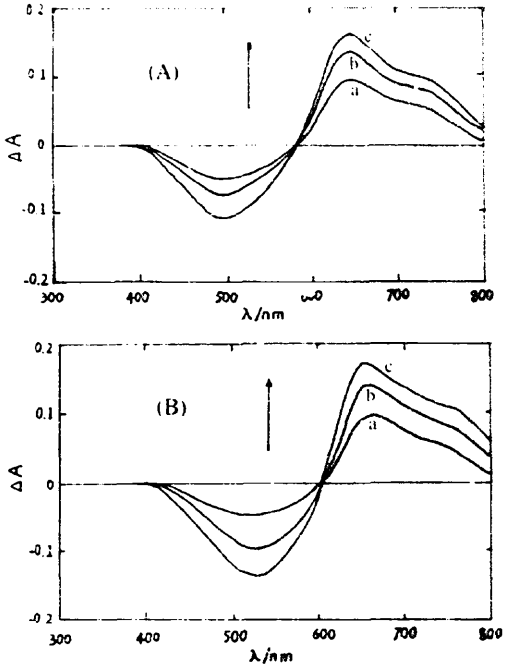

Fig. 4. UV–visible absorption difference spectra obtained 120 s (a), 240 s (b) and 360 s (c) after irradiation of a solution of 5-RS-HB (spectrum A) and 5,8-RS-HB (spectrum B) in deaerated DMSO. Bleaching of 5-RS-HB and 5,8-RS-HB is observed at 420–580 nm, and the characteristic absorption peaks due to the radical anions of 5-RS-HB and 5,8-RS-HB are observed at 645 nm and 655 nm respectively.

The decay kinetics of the 5-RS-HB and 5,8-RS-HB radicals were measured by recording the decrease in the ESR signal amplitude from its steady state level after illumination had been extinguished. The half-life of these radicals is approximately 10 min, and can be satisfactorily described by a second-order reaction constant. The semiquinone radical anions of 5-RS-HB and 5,8-RS-HB were very long lived, and therefore experiments were carried out using a scanning UV–visible spectrometer. When 5-RS-HB or 5,8-RS-HB in a deaerated solution of DMSO was irradiated, the colour of the sample changed from purple to green. The difference spectra obtained after irradiation of the samples are illustrated in Fig. 4: the absorption at 655 nm and 645 nm can be attributed to the 5,8-RS-HB and 5-RS-HB radicals respectively for the following reasons:

1. identical absorbance peaks were formed in the presence of a reductant (such as *N,N*-diethylaniline, *N*-ethylaniline and aniline) on irradiation with deaeration;
2. when the air was made to re-enter the photoirradiation system, the absorbance peak at 655 nm or 645 nm disappeared completely and the product returned quantitatively to 5,8-RS-HB or 5-RS-HB;
3. the decay constants of absorption at 655 nm and of the ESR signal of the 5,8-RS-HB radical, or the absorption at 645 nm and the ESR signal of the 5-RS-HB radical, were consistent in the dark.

4. Discussion

In this work, we have shown that both 5-RS-HB and 5,8-RS-HB in DMSO solution, on irradiation, can generate a radical in the absence of electron donors. The 5-RS-HB or 5,8-RS-HB concentration exerts a strong effect on the generation of the semiquinone radical. The strong concentration effect indicates that both the 5-RS-HB and 5,8-RS-HB radicals may be generated by intermolecular electron transfer between the ground and excited state species according to

$$5\text{-RS-HB} + 5\text{-RS-HB}^{*} \rightarrow 5\text{-RS-HB}^{\bullet -} + 5\text{-RS-HB}^{\bullet +} \quad (1)$$

It has been observed that HB, the parent molecule of 5-RS-HB and 5,8-RS-HB, can form an excimer at high concentration. Nuclear magnetic resonance (NMR) studies suggest that the excimer has a sandwich-like configuration in which the quinonoid moiety of HB overlaps with the phenolic part of another HB molecule [15]. This structure is favourable for electron transfer because the electron donor (phenolic moiety) and the electron acceptor (quinonoid moiety) are positioned relatively close to one another and are better oriented compared with the free-distributed mode of a dilute solution. Due to the analogous chemical structure of 5-RS-HB, 5,8-RS-HB and HB, the light stimulation of the ESR signals of 5-RS-HB or 5,8-RS-HB may also be explained by an intermolecular electron transfer mechanism. On irradiation of the sample, an excited state is formed which may transfer its electron to 5-RS-HB or 5,8-RS-HB in the ground state to give the semiquinone cation and anion radical. This mechanism was postulated to occur on photoexcitation of concentrated solutions of quinone (1×10^{-3} mol dm^{-3} or more) in the absence of electron donors [15]. An additional explanation for the ease of generation of the semiquinone radical of 5-RS-HB or 5,8-RS-HB in the absence of electron donors may be due to the long chains at position 5,8. Interaction results in a short distance between the 5,8-RS-HB or 5-RS-HB molecules, especially at high concentration (possibly stacked intermolecularly), making the electron transfer process more efficient, so that the absorbance of the semiquinone radical can be observed in the steady state absorption spectra.

If both the 5-RS-HB and 5,8-RS-HB radicals are produced via Eq. (1), the ESR signal may be due to the 5-RS-HB/5,8-RS-HB semiquinone radical anion, the 5-RS-HB/5,8-RS-HB radical cation or the composite spectrum of both radical species, depending on the reaction conditions and relative lifetimes of these radicals. It is observed that the ESR signal of the 5-RS-HB or 5,8-RS-HB radical is greatly intensified by the addition of electron donors. These reduced substrates, which are much richer in electrons than both 5-RS-HB and 5,8-RS-HB, may transfer an electron directly to the excited state via

$$5\text{-RS-HB(5,8-RS-HB)} + D \rightarrow 5\text{-RS-HB}^{\bullet -}\text{(5,8-RS-HB}^{\bullet -}\text{)} + D^{\bullet +} \quad (2)$$

and thus enhance efficiently the concentration of the semiquinone radical anion. Furthermore, the same ESR spectrum is also recorded when 5-RS-HB or 5,8-RS-HB is reduced by electrolytic methods or by sodium dithionite reactions, both of which are accepted as reliable methods for the production of semiquinone radicals from quinones. Therefore the observed ESR spectrum is considered to originate from 5-RS-HB$^{\bullet -}$ or 5,8-RS-HB$^{\bullet -}$.

The decay kinetics of both 5-RS-HB$^{\bullet -}$ and 5,8-RS-HB$^{\bullet -}$ can be satisfactorily described by second-order reaction constants, indicating that the termination of the semiquinone radical anion is via a radical–radical annihilation reaction. In general, the radical cation of quinone is difficult to detect in common organic solvents due to its strong oxidizing ability [17].

The spin trapping experiments on irradiation of 5-RS-HB or 5,8-RS-HB in DMSO using DMPO point to the presence of a superoxide molecule. The absence of an ESR signal due to the DMPO–$O_2^{\bullet -}$ adduct in the presence of SOD further confirms the formation of superoxide molecules. The generation of superoxide may be rationalized by one of the following processes

$$5,8\text{-RS-HB}^{\bullet -}\text{(5-RS-HB}^{\bullet -}\text{)} + O_2 \rightarrow 5,8\text{-RS-HB(5-RS-HB)} + O_2^{\bullet -} \quad (3a)$$

$$^3\text{5,8-RS-HB(}^3\text{5-RS-HB)} + O_2 \rightarrow 5,8\text{-RS-HB}^{\bullet +}\text{(5-RS-HB}^{\bullet -}\text{)} + O_2^{\bullet -} \quad (3b)$$

$$^3\text{5,8-RS-HB(}^3\text{5-RS-HB)} + O_2 \rightarrow {}^1O_2 + 5,8\text{-RS-HB(5-RS-HB)} \quad (3c1)$$

$${}^1O_2 + 5,8\text{-RS-HB(5-RS-HB)} \rightarrow 5,8\text{-RS-HB}^{\bullet +}\text{(5-RS-HB}^{\bullet -}\text{)} + O_2^{\bullet -} \quad (3c2)$$

In process (3a), the excited state of 5,8-RS-HB or 5-RS-HB, formed on irradiation, collides with another molecule in the ground state, resulting in electron transfer leading to the generation of the semiquinone radical anion; this then transfers its electron to oxygen, producing the original sensitizer and the superoxide radical anion. Radical anions derived from anthraquinone or adriamycin react rapidly with oxygen with a rate constant of the order of 10^8 dm^3 mol^{-1} s^{-1} [18,19]. Alternatively, process (3b) may produce the superoxide rad-

ical. In addition to these type I mechanisms, a type II mechanism (process (3c)) may take place. This process involves the primary generation of singlet oxygen which, in turn, oxidizes a ground state sensitizer. Because the addition of a $^1O_2$ scavenger, such as sodium azide and 1,4-diazabicyclo[2.2.2]octane (DABCO), does not lead to a decrease in the ESR signal intensity of the adduct, process (3c) is probably not involved in the formation of $O_2^{\cdot -}$ to a significant extent. In addition, it was also found that DEA (3 mM) results in a 15-fold enhancement of $O_2^{\cdot -}$ production; at the same time, when DEA (1 mM) is added to an Ar-saturated DMSO solution containing 5,8-RS-HB (1 mM), the intensity of the ESR spectrum due to 5,8-RS-HB$^{\cdot -}$ increases by 15-fold when compared with the spectrum of the sample in the absence of DEA. When an Ar-saturated DMSO solution containing a low concentration of 5,8-RS-HB (10 μM) is illuminated with visible light, no ESR signal of 5,8-RS-HB$^{\cdot -}$ is observed; when the solution is bubbled with oxygen in the presence of DMPO, no ESR spectrum of the DMPO–$O_2^{\cdot -}$ adduct is detected. This indicates that the formation of the DMPO–$O_2^{\cdot -}$ adduct is dependent on the presence of the semiquinone radical anion. The consistent environmental effects of formation of $O_2^{\cdot -}$ with those of 5-RS-HB or 5,8-RS-HB suggest that the semiquinone radical anion is the precursor to the formation of $O_2^{\cdot -}$ by 5-RS-HB or 5,8-RS-HB. Thus process (3a) seems to be preferred.

5. Conclusions

Our studies have indicated that mercapto-substituted HB derivatives display an ESR signal in the absence of electron donors on irradiation. This signal is attributed to the semiquinone radical anion, which is produced via intermolecular electron transfer between the excited and ground state species. In the presence of electron donors, the intensity of the ESR signal increases. On irradiation of oxygenated 5-RS-HB or 5,8-RS-HB solution in the absence of reducing agents, the generation of the $O_2^{\cdot -}$ radical was observed by the DMPO spin trapping technique, and the generation mechanism of $O_2^{\cdot -}$ via the transfer of an electron from the semiquinone radical anion to oxygen was confirmed. This work indicates that the semiquinone radical of 5-RS-HB or 5,8-RS-HB and the oxygen radical generated by the photoirradiation of the samples may be implicated in the mechanism of photodynamic activity.

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