Intemediasi in the Catalytic Cycle of Copper-Quinoprotein Amine Oxidase from Escherichia coli

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Investigations on the reductior of copper quinopro-tein amine oxidas (EC 1.4.3.6) by substrate indicate that the nature of the reduced enzyme species formed varies, as judged from the spectroscopic data reported in the literature for different enzymes and substrates. The availability of substantial amounts of overpro-duced, homogeneous Esherichia coli amine oxidase (ECAO) enabled us to investigate this aspect with a number of different approaches: quantitative titration of en-zyme with substrate, stopped-flow kinetic spectropho-tometry (anaerobic and semianaerobic), EPR spec-troscopy of stable intermediates in the catalytic cycle, and conversions with $H_2O_2$ as the oxidant. Reduction of ECAO by a variety of substrates led to spectra (UV/Vis, EPR) identical to those that have been ascribed to the semiquinone form of the topaquino cofactor. The extent of semiquinone formation was enhanced in the presence of KCN, but the properties of the artificially induced semiquinone were different from those of the spontaneously induced one, as shown by the spectroscopic data and the reactivity toward $O_2$ and $H_2O_2$. On titrating ECAO at high concentrations with substrate, evidence was obtained that disproportionation takes place of the semiquinone formed, the reaction most probably proceeding via intermolecular electron transfer, leading to a topaquino- and Cu$^{2+}$-containing en-zyme species that is able to perform substrate conver-sion. The latter, as well as OH, is probably also formed when $H_2O_2$ replaces $O_2$ as oxidant, explaining why sub-strate conversion with concomitant enzyme inactiva-tion occurs under this condition. Formation of the semiquinone was always preceded by that of a hitherto unknown species with an absorbance maximum at 400 nm. The structure proposed for this species is a proto-inated form of the aminooquinol cofactor, the Zwitter ionic structure being stabilized by amino acid residues in the active site having opposite charges. Based on the properties observed and the moment of appearance during conversions, a proposal is made for the sequence in which the three reduced enzyme species convert into each other.

Copper-quinoprotein amine oxidases (EC 1.4.3.6) consist of two identical subunits, two copper ions and one or two mole-cules of the cofactor topaquino (TPQ).$^1$ The number of TPQs, as determined by titration with hydrazines, varies from en-zyme to enzyme (Mclntire and Hartman, 1993). As will be reported elsewhere,$^2$ the enzyme investigated here, Esherichia coli amine oxidase (ECAO), contains TPQ in a ratio of 1.0 per enzyme molecule. Distance calculations based on $^{19}$F NMR data of ECAO derivatized with fluorine-substituted phenylhydrazines$^3$ as well as resonance Raman spectroscopy (Moenne-Loccoz et al., 1995) have indicated that TPQ and Cu$^{2+}$ are rather far apart from each other in this enzyme and probably also in the other amine oxidases. EPR studies (to be presented elsewhere) have shown that ECAO has only one type of Cu$^{2+}$ with three nitrogen as ligands, similar to other amine oxidas.

The mechanism of action of copper-quinoprotein amine oxi-dases has recently been reviewed (Klinman and Mu, 1994; Knowles and Dooley, 1994). Briefly, the conversion occurs in the following way (see also the reactions given below, showing the reaction steps as well as the redox couples involved, the latter causing the balances not to fit). The amine group of the substrate adds to the C-5 carbonyl group of TPQ, after which the aldehyde is released from the enzyme, yielding the cofactor in its aminooquinol form (TPQH$_2$NH$_2$) (Reaction 1); as is clearly visible in some enzymes, the latter is able to transfer one electron to the Cu$^{2+}$ in a reversible way so that the semiqui-none form of this cofactor (TPQH$^\cdot$NH$^-$; Warnecke et al. (1994)) is formed and an equilibrium exists between TPQH$_2$NH$_2$/Cu$^{2+}$ and TPQH$^\cdot$NH/Cu$^{3+}$ (Reaction 2); it has been suggested that $O_2$ reacts with the latter couple, leading to cofactor oxidation and NH$_3$ plus $H_2O_2$ release (Reaction 3).

\[ \begin{align*}
RCH,NH_2 + TPQ/Cu^{2+} & \rightarrow RCHO + TPQH_2/NH_3/Cu^{2+} \\
TPQH_2/NH_3/Cu^{2+} & \rightarrow TPQH\cdot/NH/Cu^{3+} \\
TPQH\cdot/NH/Cu^{3+} + O_2 + H_2O & \rightarrow TPQ/Cu^{2+} + H_2O_2 + NH_3
\end{align*} \]

REACTION 1.
REACTION 2.
REACTION 3.

In amino acid conversions with pyridoxal phosphate-depend-ent enzymes, the occurrence of ketimine and aldime cofactor-substrate adducts as intermediates is well established. A number of arguments exist to postulate the occurrence of similar species in the reductive half-reaction of the TPQ-dependent reaction cycle. Reductive trapping experiments of enzyme in the presence of amines (Hartmann and Klinman, 1987, 1990) have provided evidence for the formation of a Schiff base ad-
aminoethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PEA, 2-phenylethylamine.

$^1$ The abbreviations used are: TPQ, topaquino (6-hydroxydopaquinone); ECAO, Escherichia coli amine oxidase; CHES, 2-cyclohexyl-

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duct. The fact that ammonia remains attached to the cofactor after aldehyde release (Ianes and Klinman, 1991) indicates that hydrolysis occurs of the tautomeric, product Schiff base adduct. However, so far stopped-flow spectrophotometry has not provided clear evidence for the postulated intermediates. On mixing bovine plasma amine oxidase with putrescine or p-dimethylaminomethylbenzylamine (Bellelli et al., 1991), only the semiquinone form of the cofactor was observed, although the conversion of cofactor-substrate adduct into aminooquinol cofactor plus (released) aldehyde was considered to be rate-limiting in the catalytic cycle. On mixing bovine plasma amine oxidase with benzylamines substituted with electron-donating substituents at the paraposition (Hartmann et al., 1993), spectra were observed having an absorption maximum between 400 and 500 nm. However, these were ascribed to a tautomeric (quinonoid) form of the product Schiff base, whereas it was proposed that the 340-nm intermediate observed with unsubstituted benzylamine belongs to the substrate Schiff base (Hartmann et al., 1993). These results suggest that conversion of the product Schiff base occurs so fast that it cannot be detected with common stopped-flow equipment. However, the fact that reductive trapping of the substrate Schiff base and exchange of C-2 hydrogens of the substrate with water hydrogens (Summers et al., 1979; Yu, 1988; Coleman et al., 1989, 1991) have been found with certain enzyme-substrate combinations indicates that substantial levels of the postulated intermediates should be present during these conversions. This prompted us to look for detectable intermediates in the reductive half-reaction by using adequate concentrations of ECAO with good and moderate substrates like phenylethylamine (PEA), tyramine, and tryptamine as well as with methylvamine, which is not a substrate (as judged from the fact that no O2 consumption is observed in the assay) but is able to induce the semiquinone form of the cofactor (Moëna-Llocoz et al., 1995).

It has been reported (Dooley et al., 1993) that on incubating enzyme and substrate anaerobically, the amount of Cu2+ formed exceeds that of TPQH-NH. An explanation could be that disproportionation occurs according to the following.

$$2(\text{TPQH}'\cdot\text{NH}/\text{Cu}^2+) \rightarrow \text{TPQ}/\text{Cu}^{2+} + \text{TPQH}_2\cdot\text{NH}_2/\text{Cu}^{2+}$$

**Reaction 4.**

and that the TPQ/Cu2+ couple formed in this is able to oxidize substrate. In amine oxidases containing 2 TPQs/enzyme molecule, this could occur via intermolecular electron transfer. However, since an ECAO molecule contains only one TPQ, if the proposed disproportionation also applies to this enzyme, this would imply the occurrence of intermolecular electron transfer. Therefore, anaerobic titrations were carried out of ECAO with substrate at conditions presumed to be favorable for disproportionation.

The addition of H2O2, one of the reaction products of the catalytic cycle, to amine oxidase incubated with substrate under anaerobic conditions, leads to hydroxyl radical (OH') production (Castellano et al., 1993). Since H2O2 could substitute for O2 or just oxidize one of the reduced cofactor forms, the effect of this compound under anaerobic as well as that of catalase under semiaerobic conditions was studied for ECAO.

Depending on the amine oxidase used, TPQH-NH/Cu2+ is observed or not when adding substrate to enzyme under anaerobic conditions. The formation of semiquinone can be (artificially) induced by adding KCN because cyanide is able to pull the equilibrium (Reaction 2) to the right hand side by binding to Cu2+ (Dooley et al., 1991). This trick is important as it enables spectroscopic studies of the semiquinone to be performed at low temperature (the equilibrium (Reaction 2) shifts to the left hand side on lowering the temperature). However, resonance Raman spectroscopy showed already that the properties of spontaneous and artificially induced semiquinone in ECAO are dissimilar (Moëna-Llocoz et al., 1995). This was further investigated by UV/Vis and EPR spectroscopy.

**EXPERIMENTAL PROCEDURES**

Materials—All reagents were of the highest purity commercially available and were used without further purification. All solutions were prepared with demineralized water, further deionized by passage through a Waters Milli-Q system to a resistance >17 MΩ cm.

ECAO was overproduced and purified as described.2 Enzyme activity was predominantly measured using a biological oxygen monitor with 0.1 mM PEA as the substrate in 0.1 M potassium phosphate, pH 7.0, containing 2 μg/ml catalase at 37 °C. It was assumed that the O2 concentration in this mixture (1.6 ml) had a value of 0.199 mM. Enzyme concentrations were determined spectrophotometrically using a value of 1.67. Specific activities were calculated by using an oxygen concentration of 0.199 mM in a 1.6-ml assay. The purified enzyme had a specific activity of 17.6 units/mg using 200 μM PEA and 0.199 mM O2 at 37 °C. 1 unit is defined as the amount of enzyme needed to oxidize 1 μmol of substrate/min.

UV/Vis Spectroscopy of Substrate-reduced Enzyme—A cuvette equipped with a rubber septum was filled with 500 μl of 50 mM MOPS, pH 7.5, and flushed with argon for 5 min. Subsequently, 10 μl, 2.69 μM ECAO was added with a syringe through the septum, and the solution was flushed again for 1 min with argon. To reduce the fully, 1 μl of 0.1 M substrate (PEA, tryptamine, or methylvamine) in water was added with a gas-tight syringe (the substrate stock solutions were bubbled with a stream of nitrogen gas before application). The solutions were stirred with a small magnetic bar, and a continuous stream of argon was led over them. Optical absorbance spectra were measured, prior to and after the addition of substrate, with a Hewlett-Packard 8452A photodiode array spectrophotometer.

In anaerobic titration experiments with PEA, 490 μl of ECAO (375.8 μM in 50 mM MOPS buffer, pH 7.5) was titrated with aliquots of 1 μl of PEA (25 mM). Spectra were taken 2 min after the addition had taken place.

The Effect of Additives and pH on Substrate-reduced Enzyme—Enzyme was fully reduced with substrate, as described above. Subsequently, additives (1 μl of 1 M KCN or 1 μl of 0.9 M H2O2) were added anaerobically with a gas-tight syringe to the cuvette.

The pH effect on PEA-reduced enzyme was measured by mixing ECAO (5.3 μM final concentration) with 50 mM MES (pH 5.0, 5.5, and 6.0), 50 mM MOPS (pH 6.5, 7.0, 7.5, and 8.0) or 50 mM CHES (pH 8.78, 9.0, 9.5, and 10.0). The solutions were made anaerobic before the PEA was added.

Rapid Scanning Stopped-flow Spectrophotometry—Anaerobic rapid scanning stopped-flow experiments were carried out at 10, 20, and 37 °C in 0.1 M potassium phosphate, pH 7.0. ECAO (79 μM) was mixed with equal volumes of PEA (395 μM). Anaerobiosis was obtained by flushing the enzyme and substrate solutions with argon, after which (final concentrations) 10 μg/ml glucose oxidase (2 units), 2.5 μg/ml catalase, and 4 mM glucose were added before the solutions were transferred to the syringes.

Semiaerobic rapid scanning stopped-flow experiments were carried out at 20 °C in the way described above except that the solutions did not contain glucose oxidase and glucose, and the concentrations of enzyme and substrate were somewhat different (67 μM ECAO, 656 μM PEA or tyramine). Furthermore, experiments were carried out in the absence and presence of catalase (33 μg/ml).

Data acquisition was performed with a Scientific PQ/FS-53 preparative quench/stopped-flow with an EG&G Princeton Applied Research 1034 stopped-flow photodiode array spectrophotometer (model 1461). Forty absorption spectra were recorded in each experiment with an automated subtraction of a reference spectrum. The reference spectrum was defined as the absorption of the buffer with substrate and, if applicable, with glucose oxidase, catalase, and glucose. Spectra were taken from 350.1 to 605.9 nm with a 2-nm resolution and with a 6–180-ms interval.

X-band EPR Spectroscopy—EPR spectra were obtained at room temperature on a Varian E9 EPR spectrometer operating at 9.23 GHz. Spectra were taken in a quartz flat cell (2 × 5 × 45 mm) with 378 μM ECAO in 50 mM potassium phosphate buffer, pH 7. Spectra were obtained with PEA (3.7 mM) and with combinations of KCN (6 mM) and PEA (3.7 mM), and PEA (18 mM) with H2O2 (16 mM). Spectra were obtained 6 min after the mixing of substrate and additives. All EPR experiments were performed under anaerobic conditions. Enzyme and substrate solutions were flushed with argon, and the flat cell was filled in a nitrogen atmosphere glove box and sealed with Teflon stoppers.
RESULTS

Anaerobic Reduction of ECAO with Excess Substrate—Fig. 1 shows the absorption spectrum of ECAO (in all panels indicated with 0), having a shape that is typical for the oxidized form of copper-quinoprotein amine oxidases. Ten seconds after adding excess PEA or tryptamine under anaerobic condition in a cuvette, a steady state was already obtained since the spectrum taken at that moment did not change further (Fig. 1, A and B). The spectra showed the disappearance of the broad 480-nm band, which has been attributed to TPQ in the oxidized enzyme (Mure and Klinman, 1993) (Scheme 1 A), and the appearance of twin peaks at 440 and 468 nm, as well as a shoulder at 360 nm, characteristics also found in other amine oxidases and ascribed (Dooley et al., 1991) to the semiquinone in the TPQH^·*NH/Cu^·1^1^ couple (Scheme 1 E). On performing a similar experiment with methylamine (Fig. 1C), only a slight decrease of the absorbances of the 480-nm band occurred, and those in the 300–420-nm region were raised (especially around 312 and 380 nm, as judged from a difference spectrum). In the next 2.5 min, only a steady decrease at 480 nm with a concomitant increase at 312 nm was observed. A steady state was obtained, lasting for 3.5 min, after which semiquinone formation took place, a process that was completed in 3 min.

Although slight differences exist between the final spectra resulting from enzyme treated with each substrate (additional absorbance above 500 nm in the case of methylamine and around 400 nm in the case of tryptamine), the spectra ascribed
to the semiquinone are similar in shape and height. This suggests that the semiquinone was identical and induced at the same level in all cases, although the time required for its formation was very long with methylamine. Admittance of O₂ immediately converted the spectra into that of oxidized enzyme.

Anaerobic Titration of ECAO with PEA—To avoid interference of remaining O₂ with the results of anaerobic titrations of ECAO with PEA, a very high concentration of enzyme was used (184.1 nmol of TPQ, 490 μl volume) in 50 mM MOPS, pH 7.5, and the cuvette was flushed for a long time with argon. During the addition of the first four aliquots of PEA (100 nmol, Fig. 2A), a decrease of the absorbance at 480 nm occurred (10% of the total absorbance, Fig. 2B, monitored at 500 nm to avoid contribution of semiquinone formation to the absorbance value as much as possible) with no increase in absorbance in the spectral region presented and no signs for semiquinone formation. Subsequently, a substantial decrease of A₅₀₀ₙₘ occurred with a concomitant increase of A₃₅₀ₙₘ. The titration was completed after the addition of 11 aliquots of substrate (275 nmol of PEA), as judged from the observation that further additions did not lead to spectral changes. Based on the amount of enzyme used and the fact that ECAO contains only 1.0 molecule of hydrazine-titratable TPQ/enzyme molecule, it appears that 1.5 molecules of PEA are converted per TPQ molecule.

The observation that a decrease of the A₅₀₀ₙₘ value occurred during the addition of approximately the first ½ of total PEA required (4 of the 11 aliquots) means that enzyme reduction took place and not consumption of remaining O₂. Using the absorbances in Figs. 1 and 2, it is estimated that only 34% of the amount of semiquinone expected (based on the experiments shown in Fig. 1) was finally formed in the anaerobic titration (note that the experiments in Fig. 1 were performed with a 70 times lower enzyme concentration).

Factors Affecting the Semiquinone Level—Absorption spectra of ECAO measured in buffers with a pH ranging from 5 to 10 were virtually the same. However, when excess PEA was added to these samples under anaerobic conditions, the final spectra observed differed substantially (Fig. 3A). At pH 5, significantly more absorbance was present around 400 nm than at pH 7.5, whereas at pH 10 a shapeless spectrum with low absorbances was obtained. Since the spectrum of the semiquinone as such is not affected by pH, as judged from the similar values for the maxima of the twin peaks, it seems that optimal semiquinone formation occurs at pH 7.5 (Fig. 3C, taking the contribution of the 400-nm species (see below) at 468 nm into account), a lower amount is present at pH 5, and virtually nothing is present at pH 10. Difference spectra (Fig. 3B) suggest the presence of a species with a maximum at 400 nm at pH 5, and one with a maximum around 310 nm at pH 10.

The presence of 0.1 M KCl had no effect on the shape or height of the semiquinone spectrum. However, when ECAO was mixed with PEA plus KCN (2 mM final concentration) the spectrum obtained (Fig. 4) showed a 36% increase in height and a blue shift of the maxima (440 → 436 nm; 468 → 462 nm), as compared with that of the semiquinone in the absence of KCN. The same spectrum was obtained when the semiquinone was first induced by PEA, after which KCN was added. The oxidative half-reaction appeared to be blocked because admittance of O₂ did not affect the KCN-generated semiquinone spectrum.

On adding H₂O₂ to PEA-reduced ECAO, the spectrum of the semiquinone disappeared, whereas a new band appeared at 400 nm in a time span of 8 min (Fig. 5A). In the next minute, the latter disappeared, and the spectrum of oxidized enzyme came up. Virtually the same results were obtained with tryptamine as the substrate (Fig. 5B), except that the absorbances in the 300–350-nm region were rather high (which could originate from the tryptaldehyde formed). On the other hand, addition of H₂O₂ caused the immediate disappearance of the methylamine-induced semiquinone with concomitant formation of the oxidized enzyme, without any signs of an intermediate 400-nm species. Fig. 5C shows the difference spectra of the final spectra obtained of conversions taking place in the presence of H₂O₂ minus the spectrum of oxidized enzyme. The spectra show a negative band at 480 nm in the case of tryptamine and PEA. The H₂O₂-treated enzyme was still active because subsequent addition of PEA or tryptamine immediately produced the 400-nm species (but hardly semiquinone), and after the conversion went to completion, the enzyme attained the oxidized state again. However, difference spectra taken after the second round in the way indicated above showed that even a further decrease of the 480-nm absorbance had taken place. Since the spectrum of oxidized enzyme was not affected by H₂O₂, the decrease observed is related to turnover of the enzyme.

Intermediates in the Catalytic Cycle of ECAO

Rapid Scanning, Stopped-flow Spectrophotometry—ECAO was anaerobically mixed with PEA at 10, 20, and 37 °C in a stopped-flow apparatus, and the reactions were monitored with a photodiode array spectrophotometer. Within the time span between mixing and scanning (about 6 ms), even at 10 °C
complete disappearance of the 480-nm band had occurred, the 400-nm intermediate had already attained its highest level, and semiquinone formation had already started. This is concluded from the differencespectra, obtained by subtracting the subsequentscans from the first one (Fig. 6A), showing that the 400-nm species disappears with concomitant formation of the semiquinone. From the time required for the reductive half-reaction to go to completion (1.2, 0.6, and 0.2 s at 10, 20, and 37 °C, respectively) and from the spectra taken from the final state (Fig. 6B), it appears that the velocity and level of semiquinone formation attained are highest at 37 °C (note that the reliability of the spectrophotometer decreases progressively below 400 nm so that it is unclear whether the 360-nm shoulder of the semiquinone, as observed in Fig. 1, is present or not).

In the presence of catalase, the time span for the complete conversion of tyramine under conditions in which no precautions were taken to prevent entrance of O₂ in the device, causing a semiaerobic condition, a similar sequence of reactions was observed but with some deviations due to the fact that also the oxidative half-reaction took place, leading to a complete conversion of the substrate added. Fig. 7A shows that just after the first 6 ms, some absorbance at 480 nm (about 20%) is still present, the spectrum of the 400-nm species is dominating, and no indications for the presence of the semiquinone species are observed. In the next phase (between 6 ms and 0.6 s, Fig. 7B), the 480-nm absorbance decreases and the level of the 400-nm species increases, again without any indication for the presence of the semiquinone species. After that (between 0.6 and 17 s, Fig. 7C), the level of the 400-nm species dropped and semiquinone formation took place. The phases in the conversion were also detected by inspecting the traces presented in Fig. 7D, upper part, being indicative (but not conclusive) for the species involved. They suggest that 1.5 s after mixing, virtually steady state levels of the species are attained and that after about 60 s, the substrate is nearly converted, as shown by the decrease in the A₄₀₄ nm and the increase of the A₄₆₈ nm, respectively. Difference spectra in the time span from 60 to about 100 s show that the concentrations of the 400-nm species and the semiquinone decrease, whereas that of oxidized enzyme (with a maximum ostensibly shifted to the right, due to the presence of semiquinone of which the concentration decreases concomitantly with the increase of the oxidized enzyme during this phase of the conversion) increases (Fig. 7E, upper part).

In the presence of catalase, the time span for the complete conversion of tyramine under the semiaerobic condition was
enormously reduced, although the absorbance traces suggested that the same sequence of reaction steps took place (Fig. 7D, lower part). As shown in Fig. 7E, lower part, in the final phase, between 2 and 3 s after mixing, the 400-nm species is directly converted into oxidized enzyme, and the semiquinone is not detected.

X-band EPR Spectroscopy—The final state obtained after mixing high concentrations of enzyme with excess substrate anaerobically in the absence and presence of the additives mentioned above was also monitored with X-band EPR at room temperature to detect TPQH·-NH and Cu$^{2+}$. Fig. 8A shows that Cu$^{2+}$ is present in all cases but that the amount was substantially lower when PEA was added and even more in the presence of KCN or H$_2$O$_2$ as additives (Table I). The signal of an organic free radical was present in PEA-reduced ECAO, of which the intensity increased when KCN was present (Table I). On the other hand, the signal was virtually absent in the presence of H$_2$O$_2$ (the small signal seen showed a distortion of the first hyperfine line and of the g perpendicular region). As shown in Fig. 8B for PEA-reduced enzyme, the characteristic
hyperfine structure ascribed to TPQH$^\cdot$NH (Dooley et al., 1990, 1991) is present, and a $g$ value was calculated of 2.004 ± 0.0005. The addition of KCN not only lowered the Cu$^{2+}$ content further and raised that of TPQH$^\cdot$NH (Table I), but it also broadened the hyperfine lines (Fig. 8B). Furthermore, the power saturation profiles of the PEA-induced semiquinone in the absence and presence of KCN (Fig. 8C) showed a significant difference of 44 mW at half-saturation.

**DISCUSSION**

On incubating ECAO anaerobically with the substrates PEA or tyramine or with the non-substrate methylamine for the appropriate time, a stable absorption spectrum was obtained in which the contribution of that of the semiquinone, TPQH$^\cdot$NH, was clearly visible. Further evidence for formation of the semiquinone was provided by the EPR spectra taken of PEA-reduced ECAO, showing its characteristic features. Thus,
ECAO is an enzyme for which the equilibrium constant of Reaction 2 has a value enabling detection of the semiquinone. On adding KCN to the mixture, the properties of the semiquinone were affected, as judged from the induction of a blueshift in the absorption maxima and the power saturation behavior in the EPR spectrometer. The ability of KCN to act as a promoter of semiquinone formation has been ascribed (Dooley et al., 1991) to the affinity of cyanide for Cu\(^{1+}\), shifting the equilibrium (Reaction 2) to the right hand side. Although the complexation of the reduced inorganic cofactor might affect the properties of the semiquinone form of the organic cofactor, in principle, cyanide being a nucleophile, the organic cofactor could also be a candidate for reacting with cyanide. A precedent already exists since the quinone cofactor pyrroloquinoline quinone forms an adduct with cyanide at the C-5 carbonyl position, even in the presence of an excess of water (Dekker et al., 1982). However, since the absorption maxima of the semiquinone already present shifted immediately upon addition of KCN and reaction with TPQH\(_2\)NH would require replacement of the NH\(_2\) group by cyanide, the latter possibility seems unlikely. It is tentatively concluded, therefore, that cyanide affects the properties of TPQH\(^{+}\)-NH by binding to Cu\(^{1+}\). Anyhow, in view of the use of KCN as an additive in experiments to induce sufficient amounts of semiquinone at the low temperature required for certain spectroscopic techniques (e.g. ESEEM studies (Warncke et al., 1994)), it should be realized in these cases that the presence of cyanide could affect the results. It has been suggested that the TPQH\(^{+}\)-NH/Cu\(^{1+}\) is the primary species that reacts with O\(_2\) in the oxidative half-reaction (Dooley et al., 1991). Apparently, the affinity of this couple for O\(_2\) is so high that its spectrum is not observed in aerobic conversions. However, with the semiaerobic conditions used in Fig. 7, it was detected (Fig. 7D, upper part; Fig. 7E, upper part) in the phase where the level of the 400-nm species and the semiquinone steadily decreased and that of the oxidized enzyme increased. In line with this, in the presence of catalase, invoking a higher O\(_2\) tension, the semiquinone is not observed in the final phase (Fig. 7E, lower part). Similarly, the semiquinone disappears immediately on adding H\(_2\)O\(_2\), but binding of cyanide to it prevents its conversion with H\(_2\)O\(_2\) and even with O\(_2\). All these observations confirm the idea that the TPQH\(^{+}\)-NH/Cu\(^{1+}\) couple plays a role in the oxidative half part of the cycle, but it cannot yet be decided whether it is the primary species with which O\(_2\) reacts.

The results described here clearly show the presence of a 400-nm species under certain circumstances. Since it was ob-
served in substrate-reduced enzyme preparations at anaerobic conditions, especially at pH 5, and enzyme-substrate or enzyme-product adducts will be absent when the equilibrium situation is reached; the 400-nm species must be a protonated form of the reduced enzyme for which a structure is proposed in Scheme 1 (species D, indicated with the formula TPQH₂·NH₃⁺).

To stabilize the Zwitter ionic structure, active site amino acid residues of opposite charge may be involved. From its proposed place in the chain of reactions, it is clear that semiquinone (species E) formation is always preceded by that of species D, as indicated by the stopped-flow results, and that species C (TPQH₂·NH₃), having a maximum around 310 nm, is present at high pH. Why has the 400-nm species not been observed in experiments with other amine oxidases? Certain amine oxidases, e.g. bovine plasma amine oxidase (Hartmann et al., 1990), do not show semiquinone formation upon reduction of enzyme under anaerobic conditions, whereas others show high levels of semiquinone under these conditions, e.g. lentil seedling amine oxidase (Bellelli et al., 1991). Thus it could be imagined that, depending on the type of amine oxidase, formation of one of the three species of reduced enzyme proposed is favored, in that view ECAO being an enzyme stabilizing species D. Furthermore, it should also be realized that conditions like pH, temperature, and enzyme concentration affect the equilibria, which can be decisive as to whether the 400-nm species is observed or not, as illustrated here by the effect of these variables on the level of semiquinone detected in substrate-reduced ECAO.

The titration experiments at high enzyme concentrations showed that far more substrate is consumed than expected, based on the assumption that only one TPO is involved in the conversion. It must be concluded, therefore, that Cu²⁺ also participates in the reaction, in line with what was found by EPR spectroscopy of PEA-reduced ECAO in another experiment conducted at lower enzyme concentration (Table I). Since direct reaction of substrate with Cu²⁺ seems unrealistic, the disproportionation of the semiquinone as proposed in Reaction 4, providing TPO/Cu¹⁺, which converts substrate, becomes realistic. Accepting this, some observations of enzyme at high concentration with substrate are understandable: in the first ½ of the titration experiment, the TPO content decreases but no semiquinone (or 400 nm species) is formed because the conditions for disproportionation are unfavorable in this phase of the conversion; the semiquinone content at the end of the titration is only 34% from that expected, as compared with the results of titrations with a much more diluted enzyme preparation. As a consequence of the disproportionation proposed, more than one equivalent aldehyde should be produced, and part of the NH₃ should be released under anaerobic conditions, disagreeing with the findings for bovine plasma amine oxidase (Janes and Klinman, 1993). However, in view of the absence of semiquinone in this enzyme upon substrate addition (Hartmann et al., 1993), it could be reasoned that disproportionation did not occur under the conditions used for product analysis. To substantiate the view presented here, we will carry out product analysis of ECAO under conditions optimal for disproportionation.

It has been reported (Castellano et al., 1993) that anaerobic addition of H₂O₂ to substrate-reduced amine oxidases leads to OH⁻ production. Most probably, this also happens with ECAO since destruction of the enzyme occurred, observed as a decrease of the 480-nm band with PEA and tryptamine, but not with the non-substrate methylamine (Fig. 5C). Formation of OH⁻ has been proposed (Castellano et al., 1993) to occur in the following reactions:

\[
\text{Cu}^{1+} + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{OH}^- + \text{OH}^-
\]

REACTION 5. Semiquinone + H₂O₂ → TPO + OH⁻ + H₂O

REACTION 6. However, based on arguments presented below, the following reaction could also be feasible.

\[
\text{TPQ}^{+} \cdot \text{NH/Cu}^{1+} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{TPQ/Cu}^{1+} + \text{NH}_3
\]

REACTION 7.

Since the semiquinone disappeared on H₂O₂ addition (but not the KCN-induced one), as observed with UV/Vis as well as EPR spectroscopy, but the 400-nm species did not, the semiquinone seems a good candidate for the reaction with H₂O₂, the latter perhaps mimicking O₂, as has been observed for several other oxidases. The reaction proposed is in line with the finding that substrate conversion takes place (via the TPO/Cu¹⁻ formed in the reaction) and that addition of H₂O₂ to PEA-reduced ECAO increased the level of Cu¹⁻ (Table I). However, in case TPO/Cu¹⁻ does not react with H₂O₂, the reaction with H₂O₂ should be dead-ended. Since slow conversion takes place, either H₂O₂ can act as electron acceptor or a small amount of O₂ generated from decomposition of H₂O₂ is responsible for that. The slow conversion occurring under semiaerobic conditions (Fig. 7D, top part) might be due to the H₂O₂ formed, since an enormous stimulation of the rate was observed in the presence of catalase (Fig. 7D, bottom part).

The presence of catalase should also prevent enzyme inactivation, in line with the observation made for pea seedling amine oxidase (Mann, 1955). Taken together, the results for ECAO suggest that H₂O₂ formation can be a disturbing factor for conversions at high substrate concentrations but not for initial rate measurements in assays carried out under normal aerobic conditions.

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REFERENCES

Bellelli, A., Finazzi Agro, A., Fioris, G., and Brunori, M. (1991) J. Biol. Chem. 266, 20654–20657

Castellano, F. N., Zuwen, H., and Greenaway, F. T. (1993) Biochim. Biophys. Acta 1157, 162–166

Ceman, A. A., Hindsgaul, O., and Palic, M. M. (1989) J. Biol. Chem. 264, 19500–19505

Ceman, A. A., Scaman, C. H., Kang, Y. J., and Palic, M. M. (1991) J. Biol. Chem. 266, 6795–6800

Dekker, R. H., Frank, J. N., Duine, J. A., Verwiel, P. E. J., and Westerling, J. (1982) Eur. J. Biochem. 125, 69–73

Dooley, D. M., McIntire, W. S., McGuir, M. A., Coté, C. E., and Bates, J. L. (1990) J. Am. Chem. Soc. 112, 2782–2789

Dooley, D. M., McGuir, M. A., Brown, D. E., Turowski, P. W., Mcintire, W. S., and Knowles, P. F. (1991) Nature 349, 262–264

Hartmann, C., and Klinman, J. P. (1987) J. Biol. Chem. 262, 962–965

Hartmann, C., and Klinman, J. P. (1990) FEBS Lett. 261, 431–444

Hartmann, C., and Klinman, J. P. (1991) Biochemistry 30, 4605–4611

Hartmann, C., Brzozw, P., and Klinman, J. P. (1993) Biochemistry 32, 2234–2241

Hartmann, C., M., and Klinman, J. P. (1993) Biochemistry 30, 4605–4605

Klinman, J. P., and Mu, D. (1994) Annu. Rev. Biochem. 63, 299–344

Knowles, P. F., and Dooley, D. M. (1994) Amine Oxidases in Metal Ions in Biological Systems, Vol. 30 (Seigel, H. ed) pp. 361–403, Marcel Dekker, New York

Mann, P. J. G. (1955) Biochem. J. 609–620

McIntire, W. S., and Hartmann, C. (1993) Principles and Applications of Quinoproteins (Davidson, V. L., ed) pp. 97–171, Dekker, New York

Mure, M., and Klinman, J. P. (1993) Biochemistry 32, 7117–7127

Principles and Applications of Quinoproteins, Philadelphia (Davidson, V. L., ed) pp. 97–171, Dekker, New York

Munoz, D., Ochoa, P., Nakamur, N., Steinbach, V., Duine, J. A., Mure, K., Klinman, J. P., and Sanders-Lohr, J. (1995) Biochemistry 34, 7020–7026

Mure, M., and Klinman, J. P. (1993) J. Am. Chem. Soc. 115, 7117–7127

Summers, M. C., Markovic, R., and Klinman, J. P. (1979) Biochemistry 18, 1969–1979

Warncke, K., Babcock, G. T., Dooley, D. M., McGuir, M. A., and McCracken, J. (1994) J. Am. Chem. Soc. 116, 4028–4037

Yu, P. H. (1988) Biochem. Cell Biol. 66, 853–861