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Characteristics of the reaction between semiquinone radical anion of hypocrellin A and oxygen in aprotic media

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Abstract

Hypocrellin A (HA), a hydroxylperylenequinone derivative, is an efficient phototherapeutic agent. The reactivity of the semiquinone radical anion of HA ($HA^{\cdot -}$) with O_2 in aprotic medium has been studied. Although the standard redox potential of the $HA^{\cdot -}$–HA couple (−360 mV vs. a normal hydrogen electrode (NHE)) is less negative than that of the $O_2^{\cdot -}$–O_2 couple (−500 mV vs. NHE), it appears that $HA^{\cdot -}$ can still be oxidized with oxygen. The electron transfer is accompanied by a proton transfer between one of the phenolic hydroxyl groups of HA and $O_2^{\cdot -}$ yielding the conjugated base of HA (HA_b^-) and hydrogen peroxide. On the basis of the results obtained from spectrophotometric, electron spin resonance spin-trapping and CCl_4-inhibiting experiments, a reasonable reaction pathway was proposed for the oxidation of $HA^{\cdot -}$ with O_2 in aprotic media.

Keywords: Hypocrellin A; Semiquinone radical anion; Oxygen; Conjugated base; Electron transfer; Proton transfer

1. Introduction

Hypocrellin A (HA), isolated from the parasitic fungus *Hypocrella bambusae* (B. et Br) sacc [1], is an efficient phototherapeutic agent. It belongs to perylenequinone derivative bearing two hydroxyl groups peri to two carbonyl groups respectively to form strong hydrogen bonding (Fig. 1) [2].

In clinical trials, HA has produced promising results in the treatment of various skin diseases such as white lesions of vulva and vitiligo [3]. Also, damage to human erythrocyte membranes was observed when the cells were irradiated together with HA [4]. Recently, it has been shown that HA can kill tumor cells efficiently and accumulate selectively in cancer cells. Hela cells and S-180 solid tumor cells can be killed photodynamically in the presence of HA. The growth of mitochondrial ATPase and microsomal G-6-pase of hepatoma cells can be inhibited [5,6].

It has been shown that HA is an efficient singlet oxygen ($^1O_2$) generator [7,8]. However, HA has the abilities to undergo reversible oxidation–reduction and to form semiquinone radical anion ($HA^{\cdot -}$) and oxygen radicals. It is believed that not only the active oxygen species ($^1O_2$, $O_2^{\cdot -}$ and $^{\cdot}OH$) but also the semiquinone radical anion of HA ($HA^{\cdot -}$) participate in the photodynamic damage caused by

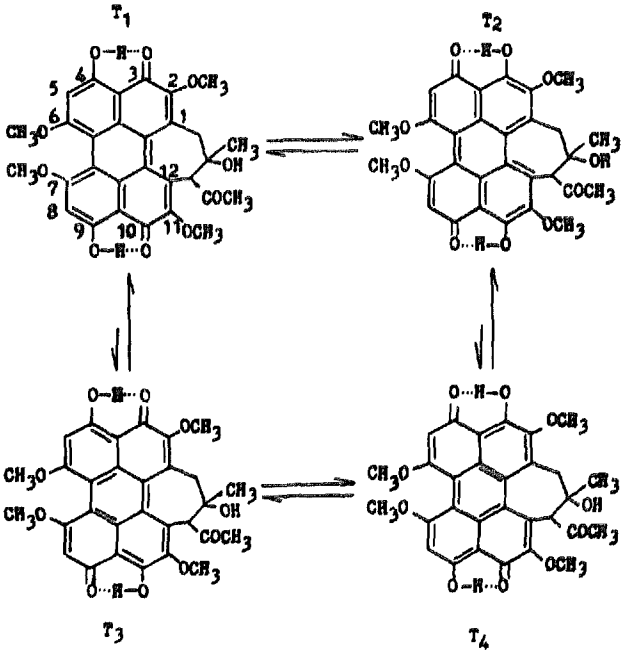

Fig. 1. The structure of HA.

HA [9,10]. $HA^{\cdot -}$ is considered to be an important intermediate in the cytotoxic reactions, through its ability to generate toxic materials such as reactive oxygen species ($O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$) and its reactions with the substrates [9,11].

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Although the spectroscopic properties of $HA^{\cdot -}$ have been well established recently [12,13], the reaction of oxygen with $HA^{\cdot -}$ has not been investigated systematically. The present study has been directed to the reactivity of $HA^{\cdot -}$ with oxygen in aprotic solvents. The solvents are dimethylsulfoxide (DMSO), dimethylformamide (DMF) and acetonitrile (MeCN). The excitation and measurement techniques used include photoreduction, chemical reduction, electron spin resonance (ESR) spin-trapping and UV–visible absorption spectroscopy. Since the electron is likely to be delocalized throughout the molecule, the semiquinone radical anion of HA may exist as several tautomers [13]. However, it is difficult to distinguish between these tautomers since they are in very rapid equilibrium. So we generally use $HA^{\cdot -}$ to represent all the forms of the semiquinone radical anions of HA throughout this paper.

2. Experimental details

2.1. Materials

HA was isolated from the fungus sacs of *Hypocrella bambusae* and recrystallized twice from acetone before use. Hypocrellin B (HB) was obtained by dehydration of HA [14]. 14-dehydroxy-15-deacetylhypocrellin A-13-sulfonate (13-SO_3Na-DDHA) was prepared using the method described previously [15]. Superoxide dismutase (SOD) was obtained from Biotech Technology Corporation, Chinese Academy of Sciences. 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Aldrich Chemical Company and stored at −20 °C under argon. 1-benzyl-1,4-dihydronicotinamide (BNAH) was a gift from Dr. G. Deng. 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 and were purged with argon, air or oxygen according to the experimental requirements.

2.2. Methods

UV–visible absorption spectra were recorded on an HP diode-array spectrophotometer model 8451A. ^{1}H nuclear magnetic resonance (NMR) spectra were measured with a Varian XL-400 spectrometer. Photolysis experiments were performed under illumination of a medium-pressure sodium lamp (400 W) on a "merry-go-round" apparatus. Light of wavelengths less than 470 nm was cut off by a long pass filter, and the apparatus was immersed in running water in a thermostat at 20 °C.

2.3. Preparations of the solutions of the semiquinone radical anion

The absorption spectrum of $HA^{\cdot -}$ in DMSO shows a strong band at 628 nm and it overlaps slightly with that of HA [13]. So the generation of $HA^{\cdot -}$ can be monitored spectrophotometrically during the preparation. The methods of photoreduction with BNAH as reductant [13] and chemical reduction with sodium dithionite powder as reductant [16] were used to prepare the solutions of $HA^{\cdot -}$. When reacting with oxygen, the semiquinone radical anion produced by photoreduction and that produced by chemical reduction gave consistent results.

In the photoreduction method [13], a DMSO solution of HA (0.035 mM) and BNAH (0.3 mM) was put in a glass cuvette with a long neck. Argon was bubbled through to remove oxygen and then the cuvette was sealed with a rubber stopper. Illumination of the argon-gassed solution was continued until the absorption due to HA was completely eliminated and a clear maximum at 628 nm developed. Then the required solution of $HA^{\cdot -}$ was obtained. During photoreduction, great care should be taken to prevent $HA^{\cdot -}$ from further reduction to the hydroquinone of HA [13].

The cuvette with a long neck was also used in the chemical reduction method with sodium dithionite powder as reductant. A DMSO mixture of HA (0.035 mM) and sodium dithionite powder (40 mg) was deaerated by purging argon gas through it and then sonicated with a sonicator. The reduction was continued until HA was all converted into $HA^{\cdot -}$ as monitored spectrophotometrically. Then the sodium dithionite powder was filtered under argon and the required argon-gassed DMSO solution of $HA^{\cdot -}$ was obtained.

2.4. Electron spin resonance spin-trapping experiments

DMPO (45 mM) was added into an argon-gassed DMSO solution of $HA^{\cdot -}$ produced by photoreduction or chemical reduction. The mixture was then put in contact with air and transferred to a quartz capillary cell for measuring its ESR spectrum. The ESR spectrum was recorded at room temperature using a Varian E-1700B spectrometer operating at 9.5 Hz (X band).

3. Results and discussion

3.1 Reaction of the semiquinone radical anion of HA with oxygen

$HA^{\cdot -}$ was prepared using the methods described in Section 2. Fig. 2 shows the absorption spectra of HA and $HA^{\cdot -}$ in argon-gassed DMSO. Absorption maxima in the visible region were identified at the following wavelengths: 468, 538 and 580 nm for HA and 628 nm for $HA^{\cdot -}$ [12,13]. Extinction coefficients found were $\epsilon_{468\,nm} = 2.94 \times 10^4\ M^{-1}\ cm^{-1}$, $\epsilon_{538\,nm} = 1.46 \times 10^4\ M^{-1}\ cm^{-1}$ and $\epsilon_{580\,nm} = 1.51 \times 10^4\ M^{-1}\ cm^{-1}$ for HA and $\epsilon_{628\,nm} = 2.38 \times 10^4\ M^{-1}\ cm^{-1}$ for $HA^{\cdot -}$ in DMSO.

The reaction of $HA^{\cdot -}$ with oxygen was followed by means of absorption spectroscopy as shown in Fig. 3. When an argon-gassed DMSO solution of $HA^{\cdot -}$ was progressively

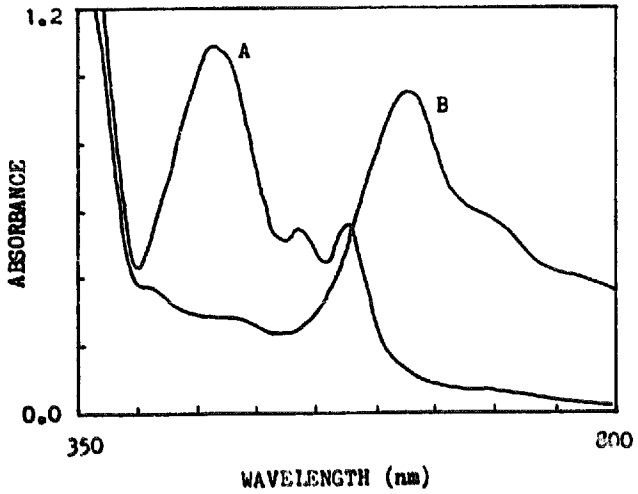


Fig. 2. Absorption spectra of HA (curve A) and $HA^{\bullet -}$ (curve B) in argon-gassed DMSO.

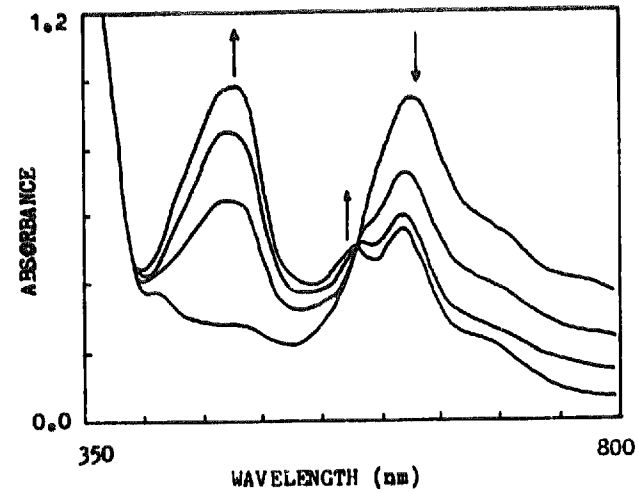


Fig. 3. Reaction of $HA^{\bullet -}$ with oxygen. Spectral changes observed during exposure of the argon-gassed DMSO solution of $HA^{\bullet -}$ mentioned in Fig. 2 to air. The arrows indicate the direction of the changes.

placed in contact with air, the sharp and strong band at 628 nm, ascribed to $HA^{\bullet -}$, decreased while the strong band at 480 nm increased with the appearance of an isosbestic point at 576 nm. Finally the absorption spectrum showed three bands at 480, 576 and 618 nm and differed markedly from that of HA (Fig. 2, curve A). During this process the colour of the solution changed from green to greenish yellow.

Since the final absorption spectrum of Fig. 3 is very similar to that of the monobase of HA reported previously [17], the product of the reaction of $HA^{\bullet -}$ with oxygen can be ascribed to the monobase of HA (HA_b^-). We checked up that the original quinone HA could be quantitatively regenerated when protons (acetic acid or nitromethane) were added to the solution of the product. This further supports the ascription. Another support for the ascription comes from the proton NMR experiments. The ^1H NMR spectrum of HA shows two sharp peaks (16.18 and 16.06 ppm in CD_3SOCD_3) in the low field, which are characteristic of the two phenolic hydroxyl protons [2]. However, only one broad peak (16.30 ppm in CD_3SOCD_3) which disappears upon treatment of the product with CD_3OD was observed in the low field of the ^1H NMR spectrum of the product. This indicates that the product possesses only one phenolic proton. Since the other part of the ^1H NMR spectrum of the product is very similar to that of HA, the ascription of the product to HA_b^- should be correct.

The oxidation of $HA^{\bullet -}$ to HA_b^- by O_2 was also observed in other aprotic solvents such as CH_3CN and DMF. The reactions of the semiquinone radical anions of HB and 13-SO_3Na-DDHA with oxygen gave similar results to that in the reaction of $HA^{\bullet -}$ with O_2.

3.2. Detection of superoxide generated from the reaction of $HA^{\bullet -}$ with O_2

In order to study the mechanism of the reaction of $HA^{\bullet -}$ with O_2, ESR spin-trapping experiments were carried out with DMPO as the spin trap. DMPO (45 mM) was added into a DMSO solution of $HA^{\bullet -}$. The mixture was then put in contact with air and introduced into a quartz capillary cell. The ESR spectrum obtained is shown in Fig. 4, curve A. This spectrum has been analyzed as a primary nitrogen triplet ($a^N = 12.8$ G); each line in the triplet is further split by a secondary proton ($a^H = 10.25$ G). Each of these lines is further split by a secondary proton ($a^H = 1.5$ G), resulting in the observed 12-line ESR spectrum which is characteristic of the DMPO superoxide radical adduct [18]. When enzyme SOD was introduced into the system (40 $\mu g\ ml^{-1}$), the ESR signal of the DMPO superoxide radical was significantly quenched

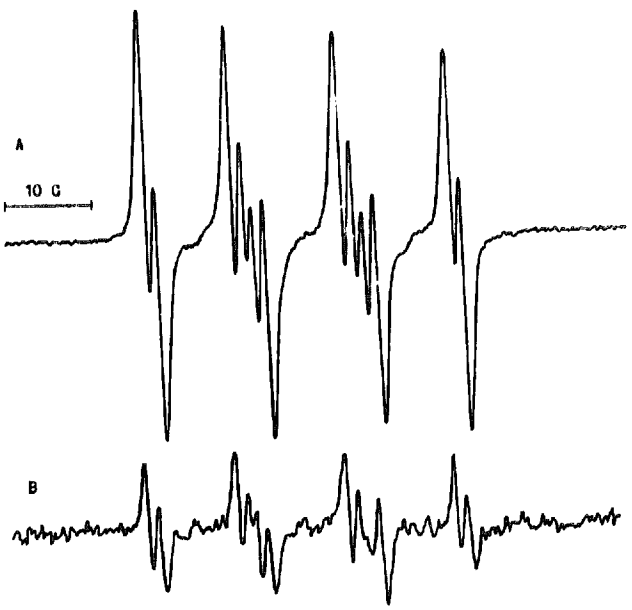
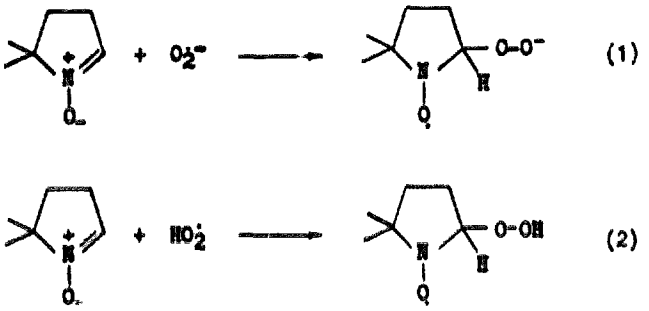


Fig. 4. ESR spectra of DMPO superoxide radical adduct obtained after a DMSO solution of $HA^{\bullet -}$ and DMPO was put in contact with air (curve A) and in the presence of SOD (40 $\mu g\ ml^{-1}$) (curve B) (spectral parameter settings: microwave power, 10 mW; time constant, 0.128 s; modulation amplitude, 1.0 G; scan width, 200 G; receiver gain, 8×10^3 for curve A and 2×10^4 for curve B).

(Fig. 4, curve B). This result confirms the correct assignment of the ESR spectrum to the DMPO superoxide radical adduct. No ESR signal was detected in the system without HA or oxygen. These results indicate that superoxide is generated in the reaction of $HA^{\cdot -}$ with O_2. Since superoxide could exist as anionic ($O_2^{\cdot -}$) and protonated ($HO_2^{\cdot}$) forms and both of them could be trapped by DMPO, the species trapped in this system might be $O_2^{\cdot -}$ and/or $HO_2^{\cdot}$ as follows:



The pK_a value of CH_3NO_2 is 17.2 [19]. A DMSO solution containing a known concentration of HA_b^- was titrated with CH_3NO_2. The final concentrations of HA_b^- and HA was obtained from optical absorbance of HA_b^- at 618 nm based on Beer's law. The pK_a of HA was estimated from the equilibrium $HA_b^- + CH_3NO_2 \rightleftharpoons HA + {}^-CH_2NO_2$. The estimated value of pK_a for HA is almost 16. This indicates that HA is a moderate acid in aprotic media [20]. It has been reported that $O_2^{\cdot -}$ in aprotic media (where it is stable) is disproportionated by the presence of protic substrates such as $HClO_4$, catechols, ascorbic acid and phenol [21]. The reaction of $O_2^{\cdot -}$ with moderate acid (such as phenol and catechols) yields the conjugated base of the substrate plus $HO_2^{\cdot}$ [21]. Therefore we infer that the monobase of HA in our system was generated from the abstraction of one of the phenolic protons of HA by $O_2^{\cdot -}$, accompanied by the formation of $HO_2^{\cdot}$ (which rapidly disproportionated to H_2O_2 and O_2). In order to confirm this inference further, the inhibition experiments were performed.

3.3. Inhibition of the formation of HA_b^- by CCl_4

Carbon tetrachloride reacts rapidly with superoxide ion in DMSO [22]:

$$CCl_4 + 6O_2^{\cdot -} \longrightarrow CO_4^{2-} + 4Cl^- + 4O_2 \qquad (3)$$

When CCl_4 (0.35 M) was added to an argon-gassed DMSO solution of $HA^{\cdot -}$ (0.035 mM), the formation of HA_b^- was completely inhibited and $HA^{\cdot -}$ was completely converted into HA after the mixture was put in contact with air. A plot of the CCl_4 quenching on the formation of HA_b^- was obtained by adding various amounts of CCl_4 to the reaction mixture (Fig. 5). Control experiments indicated that neither $HA^{\cdot -}$ nor HA_b^- could react directly with CCl_4. These results strongly confirmed that the monobase of HA was generated from the abstraction of one of the phenolic protons of HA by

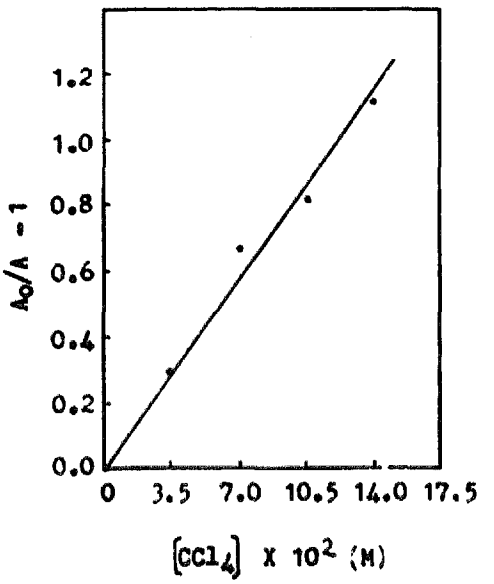


Fig. 5. Inhibition of the generation of HA_b^- by carbon tetrachloride (CCl_4), performed by adding various amounts of carbon tetrachloride to a series of argon-gassed DMSO solutions of $HA^{\cdot -}$ and then putting these solutions in contact with air. A_0 and A represent the absorbances at 618 nm in the absence and presence respectively of CCl_4 and A_0/A is the ratio of A_0 to A.

$O_2^{\cdot -}$. The inhibition of the formation of HA_b^- in the presence of CCl_4 is due to the consumption of $O_2^{\cdot -}$ by CCl_4 (Eq. (3)), which inhibits the proton transfer from HA to $O_2^{\cdot -}$.

The effects of DMPO and SOD on the formation of HA_b^- were also studied. Although DMPO is a scavenger of $O_2^{\cdot -}$ (Eq. (1)), it was observed that DMPO could not inhibit the formation of HA_b^- when a DMSO solution of DMPO (45 mM) and $HA^{\cdot -}$ (0.035 mM) was put in contact with air. This might be because the reaction between DMPO and $O_2^{\cdot -}$ is too slow ($k = 10\ M^{-1}\ s^{-1}$) and the proton transfer from HA to $O_2^{\cdot -}$ ($k = (1\text{–}2) \times 10^4\ M^{-1}\ s^{-1}$) [21] should significantly prevail over it to generate HA_b^- and $HO_2^{\cdot}$ which then reacts with DMPO (Eq. (2)). Therefore the species trapped predominantly by DMPO in our system was $HO_2^{\cdot}$ instead of $O_2^{\cdot -}$, although both of them can react with DMPO. SOD is also a good scavenger of $O_2^{\cdot -}$. However, since protons (H^+) are necessary for the SOD-catalyzed dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2, SOD cannot inhibit the formation of HA_b^- from the reaction of $HA^{\cdot -}$ with O_2, either.

3.4. Mechanistic deductions

The standard redox potential of the $HA^{\cdot -}$–HA couple was measured to be −360 mV (vs. NHE) and that of the $O_2^{\cdot -}$–O_2 redox couple has been reported to be −500 mV (vs. NHE) [23]. If only electron transfers were involved in the oxidation of $HA^{\cdot -}$ with oxygen, then $HA^{\cdot -}$ should not react with oxygen in aprotic media or should react very slowly when oxygen is in great excess since the standard redox potential of the $HA^{\cdot -}$–HA couple is less negative than that of the $O_2^{\cdot -}$–O_2 redox couple. It is the occurrence of a proton transfer accompanying the electron transfer which renders

the oxidation possible as ascertained by the production of $HA_b^{\cdot -}$. In accordance with our experimental results, a reaction pathway consisting of reactions similar to those already studied in detail in [21,24] is considered for the reaction of $HA^{\cdot -}$ with oxygen:

$$HA + e \overset{E_{HA}^0}{\rightleftharpoons} HA^{\cdot -} \quad (4)$$

$$HA^{\cdot -} + O_2 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} HA + O_2^{\cdot -} \quad (5)$$

$$HA + O_2^{\cdot -} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} HA_b^{-} + HO_2^{\cdot} \quad (6)$$

$$2HO_2^{\cdot} \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} H_2O_2 + O_2 \quad (7)$$

The equilibrium constant K_5 of reaction (5) is given by the equation $pK_5 = |E(HA^{\cdot -}\text{–}HA) - E(O_2^{\cdot -}\text{–}O_2)|/60$. According to Sawyer and coworkers [21] the pK_a of $HO_2^{\cdot}$ in DMF is 12 [21]. Assuming that the pK_a of $HO_2^{\cdot}$ in DMSO is comparable with that in DMF and the pK_a of HA is almost 16, pK_6 should be almost 4, k_6 being $(1–2) \times 10^4\ M^{-1}\ s^{-1}$ [21]. The value of $K_7 = 10^{25}$ is also given in [21], k_7 being greater than $10^7\ M^{-1}\ s^{-1}$. As both pK_5 and pK_6 are positive, reaction (7) is the driving force rendering the reaction of $HA^{\cdot -}$ with an excess of oxygen possible. The overall process can be represented as

$$2HA^{\cdot -} + O_2 \rightleftharpoons 2HA_b^{-} + H_2O_2 \quad (8)$$

The order of magnitude of K_8 is at least larger than 10^{12}. This shows theoretically that the oxidation of $HA^{\cdot -}$ can be almost complete in the presence of an excess of oxygen as experimentally observed.

4. Conclusion

In aprotic solvent, the redox potential of the $HA^{\cdot -}$–HA couple is always less negative than that of the $O_2^{\cdot -}$–O_2 couple. However, the semiquinone radical anion of HA can still be oxidized with oxygen. Our results clearly demonstrate that at first such an oxidation yields the original quinone (HA) and $O_2^{\cdot -}$; then HA transfers a phenolic hydroxy proton to $O_2^{\cdot -}$ to yield the conjugated base of HA ($HA_b^{\cdot -}$) and $HO_2^{\cdot}$ which rapidly disproportionates as shown in reaction (7). The disproportionation of $HO_2^{\cdot}$ is the driving force rendering the reaction of $HA^{\cdot -}$ with an excess of oxygen possible.

The mechanism described here is valid as long as the standard potential of the redox couple of the semiquinone radical anion of hydroxyperylenequinone ($HP^{\cdot -}$) and the original quinone (HP) ($HP^{\cdot -}$–HP) is less negative than that of the couple of $O_2^{\cdot -}$–O_2. Such a condition may not be fulfilled in the presence of water due to the potential shifts which result from the change in solvent. Particularly it is well known that the value of the potential of $O_2^{\cdot -}$–O_2 couple in water differs markedly from its value in aprotic media owing to the strong solvation of $O_2^{\cdot -}$ in water. However, the analysis of the reaction pathways in aprotic media may be of as much biological interest as the analysis of the reaction pathways in water since the photodamage caused by HA is known to occur at human erythrocyte membranes and in the mitochondrial and microsome of hepatoma cells [5,6] which are rather hydrophobic.

Acknowledgment

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