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Kinetic Study of the Quenching Reaction of Singlet Oxygen by Pyrroloquinolinequinol ($PQQH_2$, a Reduced Form of Pyrroloquinolinequinone) in Micellar Solution

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ABSTRACT: A kinetic study of the quenching reaction of singlet oxygen ($^1O_2$) with pyrroloquinolinequinol ($PQQH_2$, a reduced form of pyrroloquinolinequinone (PQQ)), $PQQNa_2$ (disodium salt of PQQ), and seven kinds of natural antioxidants (vitamin C (Vit C), uric acid (UA), epicatechin (EC), epigallocatechin (EGC), α-tocopherol (α-Toc), ubiquinol-10 ($UQ_{10}H_2$), and β-carotene (β-Car)) has been performed. The second-order rate constants k_Q ($k_Q = k_q + k_r$, physical quenching and chemical reaction) for the reaction of $^1O_2$ with $PQQH_2$, $PQQNa_2$, and seven kinds of antioxidants were measured in 5.0 wt % Triton X-100 micellar solution (pH 7.4), using UV−visible spectrophotometry. The k_Q values decreased in the order of β-Car > $PQQH_2$ > α-Toc > UA > $UQ_{10}H_2$ > Vit C ∼ EGC > EC ≫ $PQQNa_2$. $PQQH_2$ is a water-soluble antioxidant. The singlet oxygen-quenching activity of $PQQH_2$ was found to be 6.3, 2.2, 6.1, and 22 times as large as the corresponding those of water-soluble antioxidants (Vit C, UA, EGC, and EC). Further, the activity of $PQQH_2$ was found to be 2.2 and 3.1 times as large as the corresponding activity of lipid-soluble antioxidants (α-Toc and $UQ_{10}H_2$). On the other hand, the activity of $PQQH_2$ is 6.4 times as small as that of β-Car. It was observed that the chemical reaction (k_r) is almost negligible in the quenching reaction of $^1O_2$ by $PQQH_2$. The result suggests that $PQQH_2$ may contribute to the protection of oxidative damage in biological systems, by quenching $^1O_2$.

KEYWORDS: PQQ, pyrroloquinolinequinone, antioxidant activity, reaction rate, singlet oxygen, UV−vis spectrophotometry, vitamin C, catechins, α-tocopherol, micellar solution

INTRODUCTION

Pyrroloquinolinequinone (PQQ), 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-f]quinoline-2,7,9 -tricarboxylic acid, is a water-soluble quinone compound first identified as a noncovalently prosthetic group in some bacterial glucose- or alcohol dehydrogenases.[1,2] Trace amount of PQQ has been found not only in microorganisms but also in human and rat organs or tissues, especially the highest in human milk.[3,4] A further trace amount of PQQ is also found in daily foods and beverages.[5,6] PQQ has been receiving much attention in recent years, owing to its several interesting physiological functions.[7,8] PQQ is regarded to be a nutritionally important growth factor, as PQQ-deficient diets cause impaired growth, immunological defects and decreased fertility in mice.[9] PQQ is related to mitochondrial biogenesis through cAMP response element-binding protein phosphorylation and increased PGC-1alpha expression.[10,11] Moreover PQQ is related to regeneration of peripheral and central nerves. In *in vitro* experiment, PQQ enhances nerve growth factor (NGF), a neurotrophic factor responsible for the maintenance and development of peripheral nerves.[12] Regeneration of transected sciatic nerve in an *in vivo* rat model is demonstrated.[13] Recent study suggests that PQQ protects against secondary damage by attenuating inducible nitric oxide synthase (iNOS) expression following a primary physiological injury to the spinal cord.[14]

Previous works demonstrated that PQQ exhibits antioxidative capacity in *in vitro* examinations.[15,16] The reduced form of PQQ ($PQQH_2$ (pyrroloquinolinequinol), see Figure 1) is attributed to exhibit such a capacity. In experiments using cultured cells, it is reported that PQQ prevents oxidative stress-induced neuronal death.[17,18] Moreover, in *in vivo* models such as cardiovascular or cerebral ischemia models, marked decreases in the ischemia damage are reported.[19,20] Further, recently, it was found that PQQ prevents cognitive deficit caused by oxidative stress in rats.[21,22]

Lipid peroxyl radical (LOO•) and singlet oxygen ($^1O_2$) are well-known as two representative reactive oxygen species generated in biological systems. In a previous work, a kinetic study of the aroxyl (ArO•) radical-scavenging activity of $PQQH_2$ and water-soluble antioxidants was performed in 5.0 wt % Triton X-100 micellar solution (pH 7.4), using stopped-flow spectrophotometry.[23] A stable ArO• radical (2,6-di-*t*-butyl-4-(4′-methoxyphenyl)phenoxyl) was used as a model of LOO• radical, as described in previous works.[24−26] The second-order rate constant (k_s) for the reaction of ArO• with $PQQH_2$ was found to be 7.4 times as high as that of vitamin C, which is well-known as the most active water-soluble antioxidant. Furthermore, we found that $PQQNa_2$ (disodium salt of PQQ) is easily reduced to $PQQH_2$, by reacting $PQQNa_2$ with glutathione and cysteine in buffer solution (pH 7.4) under nitrogen atmosphere.

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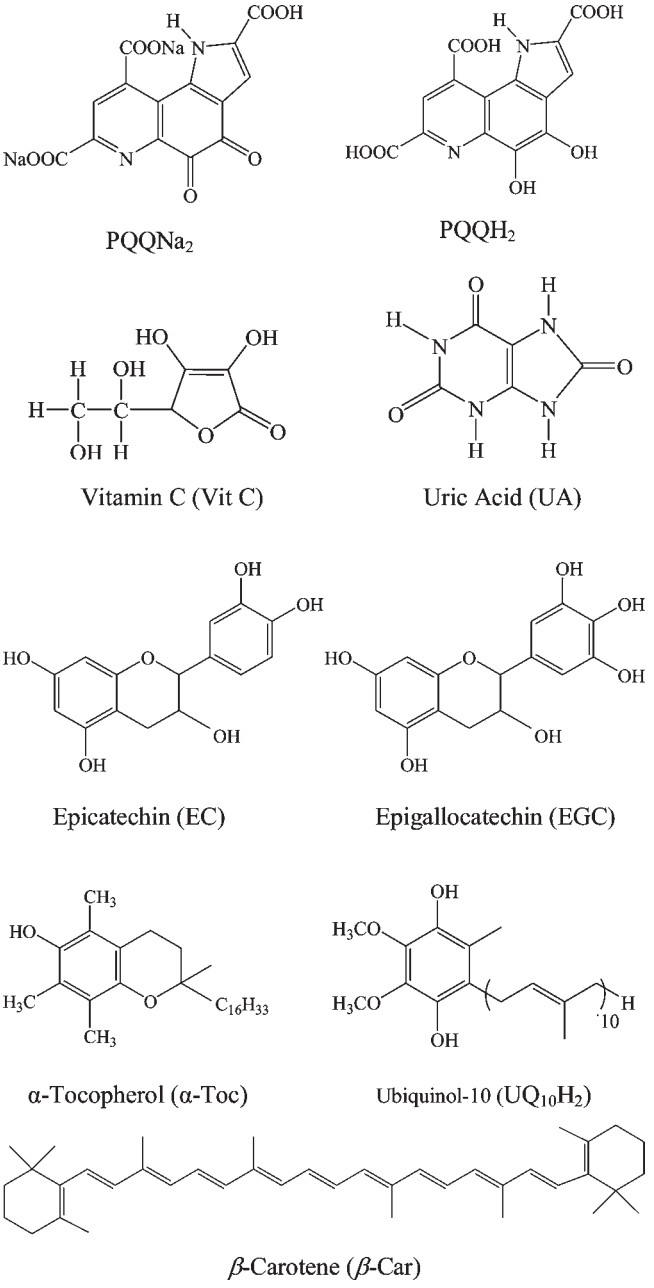
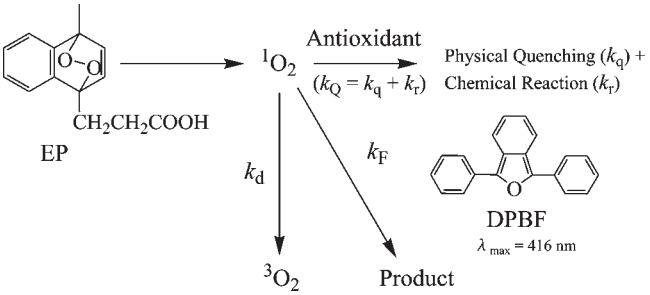


Figure 1. Molecular structures of $PQQNa_2$, $PQQH_2$, and seven kinds of natural antioxidants (vitamin C, uric acid, epicatechin, epigallocatechin, α-tocopherol, ubiquinol-10, and β-carotene).

The result suggests that PQQ exists as a reduced form throughout the cell and plays a role as antioxidant.

In the present work, a kinetic study of the quenching reaction of $^1O_2$ with $PQQH_2$ and seven kinds of natural antioxidants (see Figure 1), which are included in plasma and LDL (low-density lipoprotein),[27] was performed in 5.0 wt % Triton X-100 micellar solution (pH 7.4), using UV−visible spectrophotometry. The second-order rate constants k_Q ($= k_q + k_r$, physical quenching + chemical reaction) for the reaction of $^1O_2$ with these antioxidants (reaction 1) were measured in micellar solution, using a competition reaction method (see Scheme 1).[25] The k_Q value obtained for $PQQH_2$ was compared with those obtained for seven kinds of antioxidants. This is the first report with the kinetic study of

Scheme 1



singlet oxygen-quenching activity of $PQQH_2$.

$$^1O_2 + PQQH_2 \xrightarrow{k_Q} \text{physical quenching } (k_q) + \text{chemical reaction } (k_r) \quad (1)$$

MATERIALS AND METHODS

Materials. Commercial vitamin C (Vit C) (Wako Pure Chemicals, Japan), uric acid (UA) (Sigma-Aldrich), α-tocopherol (α-Toc) (Tokyo Kasei Chemicals, Japan), β-carotene (β-Car) (Wako Pure Chemicals, Japan), cysteine (Tokyo Kasei Chemicals, Japan), 2,5-diphenyl-3,4-benzofuran (DPBF) (Tokyo Kasei Chemicals, Japan) (see Figure 1), and Triton X-100 (Nacalai Tesque) were used as received. Epicatechin (EC) and epigallocatechin (EGC) and ubiquinone-10 (UQ_{10}) were kindly supplied by Mitsui Norin Co. Ltd. and Kaneka Corporation, respectively. Powder sample of $PQQNa_2$ was supplied from Mitsubishi Gas Chemical Company, Inc. The results of the elemental analysis, the thermogravimetry and the titration of H_2O due to Karl Fischer's reagent indicated that $PQQNa_2$ used is a monohydrate of $PQQNa_2$ ($PQQNa_2 \cdot H_2O$). The buffer solution was prepared using distilled water treated with a Millipore Q system, and its pH was adjusted to 7.4 using 0.02 M KH_2PO_4−Na_2HPO_4 buffer.

Ubiquinol-10 ($UQ_{10}H_2$) was prepared by the reduction of ubiquinone-10 (UQ_{10}) with sodium hydrosulfite in *n*-hexane under a nitrogen atmosphere.[26] 3-(1,4-Epidioxy-4-methyl-1,4-dihydro-1-naphthyl)propionic acid (endoperoxide, EP) (Scheme 1) was synthesized according to the method reported in previous works.[28−30]

Preparation of $PQQH_2$. The reduction of PQQ (or $PQQNa_2$) to $PQQH_2$ was performed under nitrogen atmosphere by using several reducing agents (such as (i) cysteine, (ii) $NaBH_4$, (iii) glutathione, and (iv) H_2 (PtO_2)), giving the same UV−vis spectrum of $PQQH_2$, as described in a previous work (see Table 1 in ref 23). In the present work, $PQQH_2$ was prepared by the reduction of $PQQNa_2$ with cysteine. $PQQNa_2$ is stable in 5.0 wt % Triton X-100 micellar solution (0.02 M phosphate buffer, pH 7.4) under air. However, $PQQH_2$ is unstable in micellar solution under air, and easily oxidized to PQQ, as reported in previous works.[23,31,32] Consequently, the reduction of $PQQNa_2$ to $PQQH_2$ in micellar solution and the measurement of the UV−vis absorption spectrum were performed under strictly deaerated and nitrogen-substituted conditions by using Hamilton 1000 series gastight syringe and sealing cap, to avoid an oxidation of $PQQH_2$.

By adding 5.0 wt % Triton X-100 micellar solution (pH 7.4) of cysteine (1.26×10^{-3} M) to the micellar solution of $PQQNa_2$ (1.07×10^{-4} M) (1:1 in volume) at room temperature, the absorption spectrum of $PQQNa_2$ (see Figure 2A) disappeared rapidly, and changed to that of $PQQH_2$ (see Figure 2B) with absorption peaks at λ_{max} = 304 and 506 nm. The absorbance of $PQQH_2$ shows a maximum at $t \sim 50$ min, and then decreases gradually.

Table 1. Values of UV−Visible Absorption Maxima (λ^i_{max}) and Molar Extinction Coefficients (ε_i) of $PQQNa_2$, $PQQH_2$, and DPBF in Triton X-100 Micellar Solution (5.0 wt %, pH 7.4) and Homogeneous Buffer Solution (pH 7.4)

	solution	λ^1_{max}/nm (ε_1/M^{-1} cm^{-1})	λ^2_{max}/nm (ε_2/M^{-1} cm^{-1})	λ^3_{max}/nm (ε_3/M^{-1} cm^{-1})	λ^4_{max}/nm (ε_4/M^{-1} cm^{-1})	λ^5_{max}/nm (ε_5/M^{-1} cm^{-1})
$PQQNa_2$	micellar solution			331 (10600)	477 (706)	
$PQQNa_2$	buffer solution[a,b]	249 (26600)	267 sh (20500)	331 (12700)	477 (692)	
$PQQH_2$	micellar solution	304 (32300)	340 sh (11000)	405 sh (2320)	506 (990)	416 sh[c] (2540)
$PQQH_2$	buffer solution[a,b]	304 (40000)	340 sh (11500)	405 sh (2410)	499 (1170)	
DPBF	micellar solution		314 (6810)	327 (6720)		416 (19800)

[a] The reduction of $PQQNa_2$ was performed by cysteine in buffer solution (pH 7.4) (see ref 23). [b] The values (λ^i_{max} and ε_i, $i = 1-4$) in buffer solution (pH 7.4) reported in a previous work (ref 23). [c] The ε_5 value at λ^5_{max} = 416 nm was used for the baseline correction.

Measurements of Rate Constants (k_Q). Measurements of rate constants (k_Q) were performed in 5.0 wt % Triton X-100 micellar solution (pH 7.4), by using a Shimadzu UV−vis spectrophotometer (UV-1800), equipped with a six-channel cell-positioner and an electron-temperature control unit (CPS-240A). Measurements of UV−vis absorption spectra were performed under nitrogen atmosphere, to avoid the degradation of antioxidants ($PQQH_2$, Vit C, UA, EC, EGC, α-Toc, $UQ_{10}H_2$, and β-Car) and DPBF. All of the measurements were done in a sealed system using a cuvette with a sealing cap. The rate constant (k_Q) was determined by analyzing the first-order rate constant (S) of the decay curve of DPBF with antioxidants, as described in Results. As reported in a previous work,[33] the measurements of the k_Q values for α-Toc and 8 kinds of carotenoids including β-Car were repeated three times in ethanol:chloroform:D_2O (50:50:1, v/v/v) solution, and experimental errors in the rate constants (k_Q (average)) were estimated to be <5%. Experimental errors in rate constants (k_Q (average)) obtained in micellar solution were larger and estimated to be <8%, as described in Results. All measurements were performed at 35.0 ± 0.5 °C.

The production of $^1O_2$ due to the thermal decomposition of EP occurs at 25 °C. Consequently, sample preparation was performed by adding 1.00 mL of EP solution to 2.00 mL of solution including DPBF and an antioxidant in a quartz cuvette at ∼20 °C to avoid the decomposition of EP, and measurements of the UV−vis absorption spectra were then started at 35 °C. We took about 5 min to prepare solutions of six cuvettes. About 3 min was necessary before the solution temperature in the cuvette rose from ∼20 to 35 °C.

RESULTS

UV−Vis Absorption Spectra of $PQQNa_2$ and $PQQH_2$ in Micellar Solution. 5.0 wt % Triton X-100 micellar solution (pH 7.4) shows a strong absorption in the λ = 300−400 nm region, because aromatic phenyl ring moiety is included in Triton X-100 molecule (Figure 6 in ref 23). Therefore, the absorption spectrum of $PQQNa_2$ (see Figure 2A) was obtained by subtracting the background due to the absorption of micellar solution from that including $PQQNa_2$.

The $PQQNa_2$ is stable, and it shows absorption peaks at λ_{max} = 331 nm (molar extinction coefficient (ε) = 10600 M^{-1} cm^{-1}) and 477 (706) (Figure 2A) in 5.0 wt % Triton X-100 micellar solution (pH 7.4), as listed in Table 1. The λ^i_{max} and ε_i values (i = 1−4) of $PQQNa_2$ in 0.05 M phosphate buffer solution were reported in a previous work[23] (see Table 1). The λ^i_{max} and ε_i values (i = 3 and 4) of $PQQNa_2$ obtained in micellar solution show good accordance with the corresponding those in buffer solution, suggesting that the interaction between $PQQNa_2$ and Triton X-100 molecules is negligible in micellar solution. $PQQNa_2$ molecules are hydrophilic and thus will be located at the outside of micelle.

The $PQQH_2$ shows absorption peaks at λ_{max} = 304 nm, 340sh, 405sh, and 506 (Figure 2B) in micellar solution (pH 7.4), as listed in Table 1. By reacting $PQQNa_2$ with cysteine, the absorption of $PQQH_2$ increases gradually, and shows a maximum at t ∼ 50 min. The molar extinction coefficient (ε_i) (i = 1−4) of $PQQH_2$ was calculated from the absorption spectra at t = 50 min, by using Lambert−Beer's equation (absorbance (A_t) = ε_1[$PQQH_2$]), where the concentration of $PQQH_2$ ([$PQQH_2$]) was assumed to be equal to that of $PQQNa_2$ at t = 0 s. The ε_i values of $PQQH_2$ obtained are listed in Table 1, together with those in 0.05 M

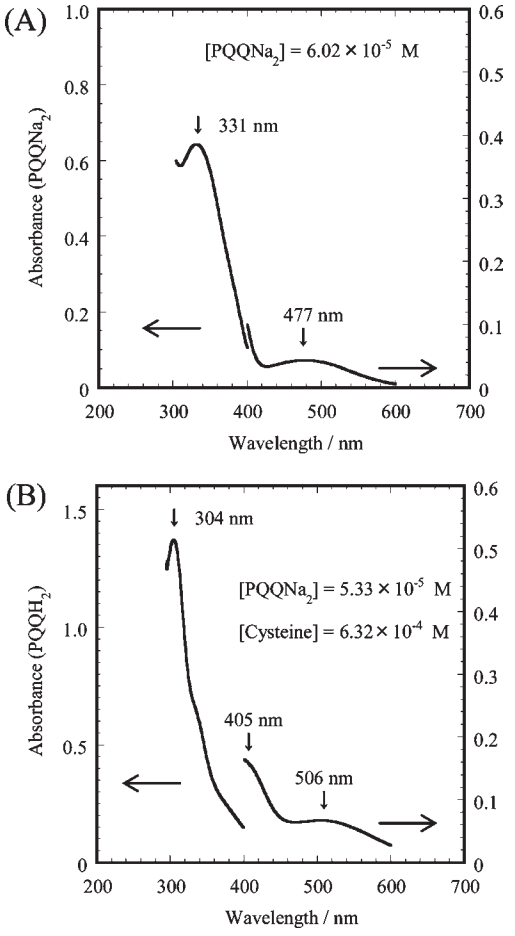


Figure 2. (A) UV−visible absorption spectrum of $PQQNa_2$ in 5.0 wt % Triton X-100 micellar solution (0.02 M phosphate buffer, pH 7.4) at 25.0 °C. [$PQQNa_2$] = 6.02 × 10^{-5} M. (B) UV−visible absorption spectrum of $PQQH_2$ obtained by the reaction of $PQQNa_2$ with cysteine in 5.0 wt % Triton X-100 micellar solution (0.02 M phosphate buffer, pH 7.4) at 25.0 °C. $[PQQNa_2]_{t=0}$ = 5.33 × 10^{-5} M and $[cysteine]_{t=0}$ = 6.32 × 10^{-4} M. In panels A and B, the absorption due to micellar solution was subtracted from the total absorption.

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phosphate buffer solution.[23] $PQQH_2$ is comparatively stable under strict nitrogen atmosphere. On the other hand, by introducing air to the above micellar solution, the spectrum of $PQQH_2$ decreased rapidly, and changed to that of the original $PQQNa_2$. As listed in Table 1, the shift of the λ^4_{max} value from 499 nm in buffer solution to 506 nm in micellar solution was observed for $PQQH_2$, although the shift of the $\lambda_{max}{}^1$ value at 304 nm was not observed. The result suggests that the $PQQH_2$ molecules are located at the surface of micelle by the interaction between $PQQH_2$ and Triton X-100 molecules.

On the other hand, DPBF is insoluble in buffer solution, and will be localized at the inside of the micelle. DPBF shows an absorption peak at λ_{max} = 416 nm (see Table 1).

The Overall Rate Constants (k_Q) for the Reaction of $^1O_2$ with $PQQH_2$, $PQQNa_2$, and Seven Kinds of Natural Antioxidants in Micellar Solution. Singlet oxygen was generated by the thermal decomposition of the endoperoxide (EP) (see Scheme 1).[25,34−36] DPBF was used as a standard compound. The overall rate constants k_Q ($= k_q + k_r$) for the reaction of $^1O_2$ with $PQQH_2$, $PQQNa_2$, and seven kinds of antioxidants (Vit C, UA, EC, EGC, α-Toc, $UQ_{10}H_2$, and β-Car) were determined in micellar solution by eq 2 derived from the steady-state treatment of Scheme 1:[37,38]

$$S_{blank}/S_{antioxidant} = 1 + (k_Q/k_d)[antioxidant] \quad (2)$$

where S_{blank} and $S_{antioxidant}$ are slopes of the first-order plots (that is, ln(absorbance) vs t plots) of disappearance of $^1O_2$ acceptor, DPBF, in the absence and presence of antioxidants, respectively. k_d is the rate of natural deactivation of $^1O_2$ in micellar solution.

Figure 3 shows an example of the reaction between DPBF (6.65×10^{-5} M) and EP (1.03×10^{-3} M) in the absence and presence of Vit C ($0-2.97 \times 10^{-3}$ M) in micellar solution at 35 °C. By the reaction, the disappearance of DPBF at λ_{max} = 416 nm due to the chemical reaction between DPBF and $^1O_2$ produced was observed. The S_{blank} and $S_{antioxidant}$ values were obtained by analyzing the decrease in absorbance at 416 nm of the DPBF. A plot of $S_{blank}/S_{antioxidant}$ vs the concentration of Vit C ([Vit C]) is shown in Figure 4A. The k_Q/k_d value obtained is listed in Table 2. The rate constant (k_Q) was tentatively calculated by using the value of k_d in 1.0 wt % Triton X-100 micellar solution ($k_d = 2.17 \times 10^5$ s^{-1}).[39] The k_Q value obtained is 1.56×10^8 M^{-1} s^{-1}.

Similarly, solutions containing EP (9.82×10^{-4} M), DPBF (4.29×10^{-5} M), and various amounts of $PQQH_2$ ($0-9.35 \times 10^{-3}$ M) in micellar solution were reacted at 35 °C (see Figure 5A. The disappearance of DPBF was measured at 416 nm. However, as the absorption of $PQQH_2$ overlaps with that of DPBF at 416 nm (see Figure 2B), the correction of the baseline is necessary for each decay curve. The correction of the baseline for each decay curve of DPBF was performed by taking the absorbance of this baseline into account and using Lambert−Beer's equation (absorbance = $\varepsilon[PQQH_2]$). The decay curves corrected are shown in Figure 5B. A plot of $S_{blank}/S_{antioxidant}$ vs $[PQQH_2]$ is shown in Figure 4A. The k_Q/k_d and k_Q values obtained are 4.54×10^3 M^{-1} and 9.85×10^8 M^{-1} s^{-1}, respectively. The measurement of the rate constant for the reaction of $PQQNa_2$ with $^1O_2$ was also performed in micellar solution at pH 7.4, showing that the reaction between $PQQNa_2$ and $^1O_2$ is negligible (data are not shown).

Similar measurements were performed for UA, EC, EGC, α-Toc, $UQ_{10}H_2$, and β-Car, where the baseline correction was performed only for β-Car, because antioxidants except for β-Car do not show absorption at 416 nm. The $S_{blank}/S_{antioxidant}$ vs [antioxidant] plots are shown in Figures 4A and 4B. The k_Q/k_d and k_Q values obtained are listed in Table 2. For instance, the k_Q/k_d value (2.10×10^3 M^{-1}) obtained for α-Toc showed good accordance with that (2.20×10^3 M^{-1}) reported for α-Toc in a previous work.[40] The experimental error in k_Q/k_d value was estimated to be ±8% at maximum.

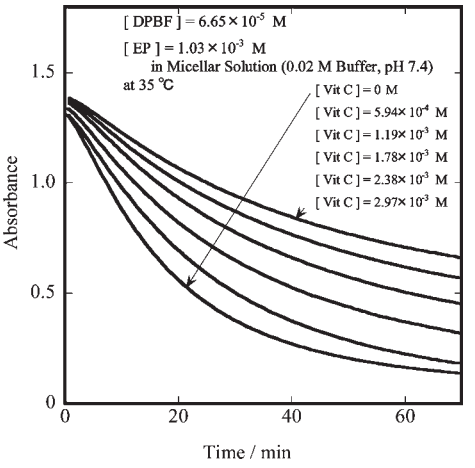

Figure 3. Change in absorbance of DPBF at 416 nm during the reaction of DPBF with $^1O_2$ in the absence and presence of vitamin C in 5.0 wt % Triton X-100 micellar solution (0.02 M phosphate buffer, pH 7.4) at 35 °C. $[DPBF]_{t=0} = 6.65 \times 10^{-5}$ M and $[EP]_{t=0} = 1.03 \times 10^{-3}$ M. The values of $[Vit\ C]_{t=0}$ are shown.

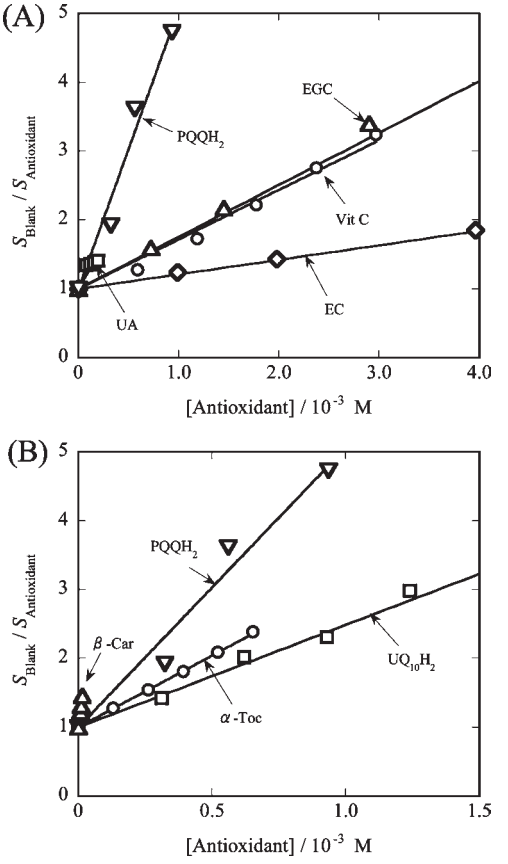

Figure 4. (A) Plot of $S_{blank}/S_{antioxidant}$ vs concentrations of antioxidants ($PQQH_2$, Vit C, UA, EC, and EGC). (B) Plot of $S_{blank}/S_{antioxidant}$ vs concentrations of antioxidants ($PQQH_2$, α-Toc, $UQ_{10}H_2$, and β-Car).

Table 2. The k_Q/k_d and k_Q Values for $PQQH_2$ and Natural Antioxidants (AOs) in Micellar Solution at 35.0 °C, the k_Q Values in Ethanol, and Relative Rate Constants (k_Q(AO)/k_Q(Vit C) and k_Q(micelle)/k_Q(ethanol))

antioxidant	k_Q/k_d[a] /M^{-1}	k_Q[b] /M^{-1} s^{-1}	k_Q(AO) /k_Q(Vit C)	k_Q[c] /M^{-1} s^{-1}	k_Q(micelle)/k_Q(ethanol)
	in micelle	in micelle	in micelle	in ethanol	
$PQQNa_2$	no reaction			insoluble	
$PQQH_2$	4.54×10^3	9.85×10^8	6.32		
Vit C	7.18×10^2	1.56×10^8	1.00	insoluble (1.6×10^8)[d]	(0.98)
UA	2.03×10^3	4.41×10^8	2.83	insoluble (3.6×10^8)[e]	(1.2)
EC	2.08×10^2	4.51×10^7	0.290	1.32×10^7	3.41
EGC	7.39×10^2	1.60×10^8	1.03	1.72×10^7	9.30
α-Toc	2.10×10^3 (2.20×10^3)[f]	4.56×10^8	2.92	2.06×10^8	2.21
$UQ_{10}H_2$	1.46×10^3	3.17×10^8	2.03	1.58×10^8	2.01
β-Car	2.91×10^4	6.31×10^9	40.5	1.58×10^{10}	0.399

[a] Experimental errors in the k_Q/k_d values were estimated to be <8%. [b] The k_Q value was calculated using the k_d value ($=2.17 \times 10^5$ s^{-1}) obtained in 1.0 wt % Triton X-100 micellar solution at 24 °C (see ref 39). [c] Experimental errors in the rate constants (k_Q) were estimated to be <5%. [d] The value in buffer solution (pH 7.4) (see ref 46). [e] The value in buffer solution (pH 7.4) (see ref 47). [f] The value reported in a previous work (see ref 40).

As listed in Table 2, the rate constants (k_Q) of $PQQH_2$, $PQQNa_2$, and seven kinds of natural antioxidants decrease in the order of

$$\beta\text{-Car} > PQQH_2 > \alpha\text{-Toc} > UA > UQ_{10}H_2 > \text{Vit C} \sim EGC > EC \gg PQQNa_2 \quad (3)$$

in micellar solution. The k_Q value of $PQQH_2$ at pH 7.4 in micellar solution is 6.3 and 2.2 times larger than the corresponding those of Vit C and α-Toc, respectively, which are well-known as representative water- and lipid-soluble antioxidants in biological systems. However, the value of $PQQH_2$ is 6.4 times smaller than that of β-Car, which is well-known as a most active singlet oxygen quencher.

Chemical Reaction (k_r) of $PQQNa_2$ and $PQQH_2$ with $^1O_2$ in Micellar Solution. Endoperoxide (EP) was prepared by the reaction of EP-precursor (3-(4-methyl-1-naphthyl)propionic acid) with $^1O_2$ according to the method of Aubry et al.[30] The UV absorption spectra of EP-precursor and EP show absorption maxima at $\lambda_{max}(\varepsilon)$ = 299 sh (4470), 288 (6560), 279 (5340) and 233 nm (9430 M^{-1} cm^{-1}) and $\lambda_{max}(\varepsilon)$ = 229 nm (6510 M^{-1} cm^{-1}), respectively, in ethanol solution.[38] The result of the measurement of the UV spectrum of EP indicates that the powder sample of EP includes 95.6% EP and 4.4% EP-precursor unreacted.

Figure 6 shows an example of the reaction between $PQQH_2$ (9.58×10^{-4} M) and EP (9.77×10^{-4} M) in 5.0 wt % Triton X-100 micellar solution at 35 °C. The spectra were recorded at 5 min intervals, and the scanning was repeated 12 times. At 35 °C, EP decomposes and generates singlet oxygen and EP-precursor. However, the absorption of $PQQH_2$ at 506 nm shows almost no change in the intensity. The chemical reaction between $^1O_2$ and $PQQH_2$ is very slow, and the measurable change in the absorption spectrum of $PQQH_2$ was not observed. Consequently, the k_Q value obtained for $PQQH_2$ is thought to be due to physical quenching (k_q), that is, $k_Q \approx k_q$. Similar measurement was performed for the reaction between EP and $PQQNa_2$. The chemical reaction expected was not observed.

DISCUSSION

Singlet oxygen ($^1O_2$) has attracted much attention as a biological oxidant. $^1O_2$ is generated by the reaction of triplet sensitizers with molecular oxygen, $^3O_2$ (type II photosensitization reaction),[41,42] and by the biochemical reactions in cells and tissues exposed to oxidative stress.[43,44] Due to its high reactivity, it oxidizes many biological substances.

In the present work, the measurement of the quenching rate of $^1O_2$ with nine kinds of natural antioxidants, including $PQQH_2$ and $PQQNa_2$, has been performed in micellar solution. As listed in Table 2, the rate constants (k_Q) decrease in the order of eq 3. $PQQH_2$ is a water-soluble antioxidant. The k_Q value of $PQQH_2$ at pH 7.4 is 6.3, 2.2, 6.1, and 22 times as large as the corresponding those of water-soluble antioxidants (Vit C, UA, EGC, and EC). Further, the k_Q value of $PQQH_2$ is 2.2 and 3.1 times as large as the corresponding those of lipid-soluble antioxidants (α-Toc and $UQ_{10}H_2$). On the other hand, the k_Q value of $PQQH_2$ is 6.4 times as small as that of β-Car. Carotenoids including β-Car are well-known as most active singlet oxygen quenchers.

Five kinds of antioxidants (Vit C, UA, α-Toc, $UQ_{10}H_2$, and β-Car) used in the present work are included in plasma and LDL.[27] EC and EGC appear in plasma after oral ingestion.[45] Consequently, kinetic studies of the quenching reaction of $^1O_2$ have been performed for these antioxidants in homogeneous solutions. The rate constants (k_Q) obtained for EC, EGC, α-Toc, $UQ_{10}H_2$, and β-Car in ethanol are listed in Table 2.[25,34,35] As Vit C and UA are insoluble in ethanol, the k_Q values obtained in buffer solution (pH = 7.4) were used for the comparison.[46,47] In the present work, first, we tried to measure the reaction rate of $PQQH_2$ and $PQQNa_2$ in ethanol solution. However, we were unsuccessful in determining the reaction rate, because $PQQNa_2$ is insoluble in ethanol. Similarly, we could not determine the reaction rate of $PQQH_2$ in phosphate buffer solution, because DPBF is insoluble in buffer solution. As listed in Table 2, the k_Q values of these antioxidants in homogeneous solution decrease in the order of

$$\beta\text{-Car} > UA > \alpha\text{-Toc} > UQ_{10}H_2 \sim \text{Vit C} > EGC > EC \quad (4)$$

The order of the rate constants in homogeneous solution is the same as that in micellar one (see eq 3), except for the case of UA and Vit C.

The quenching rate (k_Q) of $^1O_2$ increases with increasing the polarity (dielectric constant (ε)) of solvent (see eq 5), as reported by Gruszka et al.[48]

$$k_Q = k_Q^0 + C\varepsilon \quad (5)$$

Consequently, UA and Vit C will show smaller k_Q values in ethanol (ε = 25.3) than those in D_2O solution (ε = 87.65), if we

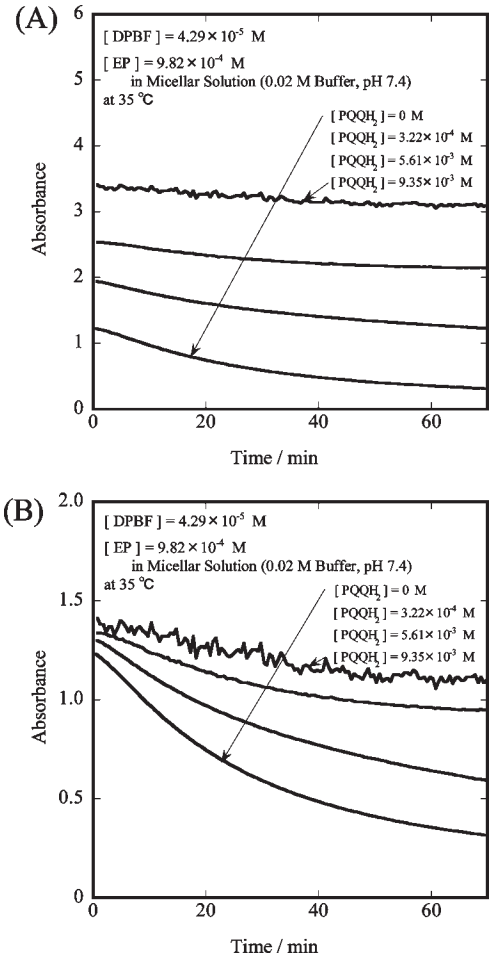

Figure 5. (A) Change in absorbance of DPBF at 416 nm during the reaction of DPBF with $^1O_2$ in the absence and presence of $PQQH_2$ in 5.0 wt % Triton X-100 micellar solution (0.02 M phosphate buffer, pH 7.4) at 35 °C. $[DPBF]_{t=0} = 4.29 \times 10^{-5}$ M and $[EP]_{t=0} = 9.82 \times 10^{-4}$ M. The values of $[PQQH_2]_{t=0}$ are shown. (B) Change in absorbance of DPBF, where the correction of baseline due to $PQQH_2$ was performed (see text).

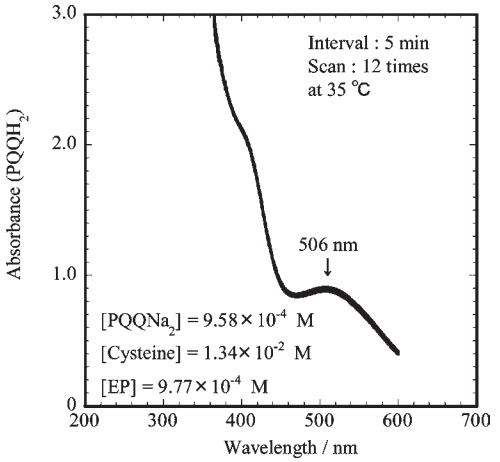

Figure 6. The measurement of the chemical reaction between $PQQH_2$ and EP in micellar solution at 35 °C. $PQQH_2$ was prepared by the reduction of $PQQNa_2$ with cysteine in micellar solution at 25 °C (see Figure 2B). $[PQQNa_2] = 9.58 \times 10^{-4}$ M, [cysteine] = 1.34×10^{-2} M, and $[EP] = 9.77 \times 10^{-4}$ M.

may measure the reaction rates in ethanol. The result suggests that the orders of the rate constants of these antioxidants in homogeneous ethanol and inhomogeneous micellar solutions (eqs 3 and 4) are comparable with each other.

Further, the rate constants (k_Q) in homogeneous solution are similar to the corresponding rate constants in micellar solution. For instance, the k_Q values (1.56×10^8 and 4.41×10^8 M^{-1} s^{-1}) of water-soluble Vit C and UA in micellar solution are similar to the corresponding values (1.6×10^8 and 3.6×10^8 M^{-1} s^{-1}) in buffer solution. The water-soluble (and ethanol-insoluble) antioxidants (Vit C and UA) will be localized at the outside of the micelle, and exist in water region. Therefore, the concentration of these antioxidants in micellar solution will be similar to that in homogeneous buffer solution, suggesting that the rate constants (k_Q) in micellar and buffer solution are similar to each other. Consequently, the k_Q value of water-soluble $PQQH_2$ in buffer solution will also be similar to that (9.84×10^8 M^{-1} s^{-1}) in micellar solution, although we could not determine the k_Q value in ethanol and buffer solutions, because of the solubility reason.

On the other hand, α-Toc, $UQ_{10}H_2$, and β-Car are lipophilic, and thus will be localized at the inside of micelle. Consequently, the local concentration of these antioxidants in 5.0 wt % Triton X-100 micelle will become about 20 times as large as that in homogeneous ethanol solution, if we assume that the density of the part of micelle is 1 g/mL and the volume that Triton X-100 molecules (5.0 wt %) occupy in micellar solution is 5.0% of the total volume. We can expect that the k_Q values of these lipophilic antioxidants in micellar solution are 20 times as large as the corresponding ones in ethanol solution, as observed for the reaction of ArO• radical with α-, β-, γ-, δ-Toc and $UQ_{10}H_2$.[26] However, as listed in Table 2, the ratios of reaction rates, k_Q(micelle)/k_Q(ethanol), of α-Toc, $UQ_{10}H_2$, and β-Car in ethanol and micelle solution are 2.2, 2.0, and 0.40, respectively. As shown in eq 5, the reaction rate (k_Q) changes remarkably depending on the polarity (that is, ε) of the reaction field. If the lipophilic α-Toc, $UQ_{10}H_2$, and β-Car molecules react with $^1O_2$ at nonpolar reaction field such as *n*-hexane (ε = 1.89), the k_Q values will decrease notably compared to the corresponding one in polar ethanol (ε = 25.3).[48] This will be one important reason why the ratios, k_Q(micelle)/k_Q(ethanol), are much smaller than 20. It is interest that the singlet oxygen quenching-activity (and the relative ratio of the activity) of water- and lipid-soluble antioxidants in homogeneous solution is similar to the corresponding activity in inhomogeneous micellar solution. However, the detailed reason is not clear at present.

$^1O_2$ is an active oxidant and reacts with a wide variety of biological molecules including lipids, sterols, proteins (amino acids), DNA, and RNA, thus inducing cell death and mutations.[41,42] For instance, the quenching rates are 1.9×10^5 M^{-1} s^{-1} for methyl linolenate in pyridine, 5.7×10^4 M^{-1} s^{-1} for cholesterol in C_6D_6, and 5.1×10^5 M^{-1} s^{-1} for DNA in H_2O (pH 7.0). The k_Q values obtained for the above antioxidants ($1.32 \times 10^7 \sim 1.58 \times 10^{10}$ M^{-1} s^{-1} in ethanol and $4.51 \times 10^7 \sim 6.32 \times 10^9$ M^{-1} s^{-1} in micellar solution) are 2 to 4 orders of magnitude larger than those for methyl linolenate and DNA. The result suggests that these antioxidants may contribute to the protections of the lipid peroxidation and the degradation of DNA in biological systems, by quenching $^1O_2$.

Proteins are present in most biological systems at high concentrations, react rapidly with $^1O_2$, and hence are major targets (see Table 1 in ref 41). Of the common amino acids

present in proteins, only Trp, His, Tyr, Met and Cys react at considerable rates ($k_Q = 0.8 \times 10^7 \sim 3.7 \times 10^7$ M^{-1} s^{-1}) at physiological pH range.[41,42] The reactions of $^1O_2$ with proteins occur mainly via the chemical rather than physical routes. The values are similar to those of EC and EGC in ethanol and micellar solutions, and one to three orders of magnitude smaller than those of Vit C, $UQ_{10}H_2$, UA, α-Toc, $PQQH_2$, and β-Car, suggesting that these antioxidants may contribute to the protection of the degradation of proteins in biological systems.

As described in the Introduction, PQQ was found in daily foods and beverages.[5,6] Trace amounts of PQQ have been found not only in microorganisms but also in human and rat organs or tissues, especially the highest in human milk.[3,4] Furthermore, PQQ exists as the reduced form ($PQQH_2$) in a variety of tissues.[23] In the present work, the second-order rate constants k_Q ($k_Q = k_q + k_r$, physical quenching and chemical reaction) for the reaction of $^1O_2$ with $PQQH_2$, $PQQNa_2$, and seven kinds of antioxidants were measured in micellar solution (pH 7.4). The k_Q values decreased in the order of eq 3. $PQQH_2$ showed high activity for the quenching of singlet oxygen in micellar solution. Furthermore, it was observed that the chemical reaction (k_r) is almost negligible in the quenching reaction of $^1O_2$ by $PQQH_2$. The rate constant (k_Q) of $PQQH_2$ was found to be much larger than those (k_r) for the chemical reaction of biological molecules (lipids, proteins (amino acids), and nucleic acids) with $^1O_2$. The results suggest that $PQQH_2$ may contribute to the protection of oxidative damage in biological systems, by quenching $^1O_2$.

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