Mechanistic study of amine oxidation catalysed by quinonoid cofactors

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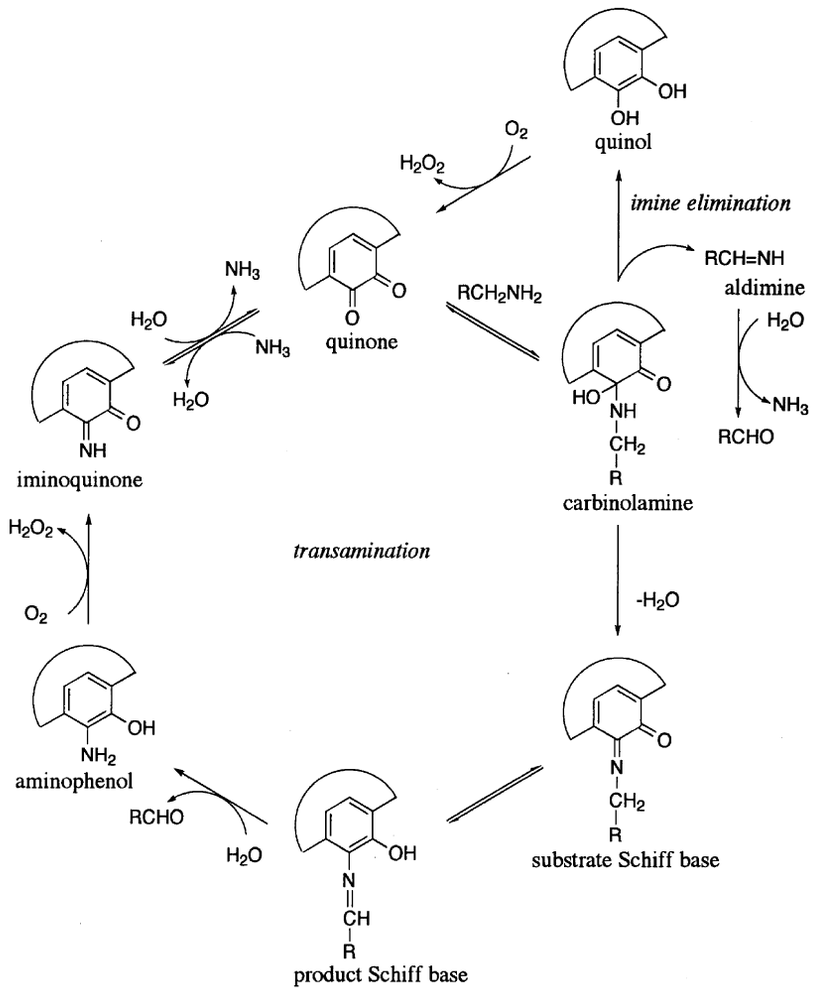
A kinetic study was performed on the oxidative deamination of benzylamine (BzAm) catalysed by pyrroloquinoline quinone (PQQ), a tryptophan tryptophylquinone analogue [3-methyl-4-(3′-methylindol-2′-yl)indole-6,7-dione; TTQ_a], and a topa quinone analogue (6-hydroxydopa quinone; TPQ_a) using high-performance liquid chromatography under aerobic conditions and cyclic voltammetry under anaerobic conditions. The overall kinetics are second-order in the quinone and BzAm concentrations and independent of the electron acceptors used (dioxygen under aerobic conditions and electrode under anaerobic conditions). The second-order rate constants *vs.* pH profiles are explained in terms of the acid dissociation of BzAm and the quinones, yielding pH-independent second-order rate constants, which are in the order $TTQ_a > PQQ \gg TPQ_a$. Half-wave potentials of steady-state catalytic voltammograms are more positive than the redox potentials of the free quinones, indicating that the BzAm-reduced quinones are not quinols but the corresponding aminophenols, which are oxidized to iminoquinones. Iminoquinone forms of PQQ and TTQ_a exhibit lower catalytic activity compared with the quinone forms. The reaction rate of TTQ_a slows down with the time, while those of PQQ and TPQ_a are practically independent of the reaction time within a 15 min period. The diminished rate of TTQ_a is reasonably ascribed to slow hydrolysis of imino-TTQ_a to TTQ_a. Generation of the iminosemiquinone radical during the catalytic reactions is evidenced by EPR spectroscopic measurements. The significance of radical generation is discussed in the light of autoxidation of the aminophenols. All these results are interpreted well by a transamination mechanism. In addition, an asymmetric *ortho*-quinone structure is proposed to be important in catalytic amine oxidation activity, and the relatively low catalytic activity of free TPQ_a is ascribed to the contribution of a *para*-quinone-type electronic structure. On the basis of these results, a role for Cu^{II} is proposed as a transient intramolecular electron acceptor in copper amine oxidases to enhance the reactivity toward dioxygen through the generation of the imino-TPQ semiquinone and as a weak ligand in TPQ-containing amine oxidases making the TPQ cofactor bear an *ortho*-quinone-type electronic structure.

In the past decade and a half, three quinonoid redox cofactors, pyrroloquinoline quinone (PQQ),[1] topa quinone (TPQ)[2] and tryptophan tryptophylquinone (TTQ)[3] have been discovered in several oxidoreductases. TPQ-containing enzymes such as bovine plasma amine oxidase and TTQ-containing methylamine dehydrogenase are involved in oxidative deamination of amines. Recently, a new quinonoid cofactor, lysine tyrosylquinone (LTQ), has also been discovered in lysyl oxidase.[4] It is also well known that free PQQ works as a catalyst of amine oxidation, although there exists no evidence for amine oxidation activity in PQQ enzymes.[5] Historically, those amine-oxidizing quinoenzymes were believed to contain PQQ or PQQ derivatives as organic cofactors by the late 1980s[5] and extensive studies were carried out on the mechanism of the oxidation of amines by free PQQ and its analogues.[6,7] After the discovery of TPQ and TTQ, several analogues of TPQ[8,9] and TTQ[10] were synthesized and model reactions of amine oxidases–dehydrogenases were examined for analogues of TPQ[8,9,11] and TTQ.[10]

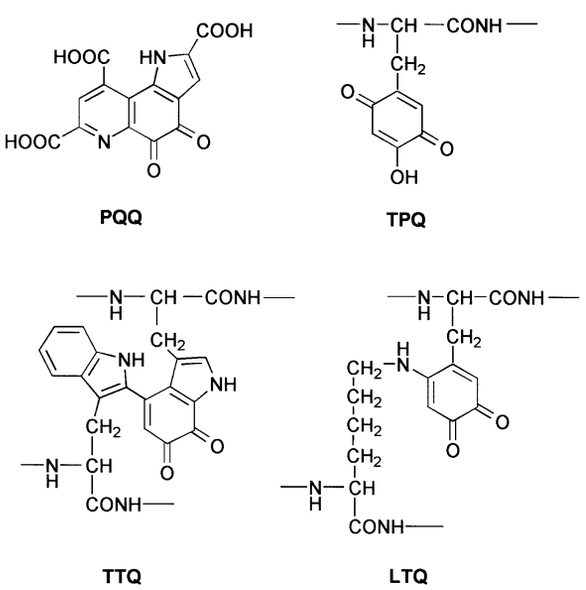
Our current understanding of the ionic mechanisms proposed so far for quinone-catalysed aerobic deamination is outlined in Scheme 1. The mechanisms involve a carbinolamine intermediate. The transamination mechanism[6–9,12–15] is the most popular and involves a tautomerization between a substrate Schiff base and a product Schiff base, the latter being hydrolysed to yield an aldehyde product and an aminophenol. The aminophenol is autoxidized to an iminoquinone, which is in turn hydrolysed to the quinone and ammonia. The alternative is an imine elimination mechanism proposed first for PQQ,[6] in which the carbinolamine is directly converted to the quinol and an aldimine without generating aminophenol intermediate. The quinol is autoxidized to regenerate the quinone, while the aldimine is hydrolysed to give aldehyde and ammonia as the products. It was difficult to distinguish between the two mechanisms by product analysis under anaerobic single turnover conditions because of the intermolecular electron exchange between (imino)quinone type species and their fully reduced species.[9b]

Although semiquinone and iminosemiquinone radicals do not explicitly appear in Scheme 1, several EPR spectroscopic studies have provided evidence of the generation of (imino)-semiquinone radical intermediates during the catalytic reaction of TPQ-dependent amine oxidases[11a,16–18] and TTQ-dependent methylamine dehydrogenases.[15,19,20] Radical generation might be explained by considering the intermolecular electron exchange between the oxidized and reduced (imino)quinone species,[9b] but as an alternative a sequential pathway involving iminosemiquinone as a catalytic intermediate is suggested.[8d,11a,15–17,19] Radical generation was also discussed based on the imine elimination mechanism.[20]

Non-blue copper amine oxidases (involving lysyl oxidase) contain neither PQQ nor TTQ but TPQ or LTQ as the organic cofactor. However, there is no clear evidence concerning specific chemical reactivity of reduced TPQ (or LTD) toward dioxygen compared with the other quinonoid cofactors in model reactions. Rather, it was reported that the autoxidation process of substrate-reduced TPQ analogues was the rate-determining step of the catalytic amine oxidation in acetonitrile,[8a] which might lead to an earlier proposal that Cu^{II} ion near the TPQ cofactor accelerates the oxidative regeneration of the substrate-reduced cofactor.[21] However, the clear role of Cu^{II} in the



Scheme 1 Transamination and imine-elimination mechanisms proposed for quinone-catalysed aerobic oxidation of BzAm



catalytic cycle remains to be elucidated. In model reactions, the catalytic activity of TPQ analogues was inhibited by addition of Cu^{II},[9b] while a TPQ analogue complexed with Cu^{II}–bipyridine was reported to exhibit enhanced catalytic activity.[11b] The role of Cu^{II} as a Lewis acid was also proposed.[22] In order to clarify the function of Cu^{II}, it is important to take into account the finding of a Cu^{I}–TPQ semiquinone state in the substrate-reduced amine oxidases.[16] The redox state is generated by the intramolecular electron transfer from the fully reduced TPQ to Cu^{II} and is in equilibrium with the Cu^{II}–TPQ quinol state.[16]

It is well known that 2-hydroxy-1,4-benzoquinones as free TPQ analogues are deprotonated above pH 5 and that the anionic form has an electronic structure between *ortho*- and *para*-quinones,[11d] though the active form has usually been depicted as a *para*-quinone. The structure seems to be in marked contrast to PQQ, TTQ and LTQ with *ortho*-quinone structures. This point has also been questioned.[8,11d]

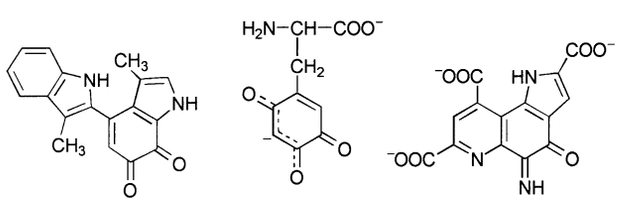
Most studies on model reactions in amine oxidation have been directed to one of the quinonoid cofactors. In order to gain further insight into the above questions and to understand the general function of the quinonoid cofactors in amine oxidation and also Cu^{II} in amine oxidases, it might be useful to compare the chemical function of the cofactor analogues. This article describes studies on the catalytic function of several quinonoid compounds under aerobic and anaerobic conditions using high-performance liquid chromatographic (HPLC) and electrochemical techniques. Kinetic results were explained well by the transamination mechanism. EPR spectroscopy was also applied to detect radical species during model catalytic reactions, which revealed the generation of the iminosemiquinone intermediate. The iminosemiquinone generation, which can be ascribed to the comproportionation reaction between the aminophenol and iminoquinone in model reactions, is explained as an essential process in autoxidation of the reduced quinones. In addition, the significance of the asymmetric *ortho*-quinone structure in the catalytic activity is proposed. On the basis of the present study, we will discuss roles of Cu^{II} in copper amine oxidases as a tentative intramolecular electron acceptor in amine oxidases to facilitate the reoxidation with dioxygen

and also as a weak ligand to make TPQ bear an *ortho*-quinone type electronic structure in TPQ-containing amine oxidases.

Experimental

PQQ was used as received from Sigma and was dissolved in distilled water at *ca.* 10^{-3} M by sonication and used as a stock solution. 3-(2,4,5-Trihydroxyphenyl)alanine (6-hydroxydopa, topa) from Aldrich was used as a reduced analogue of TPQ (TPQ_a). Stock solutions of the reduced TPQ_a were prepared just before use with 0.1 M HCl to avoid autoxidation and succeeding degradation. The reduced TPQ_a is quickly autoxidized under slightly alkaline conditions and a specific oxidizing agent is not required to convert it to TPQ_a in our experiments. 3-Methyl-4-(3′-methylindol-2′-yl)indole-6,7-dione as a TTQ analogue (TTQ_a) was donated from Professor S. Itoh. Dimethyl sulfoxide (DMSO) solutions of TTQ_a were used as stock solutions. Benzylamine (BzAm) was the substrate. Other chemicals are of reagent grade quality. Buffer solutions used were 0.1 M phosphate buffer (pH 6–9, 12–13) and 0.1 M carbonate buffer (pH 9–11), of which the ionic strength was adjusted to 0.5 M with KCl. For the generation of iminoquinone, 0.1–1.0 M ammoniacal buffers (pH 9.5) were used.

Aerobic oxidation of BzAm catalysed by quinones was carried out at 25 °C. The reaction was started by addition of an aliquot of quinone stock solution into 0.5 ml of a buffer solution containing a given amount of BzAm (0.025–0.5 M). The reaction solutions were opened to the atmosphere under stirring. 20 μl portions of the reaction solution were periodically subjected to HPLC analysis to determine benzaldehyde (BzAl) by the absolute calibration method. The HPLC experiments were performed on an HPLC system consisting of a Shimadzu LC-10AD chromatograph pump, a Shimadzu DGU-4A degasser, a Rheodyne 7125 sample injector, a Shimadzu SPD-10AD UV–VIS spectrophotometer and a Shimadzu C-R7A ChromatoPac data processor. A Develosil C_{18}-A-5 column (150 × 4 mm, Nomura Chemicals) was used for the HPLC separation at room temp. with a mobile phase consisting of methanol and 0.61 M phosphoric acid (7:3, v/v) at a flow rate of 1.0 ml min^{-1}. Under these conditions, BzAl was detected at a retention time of 2.7 min at a detection wavelength of 250 nm.



TTQ_a TPQ_a imino-PQQ

Electrochemical experiments were carried out on a BAS 100B electrochemical analyser with a three-electrode system consisting of a glassy carbon (GC) working electrode with an inner diameter $\Phi = 3.0$ mm (BAS), a platinum plate auxiliary electrode and an Ag/AgCl/saturated KCl reference electrode. The GC electrode was polished with 0.05 μm alumina powder, sonicated to remove it and washed with water. An aliquot of quinone stock solution was injected into 1.0 ml of a buffer solution. In experiments using TTQ_a, DMSO was added to the buffer solution to 20% (v/v), the pH of which was measured with a conventional pH meter without correction. All experiments were carried out at 25 °C under a moist nitrogen or argon atmosphere. Steady-state kinetics of anaerobic quinone-catalysed oxidation of BzAm were followed by cyclic voltammetry or constant potential amperometry. The diffusion constants of quinones and the effective surface area of the electrode were determined by potential step chronoamperometry.

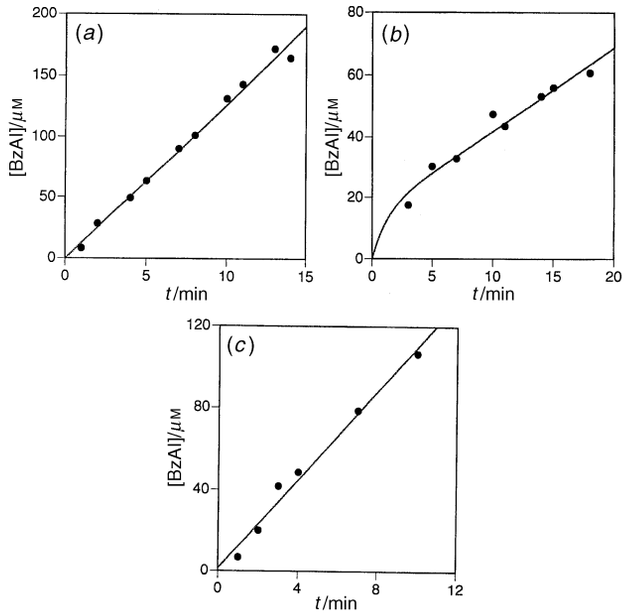


Fig. 1 Time course of the BzAl generation in aerobic oxidation of BzAm catalysed by (*a*) 37 μM of PQQ ([BzAm] = 0.12 M), (*b*) 18 μM of TTQ_a ([BzAm] = 0.12 M), and (*c*) 1.03 mM TPQ_a ([BzAm] = 0.106 M) at pH 9.5 (carbonate buffer). The solid line in (*b*) is generated from eqn. (9). See text for details.

EPR spectra were recorded on a JEOL JES-RE2X spectrophotometer or a Nikkiso ES-10A spectrophotometer with a quartz flat cell or glass capillary cell with $\Phi = 0.7$ mm. The magnitude of the modulation (100 kHz) was chosen to be as low as possible to optimize the resolution and the signal-to-noise ratio of observed spectra. The microwave power was set to 1–2 mW. UV–VIS spectra were recorded with a Shimadzu UV-2500(PC)S. Other details of electrochemical and spectroscopic experiments were described in previous papers.[10b,11d,23] Non-linear least-squares analysis was carried out using a Delta Graph Pro 3.5 program (Delta Point).

Results and discussion

PQQ-catalysed aerobic oxidation of BzAm

The catalytic function of PQQ in the aerobic oxidation of BzAm was examined by using HPLC. The reaction was followed at relatively short intervals (1–20 min) to minimize possible effects of side reaction(s) such as oxazole formation and oxidative degradation. The HPLC analysis of the reaction solution indicated the generation of BzAl. The concentration of the generated BzAl ([BzAl]) evaluated from the HPLC peak height increased linearly with the reaction time (t) and exceeded the total PQQ concentration ($[PQQ]_t$) after certain periods [Fig. 1(*a*)]. The steady-state rates evaluated from the slope of the linear plots were first-order both in the initial BzAm concentration ($[BzAm]_t$, 0.025 to 0.2 M) and in $[PQQ]_t$ (10–50 μM). The steady-state second-order rate constant ($k_{2,Q}$) was calculated to be 3.6×10^{-2} dm^3 mol^{-1} s^{-1} at pH 9.5. Such second-order reaction characteristics were observed even in single turnover oxidation of BzAm with PQQ under anaerobic conditions in the presence of cetyltrimethylammonium bromide (CTAB) micelle.[24]

Values of $k_{2,Q}$ showed a bell-type pH dependence centred around pH 10, as depicted in Fig. 2(*a*). The decrease in $k_{2,Q}$ on the less basic side (pH < 9) is reasonably ascribed to a decrease in the mole fraction of the free base form of BzAm as an active nucleophilic substrate due to the protonation ($pK_{a,BzAm} = 9.35$,[25] $K_{a,BzAm}$: the acid dissociation constant of benzylammonium ion). On the other hand, the decrease in $k_{2,Q}$ on the more basic side (pH > 10) is attributable to a decrease in the mole fraction of the catalytically active PQQ as a true catalyst due to the acid

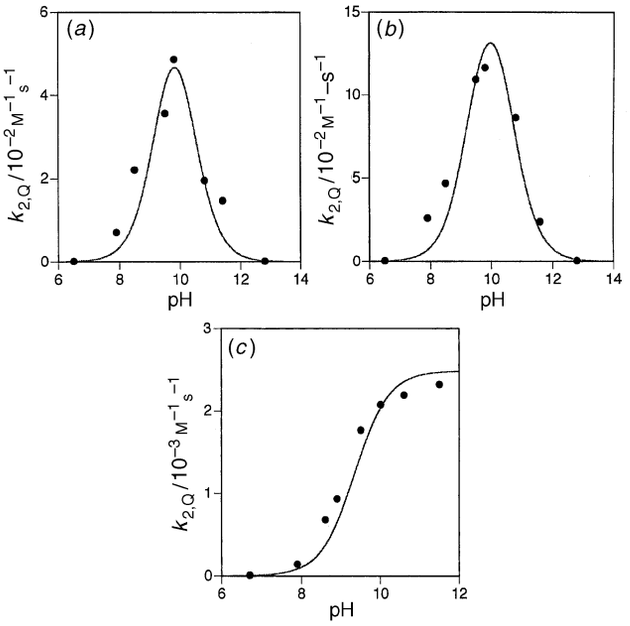


Fig. 2 Second order rate constants between quinones and BzAm ($k_{2,Q}$) as a function of pH. (*a*) PQQ, (*b*) TTQ_a, and (*c*) TPQ_a. Solid lines represent regression curves obtained based on eqn. (1), for which the parameters used are given in the text.

dissociation of the pyrrole NH moiety ($pK_{a,Q} = 10.3$,[23] $K_{a,Q}$: the acid dissociation constant of PQQ). The significance of the pyrrole NH moiety in the catalytic function of PQQ was demonstrated by the fact that the methylation of the pyrrole NH moiety caused a drastic decrease in the catalytic activity of PQQ,[26] though the dissociation of PQQ ($pK_{a,Q} = 10.3$) might be assigned to the hydration of the quinone moiety (pseudo-base formation).[7b] On the basis of these considerations, $k_{2,Q}$ can be expressed as a function of the proton activity (a_H) by eqn. (1),

$$k_{2,Q} = k^{\circ}_{2,Q} \frac{a_H K_{a,BzAm}}{(a_H + K_{a,Q})(a_H + K_{a,BzAm})} \qquad (1)$$

where $k^{\circ}_{2,Q}$ is the pH-independent second-order rate constant between the active PQQ and free BzAm. The value of $k^{\circ}_{2,Q}$ was optimized to be 8.3×10^{-2} dm^3 mol^{-1} s^{-1} by non-linear least squares analysis based on eqn. (1) using the above values of $K_{a,Q}$ and $K_{a,BzAm}$. The calculated curve depicted in Fig. 2(*a*) reproduces the experimental data. This fact supports the validity of eqn. (1). The optimized $k^{\circ}_{2,Q}$ value is comparable with the related values reported for anaerobic single turnover oxidation of BzAm by PQQ in the presence of CTAB (5.4×10^{-1} dm^3 mol^{-1} s^{-1} at 35 °C, pH 10.1 without correction for $K_{a,Q}$),[24] by 9-decarboxy-PQQ (*ca.* 2×10^{-2} dm^3 mol^{-1} s^{-1} at 25 °C)[7c] and by 7,9-didecarboxy-PQQ (*ca.* 7×10^{-2} dm^3 mol^{-1} s^{-1} at 30 °C).[7b]

PQQ-catalysed electrochemical oxidation of BzAm

The catalytic oxidation of BzAm with PQQ was also studied under anaerobic conditions using electrochemical techniques. Fig. 3(*a*) shows cyclic voltammograms of PQQ at a GC electrode. In the absence of BzAm, PQQ gave one pair of quasi-reversible waves with a midpoint potential (E_m) of −0.27 V (*vs.* Ag/AgCl) ascribed to an overall two-electron redox reaction of the quinone moiety. In this experiment, the initial potential was −0.55 V, where PQQ near the electrode surface is reduced to the quinol form as judged by the appearance of the reduction current on the anodic scan in the potential range more negative than E_m of PQQ (−0.55 to −0.35 V). In the presence of excess of BzAm, the voltammogram exhibited typical steady-state catalytic behaviour. Under the present conditions, the reduction current on the anodic scan in the potential range became negligibly small, indicating that most of PQQ in solution had been

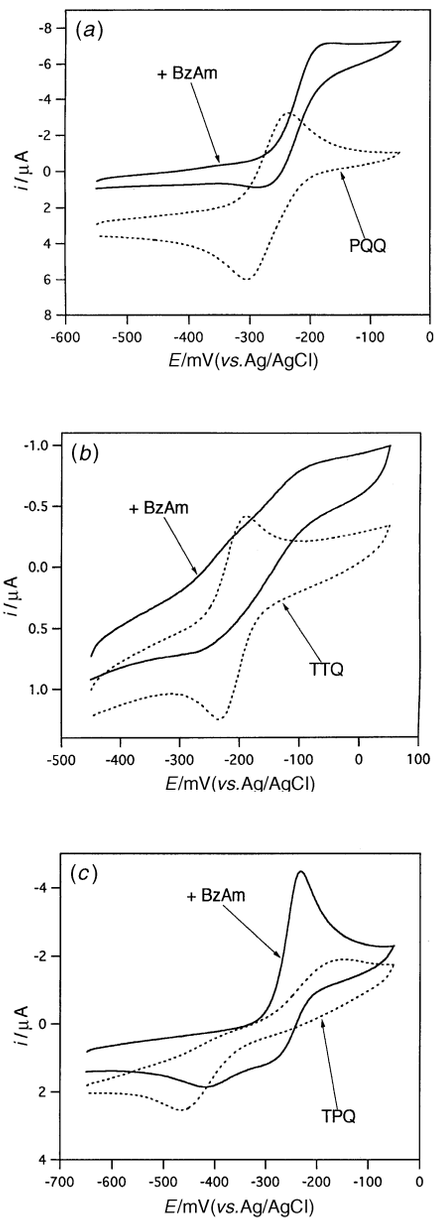


Fig. 3 Cyclic voltammograms of (*a*) PQQ (0.726 mM), (*b*) TTQ_a (6.72 μM) and (*c*) TPQ_a (0.235 mM) in the absence and presence (0.5 M) of BzAm at pH 9.5 (carbonate buffer) at a sweep rate of 5 mV s^{-1}. All voltammograms were recorded from the negative potential side.

reduced by BzAm before the voltammetric measurement. During the catalytic electrode process, BzAm is oxidized by the electrochemically generated PQQ yielding BzAl and the reduced form of PQQ (which is the aminophenol as described later). The BzAm-reduced PQQ is reoxidized at the electrode surface, that is, the final electron acceptor is the electrode in place of dioxygen in the anaerobic catalysis.

According to the theory of homogeneous electrocatalysis in the presence of excess amounts of substrates,[27] the limiting steady-state current (i_s) observed at sufficiently positive potentials, where the reoxidation of the BzAm-reduced PQQ is diffusion-controlled, allows direct measurement of the steady-state kinetics between the quinone and BzAm as given by eqn. (2), where n and D are, respectively, the number of the

$$i_s = nFA\sqrt{Dk_{2,Q}[\mathrm{BzAm}]_t}[\mathrm{PQQ}]_t \qquad (2)$$

electrons ($n = 2$ in this case) and the diffusion constant of PQQ, F and A being the Faraday constant and the electrode surface area, respectively. Experimental values of i_s satisfied well the

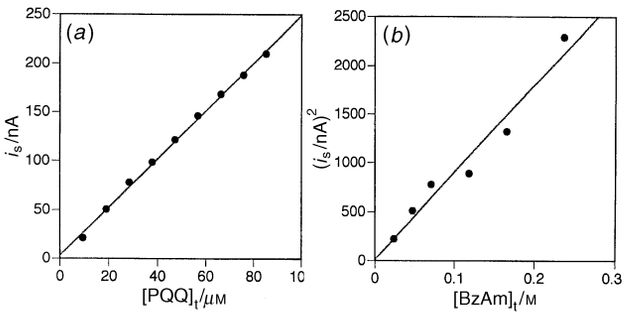


Fig. 4 Steady-state limiting catalytic current (i_s) of PQQ-catalysed oxidation of BzAm as functions of (*a*) $[PQQ]_t$ and (*b*) $[BzAm]_t$ at pH 9.5

$[PQQ]_t$ and $[BzAm]_t$ dependencies predicted by eqn. (2) as evidenced by Fig. 4. On evaluating the value of $A\sqrt{D}$ by independent potential step chronoamperometry of PQQ in the absence of BzAm,[28] $k_{2,Q}$ was calculated to be 3.7×10^{-2} dm^3 mol^{-1} s^{-1} at pH 9.5, which is very close to that obtained by HPLC in the aerobic catalytic reaction (see above).

The result implies that the overall steady-state catalytic rate is independent of the electron acceptors (dioxygen or electrode) and then that the reoxidation of the BzAm-reduced PQQ is not the rate-determining step in aerobic turnover catalysis. This consideration is also supported by kinetic studies concerning autoxidation of reduced quinones, in which the second-order rate constant between reduced PQQ and dioxygen (k_{ox}) was electrochemically evaluated to be 3.4×10^3 dm^3 mol^{-1} s^{-1} at pH 7 and it was found to increase by approximately one order of magnitude by increase in pH up to 9.5.[29] Therefore, it can be concluded that the aerobic reoxidation process is much faster than the overall catalytic turnover process, that is, $k_{ox}[O_2] \gg k_{2,Q}[BzAm]$ ($[O_2]$: dissolved dioxygen concentration) under the present experimental conditions ($[O_2] = 0.25$ mM).

Another interesting feature of the catalytic voltammogram is that the half-wave potential of the sigmoidal catalytic wave is *ca.* 40 mV more positive than E_m of free PQQ at pH 9.5 [Fig. 3(*a*)]. If the electrochemically oxidized species were the quinol, the half-wave potential would coincide with or be close to E_m of free PQQ.[30] Considering the fact that the redox potential of the imino-PQQ–aminophenol redox couple is more positive by 34 mV than that of the free PQQ–quinol couple at pH 9.2,[31] the electrochemically oxidized species, *i.e.* BzAm-reduced PQQ, can be reasonably assigned to the aminophenol form. This strongly supports the transamination mechanism: PQQ is reduced by BzAm into the aminophenol form, which is electrochemically oxidized to generate imino-PQQ at more positive potentials than that of the redox potential of PQQ. Imino-PQQ is quickly hydrolysed to PQQ in the absence or even in the presence of extremely low concentrations of catalytically generated ammonia.

Catalytic activity of imino-PQQ

In the above discussion, the catalytic activity of imino-PQQ was ignored. We then examined the catalytic activity of imino-PQQ under aerobic conditions in the presence of excess of BzAm. PQQ is reversibly converted into imino-PQQ in the presence of ammonia[31] and the rate of the conversion between them was too fast to be followed by conventional spectrophotometry. At 0.12 M total ammonia concentration and at pH 9.5, the generated BzAl concentration evaluated by HPLC increased linearly with the reaction time at least up to 15 min. However, the slope was diminished to one half that in the absence of ammonia. The apparent second-order rate constant ($k_{2,a}$) was 1.8×10^{-2} dm^3 mol^{-1} s^{-1}.

Assuming the (pseudo-)equilibrium between PQQ and imino-PQQ depending on the concentration of ammonia ($[NH_3]_t$) during the catalytic turnover, the concentration ratio of free imino-PQQ against free PQQ ([IPQQ]/[PQQ]) can be calculated to be 5.3 at $[NH_3]_t = 0.12$ M and pH 9.5 by eqn. (3),

$$\frac{[IPQQ]}{[PQQ]} = K_{IM}[NH_3]_t \frac{K_{a,A}}{K_{a,A} + a_H} \quad (3)$$

where K_{IM} is the constant of the imino-PQQ formation from PQQ and free ammonia (69 dm^3 mol^{-1} [31]) and $K_{a,A}$ is the acid dissociation constant of ammonium ion ($pK_{a,A} = 9.25$ [32]). On the other hand, the apparent catalytic rate constant in the presence of ammonia would be expressed by eqn. (4), where $k_{2,I}$ is

$$k_{2,a}[PQQ]_t = k_{2,Q}[PQQ] + k_{2,I}[IPQQ] \quad (4)$$

the catalytic activity of imino-PQQ. Substituting $k_{2,a}$, $k_{2,Q}$ and [IPQQ]/[PQQ] assessed above, $k_{2,I}$ can be calculated as 1.5×10^{-2} dm^3 mol^{-1} s^{-1}, which is *ca.* 40% of $k_{2,Q}$. This may imply that imino-PQQ itself also acts as a catalyst, during which imino-PQQ would be directly converted to the substrate Schiff base by transimination, as suggested first in TPQ model study.[9b] During catalytic turnover in the absence of ammonia, however, the effective catalyst is not imino-PQQ but free PQQ, because the amount of the generated ammonia is very small and then [IPQQ] is negligibly small as expected from eqn. (3).

Catalytic function of TTQ_a

TTQ_a also acts as a catalyst for the oxidation of BzAm.[10] In contrast with PQQ, slopes of [BzAl] *vs. t* plots monitored by HPLC decreased with *t*, as shown in Fig. 1(*b*). The second-order reaction rate constant between TTQ_a and BzAm ($k_{2,Q}$) evaluated from the initial slope was 1.1×10^{-1} dm^3 mol^{-1} s^{-1} at pH 9.5 and approximately three times larger than that of PQQ. The $k_{2,Q}$ values exhibited a bell-type pH dependence as in the case of PQQ [Fig. 2(*b*)]. The pH dependence was explained by eqn. (1) (modified for TTQ) using $pK_{a,Q} = 10.6$ (or 10.9) [10b] for TTQ_a as depicted by the solid curve in Fig. 2(*a*). The value of pH-independent $k^\circ_{2,Q}$ was optimized to be 2.0×10^{-1} dm^3 mol^{-1} s^{-1}. Interestingly, a similar bell-type pH dependence was reported in the catalytic activity of TPQ-containing methylamine dehydrogenase (*Paracoccus denitrificans*), although the optimum pH was *ca.* 7.5 and an apparent pK_a of methylamine dehydrogenase was estimated to be 8.2 by spectroscopic titration.[33] The difference in the optimum pH (or pK_a) between free TTQ_a and the methylamine dehydrogenase is not clear, but it might suggest that the active pocket of the enzyme provides more basic conditions compared with the bulk solution.

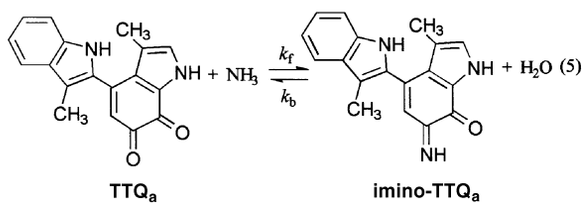
TTQ_a gave a steady state cyclic voltammogram in the presence of BzAm under anaerobic conditions, although a small hump appeared on the foot of the sigmoidal catalytic wave (*ca.* −0.23 V) as shown in Fig. 3(*b*). Analysis of the limiting catalytic current according to eqn. (2) (modified for TTQ) yielded a $k_{2,Q}$ value of 1.8×10^{-1} dm^3 mol^{-1} s^{-1}. This value is comparable with that evaluated using the HPLC method under aerobic conditions, although there might be some overestimation in the electrochemical evaluation because of the existence of unreacted TTQ_a in the bulk solution (see below).[34] The present result supports the fact that the aerobic reoxidation of the BzAm-reduced TTQ_a is not the rate-determining process, as concluded for PQQ.

In the voltammetric measurements, the total concentration of TTQ_a was one order of magnitude lower than that of PQQ in Fig. 3(*a*), because of the lower solubility of TTQ_a. This is the reason why the catalytic current was smaller than that in the case of PQQ in spite of the stronger catalytic activity of TTQ_a. Under the present conditions, some part of TTQ_a remained unreacted in the bulk solution, as evidenced by the reduction current on the anodic scan in the potential range more negative than E_m of TTQ_a (−0.45 to −0.3 V), during which the quinol form of TTQ_a is accumulated near the electrode surface. Thus,

the appearance of the small hump at *ca.* −0.23 V is reasonably ascribed to the direct oxidation of the electrochemically generated TTQ_a quinol and in turn the half-wave potential of the catalytic wave can be concluded to be more positive than E_m of free TTQ_a. This implies that the electrochemically oxidized species in the catalytic reaction is the aminophenol form of TTQ_a, on the basis of the fact that the redox potential of the imino-TTQ_a–aminophenol couple is more positive than that of the free TTQ_a–quinol couple.[10b] These arguments strongly support transamination as the mechanism, as in the case of PQQ.

The catalytic activity of imino-TTQ_a was also studied. TTQ_a can be reversibly converted into imino-TTQ_a with an iminoquinone formation constant: $K_{IM} = 24$ dm^3 mol^{-1}, but the forward and backward rate constants ($k_f = 2.8 \times 10^{-2}$ dm^3 mol^{-1} s^{-1} and $k_b = 1.2 \times 10^{-3}$ s^{-1}) are much smaller than those of PQQ.[10b]

The catalytic oxidation of BzAm in ammonia-containing buffer was then carried out after pre-incubation of TTQ_a for sufficient reaction periods (5–10 min) to attain equilibrium described by eqn. (5). The $k_{2,a}$ value was decreased down to



TTQ_a **imino-TTQ_a**

4.0×10^{-2} dm^3 mol^{-1} s^{-1} in the presence of 1.0 M of total ammonia at pH 9.5, where the free concentration ratio of imino-TTQ_a to TTQ_a is calculated to be 15.4 according to eqn. (3) (modified for TTQ_a). As a result, the second-order catalytic constant of imino-TTQ_a ($k_{2,I}$) is evaluated as 3.5×10^{-2} dm^3 mol^{-1} s^{-1} at pH 9.5. Such ammonia-dependent reduction of the catalytic activity might be in part responsible for the inhibition effects of (high concentrations of) ammonia (or ammonium ion) toward some methylamine dehydrogenases[35,36] and copper amine oxidases.[16c]

Turning our attention to the decreasing slope of the [BzAl] *vs.* *t* plot in the absence of ammonia [Fig. 1(*b*)], we propose that this arises from the slow hydrolysis rate of imino-TTQ_a to TTQ_a as well as the low catalytic activity of imino-TTQ_a compared with free TTQ_a. In order to verify our hypothesis, we attempted to interpret the curved characteristics of the [BzAl] *vs.* *t* plot from the kinetic point of view. On the basis of the transamination mechanism, it may be considered that free TTQ_a is converted to imino-TTQ_a with a pseudo-first-order rate constant of $k_{1,Q}$ ($= k_{2,Q}[\mathrm{BzAm}]_t$) during the catalytic reaction in the presence of an excess amount of BzAm under aerobic conditions, while free imino-TTQ_a is hydrolysed to regenerate TTQ_a with a rate constant k_b, eqn. (6).

$$TTQ_a \underset{k_b}{\overset{k_{1,Q}}{\rightleftharpoons}} \text{imino-}TTQ_a \qquad (6)$$

When we assume that the sum of the concentration of free TTQ_a and free imino-TTQ_a ($[TTQ_a]^* = [TTQ_a] + [ITTQ_a]$) is constant during the steady-state catalytic reaction, the time-dependent concentration of free TTQ_a ($[TTQ_a]$) in the absence of ammonia is given by eqn. (7), where $k = k_{1,Q} + k_b$. The

$$[TTQ_a] = \frac{k_b + k_{1,Q}\exp(-kt)}{k}[TTQ_a]^* \qquad (7)$$

observed catalytic rate (v_c) is expressed as the sum of the contributions from TTQ_a and imino-TTQ_a, eqn. (8), where $k_{1,I}$

$$v_c = k_{1,Q}[TTQ_a] + k_{1,I}[ITTQ_a] \qquad (8)$$

($= k_{2,I}[\mathrm{BzAm}]_t$) is the pseudo-first-order catalytic rate constant of imino-TTQ_a. Then the concentration of the generated BzAl ($[\mathrm{BzAl}] = \int v dt$) is expressed by eqn. (9).

$$[\mathrm{BzAl}] = \frac{k_{1,Q}[TTQ_a]^*}{k} \times \left[(k_b + k_{1,I})t + \frac{(k_{1,Q} - k_{1,I})}{k}\{1 - \exp(-kt)\}\right] \qquad (9)$$

The experimental data can be reproduced by eqn. (9) using $k = 8.5 \times 10^{-3}$ s^{-1}, $k_{1,Q}(k_b + k_{1,I})[TTQ_a]^* = 3.7 \times 10^{-10}$ mol dm^{-3} s^{-2} and $k_{1,Q}(k_{1,Q} - k_{1,I})[TTQ_a]^* = 1.2 \times 10^{-9}$ mol dm^{-3} s^{-2}, as indicated by the solid curve in Fig. 1(*b*). Based on the kinetic results described above, the corresponding constants are expected as follows: $k = 1.3 \times 10^{-2}$ s^{-1}, $k_{1,Q}(k_b + k_{1,I})[TTQ_a]^* = 5.9 \times 10^{-10}$ mol dm^{-3} s^{-2} and $k_{1,Q}(k_{1,Q} - k_{1,I})[TTQ_a]^* = 1.1 \times 10^{-9}$ mol dm^{-3} s^{-2}, assuming that $[TTQ_a]^*$ is equal to the total concentration of TTQ_a. Acceptable agreement between the two sets of the three constants supports our hypothesis. In the case of PQQ, k_b is sufficiently large compared with $k_{1,Q}$ and $k_{1,I}$, resulting in $[\mathrm{BzAl}] \cong k_{1,Q}[\mathrm{PPQ}]^* t$. This can explain the linear correlation between [BzAl] and *t* [Fig. 1(*a*)].

Catalytic function of TPQ_a

HPLC analysis of the reaction mixture of TPQ_a and BzAm under aerobic conditions revealed the catalytic activity of TPQ_a for the aerobic oxidation of BzAm. A plot of [BzAl] *vs.* *t* was almost linear [Fig. 1(*c*)], the slope of which yields the second-order rate constant ($k_{2,Q}$) of TPQ as 2.0×10^{-3} dm^3 mol^{-1} s^{-1} at pH 9.5. In contrast to PQQ and TTQ_a, TPQ_a exhibited a sigmoidal pH dependence as shown in Fig. 2(*c*). This is reasonably explained by the fact that TPQ_a does not undergo any acid dissociation in the pH range examined.[11d] The experimental data were reproduced by eqn. (1) under the condition of $K_{a,Q} \ll a_H$, giving a pH-independent $k^\circ_{2,Q}$ value of 2.5×10^{-3} dm^3 mol^{-1} s^{-1}, which is two orders of magnitude smaller than those of TTQ_a. TPQ_a is also considered to be converted to imino-TPQ_a in the presence of ammonia,[8a,11d] but imino-TPQ_a is very unstable. Therefore, we could not evaluate the catalytic activity of imino-TPQ_a. The linear [BzAl] *vs.* *t* relation is suggestive of immediate hydrolysis of imino-TPQ_a, as in the case of PQQ.

Since the catalytic activity of TPQ_a is very low compared with PQQ or TTQ_a, increased concentration of TPQ_a as well as BzAm was required to produce cyclic voltammograms with catalytic characteristics [Fig. 3(*c*)]. The voltammogram showed a steady-state limiting catalytic wave at more positive potentials than −0.15 V. Analysis of the limiting catalytic current obtained by constant potential amperometry at 0 V based on eqn. (2) (modified for TPQ_a) yielded a $k_{2,Q}$ value of 3.0×10^{-3} dm^3 mol^{-1} s^{-1}, which is comparable with that evaluated by the HPLC method under aerobic conditions. This implies also that the autoxidation process is not the rate-determining step. In addition to the catalytic wave, the voltammogram exhibited a large oxidation peak at *ca.* −0.25 V and a small unexpected reduction peak at *ca.* −0.4 V. These peaks are not assigned to TPQ_a, since the peak potentials are different from those of free TPQ_a as judged from Fig. 3(*c*). The heights of the unexpected anodic peak increased while the initial potential (−0.65 V) was applied before scanning and diminished in the succeeding second scan. Similar behaviour in the presence of BzAm was also observed for 3,5-di-*tert*-butyl-1,2-benzoquinone, which is a well-known catalyst for the BzAm oxidation.[37] Considering the relatively small $k_{2,Q}$, these unexpected peaks may be assigned to some reaction intermediate, most probably the substrate Schiff base with possible electroactivity due to its iminoquinone-type electronic structure. The substrate Schiff base as well as the product Schiff base can be generated in the bulk reaction between TPQ_a and BzAm before the potential scan. If the

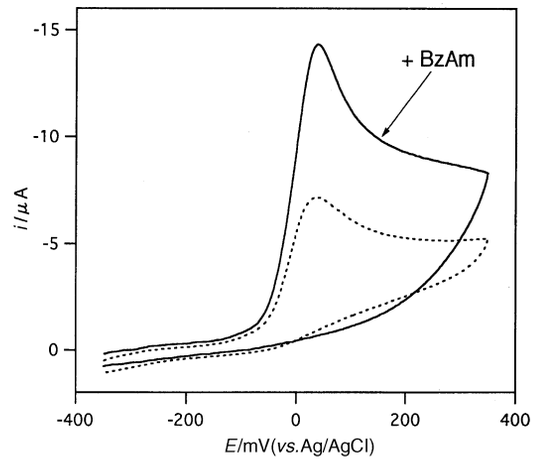


Fig. 5 Cyclic voltammograms of dopamine (0.60 mM) in the absence and presence (0.5 M) of BzAm at pH 9.5 (carbonate buffer) with a sweep rate of 5 mV s^{-1}

hydrolysis process of the product Schiff base is very slow, the substrate Schiff base near the electrode surface will be accumulated as its reduced form at the initial potential (−0.65 V). In the first anodic scan, the reduced substrate Schiff base will be reoxidized to generate the anodic peak. The concentration of the oxidized form of the substrate Schiff base will decrease due to the catalytic cycle at positive potentials. Then the reduction peak at *ca.* −0.4 V will become smaller than the oxidation peak at *ca.* −0.25 V. Further study is in progress. This consideration implies that the rate-determining step is the hydrolysis process of the product Schiff base. This is in accord with the conclusion presented for copper amine oxidase.[14]

Significance of *ortho*-quinone structure

Catalytically active quinonoid compounds reported so far involve PQQ and its analogues,[6,7] TTQ_a,[10] 3,5-di-*tert*-butyl-1,2-benzoquinone[37] and several TPQ analogues such as 2-hydroxy-1,4-benzoquinones including TPQ_a,[8,9,11] 4-*tert*-butyl-1,2-benzoquinone and 4-methoxy-5-*tert*-butyl-1,2-benzoquinone.[8b] All these quinonoid compounds as well as LTQ have the *ortho*-quinone-type electronic structure except 2-hydroxy-1,4-benzoquinones, of which the deprotonated form has an electronic structure between *ortho*- and *para*-quinones.[11d] In contrast, there exists no clear evidence to support such catalytic activity of *para*-quinones.[8]

Considering these properties, we propose the hypothesis that some *ortho*-quinone structure is important in catalysts in amine oxidation. Thus, we examined the catalytic activity of several quinones by cyclic voltammetry. Fig. 5 shows cyclic voltammograms of dopamine in the absence and presence of BzAm. Dopamine is electrochemically oxidized to dopamine quinone as an *ortho*-quinone. Although dopamine quinone is not so stable and labile to a succeeding chemical reaction and then dopamine gives an irreversible wave,[38] the anodic wave of dopamine increased on the addition of BzAm, suggesting that dopamine quinone can act as a catalyst in part in the electrochemical measurements, most probably competitively with the succeeding degradation. In contrast, however, cyclic voltammograms of benzene-1,2-diol and 9,10-phenanthrenequinone were not affected by the addition of BzAm, suggesting that symmetric *ortho*-quinones are catalytically inactive. Several *para*-quinones involving 2-methyl-1,4-naphthoquinone, 1,4-benzoquinone, 2,6-dimethyl-1,4-benzoquinone and 2-methyl-1,4-benzoquinone were also inert for BzAm oxidation. Therefore, we can propose that non-equivalent electronic characteristics of two carbonyl groups in *ortho*-quinones would be essential for amine oxidation catalysts. Low catalytic activity (*i.e.* low value of pH-independent catalytic constant $k^{\circ}_{2,Q}$) of TPQ_a compared with PQQ and TTQ_a is reasonably ascribed to the contribution of *para*-quinone-type electronic structure.

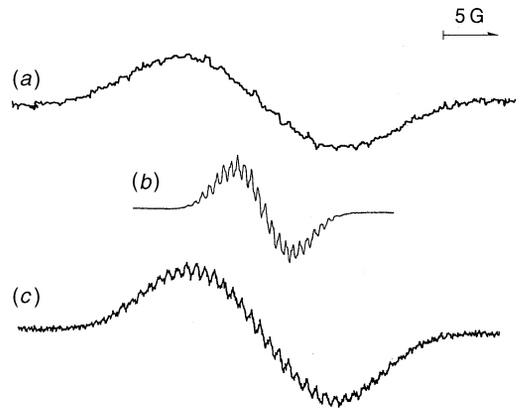


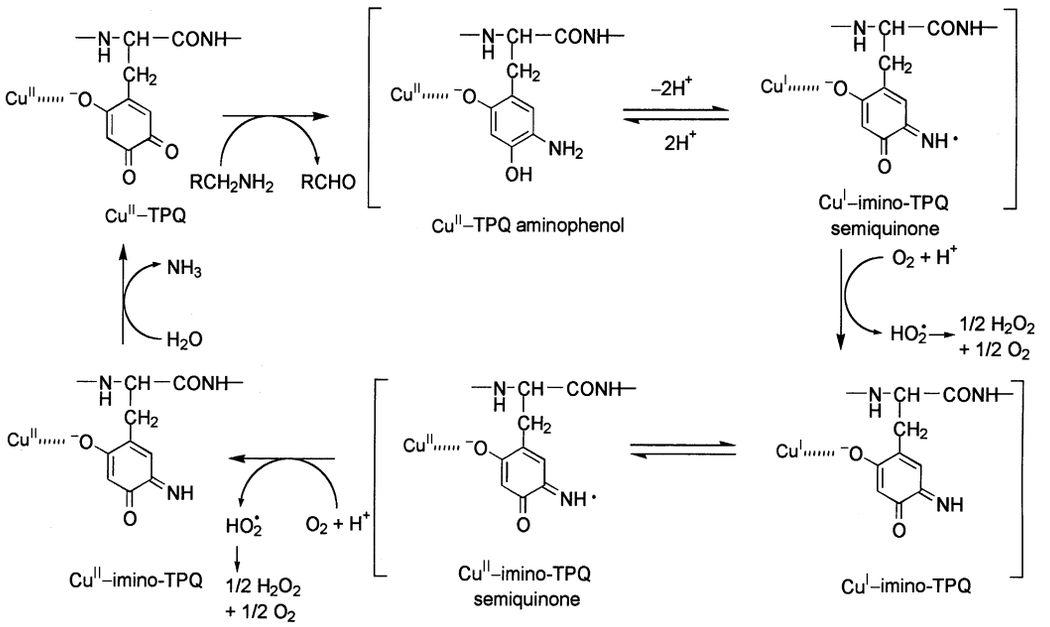
Fig. 6 EPR spectra of (*a*) TTQ_a in the presence of 0.5 M BzAm in pH 9.5 carbonate buffer containing 30% (v/v) DMSO under aerobic conditions (modulation 50 mG), (*b*) free TTQ_a semiquinone radical generated by half-reduction of TTQ_a with $NaBH_4$ in methanol under deaerated conditions (modulation: 40 mG), (*c*) imino-TTQ_a semiquinone radical generated by half-reduction of TTQ_a with $NaBH_4$ in 2.0 M ammoniacal buffer containing 30% (v/v) DMSO at pH 9.5 under deaerated conditions (modulation: 200 mG). 1 G = 0.1 mT.

EPR spectral measurements during the catalytic reaction

There is evidence for the generation of semiquinone and/or iminosemiquinone radical intermediate during the catalytic reaction of TTQ-dependent methylamine dehydrogenase[15,19,20] and TPQ-dependent amine oxidases.[16,17] Therefore, we attempted to detect and characterize the radical intermediate during the model reactions.

A radical intermediate during the TTQ_a-catalysed oxidation of BzAm was easily detected by EPR measurements, in which the reaction was carried out under aerobic conditions. Fig. 6 shows the observed EPR spectrum during the catalytic reaction as well as those of the TTQ_a and imino-TTQ_a semiquinone radicals. The total spectral width of the imino-TTQ_a radical is broadened compared with that of the TTQ_a radical due to the additional coupling caused by the NH group incorporated into imino-TTQ_a. The total spectral width of the radical species detected at a modulation width as low as possible during the catalytic reaction is almost equal to that of the imino-TTQ_a radical. Thus, the radical observed during the catalytic turnover is reasonably assigned to the imino-TTQ_a semiquinone radical generated by a one-electron oxidation of the aminophenol and/or by a one-electron reduction of imino-TTQ_a. Similar broadened spectra might be expected for a one-electron reduced radical of the substrate Schiff base generated by the reduction with the aminophenol or quinol.[9b] However, this process, if at all, does not seem to be the predominant one to produce the radical species during the catalytic reaction. Such broadened EPR spectra indicating the incorporation of the substrate nitrogen atom were observed for the BzAm oxidation catalysed by PQQ, TPQ_a and 3,5-di-*tert*-butyl-1,2-benzoquinone in this work and have already reported for other TPQ model compounds[11a] as well as methylamine dehydrogenases[15,19b] and amine oxidases.[17] Iminosemiquinone radical generation also supports the transamination mechanism.

Methylamine dehydrogenase donates two electrons of the substrate sequentially to the one-electron carrier protein, amicyanine. Then, the generation of the radical intermediate derived from the TTQ cofactor is reasonable.[15] On the other hand, in model reactions of aerobic BzAm oxidation, the radical can be simply interpreted to be generated *via* the comproportionation reaction between the aminophenol and the iminoquinone generated by autoxidation of aminophenol (or trace of quinone in the first catalytic turnover process). However, we suppose more positively that the semiquinone radical is essential in the catalytic process when the electron acceptor is dioxygen considering the fact that the autoxidation of quinones[29,39]



Scheme 2 Proposed mechanism of the reoxidation process of copper amine oxidases

as well as flavins[40] takes place *via* one-electron transfer from semiquinone radicals to dioxygen. This is also consistent with the fact that the fully reduced state of methylamine dehydrogenase (bacterium W3A1[41] and *Paracoccus denitrificans*[42]) is inert to dioxygen, while the half-reduced state is reoxidized by dioxygen, though dioxygen is not the native electron acceptor of the enzyme.

Proposed role of Cu^{II} in copper amine oxidases

In the model reactions, the radical intermediate essential for the autoxidation would be generated by the comproportionation reactions as described above. However, the rate of such comproportionation reactions would become very slow at extremely low concentrations of TPQ-(or LTQ-)containing amine oxidases and then we suppose that there exists another pathway to generate the radical intermediate in the amine oxidase reaction. It has been evidenced that the Cu^{II}–fully reduced TPQ (aminophenol) state in amine oxidases is in equilibrium with the Cu^{I}–imino-TPQ radical state[16] by the intramolecular electron transfer. This seems to be the alternative to the comproportionation reaction. That is, Cu^{II} can be considered to serve as a transient one-electron acceptor to generate the iminosemiquinone radical, which is quickly reoxidized by dioxygen to yield Cu^{I}–TPQ or Cu^{II}–TPQ iminosemiquinone; the latter will be further reoxidized by dioxygen. The proposed mechanism for the reoxidation process of the substrate reduced TPQ is illustrated in Scheme 2. This mechanism can well explain the recent evidence that Cu^{II}-lacking lentil amine oxidase apoprotein does not generate its semiquinone on reduction with substrates, leading to accumulation of the aminophenol form.[14] The second step of the reoxidation process can be considered as the reduction of perhydroxyl radical $HO_2^{\bullet}$ (or superoxide anion radical $O_2^{\bullet -}$) by Cu^{I}, in which the driving force is concerned with the sufficiently positive redox potential of $HO_2^{\bullet}$ (or $O_2^{\bullet -}$) as suggested by Klinman.[8d] The role of Cu^{II} in LTQ-containing lysyl oxidase might be also explained by a similar mechanism. Furthermore, the intramolecular electron transfer from the TPQ aminophenol to Cu^{II} in copper amine oxidases seems to resemble the intermolecular electron transfer from the reduced TTQ to amicyanine as a native one-electron acceptor in methylamine dehydrogenase.[15]

In order for the intramolecular electron transfer in TPQ-dependent amine oxidases to proceed, it would be essential for Cu^{II} to locate close to TPQ. This is evidenced by earlier distance mapping experiments:[43] Cu^{II} lies in the vicinity of the C^4–O moiety of the TPQ cofactor in the amine oxidases. Therefore, Cu^{II} might coordinate weakly with TPQ to stabilize deprotonated TPQ. Such coordination will make TPQ bear an *ortho*-quinone-like electronic structure, as depicted in Scheme 2. We suppose that this is also an important feature in TPQ-dependent amine oxidases to exhibit the catalytic amine oxidation activity of the TPQ cofactor.

Conclusion

The kinetic and voltammetric behaviour in quinone-catalysed BzAm oxidation presented here is well interpreted by the transamination mechanism. In such quinone-mediated amine oxidation, asymmetric *ortho*-quinone electronic structure seems to be significant, although some further study is required for a definitive conclusion. We have successfully explained the role of the iminosemiquinone radical as a reaction intermediate in the autoxidation. Based on these considerations, we have finally proposed the roles of Cu^{II} as a transient intramolecular electron acceptor in copper amine oxidases to accelerate the reoxidation with dioxygen through the generation of the imino-TPQ semiquinone and also as a weak ligand in TPQ-containing amine oxidases to make the TPQ cofactor bear an *ortho*-quinone-type electronic structure.

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