Spectroscopic Studies on the Mechanism of the Topa Quinone Generation in Bacterial Monoamine Oxidase

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ABSTRACT: Electron paramagnetic resonance (EPR), circular dichroism (CD), and optical absorption spectroscopies have been used to investigate the copper-dependent autoxidation process generating the 6-hydroxydopa (topa) quinone cofactor in the recombinant phenethylamine oxidase from *Arthrobacter globiformis*. The cupric ion bound to the copper/topa quinone-less, inactive enzyme is first reduced to Cu(I), as inferred from the spectroscopic features observed under strictly anaerobic conditions. Cu(I) is also detectable chemically with a Cu(I)-specific chelating agent, bathocuproinedisulfonate. Introduction of a limited amount of oxygen then leads to the formation of a paramagnetic species ($g = 2.004$) that is stable for over several to 10 min but vanishes swiftly upon addition of sufficient oxygen. Strikingly, the hyperfine EPR structure of the organic radical is almost identical with that of the topa semiquinolamine observed in the copper/topa quinone-containing, active enzyme anaerobically reduced with substrate. Concomitant with the generation of topa quinone exhibiting characteristic optical absorption and CD bands under fully aerobic conditions, the bound copper finally shows EPR signals typical of nonblue, type II Cu(II) and optical absorption around 700 nm with negative CD above 700 nm. None of these spectral changes are evoked in the binding of Cu(II) to the Tyr382 → Phe mutant enzyme, indicating that the precursor Tyr382 to topa quinone participates in the initial reduction of bound copper and serves as the origin of the transiently formed semiquinone radical. The prosthetic cupric ion plays an essential role, by changing its redox state, in the oxidative modification of the tyrosyl phenol ring, leading to topa quinone.

Copper-containing amine oxidases (EC 1.4.3.6) catalyze the oxidation of various biogenic primary amines to the corresponding aldehydes, ammonia, and hydrogen peroxide [for recent reviews, see McIntire and Hartmann (1993), Knowles and Dooley (1994), and Klinman and Mu (1994)]. Besides copper, the enzymes also contain a covalently bound organic cofactor, whose structure in the bovine plasma enzyme has recently been identified as the quinone of 3-(2,4,5-trihydroxyphenyl)-L-alanine (6-hydroxydopa; topa[1]), integrated in the polypeptide chain (Janes et al., 1990). In the genes subsequently cloned and sequenced for the enzymes from various types of organisms, the quinone cofactor is encoded by a tyrosine codon (Zhang et al., 1993; Mu et al., 1994; Azakami et al., 1994), implicating that the topa quinone cofactor is generated from the precursor tyrosyl residue by a hitherto unknown mechanism of posttranslational modification (Mu et al., 1992; Cai & Klinman, 1994). More recently, we have demonstrated, using the copper/topa quinone-less, inactive form of the recombinant phenethylamine oxidase from *Arthrobacter globiformis* (Tanizawa et al., 1994), that the topa quinone cofactor is generated by copper-dependent autoxidation of the precursor Tyr (Matsuzaki et al., 1994). Thus, it has been revealed for the first time that the posttranslational modification generating topa quinone requires, at least *in vitro*, no external enzymic systems except for the prosthetic metal ion.

To further elucidate the mechanism of the topa quinone generation in the recombinant phenethylamine oxidase, we have investigated the copper-dependent autoxidation process by EPR, CD, and optical absorption spectroscopies. In this report we provide spectroscopic and chemical evidence for the change of the redox state of the bound copper and the transient formation of a semiquinone radical during the topa quinone generation. It has been suggested that the bound copper participates, by changing its redox state, Cu(II) ⇌ Cu(I), in the two-step oxidation of the phenol ring of the precursor Tyr382 to topa quinone. Furthermore, the semiquinone radical formed in a later phase of the topa quinone generation has a hyperfine EPR structure almost identical with that of the topa semiquinolamine radical observed when the copper/topa quinone-containing, active enzyme is anaerobically reduced by a substrate, suggesting the close mechanistic relationship between the topa quinone generation and the amine oxidase catalysis.

EXPERIMENTAL PROCEDURES

Purification and Assay of Copper/Topa Quinone-less Enzyme. The recombinant phenethylamine oxidase from *A. globiformis* overproduced in *Escherichia coli* cells grown in a copper-depleted medium (Tanizawa et al., 1994) was purified in the copper/topa quinone-less, inactive form to >99% homogeneity on SDS gels and assayed after incuba-

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[1] Abbreviations: CD, circular dichroism; EPR, electron paramagnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; topa, 3-(2,4,5-trihydroxyphenyl)-L-alanine.

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tion at 30 °C for 30 min with a 5−10 times molar excess of $CuSO_4$ over the enzyme subunit (to generate the topa quinone cofactor), as described previously (Matsuzaki et al., 1994). Specific activity of the purified, copper-activated enzyme was in the range of 40−50 units/mg of protein, which is much higher than that of the previous preparations (20−24 units/mg; Matsuzaki et al., 1994), probably because partial inactivation of the enzyme could be avoided by the prompt purification presently employed. Protein concentrations were determined spectrophotometrically at 280 nm using extinction coefficients of 12.3 and 13.2 for 1% solutions of the copper/topa quinone-less, precursor enzyme and the copper/topa quinone-containing, active enzyme, respectively (Matsuzaki et al., 1994). For calculation of the subunit concentration of the dimeric enzyme, a molecular weight of 70 600 (Tanizawa et al., 1994) was used; the enzyme concentrations are expressed in terms of the subunit concentration (molar basis) throughout the present studies. The Tyr382 → Phe mutant enzyme, in which Phe is substituted for the precursor Tyr382 to topa quinone (Tanizawa et al., 1994), was also purified to >99% homogeneity in the copper-free form by the same procedure as for the wild-type enzyme (Matsuzaki et al., 1994).

Copper Analysis. The copper contents in the enzyme were analyzed with a Nippon Jarrell-Ash AA-880 mark II atomic absorption spectrophotometer (acetylene/air flame) at 324.8 nm. Before copper analysis, the enzyme samples were dialyzed thoroughly against 50 mM Hepes, pH 6.8, containing 3 mM EDTA, followed by further dialysis against buffer alone, to remove the unbound copper. The cuprous ion was quantitated photometrically with a Cu(I)-specific chelating agent, disodium bathocuproinedisulfonate (Dojindo Laboratories Ltd., Kumamoto, Japan), which forms a 2:1 complex with Cu(I) in an aqueous solution (ϵ = 12 000 $M^{-1}\cdot cm^{-1}$ at 483 nm). The chelating agent (10 mM) dissolved in 50 mM Hepes, pH 6.8, was added at a final concentration of 1 mM to the enzyme solution under anaerobic conditions (see below).

Spectroscopic Measurements. To establish strictly anaerobic conditions, all solutions of enzyme, copper, buffer, and substrate were deaerated by keeping for at least 5−6 h in a bench-top glove box filled with 99.999% Ar and mixed in the box with further flushing with Ar for 3−5 min, immediately before spectroscopic measurements. EPR spectra were measured at room temperature or 77 K with a JEOL JES-FE1X EPR spectrometer. For measurements at room temperature, approximately 200 μL of sample was drawn into a thin-walled flat quartz capillary (0.3 mm × 4 mm × 45 mm), and the capillary was tightly sealed with Parafilm; oxygen diffusion into the samples during the measurements was found to be negligible. For introducing a limited amount of the dissolved oxygen during the measurements, an appropriate volume of water saturated with O_2 gas (oxygen concentration, about 1.4 mM at 20 °C, 1 atm) was added to the cell (or capillary). g values were calibrated with lithium tetracyanoquinodimethane (TCNQ) as an external standard. Low-temperature EPR spectra were quantitated by comparing double integrals of spectra with that of the standard spectrum of 0.4 mM [Cu(2,2′-bipyridyl)$(H_2O)_2$]$(NO_3)_2\cdot H_2O$ recorded under similar conditions. Concentrations of organic radicals were estimated from EPR signals of 2,2-diphenyl-1-picrylhydrazine hydrate (Aldrich). Optical absorbance spectra were measured with a Hewlett-Packard 8452A diode-array spectrophotometer. CD measurements were carried out with a JASCO J-500A spectropolarimeter attached with a DP-500 data processor.

RESULTS

Anaerobic Binding of Copper to Topa Quinone-less Enzyme. The recombinant phenethylamine oxidase overproduced and purified in the absence of copper as described previously (Matsuzaki et al., 1994) contained less than 0.01 mol atom of copper/mol of subunit on atomic absorption analysis and had a specific activity of only about 1% of the maximal level (40−50 units/mg of protein) that could be obtained by incubation with excess cupric ions. The EPR (Figure 1a), optical absorbance (Figure 2a), and CD (Figure 3a) spectra are also consistent with the absence of both copper and topa quinone in the inactive enzyme.

Concomitant with the dramatic activation of the inactive enzyme by aerobic incubation with cupric ions, the topa quinone cofactor is generated through autoxidation of the precursor Tyr382 (Matsuzaki et al., 1994). Therefore, to examine the initial binding of copper and its redox state before the autoxidation reaction starts, spectroscopic measurements have been performed under strictly anaerobic conditions. Figure 1b represents the X-band EPR spectrum of the enzyme anaerobically mixed with a 0.8 mol equiv of $CuSO_4$/mol of subunit, measured at liquid nitrogen temperature (77 K); $CuSO_4$ in less than the stoichiometric amount was added to suppress EPR signals due to free or adventitiously bound Cu(II). When compared with the low-temperature EPR spectrum of the bipyridyl-chelated cupric ion at the same concentration (0.4 mM) exhibiting typical Cu(II) signals (Figure 1d), it is evident that a major portion of the enzyme-bound copper is converted into an EPR-silent form, most probably Cu(I), with added Cu(II) accounting for only about 0.07 mM as estimated by double integration of the EPR spectrum (Figure 1b). This suggests that the added copper is bound readily to the enzyme and reduced to Cu(I) in the absence of dioxygen.

To detect Cu(I) chemically, we used a cuprous ion-specific chelating agent, bathocuproinedisulfonate. The enzyme anaerobically reconstituted with Cu(II) in the presence of the chelating agent exhibited an intense orange color due to the formation of the Cu(I)−reagent (1:2) complex, which was not formed with the copper/quinone-containing active enzyme, irrespective of the presence of the dissolved oxygen. The amount of Cu(I) formed in the anaerobically reconstituted enzyme was estimated to be about 74% of the amount of added Cu(II) from the 483-nm absorbance of the complex, in good agreement with the value (83%) calculated from the EPR spectrum. The CD specrum shown in Figure 3b also indicates the reduction of Cu(II) to Cu(I), which exhibits no CD in the wavelength region of 450−800 nm; the bound Cu(II) in the fully oxidized (activated) enzyme exhibits negative CD in the 700−800-nm region arising from $d \rightarrow d$ transitions (vide infra).

The EPR spectrum of the Tyr382 → Phe mutant enzyme anaerobically mixed with a 0.8 mol equiv of $CuSO_4$ (Figure 1e) shows that most of the bound copper remains in the Cu(II) state, although the binding mode of copper appears heterogeneous by EPR spectroscopy [double integration of the spectrum gave the Cu(II) concentration of about 0.6 mM, which was probably overestimated due to the aberrant

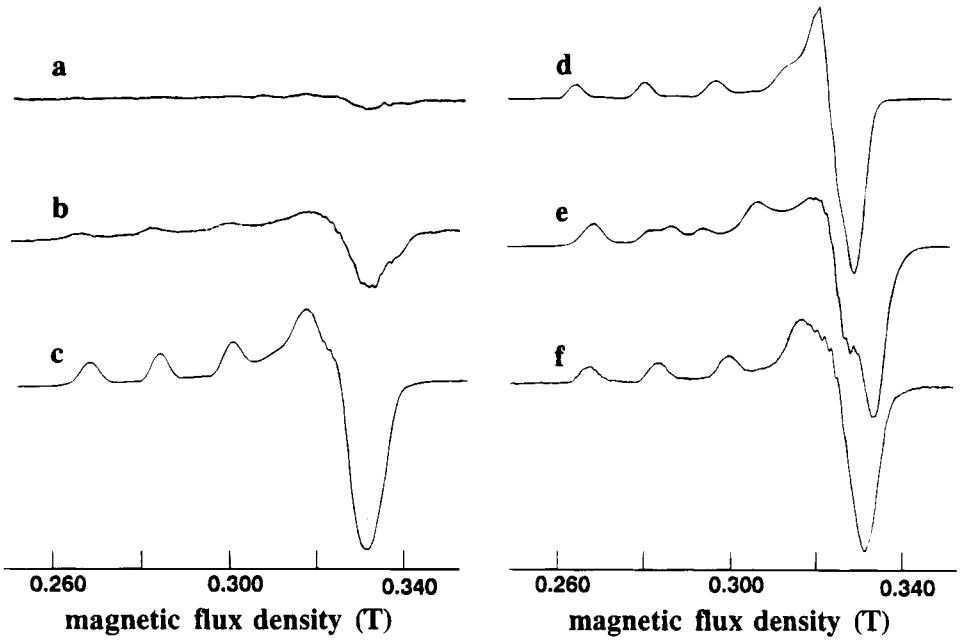


FIGURE 1: Low-temperature EPR spectra of phenethylamine oxidase before and after reconstitution with cupric ions. Spectra were obtained with approximately the same sample volumes of (a) the purified copper/topa quinone-less, inactive enzyme (0.5 mM) in 50 mM Hepes, pH 6.8; (b) the enzyme (0.5 mM) anaerobically mixed with 0.4 mM $CuSO_4$; (c) the fully oxidized (activated) enzyme (0.4 mM; the copper content after further addition of copper to 0.8 mM followed by dialysis against 3 mM EDTA was 1.05 mol atom/mol of subunit by atomic absorption and 0.95 spin/mol of subunit from the EPR spectrum); (d) the standard Cu(II) solution (0.4 mM $[Cu(2,2'\text{-bipyridyl})(H_2O)_2](NO_3)_2 \cdot H_2O$); (e) the Tyr382 → Phe mutant enzyme (0.5 mM) anaerobically mixed with 0.4 mM $CuSO_4$; and (f) the oxidized Tyr382 → Phe mutant enzyme (0.33 mM) treated with excess copper followed by dialysis against 3 mM EDTA, containing 0.91 mol atom of copper/mol of subunit. All spectra were measured at 77 K; microwave power, 5 mW; frequency, 9.28 GHz; modulation, 0.63 mT; amplitude, (a–d) 400, (e) 160, (f) 500.

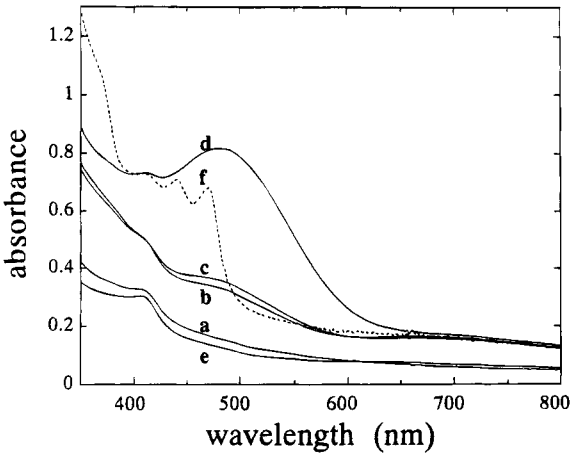


FIGURE 2: Optical absorbance spectra of phenethylamine oxidase during the topa quinone generation. Spectra were obtained with (a) the purified copper/topa quinone-less, inactive enzyme (0.5 mM) in 50 mM Hepes, pH 6.8; (b) the enzyme (0.5 mM) anaerobically mixed with 0.5 mM $CuSO_4$; (c) the copper-reconstituted enzyme (0.5 mM) with about 0.3 mM concentration of the dissolved oxygen; (d) the fully oxidized (activated) enzyme (0.5 mM; copper content by atomic absorption, 1.03 mol atom/mol of subunit); (e) the Tyr382 → Phe mutant enzyme (0.5 mM; copper content, 0.97 mol atom of copper/mol of subunit); and (f) the copper/topa quinone-containing, active enzyme (0.5 mM) reduced with 1.5 mM phenethylamine sulfate under anaerobic conditions (dotted line).

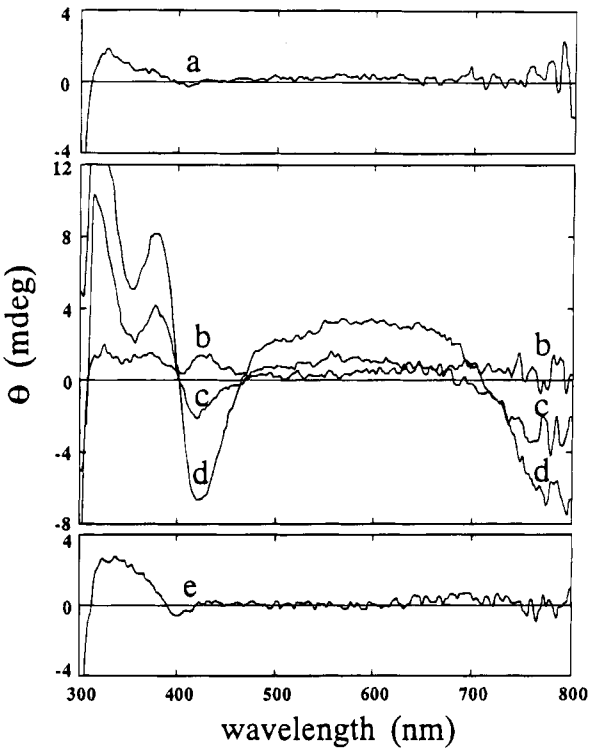


FIGURE 3: CD spectra of phenethylamine oxidase during the topa quinone generation. Enzyme samples for spectra a–e correspond to those in Figure 2.

signals]. Also, Cu(I) was not detected at all with the chelating agent. A reasonable conjecture derived from this observation would be that the precursor Tyr382 to topa quinone participates in the initial reduction of Cu(II) to Cu(I) on binding to the wild-type enzyme.

Introduction of a Limited Amount of Oxygen. To the enzyme solution anaerobically reconstituted with copper was then introduced a limited amount of the dissolved oxygen (about 0.6 mol equiv to the subunit) to initiate the oxidative modification of the precursor Tyr, and the changes of EPR spectra were recorded at room temperature. Accompanying the gradual increase in EPR signals ascribed to Cu(II), an

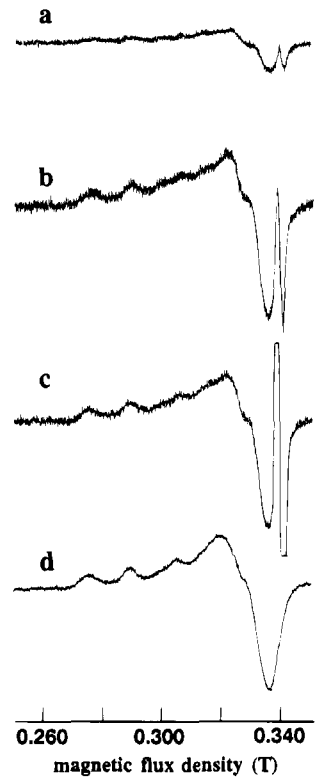


FIGURE 4: Room temperature EPR spectra of the copper-reconstituted enzyme measured after introduction of a limited amount of the dissolved oxygen. Spectra were obtained in a flat quartz capillary containing the topa quinone-less, inactive enzyme (1.2 mM) and 2.0 mM $CuSO_4$ in 50 mM Hepes, pH 6.8, prepared and mixed in an Ar atmosphere glove box; measured at (a) 2 min, (b) 10 min, and (c) 20 min after introduction of about 0.9 mM final concentration of the dissolved oxygen and (d) immediately after exposure to excess air. Frequency, 9.46 GHz; power, 5 mW; modulation, 0.63 mT; amplitude, (a) 2000, (b) 5000, (c) 3200, (d) 2500; room temperature.

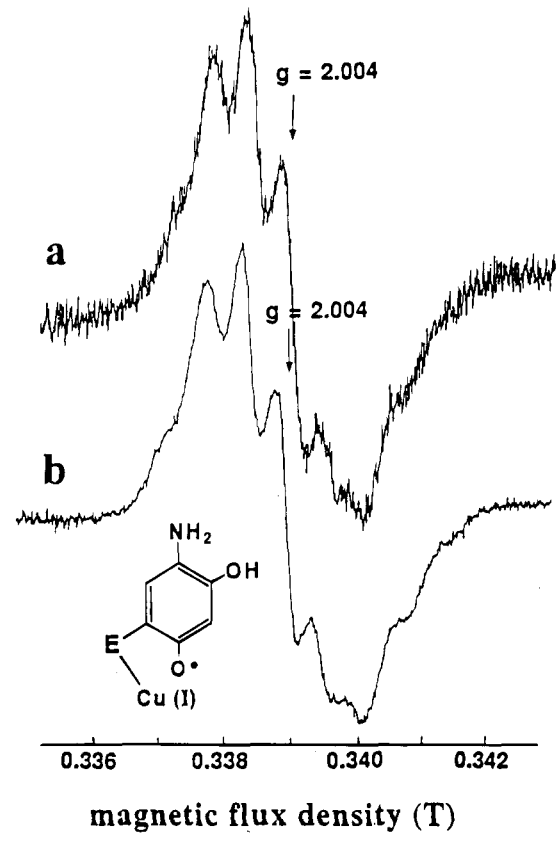


FIGURE 5: Hyperfine EPR structures of the free radical species generated in the copper-reconstituted enzyme under slightly aerobic conditions and in the copper/topa quinone-containing, active enzyme anaerobically reduced by substrate. Spectrum a was obtained at 20 min after introduction of a limited amount of the dissolved oxygen to the topa quinone-less, inactive enzyme anaerobically mixed with cupric ions (enzyme, copper, and oxygen concentrations, same as in Figure 4); frequency, 9.46 GHz; power, 3 mW; modulation, 0.08 mT; amplitude, 4000; room temperature. Spectrum b was obtained with the copper/topa quinone-containing, active enzyme (0.9 mM) reduced with 1.5 mM phenethylamine sulfate under anaerobic conditions; frequency, 9.46 GHz; power, 5 mW; modulation, 0.08 mT; amplitude, 1000; room temperature. The proposed structure for the semiquinone radical catalytic intermediate (spectrum b) is also shown.

EPR signal centered at $g = 2.004$ appeared with time (Figure 4a,b), indicating the formation of a free radical species. The organic radical was not observed on introduction of excess air, presumably because it disappears very rapidly in the presence of sufficient oxygen, as shown later. The time needed for the maximal formation of the radical and the magnitude of the EPR signal were variable, depending on the concentrations of the enzyme (1.0−1.5 mM subunit) and Cu(II) (mole equivalent to or a slight excess over the enzyme subunit) relative to the concentration of the added oxygen, which could not be increased to that close to the enzyme concentration due to the limitation of the introducible volume of the O_2-saturated water (dissolved O_2 concentration, about 1.4 mM at 20 °C, 1 atm) in keeping the high enzyme concentration. However, typically at about 20 min after introduction of a substoichiometric amount of oxygen, the magnitude of the EPR signal reaches the maximum level (Figure 4c), being high enough to resolve the hyperfine splitting as described below. The radical species was stably observed in the room temperature EPR for over several to 10 min unless the enzyme solution was further exposed to air. The absorption and CD spectra of the enzyme measured under similar conditions indicated that very little topa quinone was yet generated (Figures 2c and 3c) but a considerable portion of the bound copper was in the Cu(II) state with negative CD above 700 nm (Figure 3c), which appeared to correspond to about 0.4 spin/mol of subunit from the EPR signals (Figure 4c). The radical species is thus thought to be a partially oxidized intermediate before topa quinone. Introduction of a limited amount of oxygen to the Tyr382 → Phe mutant enzyme anaerobically mixed with cupric ions did not result in the formation of such radical species (data not shown), revealing that the organic radical derives from the precursor Tyr382.

Surprisingly, the EPR signal of the radical species at $g = 2.004$ (Figure 4c) was almost identical with that observed when the copper/topa quinone-containing, active enzyme was incubated with the substrate phenethylamine under anaerobic conditions, having the same g value (not shown). The hyperfine structures are also indistinguishable from each other (Figure 5a,b), although the spin quantitation indicated that the concentration of the transient radical corresponded to only about 5% of the enzyme concentration, while that of the substrate-derived radical accounted for about 40% of the enzyme concentration with a comparable amount of the bound copper being detectable as Cu(I) by the chelating agent. Quite similar substrate-derived radicals have previously been reported for several copper amine oxidases anaerobically reduced with substrate [e.g., Dooley et al.

(1991) and Pedersen et al. (1992)] and assigned to a topa semiquinone/Cu(I) complex, which is in temperature-dependent rapid equilibrium with the $2e^-$-reduced topa hydroquinone/Cu(II) complex (Turowski et al., 1993). Since identical hyperfine splittings have been observed independently of the kinds of amine substrate and enzyme used, the topa semiquinone radical is believed to be associated with a common intermediate in the amine oxidase catalysis, structurally referred to as topa semiquinolamine (or aminosemiquinone) (Figure 5), in which the amino group is derived from substrate (Bellelli et al., 1991; Warncke et al., 1994). It should be noted, however, that the present reaction system for the topa quinone generation contains none of the amine substrates and exogenous agents known to reduce the bound copper and the quinone cofactor such as sodium dithionite (Yamada et al., 1965; Mondovi et al., 1967).

As has been reported previously (Dooley et al., 1991; Bellelli et al., 1991; Pedersen et al., 1992; Turowski et al., 1993), the substrate-derived semiquinone radical displays a characteristic absorption spectrum consisting of two sharp transitions at 468 and 438 nm and a major shoulder around 360 nm (Figure 2f). However, we have so far been unable to observe such absorption bands with the copper-reconstituted enzyme under slightly aerobic conditions (Figure 2c), probably because the concentration of the radical species formed is rather low as described above.

Exposure to Excess Air. Introduction of excess air to the above sample in the slightly aerobic conditions resulted in an immediate disappearance of the free radical species (Figure 4d) and led finally to the generation of topa quinone with an intense absorption band at 475 nm (Figure 2d) and a distinct positive CD band around 370 nm and a negative CD band near 420 nm (Figure 3d). In addition, the bound copper now shows EPR signals (Figure 1c) that indicate the axial symmetry of Cu(II) with spin Hamiltonian parameters $g_{||} = 2.29$, $g_{\perp} = 2.06$, and $A_{||} = 16.0$ mT. Broad and weak absorption around 700 nm (Figure 2d) and negative CD above 700 nm (Figure 3d) are probably due to the $d \rightarrow d$ transitions of the tetragonal Cu(II) bound to the metal center; the copper contents in several preparations of the fully oxidized (activated) enzyme were determined to be 0.95−1.05 mol atom/mol of subunit by atomic absorption and close to 1.0 spin/mol of subunit from EPR spectra (Figure 1c). These magnetic and electronic spectral features of the bound copper are typical of nonblue, type II copper and fully accord with those reported previously for other copper amine oxidases including the resting (oxidized) form of bovine serum amine oxidase (Suzuki et al., 1983, 1986) and methylamine oxidase of *Arthrobacter* P1 (Dooley et al., 1990) and those of the recombinant phenethylamine oxidase overproduced in the presence of cupric ions and purified in the copper/quinone-containing active form (Matsuzaki and Tanizawa, unpublished results). The two Cu(II) ions in the dimeric enzyme appear to be equivalent by EPR spectroscopy, and each copper atom is probably coordinated with equatorial nitrogen/oxygen ligands, most likely derived from three conserved histidine imidazoles and an H_2O molecule, as suggested in numerous studies of other copper amine oxidases [e.g., Scott and Dooley (1985) and Dooley et al. (1990)].

It is noteworthy that the Tyr382 → Phe mutant enzyme reconstituted with copper and exposed to air exhibits no characteristic absorption and CD bands in the 600−800-nm region (Figures 2e and 3e), even though it contains around 0.9 mol atom of copper/mol of subunit on atomic absorption analysis and shows EPR signals of Cu(II) ($g_{||} = 2.30$, $g_{\perp} = 2.06$, and $A_{||} = 16.0$ mT) equivalent to about 0.8 spin/mol of subunit (Figure 1f), indicating that the tetragonal structure of the metal center is similar to that of the wild-type enzyme. A possible interpretation of these results is that the coordination state, in particular afforded by axial ligands, of the Cu(II) site in the mutant enzyme is somehow distorted not to exhibit $d \rightarrow d$ transitions characteristic of the amine oxidase-bound copper. Combined with the fact that the topa quinone cofactor is not generated in the Tyr382 → Phe mutant enzyme (Matsuzaki et al., 1994), it is therefore suggested that topa quinone needs to be present for the bound Cu(II) to display proper spectral features in the 600−800-nm region. The spectral feature of the wild-type enzyme further implies a close electronic interaction between Cu(II) and topa quinone, whose distance has been estimated to be approximately 3.0 Å in the active site of copper amine oxidases (McGuirl et al., 1991).

DISCUSSION

Spectroscopic and chemical evidence presently obtained clearly shows that the Cu(II) bound anaerobically to the copper/quinone-less inactive form of the recombinant phenethylamine oxidase undergoes $1e^-$ reduction to Cu(I) (Figure 1b; detection with bathocuproinedisulfonate). This Cu(II) reduction occurs prior to the initiation of the oxidative modification of the precursor Tyr382 to topa quinone that proceeds in multiple steps comprising at least two first-order processes (Matsuzaki et al., 1994). Since the copper anaerobically bound to the Tyr382 → Phe mutant enzyme is not reduced to Cu(I) (Figure 1e), it is reasonable to assume that the single electron in the initial reduction of Cu(II) bound to the wild-type enzyme is provided from the phenol group of Tyr382. The assignment of tyrosine to an electron donor is consistent with its lowest E_0' (+0.93 V vs NHE; therefore most easily oxidized) among the 20 common amino acid constituents of proteins in neutral, aqueous solution (DeFelippis et al., 1989, 1991). However, the tyrosyl radical that should be formed on donation of $1e^-$ to Cu(II) has been undetectable so far in the present EPR measurements performed at both room temperature and 77 K [although a trace amount of an organic radical has been detected in an anaerobic EPR measurement at 77 K in the presence of bathocuproinedisulfonate added to stabilize the radical by chelating Cu(I); Matsuzaki et al., unpublished results]. It is even unknown whether the tyrosyl radical is formed but is undetectable with a very short lifetime or not formed at all. Alternatively, if Tyr382 is not a direct electron donor in the Cu(II) to Cu(I) reduction, another protein group, most likely one of the potentially redox active amino acids, such as tyrosine (except for Tyr382), cysteine, cystine, or tryptophan, has to be considered as an electron donor to Cu(II), but then its redox capability must be impaired somehow in the Tyr382 → Phe mutant enzyme, which seems inconceivable. The EPR inaccessibility also cannot be explained. Further studies are thus needed to identify the electron donor in the Cu(II) reduction and to clarify the fate of the resulting $1e^-$-deficient species in the absence of the dissolved oxygen.

Type III copper proteins, such as tyrosinase and hemocyanin, have a dinuclear Cu(II) site, being diamagnetic due to the strong antiferromagnetic coupling between the two

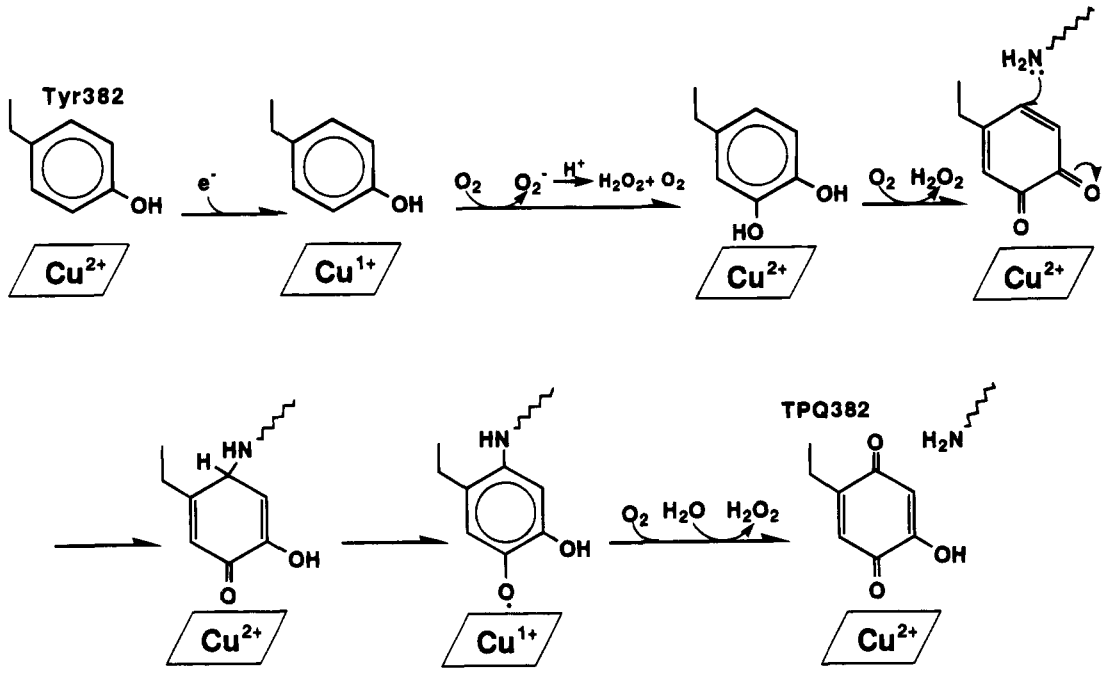


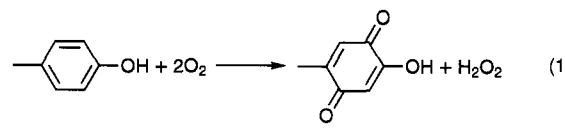
FIGURE 6: Postulated mechanism of the topa quinone generation through the Cu(I)/semiquinone intermediate. The semiquinone radical observed in this study corresponds to the species shown in the middle of the reaction scheme (lower row) and is covalently linked to a hypothetical lysyl ϵ-amino group of the protein. The positions of the oxygen and nitrogen substituents on the ring are interchangeable. Topa quinone (TPQ) is depicted as the *p*-carbonyl tautomer (Janes et al., 1990).

Cu(II) ions and capable of reducing O_2 ($2e^-$ reduction) to a peroxide ion, O_2^{2-}, bound in a symmetric coordination mode (Himmelwright et al., 1980; Loehr, 1988). By contrast, the copper site in amine oxidase is mononuclear, and hence the bound Cu(II) is generally inert with O_2. Nevertheless, once it is reduced to Cu(I) as described above, O_2 would readily accept an electron from Cu(I), yielding a superoxide anion, O_2^-; indeed, Cu(I) is usually very reactive toward O_2 (Greenwood & Earnshaw, 1972). In addition, evidence for a bound superoxide intermediate, produced from O_2 with the presumptive Cu(I) during the oxidative phase of the amine oxidase catalytic cycle, has been presented in several independent studies (Rotilio et al., 1970; Younes & Weser, 1978; Dooley et al., 1984). Although the formation of O_2^- in the topa quinone generation is devoid of direct proof, we have observed that a radical scavenger, ascorbic acid, does inhibit the topa quinone generation, though partially (Matsuzaki and Tanizawa, unpublished results). However, superoxide dismutase had apparently no effect, suggesting that the O_2^- formed, if any, is well sequestered from solvent or even firmly bound to Cu(II).

The O_2^- produced (or more likely H_2O_2 formed thereof by proton abstraction) would then hydroxylate the phenol ring of Tyr382 to form dihydroxyphenylalanine (dopa), as depicted in Figure 6. Notwithstanding that the ring hydroxylation that occurs first at either position (ortho or meta relative to the tyrosine hydroxyl group) is unclear at present, following oxidation of dopa to dopa quinone presumably results in an electron deficiency at the para position of the carbonyl group so that the subsequent hydroxylation could easily proceed at this position, leading to topa. Indeed, recent studies by Mure and Klinman (1993) using synthetic model compounds have demonstrated that the quinone of dopa hydantoin rapidly undergoes 1,4-addition of water under alkaline conditions to give topa.

On the other hand, we have found the formation of an organic radical in the presence of a limited amount of the dissolved oxygen (Figures 4c and 5a), which appears kinetically to be an intermediate formed in a later stage of the topa quinone generation. Despite the fact that no amine substrate is included in the present system, the hyperfine structure of the radical species remarkably resembles that of the topa semiquinolamine formed in the copper/topa quinone-containing, active enzyme anaerobically reduced with substrate (Figure 5b) but is clearly distinct from those of tyrosyl radicals reported previously [e.g., Sjöberg et al. (1978) and Hoganson and Babcock (1992)]. Accordingly, provided that the identity of hyperfine EPR signals can be regarded as indicating the structural similarity between the radical species formed under slightly aerobic conditions and the substrate-derived topa semiquinolamine catalytic intermediate (Warncke et al., 1994), one has to assume the involvement of an amino group of the protein (i.e., a lysyl residue), instead of the substrate amino group, in the transiently formed semiquinone structure. The formation of this semiquinolamine-like radical has thus led us to propose, although without direct evidence, the participation of a lysyl ϵ-amino group in the topa quinone generation (Figure 6), rather than the direct hydration as suggested in the model studies.

The minimum formula for the topa quinone generation (eq 1) indicates the net $2e^-$ oxidation of the precursor Tyr with simultaneous $2e^-$ reduction of O_2 to H_2O_2. This



(1)

requires that the $1e^-$ reduction of O_2 through the mononuclear Cu(II) site must occur at least twice in the postulated mechanism without formation of O_2^{2-}. Therefore, by analogy with the semiquinolamine intermediate accounting for the Cu(I) state in amine oxidase catalysis (Dooley et al.,

1991; Pedersen et al., 1992; Turowski et al., 1993) [note that Cu(I) formed in the substrate-reduced enzyme was indeed detected chemically in the present studies], the formation of the radical species in a later phase of the topa quinone generation (Figure 5) well rationalizes the second $1e^-$ reduction of Cu(II) to Cu(I) (Figure 6). Thus, on further supply of oxygen, O_2^- would again be formed by Cu(I) and reacts with the semiquinone radical, finally leading to the fully oxidized topa quinone. This last step mimics the oxidative phase of the catalytic cycle of copper amine oxidases described in most recent literatures (Hartmann & Klinman, 1991; Bellelli et al., 1991; Turowski et al., 1993) and thereby suggests the close mechanistic relationship between the topa quinone generation and the amine oxidase catalysis, in both of which the prosthetic metal, copper ion, serves as an electron mediator by changing its redox state, Cu(II) $\rightleftharpoons$ Cu(I).

The proposed scheme for the topa quinone generation in phenethylamine oxidase (Figure 6) does not necessarily represent the reaction involved in the topa quinone formation in the cell but would certainly provide for the first time a clue to elucidate the mechanism of biogenesis of the unique redox amino acid cofactor that has emerged in a wide range of copper amine oxidases (McIntire & Hartmann, 1993). Finally, attempts to obtain chemical and kinetic evidence further detailing and confirming the proposed mechanism are in progress, e.g., by examining the incorporation of ^{18}O from $^{18}O_2$ or $H_2{}^{18}O$ into topa quinone, determining the stoichiometry of O_2 consumption, H_2O_2 production, and topa quinone formation, and identifying the postulated lysyl residue involved in the semiquinone radical.

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