Investigation of Spectroscopic Intermediates during Copper-Binding and TPQ Formation in Wild-Type and Active-Site Mutants of a Copper-Containing Amine Oxidase from Yeast†

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Received September 24, 1999; Revised Manuscript Received January 12, 2000

ABSTRACT: Copper amine oxidases possess the unusual ability to generate autocatalytically their organic cofactor, which is subsequently utilized in turnover. This cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ), is formed within the active site of these enzymes by the oxidation of a single tyrosine residue. *In vitro*, copper(II) and oxygen are both necessary and sufficient for the conversion of tyrosine to TPQ. In this study, the biogenesis of TPQ has been characterized in an amine oxidase from *Hansenula polymorpha* expressed as the apo-enzyme in *Escherichia coli*. With the WT enzyme, optical absorbances which are copper or oxygen dependent are observed and characterized. Active-site mutants are used to investigate further the nature of these spectral species. Evidence is presented which suggests that tyrosine is activated for reaction with oxygen by liganding to Cu(II). In the following paper in this issue [Schwartz, B., Dove, J. E., and Klinman, J. P. (2000) *Biochemistry 39*, 3699-3707], the initial reaction of precursor protein with oxygen is characterized kinetically. Taken together, the available data suggest a mechanism for the oxidation of tyrosine to TPQ where the role of the copper is to activate substrate.

Copper amine oxidases (CAOs)[1] constitute a ubiquitous class of enzymes which catalyze the oxidation of primary amines to the corresponding aldehydes, concomitant with the reduction of dioxygen to hydrogen peroxide (*1*). These enzymes allow prokaryotes and fungi to use primary amines as the sole source of nitrogen for growth. In plants, hydrogen peroxide generated by CAOs during enzymatic turnover has been shown to be important for cell wall formation and wound healing (*2*, *3*). A role for CAOs in mammals has not been as well-defined, but existing proposals have focused on two possiblities. The initial suggestion was that CAOs function to metabolize biogenic amines (*3*). Recently, evidence of hydrogen peroxide involvement in cell-signaling pathways (*4*−*6*) has raised the possibility of a role for CAOs in cellular homeostasis.

CAOs are homodimers with each subunit containing a mononuclear copper and 2,4,5-trihydroxyphenylalanine quinone (TPQ or topa quinone) as cofactors (*7*). Catalysis has been shown to proceed via ping-pong kinetics (*1*) with the following half reactions:

$$TPQ_{ox} + R{-}NH_3^+ \rightarrow TPQ_{red} + R{-}CHO \text{ (Reductive)}$$

$$TPQ_{red} + O_2 \rightarrow TPQ_{ox} + H_2O_2 + NH_4^+ \text{ (Oxidative)}$$

The catalytic mechanism has been extensively studied (*8*, *9*), but much less is known about the biogenesis of the cofactor.

TPQ is formed by the oxidation of a tyrosine within the active site (*10*). Crystal structures of holo-enzymes from various sources have shown that the copper is liganded by three histidines and two waters and that the plane of the TPQ ring is approximately 4−5 Å away from the copper (*11*−*14*). A crystal structure of the apo-enzyme from *Arthrobactor globiformis* (AGAO) has also been solved, indicating no significant differences in the backbone between the apo- and holo-enzymes. The main structural difference in the apo-enzyme is a rotation of the precursor tyrosine side chain relative to the position of the mature cofactor, such that the tyrosyl oxygen is pointed toward the vacant copper-binding site (*13*). Under certain conditions, the mature TPQ has also been shown to rotate toward the copper site in a manner similar to the precursor tyrosine. In this case, TPQ can then ligand the copper through its 0−4 oxygen, though this is a catalytically inactive conformation (*13*, *14*).

TPQ is generated autocatalytically, requiring only copper and oxygen to initiate the reaction (*15*, *16*). Freeman and co-workers have suggested a mechanism for biogenesis based largely on structural data (*13*). The mechanism (Scheme 1)

† This work was supported by a National Institutes of Health Grant (GM 39296) to J.P.K. J.E.D. was supported by a Training Grant from the National Institutes of Health (GM 08295-10). B. Schwartz was supported by a National Institutes of Health Postdoctoral Fellowship (GM 18813). N.K.W. was supported by an Australian National Health and Medical Research Council C. J. Martin Postdoctoral Fellowship.

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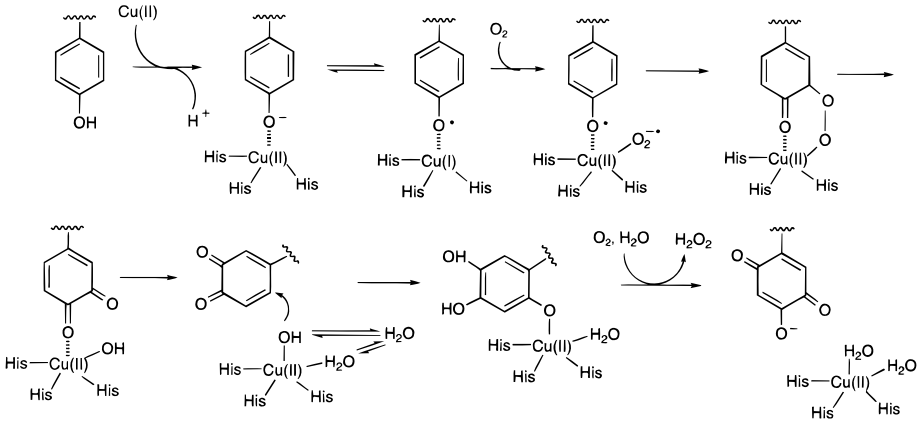
[1] Abbreviations: WT, wild-type; HPAO, *Hansenula polymorpha* amine oxidase; CAO, copper amine oxidase; TPQ, topa quinone or 2,4,5-trihydroxyphenylalanine quinone; AGAO, *Arthrobactor globiformis* amine oxidase; PCD, protocatechuate 3,4-dioxygenase; PCA, protocatechuate; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); ICP-AES, inductively coupled plasma-atomic emission spectroscopy; EPR, electron paramagnetic resonance; LMCT band, ligand to metal charge-transfer band; DEANO, diethylamine nitric oxide, sodium salt.

10.1021/bi992225w CCC: $19.00
Published on Web 03/10/2000

Scheme 1: Previously Proposed Mechanism for TPQ Biogenesis [adapted from Wilce et al. (*13*)]



has the precursor tyrosine liganded to Cu(II) in the absence of dioxygen. Tyr-Cu(II) is in equilibrium with tyrosyl radical-Cu(I). Molecular oxygen reacts with Cu(I) and tyrosyl radical to form a peroxy intermediate, which subsequently decomposes to give dopa quinone. Nucleophilic attack of hydroxide generates the reduced form of TPQ, which can then be oxidized by O_2 to give the fully oxidized cofactor. This mechanism is consistent with the stoichiometry of molecular oxygen consumption and hydrogen peroxide production measured during biogenesis in AGAO (*17*). It must be considered speculative, however, since no evidence for any of the proposed intermediates has been reported.

The amino acids that precede and follow the precursor tyrosine have been shown to form a completely conserved concensus sequence of N-Y-D/E in the active site of copper amine oxidases (*10*). Mutations of the conserved N404 and E406 in the *Hansenula polymorpha* amine oxidase[2] (HPAO) (*18*) suggest that both residues play a role in catalysis by limiting the flexibility of the mature cofactor (*19*, *20*). In AGAO, mutations of these consensus site residues were shown to decrease the rate of catalysis and biogenesis, but no detailed study was carried out to determine the cause of this decrease in rate (*21*).

Though there have been many active-site mutants of CAOs that retain partial activity, all the mutations of the copper-binding ligands have resulted in inactive enzymes. These mutations have been designed to disrupt copper binding and, as copper is essential for biogenesis, result in protein which is unable to form TPQ (*15*, *22*). In this study, a mutation which alters the properties of the copper site, but still supports copper binding and biogenesis, has been generated in order to examine the role of the copper in biogenesis. Characterization of this mutant (H624C), together with WT HPAO, E406Q, and N404D, provides new information about the nature of the species leading to TPQ. A mechanism for biogenesis is proposed based on our findings, as well as kinetic results presented in the accompanying paper in this issue (*23*).

EXPERIMENTAL PROCEDURES

Mutagenesis, Escherichia coli Expression, and Protein Purification. Mutagenesis of HPAO was performed as previously described (*20*) using the Chameleon Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene Cloning Systems) in the pDB20 plasmid. The mutagenic primers were 5′-GCT GCC AAT TAC **C**AG TAC TGT CTG TAC TGG G-3′ for E406Q, 5′-ATA TTT ACT GCT GCC **G**AT TAC GAG TAC TGT CTG-3′ for N404D, and 5′−C CAT ACT TTC GGT ATC ACC **TG**T TTC CCA GCT CCT GAG G-3′ for H624C. The mutated codon is underlined and the mutated nucleotides are shown in bold. Confirmation of the mutation was obtained by sequencing of approximately 50 bases around the mutation using a T7 Sequenase$_{v.\ 2.0}$ Quick-Denature Plasmid DNA Sequencing Kit (Amersham Life Science, Inc.) or by sequencing at the UC Berkeley DNA Sequencing Facility. Subcloning of the mutated gene from pDB20 into the pKW3 expression vector (*24*) was performed as previously described (*25*). BL21(DE3) cells were transformed with pKW3 plasmids containing the mutated amine oxidase genes.

Growth of BL21(DE3)/pKW3 cells and subsequent protein purification followed previously reported procedures (*24*) with the following modifications. To begin cell growth, either 18 cultures of 450 mL in plastic Erlenmeyer flasks or 12 cultures of 1.5 L in plastic Fernbach flasks were innoculated with a single colony from plates grown overnight. Following the gel filtration column during protein purification, fractions were pooled and concentrated against 50 mM HEPES, pH 7.0, and dialyzed against the same buffer two more times for at least 4 h to remove the copper chelators. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories) with bovine serum albumin as a standard. To determine the stoichiometry of bound copper, Cu(II) was added to aerobic apo-enzyme and incubated for 30 min. To remove any unbound copper, the sample was washed three times with an excess of buffer using a microcon (Amicon). Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was performed on an Optima 3000 DV (Perkin-Elmer).

TPQ Biogenesis Monitored Spectrophotometrically. UV−vis spectra were taken using an HP8452A (Hewlett-Packard) spectrophotometer fitted with a constant-temperature bath. Biogenesis reactions were followed spectrophotometrically in a quartz cuvette containing 150 μL of 40 μM apo-HPAO, 50 mM HEPES, pH 7.0, at 25 °C. Metal stock solutions were prepared in Milli-Q water from $CuCl_2$ and $ZnCl_2$. To minimize Cu(II)-induced precipitation of the protein, either stoichiometric or slightly substoichiometric amounts (0.7−

[2] *Hansenula polymorpha* has been reclassified as *Pichia angusta*. HPAO will still be used to refer to the enzyme, to be consistent with previous papers and to avoid confusion in the amine oxidase nomenclature.

0.8 equiv) of Cu(II) were added to initiate biogenesis, as indicated. For the aerobic addition of Cu(II), reactions were followed until no further absorbance changes were observed. For the aerobic addition of Zn(II), a slight excess was added (1.2 equiv). There were no absorbance changes, so Zn(II) was simply incubated with apo-HPAO for approximately 15 min before the addition of Cu(II).

Anaerobic metal solutions were prepared by bubbling with argon, scrubbed with a basic pyrogallol solution (Sigma), for 30−60 min. Anaerobic enzyme solutions were prepared as previously described (*25*) with scrubbed argon blown over the enzyme solution in a cuvette on ice for at least 60 min. After the enzyme was allowed to equilibrate at 25 °C, an anaerobic solution of Cu(II) was added through a septum via a gastight syringe (Hamilton Company). Copper binding was allowed to proceed until no further absorbance changes were observed. During this time there was no detectable leakage of oxygen, judged by the lack of any absorbance increase at 480 nm (which would indicate TPQ formation). Biogenesis was initiated by bringing the enzyme solution into the bulbous part of the cuvette and blowing air over the top of the solution while swirling for 15−30 s to ensure complete aeration. Further aeration was not found to alter the kinetics.

DEANO (Molecular Probes) was used to generate 2 equiv of nitric oxide. Following anaerobic binding of Cu(II) to apo-HPAO, 3 sequential equiv of DEANO was added anaerobically 20 min apart via a gastight syringe. The solution was exposed to air 20 min following the third DEANO addition, and TPQ formation was followed at 480 nm. As a control for nitric oxide access to the active site, holo-WT was incubated anaerobically with methylamine. This results in the formation of the semiquinone. Two equivalents of DEANO were added anaerobically 20 min apart, and the reaction was allowed to proceed for 20 min. The reaction was then exposed to air.

TPQ Biogenesis of H624C Monitored by Oxygen Consumption. Oxygen consumption was measured using a YSI model 5300 Biological Oxygen Monitor (Yellow Springs Instrument Co., Inc.). Calibration was performed as described (*23*). Assays were monitored in 50 mM HEPES, pH 7.0, 1 mL total volume at 25 °C. The concentration of apo-H624C was 20 μM. After equilibration to a constant baseline, stoichiometric copper was added to initiate biogenesis.

Determination of Rate Constants. Spectrophotometric absorbances were normalized for background at 800 nm. The increase in 480 nm absorbance for WT, as well as the decay at 380 nm for all enzyme forms, was fit to a single-exponential equation using KaleidaGraph 3.0.2 to determine k_{obs}. For H624C, the rate constant for the formation of TPQ was calculated from the linear initial rate. The linear rate was taken from 100 min until 300 min (see Results). Calculation of a rate constant from initial rate requires an accurate estimate of the concentration of reactive enzyme. If we use final yields of TPQ as a measure of reactive enzyme, this is approximately 0.2 of total protein concentration for H624C.

Data for WT are reported as the average of at least three measurements, and errors are based on a 95% confidence interval using the Student's *t* distribution. Errors for E406Q, N404D, and H624C were estimated to be the same percentage of the rate as was observed for WT.

Due to overlapping absorbances between the intermediate and TPQ, global fitting was necessary to determine the rate constants for the formation and decay of the 350 nm intermediate observed with WT enzyme and the 390 nm intermediate observed with H624C. In the case of H624C, more data points than shown could not be obtained due to apparent photosensitivity of the enzyme in the diode array. Data were fit using SPECFIT/32 for Windows, Version 3.0 (Spectrum Software Associates), to the model A → B → C. The errors reported for the respective rate constants, k_1 and k_2, were generated by the fitting program.

No oxygen consumption was observed with H624C, so a limit for an observable rate constant was calculated as described for N404D (*23*).

RESULTS

Copper-Dependent Spectral Features in WT, E406Q, and N404D. Upon addition of Cu(II) to aerobic apo-enzyme, there is an increase in absorbance at 380 nm during the mixing time of the experiment (approximately 3 min due to reversible protein precipitation upon initial incubation of the enzyme with copper), which decays on approximately the same time scale as TPQ formation in WT enzyme (Figure 1A and Table 1). The same absorbance feature and rate of decay are seen upon addition of Cu(II) to anaerobic apo-enzyme (data not shown).

With E406Q and N404D, the 380 nm absorbance appears quickly, analogous to what is seen with WT. The absorbance also decays on roughly the same time scale as WT (Table 1). Since TPQ formation is retarded, the decay of the 380 nm species is more rapid than the appearance of cofactor for the consensus site mutants (Figure 1, panels B and C, and Table 1); thus, the 380 nm species decay is not kinetically coupled to TPQ formation.

Zn(II) is known to bind at the active site of apo-HPAO and prevent the formation of TPQ, because it cannot be displaced by copper (*24*). If apo-enzyme is preincubated with Zn(II), the addition of Cu(II) still leads to a rapid increase at 380 nm, followed by a decay at the same rate as in the absence of Zn(II). However, in this case there is no concomitant production of TPQ (Figure 2).

To test if the 380 nm species is only seen in enzyme without TPQ, Cu(II) was added to holo-HPAO. Biogenesis was initiated with Cu(II); after biogenesis was complete, the same amount of copper was added to the holo-enzyme. The 380 nm species was again observed, and the extent of absorbance, as well as the decay rate, was approximately the same (data not shown). Addition of a third equivalent of Cu(II) was found to irreversibly precipitate the protein.

Oxygen-Dependent Spectral Features in WT. When Cu(II) is prebound anaerobically and the 380 nm species is allowed to decay, biogenesis can then be initiated by exposure to oxygen. Under these conditions, an absorbance feature can be seen at 350 nm following exposure to oxygen. The 350 nm species can be seen at early time points relative to TPQ formation (Figure 3A). This spectral feature decays concomitant with TPQ formation (Figure 3B). The kinetics of the appearance and disappearance of the 350 nm species, as well as TPQ formation, are shown in Figure 3C. The rate constant for TPQ formation is 0.068 ± 0.002 min^{-1}.

Data for the kinetics of the intermediate species could be fit by rate constants of either 0.51 ± 0.01 and 0.076 ± 0.003

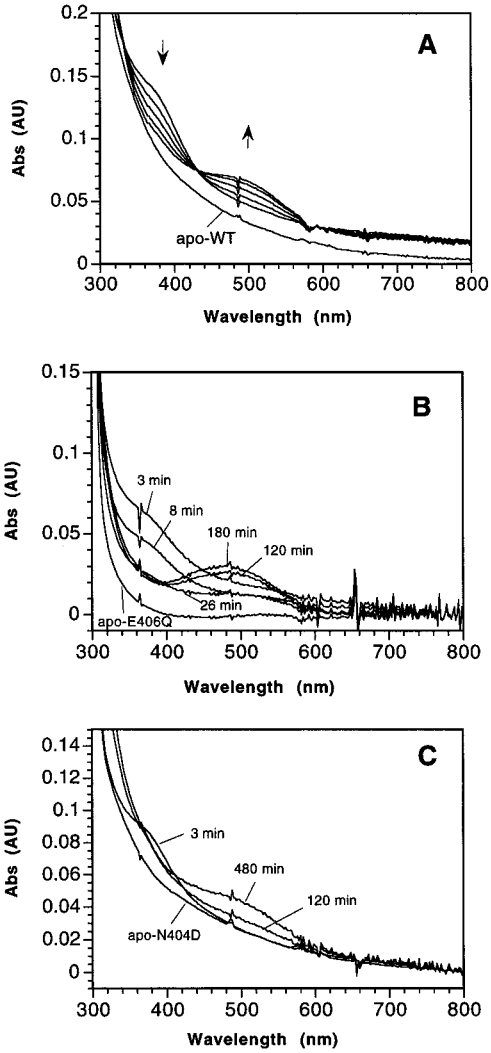


FIGURE 1: UV−vis spectra showing the time course for changes in 380 nm absorbance and 480 nm absorbance (TPQ) with the aerobic addition of Cu(II) at pH 7.0, 25 °C. (A) WT HPAO; the timepoints shown are 0 (apo-WT), 3, 8, 15, 26, and 40 min. The direction of change is indicated by the arrows. (B) E406Q; the timepoints shown are 0 (apo-E406Q), 3, 8, 26, 120, and 180 min. (C) N404D; the timepoints shown are 0 (apo-N404D), 3, 120, and 480 min.

Table 1: Kinetics of Cu-Dependent Spectroscopic Changes during Biogenesis[a]

	decrease, k_{380} (min^{-1})	increase, k_{480} (min^{-1})
WT HPAO	$(6 \pm 1) \times 10^{-2}$	$(8 \pm 3) \times 10^{-2}$
E406Q	$(8 \pm 1) \times 10^{-2}$	$(1.3 \pm 0.5) \times 10^{-2}$
N404D	$(3.4 \pm 0.6) \times 10^{-2}$	$(6 \pm 2) \times 10^{-4}$

[a] Assays were conducted at 25 °C and pH 7.0. Reactions were begun by the addition of substoichiometric copper (0.7−0.8 equiv) to an air saturated enzyme solution.

min^{-1} or 0.076 ± 0.003 and 0.51 ± 0.01 min^{-1} for the growth and decay of the species, respectively. These two fittings predict different maximal concentrations of intermediate and, therefore, different extinction coefficients for the intermediate. The first case, a fast formation and slow decay, predicts an extinction coefficient of approximately 3200 M^{-1} cm^{-1}. The second case, a slow formation and fast decay, predicts an extinction coefficient of approximately 23 000 M^{-1} cm^{-1}.

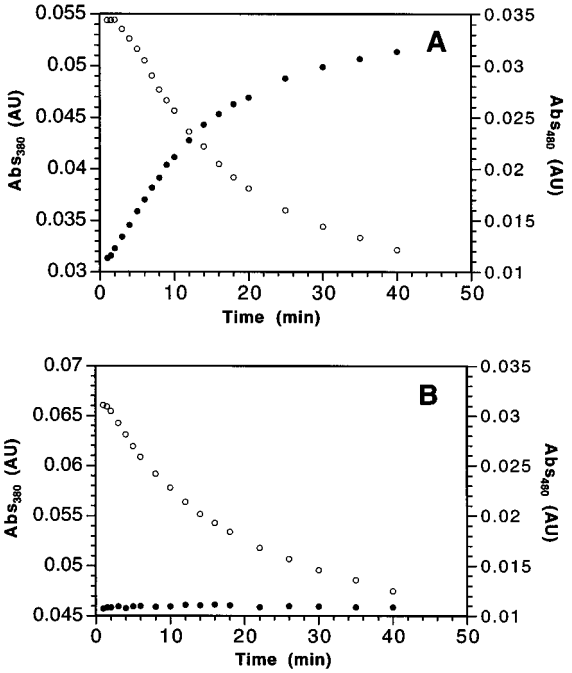


FIGURE 2: Effect of pre-binding Zn(II) on TPQ biogenesis. (A) Kinetics of the change in 380 nm absorbance (○) and 480 nm absorbance (●) following the addition of copper aerobically to apo-WT HPAO. (B) Kinetics of the change in 380 nm absorbance (○) and 480 nm absorbance (●) when Cu(II) is added to Zn(II)-containing WT HPAO.

The rate of oxygen consumption at pH 7.0, 25 °C is 0.1 ± 0.03 min^{-1} (*23*).

Nitric oxide can be used to trap a transiently formed tyrosyl radical, as in the case of prostaglandin H synthase (*26*). DEANO has been used to generate nitric oxide which then reacts with tyrosyl radical, though in some cases this has been found to be a reversible reaction (*26*). Apo-HPAO[3] with prebound Cu(II) was incubated anaerobically with DEANO. Subsequent exposure to oxygen was found to result in a normal extent and rate of TPQ formation. As a control, the semiquinone of TPQ was formed by anaerobic incubation of the mature enzyme with methylamine. In this case, the anaerobic addition of DEANO resulted in a bleaching of the characteristic semiquinone absorbance, and the oxidized cofactor was not regenerated upon exposure to air (data not shown). These data indicate that nitric oxide has access to the active site of mature protein. Yet, no effect of nitric oxide can be discerned on biogenesis, appearing to rule out any significant accumulation of a tyrosyl radical in the course of TPQ formation.

Oxygen-Dependent Spectral Features in E406Q and N404D. With E406Q and N404D, no oxygen-dependent spectral absorbances other than TPQ formation were detectable when initiating biogenesis with oxygen after prebinding Cu(II) anaerobically (data not shown).

Copper-Dependent Spectral Features in H624C. ICP-AES analysis indicates that H624C binds one copper per subunit. When copper is added to apo-H624C either aerobically or anaerobically, the 380 nm species described above is again

[3] Apo-HPAO refers to enzyme containing precursor tyrosine. If a metal has been added, this is indicated. Holo-HPAO refers to enzyme containing Cu and TPQ. Precursor tyrosine refers to Y405 which is modified to form TPQ.

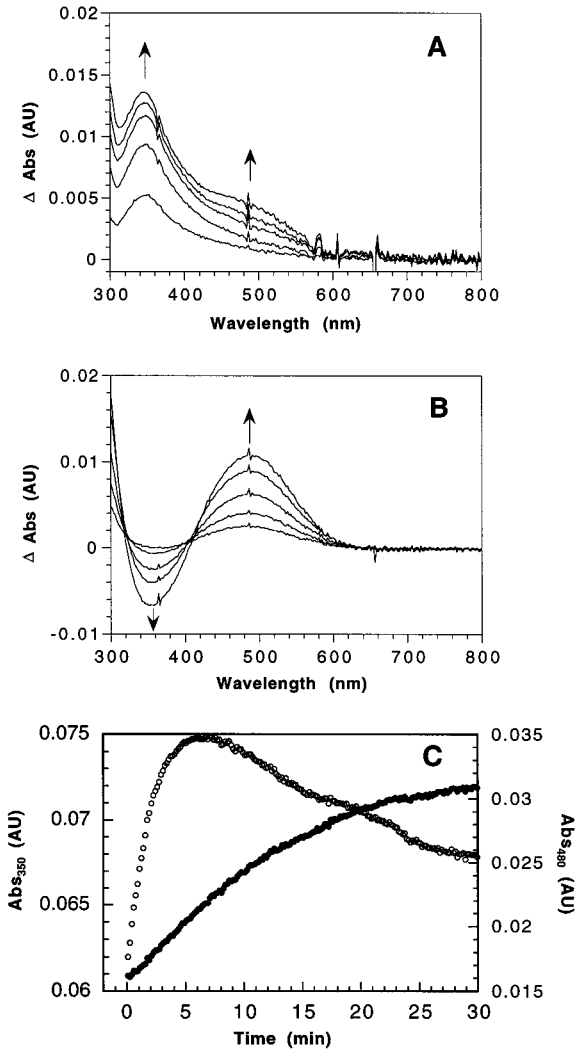


FIGURE 3: Spectroscopic changes following oxygenation of WT-HPAO, with Cu(II) prebound anaerobically, at pH 7.0, 25 °C. (A) Absorbance changes following exposure to O_2. Spectra are at 1, 2, 3, 4, and 5 min following exposure to oxygen; the spectrum before oxygenation has been subtracted. Arrows indicate the direction of change. (B) Absorbance changes during the decay of the 350 nm species. Spectra are 8, 10, 14, 20, and 30 min following the introduction of O_2; the spectrum at 5 min after aeration has been subtracted. Arrows indicate the direction of change. (C) Kinetics of the reaction with O_2 at 350 nm (○) and 480 nm (●).

seen to appear and decay at roughly the same rate as WT (data not shown). In this case, though, another absorbance is also seen at approximately 400 nm, which persists anaerobically (Figure 4A). The inset spectrum illustrates the concurrent increase in absorbance at 400 nm and decay in absorbance at 380 nm (Figure 4A). If this absorbance results from stoichiometrically bound copper, which is supported by Cu analysis, then the species can be estimated to have an extinction coefficient of approximately 800 M^{-1} cm^{-1}.

Zn(II) was used as a probe to determine if the 400 nm absorbance is specific to the active site of H624C. If Zn(II) is prebound to the enzyme anaerobically followed by the anaerobic addition of Cu(II), the 380 nm species is observed but no absorbance is seen at 400 nm (Figure 4B). In this case, the inset spectrum reveals a decay at 380 nm with no new spectral change above 400 nm (Figure 4B).

Oxygen-Dependent Spectral Features in H624C. When copper is prebound anaerobically and then oxygen is used

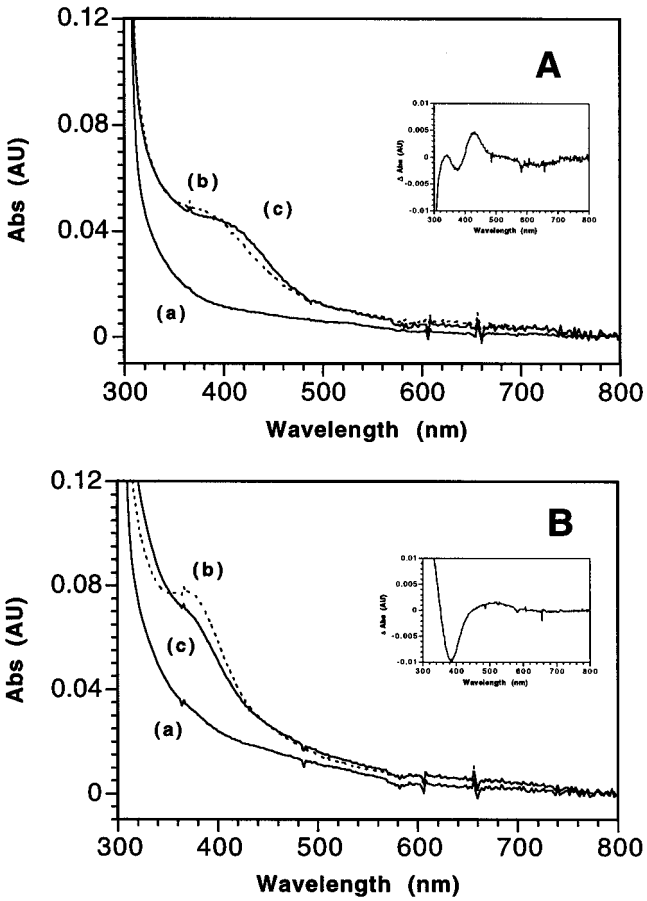


FIGURE 4: Anaerobic Cu(II)-dependent spectroscopic changes with H624C at pH 7.0, 25 °C. (A) Cu(II) added anaerobically to apo-H624C: (a) apo-H624C (b) 1 min after addition of Cu(II) (dashed line) (c) 60 min after addition of Cu(II). Inset shows curve c subtracted from curve b. (B) Cu(II) added anaerobically after prebinding Zn(II) to apo-H624C: (a) Zn-H624C, (b) 1.5 min after addition of Cu(II) (dashed line), (c) 60 min after addition of Cu-(II). Inset shows curve c subtracted from curve b.

to initiate the biogenesis in H624C, an absorbance grows in at 390 nm (Figure 5A). This absorbance can then be seen to decay concomitant with TPQ formation (Figure 5B). The wavelength for the decay of the 390 nm band appears red shifted to 410 nm. This is attributed to overlapping absorbance from the TPQ-band below 400 nm. The kinetics for this reaction are shown in Figure 5C. The smaller apparent decrease in absorbance at 410 nm relative to the increase at 480 nm is once again attributed to overlapping absorbances in this region of the spectra. The rate constant for the formation of TPQ, fit from the linear increase from 100 to 300 min, is 0.0011 ± 0.0007 min^{-1}. The absorbance increase at 480 nm before 100 min corresponds to background absorbance from the 390 nm species rather than a product peak at 480 nm (see Figure 5A).

Again, two kinetic scenarios successfully fit the data. The first possibility was a fast formation and a slow decay of the intermediate with rate constants of 0.042 ± 0.002 and 0.0011 ± 0.0001 min^{-1}, respectively. This would predict an intermediate with an extinction coefficient of 2000 M^{-1} cm^{-1} at 390 nm. Alternatively, the data can be fit by a slow formation and a fast decay of the intermediate with rate constants of 0.0011 ± 0.0001 and 0.042 ± 0.002 min^{-1}, respectively. In this case, the intermediate is predicted to

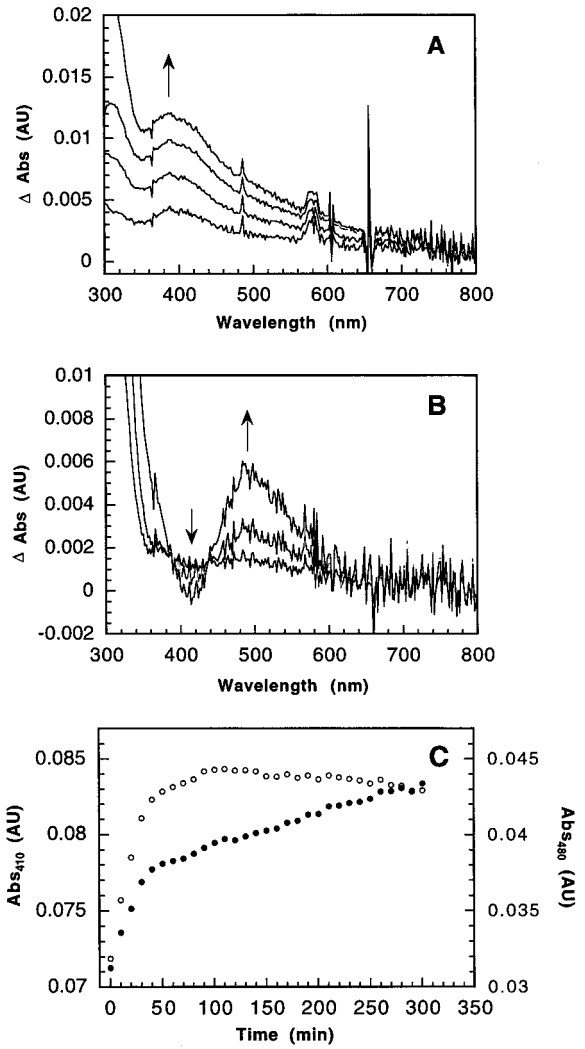


FIGURE 5: Spectroscopic changes following oxygenation of H624C, with Cu(II) prebound anaerobically, at pH 7.0, 25 °C. (A) Absorbance changes following exposure to O_2. Spectra are 10, 20, 30, and 60 min following exposure to O_2; the spectrum before oxygenation has been subtracted. Arrow indicates the direction of change. (B) Absorbance changes during the decay of the 390 nm species. Spectra are 120, 180, and 300 min following exposure to O_2; the spectrum at 60 min after aeration has been subtracted. Arrows indicate direction of change. (C) Kinetics of reaction with O_2 at 410 nm (○) and 480 nm (●).

have an extinction coefficient of 88 000 M^{-1} cm^{-1} at 390 nm.

An attempt was made to determine the rate constant for oxygen consumption with H624C. No rate was detectable, so that only an upper limit of <0.003 min^{-1} could be determined.

DISCUSSION

The mechanism of TPQ biogenesis is one of the compelling questions in the copper amine oxidase field. Detection of intermediates in the reaction would provide a much needed handle for studying the mechanism. Additionally, very little is known about the roles that active-site residues play in facilitating the oxidation of the precursor tyrosine. An examination of spectroscopic changes during TPQ formation can begin to address these issues.

Copper-Dependent Absorbance in WT Is Not an Intermediate in Tyrosine Oxidation. Upon aerobic Cu(II) addition to apo-enzyme, the dominant spectral feature is a rapid increase and subsequent decay of absorbance at 380 nm. The decay rate is roughly similar to the rate of biogenesis, suggesting the accumulation of a biogenesis intermediate. The observation of the same absorbance under anaerobic conditions requires that this intermediate be present before any reaction with oxygen. This led to the idea that the absorbance might represent the active species which reacts with oxygen. A LMCT band resulting from the precursor tyrosine liganded to copper was considered a possiblity based on crystal structures showing the mature cofactor as a ligand to copper under certain conditions (*13*, *14*) and the apo-structure of a CAO in which tyrosine is pointing toward the vacant copper-binding site (*13*).

To test this possibility, the mutant enzymes E406Q and N404D were used to examine the relationship between the rate of 380 nm decay and the rate of biogenesis. In both mutants, the 380 nm species decays at roughly the same rate as was seen with WT, even though the biogenesis is slowed approximately 1 and 2 orders of magnitude, respectively. Additionally, with E406Q, the rate of oxygen consumption has been determined to be close to the rate of cofactor formation. Therefore, the 380 nm decay is distinct both from the consumption of oxygen and the formation of cofactor. This clearly precludes the 380 nm species from being the species that reacts with oxygen.

Further experiments were used to investigate the relationship between the 380 nm species and the active site of the apo-enzyme. In HPAO, it has been shown that when Zn(II) binds at the active site, it cannot be displaced by Cu(II); additionally, preincubation of apo-enzyme with Zn(II) blocks biogenesis (*24*). Thus, addition of Cu(II) to apo-enzyme preincubated with Zn(II) would not yield a 380 nm absorbance if this species results from copper binding at the active site. In fact, prebinding of Zn(II) has no effect on the appearance or decay rate of the 380 nm species (Figure 2). This result clearly shows that the 380 nm species arises from an interaction of copper distinct from the active-site metal-binding site.

To determine if the absorbance was even specific for the apo-enzyme, copper was added a second time after biogenesis was complete. The 380 nm absorbance again appeared and decayed similarly to addition of Cu(II) to the apo-enzyme. This rules out the involvement of the precursor tyrosine in the absorbance, since at this point the tyrosine has already been oxidized to TPQ.

Crystallographically, no additional metal-binding site was identified in HPAO (*12*), though second metal-binding sites have been seen in crystal structures of other copper amine oxidases (*11*, *13*, *14*). Also, preliminary resonance Raman experiments fail to reveal any resonance-enhanced modes upon excitation of the 380 nm absorbance band (E. L. Green and J. Sanders-Loehr, unpublished work). While the 380 nm absorbance has been clearly shown to result from an interaction outside the active site of HPAO, the cause of the absorbance remains unclear.

Oxygen-Dependent Intermediate Is Observed in WT-HPAO. As no intermediates in tyrosine oxidation were observed upon anaerobic addition of Cu(II), we investigated whether any intermediates could be observed following the addition of oxygen to apo-WT preincubated anaerobically

with Cu(II). An absorbance can be seen at 350 nm following exposure to oxygen. This species forms and decays rapidly enough to be on the pathway of the reaction (Figure 3), which suggests that it is an intermediate in TPQ formation. Knowing the chemical nature of the species could provide very important evidence regarding the mechanism of biogenesis.

The kinetics of the intermediate formation can be fit with rate constants of 0.51 ± 0.01 and 0.076 ± 0.003 min^{-1}. These rate constants are consistent with the observed rate of TPQ formation of 0.068 ± 0.002 min^{-1}. Fitting the data cannot distinguish the rate constant of formation from the rate constant for decay of the intermediate. The extinction coefficient predicted for the intermediate, though, can indicate whether the kinetics are reasonable for the reaction. The predicted extinction coefficients for the 350 nm species are 3200 M^{-1} cm^{-1} if there is a fast formation and slow decay and 23 000 M^{-1} cm^{-1} if there is a slow formation and fast decay.

Model compounds of the species expected to be intermediates in this reaction give a foundation for determining what a reasonable extinction coefficient would be for this reaction. In model compounds, the following absorbances (and extinction coefficients) have been determined: tyrosyl radical absorbs at 407 nm (3200 M^{-1} cm^{-1}) (*27*), tyrosinate-Cu(II) LMCT band absorbs around 340−530 nm (1000−3000 M^{-1} cm^{-1}) (*28*), Cu(II)-superoxo species absorb around 350−410 nm (2000−8000 M^{-1} cm^{-1}) (*29*), dopa quinone absorbs at 386 nm (1100 M^{-1} cm^{-1}) (*30*), and topa absorbs between 295 and 315 nm (*31*) (3200 M^{-1} cm^{-1}; M. Mure, unpublished work).

An extinction coefficient of 23 000 M^{-1} cm^{-1} is significantly higher than the extinction coefficient of any of the expected intermediates in the reaction, making a slow formation and fast decay of the 350 nm species an extremely unlikely scenario. We therefore conclude that the 350 nm species forms at a rate of 0.51± 0.01 min^{-1}, decays at a rate of 0.076 ± 0.003 min^{-1}, and has an extinction coefficient of 3200 M^{-1} cm^{-1}.

The experimental λ_{max} and extinction coefficient are consistent with either a Cu(II)−tyrosinate or a Cu(II)−superoxo species. To form a Cu(II)−superoxo intermediate, tyrosyl radical would have already been formed. Tyrosyl radical should have a detectable visible absorbance; however, we only see a single absorbance at 350 nm, apparently ruling out a Cu(II)−superoxo species. Additionally, comparison of the rate of formation of the 350 nm species, 0.51 ± 0.01 min^{-1}, with the rate of oxygen consumption, 0.1 ± 0.03 min^{-1}, demonstrates an unexpected result: although the 350 nm species requires the presence of oxygen for formation, *it is formed more rapidly than oxygen in consumed.* This kinetic information greatly reduces the possiblities for the identity of the 350 nm species. Before the first reaction with oxygen, there is only tyrosine and Cu(II), i.e., a Cu(II)−superoxo species cannot be formed unless this species is in rapid equilibrium with free O_2. Thus, on the basis of the spectral properties of the intermediate and the kinetics of the reaction of oxygen, we propose that the 350 nm intermediate is a Cu(II)−tyrosinate species.

It would be best to confirm the nature of the species directly by resonance Raman spectroscopy. Unfortunately, the absorbance was not amenable to study by resonance Raman spectroscopy because of the low wavelength, small extent of absorbance, and short time scale of accumulation. Instead, site-directed mutagenesis was utilized as a method for prolonging or altering the 350 nm species and ultimately allowing a kinetic and spectroscopic characterization of the precursor to TPQ.

Role of Consensus Site Residues in Biogenesis. N404D and E406Q, mutants of the residues immediately preceding and following the precursor tyrosine, respectively, were shown to decrease the rate of biogenesis (Table 1). During catalysis, these residues have been postulated to position the mature cofactor (*19*−*21*). If their role in biogenesis is to position an intermediate in tyrosine oxidation, mutants at these positions may show very different time-dependent changes in absorbance. In fact, when biogenesis is initiated with oxygen after prebinding Cu(II) anaerobically, no oxygen-dependent absorbances accumulate, not even the 350 nm absorbance seen with WT. As all of the oxidized intermediates in the reaction are expected to have visible absorbance (see above), this suggests that the slow rate of biogenesis in these mutants is due either to the accumulation of an unreactive form of the precursor tyrosine or other deformations within the active site. This points toward a role for each of these residues in positioning the precursor tyrosine for the first step of biogenesis in the WT enzyme.

H624C Binds Copper Stoichiometrically. In contrast to alterations at the N404 and E406 side chains, a mutation was made to one of the Cu(II) ligands with the hope of increasing the extent of accumulation of the 350 nm species. His-624 was mutated to a cysteine because cysteine is known to ligand Cu(II) in other enzymes (*32*, *33*). Position 624 was chosen specifically because there is crystallographic evidence suggesting mobility of this copper-binding residue (*13*), which might permit a change in the length of the ligand.

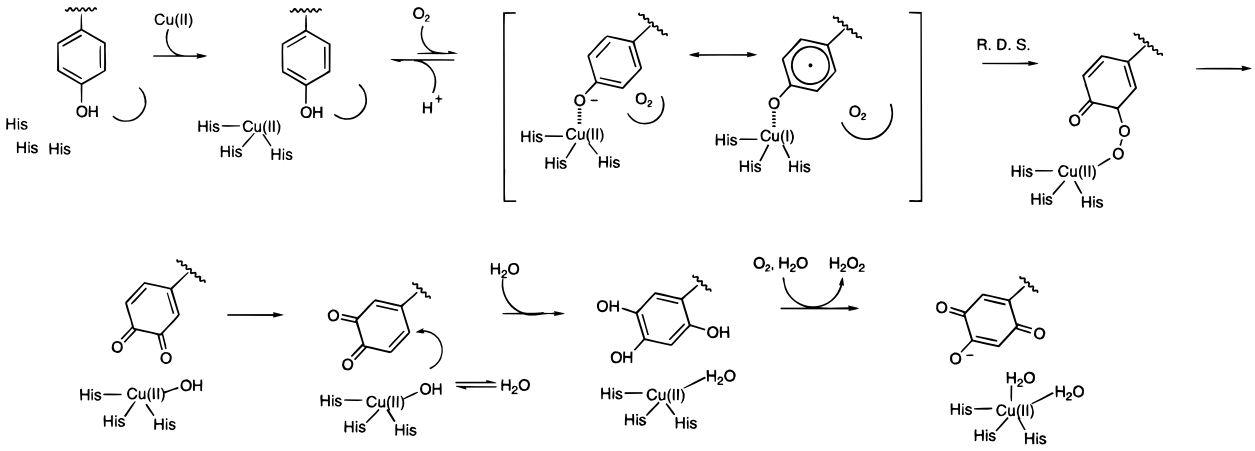
A copper-dependent absorbance which persists at 400 nm is seen when Cu(II) is added anaerobically to apo-H624C. The fact that this absorbance is not observed when Cu(II) is added to apo-enzyme prebound with Zn(II) argues strongly that the 400 nm absorbance results from an interaction in the normal copper-binding site of the enzyme.

This is supported by work on a copper−zinc superoxide dismutase (CuZnSOD), where mutation of one of the nonbridging Cu(II) ligands from histidine to cysteine resulted in an absorbance at 406 nm, with an extinction coefficient of 1250 M^{-1} cm^{-1}(*32*). In that case, the assignment of this absorbance as a Cys to Cu(II) LMCT band was confirmed by EPR and resonance Raman spectroscopy (*32*, *33*). The result in CuZnSOD is in excellent agreement with both the maximal wavelength and the extinction coefficient for the absorbance seen with H624C, 400 nm and 800 M^{-1} cm^{-1}, respectively. The 400 nm absorbance is, therefore, tentatively assigned as a Cys to Cu(II) LMCT band. In future work, EPR and resonance Raman spectroscopy will be used to characterize further the Cys−Cu(II) species.

Oxygen-Dependent Intermediate Is Observed in H624C Biogenesis. An oxygen-dependent absorbance accumulates at 390 nm during H624C biogenesis. This species is kinetically competent to be an intermediate in the biogenesis; it appears and decays before the formation of TPQ (Figure 5).

Again, two possible scenarios were found to fit the kinetics of the 390 nm species. A fast formation and slow decay of

Scheme 2: New Proposal for TPQ Biogenesis Mechanism[a]



[a] R. D. S. indicates rate-determining step.

the 390 nm band, 0.042 ± 0.002 and 0.0011 ± 0.0001 min^{-1}, suggests a species with an extinction coefficient of 2000 M^{-1} cm^{-1}. Alternatively, a slow formation and fast decay, 0.0011 ± 0.0001 and 0.042 ± 0.002 min^{-1}, suggests 88,000 M^{-1} cm^{-1} as the extinction coefficient for the 390 nm species. Both scenarios are consistent with the observed rate of TPQ formation of 0.0011 ± 0.0007 min^{-1}.

A comparison of these two possible scenarios with known extinction coefficients for species expected to be intermediates in biogenesis again provides a basis for evaluating their plausibility (see above). On the basis of this information, it was concluded that an extinction coefficient of 88 000 M^{-1} cm^{-1} was unreasonably high for an intermediate in TPQ formation. Thus, the rate constants for formation and decay of the 390 nm species are concluded to be 0.042 ± 0.002 and 0.0011 ± 0.0001 min^{-1}, respectively. Comparison of the rate of formation of the 390 nm species, 0.042 ± 0.002 min^{-1}, with the rate of oxygen consumption in H624C, <0.003 min^{-1}, is also quite informative. It can be seen that this intermediate is formed much more rapidly than O_2 is consumed, appearing to eliminate species that follow the reaction with oxygen and once again implicating a Cu(II)−tyrosinate intermediate.

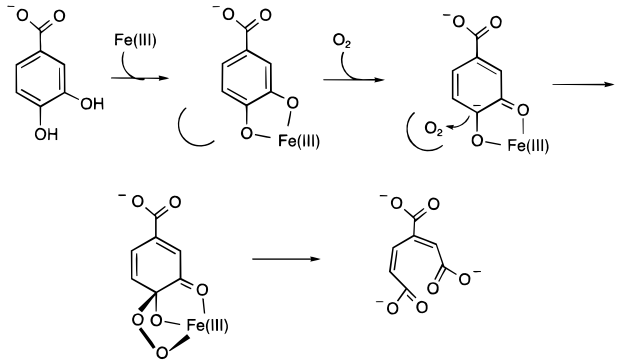
Cu(II)−Tyrosinate Is Proposed To Be the Intermediate Observed in WT-HPAO and H624C Biogenesis. One effect of changing the Cu(II) ligand from His to Cys will be to decrease the Lewis acidity of the Cu(II). Using Fe(III)-catecholate model compounds which react with molecular oxygen without any detectable reduction in Fe(III) by EPR, it has been shown that decreases in Lewis acidity can slow the reaction with molecular oxygen (*34*).

If this example is extrapolated to HPAO, then a decrease in Lewis acidity of the active-site copper will decrease the reactivity of the Cu(II)−Tyr species toward oxygen and, thus, prolong its lifetime. This trend is observed with the WT and H624C mutant. In WT-HPAO, the rate of decay of the 350 nm species is 0.076± 0.003 min^{-1}, and the rate of oxygen consumption is 0.1 ± 0.03 min^{-1}. In H624C, where the Lewis acidity of the Cu(II) is expected to be decreased, the rate of decay of the 390 nm species is 0.0011 ± 0.0001 min^{-1}, and the rate of oxygen consumption in H624C is <0.003 min^{-1}. Thus, the rate of breakdown of the proposed Cu(II)−Tyr intermediate and the rate of the reaction of oxygen are both slowed about 100-fold. The λ_{max} for the species is 40 nm red shifted in the H624C mutant, but this is not surprising for Tyr−Cu(II) CT bands which are known to show variable absorbances depending on the geometry and ligands at the copper site (*28*). It seems quite reasonable that the 350 nm intermediate seen with WT and the 390 nm intermediate seen with H624C are the same Tyr−Cu(II) species.

New Proposed Mechanism. On the basis of the evidence supporting a Tyr−Cu(II) species as an intermediate in biogenesis and, importantly, that the presence of oxygen is required for, but not consumed during the formation of this intermediate, a new mechanism for the biogenesis of TPQ is proposed (Scheme 2). Cu(II) enters the metal-binding site prior to O_2; at this point, the precursor tyrosine is not a ligand to Cu(II). When O_2 is introduced, it affects the positioning of the precursor tyrosine such that it becomes a ligand of Cu(II). This is proposed to be the step which is slowed in the consensus site mutants, E406Q and N404D. This positioning effect might be caused by O_2 binding into a hydrophobic pocket in the active site of the enzyme, as has been suggested to occur during catalysis (*9*). This tyrosinate−Cu(II) species has partial tyrosyl radical−Cu(I) character which facilitates the reaction with dioxygen. A very small extent of radical character is consistent with the inability to trap tyrosyl radical with nitric oxide. In a rate-determining step, oxygen reacts with the tyrosine and copper unit. This is proposed to be the step in biogenesis slowed by the copper ligand mutant, H624C. We cannot currently distinguish between a mechanism in which the oxygen reacts first with Cu(I) and a mechanism where the oxygen reacts first at the tyrosyl radical. In either case, a bridging peroxy intermediate is expected to form rapidly. The peroxy species can breakdown to give dopa quinone and Cu(II)-hydroxide. Nucleophilic attack of hydroxide on dopa quinone generates the reduced form of TPQ which can be oxidized by molecular oxygen to give the oxidized, resting form of cofactor. This mechanism for the biogenesis of TPQ is consistent with a detailed kinetic investigation of the reaction of precursor protein with oxygen (*23*) and shows some intriguing similarity to the proposed mechanism for the oxidative half reaction of catalysis (*9*, *23*).

Comparison with Other Oxygen-Utilizing Metalloenzymes. Detailed studies on protocatechuate 3,4-dioxygenase (PCD)

Scheme 3: Proposed Catalytic Mechanism for Protocatechuate 3,4-Dioxygenase [adapted from Orville et al. (*35*)]



have elucidated some interesting mechanistic features which are analogous to the current proposal for TPQ biogenesis. The proposed mechanism for PCD is shown in Scheme 3 (*35*). The substrate, protocatechuate (PCA), binds to the enzyme and is coordinated to Fe(III). The binding of substrate opens up a pocket for oxygen to bind near PCA, but not at the Fe(III) site. The reaction of PCA and oxgyen results in a peroxy species which can now coordinate to Fe(III). Further reaction results in the ring opened product.

There are two key similarities between the PCD mechanism and the proposed TPQ biogenesis mechanism. The first is the use of a redox metal for organic substrate activation. The second is an initial binding of molecular oxygen at a nonmetal site. In the case of PCD, it's proposed that the liganding of the substrate by the metal causes the formation of the oxygen-binding pocket (*35*). In the case of TPQ biogenesis, it is proposed that the oxygen binding at a pocket in the active site causes the liganding of the substrate tyrosine to copper.

ACKNOWLEDGMENT

We thank Dr. Joann Sanders-Loehr, Dr. Pierre Moënne-Loccoz, and Ed Green for helpful discussion and advice, and Stephen Mills for ICP analysis.

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BI992225W