Biochemistry

 Volume 36, Number 8 February 25, 1997

Accelerated Publications

Mechanistic Studies of Topa Quinone Biogenesis in Phenylethylamine Oxidase†

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Received November 21, 1996; Revised Manuscript Received January 10, 1997⊗

ABSTRACT: An alternative purification for apophenylethylamine oxidase from *Arthrobacter globiformis* has been developed, which avoids the use of possible contaminants that may interfere with the topa quinone (TPQ) self-processing reaction. The binding of Cu(II) and the kinetics of TPQ formation in these enzyme preparations have been reinvestigated. Our results show that Cu(II) is *not* significantly reduced when added to the apoprotein under anaerobic conditions. The Cu(II) EPR and circular dichroism spectra of the initially formed complex are different from the spectra of the mature Cu(II)/TPQ-containing protein, indicating that the active site structure must be altered during TPQ formation. The kinetics we observe are cleanly first-order in protein [measured subsequent to Cu(II) binding] when dioxygen is present in pseudo-first-order excess (k_{obs} = 1.5 min^{-1}). We found no rate dependence on copper, so long as one copper per subunit was present. This indicates that tyrosine oxidation to give TPQ depends only on the copper that is bound in the active site. These results differ from those originally reported; an alternative mechanism, which involves attack of an activated copper−oxygen species on a tyrosine radical intermediate, is proposed for TPQ formation.

Copper amine oxidases catalyze the oxidative deamination of primary amines by dioxygen in a two-step aminotransferase reaction to produce hydrogen peroxide, ammonia, and aldehydes (Klinman & Mu, 1994; Knowles & Dooley, 1994). The active site of these amine oxidases contains both a histidine/H_2O-ligated copper and topa quinone (TPQ),[1] a protein-derived organic cofactor (Klinman *et al*., 1991; Janes *et al*., 1990; Cooper *et al*., 1992; Brown *et al*., 1991). The TPQ cofactor binds and oxidizes substrate during the first step of the catalytic cycle, and copper appears to be involved in the reduction of molecular oxygen and reoxidation of TPQ during the second step (Dooley *et al*., 1991a; Warncke *et al*., 1994; Klinman & Mu, 1994; Knowles & Dooley, 1994). The precursor of TPQ was found to be a specific tyrosine residue in the highly conserved sequence Thr-X-X-Asn-Tyr-(TPQ)-Asp/Glu-Tyr (Mu *et al*., 1992; Janes *et al*., 1992; Matsuzaki *et al*., 1994). Recent crystal structure data from *Escherichia coli* (ECAO) and pea seedling (PSAO) amine oxidases show that the TPQ cofactor is in close proximity to the copper ion, but not ligated in the active enzyme (Parsons *et al*., 1995; Kumar *et al*., 1996).

Preliminary studies have shown that the generation of TPQ occurs in a post-translational, self-processing reaction that requires the addition of both Cu(II) and O_2 to the unprocessed apoenzyme (Cai & Klinman, 1994a; Matsuzaki *et al*., 1994).

† This work was supported by National Institutes of Health Grant GM27659 to D.M.D. and NRSA Fellowship GM17706 to C.E.R., and Grants-in-Aid for Scientific Research 08249224 and 08458196 from the Ministry of Education, Science, Sport, and Culture of Japan to K.T.

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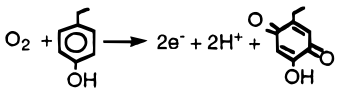
⊗ Abstract published in *Advance ACS Abstracts*, February 15, 1997.

[1] Abbreviations: TPQ, topa quinone or 6-hydroxydopa quinone; PEAO, phenylethylamine oxidase; DDC, *N*,*N*-diethyldithiocarbamic acid; PMSF, phenylmethanesulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; HRP, horseradish peroxidase; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid).

S0006-2960(96)02883-8 CCC: $14.00

The 6e$^-$ oxidation of the precursor Tyr to form TPQ formally requires the cleavage of the O−O bond of one molecule of dioxygen (eq 1) and the 2e$^-$ reduction of a second molecule of dioxygen to peroxide (eq 2). Hydrogen peroxide production during TPQ formation has not yet been confirmed. Recent isotope labeling experiments show that the C2 oxygen of TPQ is derived from solvent (Nakamura *et al*., 1996). Mutagenesis studies have shown that the copper-ligating histidine residues are required for TPQ formation, suggesting that coordinated copper is involved in the biogenesis reaction (Cai & Klinman, 1994b). Replacement of the flanking amino acids in the consensus sequence can significantly affect the rate of TPQ formation. For example, in histamine oxidase, replacement of the conserved Asn with Asp or Gln caused a 10^3-10^4-fold reduction in the rate of TPQ formation, replacement of the conserved Asp with Asn caused a 50-fold decrease, but replacement of the flanking Tyr did not change the rate of TPQ formation (Choi *et al*., 1996).



(1)

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2 \quad (2)$$

Previous studies indicated that the rate of tyrosine oxidation to TPQ was dependent on copper; the kinetics were consistent with two parallel first-order processes (Matsuzaki *et al*., 1994). When the apoprotein is treated with Cu(II) under anaerobic conditions, nearly all the copper appeared to be reduced to Cu(I); however, there was no spectroscopic evidence for the corresponding "reducing equivalent" that was needed to form the Cu(I) (Matsuzaki *et al*., 1995).

We have recently purified recombinant copper-deficient phenylethylamine oxidase (PEAO) from *Arthrobacter globiformis* using a procedure to avoid small molecule contaminants (dithiothreitol and ammonium sulfate) that may affect the TPQ formation reaction. Here we present spectroscopic and kinetics data on TPQ formation, which leads to the formulation of an alternative mechanism.

MATERIALS AND METHODS

Purification of Unprocessed Apoenzyme. To remove traces of metal ions, all glassware was washed with sulfuric acid and all plasticware soaked in 1 M EDTA. All solutions were made using water from a NANOpure system (Barnstead) with resistivity greater than 17 MΩ cm^{-1}, and were passed through a Chelex (Biorad) column. *E. coli* BL21-(DE3) cells transformed with pPEAO (Tanizawa *et al*., 1994) were grown and harvested according to the published procedure (Matsuzaki *et al*., 1994). ApoPEAO was purified by a modification of this procedure. Harvested cells were suspended in 50 mM potassium phosphate buffer (pH 6.8) containing 1 mM EDTA and 1 mM DDC (buffer A) with 0.1 mg/mL PMSF and 0.1 mg/mL TPCK, and were frozen at −80 °C. The cells were then thawed and disrupted by ultrasonic disintegration. The resulting lysate was centrifuged at 8000 rpm for 20 min. The supernatant was saved and the remaining cell pellet resuspended, disrupted by ultrasonic disintegration, and recentrifuged. The supernatants were combined, diluted with 5 mM potassium phosphate buffer (pH 6.8, with 1 mM EDTA, and 1 mM DDC) to lower the conductivity, and added to 500 mL of DEAE-Sepharose fast flow gel (Sigma) which had been preequilibrated with buffer A. This batch gel was loaded into a gravity column and washed with approximately 1000 mL of buffer A to remove unbound protein. Protein was eluted from the column by washing with 500 mM potassium phosphate buffer (pH 6.8, with 1 mM EDTA and 1 mM DDC) and collected in 30 mL fractions. Fractions with activity were combined and dialyzed overnight against 2 × 4 L of buffer A. Using a Biocad-Sprint Perfusion Chromatographic system (PerSeptive Biosystems), the enzyme solution was loaded onto a 52 mL (20 mm × 165 mm) POROS 50 DEAE column (PerSeptive Biosystems) which had been equilibrated with 50 mM potassium phosphate buffer (pH 6.8) and 1 mM EDTA (buffer B). Protein was eluted using a multistep gradient of 0 to 0.5 M KCl in buffer B at a flow rate of 16 mL/min. High-activity fractions were combined, concentrated using a YM30 ultrafiltration membrane (Amicon), and loaded onto a 8 mL monoQ (10 mm × 100 mm) HR anion exchange column (Pharmacia) which had been preequilibrated with 20 mM sodium acetate buffer (pH 5.2 with 1 mM EDTA). Protein was eluted at 3 mL/min using an approximately linear gradient of 0 to 0.5 KCl M in 20 mM sodium acetate buffer (pH 5.2 with 1 mM EDTA). Fractions of the highest activity were combined and dialyzed against 2 × 4 L of 50 mM HEPES (pH 6.8) overnight. Protein was stored in small aliquots at −80 °C until use. Typical yields from 20 g wet cells were 80−100 mg of apoprotein with a specific activity of 28−33 units/mg after incubation with copper, as described below.

Protein Assay. Phenylethylamine oxidase activity was measured by the production of H_2O_2 using a HRP-coupled assay (Szutowicz *et al*., 1984). Protein aliquots were incubated at 30 °C with 0.5 mM $CuSO_4$ for 30 min before assaying. Assays were run at 30 °C in 100 mM phosphate buffer (pH 7.0) with 22 purpurogalin units of HRP, 2.0 mM ABTS, and 0.1 mM phenylethylamine sulfate as substrate. Oxidization of ABTS was monitored at 414 nm. One unit of activity corresponds to an absorbance change of 24.6 ΔA/min. Protein concentrations were determined spectroscopically at 280 nm ($\epsilon^{1\%} = 12.3$ for apoprotein and 13.2 for Cu−TPQ-containing protein; Matsuzaki *et al*., 1994) or by a Bradford assay using a molecular mass of 70 600 g per subunit (Tanizawa *et al*., 1994).

Spectroscopic Studies. UV−visible spectra were obtained on a Hewlett-Packard diode array spectrometer (HP8452A) equipped with a circulating cell holder attached to a water bath. EPR spectra were run on a Bruker ER220D SRC instrument. CD spectra were run on a Jasco J-710 spectropolarimeter. For EPR and CD, solutions of apoprotein were purged with argon gas for approximately 2 h before use. An anaerobic $CuSO_4$ stock solution was subsequently added to the protein solution (just under 1 equiv of copper per subunit), and the mixture was allowed to stand for at least 1 h to ensure complete copper uptake. Anaerobic copper−enzyme solutions were either prepared directly in a spectrophotometer cell (CD and UV−vis) or transferred into a purged tube via a gas-tight syringe (EPR). EPR spectra were integrated against a 0.68 mM $CuSO_4$/2 M $NaClO_4$ standard and were simulated using the program JDLABS (provided by J. D. Lipscomb, Department of Biochemistry, University of Minnesota, Minneapolis, MN 55414.)

Kinetic Studies. For each kinetic run, protein solution was added to a 1 cm cuvette and made anaerobic by purging with argon for approximately 2 h. A stock solution of anaerobic

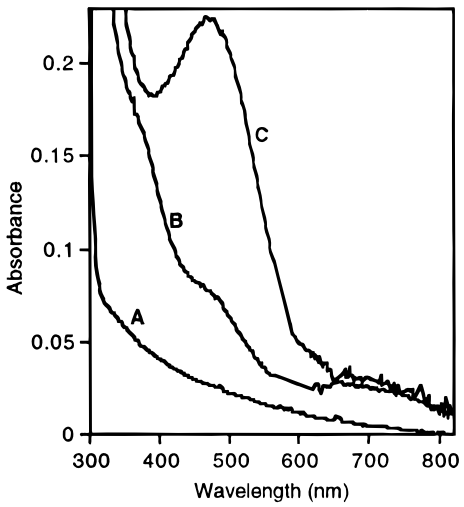


FIGURE 1: Absorption spectra of PEAO: (A) apo-PEAO (0.11 mM), (B) apo-PEAO (0.11 mM) anaerobically incubated with $CuSO_4$, and (C) apo-PEAO (0.11 mM) aerobically incubated with $CuSO_4$.

copper sulfate was then added to give a total volume of 700 μL with final concentrations of 0.1 mM enzyme and 0.1−0.6 mM Cu. The enzyme was allowed to incubate with copper for at least 30 min, and the reaction was started with the addition of a slow O_2 purge.[2] Spectra were collected every 5−6 s with vigorous shaking between each spectrum to keep the solution saturated with oxygen. The reaction was monitored for the formation of TPQ at 474 nm. End points were estimated using a Kedzy-Swinbourne analysis (Kezdy *et al*., 1958; Swinbourne, 1960), and data were fit to standard linear first-order kinetic equations; $\ln[(A_t - A_\infty)/(A_o - A_\infty)] = -k_{obs}t$, where A_t is the absorbance at 474 nm at time t, A_∞ is the end point absorbance calculated from the Kedzy−Swinbourne analysis, and A_o is the initial absorbance. Data sets that were used were linear to at least three half-lives.

RESULTS

Purification. Phenylethylamine oxidase was purified to homogeneity from *E. coli* BL21(DE3)/pPEAO2 cells (Tanizawa *et al*., 1994) in a procedure adapted from Matasuzaki *et al.* (1994). The use of high levels of ammonium sulfate was avoided because it has been shown to deactivate *E. coli* amine oxidase (Parsons *et al*., 1995). Dithiothreitol (DTT) was not used because it is a known metal reductant. Unless incubated with copper, the purified apoprotein had almost no activity (1 unit/mg) and had no detectable absorption peaks in the visible spectrum (Figure 1A), no observable EPR signal (Figure 2A), and no CD intensity in the visible wavelength region (Figure 3A).

Aerobic Incubation of PEAO with Cu(II). After aerobic incubation of the apoprotein with 0.5 mM copper for 30 min, the activity increased to 28−33 units/mg, showing that active enzyme could be formed. To generate fully oxidized, TPQ-containing protein for spectroscopic studies, apoprotein was incubated with excess copper under aerobic conditions and extensively dialyzed to remove any unbound copper. This

[2] Oxygen solubility at 640 mmHg (Bozeman pressure) at 30 °C is 0.2 mM (Weiss, 1970). Vigorous shaking of the reaction mixture under a pure oxygen atmosphere ensured that the concentration of oxygen remained constant throughout the reaction.

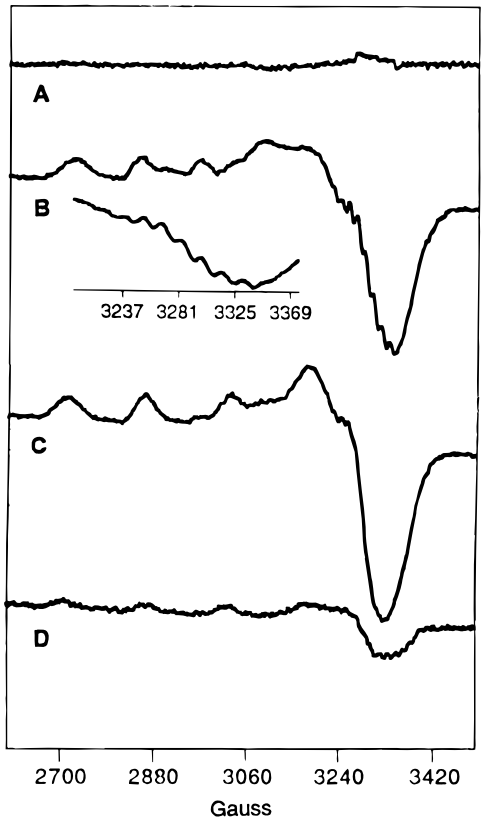


FIGURE 2: EPR spectra of PEAO. (A) Apo-PEAO (0.20 mM). (B) Apo-PEAO anaerobically incubated with 1 equiv of $CuSO_4$ (0.20 mM); $g_{||} = 2.32$, $A^{Cu}_{||} = 141$ G, $g_\perp = 2.07$, and $A_\perp = 14$ G. (Inset) Expanded view of $A_\perp$ hyperfine. (C) Apo-PEAO aerobically incubated with 1 equiv of $CuSO_4$ (0.20 mM); $g_{||} = 2.29$, $A^{Cu}_{||} = 175$ G, $g_\perp = 2.07$. Our spectrum is very similar to the previously published spectrum (Matsuzaki *et al*., 1995). (D) Apo-PEAO anaerobically incubated with 1 equiv of each $CuSO_4$ and DTT (0.20 mM). All spectra were run at 77 K, 9.4 GHz, and 5 mW, with 10 scans.

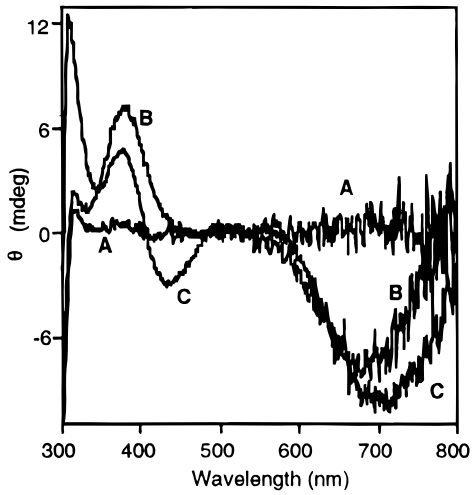


FIGURE 3: CD spectra of PEAO: (A) apo-PEAO (0.16 mM), (B) apo-PEAO (0.13 mM) anaerobically incubated with $CuSO_4$, and (C) apo-PEAO (0.22 mM) aerobically incubated with $CuSO_4$, scaled to the proportional concentration of that in panel B.

fully oxidized TPQ-containing enzyme is bright pink; it has a visible absorption maximum at 474 nm, which has been assigned to a TPQ transition (Figure 1C), and an axial Cu(II) EPR signal (Figure 2C). The negative CD band in the 700−800 nm wavelength region arises from a copper(II) d−d transition (Dooley *et al*., 1990), and the negative CD band

in the 400−500 nm region corresponds to the TPQ absorption feature (Figure 3C), as previously reported (Matsuzaki *et al*., 1995).

Anaerobic Incubation of PEAO with Cu(II). Incubation of the apoprotein with Cu(II) under strictly anaerobic conditions led to the formation of a light yellow species. TPQ is not yet formed as shown by the lack of a 474 nm absorption band (Figure 1B) and the lack of intensity in the CD spectrum in the 400−500 nm wavelength region (Figure 3B). The low-temperature EPR spectrum shows a typical copper(II) signal with a multiline superhyperfine pattern (Figure 2B); this signal is slightly different than that of the fully oxidized, active enzyme. Quantitation of this signal by double integration indicates that more than 90% of the copper is still oxidized under anaerobic conditions (based on double integration of an identically-prepared sample exposed to oxygen).[3] The CD spectrum exhibits negative intensity in the 700−800 nm region, which is assigned to a copper d−d transition (Figure 3B). This CD band is slightly blue-shifted compared to the peak in the CD spectrum of the fully oxidized TPQ-containing protein. This spectroscopic evidence shows that, when copper is added anaerobically to apoPEAO, the majority of it remains oxidized, not reduced, as previously reported (Matsuzaki *et al*., 1995).

To test if contaminating DTT could have caused the initial reduction to copper(I) in previous reports, we anaerobically incubated the apoenzyme with copper to allow the enzyme−copper complex to form, then added 1 equiv of DTT. Integration of the EPR signal of the DTT-treated enzyme−copper complex detected less than 15% of the added copper, demonstrating that DTT can reduce enzyme-bound copper (Figure 2D). When these samples were exposed to oxygen, the pink color of TPQ rapidly developed, showing that tyrosine oxidation occurs in this chemically reduced enzyme.

Kinetic Analysis of TPQ Formation. To simplify the kinetic analysis of the TPQ formation reaction, kinetic runs used enzyme that had been preincubated with various amounts of copper (0.1, 0.2, 0.4, and 0.6 mM) so that the initial rate of copper uptake and binding to the protein would not affect the subsequent kinetics. The formation of TPQ was monitored by the change in absorption at 474 nm (Figure 4); no other spectroscopically observable species was detected during the reaction. The rate law, $d[TPQ]/dt = k_{obs}[Cu][E-Tyr382]$, where $k_{obs} = k[O_2]$, was initially assumed to be appropriate under O_2-saturated conditions. The increase in the absorbance at 474 nm was fit to a first-order rate equation (Figure 5), yielding similar values of k_{obs} ($k_{obs} = 1.5 \pm 0.2$ min $^{-1}$), regardless of the amount of copper used (Table 1), indicating that the rate order in copper is zero. When 1 equiv of DTT was added to the reaction mixture, the rate of TPQ formation also cleanly followed first-order kinetics; however, the reaction was slower ($k_{obs} \approx 1$ min $^{-1}$).

DISCUSSION

When apoPEAO is treated with copper(II) in the presence of oxygen, the TPQ cofactor is rapidly formed, yielding the fully oxidized TPQ-containing enzyme, as shown by UV−vis, EPR, and CD spectroscopy. In contrast, when apoPEAO is treated with copper(II) under anaerobic conditions, spectroscopic data are consistent with the formation of a Cu(II)−enzyme complex. The CD spectrum of this complex has a negative band in the 700−800 nm wavelength region, typical of copper(II) d−d transitions in amine oxidases (Dooley *et al*., 1990). The EPR spectrum of this enzyme−copper complex also exhibits a signal that is typical of tetragonal copper(II) complexes. Double integration of the signal indicates that more than 90% of the copper is still oxidized (when integrated against a fully oxidized PEAO sample that had been prepared identically). The multiline EPR superhyperfine ($A_\perp = 14$ G) is typical of multiple nitrogen-donor ligands, most probably the three conserved histidine imidazole groups; we suggest that these residues are copper ligands in the unprocessed enzyme. The differences between the spectra of the anaerobic enzyme−copper complex and those of the fully oxidized TPQ-containing enzyme suggest that the electronic environment of the active site copper changes as TPQ forms. Exactly how the active site structures differ is not yet known; we plan to address this with further spectroscopic and crystallographic studies. It is, perhaps, this difference in the copper electronic/structural features that allows the copper to serve two roles: tyrosine hydroxylation/oxidation during TPQ biogenesis and TPQ reoxidation during amine oxidation.

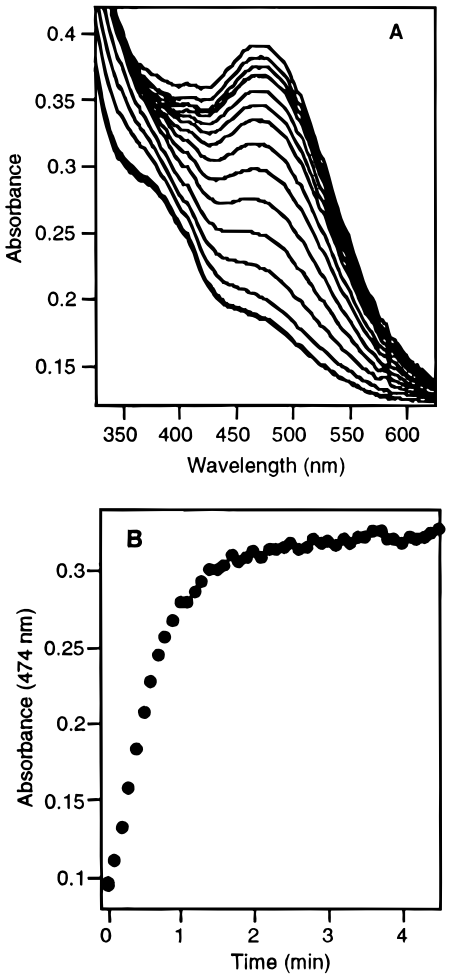


FIGURE 4: Absorption changes over time for the reaction of 0.1 mM enzyme subunit with 0.4 mM $CuSO_4$ in the presence of excess O_2: (A) full spectra (each 6 s apart) showing formation of the TPQ absorption band and (B) change of absorption at 474 nm over the time course of the reaction.

[3] Quantitation by double integration detected 70% of the added copper on the basis of the $CuSO_4/NaClO_4$ integration standard.

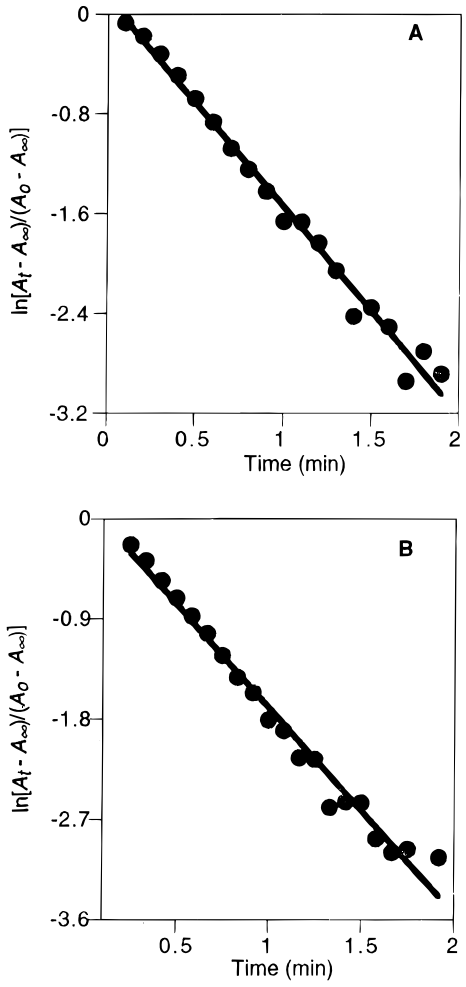


FIGURE 5: First-order kinetic fits for the reaction of 0.1 mM enzyme subunit with (A) 0.4 mM $CuSO_4$ (k_{obs} = 1.5 min^{-1}) and (B) and 0.6 mM $CuSO_4$ (k_{obs} = 1.7 min^{-1}).

Table 1: Calculated k_{obs} from Individual Kinetic Runs[a]

[Cu] (mM)	k_{obs} (min^{-1})
0.1	1.5
0.1	1.6
0.2	1.5
0.2	1.6
0.4	1.1
0.4	1.5
0.6	1.5
0.6	1.7
average	1.5
SD	0.2

[a]All runs were done at 30 °C and with 0.1 mM enzyme under a pure O_2 atmosphere.

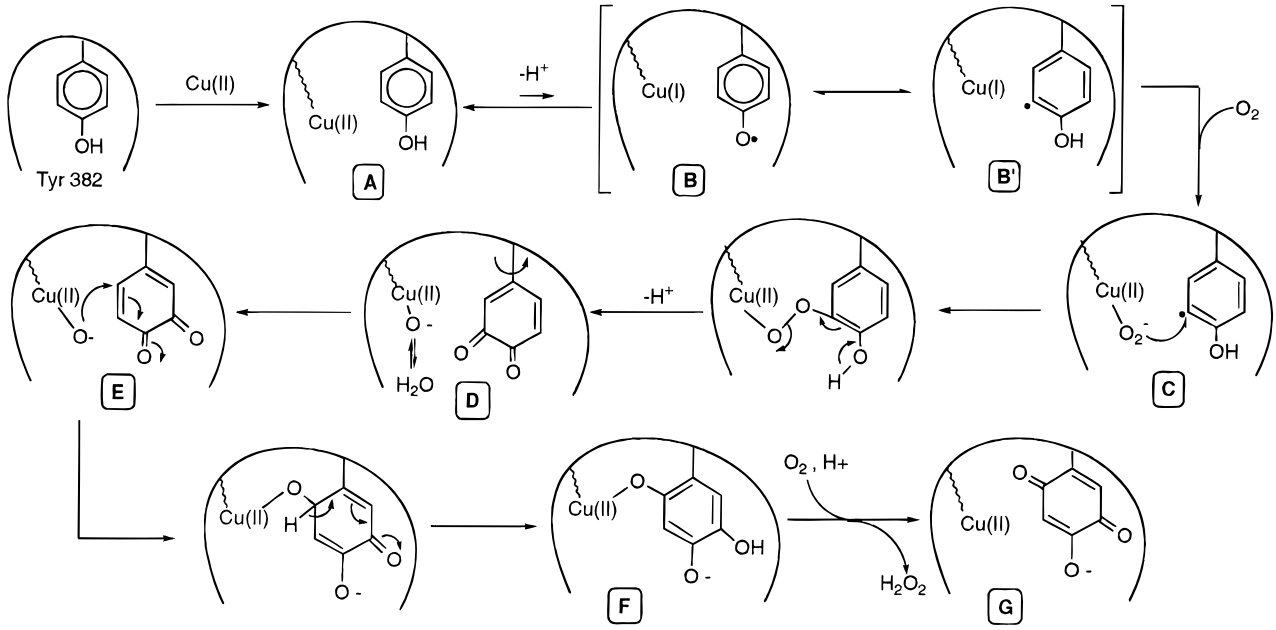
The lack of copper reduction is in marked contrast to previous reports, which indicated that nearly all the copper(II) added to anaerobic apoPEAO was reduced (Matsuzaki *et al*., 1994, 1995). The only difference between our present work and that previously reported was the use of ammonium sulfate and DTT in the purification procedure. DTT is a known reducing agent, and we have shown that it can reduce copper bound in the active site of PEAO. Contaminating DTT could be the source of the reducing equivalent in the study of Matsuzaki *et al*., which would explain why no corresponding one-electron-oxidized species was detected. The previously reported lack of Cu(II) reduction to Cu(I) in the Y382F mutant (Y382 becomes TPQ), however, is not explained by this, unless changing the active site Tyr to Phe alters the accessibility of the active site to DTT or alters the reduction potential of the bound copper so that DTT cannot reduce it (Matsuzaki *et al*., 1995);[4] this remains to be investigated. It should be noted that our results do not preclude reduction to copper(I) by Y382 as the initial step in the biogenesis reaction, as suggested by Matsuzaki *et al.* In fact, the absence of DTT eliminates the possibility that an exogenous reductant could perturb an initial copper(II)−Tyr/copper(I)−Tyr• equilibrium (*vide infra*).

The rate of formation of TPQ was found to be independent of the amount of copper added, so long as at least 1 equiv of copper per subunit was present (without copper present, no TPQ formed). This is in contrast to earlier results, which showed that the rate of formation of TPQ depended on the amount of copper added (Matsuzaki *et al*., 1994). In those studies, however, the reaction was initiated by adding an air-saturated solution of copper to the apoprotein instead of anaerobically preincubating the enzyme with copper. Thus, the observed rate dependence on copper could simply reflect the rate of copper uptake and binding by PEAO, which should depend on the availability of copper (Dooley *et al*., 1991b). The rate of TPQ formation using our protocol was more than twice as fast as when the enzyme was not preincubated with copper. While this may suggest that copper uptake is kinetically significant, the difference in oxygen concentrations may also have played a role. Preliminary experiments indicate that the reaction rate depends on oxygen concentration (unpublished results). Our kinetic analysis also differs from that of Matsuzaki *et al.* in that we can fit our data to a single first-order kinetic process with a k_{obs} of 1.5 ± 0.2 min^{-1}, rather than two parallel first order processes at lower rates.[5] This could simply reflect the more complex kinetics of monitoring copper uptake as well as TPQ formation in the previous study. In the absence of kinetically detectable protein conformational states, it is chemically reasonable that the formation of the TPQ cofactor in amine oxidases would follow a single pathway rather than multiple pathways, and hence, a single kinetic process would be observed. The observation that only 1 equiv of copper per subunit is needed for TPQ biogenesis, and that the rate of TPQ formation is independent of copper, eliminates the possibility that adventitious (i.e. unbound) copper plays a role in TPQ biogenesis. We can therefore conclude that it is the active site-bound copper that carries out the TPQ biogenesis reaction. There is no accumulation of any intermediates with low-energy electronic transitions detectable by absorption spectroscopy during TPQ formation. Assuming that any partially oxidized tyrosine species (e.g. dopa quinone) would have such transitions, it is probable that the reaction of oxygen with some initial precursor to

[4] By comparison of our EPR spectrum of apoPEAO anaerobically incubated with Cu (Figure 2C) with the EPR spectrum of the Y382F anaerobically incubated with copper (Matsuzaki *et al*., 1995), we can see that the initial copper binding in these species is different. This difference in binding could account for the lack of reduction of copper by DTT in the Y382F mutant. Also, recent crystallographic studies on apoPEAO suggest that the Y382F mutation may lead to perturbation of the copper environment (M. C. J. Wilce, D. M. Dooley, H. C. Freeman, J. M. Guss, W. S. McIntire, C. E. Ruggiero, K. T. Tanizawa, and K. Yamaguchi, unpublished results).

[5] Although our data can be fit to the two parallel first-order process equations with high accuracy, the fits are not unique, and we thus see no need to invoke an additional first-order process.

Scheme 1: Proposed Mechanism for the Biogenesis of the TPQ Cofactor in PEAO[a]



[a]Cu(II) initially binds to the apoprotein to give a Cu(II)−enzyme complex (A). This complex is in equilibrium with a Cu(I)−tyrosine radical (B). An alternate form of B, B′, is drawn to illustrate that the unpaired spin will be delocalized over the ring, activating the ring carbons. The Cu(I) ion reacts with dioxygen to form an activated oxygen complex, shown here as copper−superoxide (Fujisawa *et al*., 1994, and references cited therein) (C). This activated oxygen complex attacks the tyrosine radical to yield dopa quinone and a copper oxide (D). This copper oxide/hydroxide species may be in rapid exchange with solvent water, allowing solvent oxygen incorporation into TPQ. Rotation about the β-carbon would move the C2 ring near the copper-bound oxide (E). Nucleophillic attack by the copper oxide on the dopa quinone (which should be very susceptible to nucleophillic attack) would yield topa (F). In the presence of dioxygen, topa would then rapidly oxidize to TPQ (G). The net loss of a hydrogen, as indicated by this mechanism, is expected on the basis of the formation of TPQ, which has a pK_a of ~4 (Mure & Klinman, 1993), from tyrosine, which has a pK_a of ~10.

TPQ, rather than a later partially oxidized species, is rate-determining. With this in mind, we can envision the following minimal mechanism (eq 3):

$$[\text{Cu}-\text{E}-\text{Tyr}] \xrightarrow{k_1[\text{O}_2]} [\text{Cu}-\text{E}-\text{Tyr}]_{\text{ox}} \xrightarrow{k_2[\text{O}_2]} [\text{Cu}-\text{E}-\text{TPQ}] \quad (3)$$

where $k_2 \gg k_1$ and [Cu−E−Tyr]^{ox} is a partially oxidized enzyme complex. This would give the rate law d[Cu−E−TPQ]/dt = k_1[O_2][Cu−E−Tyr], where $k_1[O_2] = 1.5\ \text{min}^{-1}$. This rate law agrees with the hypothesis that the rate-limiting step in TPQ formation is the reaction of a copper−oxygen complex or the reaction of oxygen itself with the TPQ precursor. The kinetic order of the reaction in oxygen, presumed to be first-order here, remains to be determined.

In light of the results presented here, as well as previous work, we propose the mechanism shown in Scheme 1 for TPQ biogenesis. This mechanism agrees with both our kinetic data and the isotope labeling evidence, which demonstrates that the C2 oxygen is derived from solvent (Nakamura *et al*., 1996).[6] The initial radical species [Cu(I)−Tyr•) may be difficult to detect if the equilibrium lies strongly in favor of Cu(II)−Tyr, which is probable. The presumption of this initial equilibrium between the Cu(I)−Tyr• and Cu(II)−Tyr is not without precedence. For example, it is analogous to the Cu(I)−semiquinone radical/Cu(II)−reduced quinone equilibrium that has been shown to exist in the catalytic cycle of amine oxidases (Dooley *et al*., 1991a). Additionally, numerous other proteins purportedly use tyrosine radical species for either cofactor assembly or catalysis (Prince, 1988), including ribonucleotide reductase R2 (Sahlin *et al*., 1987; Bender *et al*., 1989), galactose oxidase (Whittaker & Whittaker, 1988, 1990), PSII (Koulougliotis *et al*., 1995; Lane Gilchrist *et al*., 1995; Hoganson & Babcock, 1992), dopamine-β-hydroxylase (Tian *et al*., 1994; Brenner *et al*., 1989), prostagladin synthase (Karthein, 1988; Lassmann, 1991), and glyoxal oxidase (Whittaker *et al*., 1996). We are currently using a variety of techniques in an attempt to observe the proposed Cu(I)−Tyr• species.

[6] The isotope labeling experiments were performed on enzyme prepared by the method of Matsuzaki *et al*. (1994). These experiments need to be redone to determine if the C2 oxygen is still derived from solvent under the conditions used here.

ACKNOWLEDGMENT

We thank Prof. K. Karlin and Prof. S. Ito for helpful discussions on possible mechanisms.

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BI9628836