Stoichiometry and Spectroscopic Identity of Copper Centers in Phenoxazinone Synthase: A New Addition to the Blue Copper Oxidase Family†

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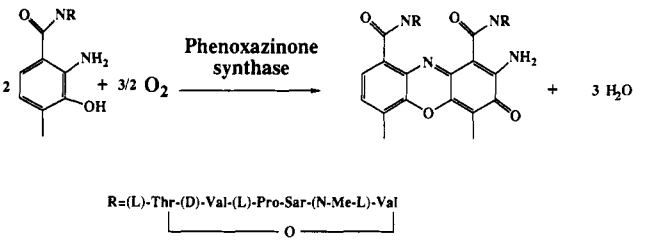
ABSTRACT: Phenoxazinone synthase catalyzes the oxidative condensation of two molecules of substituted *o*-aminophenols to the phenoxazinone chromophore of actinomycin. Cyclization occurs with the concomitant reduction of molecular oxygen to water. We have shown that the enzyme requires 4–5 copper atoms/monomer for full activity and the additional copper inhibits the enzyme. The optical absorption spectrum of phenoxazinone synthase is also dependent on the Cu per monomer ratio, and the absorption peak at 598 nm has a maximum extinction coefficient of 4000 ± 150 M^{-1} cm^{-1} at a ratio of 4–5 Cu atoms per monomer. The electron paramagnetic resonance (EPR) spectrum of enzyme as isolated with low copper content (0.8 Cu/monomer) only shows the presence of type 1 (blue) copper centers ($g_{\parallel}$ = 2.24, A = 0.0067 cm^{-1}, and $g_{\perp}$ = 2.07). Enzyme incubated with 4–5 Cu per monomer demonstrates the presence of both type 1 and type 2 copper centers with a stoichiometry of one type 1 center per monomer and the remainder bound as type 2 Cu^{2+}. Anaerobic incubation of substrate with enzyme containing five Cu atoms per subunit results in bleaching of the blue center. The EPR spectrum of the enzyme reduced under these conditions suggests that one of the type 2 Cu^{2+} centers with a $g_{\parallel}$ = 2.34, A = 0.016 cm^{-1}, and $g_{\perp}$ = 2.07 remains oxidized and is not involved in catalysis. From the spectroscopic data in this paper, phenoxazinone synthase appears to contain three functional copper atoms that can accept electrons from substrate and two additional copper atoms whose function has yet to be defined. The copper content and spectroscopic behavior of phenoxazinone synthase appear similar to many enzymes of the blue copper oxidase family.

The enzyme phenoxazinones synthase is naturally found in the bacterium *Streptomyces antibioticus* and has been cloned and overexpressed in *Streptomyces lividans* (Jones & Hopwood, 1984). Monomers of the enzyme were reported to have an estimated molecular mass of 88 000 Da (Choy & Jones, 1981), but recent analysis of the gene has led to a determination of a molecular mass of 67 500 Da (J. C. Freeman and J. J. Villafranca, unpublished results). The native enzyme is found in two forms: dimers, of low activity, and hexamers, of high activity. The oligomeric form of the enzyme depends on the age of the culture (Choy & Jones, 1981).

The enzyme catalyzes the oxidative coupling of two molecules of a substituted *o*-aminophenol to the phenoxazinone chromophore in the final step in the biosynthesis of the antineoplastic agent acinomycin D (Scheme I). The overall reaction represents a six-electron oxidative coupling that appears to take place stoichiometrically in a series of three steps (Barry et al., 1989). However, no evidence is available to establish whether electron transfer occurs in single-electron steps throughout the enzymatic cycle. Scheme II shows the probable reaction pathway consistent with the data (Barry et al., 1989).

Initial reports of the metal ion requirements for the enzyme listed copper as an inhibitory metal ion (Katz & Weissbach, 1962); later reports showed that the decrease in enzyme activity that occurred upon dialysis against cyanide was reversed by the addition of copper (Nishamura & Golub, 1969). In addition, the lag in enzyme activity exhibited in initial velocity

Scheme I: Biochemical Reaction Catalyzed by Phenoxazinone Synthase in Vivo



kinetic experiments can be eliminated by preincubation of the enzyme with copper, indicating that copper is involved in the catalytic process (Barry, 1989).

In prior work, when the enzyme was reconstituted with Cu^{2+} (3.7 Cu/monomer), the EPR[1] spectrum showed a type 2 copper signal while the visible absorption peak had an ϵ_{600} = 1700 M^{-1} cm^{-1} (Barry et al., 1989). An absorption band with this high an extinction coefficient is not usually associated with type 2 copper centers and warranted further examination of the number and types of copper ions present in the enzyme. The data in this paper demonstrate that phenoxazinone synthase contains both type 1 (blue) and type 2 copper centers and is a new member of the blue oxidase family of enzymes.

EXPERIMENTAL PROCEDURES

All materials were reagent grade unless specified. Hydroxylapatite was purchased from Bio-Rad Laboratories (Richmond, CA).

S. lividans was grown as described by Barry et al. (1989). Purification of phenoxazinone synthase followed the method

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[1] Abbreviations: DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance.

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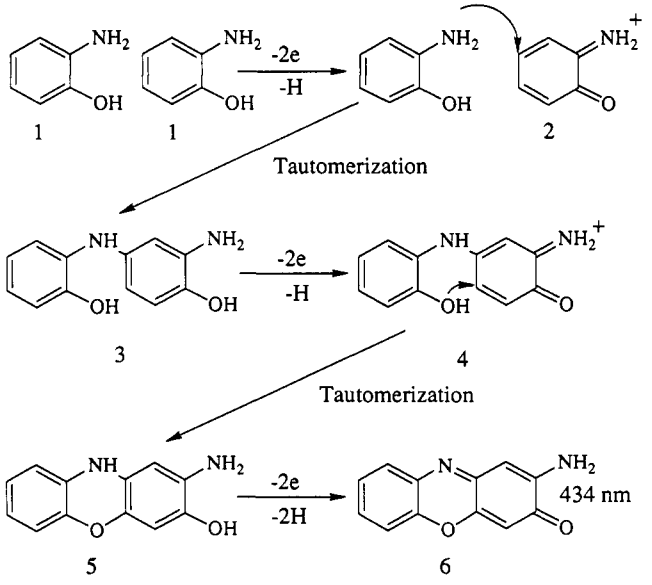
Scheme II: Reaction Pathway for Phenoxazinone Formation by an Oxidative Cascade[a]



[a] The step showing formation of compound **6** (the six-electron oxidation final product) from compound **5** (the four-electron oxidation product) takes place readily in the presence of atmospheric oxygen and does not require the presence of enzyme. Adapted from Barry et al. (1989).

of Barry et al. (1989) with the addition of an ammonium sulfate fractionation step following the hydroxylapatite chromatography step. The phenoxazinone synthase containing fractions were concentrated to approximately 2 mg/mL, and 50% ammonium sulfate (in water) was added at 4 °C over 30 min to reach a final concentration of 20%. The solution was stirred for an additional 30 min and then pelleted at 14500*g*. Protein was dissolved in 10 mL of 100 mM sodium phosphate, pH 6.0, followed by dialysis against 3 × 1 L of the same buffer at 4 °C.

Copper chloride in water was added to isolated enzyme in stoichiometric amounts, incubated overnight at 4 °C, and assayed for activity. These enzyme samples were analyzed for copper content, and spectroscopic parameters were obtained. Freshly isolated enzyme typically contained less than one Cu per monomer.

Protein determinations were obtained using the BCA assay from Pierce (Rockford, IL) with bovine serum albumin as a standard. Recent nucleotide sequencing of the cDNA encoding the phenoxazinone synthase gene provides a molecular mass of 67 500 Da for the mature protein (J. C. Freeman and J. J. Villafranca, unpublished results); this molecular mass was used to calculate the enzyme to copper ratios in the present paper.

Copper content was determined using a Perkin-Elmer 1100B atomic absorption spectrophotometer equipped with a Perkin-Elmer HGA 700 graphite furnace and a Perkin-Elmer AS-70 autosampler. Matrix effects were found to be negligible for samples containing less than 10 mM phosphate. Furnace conditions were 130 °C preconditioning, 1100 °C conditioning, and 2650 °C for atomization of the sample.

EPR spectra were taken on an ESP300E spectrometer from Brüker Instruments Inc, with the frequency set at 9.43 GHz. Standard conditions for spectral acquisition include 63 mW of power, center field at 3000 G, and a sweep width of 1000 G. Spectra shown are a compilation of eight scans. Spin counting (spectral integration) was performed by the technique of Broman et al. (1962) using CuEDTA as the standard. Anaerobic samples were alternately purged with argon and evacuated for several cycles. When substrate was added to

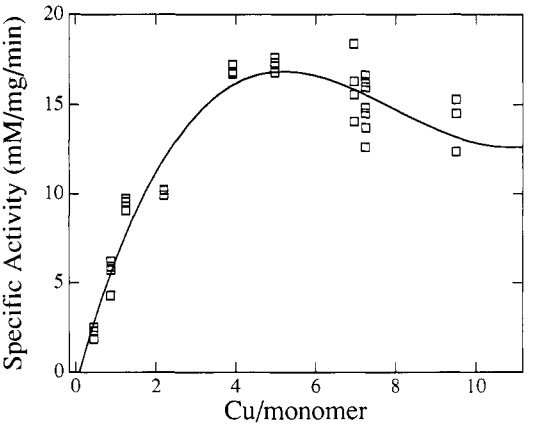


FIGURE 1: Specific activity of phenoxazinone synthase plotted against copper content per subunit. The activity was measured as described under Experimental Procedures.

enzyme under anaerobic conditions, a 100-μL syringe was used.

Optical spectra were obtained on either a Varian Cary 2200 interfaced to an IBM PS 2 using Spectra Calc software from Galactic Industries Co. or a Gilford Response spectrophotometer.

Enzyme activity was followed by monitoring the conversion of 2-aminophenol to phenoxazinone at 435 nm (ϵ = 23 200 M^{-1} cm^{-1}). Data were obtained on a Varian Cary 220 and analyzed using the subroutine StatPack contained within the Spectra Calc program. Assays were performed at 25 °C. In a typical experiment, 20 μL of 250 mM *o*-aminophenol in DMSO was added to 980 μL of 100 mM sodium acetate, pH 5.0, in a semimicrocuvette, and mixed by inversion. Phenoxazinone synthase (1–5 μg) was added to the cuvette, the reaction was initiated by inversion, and the cuvette was inserted into the spectrophotometer. The time elapsed between the mixing of the sample and data collection was less than 5 s. Linear steady-state rates were typically reached after 2 min, and the rates shown in Figure 1 were calculated using the data from 3–4 min.

For anaerobic incubation experiments, enzyme samples were sealed in optical semimicrocuvettes fitted with septa and purged with argon for 30 min. Substrate solutions and control solutions (DMSO without substrate) were purged with argon, withdrawn using a 100-μL syringe, and added to the cuvette; optical spectra were taken before and after the addition. Samples for mass spectral analysis were prepared by injecting 200 μL of methylene chloride (previously purged with argon) into the capped cuvette. The methylene chloride layer was removed by syringe and injected into a sealed tube purged with argon. After evaporation to dryness with a stream of argon, the solid residue was dissolved in 10 μL of methylene chloride. One microliter of the sample was analyzed using a Kratos Instruments mass spectrometer equipped with a direct insertion probe heated from 0 to 300 °C. Ionization was carried out using the electron impact method with an energy level of 60 eV and a source temperature of 200 °C.

RESULTS

Enzyme Activity vs Copper Content. Experiments were conducted to determine the stoichiometric amount of copper necessary to fully activate phenoxazinone synthase. $CuCl_2$ was added to enzyme samples as described under Experimental Procedures, and the activity was measured. The steady-state specific activities are plotted against the copper content per subunit of the enzyme as shown in Figure 1. Maximum activity occurs at approximately 4–5 Cu atoms per subunit, and a

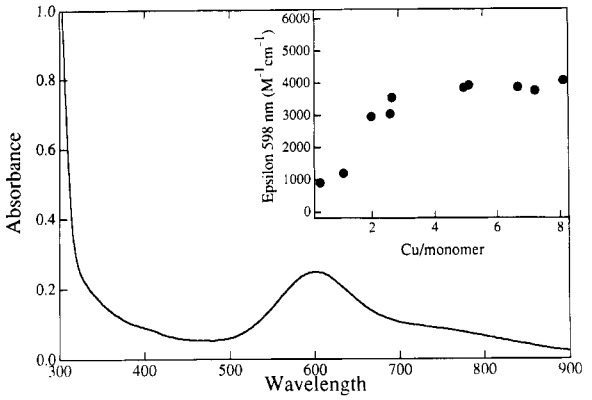


FIGURE 2: Ultraviolet/visible spectra of phenoxazinone synthase containing 5.2 coppers/monomer. The spectrum shows a λ_{max} at 598 nm typical of blue copper protein. The shoulder at 412 nm varies in size depending on preparation and does not seem to be related to copper content or activity. The inset graph is a plot of ϵ values vs copper/monomer at 598 nm.

slight decrease in specific activity was observed in samples incubated with more than five Cu atoms per subunit. This decrease in activity at high copper levels agrees with the early reports of Cu as an inhibitory metal ion (Katz & Weissbach, 1962).

Spectroscopic Identity of the Copper Sites. To explore the different possible environments of the enzyme-bound copper ions, both optical and EPR spectroscopic experiments were conducted. Figure 2 shows the absorbance spectrum of phenoxazinone synthase containing five Cu^{2+} per monomer. The inset is a plot of the extinction coefficient at 598 nm as a function of copper content per monomer. The extinction coefficient for the protein increases with the addition of Cu^{2+} until approximately four Cu/monomer and then levels off. The highest value for the extinction coefficient (ϵ) (4000 ± 150 M^{-1} cm^{-1}) was obtained at a Cu/enzyme ratio that corresponds to the ratio that gave the highest specific activity. The optical spectrum is typical of a number of blue copper proteins with a peak centered at 598 nm and a weak shoulder at approximately 750 nm. The visible portion of the spectrum resembles that of the blue copper-containing proteins ascorbate oxidase, ceruloplasmin, and azurin (Dooley et al., 1981; Solomon et al., 1974; Deinum et al., 1974; Nakamura et al., 1968; Brill et al., 1968). The spectrum does not show the prominent 330-nm band found in the blue copper oxidases corresponding to the type 3 copper center present in those systems (Sakuei et al., 1985, 1986; Mondovi & Aivigliano, 1984; Reinhammer & Malmstrom, 1982; Avigliano et al., 1979; Deinum et al., 1974; Malken et al., 1969). The best preparations of enzyme consistently had low absorbance at 330 nm (Figure 2).

Figure 3 (spectrum A) shows the EPR spectrum of the protein as isolated containing 0.8 Cu/subunit. The spectrum is that of a type 1 (blue) copper center with axial symmetry. The spectral parameters obtained are $g_{\|} = 2.24$ with $A = 70$ G (0.0067 cm^{-1}) and $g_{\perp} = 2.07$. Figure 3 (spectrum B) shows phenoxazinone synthase containing 5.2 equivalents of copper per monomer. The spectrum shows Cu^{2+} with axial symmetry [$g_{\perp} = 2.07$ and $g_{\|} = 2.34$ with $A = 165$ G (0.015 cm^{-1})], but both type 1 and type two copper signals are evident, with the type 2 copper signal being dominant. Integration of the Cu signals in protein containing five Cu per monomer indicate one spin per Cu as previously observed by Barry et al. (1989) with phenoxazinone synthase containing 3.7 coppers per subunit.

Anaerobic Incubation of Enzymic with Substrate. Phenoxazinone synthase was incubated with substrate under

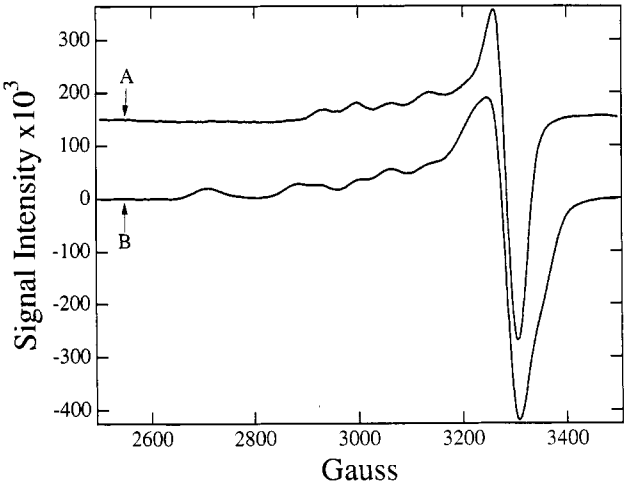


FIGURE 3: EPR spectra of phenoxazinone synthase in arbitrary intensity units. Spectrum A is the EPR signal of phenoxazinone synthase as isolated containing 0.8 coppers per monomer (offset from 0 intensity). The spectrum shows $g_{\|}$ of 2.24 and $A\|$ of 70 G (0.0067 cm^{-1}). The $g_{\perp}$ value is 2.07. Spectrum B is the EPR signal of phenoxazinone synthase containing 5.7 copper per monomer. Two sets of $g_{\|}$ values can be assigned to the spectrum, one with $g_{\|} = 2.24$ and $A = 70$ G (0.0067 cm^{-1}) typically associated with type 1 copper centers and one with $g_{\|} = 2.34$ and $A = 165$ G (0.015 cm^{-1}) normally found associated with type 2 copper centers. Spin counting indicates 1.1–1.3 spins per copper.

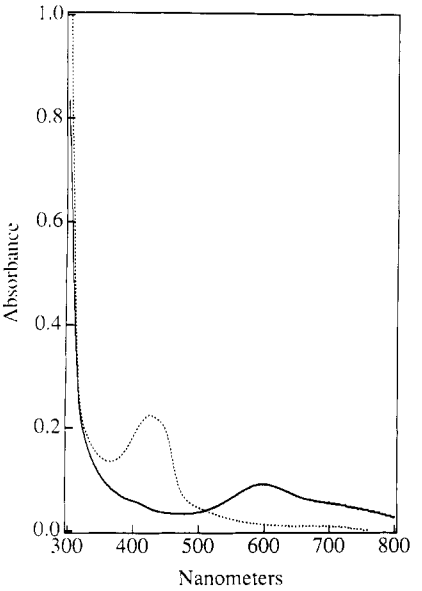


FIGURE 4: Ultraviolet and visible spectra from 300 to 800 nm of phenoxazinone synthase containing 5.2 coppers/monomer under anaerobic conditions. The spectra are from experiments before (solid line) and after (dashed line) the addition of 2-aminophenol. The 598-nm band associated with the blue copper center is bleached, and an absorption band similar to product appears at 425 nm. If the band is considered to be product, 9.5 ± 1 nmol of product was produced in the presence of 22 ± 2 nmol of phenoxazinone synthase.

anaerobic conditions to determine if the enzyme would accept electrons from substrate in the absence of oxygen. The absorption band at 598 nm was monitored under anaerobic conditions, and a reduction in intensity was observed (Figure 4). Under these experimental conditions, an absorption band appears in the 425–435-nm region that resembles the spectrum of the product (Barry et al., 1989). To identify the chemical nature of the product (Scheme II, **5** and **6**), the incubation solution was extracted with methylene chloride and molecular mass analysis obtained of the organic soluble extract. The mass spectrum showed two prominent peaks, one at 109 Da, appropriate for 2-aminophenol molecular ion, and the other at 212 Da, appropriate for phenoxazinone molecular ion. Using

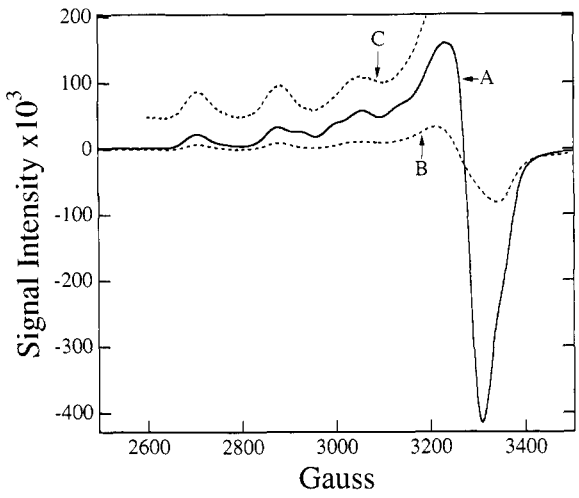


FIGURE 5: EPR spectra of phenoxazinone synthase obtained under anaerobic conditions. The solid line (spectrum A) represents the spectrum of enzyme before the addition of substrate, and the dashed line (spectrum B) is the spectrum after substrate addition. Spectrum C is a 5-fold expansion of the $g_\parallel$ region of spectrum B. Spin areas in arbitrary units are 7.19×10^9 for spectrum A and 2.77×10^9 for spectrum B. For spectrum B, the spectroscopic values are $g_\parallel = 2.28$, $A = 175$ G (0.016 cm^{-1}), and $g_\perp = 2.07$.

the extinction coefficient of phenoxazine at 435 nm to determine the quantity of product, 22 ± 2 μmol of enzyme produced 9.5 ± 1 μmol of product in one experiment, and 112 ± 2 μmol of enzyme produced 57 ± 2 μmol of product in another experiment. Absorbance in the 330-nm region of the spectrum was obscured by product formation in contrast to the behavior of ceruloplasmin, laccase, and ascorbate oxidase where the reduction of those enzymes showed changes at 330 nm (Sakurei et al., 1986; Deinum et al., 1974).

Figure 5 shows the EPR spectra of phenoxazinone synthase containing five Cu/monomer under anaerobic conditions before (A, solid line) and after (B, dashed line) the addition of substrate. A 5-fold vertical expansion of the $g_\parallel$ region of spectrum B is also shown [C, dashed line; $g_\parallel = 2.28$, $A = 175$ G (0.016 cm^{-1}), and $g_\perp = 2.07$]; the signal attributed to blue copper is absent under these conditions. Integration of the lowest field Cu signal shows that 50% of the type 2 signal disappears after reduction with substrate. When the total integrated spin for all of the Cu signal is obtained, the data show that 60 ± 5% of the copper was reduced by the addition of substrate. Conversion of the spin values to copper equivalents indicates that three Cu^{2+} per monomer are reduced by the addition of substrate, one type 1 blue copper and two type 2 coppers.

DISCUSSION

This paper reports our investigation of the stoichiometry of copper binding to phenoxazinone synthase as well as the spectroscopic properties of bound copper. The kinetic data shown in Figure 1 strongly suggest that phenoxazinone synthase requires 4–5 copper atoms/monomer for full activity. Additional copper above this level produces an inhibitory effect for reasons that remain unclear. The decrease in rate at higher copper concentrations was also reported in previous studies of the enzyme (Katz & Weissbach, 1962). Optical spectra of the enzyme show that the value of the extinction coefficient at 598 nm (due to the blue copper center) is correlated with specific activity. The conclusion that can be reached is that active enzyme contains a type 1 blue copper center. The visible absorption envelope of phenoxazinone synthase is characteristic of a number of blue copper containing proteins, and the assignment of the electronic transitions should be similar (Gray & Solomon, 1982; Solomon et al., 1980). Preliminary resonance raman data agree with the presence of a blue copper center but do not exclude organic cofactors (D. Dooley, personal communication).

The EPR spectra shown in Figure 3A indicate that the most tightly bound copper has characteristics of a type 1 copper center with typical $A_\parallel$ values. When Cu^{2+} is successively added to apoenzyme, a type 2 Cu EPR signal rapidly obscures the type 1 copper signal. The data are consistent with additional copper above one atom per monomer binding sequentially in type 2 copper sites in an individual enzyme molecule. This sequential binding pattern is found with ascorbate oxidase (Savini et al., 1985), but another binding order was found for ceruloplasmin (Aisen & Morell, 1965). Previous characterization of phenoxazinone synthase did not identify this protein as containing a blue copper center (Barry et al., 1989), but the present data show that the type 2 copper signal can readily obscure the EPR signal due to the type 1 center. It was noted previously that the EPR spectrum in the high copper/monomer sample contained more than one signal. Additionally, we have found that storage of the enzyme with low copper content at low protein concentrations results in the loss of the type 1 signal (data not shown).

The ultraviolet region of the optical spectrum does not show a band at 330 nm that has in the past been attributed to type 3 spin-paired copper centers. From our spin counting experiments, all the copper centers appear to be EPR active. Additionally, there is no bleaching in the 330-nm region under anaerobic conditions with the addition of substrate as seen in the other blue oxidases (Avigliono et al., 1978). These results would preclude the presence of a prototypical type 3 binuclear copper center in phenoxazinone synthase.

The data in this paper suggest that phenoxazinone synthase has a metal ion stoichiometry of one type 1 copper with an extinction coefficient of 4000 M^{-1} cm^{-1}, which is somewhat high but not unprecedented (Freedman & Peisach, 1984), and 3–4 type 2 copper centers. From the EPR experiments, three of the type 2 centers seem to be involved in catalytic activity, and two can be readily reduced by substrate under anaerobic conditions. Establishing the exact stoichiometry of metal ion sites involved in copper-containing enzymes is typically a difficult process as exemplified by the cases of ascorbate oxidase (Lee & Dawson 1978) and dopamine β-hydroxylase (Ash et al., 1984), and a final determination for phenoxazinone synthase may not be realized until the crystal structure is solved.

Some details of the catalytic mechanism have derived from this study. The type 1 copper in phenoxazinone synthase can be reduced by substrate (Figures 4 and 5), and this process is likely to be the path by which electrons enter the copper centers. A similar mechanism has been demonstrated for ascorbate oxidase, and direct reduction of enzyme by substrate is assumed to be the mechanism for the other blue oxidases (Meyer et al., 1991; Farver & Pecht, 1984). Scheme II shows the oxidative cascade proposed by Barry et al. (1989). The mass spectral data and the absorbance change in the spectrum at 425 nm seen in Figure 4 suggest that phenoxazinone synthase contains a sufficient number of electron acceptors to complete the four-electron oxidation of amino phenol to the phenoxazinone chromophore **5** shown in Scheme II. Spin counting data from the anaerobic EPR experiment shown in Figure 5 indicate that three electrons from each aminophenol are transferred to the copper centers in phenoxazinone synthase under anaerobic conditions. Without the terminal electron acceptor O_2 present, the coupled product (**4** or **5**) most likely leaves the enzyme and disproportionates to the chromophore

observed in Figure 4 (confirmed by mass spectral data to be a coupled product). Barry et al. (1989) demonstrated that the predicted product of the four-electron oxidation (structure **5**) is oxidized readily in air to the final product 2-aminophenoxazinone (structure **6**). Thus, the enzyme may not catalyze the final two-electron transfer in Scheme II.

Blue copper oxidases constitute a small group of enzymes responsible for substrate-specific oxidation of quinols, phenols, and aminophenols. These enzymes contain all three types of copper centers designated type 1 or blue, EPR detectable $\epsilon_{600} = 10^3$, type 2, EPR detectable $\epsilon_{600} = 10^1$, and type 3, EPR nondetectable (Reinhammar & Malmström, 1982). Additionally, nitrous oxide reductase contains type 1 and type 3 copper centers (Dooley et al., 1987). Recent studies reveal significant protein sequence and structural homology among small blue copper proteins (azurins, plastocyanins), the blue copper oxidases, and nitrous oxide reductase (Fenderson et al., 1991; Messerschmidt & Huber, 1990; Ohkawa et al., 1989). All of the blue oxidases catalyze the oxidation of aminophenols to polymers (Tanaka & Murao, 1983; Reinhamer & Malmstrom, 1982; Fahraeus & Ljunggren, 1961) and contain 4–8 copper atoms per subunit (Marchesini & Kroneck, 1979; Deinum & Vanngard, 1973; Malmstrom et al., 1970, 1968). The copper content of phenoxazinone synthase, the substrate (an aminophenol), and product (a dimerized azophenone) make it highly likely that an evolutionary relationship exists between the blue oxidases and phenoxazinone synthase.

It is probable that the ancestral blue copper oxidase contained the copper stoichiometry found in laccase (Messerschmidt & Huber, 1990). As enzymes of this class diverged, the number and types of copper centers may have changed to satisfy the chemical needs of the reaction being catalyzed. Ascorbate oxidase and ceruloplasmin show an increase in the number of blue centers in the protein, while nitrous oxide reductase has lost the type 2 copper center. Phenoxazinone synthase may have converted the oxygen reduction site from a type 3 site into two type 2 centers.

The lack of a type 3 copper center in phenoxazinone synthase is unusual because the blue oxidases use this center as the site of O_2 reduction (Sakurai et al., 1986; Avigliano et al., 1978). However, the requirement of type 3 copper centers for the reduction of O_2 is not strict as there are a number of copper-containing proteins that use a type 2 site for the reduction of O_2 to the $2O^{2-}$ oxidation state. Most notably, dopamine β-hydroxylase has been shown to oxidize quinols to quinones while reducing O_2 to water (Kim & Klinman, 1991). Future experiments are aimed at obtaining additional spectroscopic data to characterization the copper centers as well crystallization of the enzyme for three-dimensional analysis.

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