Spectroscopic Observation of Intermediates Formed during the Oxidative Half-Reaction of Copper/Topa Quinone-Containing Phenylethylamine Oxidase†

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ABSTRACT: The catalytic reaction of copper/topa quinone (TPQ) containing amine oxidase consists of the initial, well-characterized, reductive half-reaction and the following, less studied, oxidative half-reaction. We have analyzed the oxidative half-reaction catalyzed by phenylethylamine oxidase from *Arthrobacter globiformis* (AGAO) by rapid-scan stopped-flow measurements. Upon addition of dioxygen to the substrate-reduced AGAO at pH 8.2, the absorption bands derived from the semiquinone (TPQ_{sq}) and aminoresorcinol forms of the TPQ cofactor disappeared within the dead time (<1 ms) of the measurements, indicating that the reaction of the substrate-reduced enzyme with dioxygen is very rapid. Concomitantly, an early intermediate exhibiting an absorption band at about 410 nm was formed, which then decayed with a rate constant of $390 \pm 50\ s^{-1}$. This intermediate was detected more prominently in the reaction in D_2O buffer (pD 8.1) and was assigned to a Cu(II)-peroxy species. The assignment was based on the observation that addition of H_2O_2 to the substrate-reduced AGAO under anaerobic conditions led to the formation of a new band at about 415 nm, accompanied by partial quenching of absorption bands derived from TPQ_{sq}. Other intermediates exhibiting absorption bands at about 310 and 340 nm were also observed in the oxidative half-reaction. Kinetics of the disappearance of these latter bands did not correspond with that of the Cu(II)-peroxy band at 410 nm but did well with that of the increase of the 480 nm absorption band due to the reoxidized TPQ. Rapid increase of the absorption in the 320−370 nm region was also observed for the reaction of the substrate-reduced, Ni-substituted enzyme with dioxygen. On the basis of these results, a possible mechanism is proposed for the oxidative half-reaction of the bacterial copper amine oxidase.

Copper amine oxidases (EC 1.4.3.6) are ubiquitously distributed from bacteria to higher organisms, catalyzing the oxidation of various primary amines to their corresponding aldehydes (*1*, *2*). The enzymes are commonly homodimers of identical subunits with a molecular weight ranging from 70000 to 95000 and contain a prosthetic copper ion together with a redox-active organic cofactor, 2,4,5-trihydroxyphenylalanine quinone (topa quinone, TPQ)[1] in each subunit. The TPQ cofactor is linked to the polypeptide chain as a modified amino acid residue (*3*) and is produced from a precursor tyrosine residue by posttranslational modification that proceeds in a copper-dependent self-processing reaction of the protein itself (*4*−*6*). According to the X-ray crystallographic structures determined so far for the enzymes from pea seedling (*7*), *Escherichia coli* (*8*, *9*), *Arthrobacter globiformis* (*10*), and yeast *Hansenula polymorpha* (*11*), they all have very similar main-chain folding as well as active site structures. The Cu(II) ion is coordinated with four equatorial ligands (three conserved histidine residues and one water molecule) and an axial ligand (another water), placed in a distorted square-pyramidal geometry. The TPQ cofactor is connected indirectly to the Cu(II) ion through a hydrogen-bonding network involving several active site water molecules (*7*−*11*).

The catalytic reaction of amine oxidases proceeds by a ping-pong transamination mechanism, consisting of the initial oxidative deamination of the amine substrate (reductive half-reaction) and the subsequent two-electron reduction of molecular oxygen to hydrogen peroxide (oxidative half-reaction) (Figure 1) (*1*, *2*). It has been well established that the TPQ cofactor participates in the reductive half-reaction by forming a covalent adduct with the amine substrate (substrate Schiff base complex) (*12*−*14*). The substrate Schiff

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[1] Abbreviations: AGAO, *Arthrobacter globiformis* phenylethylamine oxidase; TPQ, topa quinone; TPQ_{red}, topa quinone in the reduced form; TPQ_{sq}, topa semiquinone; TPQ_{ox}, topa quinone in the oxidized form; Cu_{dep}, copper depleted.

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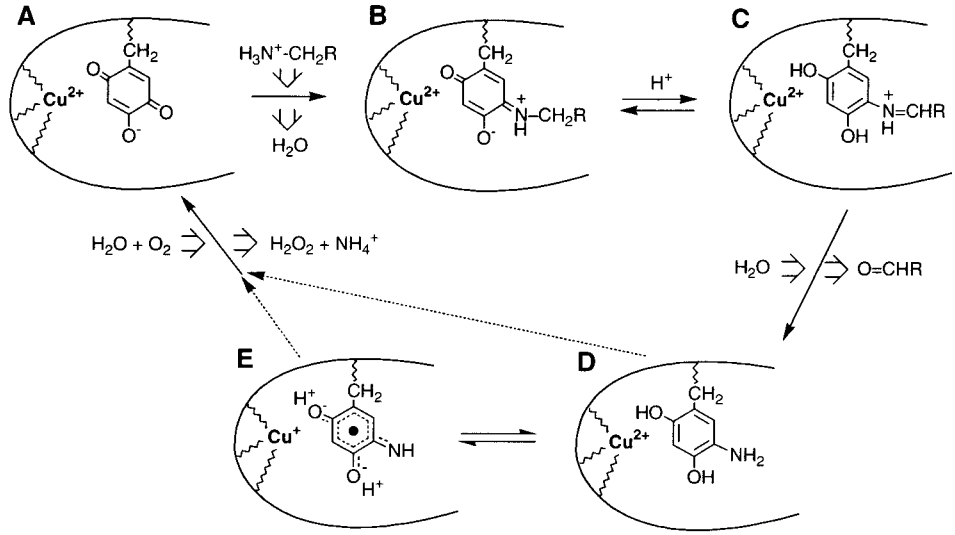

FIGURE 1: Mechanism of the overall reaction of copper amine oxidase.

base is then deprotonated by an active site base, assigned to an invariant aspartate residue, to yield the reduced cofactor in a product Schiff base complex. Subsequent hydrolysis of the Schiff base releases the product aldehyde, leaving an aminoresorcinol form of the reduced cofactor (TPQ_{red}). The produced TPQ_{red} is eventually reoxidized by dioxygen to the initial oxidized state (TPQ_{ox}) in the oxidative half-reaction, liberating ammonia. In contrast, the role of copper in the reaction cycle still remains controversial, although its essentiality for the catalytic activity has been demonstrated in earlier studies (*15*, *16*).

Presumed participation of the copper ion in the oxidative half-reaction was first suggested from the observation of a Cu(I)/topa semiquinone (TPQ_{sq}) state in the optical absorption and room temperature EPR studies on the enzymes anaerobically reduced with their amine substrates (*17*). The Cu(I)/TPQ_{sq} state has subsequently been characterized in detail for several amine oxidases (*18*−*23*), mainly focusing on the TPQ_{sq} species with absorption maxima at about 360, 430, and 465 nm and hyperfine splitting of the $g \sim 2$ signal characteristic for a semiquinone radical (*17*). Because the intramolecular one-electron transfer from TPQ_{red} to Cu(II) to form the Cu(I)/TPQ_{sq} state is extremely rapid with a rate constant as large as 20000 s^{-1} and Cu(I) has higher reactivity with O_2 than Cu(II), the Cu(I)/TPQ_{sq} state has been proposed as a kinetically competent intermediate with Cu(I) directly reducing O_2 in the oxidative half-reaction (*18*). However, Su and Klinman have argued in a recent kinetic study with the bovine plasma enzyme that the initial one-electron reduction of O_2 occurs from the Cu(II)/TPQ_{red} species and not from the Cu(I)/TPQ_{sq} species in the oxidative half-reaction (*24*). Analysis of azide inhibition for the yeast enzyme has also supported the proposed mechanism with O_2 binding off the copper ion prior to the reaction (*25*). On the basis of these and other studies including kinetic analysis of the metal-substituted yeast enzyme (*26*), the Cu(II) ion has been suggested to provide electrostatic stabilization of the superoxide anion formed by the rate-limiting, direct electron transfer from TPQ_{red} to O_2, instead of being reduced to Cu(I).

To further clarify the mechanism of the oxidative half-reaction and the role of the Cu(II) ion in the catalysis, we have investigated the reaction of the substrate-reduced amine oxidase from *A. globiformis* (AGAO) with O_2. In this paper, we report spectrophotometric detection of an intermediate that can be assigned to the enzyme with a peroxide species bound to Cu(II). The Cu(II)-peroxy species observed spectrophotometrically for the first time may be similar to that identified in a recent crystal structure by Wilmot et al. (*27*). A possible mechanism of the oxidative half-reaction by the bacterial enzyme is also proposed, including the reoxidation of the TPQ cofactor as the major rate-determining step, unlike in the reactions of yeast and bovine plasma enzymes.

EXPERIMENTAL PROCEDURES

Enzyme Purification and Assay. Recombinant AGAO overproduced in *E. coli* cells was purified in the Cu(II)/TPQ-containing, holo form according to the previously published methods (*4*, *28*, *29*) by adding 100 μM $CuSO_4$ to the buffers used for cell disruption and subsequent dialysis. Purification was performed with particular cautions to obtain spectrophotometrically pure preparations thoroughly devoid of impurity proteins that had strong absorption around 400 nm likely due to the bound heme in catalase (S. Kishishita, unpublished observations). Solutions containing enzymes with a specific activity of >50 units/mg of protein were collected and concentrated by ultrafiltration to the desired enzyme concentrations. AGAO activity was determined with 2-phenylethylamine as a substrate by the peroxidase-coupled assay (*4*). Protein concentrations were determined by the absorbance at 280 nm (*4*) and calculated as subunit concentrations.

Preparation of Ni-Substituted AGAO. Cu-depleted (Cu_{dep}) AGAO was prepared essentially according to the method described by Suzuki et al. (*16*) with some modifications for keeping anaerobic conditions more strictly: Holo-AGAO was dialyzed at 4 °C for 2−3 h against 500 mL of 50 mM HEPES buffer, pH 6.8, containing 50 mM sodium dithionite. Before each dialysis, HEPES and potassium phosphate buffers used were freed from contaminating metal ions by passing through a Chelex resin column (Bio-Rad) and purged for a minimum of 1 h with pyrogallol-scrubbed Ar gas as described (*26*). Solid KCN was then added to the buffer to a final concentration of 10 mM, and dialysis was continued over-

night in a glovebag filled with oxygen-free Ar gas. The remaining cyanide was removed by extensive anaerobic dialysis against 1 L of metal-free 50 mM HEPES buffer, pH 6.8. The Cu_{dep} AGAO obtained was found to contain less than 0.004 mol of copper/mol of enzyme subunit by metal analysis with a Shimadzu AA-6400G atomic absorption spectrophotometer and to possess only 0.04% of the original activity when assayed without addition of Cu(II). Reconstitution of Cu_{dep} AGAO with Ni(II) ion was accomplished by addition of 0.5 mM $NiCl_2 \cdot 6H_2O$ (99.999%, Aldrich) to 0.1 mM Cu_{dep} AGAO in 50 mM HEPES buffer, pH 6.8 (total volume, 1 mL), and incubation at 30 °C for 3 h. During the incubation, the TPQ cofactor that had been reduced by dithionite to a hydroxyquinol form was gradually reoxidized to TPQ_{ox} (S. Kishishita, unpublished results). The Ni-substituted AGAO thus obtained was dialyzed against 1 L of metal-free 50 mM HEPES buffer, pH 6.8, containing 1 mM EDTA and finally against 1 L of metal-free 100 mM potassium buffer, pH 7.3.

Spectroscopic Measurements. All measurements were done in 100 mM potassium phosphate buffers, pH 6.0, 7.3, or 8.2, as indicated. For the experiments in D_2O buffers, appropriate amounts of potassium phosphates, monobasic and dibasic, were dissolved in D_2O (99.9 atom % for deuterium, Sigma) to give pD 8.1 (uncorrected value of a pH meter) at a final concentration of 100 mM. To set up conditions as anaerobic as possible, all of the enzyme and substrate solutions were deaerated using a vacuum line and repressurized with N_2 gas, and a gas-tight syringe was used to handle the solutions. Sample solutions were transferred to a quartz cell through a silicone rubber septum using a gas-tight syringe. The substrate-reduced enzyme was prepared by addition of the substrate (2-phenylethylamine) under anaerobic conditions; for preventing the catalytic turnover by excess substrate in the stopped-flow measurements of the oxidative half-reaction, an equimolar amount of substrate was added, while for consuming all of the trace of dissolved oxygen and converting the enzyme completely into the reduced form in the steady-state measurements, a 10-fold molar excess of substrate was used (see figure legends for details of enzyme and substrate concentrations). In the former case, the enzyme was anaerobically preincubated with substrate for a sufficient period (>20 min) to ensure that the equilibrium was reached before the reaction was started with dioxygen. The reduction was sometimes incomplete, as judged from the remaining TPQ_{ox}-derived absorption at 480 nm of the initial enzyme solution, partly due to the consumption of the substrate by significant turnovers with a trace of dissolved oxygen. Therefore, spectral data were reported in most cases as difference spectra by subtracting the spectrum of the TPQ_{ox} enzyme obtained in each experiment.

Pre-steady-state measurements were done at 5 °C with Unisoku RSP-601-03 rapid-scan stopped-flow equipment with a mixing cell volume of 40 μL. Typically, equal volumes (about 30 μL each) of the enzyme solution and the oxygen-saturated buffer ($[O_2]$ = ca. 1 mM at 15 °C) were mixed to initiate the oxidative half-reaction, and the spectra were recorded at every 1 ms in a wavelength region of 300−600 nm and averaged in a 2−10-ms time span to obtain a spectrum after mixing. The first-order rate constants for the absorption changes were obtained by extraction of the data from fixed wavelengths and least squares exponential fitting using Igor Pro Version 3.1 (WaveMetrics, Inc.). UV−vis absorption spectra of the steady-state enzymes were recorded at 20 °C with a Shimadzu UV 3101PC spectrophotometer. For the reaction of the enzyme with H_2O_2, the enzyme solution was mixed anaerobically with H_2O_2 in the presence and absence of substrates.

RESULTS

Reaction of Substrate-Reduced AGAO with O_2. In the absorption spectral changes recorded after mixing the substrate-reduced enzyme with the O_2-saturated buffer at pH 8.2, a new transient absorption band emerged at about 410 nm, which decayed with a rate constant of 390 ± 50 s^{-1} (Figure 2A). This 410 nm transient absorption band disappeared faster at pH 7.3 (Figure 2B) and was detected more prominently in D_2O buffer (Figure 2C), where the reaction proceeded slower with a reduced decay rate of 190 ± 40 s^{-1}. Other slower absorption changes were also observed around 300−380 and 480 nm for the reaction of the substrate-reduced AGAO with dioxygen.

To see the absorption changes more precisely, the time dependence of the absorption changes at selected wavelengths (310, 340, 400, and 480 nm) was analyzed, and the traces are shown in Figure 3. The absorption increase at 480 nm (curve d) was due to the formation of reoxidized TPQ (TPQ_{ox}). The wavelength at 400 nm, rather than at 410 nm, was chosen to exclude the influences from the slowly increasing strong absorption band of TPQ_{ox} (see Figure 2); the decay rates would also be estimated more accurately at a wavelength close to the isosbestic point (~390 nm, Figure 2A) for the slower emerging species. The absorption at 310 and 340 nm (curves a and b) increased slightly during 0−20 ms in the H_2O buffers, but in this time span, their rate constants did not correspond well with those of the absorption decrease at 400 nm (curve c), which suggested a different nature of the 310 and 340 nm species from the 400 nm species. The rate constants of the absorption decrease at 310 and 340 nm observed after 20 ms were about 13, 12, and 9 s^{-1} for the reactions at pH 7.3, pH 8.2, and in D_2O buffer (pD 8.1), respectively, which corresponded very well to the absorption increase at 480 nm due to the formation of TPQ_{ox} (Table 1) in each measurement. The similarities of these latter rate constants indicate that the 310 and 340 nm absorption bands are both related to the direct precursor to TPQ_{ox}.

Absorption Spectra of TPQ_{ox}, TPQ_{red}, and TPQ_{sq}. A steady-state absorption spectrum of the TPQ_{sq} species obtained by anaerobic reduction with substrate is depicted in Figure 4B (curve a), together with the spectra obtained immediately (curve b) and sufficiently long (until reaching a steady state, curve c) after mixing with O_2 in the D_2O buffer, where the reaction proceeds slowly. The TPQ_{sq} species exhibited absorption bands at about 365, 440, and 470 nm (curve a), typical for the bands of the semiquinone radical as reported previously (*17*). Immediately after mixing the substrate-reduced AGAO with O_2-saturated buffer, none of these TPQ_{sq}-related bands was observed (curve b; see also Figure 2). These results thus indicate that the substrate-reduced enzyme reacts very rapidly with O_2, faster than the mixing dead time (<1 ms) of the stopped-flow measurement.

On the basis of our previous estimation (*29*), TPQ_{sq} accounted for about 10% of the total TPQ species in the

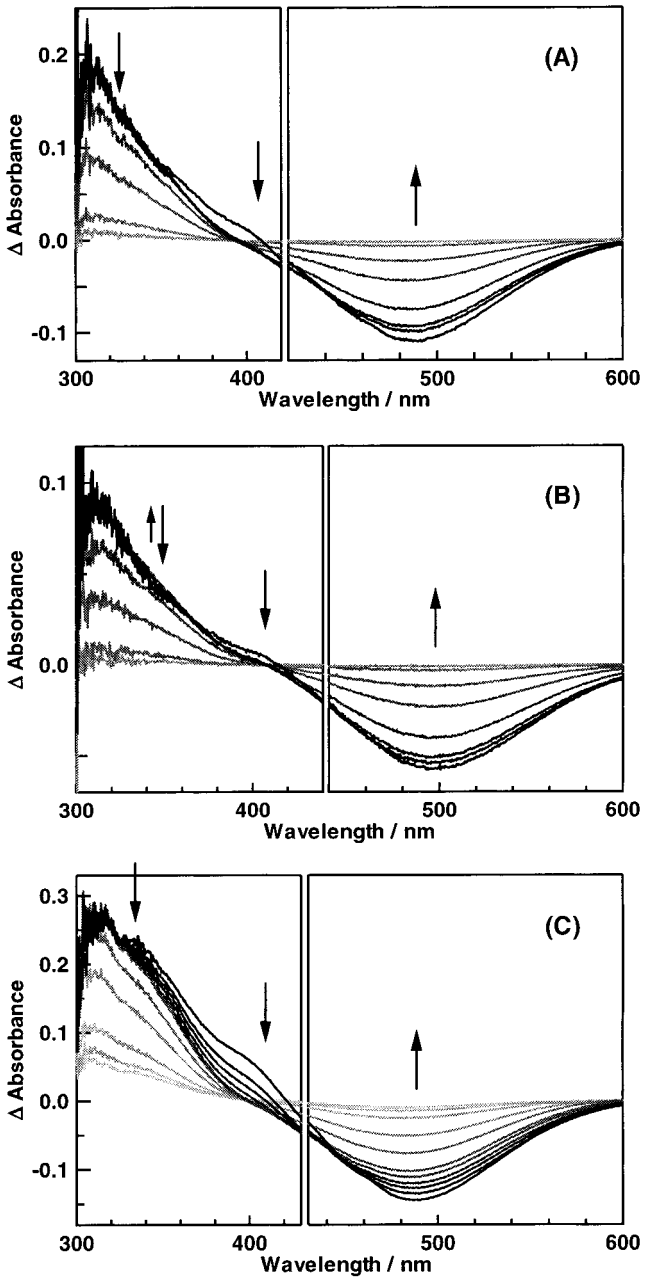

FIGURE 2: Rapid-scan stopped-flow spectral measurements of the reaction of the substrate-reduced AGAO with O_2. Prior to the oxidative half-reaction, the enzyme was reduced anaerobically with an equimolar amount of 2-phenylethylamine in 100 mM H_2O buffer at pH 8.2 (A) and pH 7.3 (B) and in D_2O buffer (pD 8.1) (C). Absorption spectra are shown as difference spectra with each raw spectrum subtracted with the corresponding spectrum of reoxidized AGAO. Difference spectra in (A) and (B) are at 1, 5.5, 8.5, 12.5, 21, 53, 98, and 198 ms after the mixing, and those in (C) are at 1, 5.5, 8.5, 12.5, 19.5, 26.5, 53, 98, 198, 298, and 398 ms after the mixing. Darker spectra represent earlier times. Subunit concentrations of AGAO were 135 and 130 μM for measurements in H_2O and D_2O buffer, respectively. Measured at 5 °C.

substrate-reduced enzyme; the rest is in the $2e^-$-reduced aminoresorcinol form (TPQ_{red}). TPQ_{red} has been reported to exhibit an absorption band at 310 nm (*13*, *30*). In the substrate-reduced AGAO, the 310 nm absorption of TPQ_{red} is clearly seen in the difference spectrum (Figure 4A, curve a). Upon being mixed with O_2, an immediate disappearance of the TPQ_{red}-derived absorption band is also evident from the difference spectrum (curve b) (see also Figure 2). Although we cannot conclude whether the Cu(I)/TPQ_{sq} or Cu(II)/TPQ_{red} couple reacts first with O_2 in the oxidative half-reaction, these results are consistent with the extremely rapid $1e^-$ transfer from TPQ_{red} to Cu(II) [and from Cu(I) to TPQ_{sq}] with a rate constant of 20000 s^{-1} (*18*).

Reaction of Substrate-Reduced AGAO with H_2O_2. To infer the identity of the 410 nm species transiently formed (Figure 2A), we then examined the effect of one of the reaction products, H_2O_2, on the absorption bands of TPQ_{sq} formed at pH 8.2. When H_2O_2 was added to the substrate-reduced enzyme in the steady state, the intensities of absorption bands of TPQ_{sq} at 365 and 470 nm (Figure 5A, curve a) decreased significantly (to about 57% of no addition), while a new peak emerged at about 415 nm (Figure 5A, curve b). A difference spectrum obtained by subtraction of curve a multiplied by 0.57 from curve b revealed a new broad peak centered at 415 nm and a shoulder band at about 330 nm (curve c); the factor of 0.57 used for multiplying curve a was based on an assumption that the relative magnitudes of the TPQ_{sq}-related absorption bands were constant irrespective of the actual amount of the TPQ_{sq} species. In contrast, addition of H_2O_2 at pH 6.0 resulted in no emergence of the 415 nm peak in the difference spectrum (Figure 5B, curve c), even though the TPQ_{sq}-derived absorption bands, shifted to 436 and 467 nm, similarly decreased (Figure 5B, curve a → b). Thus, the 415 nm peak resulting from the addition of H_2O_2 at pH 8.2 appears to be unstable or not formed at a pH lower than neutral or slightly alkaline regions. This feature agrees with the properties of the "end-on" Cu-peroxy species formed in inorganic compounds (*31*), which is readily protonated and, hence, leads to the assignment of the 410−415 nm absorption band to the Cu-peroxy species. Similar absorption bands have been reported for the Cu-peroxy species formed in a multicopper enzyme, laccase, with λ_{max} at about 340 and 400 nm (*32*).

Reaction of Substrate-Reduced, Ni-Substituted AGAO with O_2. The results described above suggest that the first detectable product in the oxidative half-reaction is the $2e^-$-reduced peroxide bound to Cu(II) and that the metal ion plays a role in stable binding of the peroxide for the subsequent protonation and release of hydrogen peroxide. To examine this hypothesis, we prepared a copper-depleted (Cu_{dep}) AGAO and reconstituted it with a nickel ion, as described in Experimental Procedures. The Ni-substituted enzyme was found to have a very low but detectable catalytic activity, higher than that of the almost inactive Cu_{dep} enzyme (S. Kishishita et al., unpublished). In the anaerobic reaction of the Ni-substituted enzyme with a molar equivalent of substrate, the 480 nm band of TPQ_{ox} disappeared very rapidly (data not shown), suggesting that the reductive half-reaction proceeds independently of the bound metal ion. However, absorption bands of TPQ_{sq} were not observed (Figure 6, inset), in agreement with the unfeasible $1e^-$ transfer from TPQ_{red} to Ni(II). In the absorption spectra obtained by the reaction of the substrate-reduced, Ni-substituted enzyme with O_2, a new broad absorption emerged at 320−370 nm (Figure 6), which could be deconvoluted to at least two absorption bands. One of such putative bands at about 320 nm is formed very rapidly in an early stage of the reaction (<2.5 ms), and another one at about 360 nm is formed more slowly with a rate constant of 0.17 ± 0.04 s^{-1}, thereby shifting the peak position to a longer wavelength. The intensities of these

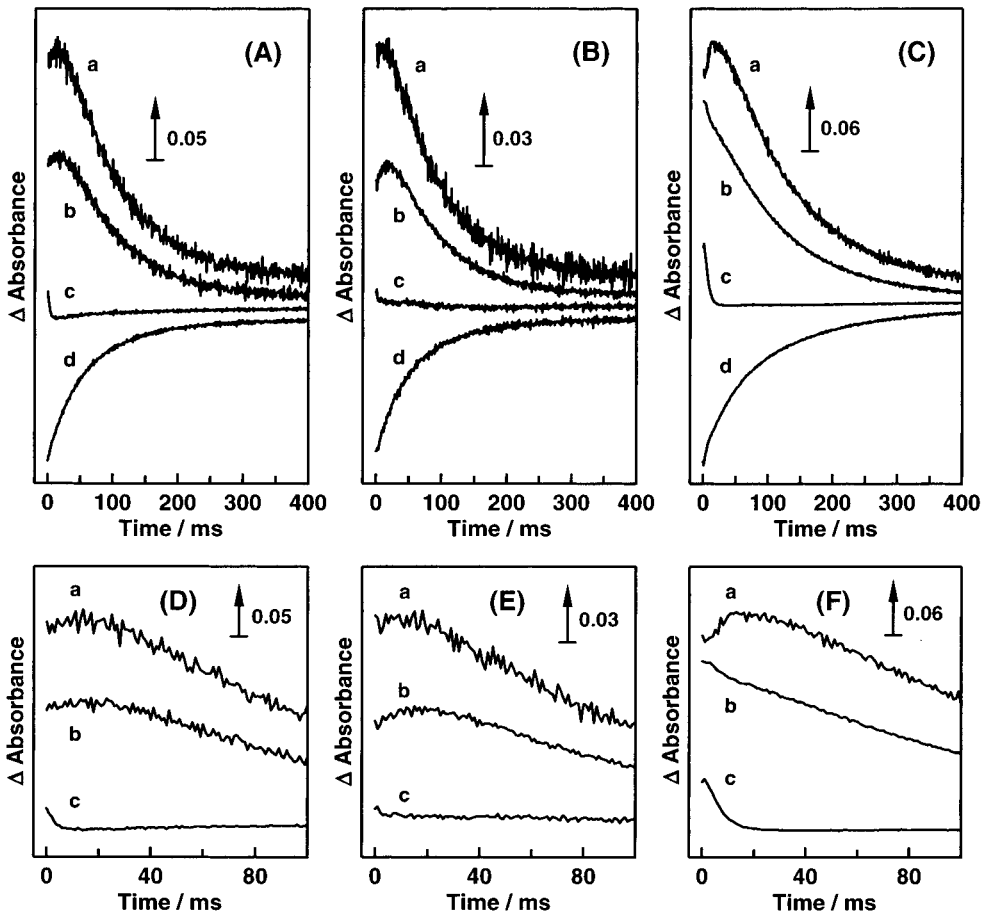

FIGURE 3: Traces of absorbance changes at fixed wavelengths. The wavelengths are 310 (a), 340 (b), 400 (c), and 480 (d) nm for the reaction of the substrate-reduced AGAO with O_2 at pH 8.2 (A, D) and pH 7.3 (B, E) and in D_2O buffer at pD 8.1 (C, F). The level of absorbance at each wavelength is offset for clarity. Experimental conditions were the same as those used in Figure 2.

Table 1: Rate Constants for the Pre-Steady-State Reaction of Substrate-Reduced AGAO with O_2

wavelength (nm)	increase or decrease (time span)	k_{obs} (s^{-1}) pH 8.2/ H_2O	pH 7.3/ H_2O	pD 8.1/ D_2O
310	increase (0−20 ms)	51 ± 7	64 ± 7	−[a]
310	decrease (30−400 ms)	12.0 ± 0.5	12.9 ± 0.5	8.9 ± 0.3
340	increase (0−20 ms)	52 ± 7	58 ± 7	−[b]
340	decrease (30−400 ms)	12.0 ± 0.4	13.1 ± 0.4	9.2 ± 0.3
400	decrease (0−20 ms)	390 ± 50	−[c]	190 ± 40
480	increase (0−400 ms)	12.0 ± 1.5	13.0 ± 1.5	8 ± 1

[a] Inapplicable to a single-exponential fit (Figure 3F, curve a). [b] No increase (Figure 3F, curve b). [c] Absorption changes were too small to be fitted (Figure 3E, curve c).

bands did not decrease within 30 s of the stopped-flow measurement (Figure 6) but instead did gradually later on (in several minutes), concomitantly with the slow increase of the 480 nm band of TPQ_{ox} (not shown). The absorption at 320−370 nm was absent before the reaction of the substrate-reduced, Ni-substituted enzyme with O_2 was started (Figure 6, inset). The 320−370 nm absorption observed in the oxidative half-reaction thus appears to correspond to the 310 and 340 nm bands observed with the native Cu enzyme and is assignable to the immediate precursor to TPQ_{ox}. It is also likely that the low activity of the Ni-substituted enzyme correlates with the impairment of the later processes of the oxidative half-reaction.

DISCUSSION

Cu-Peroxy Intermediate. The peak position and the broad feature of the 410 nm absorption band, transiently observed in the oxidative half-reaction, corresponded well with those of the 415 nm Cu-peroxy absorption band obtained by the nonproductive reaction of the substrate-reduced AGAO with H_2O_2. Like the 415 nm Cu-peroxy absorption band, the transient absorption band at 410 nm was also observed more prominently at higher pH. Therefore, we assign this transient band to the Cu-peroxy species formed during the oxidative half-reaction. The solvent deuterium isotope effect, showing the involvement of proton transfer in the decay of the 410 nm species, also supports the assignment of the 410 nm absorption band to the Cu-peroxy species. Consistent with this assignment, an electron density peak close to the copper ion was found and modeled as a peroxy species bound to Cu(II) in the recent X-ray crystallographic study of the reduced form of the enzyme from *E. coli*, to which a trace of O_2 was introduced (*27*).

While the 415 nm Cu-peroxy absorption band emerged upon addition of H_2O_2 to the substrate-reduced AGAO at pH 8.2, the intensities of the TPQ_{sq}-related absorption bands decreased significantly (Figure 5). Both the copper ion and

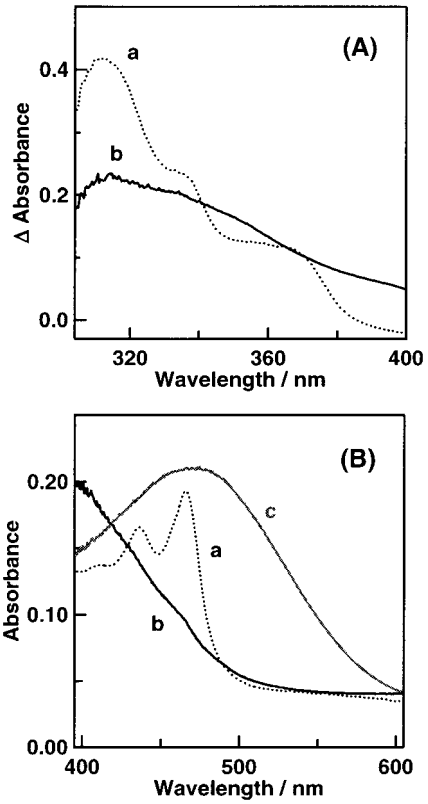

FIGURE 4: Absorption spectra of AGAO. A steady-state absorption spectrum of the substrate-reduced enzyme (a, dotted curve) and those obtained in the reaction of the substrate-reduced AGAO with O_2 immediately (1 ms) (b, dark curve) and sufficiently long (398 ms) (c, gray curve) after the mixing are shown as difference spectra, obtained by subtracting the spectrum of the reoxidized enzyme, in the 300−400-nm region in (A) and as the raw spectra in the 400−600 nm region in (B). Subunit concentration was 130 μM. Measured in D_2O buffer (pD 8.1) at 5 °C.

the TPQ cofactor of the substrate-reduced enzyme are reported to be in an equilibrium, Cu(II)/TPQ_{red} ⇆ Cu(I)/TPQ_{sq}, intramolecularly transferring 1 e^- (*18*). On the other hand, it is conceivable that the anionic peroxy species is more preferentially bound to Cu^{2+} than to Cu^+ having less positive charge. Thus, the decrease of the absorption intensity of TPQ_{sq}-related absorption bands is probably due to the shift of the Cu(II)/TPQ_{red} ⇆ Cu(I)/TPQ_{sq} equilibrium toward the former by binding of H_2O_2 to Cu(II). The 415 nm absorption band, however, was not observed upon addition of H_2O_2 to the oxidized AGAO even at pH 8.2 (data not shown). The observation that the Cu-peroxy species is generated only in the presence of substrate shows that it is more stable in the TPQ_{red} state than in the TPQ_{ox} state, even though the copper ion is Cu(II) in both states.

In our previous study (*29*), carbon monoxide was shown to bind with the Cu(I)/TPQ_{sq} species of the substrate-reduced AGAO and thereby shift the Cu(II)/TPQ_{red} ⇆ Cu(I)/TPQ_{sq} equilibrium toward the latter. These results and those obtained in this study thus show that the Cu(II)/TPQ_{red} ⇆ Cu(I)/TPQ_{sq} equilibrium shifts to either direction by the interaction of the copper ion with a small molecule, depending on the copper redox state to which the molecule binds. This equilibrium was also reported to be temperature-dependent (*18*).

TPQ-Related Intermediates. Although the intensities of the absorption at 310 and 340 nm decreased with similar rates

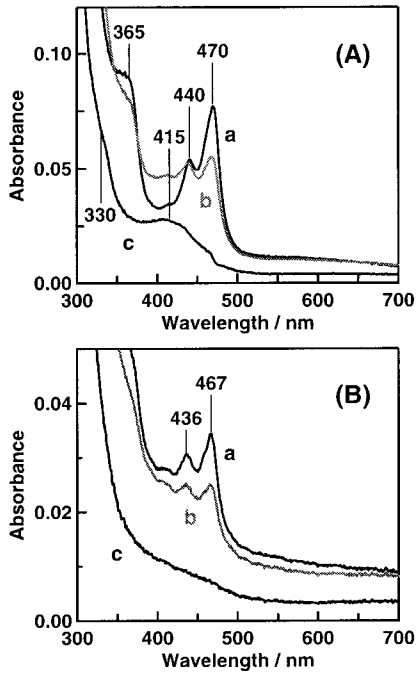

FIGURE 5: Effect of H_2O_2 on absorption spectra of AGAO. The enzyme was anaerobically reduced with 2-phenylethylamine (10 equiv to the subunit) in the absence (a, dark curve) and presence (b, gray curve) of H_2O_2 (50 equiv to the subunit) at pH 8.2 (A) and pH 6.0 (B). The difference spectra (c) were obtained by subtracting spectrum a from spectrum b, where spectrum a was multiplied by a factor of 0.57 and 0.52 for the reaction at pH 8.2 and 6.0, respectively, to remove the influence of the TPQ_{sq}-derived absorption bands. Enzyme subunit concentrations were 46 and 37 μM for the measurements at pH 8.2 and 6.0, respectively. Measured at 20 °C.

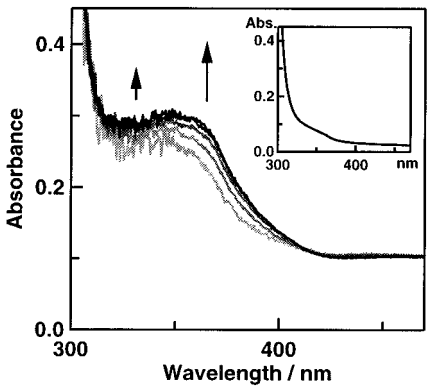

FIGURE 6: Rapid-scan stopped-flow spectral measurements of the reaction of the substrate-reduced, Ni-substituted AGAO with O_2. The enzyme was reduced in advance with a molar equivalent of substrate (2-phenylethylamine). Subunit concentration was 135 μM. Absorption spectra are at 2.5 ms and 4.2, 8.3, 16.5, and 30.8 s after the mixing. Darker spectra represent later times. Inset: Absorption spectrum of the substrate-reduced, Ni-substituted AGAO (40 μM). Measured in 100 mM potassium phosphate buffer, pH 7.3, at 5 °C.

after 20 ms, the absorption increase at 310 nm did not correspond well with that at 340 nm in the initial phase (0−20 ms) of the oxidative half-reaction (Figure 3). In the 0−20 ms phase, the absorption increase was more prominent at 340 nm than that at 310 nm at pH 7.3 (Figure 3E) and vice versa at pH 8.2 (Figure 3D), whereas in D_2O buffer the absorption increase was not observed at 340 nm but was observed significantly at 310 nm (Figure 3F). These differences suggest that the 310 and 340 nm absorption bands are

different from each other in the protonation state, but both are attributed to the direct precursors to TPQ_{ox}, as they have decay rates similar to those of the TPQ_{ox} formation in the later phase of the oxidative half-reaction (Table 1). Since the relative magnitude of the 340 nm band over the 310 nm band is slightly higher at pH 7.3 than at pH 8.2 (A_{340}/A_{310} = 0.63 at pH 7.3 and 0.58 at pH 8.2; cf. 53 ms spectra in Figure 2A,B), the 340 nm band may be assigned to the protonated form of the TPQ iminoquinone intermediate and the 310 nm band to the deprotonated one. A similar TPQ iminoquinone intermediate has been identified in the crystal structure of the *E. coli* enzyme (*27*). However, on the basis of the model studies (*33*), the iminoquinone paired with an ammonium ion at C4−O^- (numbering according to the enzyme-linked TPQ) has a λ_{max} at 454 nm, while if there were a hydrogen bonding between the 4-oxyanion and the hydrogen atom on the imino nitrogen, the λ_{max} is blue shifted to ∼350 nm by electron localization. The deprotonated iminoquinone formed by the reaction with ammonia of bovine serum amine oxidase (*30*) and of an active site mutant of the yeast enzyme (*34*) also shows an absorption band at ∼430−450 nm. Moreover, the ∼340−350 nm absorption observed in the reactions of the yeast wild-type enzyme with ammonia and of a mutant with benzylamine under turnover conditions was ascribed to a localization of the cofactor oxyanion induced by binding of a cationic species at the active site and not to covalent adduct formation (*34*). Therefore, the 340 nm species observed in the later phase of the oxidative half-reaction of AGAO has a possibility of the charge-localized TPQ_{ox}, formed after the hydrolysis of the iminoquinone intermediate and still bound with ammonia.

In the initial phase (0−10 ms) of the oxidative half-reaction in D_2O buffer, where the solvent isotope effect would predominate on the proton-transfer process, the rapid absorption increase at 310 nm appears to correspond with the rapid absorption decrease at 400 nm (k_{obs} = 190 s^{-1}) (Figure 3F), although the rate constant for the initial increase at 310 nm was not determined due to the inapplicability of the data to a single exponential fit (Table 1). These results thus suggest that the absorption changes during 0−10 ms are associated with proton transfer from the protonated intermediate (absorbing at 340 nm) to the Cu-peroxy species, generating the deprotonated intermediate (absorbing at 310 nm) and H_2O_2.

Rapid generation of the broad 320−370 nm absorption in the reaction of the substrate-reduced, Ni-substituted AGAO with O_2 (Figure 6) indicates that the Ni enzyme can also form an intermediary species during the oxidative half-reaction, as in the reaction of the Cu enzyme. Thus it is concluded that the initial reaction of the substrate-reduced enzyme with O_2 proceeds irrespective of the types of bound metal ion, although the rate is significantly affected. This is consistent with the recently proposed mechanism, in which the electrons are passed directly from TPQ_{red} to the prebound dioxygen without the need for prior reduction of the metal (*24*, *26*). The ratio of A_{340}/A_{310} in the reaction of the substrate-reduced, Ni-substituted enzyme with O_2 was close to 1 (Figure 6), in contrast to those less than 1 in the reaction of Cu AGAO (Figure 2). These differences of A_{340}/A_{310} may reflect the initial populations of the two forms (protonated and deprotonated) of the intermediate and are probably associated with the differences in the metal coordination

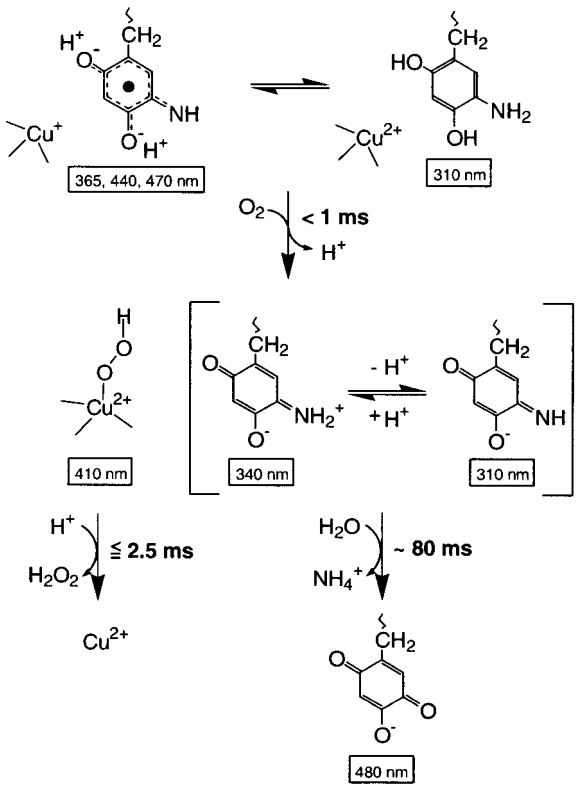

FIGURE 7: Presumed mechanism of the oxidative half-reaction of AGAO.

structure, as revealed by the crystal structure of the Ni-substituted enzyme with an additional metal-coordinating water molecule (S. Kishishita et al., unpublished results).

Collectively, we conclude that the final formation of the oxidized, charge-delocalized TPQ cofactor is the major rate-determining step of the oxidative half-reaction of AGAO. This conclusion obtained with the bacterial enzyme is in marked contrast to that drawn from the recent studies with the enzymes from eukaryotic sources (*24*, *26*), in which the first $1e^-$ transfer from TPQ_{red} to dioxygen has been shown to be rate limiting in the oxidative half-reaction. Although the reason for this discrepancy is unclear without notable differences in the active site structures of all copper amine oxidases whose crystal structures have been solved (*7*−*10*), a subtle difference in the hydrogen-bonding network around the active site (including water molecules coordinating and noncoordinating to the metal ion) may affect the efficiency of proton transfer involved in the oxidative half-reaction such that other processes involving proton transfer become the slowest step. Further, a Cu-peroxy species coexisting with a presumed TPQ iminoquinone intermediate has been trapped by flash-freezing of the reacting crystal of the enzyme from *E. coli* (*27*), which itself implicates that the generation of the oxidized TPQ cofactor is the rate-limiting step of the oxidative half-reaction of the bacterial enzyme.

Presumed Mechanism of the Oxidative Half-Reaction. A possible mechanism of the oxidative half-reaction of AGAO is depicted in Figure 7. In the initial stage, the substrate-reduced AGAO with the copper ion and TPQ in the equilibrium, Cu(II)/TPQ_{red}/Cu(I)/TPQ_{sq}, reacts very rapidly with O_2, resulting in the immediate disappearance of both the TPQ_{sq}- and TPQ_{red}-derived absorption bands. Nearly simultaneously, the Cu-peroxy species absorbing at 410 nm

and the TPQ-related intermediates absorbing at 310−340 nm are formed. The Cu-peroxy species then captures protons from the protonated intermediate and releases H_2O_2 in a millisecond time scale at pH 8.2. This latter reaction appears to proceed slower at higher pH and in D_2O buffer due to the necessity of proton transfer. Although there are some differences in the absorption wavelengths of the TPQ iminoquinone species as described above, the TPQ-related intermediates absorbing at 310 and 340 nm are assigned tentatively to the deprotonated and protonated iminoquinone intermediates, respectively, in Figure 7. In the subsequent step, the presumed iminoquinone is hydrolyzed, liberating the final product ammonia and regenerating TPQ_{ox}. The final formation of the oxidized, charge-delocalized TPQ cofactor should be the major rate-determining step of the oxidative half-reaction of AGAO, as it is observed spectrophotometrically as the slowest step.

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