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Reaction of Reduced PQQ ($PQQH_2$) and Molecular Oxygen

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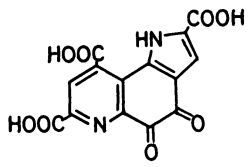
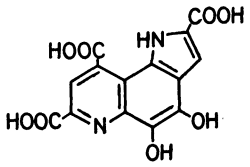
Reduced PQQ ($PQQH_2$) is prepared by the reaction of PQQ with thiophenol, 1-benzyl-1,4-dihydronicotinamide (BNAH), sodium dithionite, or sodium borohydride. Oxidation of $PQQH_2$ to PQQ by molecular oxygen in aqueous solutions is investigated kinetically. The oxidation is accelerated gradually with proceeding of the reaction, which may be attributed to the side reaction of $PQQH_2$ and H_2O_2 produced by the reaction of $PQQH_2$ and O_2. As in fact, the yield of H_2O_2 is found to be 50% based on $PQQH_2$. Initial rate is first-order in oxygen concentration. The pH-rate profile suggests that an active species in the reaction is $PQQH^-$. Autocatalysis of $O_2^{\cdot-}$ and PQQ itself is scarcely detected in this reaction. The mechanism of the oxidation is also discussed.

It has been well-known that copper-containing amine oxidases constitute important sources of H_2O_2 in biological systems.[1] However, the reaction of this group of enzymes with O_2 has been less explored, because the true character of the second prosthetic group of the enzymes has not been clear. Meanwhile, Ameyama and Duine simultaneously indicated the possible occurrence of PQQ (4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid, a newly discovered coenzyme) in copper-containing amine oxidases.[2,3]



PQQ



$PQQH_2$

We have already demonstrated the autorecycling PQQ-catalyzed oxidation of amines, which can be regarded as a model system of PQQ-containing amine oxidases.[4] And in the preceding paper, we investigated the mechanism of the reaction between PQQ and an amine under anaerobic conditions.[5] Moreover, existence of reduced PQQ (4,5-dihydroxy-1*H*-pyrrolo[2,3-*f*]-quinoline-2,7,9-tricarboxylic acid, $PQQH_2$) and its importance in a quinoprotein have also been reported by Duine and his co-workers.[6] Thus in this paper, we wish to investigate the reaction of $PQQH_2$ and O_2 in order to clarify the redox systems of PQQ in more detail. The reaction of hydroquinone and catechol derivatives with O_2 has not been studied so widely that the mechanism has not been clear.[7,8]

Experimental

Ultraviolet and visible absorption spectra were recorded on Shimadzu UV-240 spectrophotometer equipped with a temperature controlled cell holder, Shimadzu TCC-240. Measurements of pH were performed by using Horiba pH-meter F-8. Oxygen concentrations were determined by using Unicon oxygen meter. Superoxide dismutase (SOD) was obtained commercially, and catalase was supplied by Dr. K. Kobayashi (Osaka University). Other chemicals used were purchased at the highest purities available.

Preparation of $PQQH_2$ (quinol). Reduction of PQQ to $PQQH_2$ was performed with thiophenol, 1-benzyl-1,4-dihydronicotinamide (BNAH),[9] sodium dithionite, or sodium borohydride (Table). A typical procedure is as follows. After quantitative hydrolysis of PQQTME[10] (trimethyl ester of PQQ, 12.7 mg, 3.4×10^{-2} mmol) to PQQ in 0.05 M Na_2CO_3 (10 ml (1 M=1 mol dm^{-3})) at 30 °C for 24 h, the solution was neutralized (pH 6.8) with 2 M HCl. The solution was placed in the bottom of a Thunberg vessel (30 ml), and 10-fold excess of thiophenol (0.34 mmol) in acetonitrile (3 ml) was deposited in the side arm of the vessel. Both solutions were degassed for 30 min by bubbling N_2 through them, which were then mixed to start the reaction. After 2 h, the reaction mixture was acidified with 2 M HCl (1 ml), and was stood overnight. The precipitated product was collected by centrifugation and was washed with acetonitrile (5 ml). The product was dried in vacuo over P_2O_5, and recrystallized from dimethyl sulfoxide–acetonitrile (84% yield). The spectral data of the product were in good agreement with those reported by Duine and his co-workers.[6] BNAH, $Na_2S_2O_4$, $NaBH_4$ could also be reacted in the same way, and $PQQH_2$ was obtained in 77, 99, and 99% yield, respectively. Reduction of PQQ with phenylhydrazine (pH 3.1) and catalytic hydrogenation of PQQ (H_2 (1 atm)/PtO_2, pH 6.7) were carried out in the similar way reported elsewhere (82 and 65%, respectively).[6]

Oxidation of $PQQH_2$ by Molecular Oxygen. The reaction between $PQQH_2$ and O_2 was performed in 0.1 M acetate, phosphate, and carbonate buffer solutions (μ=0.3 with KCl, containing 5% dimethyl sulfoxide) at 30 °C under air atmosphere. An air saturated buffer solution (2.85 ml, $[O_2]=2.28\times10^{-4}$ M) was placed into a cuvette, and the reaction was started by adding 0.15 ml of an anaerobic DMSO solution of $PQQH_2$ (2.86×10^{-4} M). The reaction was followed by observing the disappearance of $PQQH_2$ at 320 nm. Hydrogen peroxide product was determined by adding 1 M NaI aqueous solution (0.5 ml) to the final reaction mixture, and monitoring the appearance of I_3^- at 353 nm (ε=25000 $M^{-1}cm^{-1}$). The rate constant was found to be identical with that for the reaction of I^- with authentic H_2O_2 at the pH employed. The yield of H_2O_2 was determined from the increase of A_{353} and was calculated to be about 50% based on $PQQH_2$. Oxygen concentration were controlled by mixing a O_2-saturated buffer solution and a N_2-saturated one.

Results and Discussion

Synthesis of $PQQH_2$. PQQ is reduced in the reac-

Table. Synthesis of $PQQH_2$[a]

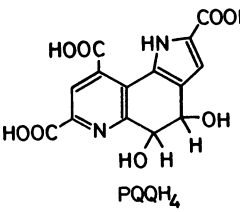
Reductant[b] (Solvent, ml)	pH[c]	Time h	Isolated Yield[d] %
PhSH (CH_3CN, 3 ml)	6.8	2	84
BNAH (CH_3CN, 3 ml)[e]	6.8	3	77
$Na_2S_2O_4$ (H_2O, 1 ml)	11.4	5	99
$NaBH_4$ (H_2O, 1 ml)	7.2	4	99
$PhNHNH_2$ (CH_3CN, 3 ml)	3.1	3	82
H_2(1 atm)/PtO_2	7.0	5	65

a) PQQ was generated by hydrolysis of PQQTME (0.032—0.036 mmol) in 0.05 M Na_2CO_3 (10 ml). b) 10-fold excess over PQQ. c) The pH of a PQQ-aqueous solution was adjusted with 2 M HCl. d) Based on PQQ. e) In the dark.

tion with amines, but a mixture of the quinol and the aminophenol was obtained.[5] On the other hand, we found that only $PQQH_2$ (quinol) was produced in the reaction of PQQ with thiophenol,[11] BNAH, $Na_2S_2O_4$, and $NaBH_4$ (Table). Formation of PhSSPh and BNA^+ was detected in the reaction of PQQ with PhSH and BNAH by HPLC, respectively.

It should be noted that 99% of $PQQH_2$ was isolated in the reaction with $NaBH_4$, though Duine and his co-workers reported the formation of $PQQH_4$ in the reaction with $NaBH_4$.[12] Probably, they used so excess $NaBH_4$ over PQQ that further reduction might take place. The reaction with $PhNHNH_2$ (under acidic conditions) and catalytic hydrogenation (H_2/PtO_2, under neutral conditions) could be applied to the preparation of $PQQH_2$, but removal of the catalyst from the reaction mixture caused the autoxidation in the latter case.

The present methodology was applicable to the preparation of reduced PQQTME (trimethyl 4,5-dihydroxy-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylate, $PQQTMEH_2$) which was obtained in 80% yield in the reaction of PQQTME and PhSH in acetonitrile under anaerobic conditions for 24 h.[13] BNAH, $NaBH_4$, and $PhNHNH_2$ were also applicable to the prepara-



$PQQH_4$

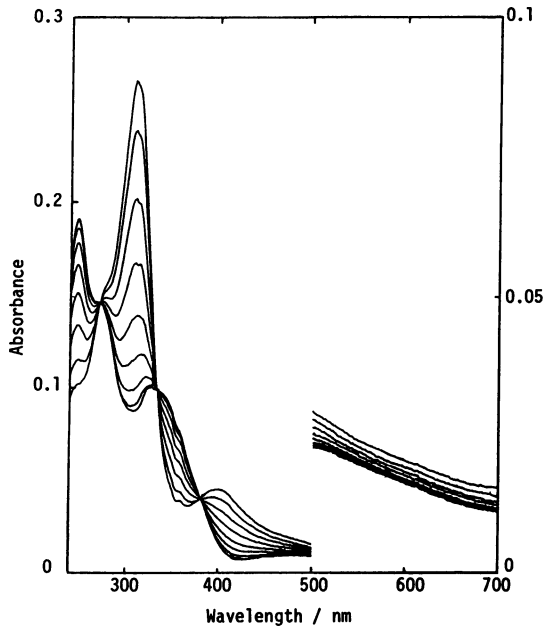


Fig. 1. Spectral change along the progress of the reaction of $PQQH_2$ (1.5×10^{-5} M), and O_2 (2.28×10^{-4} M), in 0.1 M acetate buffer (pH 3.81, μ=0.3 with KCl, containing 5% DMSO) at 30°C.

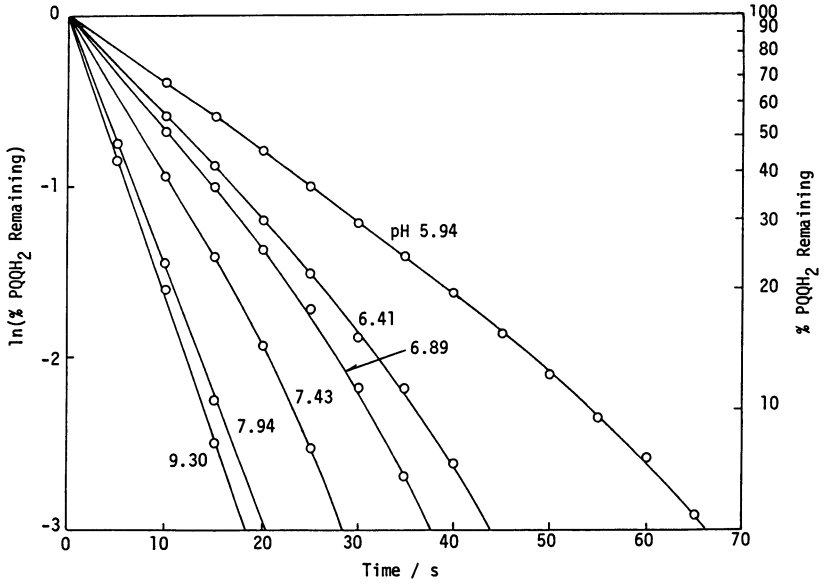


Fig. 2. Time course of the oxidation of $PQQH_2$ (1.5×10^{-5} M), by O_2 (2.28×10^{-4} M), in 0.1 M acetate (pH 5.94), phosphate (pH 6.41, 6.89, 7.43, 7.94), and carbonate (pH 9.30) buffer solutions (μ=0.3 with KCl, containing 5% DMSO) at 30°C.

tion of PQQTMEH$_2$ (70, 65, and 82% yield, respectively).

Oxidation of PQQH$_2$ by O$_2$ was studied under pseudo-first-order conditions of $[PQQH_2]_T=1.5\times10^{-5}$ M under air atmosphere ($[O_2]=2.28\times10^{-4}$ M at 30°C) in 0.1 M buffer solutions (μ=0.3 with KCl, containing 5% DMSO). The progress of the reaction was followed by monitoring the disappearance of PQQH$_2$ at 320 nm (Fig. 1). PQQH$_2$ was oxidized rapidly to PQQ with isosbestic points at 272, 333, and 379 nm at pH 3.81 (0.1 M acetate buffer containing 5% DMSO) and the yield of H_2O_2 was determined to be about 50% based on PQQH$_2$ by iodometric titration. An intermediate such as a semiquinone or a covalent adduct of O$_2$ to PQQH$_2$ was not detected by UV-visible absorption spectroscopy in the course of the reaction.

The reaction did not follow the first-order kinetics, namely it is accelerated gradually with proceeding of the reaction (Fig. 2). Thus, the initial rate constant (k_i) was calculated from the initial slope of the pseudo-first-order plots. The initial rate (pH 6.90) was first-order in oxygen concentration (Fig. 3). As shown in Fig. 2, the rate increases as the pH is increased from 5.94 to 9, though the dependence of the initial rate on pH is somewhat complicated. The acid-base dissociation constant (K_a) for the quinol function of PQQH$_2$ was determined spectrophotometrically to be 7.08×10^{-10} M (pK_a=9.15) under the anaerobic kinetic conditions. Moreover, PQQH$_2$ was relatively stable in an organic solvent such as dimethyl sulfoxide even under aerobic conditions. These results indicate that PQQH$^-$ is considered to be an active species in the oxidation of PQQH$_2$ by O$_2$ as shown in Eq. 1.

$$PQQH_2 \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} PQQH^- \xrightarrow{k_2[O_2]} PQQ + H_2O_2 \qquad (1)$$

$$pK_a = 9.15$$

Why does the reaction deviate from the first-order rate law? To solve the problem, the reaction was carried out in the presence of catalase (10^{-7} M) under the same conditions (Fig. 4). In this case, the reaction followed the first-order rate law and such a deviation was not observed. On the other hand, the presence of SOD (superoxide dismutase, 10^{-7} M) in addition to catalase hardly affected the time course compared with the case of autoxidation of reduced flavins.[14] Furthermore, the presence of PQQ (1.5×10^{-5} M) in the reaction mixture also did not alter the time course of the reaction. From these results, we consider that the main cause for the deviation from the pseudo-first-order rate law is a side reaction of PQQH$_2$ with H_2O_2 (Eq. 4) formed by the reaction of PQQH$_2$ and O$_2$ (Eq. 3). As in fact, the yield of H_2O_2 was found to be about 50% based on PQQH$_2$, and PQQH$_2$ actually reacted with H_2O_2 comparably

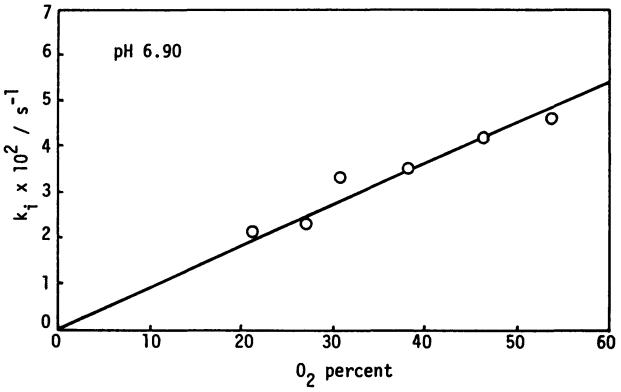


Fig. 3. Initial rate constant (k_i) for the O$_2$ oxidation of PQQH$_2$ (1.5×10^{-5} M) plotted as a function of O$_2$ percent (determined by oxygen meter based on O$_2$- saturated solution: 100%) in 0.1 M phosphate buffer (pH 6.90, μ=0.3 with KCl, containing 5% DMSO) at 30°C.

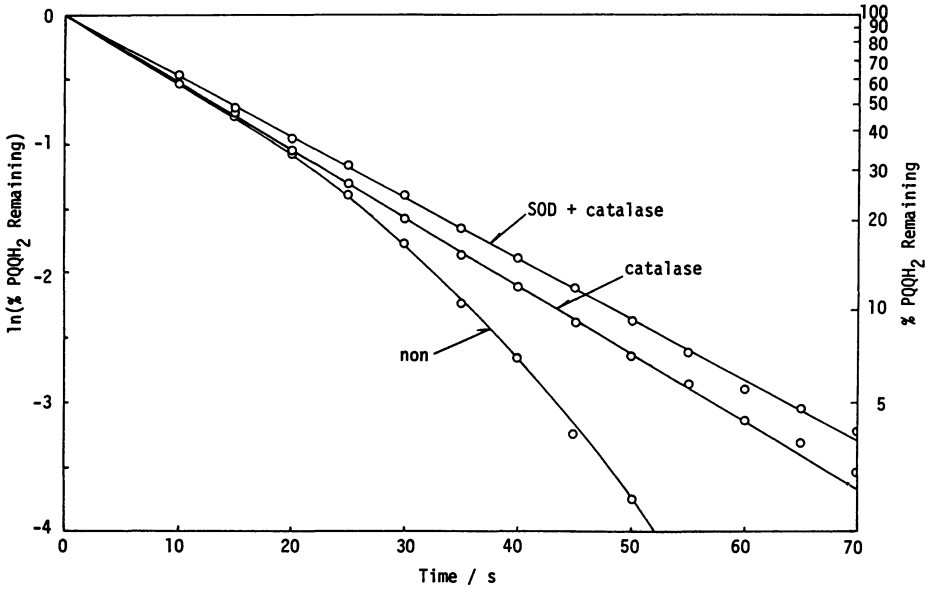


Fig. 4. Effect of catalase (10^{-7} M) and superoxide dismutase (SOD, 10^{-7} M) on the oxidation of PQQH$_2$ (1.5×10^{-5} M) by O$_2$ (2.28×10^{-4} M) in 0.1 M phosphate buffer (pH 6.90, μ=0.3 with KCl, containing 5% DMSO) at 30°C.

fast in the absence of O_2. On the other hand, there may be little participation of autocatalysis by $O_2^{\cdot-}$ and PQQ itself (Eq. 5—7).

$$PQQH_2 + O_2 \longrightarrow PQQ + H_2O_2 \quad (3)$$

$$PQQH_2 + H_2O_2 \longrightarrow PQQ + 2H_2O \quad (4)$$

$$PQQH_2 + PQQ \rightleftharpoons 2PQQH^{\cdot} \quad (5)$$

$$PQQH^{\cdot} + O_2 \longrightarrow PQQ + O_2^{\cdot-} + H^+ \quad (6)$$

$$PQQH^{\cdot} + O_2^{\cdot-} \xrightarrow{H^+} PQQ + H_2O_2 \quad (7)$$

As molecular oxygen contains two unpaired electrons and is paramagnetic in the ground state, the concerted two electron reduction of O_2 to H_2O_2 is highly restricted by symmetry consideration.[15] Thus, the reduction of O_2 with $PQQH_2$ is assumed to occur in two ways: (a) By binding to the electron donor, $PQQH_2$, the molecular orbitals of O_2 are perturbed, or (b) the reduction of O_2 via two univalent electron transfer (stepwise). The standard redox potential (E_0') values (pH=7.0) of $O_2/HO_2^{\cdot}$ couple has been calculated to be −0.59 V (Eq. 8).[16] On the other hand, Duine and his co-workers determined the midpoint potentials (E_m) of the quinone/semiquinone and semiquinone/quinol couples for PQQ at pH 13.0 to be −0.218 and −0.242 V, respectively (Eqs. 10, 11).[6] Although the standard redox potentials (pH 7.0) for these couples of PQQ have not been defined, they are considered to be higher than those E_m (pH 13). Thus, one electron reduction of O_2 to $O_2^{\cdot-}$ by either $PQQH_2$ or the semiquinone appears thermodynamically unfavorable. Absence of autocatalysis by $O_2^{\cdot-}$ and the semiquinone may also discard the possibility of the reduction of O_2 via two univalent electron transfer (free radical mechanism, type b). Ultimately, it reminds us a mechanism via covalent addition of O_2 to quinol carbon of $PQQH^-$ followed by elimination of H_2O_2 (type a). The mechanistic details of the reaction is currently under investigation.

$$O_2 + H^+ + e^- \longrightarrow HO_2^{\cdot} \quad E_0' = -0.59\,V \quad (8)$$

$$HO_2^{\cdot} + H^+ + e^- \longrightarrow H_2O_2 \quad E_0' = 0.98\,V \quad (9)$$

$$\text{quinone/semiquinone} \quad E_m = -0.218\,V\ (pH=13) \quad (10)$$

$$\text{semiquinone/quinol} \quad E_m = -0.242\,V\ (pH=13) \quad (11)$$

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References

1) T. P. Singer and D. E. Edmondson, "Molecular Oxygen in Biology," ed by O. Hayashi, North-Holland Publish Campany (1974), p. 315.

2) M. Ameyama, M. Hayashi, K. Matsushita, E. Shinagawa, and O. Adachi, *Agric. Biol. Chem.*, **48**, 561 (1984).

3) C. L. Lobenstein-Verbeek, J. A. Jongejan, J. Frank, and J. A. Duine, *FEBS Lett.*, **170**, 305 (1984).

4) a)Y. Ohshiro, S. Itoh, K. Kurokawa, J. Kato, T. Hirao, and T. Agawa, *Tetrahedron Lett.*, **24**, 3465 (1983); b) S. Itoh, N. Kato, Y. Ohshiro, and T. Agawa, *ibid.*, **25**, 4753 (1984).

5) S. Itoh, Y. Kitamura, Y. Ohshiro, and T. Agawa, accompanying paper in this issue.

6) J. A. Duine, J. Frank, and P. E. J. Verwiel, *Eur. J. Biochem.*, **118**, 395 (1981).

7) T. H. James, J. M. Snell, and A. Weissberger, *J. Am. Chem. Soc.*, **60**, 2084 (1938).

8) H. Musso and H. Dopp, *Chem. Ber.*, **100**, 3627 (1967).

9) D. Mauzerall and F. H. Westheimer, *J. Am. Chem. Soc.*, **77**, 2261 (1955).

10) E. J. Corey and A. Tramontano, *J. Am. Chem. Soc.*, **103**, 5599 (1981).

11) S. Itoh, N. Kato, Y. Ohshiro, and T. Agawa, *Chem. Lett.*, **1985**, 135.

12) J. A. Duine, J. Frank, and P. E. J. Verwiel, *Eur. J. Biochem.*, **108**, 187 (1980).

13) Mp (decomp) 224—247 °C; ^{1}H NMR (DMSO-d_6, δ) 4.01, 4.09, 4.17 (each s, 3H, OCH_3), 6.1 (br s, quinol OH), 7.53 (d, J=2 Hz, 1H, aromatic 3-H), 8.79 (s, 1H, aromatic 8-H); IR (Nujol, cm^{-1}) 3200—3600 (quinol OH), 3480 (NH), 1725, 1735 (ester C=O); mass spectrum m/z 374 (M^+); Anal. Calcd for $C_{17}H_{14}N_2O_8$: C, 54.55; H, 3.77; N, 7.48%. Found: C, 54.03; H, 3.67; N, 7.50%; UV spectrum (CH_3CN) λ_{max}=325 nm (ε= 30400 $M^{-1}cm^{-1}$).

14) V. Massy, G. Palmer, and D. Ballon, "On the Reaction of Reduced Flavins and Flavoproteins with Molecular Oxygen" in "Flavins and Flavoproteins, Third International Symposium," ed by H. Kamin, University Park Press, Baltimore (1971), pp. 349—361.

15) H. Taube, *J. Gen. Physiol.*, **49**, 29 (1965).

16) N. Sutin, "Oxidase and Related Redox Systems," ed by E. T. King, H. S. Mason, and M. Morrison, Wiley, New York (1965), Vol 1, p. 34.