Electron transfer interaction between hypocrellin A and biological substrates and quantitative analysis of superoxide anion radicals

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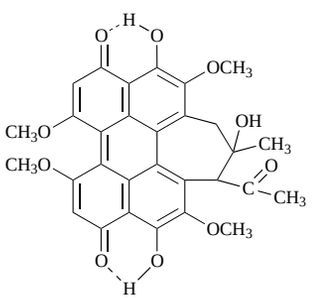
Hypocrellin A (HA), a hydroxyperylenequinone derivative, is an efficient phototherapeutic agent. Laser flash photolysis is used to produce and investigate the properties of the lowest excited triplet state (T_1) and semiquinone radical anion of hypocrellin A ($HA^{\cdot -}$) at room temperature. In the presence of some biological substrates (such as ascorbic acid and cysteine), the formation and decay of HA radical anion at different pH, attributed to the electron transfer between triplet HA and substrates, are observed. Meanwhile, the superoxide radical anion ($O_2^{\cdot -}$) production by photoactivated HA in the presence of biological substrates is 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 $O_2^{\cdot -}$ is demonstrated unequivocally to be by reaction with the superoxide dismutase. The rate of reduction of NBT is dependent not only on the concentration of NBT but also on the pH of the system. The relative quantum yield of superoxide anion radicals increases considerably in alkaline solution: $\varphi_{O_2^{\cdot -}} = 6.97 \times 10^{-3}$ at pH 9.0 as compared with $\varphi_{O_2^{\cdot -}} = 2.49 \times 10^{-4}$ at pH 5.8 in the presence of ascorbic acid. Based on the experimental results, electron transfer (Type I) mechanism may play a hitherto unrecognized role in the photodynamic interaction between HA and some biological substrates.

Introduction

Hypocrellin A (HA) isolated from the parasitic fungus (*B. et Br*) *Sacc*. is an efficient phototherapeutic agent. It is a perylenequinone derivative bearing two hydroxy groups *peri* to two carbonyl groups, which form strong hydrogen bonds.[1,2] In clinical trials, HA has produced promising results as an antitumor agent.[3–5] It exhibits several advantages over the presently widely used hematoporphyrin derivatives (HPD), *i.e.* ready preparation and easy purification relative to HPD, low aggregation tendency, strong red light absorption and significantly reduced normal-tissue photosensitivity because of its rapid metabolism *in vivo*.[6] As a result, hypocrellin A has been successfully employed in the clinical photodynamic therapy (PDT) treatment of certain skin diseases, such as white lesion of vulva, keloid, vertiligo, psorasis, tinea capitis and lichen amyloidisis,[7–9] without observation of the prolonged normal-tissue photosensitivity that occurs with porphyrin photosensitizers.[6] Recently, it has been shown that HA can kill tumor cells efficiently and accumulate selectively in cancer cells.



Hypocrellin A

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.[11,12] Moreover, previous studies have shown that, on visible light irradiation, HA undergoes an energy transfer reaction in organic solvent, leading to the generation of singlet oxygen. However, very few studies have been reported on the photodestruction mechanism of various biological substrates by HA. The visible-light illumination of oxygen-saturated HA-containing biological substrates in DMSO led to damage of these substrates even in the presence of singlet oxygen quenchers, such as NaN_3 and 1,4-diazabicyclo[2,2,2]octane (DABCO), although HA has been identified as a new efficient singlet oxygen generator.[11] Usually $^1O_2$ is thought to play a key role in the photosensitization of tumors; 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 tumors. These considerations prompted our interest in the mechanism of interaction between HA and some biological substrates.

Although there are hints in the literature that $O_2^{\cdot -}$ should be generated by HA *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[15,16] appeared to be more suitable for the quantitative analysis of $O_2^{\cdot -}$ than 5,5-dimethyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (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.* considered that the semiquinone radical anion of HA could be produced and observed only in neutral or basic solution in the presence of ascorbic acid.[13a] In this present work, laser flash photolysis was used to investigate the properties of the semiquinone radical anion of HA in the presence of various biological substrates, such as ascorbic acid and cysteine, at different pH. Meanwhile, superoxide radical anion was also observed and studied by EPR. The purpose of this study was to elucidate the mechanism of photodynamic interaction of HA with some biological substrates, confirm the production of HA radical anion in weak acidic medium and quantify the formation of $O_2^{\cdot -}$ using NBT trapping method.

Results and discussion

Generation of triplet states

Direct excitation of HA in CH_3CN in the absence of biological substrates produced a detectable amount of triplet state.[17]

Table 1 ^{3}HA decay constants (k_d) and $HA^{\bullet -}$ radical formation (k_f) and decay (k_d) constants in the presence of biological substrates. [HA] = 1.2×10^{-5} mol dm^{-3}

Biological substrate	$k_d(^3HA)/10^{-7}$ M^{-1} s^{-1}	$k_f(HA^{\bullet -})/10^{-7}$ M^{-1} s^{-1}	$k_d(HA^{\bullet -})/10^{-5}$ M^{-1} s^{-1}
Ascorbic acid	5.12 ± 0.22	4.98 ± 0.30[a] (5.02 ± 0.21)[b]	1.41[a] (3.52[b])
Cysteine	3.52 ± 0.18	3.75 ± 0.20[a] (3.61 ± 0.18)[b]	1.03[a] (2.53[b])
Tryptophan	3.42 ± 0.11	3.21 ± 0.19[a] (3.30 ± 0.23)[b]	0.86[a] (1.82[b])
Methionine	3.22 ± 0.23	3.45 ± 0.24[a] (3.20 ± 0.24)[b]	0.93[a] (2.04[b])
Kynurenine	1.45 ± 0.22	1.32 ± 0.18[a] (1.40 ± 0.25)[b]	0.42[a] (1.28[b])
Indole	1.98 ± 0.24	2.12 ± 0.17[a] (2.03 ± 0.19)[b]	0.59[a] (1.37[b])

[a] In the absence of oxygen. [b] In the presence of oxygen.

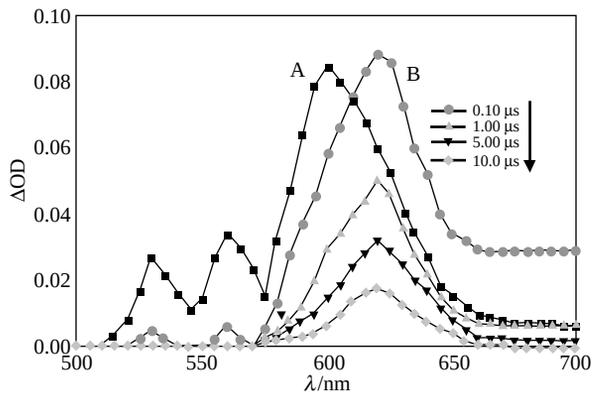


Fig. 1 A: Time-resolved transient differential absorption of HA in $CHCN_3$ at room temperature ([HA] = 1.2×10^{-4} mol dm^{-3}, Ar saturated for 30 min, λ_{exe} = 532 nm). B: Time-resolved transient differential absorption spectra of the interaction between HA and ascorbic acid in CH_3CN at room temperature ([HA] = 1.2×10^{-4} mol dm^{-3}, DEA = 3.5×10^{-3} mol dm^{-3}, λ_{exe} = 532 nm). The time delay after the pulses are shown above the spectra.

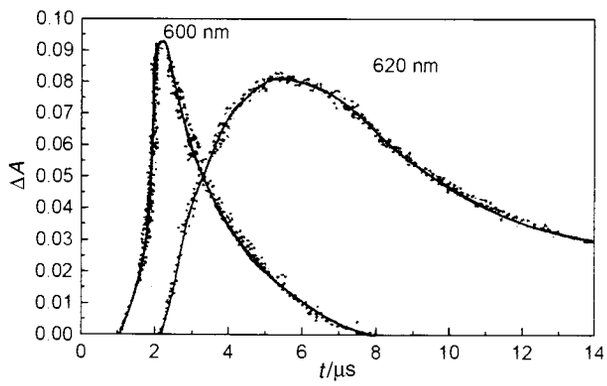


Fig. 2 The absorption–time profile of the decay of ^{3}HA* at 600 nm and formation of $HA^{\bullet -}$ at 620 nm in the presence of HA (1.2×10^{-4} M) and ascorbic acid (3.0×10^{-3} M) in acetonitrile, pH = 8.0

Fig. 1A shows the time-resolved absorption spectra of the transients produced after 532 nm laser excitation of a deaerated solution of HA in $CHCN_3$. 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 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 (λ_{exe} = 355 nm). 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.

Formation of semiquinone radical anion in the presence of biological substrates

The aromatic amine *N*,*N*-diethylaniline (DEA), known to be an excellent electron donor, has often been used to study radical anions. The interaction between HA* and DEA has been investigated by laser flash photolysis.[13b] There was no evidence of T–T absorption and the transient observed might be $HA^{\bullet -}$, reaction (1). The time-resolved transient absorption spectra

$$HA^* + DEA \longrightarrow HA^{\bullet -} + DEA^{\bullet +} \qquad (1)$$

after irradiation of HA in the presence of ascorbic acid, recorded at different times after the flash, is shown in Fig. 1B. The absorption–time profiles of the decay of HA triplet at 600 nm and the formation of radical anion ($HA^{\bullet -}$) at 620 nm are shown in Fig. 2. At the same time, the measured formation rate of $HA^{\bullet -}$ is identical, within the experimental error (5–10%), to the HA triplet decay rates in each case as given in Table 1. Photophysical and electrochemical properties of HA and biological substrates are listed in Table 2. The standard free energy change ΔG_{et} of electron transfer between the triplet state of HA and biological substrates calculated from Weller's equation is less than zero, thus the above electron transfer process is feasible thermodynamically. With the knowledge that the quenching of the HA triplet by ascorbic acid or other reductant biological substrates is an electron transfer process, the quenching rates in each case become the electron transfer rate (k_{et}).

It is known that semiquinone radical anion is quite basic and can be easily protonated to semiquinone radical in acidic conditions. The protonation may catalyze the disproportionation of the radical anion and the transfer of the second electron to give the two-electron reduction product and the starting quinone. In the presence of ascorbic acid, the radical anion of HA ($HA^{\bullet -}$) absorbing at 620 nm (Fig. 1B) grows as the only product formed from the HA triplet. The formation and decay kinetics of the radical anion ($HA^{\bullet -}$) monitored at 620 nm are also investigated. Double exponential analysis was used to fit the fast (formation) and the slow (decay) component. We examined the influence of O_2 on the kinetic rate constants of $HA^{\bullet -}$ formation and disappearance in the presence of reductant biological substrates, and the results are listed in Table 1. It is evident that the rate constant of $HA^{\bullet -}$ formation was the same both in the absence and in the presence of oxygen, although the rate of $HA^{\bullet -}$ disappearance was found to increase in the presence of oxygen. From these results, it can be seen that oxygen plays an important role in the stability of $HA^{\bullet -}$.

The decay of the radical anion is strongly influenced by oxygen (Table 1). The radical anion of HA, showing a lifetime >7.5 μs, has larger quenching-rate constants by oxygen than triplet. From the redox potentials for $O_2^{\bullet -}$ [$E^0(O_2/O_2^{\bullet -})$] = −0.33 V [14] and HA [$E^0(HA/HA^{\bullet -})$] = −0.55 V (CH_3CN), it is evident that, on thermodynamic consideration, $HA^{\bullet -}$ could reduce oxygen. Thus, in the presence of oxygen, $HA^{\bullet -}$ (lifetime < 2 μs) decays faster than the electron back-transfer, reaction (2), leading to

$$HA^{\bullet -} + O_2 \longrightarrow HA + O_2^{\bullet -} \qquad (2)$$

the $O_2^{\bullet -}$ radical anion and could be suggested as quenching process [reaction (2)].

Table 2 Concentration of superoxide radical anion on irradiation of HA in the presence of various biological substrates, ΔG_{et} and quantum yield of $O_2^{\bullet -}$ ($\varphi_{O_2^{\bullet -}}$) (20 °C)

Biological substrate	Oxidation potential of substrate V	ΔG_{et}/eV[a]	$[O_2^{\bullet -}]$ ppm per 10 min			$\varphi_{O_2^{\bullet -}}/s\ 10^3$		
			pH 5.8	pH 1.5	pH 9.0	pH 5.8	pH 7.5	pH 9.0
Kynurenine	0.95	−0.31	0.1	0.5	2.8	0.014	0.068	0.38
Indole	0.98	−0.28	0.18	0.8	4.5	0.025	0.11	0.62
Methionine	0.85	−0.41	0.34	1.6	8.7	0.046	0.22	1.19
Tryptophan	0.80	−0.46	0.92	4.6	25.4	0.126	0.63	3.48
Tryptophan + NaN_3	0.80	−0.46	0.97	4.7	27.3	0.133	0.64	3.74
Tryptophan + DABCO	0.80	−0.46	0.95	4.6	25.9	0.131	0.63	3.55
Cysteine	0.72	−0.54	1.22	5.5	32.1	0.167	0.75	4.40
Ascorbic acid	0.57	−0.69	1.83	9.2	50.2	0.249	1.25	6.97
Tyrosine	0.94	−0.32	0.11	0.6	2.9	0.015	0.081	0.39
Aspartic acid	1.18	−0.08	0.05	0.3	1.4	0.007	0.041	0.19

[a] Triplet energy of HA (^3E_T = 1.84 eV) and reduction potential of HA [E(HA/HA$^{\bullet -}$) = 0.58 V] in acetonitrile solution from ref. 17.

In order to confirm that the process in reaction (2) is possible and to detect the formation of $O_2^{\bullet -}$ quantitatively, the following experiments were carried out.

Formation and detection of superoxide radical anion

Characterization of the superoxide anion. One of the most commonly used methods for the detection of superoxide anions has been the chemical trapping of NBT,[15,16] $O_2^{\bullet -}$ can be characterized by its reaction with NBT leading to the absorbance change at 560 nm, and by the inhibition of this reaction by superoxide dismutase (SOD). The increase in absorbance at 560 nm is characteristic of the reaction between NBT and $O_2^{\bullet -}$. In the presence of 20 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 is 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 with optical measurement of the production of superoxide by our system; (3) its rate constant for reaction with superoxide has been directly demonstrated to be independent of pH in the range of 5–10.[18] Control experiments indicated that HA, oxygen, biological substrates and light are essential for the reaction of NBT, and in the absence of reductant biological substrates, very slight absorbance change at 560 nm was observed when the oxygen-saturated HA solution containing NBT was irradiated for a long time. In a previous paper, Hu *et al.* have demonstrated that the HA radical anion could be generated by self-electron transfer between the ground and excited species,[13a] and thus the above phenomenon observed in the absence of reductant biological substrates may be due to the production of $O_2^{\bullet -}$ *via* electron transfer from HA$^{\bullet -}$ generated by self-electron transfer to oxygen. However, in the presence of various reductant biological substrates, such as ascorbic acid and cysteine, the change of absorption at 560 nm greatly intensified. From the results of laser flash photolysis, it was established 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 the semiquinone radical (HA$^{\bullet -}$) suggests that HA$^{\bullet -}$ could be the precursor for the formation of $O_2^{\bullet -}$. Alternatively, a direct electron transfer from substrate to singlet oxygen, yielding substrate radical cation and superoxide radical anion, should also be considered.[19] In order to test the possible contribution of singlet oxygen in the formation of $O_2^{\bullet -}$ in the presence of biological substrate, we performed the reduction of NBT with a constant concentration of HA in the presence of the singlet oxygen quenchers sodium azide, DABCO and histidine. The addition of these quenchers did not decrease the formation of $O_2^{\bullet -}$, excluding the involvement of singlet oxygen in the $O_2^{\bullet -}$ photosensitization process. Along these lines, and taking into account the above results, the probable mechanism of $O_2^{\bullet -}$ formation in the presence of biological substrates is by the reaction of semiquinone radical anion (HA$^{\bullet -}$), which is produced by electron transfer from biological substrates to HA* with oxygen. The formation yield of superoxide radical anion in the absence and presence of biological substrates were listed in Table 2.

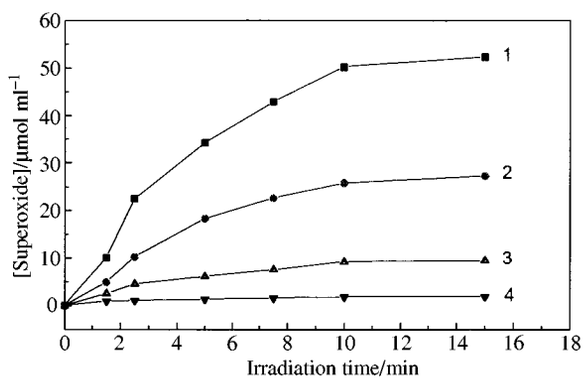


Fig. 3 Superoxide mediated by HA photosensitization in the presence of ascorbic acid as a function of pH: 1 (pH 9.0); 2 (8.2); 3 (7.5); 4 (5.8). Reaction mixtures contained 1.2×10^{-5} M HA, 3×10^{-3} M ascorbic acid with 2.0×10^{-5} M NBT.

The effect of pH on the rate of reduction of NBT in the presence of ascorbic acid containing a constant concentration of HA was also examined. A profile (Fig. 3) of the rate of $O_2^{\bullet -}$ formation in aerobic conditions as a function of pH was determined over the pH range between 5.8 and 9.0. The yield of $O_2^{\bullet -}$ was found to increase with increasing pH. It is evident that the rate of formation of $O_2^{\bullet -}$ at pH 9.0 (line 1) was over 25 times as high as at pH 5.8 (line 4). SOD completely inhibited this reaction at any pH tested.

Effect of NBT concentration on the rate of NBT reduction. The rate of trapping of superoxide by NBT, under given conditions, is dependent on the concentration of NBT in this system. If the concentration of NBT does not change appreciably in the reaction and if the reaction is not a chain event, the plot of the inverse velocity of reduced NBT formation against $[NBT]^{-1}$ should be a straight line with a constant slope and intercept. Here, the rate of accumulation of reduced NBT increased as a function of NBT concentration at pH 9 in the range (0.5–10) × 10^{-5} M. The ratio of the slope to intercept can give directly k_d/k_c, the ratio of the decay of superoxide radical to the rate of its reaction with NBT. The ratio value was found to be 1.41×10^{-4} M. Using the rate constant (k_d = 0.3 s^{-1} at pH 9) for spontaneous decay of $O_2^{\bullet -}$ in buffered solution reported in various papers,[19,20] k_c was calculated to be 2.13×10^3 M^{-1} s^{-1}. In contrast, at pH 5.8 the ratio was found to be 3.0×10^{-3} M and k_c

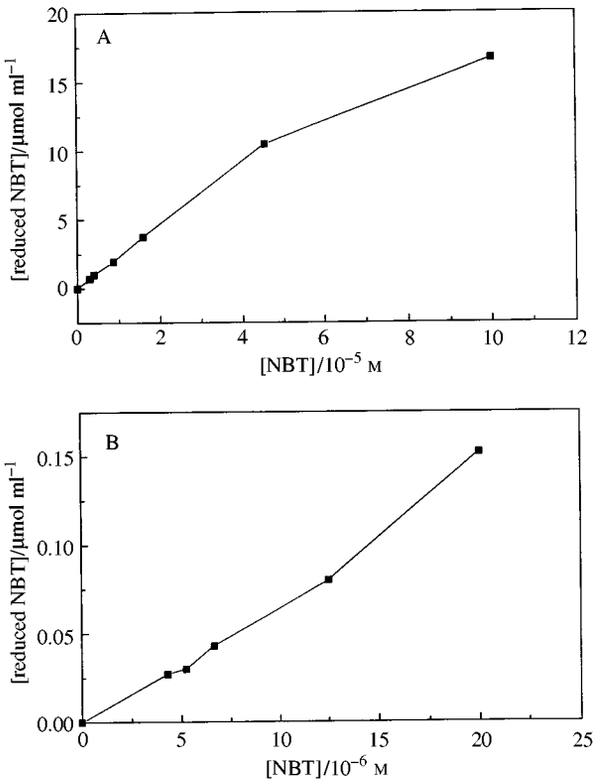


Fig. 4 Reduction of NBT as a function of its concentration after 10 min of continuous irradiation at (A) pH 9.0 and (B) pH 5.8. Reaction mixtures contain 1.2×10^{-5} M HA, 3×10^{-3} M ascorbic acid and various concentrations of NBT.

was calculated to be 7.0×10^{3} M^{-1} s^{-1} using the rate constant ($k_d = 21$ s^{-1} at pH 5).[21] From Fig. 4, it is also possible to determine the limited number of reduced NBT, *i.e.* the $O_2^{\cdot -}$ concentration, when [NBT] tends to infinity. Ten min after the start of the irradiation in the presence of 1.3×10^{-5} M HA and 2.4×10^{-5} M ascorbic acid, the value of $O_2^{\cdot -}$ concentration was found to be 5.02×10^{-5} M and 1.8×10^{-6} M at pH 9.0 and 5.8 respectively. We have determined the quantum yield $\varphi_{^1O_2}$ of $^1O_2$ by following the bleaching of 9,10-diphenylanthracene (9,10-DPA) in same system. Based on these results, the quantum yield $\varphi_{O_2^{\cdot -}}$ of $O_2^{\cdot -}$ may be evaluated using eqn. (3), where $[O_2^{\cdot -}]$ is

$$\varphi_{O_2^{\cdot -}} = \frac{[O_2^{\cdot -}]}{[^1O_2]} \varphi_{^1O_2} \qquad (3)$$

the limiting number of $O_2^{\cdot -}$ molecules 30 s after the start of irradiation when [NBT] tends to infinity, $[^1O_2]$ is the limiting number of oxidized 9,10-DPA molecules when [9,10-DPA] tends to infinity produced during the same time ($[^1O_2] = 1.8 \times 10^{-5}$ M) and $\varphi_{^1O_2}$ represents the quantum yield of $^1O_2$ ($\varphi_{^1O_2} = 0.05$). Thus, in this system, the relative quantum yield $\varphi_{O_2^{\cdot -}}$ of $O_2^{\cdot -}$ generated by HA and ascorbic acid was found to be 6.97×10^{-3} and 2.49×10^{-4} at pH 9.0 and 5.8 respectively.

In addition, formation of $O_2^{\cdot -}$ during irradiation of the oxygen-saturated HA solution (0.1 mM) containing DMPO (30 mM) and a biological substrate was confirmed by the DMPO-spin trapping method. A multiplet EPR spectrum appeared, characterized by three coupling constants, which are due to the presence of the nitrogen atom and two hydrogen atoms in the β- and γ-positions. The determined hyperfine coupling constants ($a_N = 12.8$ G, $a_{H\beta} = 10.4$ G, $a_{H\gamma} = 1.5$ G) for this EPR spectrum are consistent with previously reported values for the DMPO–$O_2^{\cdot -}$ radical adduct.[22] Addition of superoxide dismutase (SOD), a specific and efficient scavenger for superoxide, could inhibit the oxygen-dependent DMPO–$O_2^{\cdot -}$ adduct formation (Fig. 5).

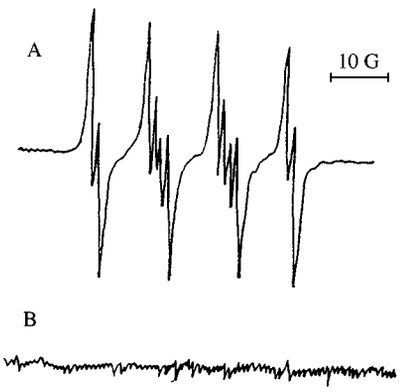


Fig. 5 Spectrum A: EPR spectrum of DMPO–superoxide radical adduct produced from the irradiation of an oxygenated DMSO solution of HA (0.1 mM) and DMPO (30 mM). Spectrum B: as A, but in the presence of SOD. (Instrumental settings: microwave power; 5.05 mW; modulation amplitude; 1.05 G; receiver gain: 2×10^4.)

Conclusions

Using laser flash photolysis and reaction of $O_2^{\cdot -}$ with NBT, the process of electron transfer from some biological substrates to HA* has been observed and the relative quantum yield of superoxide anion photoproduced by HA and a biological substrate at different pHs have been determined for the first time. According to the results described here, it seems reasonable to conclude that an electron transfer (Type 1), superoxide-mediated photodynamic mechanism may play a hitherto unrecognized role in the photodynamic interaction between HA and some biological substrates.

Experimental

Materials and methods

HA 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), 9,10-diphenylanthracene (9,10-DPA) and superoxide dismutase (SOD) were purchased from Biotech Technology Corporation, Chinese Academy of Sciences. 5,5-Dimethyl 4,5-dihydro-3*H*-pyrrole *N*-oxide (DMPO) and 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 buffered solutions were purged with argon, air or oxygen according to the experimental requirements: potassium phosphate monobasic–sodium hydroxide, pH = 5.8, sodium–potassium phosphates, pH 7.0 and pH 8.2; potassium phosphate–sodium borate, pH 9.0.

The absorbance of NBT was monitored at 560 nm using a Shimadzu 160 UV–VIS spectrophotometer and recorded at 4 min intervals. Irradiation was 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. Light intensity in the central area of the illuminator was about 10 mW cm^{-2} as determined by actinometry according to Parker.[23] The solutions examined were put in glass cuvettes with a long neck. Argon was bubbled through to remove oxygen and then the cuvettes were sealed with a rubber stopper. After illumination, their absorption spectra were measured with a UV–VIS spectrophotometer at once. The $^1O_2$-generating quantum yields were determined by the 9,10-DPA bleaching method, the details of which have been described in a recent report.[11] Time resolved transient absorption spectra were

measured with a Q-switch ND : YAG nanosecond laser apparatus (FWHM < 5 ns, 35 mJ per pulse, λ_{exe} = 532 nm or 355 nm). A xenon flash was used as an analysis flash for the detection of transients. The monitoring light passing through a grating monochromator was analyzed by a detection system of a photomultiplier tube and an oscilloscope. The samples in 10 mm × 10 mm glass cell were bubbled with highly purified argon for 30 min before measurement. Measurements of the EPR 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 EPR analysis and were illuminated with a 450 W medium-pressure sodium lamp. A long pass filter was employed to eliminated light of wavelength less than 470 nm.

Acknowledgements

Financial support from National Natural Science Foundation of China is gratefully acknowledged.

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Paper 7/02117H
Received 27*th March* 1997
Accepted 23*rd June* 1997