

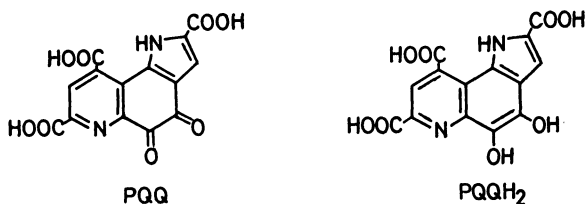
Reaction of Reduced PQQ (PQQH₂) and Molecular Oxygen

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Reduced PQQ (PQQH₂) is prepared by the reaction of PQQ with thiophenol, 1-benzyl-1,4-dihydronicotinamide (BNAH), sodium dithionite, or sodium borohydride. Oxidation of PQQH₂ 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 PQQH₂ and H₂O₂ produced by the reaction of PQQH₂ and O₂. As in fact, the yield of H₂O₂ is found to be 50% based on PQQH₂. Initial rate is first-order in oxygen concentration. The pH-rate profile suggests that an active species in the reaction is PQQH⁻. Autocatalysis of O₂⁻ 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 H₂O₂ in biological systems.¹⁾ However, the reaction of this group of enzymes with O₂ 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)}



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, PQQH₂) 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 PQQH₂ and O₂ in order to clarify the redox systems of PQQ in more detail. The reaction of hydroquinone and catechol derivatives with O₂ 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 PQQH₂ (quinol). Reduction of PQQ to PQQH₂ 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 PQQTME¹⁰⁾ (trimethyl ester of PQQ, 12.7 mg, 3.4×10⁻² mmol) to PQQ in 0.05 M Na₂CO₃ (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 N₂ 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 P₂O₅, 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, Na₂S₂O₄, NaBH₄ could also be reacted in the same way, and PQQH₂ was obtained in 77, 99, and 99% yield, respectively. Reduction of PQQ with phenylhydrazine (pH 3.1) and catalytic hydrogenation of PQQ (H₂ (1 atm)/PtO₂, pH 6.7) were carried out in the similar way reported elsewhere (82 and 65%, respectively).⁶⁾

Oxidation of PQQH₂ by Molecular Oxygen. The reaction between PQQH₂ and O₂ was performed in 0.1 M acetate, phosphate, and carbonate buffer solutions ($\mu=0.3$ with KCl, containing 5% dimethyl sulfoxide) at 30°C under air atmosphere. An air saturated buffer solution (2.85 ml, [O₂]=2.28×10⁻⁴ M) was placed into a cuvette, and the reaction was started by adding 0.15 ml of an anaerobic DMSO solution of PQQH₂ (2.86×10⁻⁴ M). The reaction was followed by observing the disappearance of PQQH₂ 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 I₃⁻ at 353 nm ($\epsilon=25000$ M⁻¹ cm⁻¹). The rate constant was found to be identical with that for the reaction of I⁻ with authentic H₂O₂ at the pH employed. The yield of H₂O₂ was determined from the increase of A₃₅₃ and was calculated to be about 50% based on PQQH₂. Oxygen concentration were controlled by mixing a O₂-saturated buffer solution and a N₂-saturated one.

Results and Discussion

Synthesis of PQQH₂. PQQ is reduced in the reac-

Table. Synthesis of PQQH₂^{a)}

Reductant ^{b)} (Solvent, ml)	pH ^{c)}	Time	Isolated Yield ^{d)}
		h	%
PhSH (CH ₃ CN, 3 ml)	6.8	2	84
BNAH (CH ₃ CN, 3 ml) ^{e)}	6.8	3	77
Na ₂ S ₂ O ₄ (H ₂ O, 1 ml)	11.4	5	99
NaBH ₄ (H ₂ O, 1 ml)	7.2	4	99
PhNHNH ₂ (CH ₃ CN, 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 Na₂CO₃ (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 PQQH₂ (quinol) was produced in the reaction of PQQ with thiophenol,¹¹⁾ BNAH, Na₂S₂O₄, and NaBH₄ (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 PQQH₂ was isolated in the reaction with NaBH₄, though Duine and his co-workers reported the formation of PQQH₄ in the reaction with NaBH₄.¹²⁾ Probably, they used so excess NaBH₄ over PQQ that further reduction might take place. The reaction with PhNHNH₂ (under acidic conditions) and catalytic hydrogenation (H₂/PtO₂,

under neutral conditions) could be applied to the preparation of PQQH₂, 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, PQQTMEH₂) which was obtained in 80% yield in the reaction of PQQTME and PhSH in acetonitrile under anaerobic conditions for 24 h.¹³⁾ BNAH, NaBH₄, and PhNHNH₂ were also applicable to the prepara-

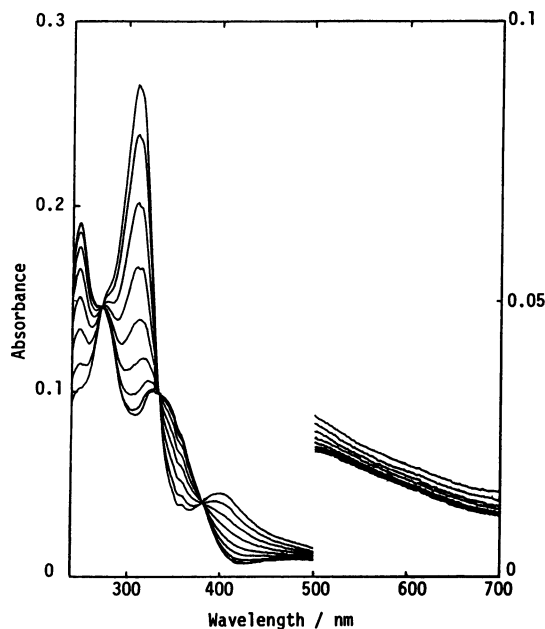
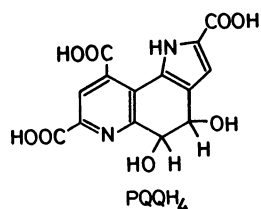


Fig. 1. Spectral change along the progress of the reaction of PQQH₂ (1.5×10^{-5} M), and O₂ (2.28×10^{-4} M), in 0.1 M acetate buffer (pH 3.81, $\mu = 0.3$ with KCl, containing 5% DMSO) at 30°C.

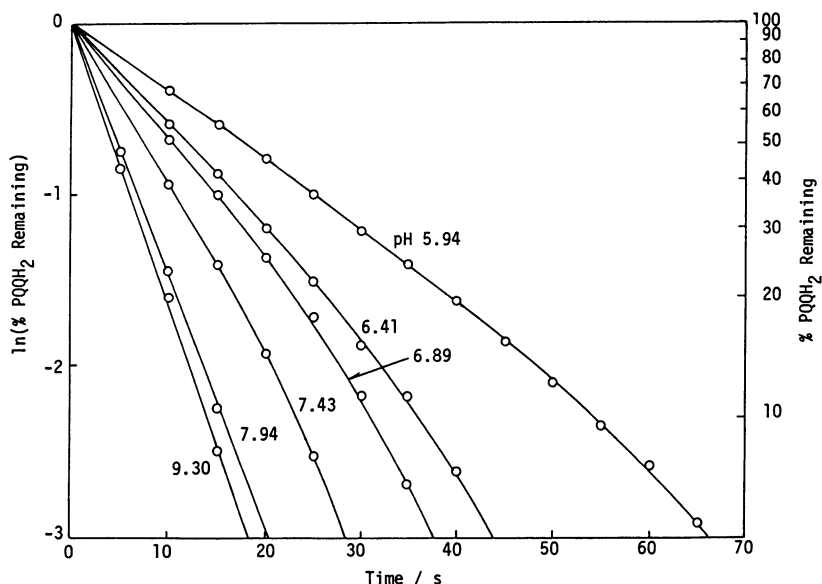
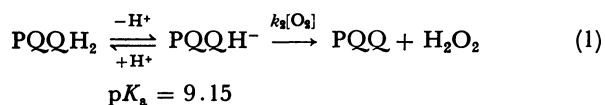


Fig. 2. Time course of the oxidation of PQQH₂ (1.5×10^{-5} M), by O₂ (2.28×10^{-4} M), in 0.1 M acetate (pH 5.94), phosphate (pH 6.41, 6.89, 7.43, 7.94), and carbonate (pH 9.30) buffer solutions ($\mu = 0.3$ with KCl, containing 5% DMSO) at 30°C.

tion of PQQTMEH₂ (70, 65, and 82% yield, respectively).

Oxidation of PQQH₂ by O₂ was studied under pseudo-first-order conditions of [PQQH₂]_T = 1.5 × 10⁻⁵ M under air atmosphere ([O₂] = 2.28 × 10⁻⁴ 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 PQQH₂ at 320 nm (Fig. 1). PQQH₂ 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 H₂O₂ was determined to be about 50% based on PQQH₂ by iodometric titration. An intermediate such as a semiquinone or a covalent adduct of O₂ to PQQH₂ 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 PQQH₂ was determined spectrophotometrically to be 7.08 × 10⁻¹⁰ M (p*K_a* = 9.15) under the anaerobic kinetic conditions. Moreover, PQQH₂ 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 PQQH₂ by O₂ as shown in Eq. 1.



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 PQQH₂ with H₂O₂ (Eq. 4) formed by the reaction of PQQH₂ and O₂ (Eq. 3). As in fact, the yield of H₂O₂ was found to be about 50% based on PQQH₂, and PQQH₂ actually reacted with H₂O₂ comparably

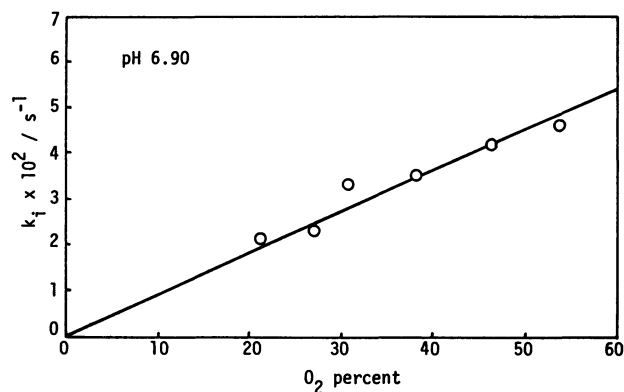


Fig. 3. Initial rate constant (*k_i*) for the O₂ oxidation of PQQH₂ (1.5 × 10⁻⁵ M) plotted as a function of O₂ percent (determined by oxygen meter based on O₂-saturated solution: 100%) in 0.1 M phosphate buffer (pH 6.90, μ = 0.3 with KCl, containing 5% DMSO) at 30 °C.

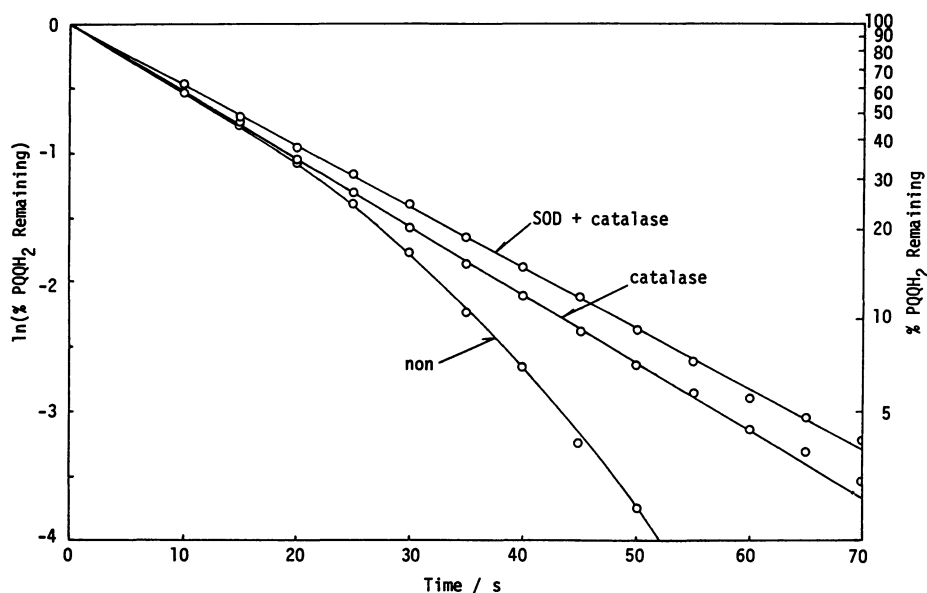
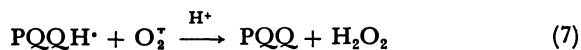
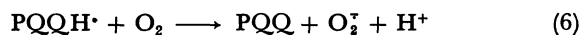
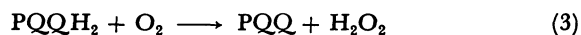
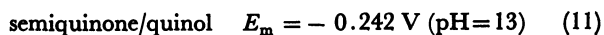
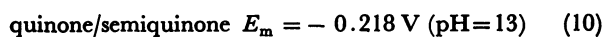
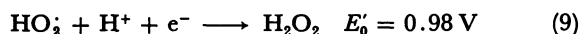
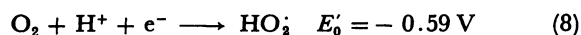


Fig. 4. Effect of catalase (10⁻⁷ M) and superoxide dismutase (SOD, 10⁻⁷ M) on the oxidation of PQQH₂ (1.5 × 10⁻⁵ M) by O₂ (2.28 × 10⁻⁴ M) in 0.1 M phosphate buffer (pH 6.90, μ = 0.3 with KCl, containing 5% DMSO) at 30 °C.

fast in the absence of O_2 . On the other hand, there may be little participation of autocatalysis by O_2^- and PQQ itself (Eq. 5—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^- 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^- by either $PQQH_2$ or the semiquinone appears thermodynamically unfavorable. Absence of autocatalysis by O_2^- 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.



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- 13) Mp (decomp) 224—247°C; 1H NMR (DMSO- d_6 , δ) 4.01, 4.09, 4.17 (each s, 3H, OCH₃), 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) $\lambda_{max}=325$ nm ($\epsilon=30400$ $M^{-1}cm^{-1}$).
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