# Stoichiometry of the Topa Quinone Biogenesis Reaction in Copper Amine Oxidases†

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ABSTRACT: The stoichiometry of the topa quinone biogenesis reaction in phenylethylamine oxidase from *Arthrobacter globiformis* (AGAO) has been determined. We have shown that the 6e$^-$ oxidation of tyrosine to topa quinone (TPQ) consumes 2 mol of $O_2$ and produces 1 mol of $H_2O_2$/mol of TPQ formed. The rate of $H_2O_2$ production is first-order ($k_{obs} = 1.0 \pm 0.2$ min$^{-1}$), a rate only slightly lower than the rate of TPQ formation directly determined previously ($k_{obs} = 1.5 \pm 0.2$ min$^{-1}$). This gives the following net reaction stoichiometry for TPQ biogenesis: E−Tyr + $2O_2$ → E−TPQ + $H_2O_2$. This stoichiometry is in agreement with recently proposed mechanisms for TPQ biogenesis, and rules out several possible alternatives.

Copper-containing amine oxidases are dimeric proteins that contain both a single active-site type(II) copper ion and a protein-derived organic cofactor, topaquinone (TPQ)[1] in each subunit (*1*−*4*). The TPQ cofactor is formed in a novel posttranslational self-processing reaction from a specific tyrosine residue located in the highly conserved sequence Thr-X-X-Asn-Tyr(TPQ)-Asp/Glu-Tyr (*5*−*7*). Initial studies have shown that the generation of TPQ requires the addition of both Cu(II) and $O_2$ to the unprocessed apoprotein, or the addition of $O_2$ to the anaerobically stabilized complex of copper and the unprocessed apoprotein (*7*−*12*). In the presence of excess oxygen, the rate of TPQ formation is consistent with a single first-order process. The rate is independent of copper concentration, as long as one copper per active site is present, indicating that only the active-site-bound copper participates in the biogenesis reaction (*11*). This is also supported by mutagenesis studies which have shown that replacement of a copper-binding His residue with Asp inhibits copper binding and prevents TPQ biogenesis (*9, 13*).

Several mechanisms for the oxidation of Tyr to TPQ in amine oxidases have been proposed (*7*−*12, 14*). To critically assess these and other possible mechanisms, the stoichiometry of the TPQ biogenesis reaction must be known unequivocally. However, the exact stoichiometry of this reaction has not been previously determined. Several distinct stoichiometries can be envisioned for this 6e$^-$ process (Figure 1). Note that Figure 1 emphasizes possible overall stoichiometries for the reaction. The various possibilities are not distinguished in terms of the mechanisms of oxygen or tyrosine activation, or the fates of the oxygen atoms in $O_2$. Initial proposals required 2 external reducing equiv: 1 reducing equiv to reduce the active site Cu(II) to Cu(I), thereby activating it for reaction with an initial dioxygen molecule; and a second reducing equivalent to give a Cu(II)−peroxo species [instead of a Cu(II)−superoxo species] as the agent for the hydroxylation of Tyr to dopa (*9*). To complete this mechanism, two more electrons reduce a second oxygen molecule to hydrogen peroxide, concomitantly oxidizing dopa to dopa quinone or topa. Subsequently, a third oxygen molecule would be reduced by the final two electrons to give TPQ and a second molecule of hydrogen peroxide as products (Figure 1A). Alternatively, the second oxygen molecule may be reduced by four electrons to give water as the only product (Figure 1B).

It is highly unlikely that there is a general requirement for reducing equivalents to be provided by the protein (e.g., via cysteine oxidations to a disulfide) because the TPQ-containing *E. coli* AO has no cysteine residues (*15*). Additionally, we and others have found that no external reductants are required for the biogenesis reaction (*10, 11*). Therefore, we have envisioned mechanisms that do not invoke external reductants. The minimum requirements for the formation of TPQ from tyrosine would be 2 mol of dioxygen consumed per mole of TPQ formed, giving 1 mol of peroxide as a product (Figure 1C). In this reaction pathway, one oxygen molecule is reduced by four electrons, which oxidizes tyrosine to the dopa quinone/topa level. A second oxygen molecule is then reduced by the final two electrons to give TPQ and a single molecule of hydrogen peroxide as products (Figure 1C). The same stoichiometry would result if the first oxygen molecule only accepted two electrons and the second accepted four electrons. Alternatively, the second oxygen molecule could accept two electrons from *each* of the active sites of the dimer, giving only water as a product (Figure 1D). This is implausible for the following reason: the dimers are connected by two bridging arms, one of which terminates close to the active site of the other subunit, but the copper ions, or the TPQ groups, are over 30 Å apart, making electron transfer between these sites highly improbable (*15*−*17*).

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[1] Abbreviations: AGAO, *Arthrobacter globiformis* amine oxidase, formerly labeled phenylethylamine oxidase (PEAO); ECAO, *E. coli* amine oxidase; PSAO, pea seedling amine oxidase; Topa, 2,4,5-trihydroxyphenylalanine; TPQ, topa quinone; DPQ, dopa quinone; HRP, horseradish peroxidase.

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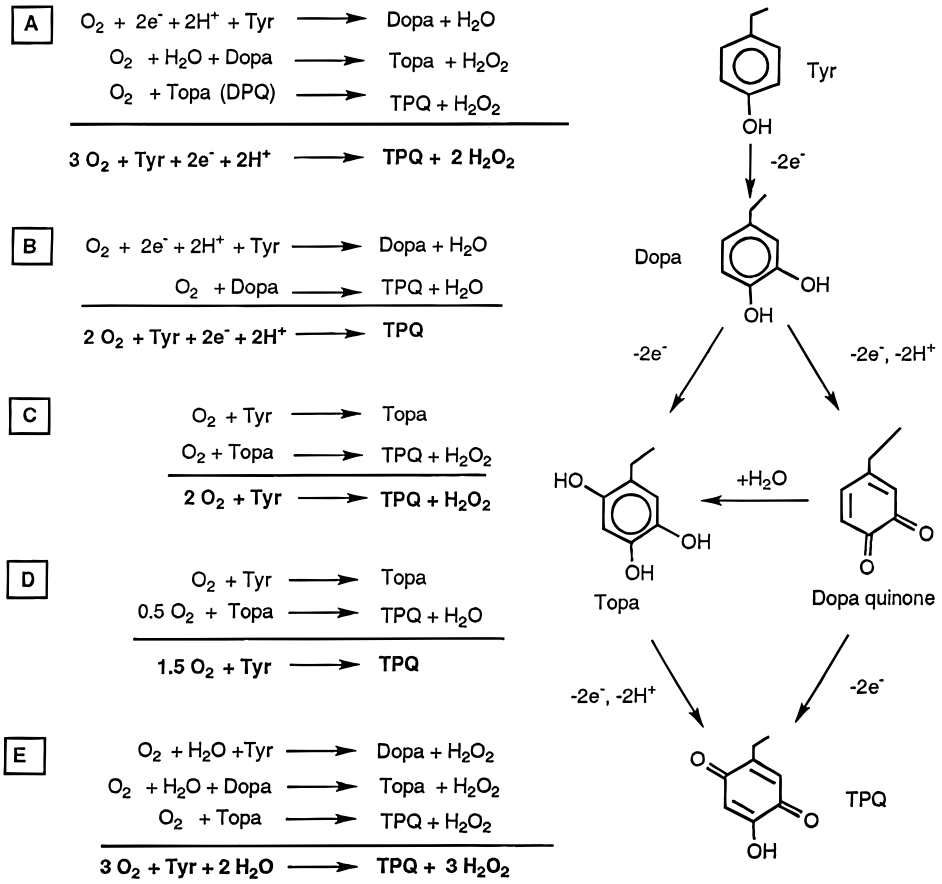



FIGURE 1: Possible stoichiometries for TPQ biogenesis. In all reactions 'Topa' can be replaced by 'DPQ + $H_2O$', giving the identical final stoichiometry.

A different stoichiometry would be obtained if only two electrons reduced each dioxygen. Thus, the oxidation of tyrosine to the dopa level would reduce the first dioxygen to peroxide, and subsequent oxidation steps would reduce the second and third equivalents of dioxygen to peroxide (Figure 1E). For completeness, we note that any one, or all, of these steps could in principle be replaced by a dioxygen accepting two electrons from each active site. It is conceivable that $H_2O_2$ produced at one site could be used as the oxidant at the second site. The resulting stoichiometries range from 3 $O_2$/0 $H_2O_2$ to 1.5 $O_2$/$H_2O_2$. As indicated above, we feel these possibilities are implausible based on the known structures of amine oxidases. Such scenarios also imply that the mechanism of tyrosine oxidation would differ between the two active sites of the dimer, but there is no evidence for this.

In summary, stoichiometries that consume from 1.5 to 3 mol of $O_2$ and produce from 0 to 3 mol of $H_2O_2$ per TPQ formed may be envisioned. These different stoichiometries have distinct mechanistic implications. Therefore, it is important to establish the stoichiometry for TPQ biogenesis under controlled conditions. The results of our determination of the stoichiometry of the TPQ formation reaction in phenylethylamine oxidase from *Arthrobacter globiformis* (AGAO) are presented herein.

## EXPERIMENTAL PROCEDURES

To remove traces of metal ions, all glassware was washed with 1:1 nitric/sulfuric acid and all plasticware soaked in 1 mM EDTA. All buffer solutions were passed through a Chelex-100 (Biorad) column. Protein purification and TPQ titrations with phenylhydrazine were previously described (*11*, *18*). All reagents were purchased from Sigma.

*$H_2O_2$ Production.* UV−visible spectra were obtained using a Hewlett-Packard Diode Array Spectrometer (HP8452A) equipped with a circulating cell holder attached to a 30 °C water bath. Hydrogen peroxide production was monitored using a spectrophotometric, coupled assay in which 4-aminoantipyrine is oxidized by HRP and condenses with vanillic acid to give a bright red quinoneimine dye (*19*). The amount of $H_2O_2$ produced is directly proportional to the change in absorbance due to dye formation ($\lambda_{max} = 496$ nm, $\epsilon = 6000$ $M^{-1}$ $cm^{-1}$). This molar extinction coefficient was determined by $H_2O_2$ titration at the exact pH and concentration conditions used in the peroxide production experiments (final dye solution = 20 units/mL HRP, 1 mM vanillic acid, 0.5 mM 4-aminoantipyrine in Hepes buffer, pH 6.8). The TPQ biogenesis reaction was initiated by the addition of copper (∼10-fold molar excess) to a cuvette containing the dye solution and the apo, unprocessed enzyme (final concentration 0.03−0.07 mM enzyme subunit, 0.5−0.8 mL total reaction volume). Alternatively, the reaction was initiated by the addition of a concentrated stock of $O_2$-saturated indicator dye solution to the enzyme preincubated with copper in Hepes buffer under anaerobic conditions. The change in absorbance at 496 nm was monitored over time. The amount TPQ formed was determined by phenylhydrazine titration. The rate of $H_2O_2$ formation was determined by fitting the data 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 496 nm at time $t$, $A_\infty$ is the end point absorbance calculated from the Kedzy−Swinbourne analysis, and $A_o$ is the initial absorbance (*20*, *21*). Data were linear to at least 3 half-lives. Control experiments using native enzyme and benzylamine as the substrate established that the expected amount of $H_2O_2$ could be detected.

*$O_2$ Consumption.* Dioxygen consumption was monitored using a computer-interfaced, Clark-type electrode (Instech). The electrode was placed in a sealed 3.8 mL vial containing the apo, unprocessed enzyme (∼0.05−0.10 $\mu$mol of enzyme subunit) in Hepes buffer, and allowed to equilibrate. After the electrode stabilized, 30−50 $\mu$L of 10 mM $CuSO_4$ was injected into the reaction flask to initiate TPQ formation. The decrease in dissolved oxygen concentration was tracked for 10 min. The amount of oxygen consumed was compared to the amount of TPQ formed (determined by direct titration of the reaction flask after the TPQ formation reaction was completed, typically 0.65−0.85 mol of TPQ/mol of subunit). Oxygen consumption during the reaction was corrected for the efficiency of the system. The efficiency was determined by using identical reaction conditions, but with a small amount of native, copper-containing amine oxidase (produced by incubating the apo enzyme with copper) in the reaction flask (∼0.001−0.003 mg/mL), and using dopamine (0.1−0.4 $\mu$mol), which is a slower substrate than phenylethylamine (*22*), to initiate the substrate-turnover reaction. This reaction is known to consume 1 mol of $O_2$/mol of amine substrate oxidized (*23*, *24*). Efficiencies were determined immediately after most experimental runs using the same batch of enzyme employed for the biogenesis experiments. Typically, 70−80% of the theoretical amount of $O_2$ consumption was detected in our system under the stated conditions. A possible reason for the <100% efficiency would be the presence of trace amounts of catalase, which would produce $O_2$ from the $H_2O_2$ product. The rate of $O_2$ consumption was estimated by fitting the data to standard linear second-order kinetic equations: $\ln([B_t]/[A_t]) = \ln([B_0]/[A_0]) + (2[B_0] - [A_0])kt$, where $[A_t]$ is the concentration of $O_2$ at time $t$, $[B_t]$ is the concentration of unreacted enzyme subunit at time $t$ (derived from the amount of $O_2$ consumed using a 2:1 stoichiometry), and $[B_0]$ and $[A_0]$ are the initial concentrations of reactants. Data were linear ($R > 0.99$) to approximately 75−80% reaction completion. A second-order analysis was employed because in some runs the total $O_2$ consumed was 15−20% of the total. Further, at the highest protein concentrations used, it was not possible to obtain a 10-fold excess of $O_2$ over protein.

## RESULTS

*$H_2O_2$ Production.* To determine if $H_2O_2$ is produced during the biogenesis of TPQ, a coupled spectrophotometric assay was used in which the absorbance change due to dye formation (496 nm) is directly proportional to the amount of hydrogen peroxide produced (Figure 2). The change in absorbance was corrected for overlapping TPQ absorbance ($\lambda_{max} = 474$ nm, $\epsilon = 2500$ $M^{-1}$ $cm^{-1}$) and for a small, but detectable background rate of quinoneimine dye formation. The absorbance at 496 nm was used to calculate the amount of peroxide produced and was compared to the amount of TPQ formed. In all runs, a value close to 1 mol of $H_2O_2$/mol of TPQ produced was measured (Table 1). The rate of peroxide production was also determined using conditions similar to those used in previous rate experiments (*11*), yielding $k_{obs} = 1.0 \pm 0.2$ $min^{-1}$ (Table 1).

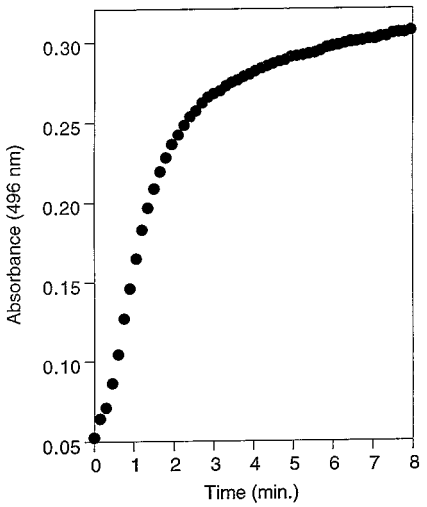



FIGURE 2: Production of $H_2O_2$ during TPQ biogenesis monitored by formation of the indicating dye at 496 nm [0.018 $\mu$mol of TPQ formed, 0.021 $\mu$mol of $H_2O_2$ produced ($\Delta A_{corrected} = 0.16$, $V = 0.8$ mL, $\epsilon = 6000$ $M^{-1}$ $cm^{-1}$)].

Table 1: Production of $H_2O_2$ during TPQ Biogenesis[a]

| run no. | TPQ formed ($\mu$mol) | $H_2O_2$ produced ($\mu$mol) | mol of $H_2O_2$/ mol of TPQ | rate ($min^{-1}$) |
|---|---|---|---|---|
| 1 | 0.017 | 0.017 | 1.0 | 0.7 |
| 2 | 0.014 | 0.017 | 1.2 | 1.0 |
| 3 | 0.018 | 0.021 | 1.2 | 1.2 |
| 4 | 0.037 | 0.030 | 0.8 | 1.2 |
| 5 | 0.027 | 0.023 | 0.9 | 1.1 |
| 6 | 0.027 | 0.028 | 1.0 | 1.0 |

[a] Average = 1.0 $H_2O_2$/TPQ, SD = 0.16; rate = 1.0 $min^{-1}$, SD = 0.20.

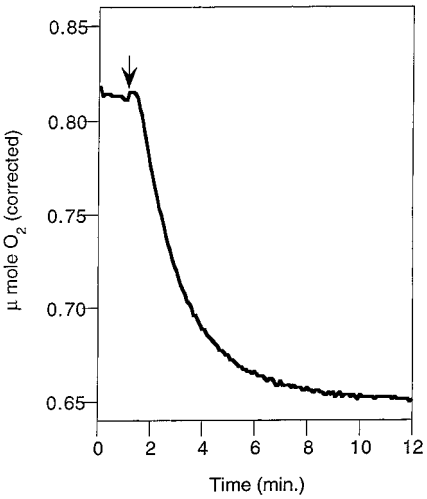



FIGURE 3: Consumption of $O_2$ during TPQ biogenesis monitored using the oxygen electrode. The arrow indicates addition of copper to the reaction (0.086 $\mu$mol of TPQ formed, 0.16 $\mu$mol of $O_2$ consumed).

*$O_2$ Consumption.* To determine the amount of $O_2$ consumed during TPQ biogenesis, the decrease in $O_2$ concentration during the TPQ formation reaction was monitored using an oxygen electrode (Figure 3). The amount of oxygen consumed, after correction for the measured electrode

Scheme 1: Biogenesis of TPQ. Adapted from (*17*)

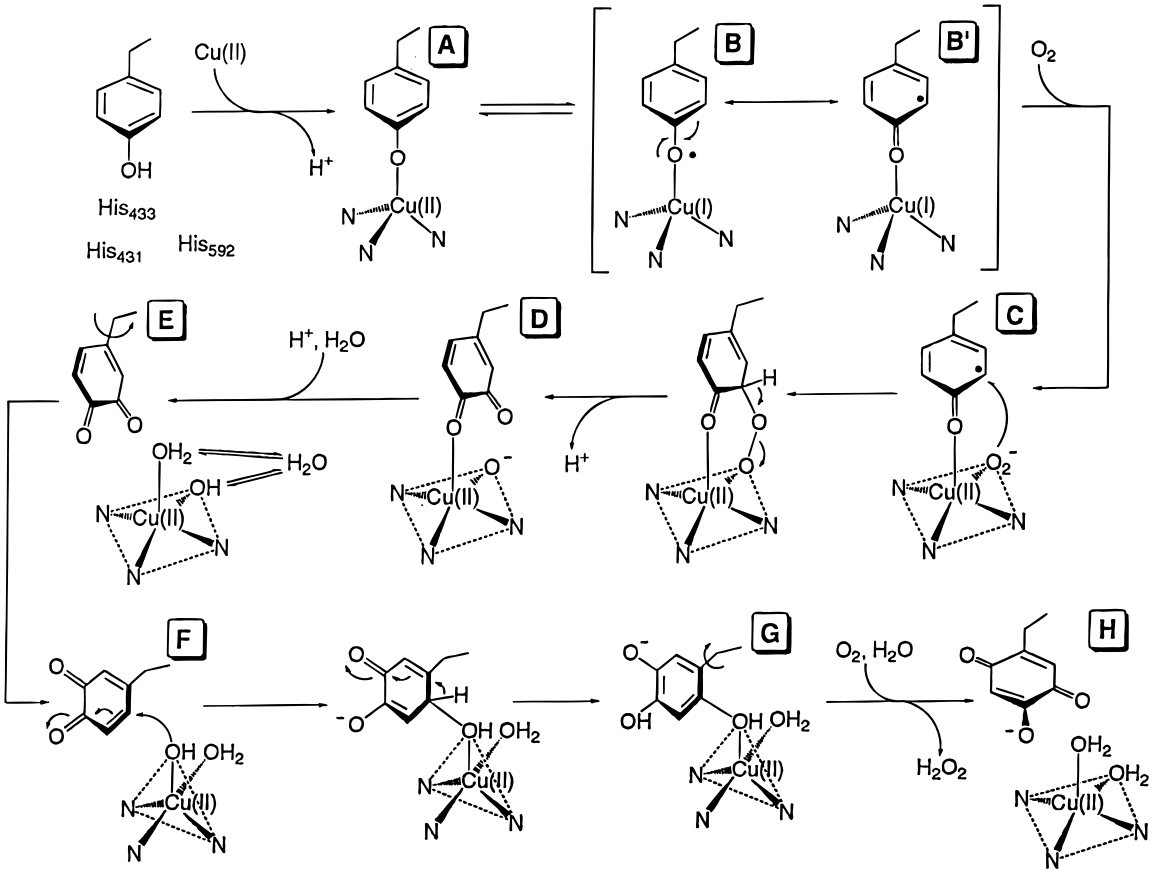



Table 2: Consumption of $O_2$ during TPQ Biogenesis[a]

| run no. | TPQ formed ($\mu$mol) | $O_2$ used ($\mu$mol) | mol of $O_2$/mol of TPQ | rate ($mM^1$ $min^{-1}$) |
|---|---|---|---|---|
| 1 | 0.13 | 0.24 | 1.9 | 3.3 |
| 2 | 0.10 | 0.18 | 1.8 | 2.8 |
| 3 | 0.052 | 0.093 | 1.8 | 1.7 |
| 4 | 0.087 | 0.16 | 1.9 | nd[b] |
| 5 | 0.047 | 0.085 | 1.8 | 3.0 |
| 6 | 0.072 | 0.17 | 2.4 | nd[b] |
| 7 | 0.074 | 0.16 | 2.2 | 2.4 |
| 8 | 0.086 | 0.16 | 1.9 | 2.5 |

[a] Average = 2.0 $O_2$/TPQ, SD = 0.21; rate = 2.6 $mM^{-1}$ $min^{-1}$, SD = 0.56. [b] Rate not determined.

efficiency, was compared to the amount of TPQ formed in the reaction. In all runs, a value close to 2 mol of $O_2$/mol of TPQ formed was determined (Table 2).[2] The inherent rate of $O_2$ consumption could not be unambiguously determined because neither reactant was in pseudo-first-order excess, and we have not yet determined the reaction order in $O_2$. However, if we make the assumption that there is an initial rate-determining step involving the reaction of the first equivalent of $O_2$ with the enzyme, we can use a second-order rate equation: $d[O_2]/dt = 2k_1[E-Tyr][O_2]$. It is inherent in this assumption that all subsequent steps, and any previous steps, are rapid. The good fit of our data to the linear second-order rate equation does not necessarily indicate it is an accurate assumption. With this caveat, the calculated rate of $O_2$ consumption was found to be $2.6 \pm 0.6$ $mM^{-1}$ $min^{-1}$ (Table 2).

[2] Without correction for the efficiency of the electrode, values of 1.4−1.8 mol of $O_2$/mol of TPQ formed were determined.

## DISCUSSION

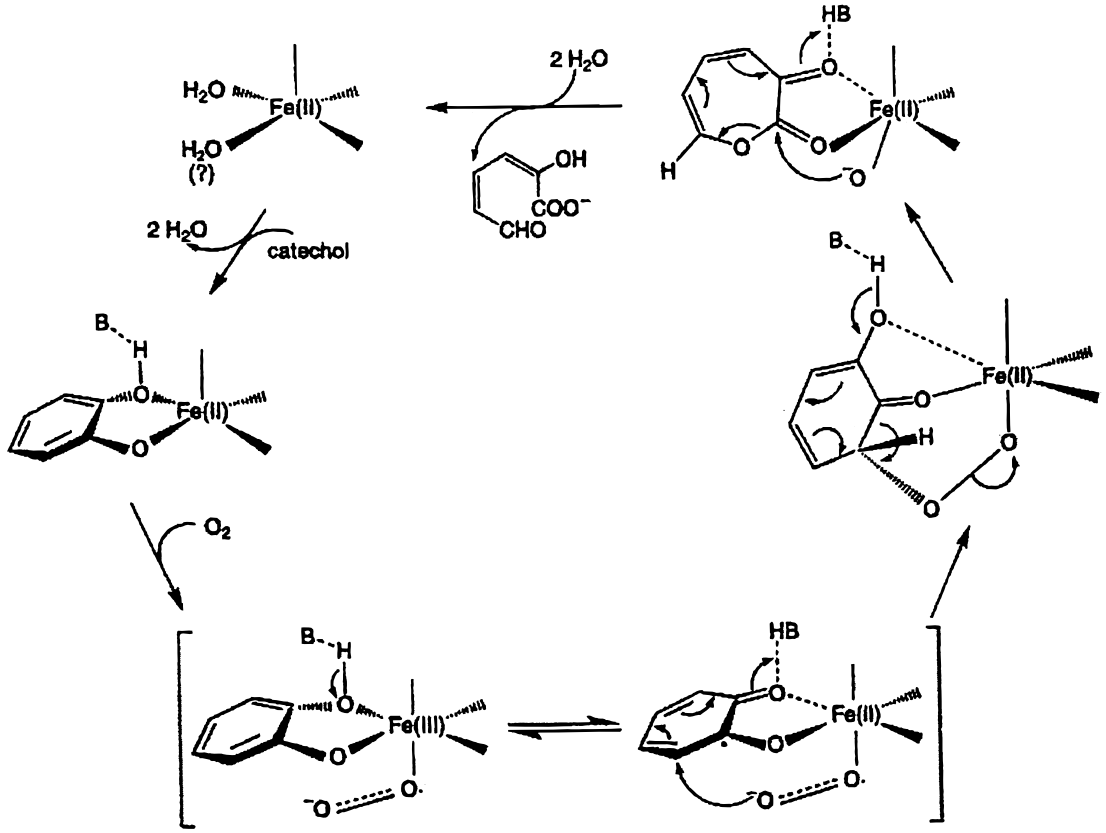
Before the adequacy of any proposed mechanism for the biogenesis of TPQ can be assessed, the exact reaction stoichiometry must be known. As outlined above, possible stoichiometries could range from 0 to 3 $H_2O_2$ produced, and from 1.5 to 3 $O_2$ consumed per mole of TPQ produced. Each of these stoichiometries would have distinct mechanistic implications.

We have shown using a coupled assay for hydrogen peroxide that the TPQ biogenesis reaction produced 1 mol of $H_2O_2$/mol of TPQ formed, and the rate of $H_2O_2$ production ($1.0 \pm 0.2$ $min^{-1}$) is only slightly less than the previously directly measured rate of TPQ formation ($k_{obs} = 1.5 \pm 0.2$). We attribute the slight disparity to the additional components in the reaction mixture, and the lack of stirring while the biogenesis reaction progressed *(11)*. The most plausible dioxygen stoichiometry consistent with a 1:1 ratio of peroxide per TPQ formed would utilize 2 mol of oxygen/TPQ. Indeed, our experiments detected the consumption of 2 mol of $O_2$ per TPQ formed (Table 2). This establishes the final reaction stoichiometry shown in eq 1:

$$2O_2 + E-Tyr \rightarrow E-TPQ + H_2O_2 \qquad (1)$$

This result is chemically satisfying because it is the simplest stoichiometry for the known reactants and products. Assuming that no electrons are shared between the active sites during the biogenesis reaction, this stoichiometry indicates

Scheme 2: Mechanism for Extradiol Ring Cleavage Catalyzed by the Fe(II)-Containing Catechol 2,3-Dioxygenase. Adapted from (*35*)




that one dioxygen is reduced by four electrons, and the second equivalent is reduced by two electrons to produce $H_2O_2$. Although the stoichiometry does not give us any insight into the order of these steps, the rate observations are informative. Because $H_2O_2$ forms at the same rate as TPQ, it must be produced during or after the rate-determining step. We believe it is produced in the final oxidation step of the reaction, when trihydroxyphenylalanine is oxidized to TPQ. Facile copper-catalyzed oxidation of reduced quinones and benzene triols by $O_2$ to yield quinones and $H_2O_2$ has been demonstrated (*25*−*28*). Keeping in mind that the rate dependence on $O_2$ still needs to be determined, and that the assumption that the initial reaction with $O_2$ is a rate-determining step and is untested (at this point), the rate of $O_2$ consumption calculated with this assumption is comparable to the rate of TPQ formation. The previously calculated pseudo-first-order rate constant, $k_{obs} = k_1[O_2] = 1.5$ min$^{-1}$ (*11*), was determined in $O_2$-saturated solution at 30 °C ([$O_2$] $\sim$ 1 mM), giving $k_1 \sim 1.5$ mM$^{-1}$ min$^{-1}$, in reasonable agreement with the calculated second-order rate constant, $2.6 \pm 0.6$ mM$^{-1}$ min$^{-1}$. The assumption of an initial slow step involving dioxygen is not unreasonable. The conversion of tyrosine to dopa is the initial and rate-determining step in the biosynthesis of catecholamine neurotransmitters (*29*). Further, the reductive activation of molecular oxygen is believed to be the rate-limiting step in the tyrosine hydroxylase reaction (*30*), and the oxidation of reduced quinones is known to be rapid, especially in the presence of metals (*25*−*28*).

We have previously proposed the mechanism shown in Scheme 1 for the biogenesis of TPQ, and this mechanism is fully compatible with the present stoichiometric results. Cu(II) binding to the apo-protein to give a Cu(II)−protein complex (**A**) has been directly demonstrated (*11*). This complex is suggested to activate the tyrosine for oxidation, as illustrated by the equilibrium with a Cu(I)−tyrosine radical (**B**). A resonance form of **B**, **B′**, is shown to depict the possibility that unpaired spin density may be delocalized over the ring, thereby activating the ring carbons. A "Cu(I)" species would be expected to react with dioxygen to form an activated oxygen complex, shown here as a copper−superoxide (*31*) (**C**). This activated oxygen complex then attacks the activated tyrosine to yield a bound dopa quinone and a copper hydroxide (**D**). Alternatively, $O_2$ might attack the activated phenolate ring directly (*32*). **D** → **G** invokes the formation of topa via nucleophilic attack on the dopa quinone intermediate. Scheme 1 outlines the role of a copper ion-based nucleophile, but it is conceivable that noncoordinated, active site water is involved. The copper hydroxide/water species (**E**) would likely be in rapid exchange with solvent water, allowing solvent oxygen incorporation into the C2 position of TPQ, as observed by resonance Raman spectroscopy using $^{18}$O-labeled water to follow isotopic exchange (*33*). Rotation about the $\beta$-carbon would be required to move the C2 ring carbon near the copper-bound hydroxide (**F**). Nucleophilic attack by the copper hydroxide on the dopa quinone (which should be very susceptible to nucleophilic attack) would yield topa (**G**). In the presence of dioxygen, topa would then rapidly oxidize to TPQ, producing $H_2O_2$ (**H**). The net loss of a hydrogen, as indicated by this mechanism, is expected based on the formation of TPQ, which has a p$K_a \sim 4$ (*34*), from tyrosine, which has a p$K_a \sim 10$.

Substantial precedent exists for the various steps in this mechanism. The initial steps in the mechanism are similar to the mechanism proposed for extradiol cleavage catalyzed by the Fe(II)-containing catechol 2,3-dioxygenase (*35*). In this case, spectroscopic data support a *monoanionic* binding mode for the catechol to iron, analogous to that suggested for tyrosine binding to copper. The mechanism proposed for the extradiol ring cleavage of catechol (Scheme 2) has clear similarities to the mechanism suggested for tyrosine oxidation in Scheme 1. Coordination of catechol to the mononuclear Fe(III) or Fe(II) centers is proposed to activate the catechol for $O_2$ attack in both intradiol-cleaving and extradiol-cleaving dioxygenases. Lipscomb, Que, and co-workers have suggested that the dianionic binding mode of the Fe(III)-containing enzymes is sufficient to activate the ring for direct attack by $O_2$, whereas monoanionic coordination to Fe(II) is postulated to favor attack of an activated Fe(III)$-O_2$- species (Scheme 2). Similarly, we suggest that tyrosine activation occurs through monoanionic coordination, and accordingly have depicted the hydroxylating agent as a Cu(II)$-O_2$- group. Alternatively, $O_2$ could directly attack the activated tyrosine (at step **C**) to give a transient alkyl peroxy radical and then coordinate to Cu to give alkylperoxo−Cu(II) species, similar to the intermediate proposed in the intradiol-cleaving catechol dioxygenase mechanism (*36*).

Recent crystal structure data from ECAO, PSAO, AGAO, and the methylamine oxidase from *Hansenula polymorpha* (*37*) show that the TPQ cofactor is in close proximity to the copper ion. However, there is considerable variation in the observed positions or orientations of TPQ within the active sites, and TPQ can clearly adopt multiple positions or conformations (*15*−*17*, *37*). In the unprocessed, apo-AGAO structure, the unmodified tyrosine is situated directly over the copper binding site, apparently in position to coordinate axially to active site bound Cu(II). Thus, the apo-AGAO crystal structure supports the hypothesis that Cu(II)−Tyr coordination is the initial step in TPQ formation (*17*). Both ECAO and AGAO can be crystallized in a form where TPQ is coordinated to the active site copper through its C4 oxygen (*17*). A comparison of the apo-AGAO structure and the form with TPQ coordinated to copper revealed that the position of coordinated TPQ and the unmodified Tyr in the apoprotein are essentially superimposable; this result further implicates direct tyrosine−copper coordination in TPQ biogenesis (*15*, *17*). In fact, all of the steps in Scheme 1 were found to be geometrically feasible when modeled into the structures of apo- and Cu-containing AGAO (*17*).

Chemical precedents for copper−oxygen reactivity and copper-catalyzed oxidations of phenols, dopa, and dopa quinone species relevant to the mechanism in Scheme 1 have been described previously (*14*, *17*, *28*, *38*, *39*). Recent modeling studies using copper in buffers at physiological pH have been interpreted as opposing direct copper involvement in the hydroxylation of dopa quinone (**E**→**F**). These results may have limited applicability to the enzyme mechanism due to the inherent insolubility of Cu(II) in aqueous or phosphate-buffered solutions near physiological pH, and the lack of characterization of the actual species involved in the chemistry (*40*).

Further understanding of the mechanism of TPQ biogenesis will require the investigation of the reaction order in $O_2$, examining isotope effects to determine more about the rate determining step(s), searching for intermediates such as the initial proposed tyrosine radical or partially oxidized dopa/DPQ species. This latter species might be chemically quenchable (*41*), or detectable by fluorescence spectroscopy (*42*). Additional study is also needed to understand the roles of other active site residues in the biogenesis reaction. For example, site-directed mutation of the conserved Asn adjacent to TPQ, and part of a hydrogen bonding network in the active site (*17*), substantially affects the rate of TPQ formation (*43*). Mutation of the conserved acidic residue (Glu/Asp) adjacent to TPQ produced variable effects, but a carboxylate group in this position does not seem to be required for TPQ biogenesis (*13*, *43*). Clearly the microenvironment of the tyrosine residue will influence the rate of the biogenesis reaction. Structural and spectroscopic studies of Cu(II) complexes of the wild-type precursor protein and active site variants should be very informative. Isotopic-labeling studies with $^{18}O_2$ would also be mechanistically informative.

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