Kinetic Study of the Antioxidant Activity of Pyrroloquinolinequinol (PQQH₂, a Reduced Form of Pyrroloquinolinequinone) in Micellar Solution

Aya Ouchi,*,† Masahiko Nakano,§ Shin-ichi Nagaoka,† and Kazuo Mukai*,†

Department of Chemistry, Faculty of Science, Ehime University, Matsuyama 790-8577, Japan, and Niigata Research Laboratory, Mitsubishi Gas Chemical Company, Inc., Niigata 950-3112, Japan

Kinetic study of the aroxyl radical-scavenging action of pyrroloquinolinequinol [PQQH₂, a reduced form of pyrroloquinolinequinone (PQQ)] and water-soluble antioxidants (vitamin C, cysteine, glutathione, and uric acid) has been performed. The second-order rate constants (k_s) for the reaction of aroxyl radical with PQQH₂ and water-soluble antioxidants were measured in Triton X-100 micellar solution (5.0 wt %) (pH 7.4), using stopped-flow and UV–visible spectrophotometers. The k_s values decreased in the order PQQH₂ > vitamin C ≫ cysteine > uric acid > glutathione. The aroxyl radical-scavenging activity of PQQH₂ was 7.4 times higher than that of vitamin C, which is well-known as the most active water-soluble antioxidant. Furthermore, PQQNa₂ (disodium salt of PQQ) was easily reduced to PQQH₂ by reaction of PQQNa₂ with glutathione and cysteine in buffer solution (pH 7.4) under nitrogen atmosphere. The result suggests that PQQ exists as a reduced form throughout the cell and plays a role as antioxidant.

INTRODUCTION

Pyrroloquinolinequinone (PQQ), 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid, has received much attention in recent years owing to its several interesting functions. PQQ is a cofactor of alcohol- and glucose-dehydrogenases in bacteria (*1–6*). PQQ is known to be a nutritionally important growth factor (*7–9*). PQQ nutritional status alters lysine metabolism and modulates mitochondrial DNA content in the mouse and rat (*10*). PQQ has also been reported to show neuroprotective effects (*11–13*). PQQ was found in many kinds of fruits and foods (*14–16*). Furthermore, the existence of small amounts of free PQQ was found in eight human organs, plasma, and urine and in three rat organs (*17*).

A previous work demonstrated that PQQ prevents mitochondrial lipid peroxidation and the inactivation of the mitochondrial respiratory chain from oxidative damage (*18*). PQQ protects against cell injury associated with oxidative stress (*19–21*). Recently, it was found that PQQ prevents cognitive deficit caused by oxidative stress in rats (*22*). Therefore, PQQ is thought to function as an antioxidant (*23, 24*). In fact, the reduced form of PQQ [PQQH₂ (pyrroloquinolinequinol), see **Figure 1**] has high free radical scavenging properties (*25*).

In the present work, a kinetic study of the aroxyl (ArO•) radical scavenging activity of PQQH₂ and water-soluble antioxidants was performed in micellar solution, using stopped-flow and UV–visible spectrophotometers. A stable ArO• radical [2,6-di-tert-butyl-4-(4′-methoxyphenyl)phenoxyl] (**Figure 1**) was used as a model of active free radicals such as LOO•, LO•, and Toc•, as described in previous works (*26–28*). The preparation of PQQH₂ was performed by the reduction of PQQNa₂ (disodium salt of PQQ, see **Figure 1**) in buffer solution at pH 7.4 under nitrogen gas atmosphere, where not only NaBH₄ but also glutathione (GSH) and cysteine (Cys) were used as reducing agents. The second-order rate constants (k_s) for the reaction of ArO• radical with PQQH₂ and water-soluble antioxidants [GSH, Cys, and uric acid (UA)] (see **Figure 1**) (reaction 1) have been measured in 5.0 wt % Triton X-100 micellar solution (pH 7.4, 0.05 M phosphate buffer). The k_s values obtained were compared with that reported for vitamin C (Vit C) (*29*), which is well-known as a typical water-soluble antioxidant. This is the first report with the kinetic study of the free radical scavenging activity of PQQH₂.

$$ArO^\bullet + PQQH_2 \xrightarrow{k_s} ArOH + PQQH^\bullet \qquad (1)$$

MATERIALS AND METHODS

Commercial glutathione (GSH) (Sigma-Aldrich), cysteine (Cys) (Tokyo Kasei), uric acid (UA) (Sigma-Aldrich), and Triton X-100 (Nacalai Tesque) were used as received. Powder sample of PQQNa₂

* Authors to whom corresponding should be addressed [fax 81-89-927-9590; e-mail (K.M.) mukai@chem.sci.ehime-u.ac.jp, (A.O.) oouchi@chem.sci.ehime-u.ac.jp].
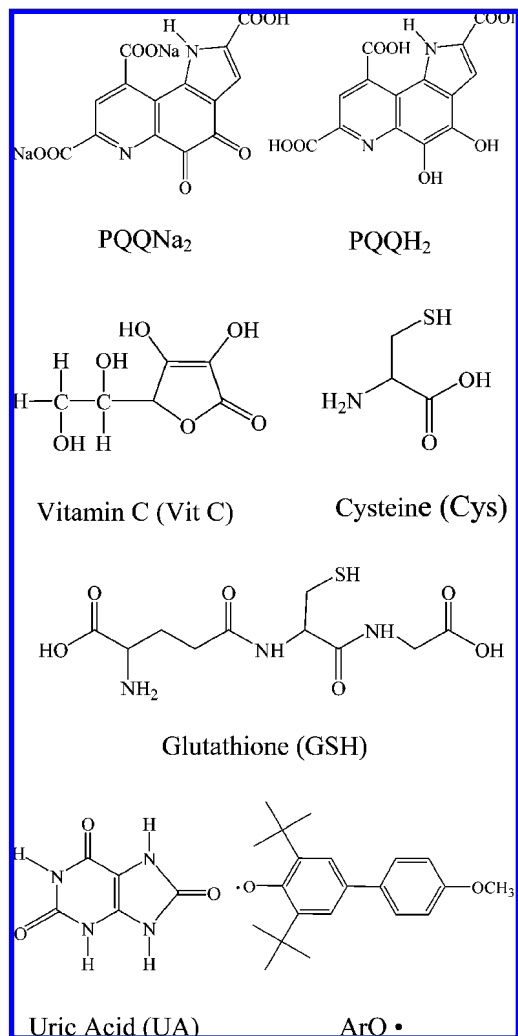† Ehime University.
§ Mitsubishi Gas Chemical Co., Inc.

Antioxidant Activity of Pyrroloquinolinequinol

J. Agric. Food Chem., Vol. 57, No. 2, 2009   451

Figure 1. Molecular structures of PQQNa₂, PQQH₂, vitamin C, cysteine, glutathione, uric acid, and aroxyl radical (ArO•).

Figure 2. (a) UV−visible absorption spectrum of PQQNa$_2$ in 0.05 M phosphate buffer solution (pH 7.4) at 25.0 °C. [PQQNa$_2$] = 7.16 × 10^{-5} M. (b) UV−visible absorption spectrum of PQQH$_2$ obtained by the reaction of PQQNa$_2$ with cysteine in 0.05 M phosphate buffer solution (pH 7.4) at 25.0 °C. [PQQNa$_2$]$_{t=0}$ = 4.45 × 10^{-5} M and [cysteine]$_{t=0}$ = 5.78 × 10^{-4} M.

was kindly supplied from Mitsubishi Gas Chemical Co., Inc. The results of the elemental analysis, the thermogravimetry, and the titration of H$_2$O due to Karl Fischer's reagent indicated that PQQNa$_2$ used is a monohydrate of PQQNa$_2$ (PQQNa$_2$ H$_2$O). ArO• radical was synthesized according to the method reported in a previous paper (*30*), and the corresponding ArO•-containing micellar dispersions were prepared according to the method reported in a previous paper (*28*). The buffer solution was prepared using distilled water treated with a Millipore Q system, and its pH was adjusted to 7.4 using 0.05 M KH$_2$PO$_4$−Na$_2$HPO$_4$ buffer.

The kinetic data were obtained with a Unisoku model RSP-1000 stopped-flow spectrophotometer by mixing equal volumes of solutions of antioxidants and ArO• under nitrogen atmosphere. The shortest time for mixing two solutions and recording the first data point (that is, dead time) was 10−20 ms. The reaction was monitored with either single-wavelength detection or photodiode array detector attached to the stopped-flow spectrophotometer. The reaction was studied under pseudo-first-order conditions, and the observed rate constant (k_{obsd}) was evaluated in the usual way using a standard least-squares analysis. If the reaction rates (k_s) were slower than 1 M^{-1} s^{-1}, the measurements were performed by using a Shimadzu UV-2100S spectrophotometer. All of the measurements were performed at 25.0 ± 0.5 °C. Experimental errors in the rate constants (k_s) were estimated to be about 10% in micellar solution.

RESULTS AND DISCUSSION

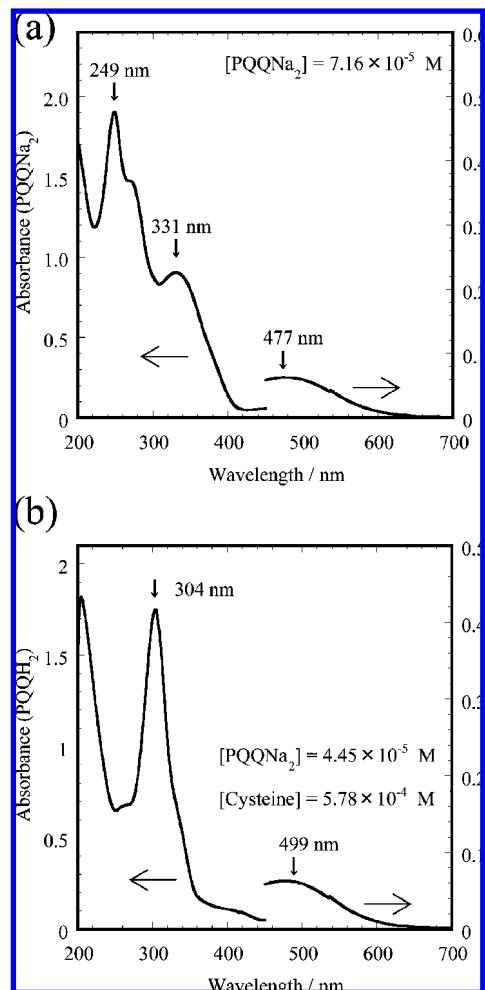**PQQNa$_2$ Is Reduced to PQQH$_2$ by the Reaction with Glutathione and Cysteine in Buffer Solution.** The PQQNa$_2$

is stable and shows absorption peaks at λ_{max} = 249 nm (ε = 26600 M^{-1} cm^{-1}), 267 sh (20500), 331 (12700), and 477 (690) (**Figure 2A**) in 0.05 M phosphate buffer solution (pH 7.4), as listed in **Table 1** (sh stands for shoulder). Catalytic oxidation of thiols by PQQ was studied in previous works by Itoh et al. (*31, 32*). The oxidation of benzenethiol and related thiol derivatives by PQQ was performed in 0.1 M phosphate buffer solution (containing 20% CH$_3$CN, pH 6.2) under anaerobic conditions, giving corresponding disulfide compounds in high yield. PQQH$_2$ is unstable in buffer solution (pH 7.4) under air and easily oxidized to PQQ, as reported in previous works (*33−35*). Consequently, in the present work, the reduction of PQQNa$_2$ to PQQH$_2$ and the measurements of the reaction rate constants were performed under strictly deaerated and nitrogen-substituted conditions by using a Hamilton 1000 series gastight syringe and sealing cap to avoid an oxidation of PQQH$_2$.

The reduction of PQQNa$_2$ was performed by using Cys and GSH as reducing agent (reaction 2). For instance, by adding the 0.05 M phosphate buffer solution of Cys (1.16 × 10^{-3} M) to the solution of PQQNa$_2$ (8.89 × 10^{-5} M) (1:1 in volume) at room temperature, the absorption spectrum of PQQNa$_2$ disappeared rapidly and changed to that of PQQH$_2$ with absorption peaks and shoulders at λ_{max} = 304, 340 sh, 405 sh, and 499 nm, as shown in **Figure 2b**. As shown in **Figure 3**, the

452 *J. Agric. Food Chem.,* Vol. 57, No. 2, 2009

Ouchi et al.

Table 1. Values of UV–Visible Absorption Maxima (λ_{max}^i) and Molar Extinction Coefficients (ε_i) of PQQNa$_2$ and PQQH$_2$ in Buffer Solution (pH 7.4) by Using Several Reducing Agents and Experimental Methods

	reducing agent (method)	λ_{max}^1/nm (ε^1/M^{-1} cm^{-1})	λ_{max}^2/nm (ε^2/M^{-1} cm^{-1})	λ_{max}^3/nm (ε^3/M^{-1} cm^{-1})	λ_{max}^4/nm (ε^4/M^{-1} cm^{-1})
PQQH$_2$	cysteine (UV–vis)[a]	304.0 (40000)	340 sh (11500)	405 sh (2410)	499 (1170)
PQQH$_2$	NaBH$_4$ (UV–vis)	304.0 (37400)	340 sh (13800)	405 sh (2610)	499.0 (1170)
PQQH$_2$	glutathione (stopped-flow)[b]	304.0 (42700)			
PQQH$_2$[c]	H$_2$ (PtO$_2$) (UV–vis)	302 (25050)			
PQQNa$_2$	(UV–vis)	249 (26600)	267 sh (20500)	331 (12700)	477 (692)
PQQ[c]	(UV–vis)	249 (18400)			

[a] UV–vis; the measurements were performed by a UV–visible spectrophotometer. [b] Stopped-flow; the measurements were performed by a stopped-flow spectrophotometer. [c] The values in 0.05 M phosphate buffer solution (pH 7.0) were reported by Duine et al. (*34*).
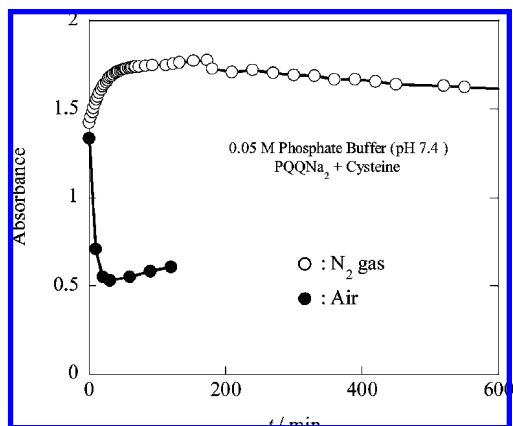

Figure 3. Time dependence of the absorbance at 304 nm of PQQH$_2$, after the reduction of PQQNa$_2$ with cysteine in 0.05 M phosphate buffer (pH 7.4) at 25.0 °C: under nitrogen atmosphere (○); under air (●). [PQQNa$_2$]$_{t=0}$ = 4.45 × 10^{-5} M and [cysteine]$_{t=0}$ = 5.78 × 10^{-4} M.

absorbance of PQQH$_2$ at 304.0 nm increases rapidly, shows a maximum at $t \sim 100$ min, and then decreases gradually. The spectrum of the PQQH$_2$ at t_{max} = 100 min is shown in **Figure 2b**. The molar extinction coefficient (ε_1) of PQQH$_2$ was calculated from the absorption spectra at t = 100 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 that reported in a previous work (*34*). The absorption spectrum of PQQH$_2$ changed to original PQQNa$_2$ after 25 h; PQQH$_2$ was comparatively stable under strict nitrogen atmosphere. On the other hand, by introducing air to the above buffer solution, the spectrum of PQQH$_2$ decreased rapidly and changed to that of the original PQQNa$_2$, as shown in **Figure 3**. Similar behavior was observed for the reaction of PQQNa$_2$ with GSH.

$$PQQ + 2RSH \rightarrow PQQH_2 + RS\text{-}SR$$

$$(RSH: \text{cysteine and glutathione}) \quad (2)$$
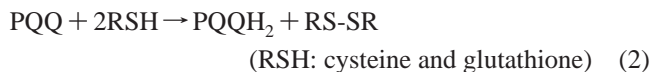
Similarly, by adding 0.05 M phosphate buffer solution of PQQNa$_2$ (4.57 × 10^{-5} M) to the powder sample of 5.79 mg of NaBH$_4$ (3.83 × 10^{-4} M) under nitrogen atmosphere at room temperature, the absorption spectrum of PQQNa$_2$ disappeared gradually and changed to that of PQQH$_2$, as observed for the reaction of PQQNa$_2$ with Cys. The values of λ_{max}^i and ε_i obtained are also listed in **Table 1**. The values of ε_1 (40000 and 37400 M^{-1} cm^{-1}) obtained by the reduction due to Cys and NaBH$_4$ are 1.63 and 1.49 times larger than that (25050 M^{-1} cm^{-1}) reported (*34*), respectively. The values of molar extinction coefficient (ε_1) are very different from that reported.

Furthermore, the molar extinction coefficient (ε_i) of PQQH$_2$ was also determined under nitrogen atmosphere by using a stopped-flow spectrophotometer. **Figure 4** shows an example
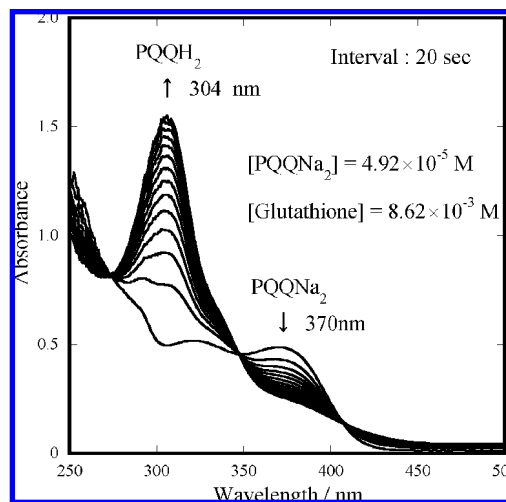

Figure 4. Change in absorption spectrum of PQQNa$_2$ and PQQH$_2$ during the reaction of PQQNa$_2$ with glutathione in 0.05 M phosphate buffer solution (pH 7.4) at 25.0 °C. [PQQNa$_2$]$_{t=0}$ = 4.92 × 10^{-5} M. [Glutathione] = 8.62 × 10^{-3} M. The spectra were recorded at 20 s intervals. The arrow indicates a decrease (PQQNa$_2$) and an increase (PQQH$_2$) in absorbance with time.

of the interaction between PQQNa$_2$ (4.92 × 10^{-5} M) and GSH (8.62 × 10^{-3} M) in 0.05 M phosphate buffer solution at 25 °C. The spectra were recorded at 20 s intervals. The arrow shows a decrease (↓) in absorbance of PQQNa$_2$ at 370 nm and an increase (↑) in absorbance of PQQH$_2$ at 304 nm with time. Three isosbestic points were clearly observed at 275, 348, and 410 nm, indicating that the reaction is simple and the contribution of side reaction is negligible. It is clear that PQQH$_2$ was produced by the reaction of PQQNa$_2$ with GSH. However, the absorption spectrum of PQQNa$_2$ [λ_{max} = 370 nm (ε_3 = 9440 M^{-1} cm^{-1}) and 320 nm (ε_3 = 9690 M^{-1} cm^{-1})] at t = 0 s (see **Figure 4**) is very different from that of PQQNa$_2$ [λ_{max}^3 = 331 nm (ε_3 = 12700 M^{-1} cm^{-1}) and λ_{max}^4 = 477 nm (ε_4 = 692 M^{-1} cm^{-1})] without GSH (**Figure 2a**). Such an absorption spectrum was also observed for the reaction of PQQNa$_2$ with Cys (data are not shown).

As Itoh et al. (*32*) reported, a similar absorption spectrum was obtained by reacting PQQ with benzenethiol (PhSH) in 0.05 M acetate buffer containing 20% CH$_3$CN. The scheme of the reduction of PQQ by PhSH was discussed by Itoh et al. As they reported, one of the possible mechanisms is that involving C-5 attack of the thiolate ion followed by breakdown to reduced PQQ and the corresponding disulfide (see the scheme in ref *32*).

Figure 5 shows the time courses of the decrease in absorbance at 370 nm of PQQNa$_2$ and the increase in absorbance at 304 nm of PQQH$_2$ observed when 0.05 M phosphate buffer solution (pH 7.4) containing PQQNa$_2$ (6.88 × 10^{-5} M) is mixed with a 0.05 M buffer solution of GSH (1.92 × 10^{-2} M) (1:1, v/v; final

Antioxidant Activity of Pyrroloquinolinequinol

J. Agric. Food Chem., Vol. 57, No. 2, 2009   **453**
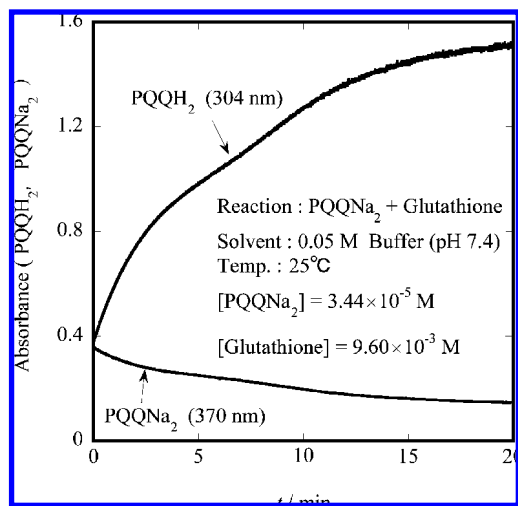

Figure 5. Change in absorbance of PQQNa$_2$ at 370 nm and PQQH$_2$ at 304 nm during reaction of PQQNa$_2$ with glutathione in 0.05 M phosphate buffer solution (pH 7.4) at 25.0 °C. [PQQNa$_2$]$_{t=0}$ = 3.44 × 10^{-5} M, and [glutathione]$_{t=0}$ = 9.60 × 10^{-3} M. The decrease and increase in the absorbance at 370 and 304 nm, respectively, are shown.

concentration of GSH of 9.60 × 10^{-3} M). At $t \sim 20$ min, the absorbance of each peak approaches the minimum and the maximum, respectively. The value of ε_1 (42700 M^{-1} cm^{-1}) for PQQH$_2$ was determined from the absorbance at $t = 20$ min. This value is similar to those obtained by the reduction due to Cys and NaBH$_4$ using UV–visible spectrophotometer (see **Table 1**). The reduction of PQQ to PQQH$_2$ is not a simple one-electron reduction, but two-electron ones. Therefore, the increase in absorbance at 304 nm of PQQH$_2$ does not follow simple first-order kinetics, showing a sigmoid curve (see **Figure 5**).

The λ_{max}^1 and ε_1 values of PQQ and PQQH$_2$ were reported by Duine et al. (*34*), who performed the reduction of PQQ by phenylhydrazine or H$_2$ in the presence of PtO$_2$ (see **Table 1**). The values of λ_{max}^1 for PQQ and PQQH$_2$ are similar to those obtained in the present work. On the other hand, both of the values of ε_1 reported for PQQ and PQQH$_2$ are 1.44 and 1.49–1.70 times smaller than those obtained in the present work. The sample of PQQNa$_2$ H$_2$O (formula weight = 392.3) used in the present work was prepared by evaporating water molecules from crude PQQNa$_2$ sample at 120 °C under vacuum (5 Torr). The X-ray structure analysis of a single crystal of PQQ compound was performed by Ishida et al. (*36*), indicating that five H$_2$O molecules are included in the PQQ compound, that is, the chemical formula of the PQQ compound is PQQNa$_2$·5H$_2$O (formula weight = 464.3). If Duine et al. used such a compound for the measurement of UV spectrum, the value of ε will decrease ∼16%. However, the details of the difference in the value of ε are not clear at present.

Free Radical Scavenging Activity of PQQH$_2$ Is 7.4 Times Higher than That of Vitamin C in Micellar Solution. ArO$^•$ was stable in the absence of PQQH$_2$ and showed absorption peaks at λ_{max} = 580 and 376 nm in aqueous Triton X-100 micellar solution (5.0 wt %) (see **Figure 6**). PQQH$_2$ was prepared by the reduction of PQQNa$_2$ (5.07 × 10^{-5} M) with GSH (6.19 × 10^{-4} M) in 0.10 M phosphate buffer (pH 7.4) under strict nitrogen atmosphere, to avoid an oxidation of PQQH$_2$, as described above. Upon addition of PQQH$_2$ in 0.10 M phosphate buffer (pH 7.4) to 10.0 wt % Triton X-100 micellar solution containing ArO$^•$ (1:1, v/v), the absorption spectrum of ArO$^•$ disappeared immediately. **Figure 6** shows an example of the interaction between ArO$^•$ (∼7.5 × 10^{-5} M) and PQQH$_2$
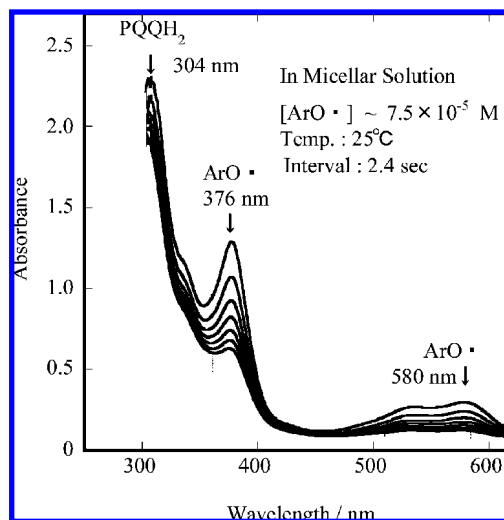

Figure 6. Change in absorption spectrum of aroxyl (ArO$^•$) during the reaction of ArO$^•$ with PQQH$_2$ at pH 7.4 in 5.0 wt % Triton X-100 micellar solution at 25.0 °C. [PQQH$_2$]$_{t=0}$ = 5.07 × 10^{-5} M. The spectra were recorded at 2.4 s intervals. The arrow indicates decreases in absorbance at 580 and 376 nm of ArO$^•$ and a decrease in absorbance at 304 nm of PQQH$_2$ with time. PQQH$_2$ was prepared by the reaction of PQQNa$_2$ with GSH in phosphate buffer solution (pH 7.4).

Table 2. Rate Constants for the Reaction of PQQH$_2$ and Water-Soluble Antioxidants with ArO$^•$ Radical in 5.0 wt % Triton X-100 Micellar Solution (pH 7.4) at 25.0 °C

antioxidant	[antioxidant]/M	k_{obsd}/s^{-1}	k_s/M^{-1} s^{-1}
PQQH$_2$ (glutathione)^a	5.34 × 10^{-4}	1.25	1.81 × 10^3
	9.35 × 10^{-4}	1.74	
	18.7 × 10^{-4}	3.62	
PQQH$_2$ (NaBH$_4$)^b	0.297 × 10^{-4}	0.121	1.92 × 10^3
	5.93 × 10^{-4}	1.04	
	11.8 × 10^{-4}	2.34	
PQQNa$_2$			<10^{-1}
glutathione	0.852 × 10^{-3}	0.574 × 10^{-3}	1.22 × 10^{-1}
	2.56 × 10^{-3}	0.816 × 10^{-3}	
	3.41 × 10^{-3}	0.878 × 10^{-3}	
cysteine	2.95 × 10^{-3}	2.52 × 10^{-3}	9.56 × 10^{-1}
	8.86 × 10^{-3}	8.17 × 10^{-3}	
uric acid	1.25 × 10^{-3}	1.05 × 10^{-3}	6.11 × 10^{-1}
	2.49 × 10^{-3}	1.87 × 10^{-3}	
	4.98 × 10^{-3}	3.34 × 10^{-3}	
vitamin C^c			2.51 × 10^2

^{a} PQQH$_2$ was prepared by reducing PQQNa$_2$ with glutathione. The concentrations of glutathione used for the reduction of PQQNa$_2$ were ≤2.40 × 10^{-2} M. ^{b} PQQH$_2$ was prepared by reducing PQQNa$_2$ with NaBH$_4$. ^{c} See ref *29*.

(5.07 × 10^{-5} M) in phosphate buffer (pH 7.4). Higher concentrations of PQQNa$_2$ (that is, PQQH$_2$) ([PQQH$_2$] ≥ 5.34 × 10^{-4} M) (see **Table 2**) were used for the measurement of the reaction rate, because the condition [PQQH$_2$] > [ArO$^•$] is necessary to determine the pseudo-first-order rate constant (k_{obsd}). By analyzing the decay curve of ArO$^•$ radical at 580 nm, the k_{obsd} value was determined. ArO$^•$ showed a slow natural decay in Triton X-100 micellar solution. Therefore, the k_{obsd} value for ArO$^•$ bleaching is given by eq 3

$$k_{obsd} = k_o + k_s[\text{PQQH}_2] \qquad (3)$$

where k_o is the rate constant for the natural decay of ArO$^•$ in the medium and k_s is the second-order rate constant for the reaction of ArO$^•$ with PQQH$_2$. These parameters are obtained by plotting k_{obsd} against [PQQH$_2$], as shown in **Figure 7**. The k_s value obtained for PQQH$_2$ at pH 7.4 is 1.81 × 10^3 M^{-1}
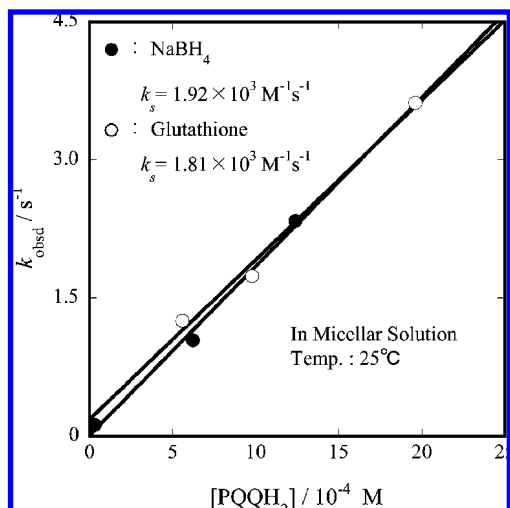
Figure 7. Dependence of pseudo-first-order rate constants (k_{obsd}) on concentration of PQQH$_2$ at pH 7.4 in 5.0 wt % Triton X-100 micellar solution. PQQH$_2$ was prepared by the reaction of PQQNa$_2$ with glutathione (○) and NaBH$_4$ (●).

s^{-1} and k_o = 0.182 s^{-1}. GSH molecules that were not consumed for the reduction of PQQNa$_2$ will remain in the reaction mixture. This GSH ([GSH] < 2.40×10^{-2} M, see **Table 2**) will also react with ArO$^\bullet$. However, the contribution of this reaction will be negligible, because the rate constant (k_s) is 4 orders of magnitude smaller than that for PQQH$_2$, as described below.

Similarly, PQQH$_2$ was prepared by the reduction due to NaBH$_4$ in 0.10 M buffer solution and reacted with 10.0 wt % Triton X-100 micellar solution containing ArO$^\bullet$ (1:1, v/v). As shown in **Figure 7**, the k_s value (1.92×10^3 M^{-1} s^{-1}) obtained is similar to that by the reduction due to GSH.

The measurement of the rate constant for the reaction of PQQNa$_2$ with ArO$^\bullet$ was also performed in Triton X-100 micellar solution at pH 7.4, showing that the reaction between ArO$^\bullet$ and PQQNa$_2$ is negligible.

Similar measurements were performed for the reaction of ArO$^\bullet$ with water-soluble antioxidants (Cys, GSH, and UA) at pH 7.4 in Triton X-100 micellar solution. The rate constants (k_s) for the reaction of Cys, GSH, and UA with ArO$^\bullet$ are expected to be much less than that for PQQH$_2$, and thus higher concentrations of antioxidants were used for the measurements (see **Table 2**). By analyzing the decay curve of ArO$^\bullet$ radical at 580 nm, the k_{obsd} values were determined. k_{obsd} versus [antioxidant] plots are shown in **Figure 8**. The k_s values obtained are listed in **Table 2**, together with that (2.51×10^2 M^{-1} s^{-1}) reported for vitamin C (*29*).

As listed in **Table 2**, the rate constants (k_s) of PQQH$_2$ and water-soluble antioxidants decrease in the order

$$PQQH_2 > Vit\ C \gg Cys > UA > GSH > PQQNa_2 \quad (4)$$

The k_s value of PQQH$_2$ at pH 7.4 is 7.4 ± 0.2 times larger than that of Vit C. The rate constants of Cys, GSH, and UA are $(1.22-9.56) \times 10^{-1}$ M^{-1} s^{-1} and are about 3–4 orders of magnitude smaller than that of PQQH$_2$ in micellar solution.

Oxidative damage of biomembrane and tissues by active oxygen free radicals and its protection by biological antioxidants have attracted much attention. Water-soluble antioxidants (Vit C, Cys, GSH, and UA) suppress free radical mediated chain oxidation of lipids of cell membrane (*37, 38*). In a previous work, the kinetic studies of the reaction of Vit
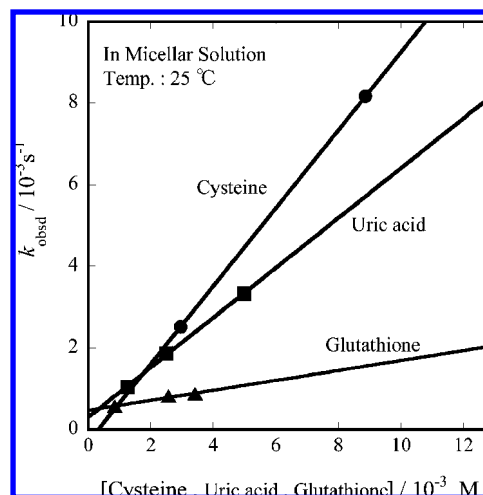

Figure 8. Dependence of pseudo-first-order rate constants (k_{obsd}) on concentration of water-soluble antioxidants (cysteine, glutathione, and uric acid) at pH 7.4 in 5.0 wt % Triton X-100 micellar solution.

C, Cys, GSH, and UA with stable 5,7-di-isopropyl-toco-pheroxyl radical were performed in 5.0 wt % aqueous Triton X-100 micellar solution, and the second-order rate constants (k_r) for these reactions were determined (*39*). The k_r value of Vit C was found to be 3–4 orders of magnitude larger than those of Cys, GSH, and UA in micellar solution. These reactions are regarded as a model for regeneration of α-TocH by the water-soluble antioxidants in human blood, and the relative contributions of each antioxidant to the total regeneration in blood were discussed.

As described in the Introduction, the reduced form of PQQ (PQQH$_2$) functions as a radical scavenger. In fact, it has been reported that PQQH$_2$ shows higher reactivity than α-tocopherol toward galvinoxyl radical and peroxyl radical in acetonitrile–DMSO (93:2, v/v) solution (*25*). PQQH$_2$ reduced the α-toco-pheroxyl radical and spared α-tocopherol in the oxidation of methyl linoleate in the same solution. These results suggest that PQQH$_2$ may act as a potent antioxidant, particularly in combination with α-tocopherol. However, the second-order rate constants for the above reactions have not been reported, because PQQH$_2$ is unstable and it is not easy to determine the concentration of PQQH$_2$ in the reaction mixture, as described above.

In the present work, first, we tried to measure the reaction rates between α-Toc$^\bullet$ and PQQH$_2$ in micellar solution. However, we were unsuccessful in determining the rate constant (k_r), because α-Toc$^\bullet$ is unstable and absorption of α-Toc$^\bullet$ at 430 nm overlaps those of PQQH$_2$ and PQQ, as shown in **Figure 2**. Therefore, the stable ArO$^\bullet$ radical having an absorption maximum at λ_{max} = 580 nm was used for the measurements of the free radical scavenging activity of PQQH$_2$. As described above, it has been found that the k_s value of PQQH$_2$ is 7.4 times larger than that of Vit C and 3–4 orders of magnitude larger than those of Cys, GSH, and UA.

Ubiquinone-10 (and -9) and PQQ are *p*- and *o*-benzoquinone derivatives, respectively. Ubiquinol-10 and -9 (the reduced forms of ubiquinone-10 and -9) are well-known as representative lipid-soluble antioxidants. Ubiquinone-10 and -9 are reduced by NADPH (and enzyme) in tissues and exist as the reduced forms in human and animal tissues (*40*). PQQ is also reduced by NADPH (*41*). On the other hand, ubiquinone-10 and -9 are not reduced by Cys and GSH in contrast to PQQ. This is because ubiquinone-9 and -10 have smaller π-electron systems than PQQ

Antioxidant Activity of Pyrroloquinolinequinol

J. Agric. Food Chem., Vol. 57, No. 2, 2009 **455**

has, and thus the reduction potentials of the former will be higher than that of the latter.

In the present work, it has been found that PQQNa$_2$ is easily reduced by GSH and Cys in buffer solution (pH 7.4), and results in PQQH$_2$. Cys is a proteinaceous thiol, and GSH is a major nonproteinaceous thiol. These thiols exist not only in plasma but also throughout the cell (*37*). These facts indicate that PQQ exists as the reduced form (PQQH$_2$) in a variety of tissues and plays a role as antioxidant. As described in the Introduction, PQQ was found in many kinds of fruits and foods and in several tissues, plasma, and urine of humans and rats (*15−17*). The ArO• radical scavenging rate constant (k_s) of PQQH$_2$ was found to be 3−4 orders of magnitude larger than those of Cys, GSH, and UA and 7.4 times larger than that of Vit C at pH 7.4 in micellar solution. The results of the present kinetic study suggest that PQQH$_2$ has high activity for the free radical cavenging (and/ or the tocopherol regeneration) and contributes to the prevention of oxidative damage in tissues.

LITERATURE CITED

(1) Duine, J. A.; Frank, J. J.; Jongejan, J. A. Glucose dehydrogenase from *Acinetobacter calcoaceticus*. A 'quinoprotein'. *FEBS Lett.* **1979**, *108*, 443–446.

(2) Salisbury, S. A.; Forrrest, H. S.; Gruse, W. B. T.; Kennard, O. A novel coenzyme from bacterial primary alcohol dehydrogenases. *Nature* **1979**, *280*, 843–844.

(3) de Beer, R.; Duine, J. A.; FrankJzn, J.; Westering, J. The role of pyrrolo-quinoline semiquinone forms in the mechanism of action of methanol dehydrogenase. *Eur. J. Biochem.* **1983**, *130*, 105–109.

(4) McIntire, W. S. Newly discovered redox cofactors: possible nutritional, medical, and pharmacological relevance to higher animals. *Annu. Rev. Nutr.* **1998**, *18*, 145–177.

(5) Yamada, M.; Elias, M. D.; Matsushita, K.; Migita, C. T.; Adachi, O. *Escherichia coli* PQQ-containing quinoprotein glucose dehydrogenase: its structure comparison with other quinoproteins. *Biochim. Biophys. Acta* **2003**, *1647*, 185–192.

(6) Toyota, H.; Mathews, F. S.; Adachi, O.; Matsushita, K. Quino-hemoprotein alcohol dehydrogenases: structure, function, and physiology. *Arch. Biochem. Biophys.* **2004**, *428*, 10–21.

(7) Killgore, J.; Smidt, C.; Duich, L.; Romero-Chapman, N.; Tinker, D.; Reiser, K.; Melko, M.; Hyde, D.; Rucker, R. B. Nutritional importance of pyrroloquinoline quinone. *Science* **1989**, *245*, 850–852.

(8) Steinberg, F. M.; Gershwin, M. E.; Rucker, R. B. Dietary pyrroloquinoline quinone: growth and immune response in BALB/c mice. *J. Nutr.* **1994**, *124*, 744–753.

(9) Steinberg, F. M.; Stites, T. E.; Anderson, P.; Storm, D.; Chan, I.; Eghball, S.; Rucker, R. B. Pyrroloquinoline quinone improves growth and reproductive performance in mice fed chemically defined diets. *Exp. Biol. Med.* **2003**, *228*, 160–166.

(10) Bauerly, K. A.; Storms, D. H.; Harris, C. B.; Hajizadeh, S.; Sun, M. Y.; Cheung, C. P.; Satre, M. A.; Fascetti, A. J.; Tchaparian, E.; Rucker, R. B. Pyrroloquinoline quinone nutritional status alters lysine metabolism and modulates mitochondrial DNA content in the mouse and rat. *Biochim. Biophys. Acta* **2006**, *1760*, 1741–1748.

(11) Jensen, F. E.; Gardner, G. J.; Williams, A. P.; Gallop, P. M.; Aizenman, E.; Rosenberg, P. A. The putative essential nutrient pyrroloquinoline quinone is neuroprotective in a rodent model of hypoxic/ischemic brain injury. *Neuroscience* **1994**, *62*, 399–406.

(12) Zhang, Y.; Feustel, P. J.; Kimberg, H. K. Neuroprotection by pyrroloquinoline quinone (PQQ) in reversible middle cerebral artery occlusion in the adult rat. *Brain Res.* **2006**, *1094*, 200–206.

(13) Hara, H.; Hiramatsu, H.; Adachi, T. Pyrroloquinoline quinone is a potent neuroprotective nutrient against 6-hydroxydopamine-induced neurotoxicity. *Neurochem. Res.* **2007**, *32*, 489–495.

(14) Van der Meer, R. A.; Groen, B. W.; van Kleef, M. A. G.; Frank, J.; Jongejan, J. A.; Duine, J. A. Isolation, preparation, and assay of pyrroloquinoline quinone. *Methods Enzymol.* **1990**, *188*, 260–283, and references are cited therein.

(15) Kumazawa, T.; Sato, K.; Seno, H.; Ishii, A.; Suzuki, O. Levels of pyrroloquinoline quinone in various foods. *Biochem. J.* **1995**, *307*, 331–333.

(16) Stites, T. E.; Mitchell, A. E.; Rucker, R. B. Physiological importance of quinoenzymes and the *o*-quinone family of cofactors. *J. Nutr.* **2000**, *130*, 719–727.

(17) Kumazawa, T.; Seno, H.; Urakami, T.; Matsumato, T.; Suzuki, O. Trace levels of pyrroloquinoline quinone in human and rat samples detected by gas chromatography/mass spectroscopy. *Biochim. Biophys. Acta* **1992**, *1156*, 62–66.

(18) He, K.; Nukada, H.; Urakami, T.; Murphy, M. P. Antioxidant and pro-oxidant properties of pyrroloquinoline quinone (PQQ): implications for its function in biological systems. *Biochem. Pharmacol.* **2003**, *65*, 67–74.

(19) Nishigori, H.; Yasunaga, M.; Mizumura, M.; Lee, J. W.; Iwatsuru, M. Preventive effects of pyrroloquinoline quinone on formation of cataract and decline of lenticular and hepatic glutathione of developing chick embro after glucocorticoid treatment. *Life Sci.* **1989**, *45*, 593–598.

(20) Zhang, Y.; Rosenberg, P. A. The essential nutrient pyrroloquinoline quinone may act as a neuroprotectant by suppressing peroxynitrite formation. *Eur. J. Neurosci.* **2002**, *16*, 1015–1024.

(21) Zhu, B.; Simonis, U.; Cecchini, G.; Zhou, H.-Z.; Li, L.; Teerlink, J. R.; Karliner, J. S. Comparison of pyrroloquinoline quinone and/ or metoprolol on myocardial infarct size and mitochondrial damage in a rat model of ischemia/reperfusion injury. *J. Cardiovasc. Pharmacol. Ther.* **2006**, *11*, 119–128.

(22) Ohwada, K.; Takeda, H.; Yamazaki, M.; Isogaki, H.; Nakano, M.; Shimomura, M.; Fukui, K.; Urano, S. Pyrroloquinoline quinone (PQQ) prevents cognitive deficit caused by oxidative stress in rats. *J. Clin. Biochem. Nutr* **2008**, *42*, 29–34.

(23) Gallop, P. M.; Henson, E.; Paz, M. A.; Greenspan, S. L.; Fluckiger, R. Acid-promoted tautomeric lactonization and oxidation-reduction of pyrroloquinoline quinone (PQQ). *Biochem. Biophys. Res. Commun.* **1989**, *163*, 755–763.

(24) Gallop, P. M.; Paz, M. A.; Fluckiger, R.; Henson, E. Is the antioxidant, anti-inflammatory putative new vitamin, PQQ, involved with nitric oxide in bone metabolism? *Connect. Tissue Res* **1993**, *29*, 153–161.

(25) Miyauchi, K.; Urakami, T.; Abeta, H.; Shi, H.; Noguchi, N.; Niki, E. Action of pyrroloquinolinequinol as an antioxidant against lipid peroxidation in solution. *Antioxid. Redox Signal.* **1999**, *1*, 547–554.

(26) Mukai, K.; Kageyama, Y.; Ishida, T.; Fukuda, K. Synthesis and kinetic study of antioxidant activity of new tocopherol (vitamin E) compounds. *J. Org. Chem.* **1989**, *54*, 552–556.

(27) Mukai, K.; Daifuku, K.; Okabe, K.; Tanigaki, T.; Inoue, K. Structure-activity relationship in the quenching reaction of singlet oxygen by tocopherol (vitamin E) derivatives and related phenols. Finding of linear correlation between the rates of quenching of singlet oxygen and scavenging of peroxyl and phenoxyl radicals in solution. *J. Org. Chem.* **1991**, *56*, 4188–4192.

(28) Mukai, K.; Tokunaga, A.; Itoh, S.; Kanesaki, Y.; Ohara, K.; Nagaoka, S.; Abe, K. Structure-activity relationship of the free-radical-scavenging reaction by vitamin E (α-, *β*-, *γ*-, *δ*-tocopherols) and ubiquinol-10: pH dependence of the reaction rates. *J. Phys. Chem. B* **2007**, *111*, 652–662.

456 *J. Agric. Food Chem.,* Vol. 57, No. 2, 2009

Ouchi et al.

(29) Mitani, S.; Ouchi, A.; Watanabe, E.; Kanesaki, Y.; Nagaoka, S.; Mukai, K. Stopped-flow kinetic study of the aroxyl radical-scavenging action of catechins and vitamin C in ethanol and micellar solutions. *J. Agric. Food Chem.* **2008**, *56*, 4406–4417.

(30) Rieker, A.; Scheffler, K. Die beteiligung von phenylresten an der aroxylmesomerie. *Liebigs Ann. Chem.* **1965**, *689*, 78–92.

(31) Itoh, S.; Kato, N.; Ohshiro, Y.; Agawa, T. Catalytic oxidation of thiols by coenzyme PQQ. *Chem. Lett.* **1985**, 135–136.

(32) Itoh, S.; Kato, N.; Mure, M.; Ohshiro, Y. Kinetic studies on the oxidation of thiols by coenzyme PQQ. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 420–422.

(33) Itoh, S.; Ohshiro, Y.; Agawa, T. Reaction of reduced PQQ (PQQH$_2$) and molecular oxygen. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 1911–1914.

(34) Duine, J. A.; Frank, J. J.; Verwiel, P. E. J. Characterization of the second prosthetic group in methanol dehydrogenase from hyphomicrobium X. *Eur. J. Biochem.* **1981**, *118*, 395–399.

(35) Itoh, S.; Ogino, M.; Fukui, Y.; Murao, H.; Komatsu, M.; Ohshiro, Y.; Inoue, T.; Kai, Y.; Kasai, N. C-4 and C-5 adducts of cofactor PQQ (pyrroloquinolinequinone). Model studies directed toward the action of quinoprotein methanol dehydrogenase. *J. Am. Chem. Soc.* **1993**, *115*, 9960–9967.

(36) Ishida, T.; Doi, M.; Tomita, K.; Hayashi, H.; Inoue, M.; Urakami, T. Molecular and crystal structure of PQQ (methoxantin), a novel coenzyme of quinoproteins: extensive stacking character and metal ion interaction. *J. Am. Chem. Soc.* **1989**, *111*, 6822–6828.

(37) Stocker, R.; Keaney, J. F., Jr. Role of oxidative modifications in atherosclerosis. *Physiol. Rev.* **2004**, *84*, 1381–1478, and references cited therein.

(38) Niki, E. Antioxidants in relation to lipid peroxidation. *Chem. Phys. Lipids* **1987**, *44*, 227–253.

(39) Mukai, K.; Isozaki, T.; Nagaoka, S. Kinetics of vitamin E regeneration by water-soluble antioxidants in micellar dispersions. *Bull. Chem. Soc. Jpn.* **2007**, *80*, 1331–1334.

(40) Kohar, I.; Baca, M.; Suarna, C.; Stocker, R.; Southwell-Keely, P. T. Is α-tocopherol a reservoir for α-tocopheryl hydroquinone. *Free Radical Biol. Med.* **1995**, *19*, 197–207.

(41) Toyama, H.; Nishibayashi, E.; Saeki, M.; Adachi, O.; Matsushita, K. Factors required for the catalytic reaction of PqqC/D which produces pyrroloquinoline quinone. *Biochem. Biophys. Res. Commun.* **2007**, *354*, 290–295.