Phenoxazinone Synthase: Mechanism for the Formation of the Phenoxazinone Chromophore of Actinomycin†

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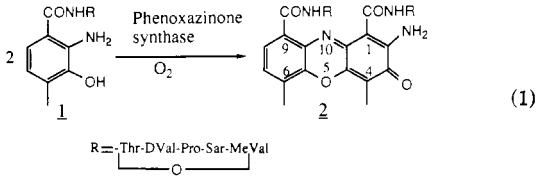
ABSTRACT: Phenoxazinone synthase is a copper-containing oxidase that catalyzes the coupling of 2-aminophenols to form the 2-aminophenoxazinone chromophore. This reaction constitutes the final step in the biosynthesis of the potent antineoplastic agent actinomycin. The mechanism of this complex 6-electron oxidation was determined by using a variety of substituted 2-aminophenols, designed to block the reaction at intermediate stages. Thus, with 3,5-di-*tert*-butyl-2-aminophenol (**16**) as substrate, the reaction was blocked at the *o*-quinone imine **17**; with 5-*tert*-butyl-2-aminophenol (**19**) as substrate, the reaction was blocked at the *p*-quinone imine **20**; and with 5-methyl-2-aminophenol (**21**) as substrate, the reaction was blocked at the dihydro-2-aminophenoxazinone **22**. These findings suggested a mechanism in which 2-aminophenoxazinone formation proceeded via a quinone imine intermediate **4** that was trapped by a second molecule of 2-aminophenol. Oxidation of the adduct **5** to the *p*-quinone imine **6** was followed by a second conjugate addition and a final 2-electron oxidation to give the product, 2-aminophenoxazinone. The role of the enzyme in the catalysis of each of these steps was examined. It was found that the second conjugate addition generated a racemic center at C4a, suggesting that this reaction did not occur at the active site. A deuterium isotope effect on the cleavage of the C4–H bond of 2-aminophenol suggested that partial dissociation of an intermediate from the enzyme occurred after the first conjugate addition. It is proposed that 2-aminophenoxazinone synthesis proceeds via a sequence of three consecutive 2-electron aminophenol oxidations and that the aminophenol moiety is regenerated during the reaction sequence by facile tautomerization reactions. Thus, what initially appears to be an impressively complex mechanism may, in fact, be ingeniously simple.

Actinomycin D (**2**) is a member of an interesting class of natural products in which the yellow-red 2-aminophenoxazinone chromophore is linked to two cyclic pentapeptides (Katz, 1967; Hollstein, 1974). These compounds are among the most potent antineoplastic agents known. Their clinical use, however, has been limited to the treatment of choriocarcinoma, Wilms tumors, rhabdomyosarcoma, and Kaposi's sarcoma due to their high toxicity (Frei, 1974). The mechanism of this cytotoxicity has been extensively studied. It has been shown that actinomycin binds to DNA by intercalation of the phenoxazinone chromophore and that the cyclic pentapeptide lactone confers sequence specificity to adjacent GC base pairs. This interaction results in highly specific inhibition of DNA-dependent RNA synthesis (Hollstein, 1974; Gale et al., 1981). In addition to the actinomycins, both xanthommatin and cinnabarin have been found to contain the phenoxazinone chromophore (Butenandt, 1957; Cavill et al., 1959).

The biosynthesis of actinomycin involves the conversion of tryptophan to 3-hydroxyanthranilic acid in a multistep sequence (Katz, 1967). The pentapeptide lactone is then attached (Keller, 1984) and the resulting 2-aminophenol (**1**) undergoes a 6-electron oxidative coupling to form actinomycin (eq 1). The latter reaction is catalyzed by phenoxazinone



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synthase. This enzyme has been previously isolated from *Streptomyces antibioticus* (Katz & Weissbach, 1962). It has a subunit molecular weight of 88 000 and in its native form is a mixture of oligomers, with the dimer and the hexamer predominating (Choy & Jones, 1981).[1] The gene coding for phenoxazinone synthase was recently cloned and overexpressed in *Streptomyces lividans* (Jones & Hopwood, 1984), thus making the enzyme available in quantity for mechanistic studies (Barry et al., 1988).

On the basis of the extensive chemistry of the phenoxazinone chromophore (Schafer, 1964), four pathways for the oxidation of 2-aminophenol to 2-aminophenoxazinone were considered as possible candidates for the enzymatic reaction (Figures 1–4).

Pathway A involves a 2-electron oxidation of 2-aminophenol (**3**) to the highly electrophilic quinone imine **4**, followed by a conjugate addition to form **5** (Figure 1). A second 2-electron oxidation gives the *p*-quinone imine **6**. A second conjugate addition is followed by a final 2-electron oxidation to the 2-aminophenoxazinone **8**. It is also possible that the conversion of **5** to **7** occurs by a conjugate addition followed by a 2-electron oxidation. In support of pathway A it has been previously demonstrated that chemically prepared quinone imine reacts with 2-aminophenol to form 2-aminophenoxazinone (Nogami et al., 1975) and that quinone imine is an intermediate in the oxidation of 2-aminophenol to 2-aminophenoxazinone by tyrosinase (Toussaint & Lerch, 1987) and ferricyanide (Brockmann & Seela, 1971).

Mechanism B considers 1-electron oxidation of two separate molecules of 2-aminophenol followed by radical coupling to give **5** (Figure 2). Conversion of **5** to 2-aminophenoxazinone

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[1] Analysis of our preparation, using a native gel, stained with aminophenol, indicated the presence of additional oligomeric forms of the enzyme.

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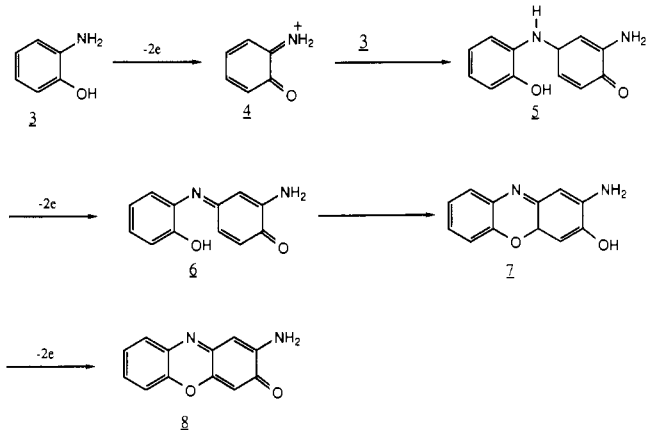


FIGURE 1: Mechanism A for the formation of 2-aminophenoxazinone.

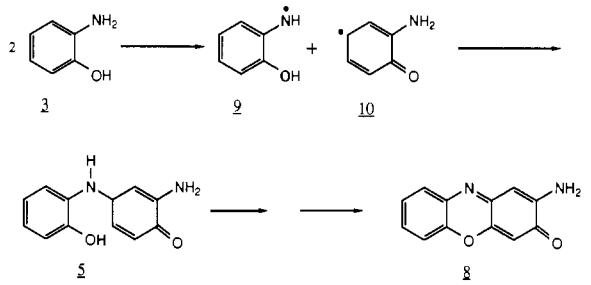


FIGURE 2: Mechanism B for the formation of 2-aminophenoxazinone.

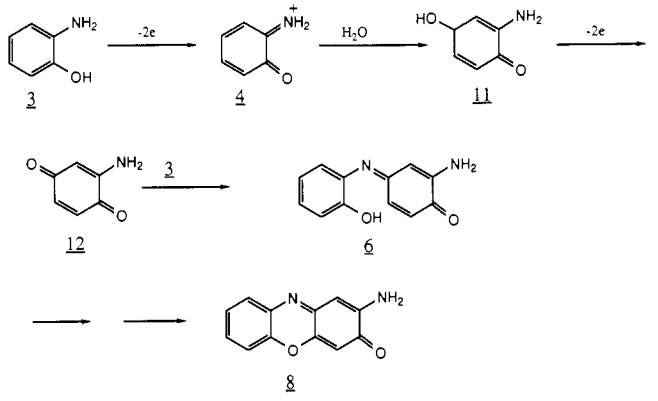


FIGURE 3: Mechanism C for the formation of 2-aminophenoxazinone.

could then proceed as in mechanism A. The active site is expected to control the regiochemistry as it is different from that observed for the coupling of the phenoxy radical in solution (Scott, 1965). The coupling of phenoxy radicals is a well-precedented step in natural product biosynthesis (Manitto, 1981). In addition, it has been demonstrated that 2-aminophenoxazinone is formed by the electrochemical 1-electron oxidation of 2-aminophenol (Harmalker & Sawyer, 1984). It is possible, however, that the 2-aminophenol radical undergoes disproportionation to the quinone imine in this case.

In mechanism C hydration of the quinone imine **4** is followed by 2-electron oxidation to give aminoquinone **12** (Figure 3). Condensation with a second molecule of 2-aminophenol results in the formation of **6**, which is then converted to 2-aminophenoxazinone as in mechanism A.

Pathway D, the final pathway considered, involves hydration of aminoquinone **12** followed by a 2-electron oxidation to **14** (Figure 4). Imine formation is followed by conjugate addition and elimination of water to give 2-aminophenoxazinone. The conversion of **14** to 2-aminophenoxazinone is precedented in the synthesis of xanthommatin (Schafer, 1964) and actinomycin derivatives (Lackner, 1970; Rinehart et al., 1977).

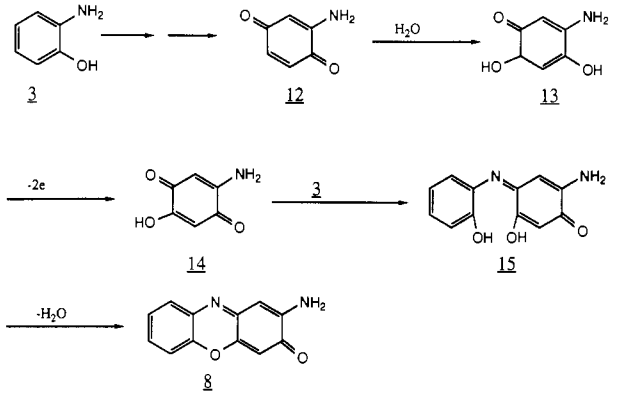


FIGURE 4: Mechanism D for the formation of 2-aminophenoxazinone.

In this paper mechanistic studies designed to differentiate between these pathways are described.

Experimental Procedures

Materials. S. lividans containing the plasmid plJ702, with a 2.45-kb insert coding for the phenoxazinone synthase gene, was a gift from George Jones of the University of Michigan (Jones & Hopwood, 1984). 2-Aminophenol, catechol, 5-methyl-2-aminophenol, 3,5-di-*tert*-butylphenol, 3-*tert*-butylphenol, 4-chloro-2-aminophenol, and 5-chloro-2-nitroanisole were obtained from Aldrich Chemical Co. All aminophenols used in this study were crystallized from deoxygenated benzene, ethanol, or ethyl acetate/hexane (7/3) until colorless unless otherwise noted. Aminoquinones **23** and **26** were prepared according to literature methods (Daruwala & Hornemann, 1977; Cameron et al., 1969). Quinone imine **4** was prepared by the oxidation of 2-aminophenol with lead dioxide (Nogami et al., 1975). Ferricyanide oxidation of 2-aminophenols was carried out by using the procedure of Butenandt (Butenandt et al., 1958). Growth media for *S. lividans* (NZ-amine, R2YE, and GGA) were as previously described (Hopwood, 1985; Gallo & Katz, 1972). Thiostrepton was from Calbiochem. Enzyme-grade ammonium sulfate was from BRL, and hydroxylapatite was from Bio-Rad. Glucose oxidase from *Aspergillus niger*, bovine liver catalase, and β-D-glucose were from Sigma Chemical Co. NMR spectra were run on a Varian XL-200 or XL-400 or on a Brucker WM-300 as noted for each spectrum. Mass spectra for the isotope effect studies were run on a Finnegan 3300 using electron impact ionization at 70 eV. HPLC was carried out on a Waters system using Radial-Pak C_{18} cartridges. UV/visible spectra were measured on a Hewlett-Packard Model 8451A diode array spectrophotometer equipped with kinetics software. FTIR measurements were performed on an IBM IR-98. Sonication was carried out by using a Heat-Systems-Ultrasonics, Inc., Model W-385 sonicator equipped with a microtip probe. Native and denaturing polyacrylamide gel electrophoresis was carried out by using standard procedures (Blackshear, 1984). Amino-terminal sequencing was carried out on an Applied Biosystems 470A protein sequencer at the Cornell Biotechnology Facility. Protein concentrations were determined by using either the Bradford (Bio-Rad) or the BCA (Pierce) assay with bovine serum albumin as a standard. Inductively coupled plasma emission spectroscopy was carried out at the Cornell Pomology Laboratory ICP Facility on a Jarell-Ash 975 plasma atom comp. The enzyme was analyzed for iron, manganese, copper, molybdenum, cobalt, chromium, and nickel. ESR spectra were recorded at 77 K on a Varian E9 spectrometer equipped with

a gas cryostat for cooled nitrogen gas (Bienert et al., 1978). Spectra were doubly integrated by using the Nicolet ESR signal averaging program. $Cu^{II}EDTA$ was used as a standard. Copper was quantitated for the ESR samples by atomic absorption spectroscopy using a Perkin-Elmer Model 2380 atomic absorption spectrometer with a HGA-400 graphite furnace and programmer.

Growth Conditions. S. lividans/plJ702 was grown and maintained on R2YE medium as previously described (Jones & Hopwood, 1984). For enzyme isolation, single colonies of *S. lividans*, obtained from R2YE plates, containing 10 μg/mL thiostrepton were screened for overexpression by patching colonies onto a filter paper grid followed by wetting with 10 mM aqueous 2-aminophenol. Colonies that turned dark yellow-red were inoculated onto an entire GGA plate. These plates were then grown to sporulation (usually about 5 days) at 30 °C and the spores collected (Hopwood, 1985). The spores were then transferred to NZ-amine medium (250 mL) and after 48 h of growth at 30 °C produced dense cultures (typically 2–5 g of cells/culture). The cells from the growth media were washed three times with saline (0.9%), transferred to GGA medium, and grown until the phenoxazinone synthase activity maximized (12–72 h). Some of the cultures did not overexpress the enzyme, presumably due to plasmid instability. Culture activity was determined by collecting the cells from 10 mL of the culture, resuspending in 50 mM Tris and 1 mM PMSF, pH 7.6 (buffer 1), sonicating (3 × 15 s bursts), and assaying for phenoxazinone synthase activity. Cultures showing high levels of enzymatic activity were combined and harvested as previously described (Jones & Hopwood, 1984).

Enzyme Isolation. The enzyme purification is summarized in Table I. All procedures were carried out at 4 °C. Cells were suspended in a minimum volume of buffer 1, sonicated in 15-s bursts to constant activity, and centrifuged at 10000*g* for 20 min. The specific activity of the resulting supernatant was typically 0.02 μmol/(min·mg) and contained about 1.0 g of protein/35 g of cells. This was treated with streptomycin sulfate and recentrifuged (10000*g*, 20 min). The supernatant was diluted to produce a final protein concentration of approximately 5 mg/mL, and powdered ammonium sulfate was slowly added, with constant stirring, to 25% saturation. This solution was centrifuged (20000*g*, 15 min) and the pellet discarded. The supernatant was brought to 50% saturation in 5% increments. The ammonium sulfate for each incremental increase was added over 20 min, and the sample was allowed to stand for 15–25 min before centrifugation. The pellet resulting from each incremental increase in ammonium sulfate concentration was isolated by centrifugation (20000*g*, 15 min), redissolved in the minimum volume of 100 mM phosphate, pH 6.0, and analyzed by SDS–PAGE. Usually the 30–35% pellet contained most of the phenoxazinone synthase with fractions within 10% containing some slightly less pure material. The purest fractions were combined, dialyzed against 10 mM phosphate, pH 6.0, and chromatographed on a short column of hydroxylapatite (approximately 5 mL/50 mg of protein). Batch elution with 50 mM phosphate, pH 6.0 (10 mL), followed by 50 mM phosphate, pH 7.4 (10 mL), and 100 mM phosphate, pH 7.4 (10 mL) resulted in good recovery of pure enzyme in the final wash.

Reconstitution with Cu(II). The enzyme was dialyzed against 200 μM copper(II) chloride in 100 mM phosphate (pH 6.5, 8 h, 4 °C) followed by dialysis against 100 mM phosphate (pH 6.5, 4 °C, 24–72 h, buffer changed every 6–8 h).

Enzyme Kinetic Assays. Kinetic assays were usually run at 37 °C in sodium acetate buffer (3-mL total volume, 100 mM, pH 5.0) containing substrate (added from a freshly prepared DMSO stock solution) and 5 μg of enzyme. Because of the reported sensitivity of the enzyme to the effects of varying ionic strength (Choy, 1980), data for the pH/rate profiles was obtained by using buffers that maintained a constant ionic strength over their useful range (Ellis & Morrison, 1982). To span the range over which the enzyme showed activity, it was necessary to use three systems that overlapped at their limits. The buffers used were as follows: *high pH range* (9.5–8.5), MES (0.1 M), Tris (0.052 M), and ethanolamine (0.052 M); *mid pH range* (8.5–4.1), acetic acid (0.05 M), Mes (0.05 M), and Tris (0.10 M); *low pH range* (4.1–3.5), citric acid (0.05 M), acetic acid (0.05 M), and Bis-Tris (0.1 M). For each buffer, the ionic strength was approximately 0.1 across the indicated pH range. 2-Aminophenoxazinone formation was monitored at 434 nm (ϵ = 23200 cm^{-1} M^{-1} for 2-aminophenoxazinone **8** in acetate buffer, pH 5.0). Kinetic data were analyzed by simple nonlinear least-squares fitting to the generalized Michaelis–Menten equation. Statistical errors were calculated directly and propagated through functions by using standard error analysis (Shoemaker et al., 1981).

Measurement of the Kinetics of Oxygen Consumption. The kinetics of oxygen consumption were determined by using the Clark electrode and the glucose oxidase/catalase system for regulating the concentration of oxygen in solution (Ando & Horie, 1971). The system was calibrated against oxygen and helium saturated buffer (100 mM acetate, pH 5.0) equilibrated at 37 °C under a constant stream of gas in the electrode chamber. Addition of known amounts of glucose was used to check the calibration and response times. The absolute concentration of oxygen in air-saturated solution was set to 0.224 mM, and the concentration in oxygen-saturated buffer was set to 1.067 mM according to the literature (Umbriet, 1964) and conformed with the values obtained by addition of known amounts of glucose. A typical procedure for a kinetic run with phenoxazinone synthase was as follows: oxygenated buffer (6.0 mL, 100 mM acetate, pH 5) was placed in the electrode chamber and equilibrated to 37 °C under a moderate flow of oxygen for 10 min. Catalase (80 μg), glucose oxidase (100 units), and phenoxazinone synthase [25 μg, specific activity 20 μmole/(min·mg)] were added, and the solution was equilibrated for 5 additional minutes after insertion of the electrode. The calculated amount of freshly prepared β-D-glucose was added in concentrated solution through a syringe port. The solution generally required about 10 min to reach a stable base line at the desired oxygen concentration. Finally, 2-aminophenol from a fresh DMSO stock solution was added to give a final concentration of 5 mM, and a linear rate of decrease of oxygen in solution was recorded for about 5 min.

Catalase Activity Measurements. To determine catalase activity the electrode was calibrated as described above and the rate of increase in oxygen concentration was monitored as a function of peroxide concentration. The rates were generally linear for a short time (1–2 min) after which time bubble formation distorted the concentration readings. The addition of air-saturated buffer (6.0 mL, 100 mM acetate, pH 5.0) and 30% hydrogen peroxide to the desired concentration was followed by an equilibration time of about 10 min after insertion of the electrode. After the electrode reading stabilized, phenoxazinone synthase (25 μg) was added via syringe to the chamber.

3,5-Di-tert-butyl-2-aminophenol (**16**). The corresponding 2-nitrophenol was prepared by lanthanum(III)-catalyzed ortho-nitration of 3,5-di-*tert*-butylphenol (Overtani et al., 1982).

The phenol (1.03 g, 5 mmol) was dissolved in 15 mL of ether. To this solution were added sodium nitrate (0.425 g, 5 mmol) and lanthanum(III) carbonate (0.06 g, 0.2 mmol) dissolved in 10 mL of 6 N HCl. The mixture was stirred for 5–8 h, extracted into ether, and dried ($MgSO_4$) and the solvent removed. The crude product was purified by flash chromatography (silica gel, hexane) to give the ortho-nitrated material (0.43 g, 1.7 mmol, 35% yield): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.30 (s, 9 H), 1.40 (s, 9 H), 6.75 (s, 1 H), 6.92 (d, J = 2 Hz, 1 H), 7.23 (d, J = 2 Hz, 1 H). This compound was reduced to the 2-aminophenol by using $SnCl_2$ as follows (Bellamy & Ou, 1984): The nitrophenol (0.42 g, 1.7 mmol) and $SnCl_2 \cdot 2H_2O$ (1.91 g, 8.5 mmol) were heated at reflux for 1.5 h in 20 mL of absolute ethanol. The reaction mixture was then cooled, poured onto ice, neutralized with 5% $NaHCO_3$, extracted with ethyl acetate, and dried ($MgSO_4$) and the solvent removed. Recrystallization from benzene gave **16** as a white crystalline solid (15.8 mg, 5%): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.30 (s, 9 H), 1.45 (s, 9 H), 3.70 (br s, 2 H), 5.30 (br s, 1 H), 6.72 (d, J = 2 Hz, 1 H) 6.90 (d, J = 2 Hz, 1 H); MS 221 (24.5), 222 (3.8), 206 (100), 57 (13.6).

5-tert-Butyl-2-aminophenol (**19**). 3-*tert*-Butylphenol (1.5 g, 10 mmol) was nitrated as described above. The crude product was chromatographed (silica gel, hexane) to give 5-*tert*-butyl-2-nitrophenol (1.46 g, 7.4 mmol, 75% yield): ^{1}H NMR (200 MHz, $CDCl_3$) δ 10.6 (br s, 1 H), 7.95 (d, J = 9.5 Hz, 1 H), 7.0 (m, 2 H), 1.23 (s, 9 H); MS 196 (6.7), 195 (52.9), 180 (100). The 2-aminophenol was formed by reduction, using hydrazine and Pd/C (Yembrick, 1961). The nitrophenol (0.27 g, 1.3 mmol) was dissolved in 5 mL of absolute ethanol. Anhydrous hydrazine (0.174 mL, 5.5 mmol) and 10% Pd/C (20 mg) were added. The ethanolic solution was refluxed for 1 h, cooled, and filtered through Celite. The ethanol was removed in vacuo, and the crude product was recrystallized from benzene to give **19** as white crystals (0.15 g, 70% yield): ^{1}H NMR (200 MHz, DMSO) δ 1.18 (s, 9 H), 4.3 (s, 2 H), 6.5 (s, 1 H), 6.53 (d, J = 9 Hz, 1 H), 6.68 (d, J = 9 Hz, 1 H), 8.75 (br s, 1 H); MS 166 (5), 165 (36), 150 (100), 133 (11).

3,6-Dimethyl-2-aminophenol (**27a**). 2,5-Dimethylphenol (1.22 g, 10 mmol) was nitrated by using the procedure described for **18** and the product purified by chromatography (silica gel, hexane/ethyl acetate 95/5) to give 3,6-dimethyl-2-nitrophenol (1.44 g, 8.6 mmol, 86% yield): ^{1}H NMR (200 MHz, $CDCl_3$) δ 10.6 (s, 1 H), 7.4 (d, J = 7.6 Hz, 1 H), 6.7 (d, J = 7.6 Hz, 1 H), 2.6 (s, 3 H), 2.3 (s, 3 H). Reduction of the nitrophenol was carried out by using the procedure described for **19**. The product was purified by chromatography (silica gel, ethyl acetate/hexane 50/50) and recrystallized from benzene to give the corresponding aminophenol (1.05 g, 7.7 mmol, 90% yield): ^{1}H NMR (200 MHz, DMSO) δ 7.8 (br s, 1 H), 6.4 (d, J = 7.5 Hz, 1 H), 6.2 (d, J = 7.5 Hz, 1 H), 4.25 (br s, 2 H), 2.1 (s, 3 H), 2.0 (s, 3 H).

4,5-Dideuterio-3,6-dimethyl-2-aminophenol (**27b**). 2,5-Dimethylphenol (1.22 g, 10 mmol) was perdeuteriated by using the procedure described for **31**. The product of this exchange was nitrated and reduced as described for **27a** to yield product that was >95% deuteriated at the 4- and 5-positions as determined by NMR analysis: ^{1}H NMR (200 MHz, DMSO) δ 7.8 (br s, 1 H), 4.3 (br s, 2 H), 2.1 (s, 3 H), 2.0 (s, 3 H).

p-Quinone Imine **29**. Compound **29** was prepared by co-oxidation of aniline and 5-methyl-2-aminophenol with ferricyanide. To a solution of aniline (465 mg, 5 mmol) and 5-methyl-2-aminophenol (615 mg, 5 mmol) in 5 mL of ethanol was added a solution of potassium ferricyanide (1.6 g, 4.8 mmol) in 20 mL of phosphate buffer (70 mM, pH 7.2). The reaction mixture was stirred for 12 h at room temperature and extracted into chloroform. The product was purified by flash chromatography (silica gel, chloroform/methanol 99/1) to give **29** as dark red crystals (12%): ^{1}H NMR (400 MHz, DMSO) δ 2.20 (s, 3 H), 5.65 (s, 1 H), 6.22 (s, 2 H), 6.50 (s, 1 H), 6.78 (d, J = 10.4 Hz, 2 H), 7.1 (t, J = 7.6 Hz, 1 H), 7.35 (dd, J = 8, 8 Hz, 2 H); MS 213 (8), 212 (42.7), 211 (55.3), 195 (13.1); FTIR 3480, 3340, 1600, 1240 cm^{-1}; UV/vis λ_{max} = 525 nm, ϵ = 4305 cm^{-1} M^{-1}.

Reduction of p-Quinone Imine **29**. An ethanolic solution of sodium borohydride was slowly added to a solution of **29** in ethanol under an argon atmosphere until the red color had disappeared. Excess borohydride was then quenched by the addition of glacial acetic acid. These stock solutions could be stored temporarily at 0–5 °C with little reoxidation and were used directly for kinetic measurements. The concentration of aminophenol **28** was determined after complete oxidation of an aliquot of the stock solution back to *p*-quinone imine **29**. Kinetic measurements were conducted by the addition of the ethanolic stock solution to assay buffer (100 mM NaOAc, pH 5.0).

Due to its oxygen sensitivity, the product was characterized in situ by NMR (prepared by reduction of **29** with sodium borodeuteride in deuteriomethanol): ^{1}H NMR (200 MHz, CD_3OD) δ 2.05 (s, 3 H), 6.65 (m, 5 H), 7.1 (m, 2 H).

Dihydro-2-aminophenoxazinone (**30**). 2-Aminophenoxazinone (**8**) (2.0 mg, 9.4 μmol) was suspended in 1 mL of absolute ethanol, and 10% Pd/C (10 mg) was added under argon. An atmosphere of hydrogen was introduced, and the suspension was stirred. The reaction mixture became homogeneous and changed color from red to colorless as the reduction went to completion (about 20 min). The Pd/C was removed by anaerobic filtration via cannula through a 1-mL syringe plugged with cotton and 2 cm of Celite. The resulting ethanolic solution was rapidly oxidized to 2-aminophenoxazinone when exposed to air. Due to its extreme oxygen sensitivity, dihydro-2-aminophenoxazinone was characterized as the diacetate. This was prepared by treating 1 mL of the reaction mixture with 500 μL of acetic anhydride. The crude product was purified by reversed-phase HPLC (C_{18}) using a sodium acetate buffer (pH 5, 100 mM)/acetonitrile gradient: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.44 (s, 6 H), 6.62 (s, 1 H), 7.4 (s, 1 H), 7.57 (m, 2 H), 7.70 (dd, J = 8, 10 Hz, 1 H), 8.03 (d, J = 10 Hz, 1 H), 8.58 (s, 1 H), 8.70 (br s, 1 H); MS 254 (24), 212 (88.9), 185 (28.9), 184 (20.7), 58 (21.2), 43 (100).

Tetradeuterio-2-aminophenol (**31**). Freshly recrystallized 2-aminophenol (150 mg, 1.4 mmol) was evaporated under vacuum three times from 2 mL of fresh deuteriomethanol to replace the exchangeable hydrogens. The aminophenol was then placed in a pressure tube along with 10.0 mL of DCl (37 wt% DCl in D_2O) and stirred at 100 °C for 7 days. After cooling to 4 °C, the solution was titrated to pH 5 with 1 N NaOH while ice was added to maintain the temperature. The precipitate was extracted with ethyl acetate, dried over $MgSO_4$, and evaporated in vacuo. Flash chromatography (ethyl acetate/hexanes 50/50), followed by repeated crystallization from degassed benzene or ethyl acetate/hexanes (30/70), gave pure material in 75% yield. Total deuterium incorporation was determined by mass spectrometry to be 92.9 ± 1.0%. The remaining 7.1% protium was shown, by NMR, to be located in the positions ortho and para to the amine. The exact amount of trideuteriated material with protium at C-5 was calculated from the NMR and mass spectra to be 16% of the total, while that with protium at C-3 was determined to be

12%. The remaining 72% was the desired tetradeuteriated 2-aminophenol: MS (average of three determinations) 109 (0.2), 110 (0.6), 111 (8.6), 112 (53.2), 113 (100.0), 114 (7.5). Corrections for normal isotopic abundance and for the M − 1 of overlapping *m/e* peaks were determined from the average of three spectra of tetraprotio-2-aminophenol. The average relative intensity for a given mass was corrected for the M + 1 of the next lower *m/e* (corrected down by 6.4% of the lower mass) and for the M − 1 of the next higher *m/e* (14.1% of the higher mass). The *m/e* intensities were corrected by starting with the highest intensity peak and correcting for its contributions to the surrounding peaks, then moving to the next most intense peak, and so on until the corrections fell within the (propagated) statistical error for a given mass. This procedure was used for deconvoluting all mass spectra described below. ^{1}H NMR (200 MHz, DMSO) δ 6.59 (s, relative area 1.0), 6.40 (s, relative area 1.4).

4-Deuterio-2-aminophenol (**32**). This compound was prepared in a manner similar to that previously used for the hydrodehalogenation of chloroaniline (Egli, 1968). 4-Chloro-2-aminophenol (1.44 g, 10 mmol, purified by recrystallization from 1.5/8.5 ethyl acetate/hexanes) was dissolved in deuteriomethanol and evaporated several times. The residue was then dissolved in 7.5 mL of deuteriomethanol under argon, and sodium deuteroxide in D_2O (2.5 mL, 10 M) was added followed by 200 mg of 5% Pd/C. While the solution was flushed with a stream of argon, sodium borodeuteride (570 mg, 13.6 mmol) was added and the reaction was allowed to stir for 48 h at room temperature. The Pd/C was removed by filtration through Celite, which was then thoroughly washed with warm methanol and water. The filtrates were combined, and the methanol was removed under vacuum. The remaining solution was cooled to 4 °C, and 20 mL of water was added. The solution was then neutralized slowly with 6 N HCl while ice was added to maintain the temperature. The precipitate was extracted with ethyl acetate, and the organic layer was separated, dried ($MgSO_4$), and evaporated to dryness under vacuum. The crude material was purified by flash chromatography (silica gel, hexanes/ethyl acetate 7.5/2.5). The product was recrystallized from benzene to give 290 mg (26%) of **32** with a deuterium incorporation at C-4 of 95.0%: ^{1}H NMR (300 MHz, DMSO) δ 8.90 (br s, 1 H), 6.61 (d, J = 8 Hz, 1 H), 6.55 (d, J = 1 Hz, 1 H), 6.37 (d, J = 8 Hz, 1 H), 4.44 (br s, 2 H); MS 111 (9.6), 110 (100), 109 (19.4), 81 (72.1), 54 (20.2).

5-Deuterio-2-aminophenol (**33**). BBr_3 treatment of 5-chloro-2-nitroanisole (McOmie & West, 1973) gave the corresponding chloronitrophenol in quantitative yield. Simultaneous reduction of the nitro group and deuteriodehalogenation were effected by adding a 2-fold excess of borodeuteride slowly over a 2-h period with cooling in an ice bath using the procedure described above for **32**. The product was purified in the same manner as for **32** and afforded in 10% yield material that was 97.1% deuteriated at C-5: ^{1}H NMR (200 MHz, DMSO) δ 8.90 (br s, 1 H), 6.61 (s, 1 H), 6.55 (AB, 2 H), 4.44 (br s, 2 H); MS 111 (7.6), 110 (100), 109 (17.1), 81 (80.2), 54 (30.6).

4a,7-Dimethyldihydro-2-aminophenoxazinone (**22**). Compound **22** was prepared by ferricyanide oxidation of 5-methyl-2-aminophenol (615 mg, 5 mmol) in 60% isolated yield (360 mg) after flash chromatography (silica gel, ethyl acetate/hexanes 60/40): ^{1}H NMR (400 MHz, DMSO) δ 2.25 (s, 3 H), 2.98 (d, J = 15 Hz, 1 H), 3.20 (d, J = 15 Hz, 1 H), 3.31 (s, 3 H), 6.03 (s, 1 H), 6.40 (br s, 2 H), 6.71 (s, 1 H), 6.80 (d, J = 8 Hz, 1 H), 7.09 (d, J = 8 Hz, 1 H); λ_{max} (100 mM acetate, pH 5) = 418 nm (ϵ = 18 200 cm^{-1} M^{-1}); MS 242 (100), 241 (13.4), 227 (70.9), 225 (12.9), 213 (14.8), 199 (18.7), 173 (18.8), 110 (13.9).

Preparation of the Mosher Amide of **22**. Racemic **22** (10 mg, 41.3 μmol) was dissolved in 5 mL of dichloromethane. To this solution were added DCC (15 mg, 72.7 μmol) and (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (60 mg, 171 μmol). The solution was stirred for 6–8 h and filtered and the solvent removed under vacuum. The crude material was purified by chromatography (silica gel, chloroform/methanol 97.5/2.5): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (s, 1.5 H), 1.28 (s, 1.5 H), 2.33 (s, 3 H), 3.1 (d, J = 11 Hz, 1 H), 3.18 (d, J = 11 Hz, 1 H), 3.49 (s, 3 H), 6.71 (s, 1 H), 3.83 (d, J = 8 Hz, 1 H), 7.3 (d, J = 9 Hz, 1 H), 7.43 (m, 3 H), 7.53 (m, 2 H), 8.21 (s, 1 H), 9.4 (d, J = 9 Hz, 1 H); MS 459 (45), 458 (96), 269 (41), 189 (100).

Enzymatic Oxidation of 3,5-Di-tert-butyl-2-aminophenol (**16**). Compound **16** (15.8 mg) was dissolved in 500 μL of DMF and added to 3 mL of assay buffer. This concentration of DMF was shown in separate experiments not to affect the rate of enzymatic oxidation of 2-aminophenol and was necessary to solubilize **16**. After incubation for 30 min at 25 °C with enzyme (275 μg), the reaction mixture was extracted with hexanes/ethyl acetate (3/2) and chromatographed (silica gel, hexane/ethyl acetate 95/5). The only product was a yellow band that was collected and identified as 3,5-di-*tert*-butyl-*o*-benzoquinone (**18**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.93 (d, J = 3 Hz, 1 H) 6.22 (d, J = 3 Hz, 1 H), 1.30 (s, 9 H), 1.25 (s, 9 H); MS 220 (1.5), 192 (30.5), 166 (36.8), 57 (100); UV/vis, λ_{max} = 420 nm (assay buffer, pH 5).

Enzymatic Oxidation of Catechol. Phenoxazinone synthase (500 μg) was added to a solution of catechol (10.0 mg, 90 μmol, recrystallized from toluene) in 26 mL of D_2O. After stirring at room temperature for 20 min, the reaction mixture was extracted with $CDCl_3$ (0.5 mL) and analyzed without purification due to the instability of the product. The UV/visible spectrum was in agreement with the published spectrum of *o*-benzoquinone (Englehard & Lüttke, 1977). In addition to catechol peaks, the NMR spectrum had peaks consistent with *o*-benzoquinone (Hollenstein & Von Philipsborn, 1973). This was chemically prepared as a standard by the oxidation of catechol with *N*-chlorosuccinimide (Durst et al., 1975): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.11 (dd, J = 10, 10 Hz), 6.44 (dd, J = 6, 6 Hz); UV/vis (ether) λ_{max} = 375 nm (ϵ = 1900 cm^{-1} M^{-1}), ($CHCl_3$) λ_{max} = 375 nm (ϵ = 1700 cm^{-1} M^{-1}).

Enzymatic Oxidation of 5-Methyl-2-aminophenol (**21**). To 6 mL of assay buffer were added a solution of **21** (3.7 mg, 30 μmol, dissolved in 50 μL of DMSO) and 100 μg of enzyme. The reaction mixture was incubated at 37 °C for 10 min, quenched by the addition of 1.0 mL of ethyl acetate/hexanes (6/4), and vigorously stirred for 1 min. The organic layer was separated and applied directly to a flash column equilibrated in the same solvent. Elution gave approximately 100 μg of a yellow material having spectroscopic properties (NMR, MS, UV/vis) identical with those of chemically prepared 4a,7-dimethyldihydro-2-aminophenoxazinone (**22**). This was converted to its Mosher amide as described above. An unstable purple compound eluted before compound **22** and was shown to rapidly convert to **22**. Under the reaction conditions described above, the rate of the nonenzymatic oxidation of **21** was 10% of the rate of the enzymatic oxidation.

To exclude the possibility of racemization after the product had formed by reversal of the conjugate addition, exchange of the C4 protons under the reaction conditions was examined. Compound **22** was dissolved in deuteriated buffer (100 mM

NaOAc, pD 5.4) for 24 h and purified as before. The product was analyzed by NMR.

Enzymatic Oxidation of 5-tert-Butyl-2-aminophenol (**19**). The reaction was carried out in 6 mL of assay buffer containing 4 mM 5-*tert*-butyl-2-aminophenol and 1.1 mg of enzyme. After 30 min at 35 °C, the reaction mixture was extracted with ethyl acetate/hexanes (1/1) and chromatographed on silica gel in the same solvent system. The reaction product was identified as **20** on the basis of the following spectroscopic parameters: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.30 (s, 9 H), 1.45 (s, 9 H), 4.43 (s, 2 H), 5.81 (s, 1 H), 6.31 (s, 1 H), 6.58 (s, 1 H), 6.80 (d, J = 8 Hz, 1 H), 6.92 (dd, J = 9 Hz, 3 Hz, 1 H), 7.17 (d, J = 3 Hz, 1 H); MS 326 (6), 149 (100); UV/vis (pH 5, assay buffer) λ_{max} = 234, 334, 566 nm.

Isotope Effect Measurements and Calculations. The buffer used in these studies was the intermediate pH range, constant ionic strength buffer described above for the pH/rate profiles. Buffer solutions were air-saturated by purging with compressed air for 0.5 h after preequilibration to 37 °C and were then titrated to the desired pH. Stock solutions of protio- and deuterio-2-aminophenol in DMSO were prepared immediately before use and were maintained on ice under argon during the experiment. Each determination was carried out in triplicate at a constant total substrate concentration (protio plus deuterio) of 5 mM in 3 mL of buffer. The initial isotopic composition was measured by removing 100 μL of the reaction mixture before enzyme addition and lyophilizing immediately. Enzyme (3.4 μg) was added, and the reaction was allowed to run for 10.0 min (approximately 1% conversion). An aliquot of the reaction mixture (2 mL) was then injected onto a C_{18} column that had been equilibrated with acetonitrile/sodium acetate buffer (pH 5, 100 mM, 32/68). Under these isocratic conditions 2-aminophenols eluted from the column in the void volume, and the product phenoxazinones were retained for 5 min. Products were collected and lyophilized. Both starting aminophenol mixtures and products were transferred to MS capillaries by using approximately 10 μL of methanol, and the solvent was removed under a stream of argon. Mass spectra for products and standards were run on the same day under identical conditions. Triplicate runs of both standards and products were averaged and deconvoluted as described above for tetradeuterio-2-aminophenol. The substrate ratio for an experiment was defined as the corrected ratio of m/e 109 (tetraprotio) to m/e 113 (tetradeuterio).

The isotope effect was calculated from the ratio of 1,4-dideuterio-2-aminophenoxazinone to 2-aminophenoxazinone (m/e 214/m/e 212) resulting from various mixtures of tetradeuterio-2-aminophenol and 2-aminophenol. The m/e 212/m/e 214 ratio was corrected for normal isotopic abundances and fragmentation of neighboring m/e peaks (2-aminophenoxazinone isolated in the same manner as the samples gave the following average corrections: M − 1 7.5 ± 1.5%; M + 1 13.6 ± 1.7%; M + 2 2.3 ± 0.3%; three runs). An additional correction to m/e 214 resulted from the coupling of tetraprotio-2-aminophenol and 3,4,6-trideuterio-2-aminophenol to form 1,4-dideuterio-2-aminophenoxazinone. This coupling was expected to manifest the same isotope effect as the corresponding coupling of tetradeuterio-2-aminophenol with tetraprotio-2-aminophenol because the isotope effect is caused by cleavage of the C4–H bond (see Results). The relative concentrations of d_0, d_4, and d_3(C5H) were calculated from deconvoluted mass spectra of the starting 2-aminophenol mixture. The products of the appropriate relative concentrations were calculated and normalized, and these values were applied as corrections to m/e 214. Errors calculated from triplicate measurements of an individual (uncorrected) m/e were propagated by using standard error propagation analysis to give the expected uncertainty in the final result. These error limits represent 68% confidence limits and reflect only the statistical error in the measured values. No corrections were applied for systematic errors. Analysis of this isotope effect for a dependence on the oxygen concentration in solution was performed by using the spectrophotometric assay, but no significant difference was observed between air-saturated and oxygen-saturated systems. When the enzymatic reaction was run in deuteriated buffer, with 2-aminophenol as substrate, incorporation of nonexchangeable deuteria into 2-aminophenoxazinone was not detected. This eliminated the possibility of exchange at C4 during the formation of 2-aminophenoxazinone.

Table I: Purification of Phenoxazinone Synthase

purification step	total act. (U)[a]	total protein (mg)	sp act. (U/mg)	yield (%)
streptomycin sulfate ppt	50.3	622	0.08	100
ammonium sulfate (35–45%)	23.1	116	0.2	46
hydroxylapatite	21.6	109	0.2[b]	43

[a] 1 U = 1 μmol/min. [b] Specific activity after Cu(II) dialysis = 8.6 U/mg.

Table II: Amino-Terminal Sequence of Phenoxazinone Synthase

H_2N-Glu-Arg-Glu-Gln-Ala-Pro-Ala-Pro-Gly-Glu-Leu-Thr-Pro-Phe-Ala-Ala-Pro-Pro-Thr-Val-Pro

Results

Enzyme Purification. Phenoxazinone synthase has been readily purified in 100-mg quantities by ammonium sulfate fractionation, followed by chromatography on hydroxylapatite (Table I). This is a simplification of the original purification scheme (Choy & Jones, 1981) due to the high level of enzyme in the recombinant strain. The resulting protein, as previously reported, had a specific activity in the range 0.2–3.3 μmol/(mg·min), had a subunit molecular weight of 88 000, and was a mixture of oligomers. The sequence of the amino terminus is shown in Table II.

Cofactor Analysis. The enzyme, as isolated, was found to contain copper as the major transition metal cofactor. The stoichiometry varied between preparations (0.5–2.0 coppers/subunit). This finding is in agreement with a previous report that prolonged dialysis of the enzyme against cyanide-containing buffer resulted in a decrease in enzyme activity that could be restored by the addition of copper (Nishimura & Golub, 1969). The enzyme as isolated showed a lag phase of variable duration (0.3–2 min).

Treatment of the enzyme with copper(II) resulted in a large increase in its specific activity and in elimination of the lag phase. This suggested that the enzyme, as initially isolated, was depleted in copper and that the lag phase was due to reconstitution of the apoenzyme containing preparation with trace quantities of copper(II) present in the assay buffer. The copper(II)-treated enzyme contained 3–7 coppers/subunit. The exact stoichiometry again varied between different preparations. The specific activity varied between preparations over the range 0.2–3.3 μmol/(mg·min) for the enzyme as isolated and 8.0–20 μmol/(mg·min) for the reconstituted enzyme. Unless otherwise stated, all kinetic experiments were performed by using the same preparation. The kinetic parameters for 2-aminophenol oxidation by this preparation before treatment with copper(II) were V_{max} = 0.2 μmol/

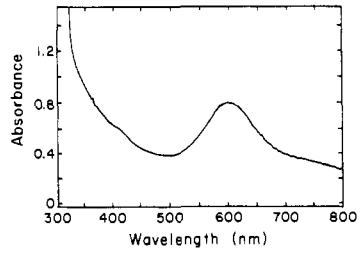


FIGURE 5: UV/visible spectrum of reconstituted phenoxazinone synthase (100 mM phosphate; pH 6.5; protein concentration, 25 mg/mL).

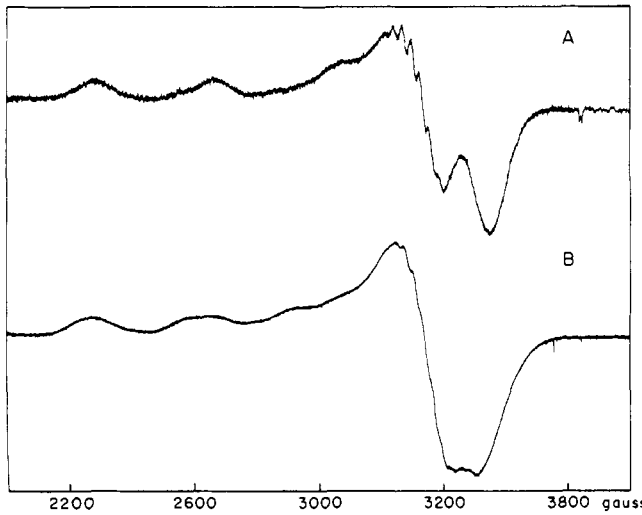


FIGURE 6: ESR spectrum of phenoxazinone synthase (a) enzyme as initially isolated (100 mM phosphate; pH 7.4; protein concentration, 15.8 mg/mL) and (b) enzyme after reconstitution with Cu(II) (100 mM phosphate; pH 6.5; protein concentration, 25 mg/mL). The spectra were recorded at 77 K.

(mg·min) and K_m = 500 μM^{-1}; the parameters after reconstitution were V_{max} = 8.6 μmol/(mg·min) and K_m = 500 μM^{-1}.

UV/Visible and ESR Spectroscopy. The UV/visible spectrum of the enzyme as isolated showed no long-wavelength absorbance, while that of the reconstituted enzyme (Figure 5) showed a single long-wavelength absorbance at 610 nm (ϵ = 1700 M^{-1} cm^{-1}, 3.7 Cu/subunit) characteristic of type 2 copper containing proteins (Spiro, 1981; Lontie, 1984). The ESR spectrum of the enzyme, as isolated, is shown in Figure 6A. This spectrum is also typical of type 2 copper containing proteins ($g_\perp$ = 2.058, $g_\parallel$ = 2.23, $A_\parallel$ = 195 G). An extra component was observed in the spectrum of the reconstituted enzyme (Figure 6B). Whether this represents catalytically active copper or nonspecifically bound copper is unknown at this point. Spin quantitation for the enzyme as isolated (0.95 ± 0.12 spin/copper, 0.4 Cu/subunit) and after reconstitution (1.1 ± 0.2 spins/copper, 3.7 Cu/subunit) eliminated the possibility of a type 3 copper center at the active site (assuming one active site per subunit). In addition, treatment of the enzyme with hydrogen peroxide did not result in an increased absorption at 350 nm. Such an absorption has been associated with a type 3 copper peroxide complex (Himmelwright et al., 1980; Skotland & Ljones, 1979).

pH/Rate Profile. The pH/rate profiles for the enzymatic reaction over the range from 3.5 to 9.5 were determined (Figure 7). The V/K and the V_{max} of the enzymatic reaction showed pH optima at 7 and 5, respectively. We have not attempted to identify catalytically important residues from these profiles because of their irregular shape and the probable interference of substrate ionization (pK_a of 2-aminophenol = 4.66; de Courville, 1966).

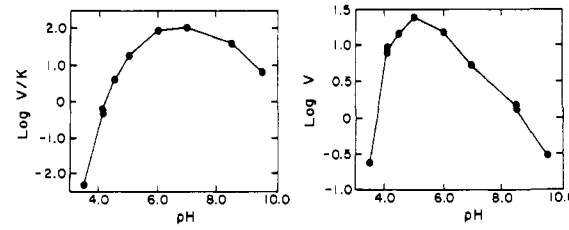


FIGURE 7: pH/rate profiles for phenoxazinone synthase. Assays were carried out as described under Experimental Procedures.

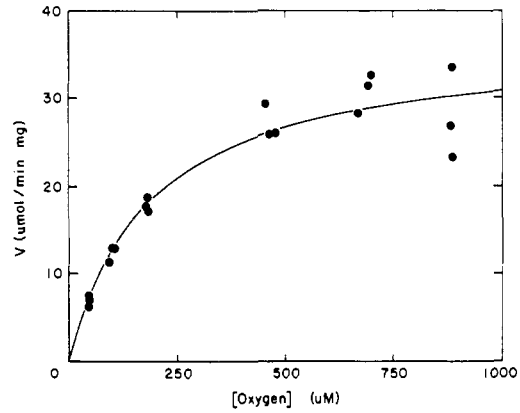
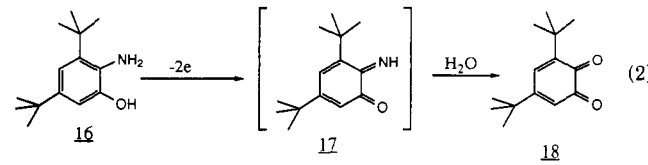


FIGURE 8: Michaelis–Menten plot for the kinetics of oxygen consumption. Assays were carried out as described under Experimental Procedures.

Stoichiometry and Kinetics of Oxygen Consumption. The kinetic parameters for oxygen consumption were measured by using a Clark electrode and found to be K_m = 207 μM and V_{max} = 36.6 μmol/(mg·min) [Figure 8; the specific activity of this enzyme preparation was 20 μmol/(mg·min)]. This K_m is close to the concentration of oxygen in air-saturated water (200 μM). Thus, typical assays have been run under oxygen-limiting conditions. In agreement with previous work (Katz & Weissbach, 1962) it was found that the ratio of dioxygen consumed to 2-aminophenoxazinone formed under V_{max} conditions was 1.5 ± 0.3.

Catalase Activity. Phenoxazinone synthase has catalase activity [K_m = 100 mM, V_{max} = 40 μmol/(min·mg)]. The reconstituted preparation showed a 3.5-fold increase in its catalase activity [K_m = 263 mM, V_{max} = 140 μmol/(min·mg)]. Reconstitution resulted in a 4.6-fold increase in the specific activity for 2-aminophenol oxidation. [The specific activity of the enzyme used for this experiment before reconstitution was 3.3 μmol/(mg·min) and after reconstitution was 15.3 μmol/(mg·min).] The parallel increase in both activities after reconstitution suggested that the catalase activity was associated with phenoxazinone synthase and not with a trace contaminant.

Mechanistic Probes for Pathways A and B. Treatment of the enzyme with 3,5-di-*tert*-butyl-2-aminophenol (**16**) resulted in the formation of the corresponding *o*-quinone **18** (eq 2).

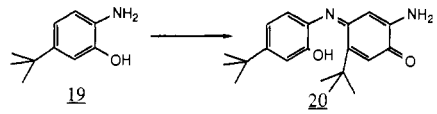


16 17 18 (2)

This experiment was suggested by the observation that 3,6-di-*tert*-butyl-2-aminophenol was oxidized to the *o*-quinone by ferricyanide under conditions where oxidation of 2-aminophenol gave 2-aminophenoxazinone (Brockmann & Seela, 1971). It was not possible to determine kinetic parameters

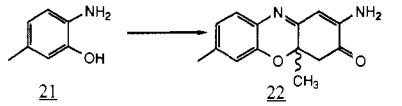
for this reaction due to the low solubility of the substrate. Phenoxazinone synthase also catalyzed the oxidation of catechol to *o*-benzoquinone. This oxidation had been previously reported, but the product was not characterized (Golub & Nishimura, 1972). It was not possible to study the potential role of the enzyme in the conversion of quinone imine **4** to 2-aminophenoxazinone due to the rapid hydrolysis of this intermediate to *o*-benzoquinone in the assay buffer.

Enzyme-catalyzed oxidation of the less sterically hindered 5-*tert*-butyl-2-aminophenol (**19**) resulted in the formation of the purple *p*-quinone imine **20** (eq 3). Kinetic parameters could not be determined due to the poor solubility of **19** in the assay buffer.



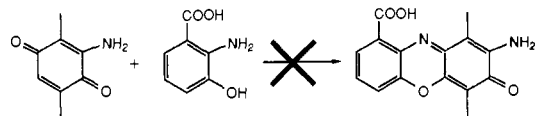
19 20 (3)

Enzyme-catalyzed oxidation of 5-methyl-2-aminophenol (**21**) resulted in the formation of the stable yellow dihydro-2-aminophenoxazinone (**22**) [V_{max} = 15 μmol/(mg·min), K_m = 197 μM; eq 4). In addition, an unstable purple compound was observed during the reaction that rapidly converted to **22**.

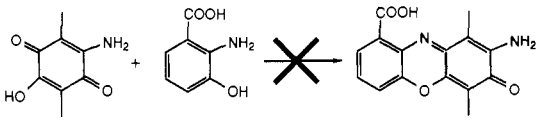


21 22 (4)

Mechanistic Probes for Pathways C and D. Treatment of a mixture of aminoquinone **23** and 3-hydroxyanthranilic acid (**24**) with the enzyme resulted only in the formation of the symmetrical 1,9-dicarboxy-2-aminophenoxazinone. None of the cross-coupled product **25** was formed (eq 5). Under the same conditions, 3,6-dimethyl-2-aminophenol was readily oxidized to the corresponding phenoxazinone by the enzyme [K_m = 800 μM, V_{max} = 1.8 μmol/(mg·min); the specific activity of this preparation was 3.9 μmol/(mg·min)]. The corresponding reaction with aminoquinone **26** also failed to give the cross-coupled product (eq 6).



23 24 25 (5)



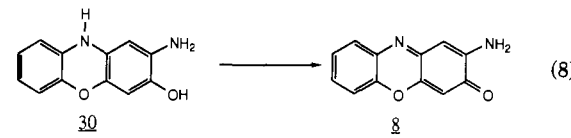
26 24 25 (6)

Oxidation of Aminophenol **28**. Phenoxazinone synthase readily catalyzed the oxidation of **28** to the corresponding *p*-quinone imine **29** (eq 7). The kinetic parameters for the

28 29 (7)

reaction were K_m = 26 μM and V_{max} = 11.1 μmol/(mg·min) [the specific activity of this preparation was 7.3 μmol/(mg·min)]. This compound proved to be quite oxygen sensitive. A 26 μM solution was oxidized at 10% of the enzymatic rate.

Oxidation of Dihydro-2-aminophenoxazinone **30**. Dihydro-2-aminophenoxazinone **30** was oxidized to 2-aminophenoxazinone so rapidly, on exposure to air, that enzymatic catalysis could not be detected (eq 8).



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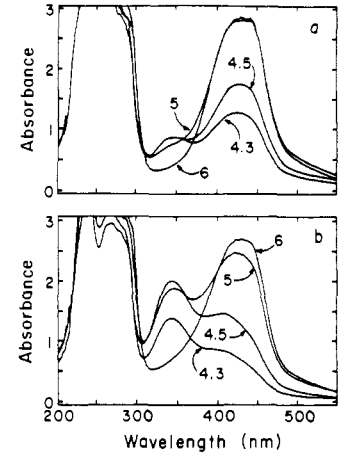

FIGURE 9: Effect of pH on 2-aminophenoxazinone formation using (a) 2-aminophenol as substrate and (b) tetradeuterio-2-aminophenol as substrate. Assays were carried out as described under Experimental Procedures.

Chirality of the Second Conjugate Addition. Racemic **22** was prepared, as a standard, by the ferricyanide-catalyzed oxidation of 5-methyl-2-aminophenol (**21**). The diastereomeric methyl groups of its Mosher amide could be readily distinguished by NMR. The corresponding product, derived from the enzymatic reaction at pH 5 and 7, was found to be racemic. The possibility of racemization of **22** after its formation by reversal of the conjugate addition was eliminated by demonstrating that the C4 protons were not exchanged when **22** was incubated in deuteriated reaction buffer for 24 h.

Deuterium Isotope Effects. The absorption spectrum of the reaction mixture, with 2-aminophenol as substrate, indicated the accumulation of a new intermediate absorbing at λ_{max} = 340 nm when the pH was lowered (Figure 9a). Enzymatic oxidation of tetradeuterio-2-aminophenol gave significantly higher concentrations of this intermediate (Figure 9b). Attempts at isolation by trapping with acetic anhydride, carbonyl diimidazole, or DCC/oxalic acid were unsuccessful, giving only complex reaction mixtures by HPLC analysis. It was observed, however, that the partially purified intermediate did not convert to 2-aminophenoxazinone under the assay conditions. Enzymatic oxidation of 4,5-dideuterio-3,6-dimethyl-2-aminophenol did not result in the formation of the 340 nm absorbing intermediate.

2-Aminophenols, monodeuteriated at the 4- and 5-positions, were synthesized to determine whether cleavage of the C4–H or the C5–H bond was responsible for the isotope effect on the formation of the 340 nm absorbing intermediate. The full isotope effect was found with 4-deuterio-2-aminophenol as substrate; no isotope effect was found with 5-deuterio-2-aminophenol as substrate.

The magnitude of the isotope effect was determined by mass spectrometry of the deuteriated 2-aminophenoxazinones resulting from incubation of mixtures of 2-aminophenol and tetradeuterio-2-aminophenol with the enzyme. This approach was taken because of the problem of deconvoluting the absorption spectrum of the reaction mixture, which in addition to 2-aminophenoxazinone contained at least one additional

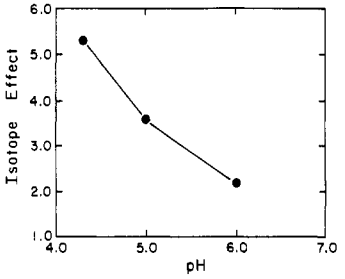

FIGURE 10: pH dependence of the isotope effect. Assays were carried out as described under Experimental Procedures.

unidentified component absorbing in the 450-nm region (Figure 9). At pH 4.3 the isotope effect was 5.3, a surprisingly large value. As expected from the absorption spectra, the magnitude of the isotope effect decreased as the pH was increased from 4.3 to 6.0 (Figure 10).

DISCUSSION

Phenoxazinone synthase catalyzes the oxidative coupling of 2-aminophenols to form the phenoxazinone chromophore. The enzyme as isolated contains 0.5–2.0 coppers/subunit, shows a lag phase, and has a specific activity in the range 0.2–3.3 μmol/(mg·min). The lag phase can be removed by dialysis against copper(II). The reconstituted enzyme contains 3–7 coppers/subunit and has a specific activity of 8.6–20 μmol/(mg·min). The enzymatic reaction has a V_{max} pH optimum at 5. Both the UV/visible spectrum and the ESR spectrum of the reconstituted enzyme are consistent with type 2 copper centers at the active site, a surprising result since tyrosinase, laccase, and ascorbate oxidase catalyze similar oxidations and all utilize a type 3 copper center (Lontie, 1984). At this point it is unknown how the type 2 copper centers of phenoxazinone synthase catalyze the transfer of four electrons from two molecules of 2-aminophenol to oxygen during the formation of 2-aminophenoxazinone. The ratio of oxygen consumed to 2-aminophenoxazinone formed was found to be 1.5 ± 0.3. This stoichiometry, however, does not preclude the formation of hydrogen peroxide as an intermediate because the enzyme also shows catalase activity.

Phenoxazinone synthase catalyzed the oxidation of a variety of substituted 2-aminophenols to the corresponding 2-aminophenoxazinones (Katz, 1967). The enzyme has proven to be an ideal candidate for mechanistic studies using modified substrates because of this lack of specificity. It was possible to distinguish among mechanisms A–D by using appropriately substituted 2-aminophenols to block the reaction at intermediate stages.

Enzyme-catalyzed oxidation of the sterically encumbered 3,5-di-*tert*-butyl-2-aminophenol (**16**) resulted in the formation of 3,5-di-*tert*-butyl-*o*-benzoquinone (**18**, eq 2). This suggested that the enzymatic reaction proceeded through the quinone imine **17** and that subsequent conjugate addition was blocked by the bulky *tert*-butyl groups. The resulting quinone imine was therefore released from the enzyme and hydrolyzed to the *o*-quinone. In support of this it was also found that the enzyme catalyzed the oxidation of catechol to *o*-benzoquinone. These observations suggested that the enzyme can catalyze 2-electron transfers from 2-aminophenol and that formation of the phenoxazinone chromophore proceeds via a quinone imine intermediate and not by the alternative radical coupling pathway.

The quinone imine intermediate **4** may undergo a sequence of hydrations/oxidations to form aminoquinones **12** and **14** (Figures 3 and 4). Alternatively, it may undergo conjugate addition with a second molecule of 2-aminophenol to form **5**. The former possibility was rendered unlikely by failure of the enzyme to catalyze the incorporation of aminoquinones **23** and **26** into 2-aminophenoxazinone, while the corresponding aminophenol was readily oxidized. While this observation did not absolutely exclude mechanisms C and D, it suggested that the enzymatic reaction followed mechanism A.

Two alternative sequences remained for the conversion of quinone imine **4** to 2-aminophenoxazinone. Intermediate **5** may be converted to dihydro-2-aminophenoxazinone **7** by a 2-electron oxidation, followed by a conjugate addition as shown in Figure 1. Alternatively, the order of these steps may be reversed. It was possible to distinguish between these pathways by using a sterically hindered substrate, in which the second conjugate addition was blocked. Enzyme-catalyzed oxidation of 5-*tert*-butyl-2-aminophenol (**19**) gave the purple *p*-quinone imine **20** (eq 3). This demonstrated that a 2-electron oxidation preceded the second conjugate addition.

Enzyme-catalyzed oxidation of 5-methyl-2-aminophenol (**21**) gave dihydro-2-aminophenoxazinone **22** in which the final 2-electron oxidation was blocked by methyl substitution at C4a. During the isolation of **22** an unstable purple intermediate was detected that rapidly converted to **22**. The similarity between the UV/visible spectrum of this intermediate and that of *p*-quinone imine **20** suggested that this compound was the *p*-quinone imine precursor to **22**.

Treatment of quinone imine **4** in acetone with 2-aminophenol (**3**) resulted in the formation of 2-aminophenoxazinone (Nogami et al., 1975). This result suggested that some of the later steps in the reaction sequence may not involve enzymatic catalysis. To explore this question, the role of the enzyme in each of the steps outlined in Figure 1 was examined.

Dihydro-2-aminophenoxazinone **30**, the more stable tautomer of **7**, was rapidly oxidized on exposure to oxygen. This reaction was so fast that we were unable to demonstrate enzyme involvement and was much faster than the overall rate of enzyme-catalyzed oxidation of 2-aminophenol to 2-aminophenoxazinone. Enzymatic catalysis of the final 2-electron oxidation was therefore not essential.

The second conjugate addition results in the formation of a chiral center at C4a. If this reaction occurred at the active site, only one stereoisomer of the product would be formed. In contrast, if it occurred in solution, the product would be racemic. The chirality at C4a of **7** could not be determined due to the instability of this intermediate. However, the stereochemistry at C4a of dihydro-2-aminophenoxazinone **22** was easily determined by NMR analysis of its Mosher amide and found to be racemic. Therefore, the second conjugate addition occurs after an intermediate has dissociated from the enzyme. The possibility of racemization at C4a due to reversal of the conjugate addition was excluded by demonstrating the absence of deuterium incorporation at C4 of **22** when it was treated with deuteriated reaction buffer for 24 h. While the kinetic parameters for the enzymatic oxidation of 2-aminophenol and 5-methyl-2-aminophenol are similar [V_{max} = 8.6 μmol/(mg·min), K_m = 500 μM^{-1}, and V_{max} = 15 μmol/(mg·min), K_m = 197 μM, respectively), suggesting the absence of unfavorable interactions between the methyl group and the enzyme, it is possible that by slowing down the second conjugate addition dissociation of the precursor to **22** from the enzyme becomes faster than the conjugate addition. Thus, for the unsubstituted aminophenol, the possibility still remains that conversion of **6** to **7** may occur at the active site.

The enzymatic reaction showed a surprisingly large deuterium isotope effect that decreased as the pH was increased

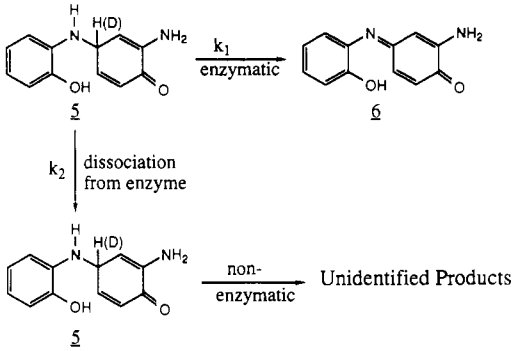

FIGURE 11: Proposed origin of the isotope effect.

(Figure 10). This isotope effect was localized to the cleavage of the C4–H bond by using regiospecifically monodeuteriated 2-aminophenols. The isotope effect, at low pH, is associated with the appearance of a new intermediate absorbing at λ_{max} = 340 nm.

An explanation for the origin of the isotope effect is outlined in Figure 11. It is proposed that a fraction $[k_2/(k_1 + k_2)]$ of intermediate **5** dissociated from the enzyme. With the deuteriated substrate, this fraction is increased due to a primary isotope effect on k_1. It is unlikely that the observed isotope effect is a V/K effect because it is improbable that **5** is in equilibrium with **3** during catalysis. As the 340 nm absorbing intermediate does not convert to 2-aminophenoxazinone, it is likely to be a decomposition product of **5** formed under acidic conditions. Our attempts to trap and characterize this intermediate have so far been unsuccessful.

The decrease in the magnitude of the isotope effect as the pH of the reaction mixture is increased from 4.3 to 6.0 (Figure 10) is consistent with the increasing magnitude of V_{max} and the decreasing magnitude of K_m that occurs over this pH range (Figure 7). The observation of an isotope effect of 2.0 at pH 6 suggests that even under optimum conditions of catalysis a partitioning of **5** between the enzymatic and the nonenzymatic pathways is occurring.

The absence of the 340 nm absorbing intermediate from the corresponding enzymatic oxidation of 4,5-dideuterio-3,6-dimethyl-2-aminophenol suggests that substitution of **5** with four methyl groups retards the dissociation of this intermediate from the enzyme. This observation raises the possibility that additional interactions between the enzyme and the natural substrate **1** may completely suppress dissociation at least at this stage.

Addition of quinone imine **4** to a solution of aminophenol (**3**) in assay buffer resulted in the formation of 2-aminophenoxazinone in low yield. Hydrolysis to *o*-benzoquinone was the major reaction. The high yield of the enzyme-catalyzed reaction indicates that the quinone imine intermediate **4** is efficiently trapped by 2-aminophenol at the active site.

The proposed pathway for the formation of 2-aminophenoxazinone (Figures 1 and 12) is a complex multistep sequence involving three 2-electron oxidations and two conjugate additions. The observation that the enzyme catalyzed the oxidation of aminophenol **28** suggested a simplification of the reaction mechanism. Regeneration of the 2-aminophenol functionality, after each of the conjugate additions, by a tautomerization (**5** to **34** and **7** to **30**, Figure 13) reduces the catalytic requirements of the enzyme to the catalysis of two sequential 2-electron aminophenol oxidations. As the reaction proceeds, the 2-aminophenols (**3**, **34**, and **30**) become progressively more electron rich, thus allowing for nonenzymatic steps toward the end of the reaction sequence. This proposal has some similarities to the mechanism for the oxidation of

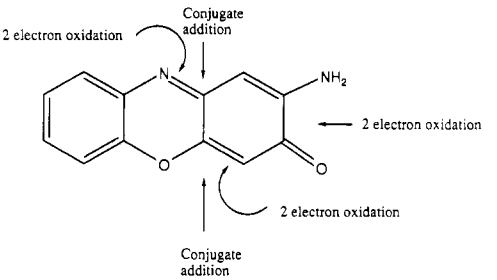

FIGURE 12: Reactions required for the assembly of the phenoxazinone chromophore.

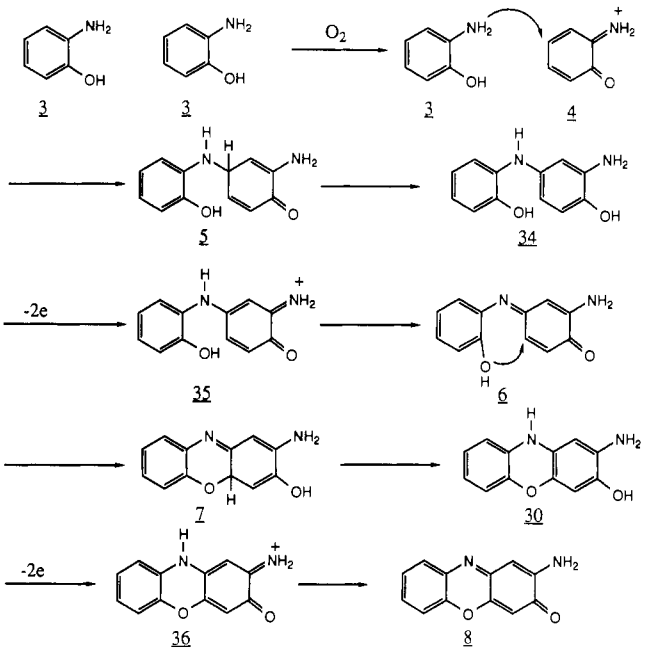

FIGURE 13: 2-Aminophenoxazinone synthesis by an aminophenol oxidative cascade.

dopa to eumelanin (Lontie, 1984).

The isotope effect on the conversion of **5** to **34** can be explained in terms of an active site designed for efficient trapping of the quinone imine **4**. For this to occur, the second 2-aminophenol must be held above or below the plane of the quinone imine at the active site. Conversion of **5** to **34** involves considerable change in the shape of the intermediate. We suggest that the active site is unable to accommodate this geometry change, thus retarding the tautomerization and facilitating dissociation of **5** from the enzyme.

Conclusions. Phenoxazinone synthesis proceeds via a quinone imine intermediate **4** that undergoes a conjugate addition, at the active site, with a second molecule of 2-aminophenol. The resulting intermediate **5** then undergoes a 2-electron oxidation to the *p*-quinoneimine **6**. This reaction occurs, in part, at the active site. A second conjugate addition followed by a final 2-electron oxidation gives the phenoxazinone chromophore. Both of these steps occur outside the active site. We propose that phenoxazinone synthesis occurs via a cascade of three consecutive 2-electron aminophenol oxidations in which the 2-aminophenol functionality is regenerated, after each conjugate addition, by a facile tautomerization reaction.

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