Characterization of the Active Site of *Arthrobacter* P1 Methylamine Oxidase: Evidence for Copper–Quinone Interactions

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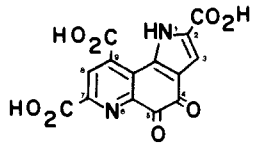
Abstract: *Arthrobacter* P1 methylamine oxidase is the first prokaryotic copper-containing amine oxidase to be discovered. Our initial characterization of the active-site structure and reactivity of methylamine oxidase is described herein. Spectroscopic studies, using a variety of techniques, of the resting (oxidized), substrate-reduced, and copper-depleted forms of methylamine oxidase have been carried out. Inhibitor binding to the copper ions and to the organic cofactor has also been investigated. Both ammonia and phenylhydrazine bind to the organic cofactor in the enzyme. Ammonia binds reversibly with K_D = 8.3 ± 0.8 mM at pH 8.51; it also inhibits the enzyme, in part by significantly decreasing the methylamine oxidase reduction rate by substrates. Phenylhydrazine covalently modifies methylamine oxidase; resonance Raman studies of the enzyme phenylhydrazone establish that the microenvironment of the derivatized cofactor is practically identical with that of the mammalian plasma amine oxidases. Hence the quinone cofactor must be covalently attached in the same way in all these enzymes. Copper removal perturbs the resonance Raman spectrum of the methylamine oxidase phenylhydrazone, providing another indication that copper and the quinone interact. EPR and CD spectroscopies strongly indicate that the Cu(II) site in methylamine oxidase has a structure very similar to that of the well-characterized eukaryotic amine oxidases. Cyanide binds rapidly and reversibly to an equatorial coordination position of methylamine oxidase Cu(II). Electron transfer between the reduced quinone and Cu(II) is shown to occur in the presence of ligands that stabilize Cu(I), generating a semiquinone and Cu(I). With CN^- as the exogenous ligand, the kinetics are first order in protein but independent of the cyanide concentration. Further, the reaction is completely reversible at low [CN^-]. This reaction may be related to the reoxidation mechanism of the substrate-reduced enzyme in the catalytic cycle.

Pyrroloquinoline quinone (PQQ), or a closely similar compound, has recently been implicated as the organic cofactor in copper-containing amine oxidases.[3] Prior to 1984, when the first evidence



for PQQ in bovine plasma and porcine kidney amine oxidase was described,[4] this coenzyme had been found only in bacteria.[5] It is possible that enzymes containing PQQ or other quinones (designated quinoproteins) may be a new, diverse, and widespread class of enzymes; possible examples include dehydrogenases,[5,6] oxidases,[4,7-12] decarboxylases,[13] hydratases,[14] and oxygenases.[15] The evidence for the presence of PQQ is not uniformly convincing, and some of these claims are likely to be erroneous. The quinone moiety may be covalently bound or readily dissociable. Copper,[3] non-heme iron,[14] and heme iron quinoproteins[6] have been identified. Copper-containing amine oxidases are the best characterized eukaryotic quinoproteins.[16] A wide variety of chromatographic, chemical, and spectroscopic evidence is consistent with the presence of covalently bound PQQ, or a related compound, in these enzymes.[3,4,7-12,16] Several intriguing questions are raised by the possible presence of PQQ in eukaryotes. Very little is known about the metabolic pathways for PQQ in higher organisms. Tyrosine and glutamate have been shown to be precursors to PQQ in bacteria,[17] but this has not been demonstrated in eukaryotic cells. There are suggestions in the literature that PQQ may be a previously unrecognized vitamin.[4,18] This possibility, and many

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other aspects of PQQ biochemistry, are currently under intensive investigation.

Eukaryotic copper-containing amine oxidases (EC 1.4.3.6) catalyze the two-electron oxidative deamination of primary amines (eq 1).[16] Generally these amine oxidases use a ping-pong

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2 \quad (1)$$

mechanism. In the first step, substrate amines reduce the quinone and the product aldehydes are released. Subsequently, O_2 reoxidizes the reduced cofactor, producing H_2O_2 and releasing NH_3. The copper is essential for the reoxidation of the substrate-reduced enzyme, although the metal may play a role in the first step as well.

Methylamine oxidase from the Gram-positive methylotrophic bacterium *Arthrobacter* P1 catalyzes an oxidative deamination reaction (eq 2) as the first step in the C_1 metabolism of methylamine.[19] Recently Duine and co-workers identified this enzyme

$$CH_3NH_2 + O_2 + H_2O \rightarrow CH_2O + NH_3 + H_2O_2 \quad (2)$$

as a copper–quinoprotein amine oxidase,[20] the first example of such an amine oxidase in bacteria. Methylamine oxidase has an M_r of 167 900 and consists of two subunits (M_r = 82 250) that are probably identical. The copper content (2 Cu ions/mol of enzyme) and the absorption spectrum of the resting (oxidized) methylamine oxidase are closely analogous to the corresponding properties of the eukaryotic copper-containing amine oxidases, but unlike the latter enzymes, methylamine oxidase is not a glycoprotein. Hence, *Arthrobacter* P1 methylamine oxidase is a very attractive enzyme for detailed structural and mechanistic studies. We anticipate that the results will be relevant to understanding structure–function correlations, not only in amine oxidases but also in other enzymes containing both metal ion and organic cofactors. An essential first step is to define the basic properties of the active site, particularly with regard to possible copper–quinone interactions, and to determine the similarities and differences among the active sites of methylamine oxidase and eukaryotic amine oxidases. We describe the results of such studies in this paper. The data clearly demonstrate that the active-site structure in copper-containing amine oxidases has been highly conserved. Notwithstanding the close structural similarity, methylamine oxidase displays some novel reactivity properties.[19,20] In one important respect, methylamine oxidase is similar to other amine oxidases in its reactions with substrates: An intermediate state, previously assigned as a semiquinone–Cu(I) form,[21,22] can also be generated in methylamine oxidase. Results presented herein establish that this form is produced by electron transfer from the reduced quinone to Cu(II), which may be mechanistically significant.

Experimental Section

General Procedures. Extreme care was taken to minimize trace metal contamination when necessary. All glassware was thoroughly acid-washed. Buffers were prepared with water obtained from a Barnstead purification system and were passed over Chelex columns. The effectiveness of these procedures was checked by atomic absorption spectroscopy. All chemicals were of the highest grade commercially available. Substrate amines were recrystallized or distilled prior to use. We found the methylamine oxidase catalyzed oxidation of benzylamine to benzaldehyde ($\Delta\epsilon_{250}$ = 12 500 M^{-1} cm^{-1}) to be a convenient assay for enzyme activity.[23] Standard assay conditions were 3.3 mM benzylamine in 0.1 M potassium phosphate buffer at pH 7.2 and 25 °C. One enzyme unit catalyzes the oxidation of 1 μmol of benzylamine/min. Protein was estimated by the Bradford assay or by using the absorption at 280 nm ($A^{1\%}_{280}$ = 18.2).[20] Electrophoresis was carried out with a Pharmacia PhastSystem by using precast gels with Coomassie Blue staining. Our standard conditions were 8–25% polyacrylamide continuous gradient gels, staining with 0.1% PhastGel Blue R, and 200–300 ng of sample applied per well. Discontinuous buffer systems, supplied by Pharmacia, were employed for both SDS and non-SDS gels. Samples for SDS gels were dialyzed against 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 5% β-mercaptoethanol, and 2.5% SDS and then heated to 100 °C for 5 min. Samples for non-SDS gels were in 0.1 M potassium phosphate buffer, pH 7.2.

Anaerobic conditions generally were achieved by vigorously purging all reagent solutions with oxygen-free argon gas; an indicating oxygen trap (Chemical Research Supplies) was used to remove oxygen from 99.999% argon. Enzyme solutions on ice were flushed with argon gas for 1.5–3 h in cuvettes fitted with septa (TCS-Medical Products) or stoppered EPR tubes. Cyanide solutions were prepared immediately prior to each experiment by transferring, via gastight syringe, a known volume of argon-saturated buffer to a septum-stoppered, argon-purged, volumetric flask containing solid KCN. Aliquots of KCN and substrate solutions were also added to the enzyme solution with a gastight syringe under a constant stream of argon. Methylamine oxidase was generally allowed to react with cyanide or substrate for several minutes prior to addition of the second reagent. The kinetics of radical formation were determined by monitoring the characteristic absorption at 460 nm. Initial rates were measured during the first 5 min of the reaction. Kinetics runs were conducted as follows. One milliliter of 11–82 μM methylamine oxidase in 0.1 M potassium phosphate, pH 7.2, was placed in a cuvette and covered with a septum. A gentle flow of argon, which had passed through an oxygen trap and a water bubbler, was blown over the protein solution for 2–3 h. The cuvette was transferred to the Cary 219 and placed under a positive argon flow as previously described. By use of a gastight syringe, a 5-μL aliquot of anaerobic substrate solution (benzylamine) was added so that the final substrate concentration was 4× that of the protein. The contents of the cuvette were gently mixed. Next, 5 μL of an anaerobic NaCN solution, prepared in buffer, was added as above so that the final CN^- concentration was either 2.5 or 5.0 mM.

After the reaction was complete, the protein was immediately put over a G-25 column and then quickly dialyzed against 500 mL of buffer, with a change of buffer after 2 h. Following this procedure, 92–100% of the specific activity and absorbance at 480 nm are regained.

Metal-depleted methylamine oxidase was prepared by dialyzing the dithionite-reduced enzyme against 10 mM KCN in Chelex-treated 0.1 M potassium phosphate buffer, pH 7.2, under anaerobic conditions for 12 h with three buffer changes. Cyanide was removed by extensive dialysis with metal-free buffer. The metal-depleted enzyme had a copper content of 0.0 ± 0.01 Cu ions/mol of enzyme, determined by flame atomic absorption spectroscopy.

Anaerobic methylamine titrations were performed by adding aliquots of approximately 4.0 mM methylamine in buffer, with a gastight syringe, to a solution of the enzyme. Buffers for the methylamine were 50 mM potassium phosphate containing 0.5 M KCl or 10 mM potassium phosphate, both at pH 7.5. Buffers for the enzymes were identical except that [KCl] = 0.45 M for the titration in 50 mM potassium phosphate. A mixture of glucose (final concentration of 0.1 M), glucose oxidase (27 μg), and catalase (4.8 μg) was included in the enzyme solution. Similar amounts of these reagents were included in the substrate solution. Prior to mixing, both solutions were made anaerobic by six pump–purge cycles with oxygen-free argon. An anaerobic cuvette fitted with a ground-glass joint to accept the gastight syringe was employed. An absorption spectrum was recorded following each addition of 1 μL of methylamine titrant. The reaction was complete within the time required to mix the solutions and initiate the absorption measurement. The end point of the titration was taken to correspond to complete bleaching of the absorbance at 500 nm. Following the titration, the exact methylamine concentration was determined enzymatically by using the methylamine dehydrogenase catalyzed reduction of phenazine ethosulfate/2,6-dichloroindophenol under an argon atmosphere. Methylamine dehydrogenase is reduced by methylamine; the reduced enzyme then transfers electrons to phenazine ethosulfate which, in turn, reduces 2,6-dichloroindophenol. The reduction of 2,6-dichloroindophenol is conveniently monitored at 600 nm (ϵ = 21 700 M^{-1} cm^{-1}). This assay was repeated 11 times and the values were averaged.

Protein Purification and Sample Preparation. *Arthrobacter* P1 (NCIB 11625) was cultured and grown as previously described, with 0.5%

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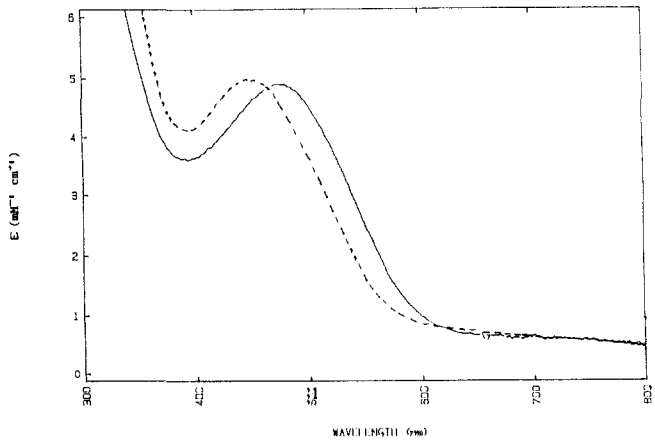

Figure 1. Absorption spectra of resting (oxidized) methylamine oxidase (—) and its complex with ammonia (---) at 22 °C in 50 mM Tris buffer, pH 8.51. The total ammonia concentration was 138 mM.

CH_3NH_4Cl as the sole carbon and energy source.[19,24] Cells were harvested in the stationary phase by centrifugation. Methylamine oxidase was purified by new procedures, to be published elsewhere.[24] The enzyme was obtained in approximately 75% overall yield and had a specific activity of 0.75 (benzylamine assay) with $A_{280}/A_{480} \cong 73$. Catalase and other heme-containing contaminants were removed by either chromatography on aminohexyl-Sepharose 4B (Pharmacia) or repetitive ammonium sulfate fractionations. Purified methylamine oxidase was homogeneous upon SDS or gradient gel electrophoresis. Enzyme samples for EPR spectroscopy were prepared as follows: Methylamine oxidase was dialyzed into 0.1 M potassium phosphate buffer, pH 7.0, and then passed over a 1 × 15 cm Chelex column, equilibrated with the same buffer, to remove trace metal ions. The enzyme was then concentrated by using a vertical, semipermeable membrane device (Bio-Molecular Dynamics) to give a final concentration of ~0.25–1.0 mM (0.5–2.0 mM copper). Samples for electronic absorption and CD spectroscopy were in 0.1 M potassium phosphate buffer, pH 7.0, and generally were concentrated to ~0.10 mM via ultrafication with an Amicon stirred cell. The phenylhydrazine derivative was prepared as previously described;[7] a literature procedure was also followed for the preparation of this derivative in a pure oxygen atmosphere,[10] which is claimed to influence the nature of the final product.[10]

Spectroscopy and Instrumentation. Absorption spectra were obtained with a Cary 219 or Cary 14 interfaced to an IBM PC/AT (On Line Instruments, Inc.) or with an H-P 8451A diode array spectrophotometer. CD spectra were recorded on a modified JASCO J40; base-line corrections and difference spectra were generated digitally on a Bascom-Turner recorder interfaced to the JASCO. Bruker 300D or Varian E-4 EPR spectrometers were used to measure EPR spectra. Microwave frequencies were measured by using the Bruker with a home-built frequency meter. A 1.0 mM Cu(II) EDTA solution (EDTA in 10% excess over [Cu(II)]) was used as the standard for double integration of the methylamine oxidase copper signal. One millimolar solutions of DPPH in toluene or aqueous 3-carbamoyl-PROXYL (Aldrich) served as the standard for double integration of the radical signal. Very low power (~50 μW) was necessary to avoid saturation of the amine oxidase radical signal; the line shape was observed to be quite sensitive to the power above saturation. Resonance Raman spectra were obtained as described previously.[7] A Perkin-Elmer 2380 was used for atomic absorption analyses.

Results

The absorption spectrum of *Arthrobacter* P1 methylamine oxidase is shown in Figure 1, as is the absorption spectrum of the ammonia complex. At pH 8.51 the dissociation constant (K_D), based on the total ammonia concentration, is 8.3 ± 0.8 mM, but at pH 6.79 K_D is 97 ± 11 mM. The calculated K_D for NH_3 binding (with the pK_a of NH_4^+ as 9.24) is 0.3 ± 0.04 mM at pH 6.79 and 1.6 ± 0.2 mM at pH 8.51. Ammonia does not perturb the Cu(II) EPR spectrum under the conditions described in Figure 1. We suggest that ammonia binds to the quinone, perhaps to generate an imino form similar to **2**, if PQQ is in fact the cofactor. The absorption spectrum of resting methylamine oxidase is very

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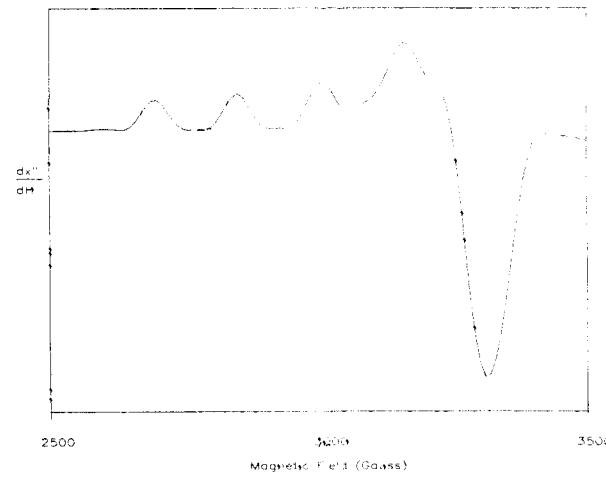

Figure 2. X-band EPR spectrum of methylamine oxidase at 77 K in 0.1 M potassium phosphate buffer, pH 7.2. The microwave power was 10 mW and the modulation amplitude was 10 G.

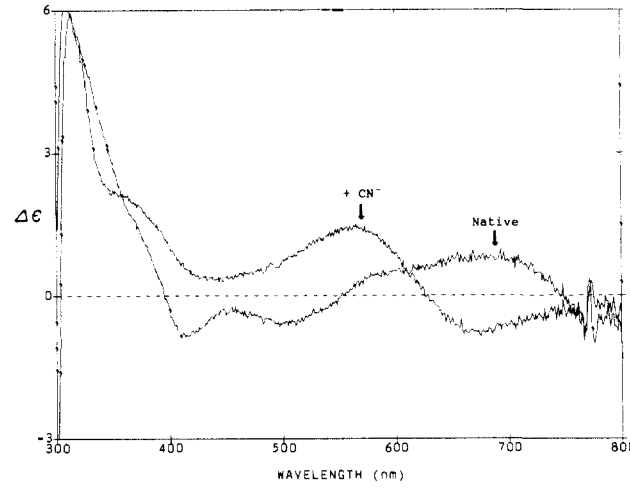

Figure 3. Circular dichroism spectra of resting methylamine oxidase and its cyanide complex at 22 °C in 0.1 M potassium phosphate buffer containing 50 mM cyanide (pH 9.0).

similar to the spectra of eukaryotic copper-containing amine oxidases. The visible region is dominated by electronic transitions associated with the quinone, although a copper–cofactor charge-transfer transition may contribute some intensity. Methylamine oxidase also displays an EPR spectrum (Figure 2) that is closely similar to the spectra of other copper amine oxidases, particularly the plant diamine oxidases. Double integration establishes the paramagnetic copper content of methylamine oxidase to be 2 Cu(II) ions/mol of enzyme; i.e., 100% of the copper is EPR detectable, in accord with results on other amine oxidases. EPR parameters, estimated from Figure 2, are $g_\perp = 2.06$, $g_\parallel = 2.229$, $A_\parallel = 165 \times 10^{-4}$ cm^{-1}. The two Cu(II) ions appear to be equivalent, by EPR spectroscopy, even at 35 GHz.[25] These parameters are typical for type 2 copper centers and indicate (N,O) coordination. Pulsed EPR results are entirely consistent with histidine imidazole and H_2O ligands.[26]

The ligand-field transitions of the Cu(II) ions in amine oxidases can be resolved by CD spectroscopy; the CD spectra of resting methylamine oxidase and its cyanide complex are displayed in Figure 3. Bands at ~800 nm (12 500 cm^{-1}) and 675 nm (14 815 cm^{-1}), and a shoulder at 590 nm (16 950 cm^{-1}), are attributable to the d–d transitions of a tetragonal Cu(II) center. These transitions shift to 675 nm (14 815 cm^{-1}) and 575 nm (17 390 cm^{-1})

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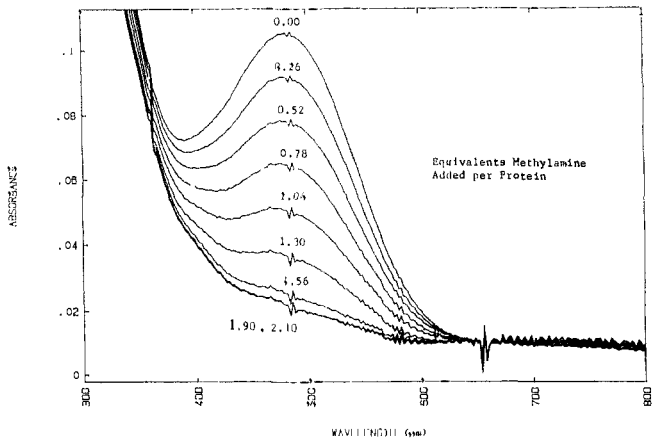

Figure 4. Electronic absorption spectral changes accompanying the anaerobic titration of methylamine oxidase with methylamine at 22 °C in 49 mM potassium phosphate buffer containing 0.45 M KCl, pH 7.5. [Enzyme] = 24.6 μM.

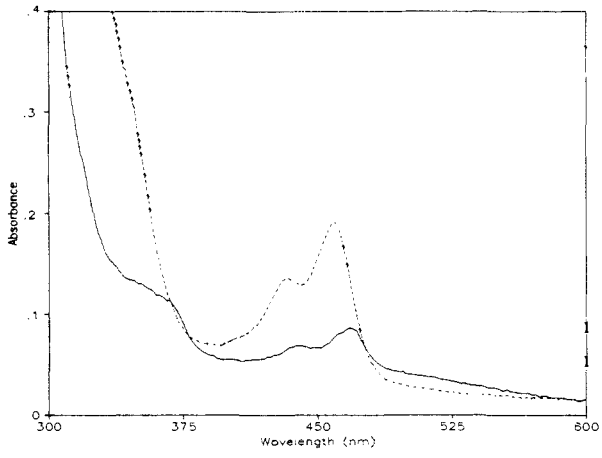

Figure 5. Absorption spectra of methylamine oxidase after (—) reduction anaerobically with benzylamine and then (---) anaerobic addition of cyanide. [Enzyme] = 11.4 μM, [benzylamine] = 50 μM, and [CN^-] = 2.5 mM in 0.1 M potassium phosphate buffer, pH 7.2, 25 °C.

in the CN^- complex (Figure 3). Shifts of this magnitude (~2500 cm^{-1}), to higher energy, are consistent with equatorial coordination of CN^- to Cu(II) in methylamine oxidase. For comparison, the d–d transitions of $[Cu^{II}(dien)CN]^+$ are centered at 545 nm (18 350 cm^{-1}), compared to 615 nm (16 260 cm^{-1}) for the aquo complex.[27] Cyanide binding shifts the ligand-field absorption envelope of the Cu(II) in Cu,Zn-SOD by 3500 cm^{-1}, i.e., from 680 nm (14 700 cm^{-1}) to 550 nm (18 180 cm^{-1}). At pH 7.0, cyanide has no effect on the visible absorption spectrum of methylamine oxidase at concentrations of 20 mM or lower, although the d–d bands resolved in the CD spectrum are perturbed. These results suggest that cyanide does not react directly with the quinone at such concentrations.

Effects of substrate on the methylamine oxidase absorption are illustrated in Figures 4–6. Two spectroscopically distinct forms of substrate-reduced methylamine oxidase can be generated. In the presence of 0.45 M KCl, complete bleaching is observed (Figure 4). The end point corresponds to a stoichiometry of 2 mol of methylamine/mol of protein, which reflects the two-electron reduction of both quinone units in methylamine oxidase. In the absence of chloride, the spectrum shown in Figure 5 results when *either* methylamine or benzylamine is added anaerobically. This must represent a different form of the substrate-reduced enzyme or a mixture of forms. Control experiments confirm that the latter spectrum may be converted to the former by jumping the KCl concentration to approximately 0.5 M and that this conversion is not induced merely by an ionic strength increase. That is,

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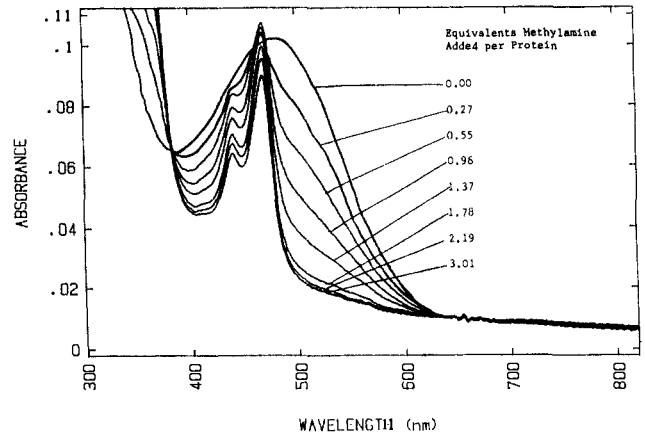


Figure 6. Electronic absorption spectral changes accompanying the anaerobic titration of methylamine oxidase with methylamine in 10 mM potassium phosphate buffer, pH 7.5, at 22 °C. [Enzyme] = 20.5 μM.

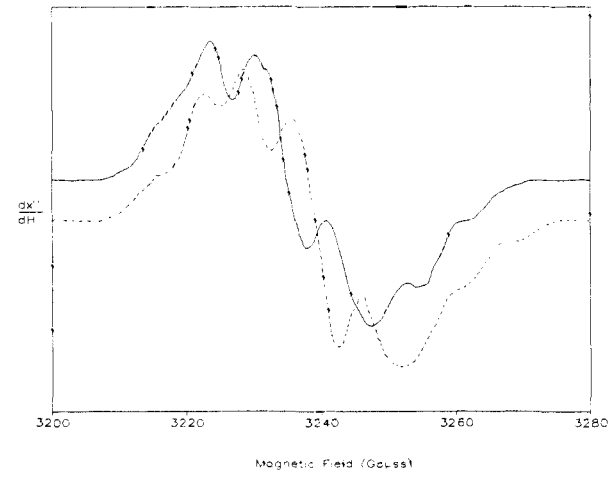
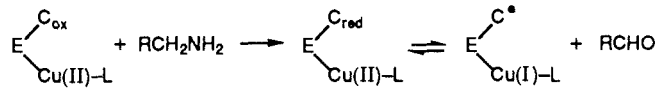


Figure 7. X-band EPR spectrum of substrate-reduced methylamine oxidase at 173 K in the presence of 21 mM KCN. [Enzyme] = 0.50 μM in 50 mM barbitol buffer, pH 8.75. Spectrometer conditions were 0.5-μW microwave power and 1.4-G modulation amplitude. (—) [^{15}N]-Methylamine; (---) [^{14}N]methylamine.

Scheme I. Possible Mechanism for Formation of an Organic Radical (Semiquinone) in Copper-Containing Amine Oxidases by Substrate Reduction[a]



[a] C = organic cofactor (PQQ); L = OH_2, CN^-.

increasing the Cl^- concentration specifically causes the spectrum shown in Figure 5 to bleach. Absorption spectral changes accompanying the anaerobic reduction of methylamine oxidase *in the absence of chloride* are shown in Figure 6. Note that the spectra of the substrate-reduced forms in Figures 5 and 6 are closely similar; the absorption spectrum of the substrate-reduced form is essentially independent of the particular substrate used. The end point of the titration shown in Figure 6 also corresponds to a 2:1 (substrate:protein) stoichiometry. The spectra of the substrate-reduced methylamine oxidase in Figures 5 and 6 are similar to the spectra of substrate-reduced diamine oxidases from plants.[28] Mammalian amine oxidases also display closely similar spectra when reduced by substrates in the presence of cyanide.[21] Cyanide perturbs the absorption spectrum of substrate-reduced methylamine oxidase, shifting λ_{max} to 460 nm and greatly in-

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Table I. Kinetics of Radical Formation[a]

expt	[enzyme], μM	[CN⁻], mM	rel rate	rel [enzyme]	rel [CN⁻]
1	11.4	2.5	0.8 ± 0.1	0.89	0.5
2	12.3	5.0	0.9 ± 0.1	0.96	1.0
3	12.8	5.0	1	1	1
4	51.2	2.5	5.0 ± 0.9	4.00	0.5
5	81.6	5.0	5.8 ± 1.0	6.40	1.0

[a] The initial rate of the absorbance increase at 460 nm was measured as described in the Experimental Section. Conditions: Ar atmosphere; 0.1 M potassium phosphate buffer (pH 7.2). The substrate (benzylamine) concentration was 4 times that of the protein in each experiment.

creasing the overall intensity (Figure 5). In the absence of exogenous ligands, substrates only slightly perturb the 77 K Cu(II) EPR spectrum of methylamine oxidase (not shown). However, in the presence of cyanide *or tert-butyl isocyanide*, the EPR spectrum shown in Figure 7 develops and the Cu(II) signal is markedly decreased. Similar observations with the porcine kidney amine oxidase have been rationalized in terms of Scheme I.[21] Although the spectra shown in Figure 7 were obtained at [CN⁻] = 21 mM, the radical EPR spectrum is fully developed at cyanide concentrations of 5 mM. Double integration indicates that the $g \simeq 2$ radical has 50–60% of the original intensity of the Cu(II) signal. There are three plausible alternatives that can account for this discrepancy: (1) the experimental errors in quantitating the radical signals are such that its intensity is significantly underestimated; (2) some intramolecular disproportionation occurs (e.g., in Scheme I, $2C^{\bullet} \rightarrow C_{red} + C_{ox}$); (3) some intermolecular disproportionation occurs. Identical signals are obtained with methylamine or benzylamine as reductant and with cyanide or *tert*-butyl isocyanide as the exogenous ligand. Therefore, this EPR signal must be associated with the quinone cofactor. This conclusion is additionally supported by the observation that the $g \simeq 2$ EPR signal is independent of the amine oxidase source; i.e., it has now been observed for enzymes isolated from bacteria, plants, and mammals.[21,28c] A comparison with the $g \simeq 2$ signal generated with [^{15}N]methylamine is shown in Figure 7. An isotope effect on the EPR signal is apparent, suggesting that the unpaired electron is partly delocalized onto the nitrogen atom derived from the substrate. Mechanistic data on copper amine oxidases are completely consistent with this inference, as the substrate-derived nitrogen is generally not released (as ammonia) until the second oxygen-dependent step, whereas the aldehyde product is released in the first step.[16,29-31] An imino form of the quinone cofactor (perhaps similar to **2**) may be an obligatory enzyme intermediate in amine oxidase catalyzed reactions.

Efforts to further characterize this radical species by both Q-band CW EPR and pulsed EPR (spin-echo envelope modulation) spectroscopy are in progress.

We have also monitored the reaction of the methylamine oxidase–cyanide complex with substrate by absorption and CD spectroscopy (Figures 5 and 8). The kinetics of formation of the radical were obtained by measuring the absorbance increase at 460 nm (cf. Figure 5) and are summarized in Table I. Clearly, the rate is independent of the cyanide concentration but first order in protein. Therefore, direct cyanide participation, and bimolecular protein reactions, are not implicated in the rate-determining step(s) for radical formation. Figure 8 demonstrates that reduction of

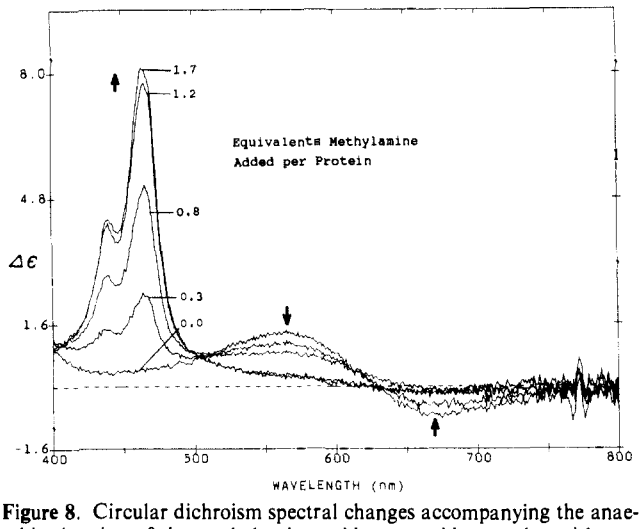

Figure 8. Circular dichroism spectral changes accompanying the anaerobic titration of the methylamine oxidase–cyanide complex with methylamine at 25 °C in 0.1 M potassium phosphate buffer, pH 9.0. [CN⁻] = 50 mM.

the methylamine oxidase copper ions is involved in radical formation. Note that the Cu(II) d–d transitions decrease in intensity as the bands characteristic of the new quinone oxidation state appear. Therefore, methylamine is reducing Cu(II) to Cu(I), abolishing the Cu(II) EPR signal and ligand-field transitions. These data rule out the possibility that the methylamine oxidase Cu(II) EPR signal is lost solely as a result of spin–spin interactions.

The relatively low extinction coefficients of the visible absorption bands, together with intrinsic fluorescence, have so far hindered our efforts to obtain resonance Raman spectra of native amine oxidases, including methylamine oxidase. Resonance Raman spectroscopy of suitably derivatized amine oxidases has proved to be an informative probe of the active-site structures around the quinone.[7-9,22] Resonance Raman spectra of the native and metal-depleted methylamine oxidase phenylhydrazine derivatives are shown in Figure 9. The spectrum of the former derivative is essentially identical, both with regard to frequencies and relative intensities, with the spectra of other amine oxidase phenylhydrazones.[7-9,22] Consequently, there can be no doubt that the organic cofactor in methylamine oxidase is identical with that found in other copper-containing amine oxidases, probably PQQ or a related compound. Generally, phenylhydrazine derivatives of metal-depleted amine oxidases are difficult to prepare, perhaps for two reasons. Carbonyl reagents such as phenylhydrazine do not react with the reduced quinone, and dithionite is used to prepare the apoprotein. Alternatively, a cyanide–quinone adduct may form during the prolonged dialysis against relatively high concentrations of cyanide necessary to remove copper. However, phenylhydrazine reacts with metal-depleted methylamine oxidases, perhaps because the quinone in methylamine oxidase is less reactive toward dithionite and cyanide.

The resonance Raman spectrum of the methylamine oxidase phenylhydrazone is not greatly perturbed by copper removal. There are some minor frequency shifts, some more substantial shifts, and additional splitting (e.g., 1362 $cm^{-1} \rightarrow$ 1313 cm^{-1} and 1456 $cm^{-1} \rightarrow$ 1440 and 1456 cm^{-1}; native → metal depleted). Note that the patterns of relative intensities in the native and metal-depleted enzyme spectra are comparable. The relative intensities in resonance Raman spectra can be sensitive to the microscopic environment of the chromophore, since these intensities are determined by the displacements of the nuclei in the excited state (or states) produced by laser excitation, projected on the ground-state normal modes.[32] Thus copper removal probably does not lead to major alterations in the environment

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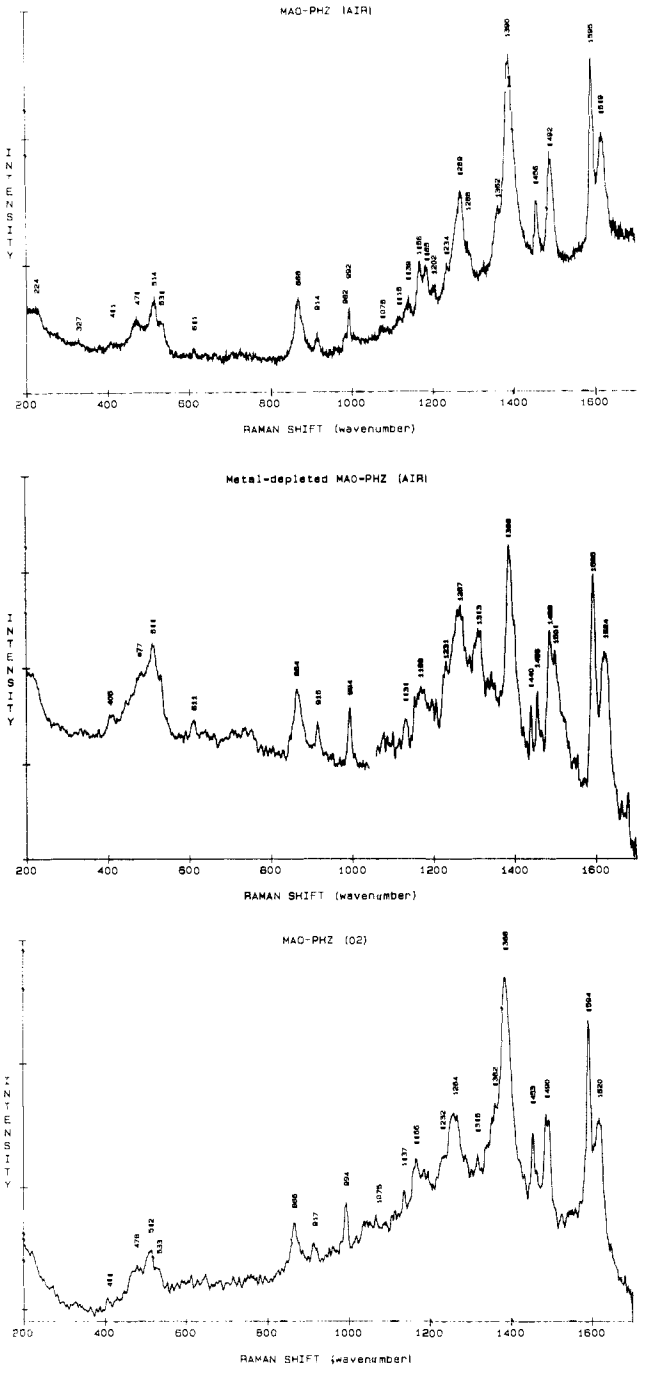

Figure 9. Resonance Raman spectra of methylamine oxidase phenylhydrazine derivatives. Top: methylamine oxidase phenylhydrazine derivative prepared as in ref 7. Middle: apo methylamine oxidase phenylhydrazine derivative prepared as in ref 7 from the apoprotein. A linear background has been subtracted and these data have been smoothed by using a standard routine. Note the break in the spectrum at 1050 cm^{-1}. More scans were collected in the 200–1050-cm^{-1} range than in the 1050–1700-cm^{-1} range in order to obtain acceptable S/N. Bottom: methylamine oxidase phenylhydrazine derivative prepared under an O_2 atmosphere as described in ref 10. A linear background has been subtracted and the spectrum smoothed by using a standard routine.

of the derivatized cofactor. We suggest that the observed differences between the native and metal-depleted phenylhydrazine derivatives may reflect specific copper–cofactor interactions, perhaps arising through coordination. Suzuki and co-workers have previously suggested, for example, that the reaction of phenylhydrazine or benzylamine with PQQ in bovine plasma amine oxidase leads to cleavage of a PQQ–copper bond.[33]

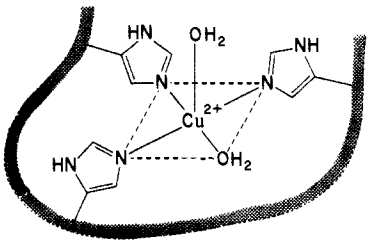

Figure 10. Model for the Cu(II) sites in copper-containing amine oxidases (ref 36).

It has been claimed[10] that phenylhydrazine reacts with amine oxidases and PQQ to give two products: an azo adduct and the tautometric phenylhydrazone, which is stated to form only in an O_2 atmosphere after several hours at room temperature. In our experience, significant protein denaturation can occur under these conditions. Moreover, phenylhydrazine is unstable in the presence of O_2, being oxidized to phenyldiimide[34] and generating phenyl radical. Nevertheless, the major products we observe in the reaction of methylamine oxidase with phenylhydrazine in air or in O_2 have identical absorption and resonance Raman spectra (Figure 9). The most logical interpretation of this result is that O_2 does not affect the identity of the major phenylhydrazine product, but it certainly can induce side reactions and hence affect the yield.

Discussion

All the data obtained to date point to very similar structures for the copper sites in methylamine oxidase and eukaryotic amine oxidases. Specifically, the Cu(II) EPR parameters, and the energies and signs of the CD bands associated with the Cu(II) d–d transitions, indicate that all copper amine oxidases contain tetragonal Cu(II) sites with nitrogen and oxygen ligands. The ligands are most probably histidine imidazole and water, although quinone coordination remains a possibility.[26,35-37] Pulsed EPR experiments and ^1H NMR relaxation measurements of H_2O exchange are consistent with imidazole and water ligands in methylamine oxidase.[26,38] Further, the Cu(II) sites in methylamine oxidase display facile ligand substitution chemistry, as judged by the rapid, reversible reaction with cyanide. The blue shifts in the Cu(II) d–d transitions indicate that exogenous ligand binding involves an equatorial coordination position. The model for the Cu(II) site structure in bovine plasma amine oxidase[36] (Figure 10) is also applicable to the Cu(II) sites in other eukaryotic amine oxidases, from both mammalian and plant sources.[39] We suggest that the model is a reasonable one for the Cu(II) sites in methylamine oxidase. If so, then the Cu(II) site structure has been conserved; in turn, this suggests, together with other data, that copper has a key functional or structural role. Two possibilities for the role of copper in catalysis have been discussed: (1) equatorially coordinated hydroxide nucleophilically assists hydride transfer from the reduced quinone to O_2;[40] (2) copper directly mediates electron transfer from the reduced cofactor to O_2.[41] We favor the latter alternative, in part for the reasons presented below.

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Resonance Raman spectra definitively establish that the cofactors in methylamine oxidase and eukaryotic copper-containing amine oxidases are identical, as previously suggested.[20,22] Independent confirmation is provided by the close similarity of the radical EPR spectra that have been obtained for methylamine oxidase (Figure 7), plant diamine oxidases, and porcine kidney diamine oxidase. Numerous experiments strongly suggest that PQQ, or a similar quinone, is the organic factor.[3,7-13,20-22,42] Differences in substrate specificity between methylamine oxidase and other copper-containing amine oxidases may be related to cofactor accessibility or substrate binding specificity, or both. Our data establish that the Cu:quinone stoichiometry is 1:1; in other words, there is one active site per subunit or two per enzyme molecule. Recent results on other copper-containing amine oxidases are also in accord with a 1:1 Cu:quinone stoichiometry,[43] although both quinones may not be fully reactive in all amine oxidases.

Questions regarding the structural and mechanistic interactions between copper and the quinone are critical to understanding catalysis in amine oxidases and warrant detailed investigation. It has been widely recognized for some time that reactions at the quinone affect or perturb the copper sites. For example, amine substrates and carbonyl reagents generally perturb the Cu(II) EPR spectrum in various amine oxidases, although only to a modest and variable extent.[16] Substrate reduction has been demonstrated to affect the affinity of the Cu(II) sites for exogenous ligands, and the energies of the associated ligand-to-metal change-transfer (LMCT) transitions, in bovine plasma amine oxidase.[44] More recently, Suzuki and co-workers have shown that the reaction of amines or phenylhydrazine with the quinone shifts the Cu(II) ligand-field transitions in this enzyme.[33] There have been relatively fewer demonstrations that copper-localized reactions perturb or influence the reactivity of the quinone cofactor. Exogeneous Cu(II) ligands, e.g., N_3^-, SCN^-, and CN^-, certainly inhibit amine oxidases and in some cases have been shown to decrease rate constants in the O_2-independent phase.[39,44,45] This suggests that copper may play some functional role in substrate reduction. In addition, it should be noted that O_2 does not reoxidize substrate-reduced, copper-depleted amine oxidases; i.e., the copper is required for oxidation of the reduced quinone in amine oxidases,[46] even though reduced PQQ in solution does react rapidly with O_2.[47]

Suzuki and co-workers have assigned a band near 480 nm ($\Delta\epsilon \simeq 600\ M^{-1}\ cm^{-1}$, observed in a difference spectrum between native and metal-depleted bovine plasma amine oxidase) to a cofactor → Cu(II) LMCT transition.[41b] CD spectra are also consistent with a Cu(II) electronic transition at this wavelength,[44,48] which could be an LMCT transition. On the other hand, ^{19}F NMR[49] and fluorescence energy transfer[50] studies by Falk and co-workers on porcine plasma amine oxidase phenylhydrazine derivatives apparently restrict the possibilities for quinone coordination to copper. Both experiments give a distance between the copper and the phenylhydrazine ring of ~11 Å, which, for example, would be inconsistent with copper coordination to N(6), the C(4) carbonyl, and the C(7) carboxylate of PQQ, or to the nitrogen atoms in the hydrazine. One can argue that the phenylhydrazine derivatives are structurally similar to the substrate-reduced enzyme and, hence, that the distances inferred from the NMR and energy-transfer experiments are only appropriate for that form of the enzyme and may be different in the resting (or some other) state. This possibility has been suggested by Suzuki.[33]

Scheme II. Proposed Mechanism for Copper-Containing Amine Oxidases[a]

$$Cu^{2+}E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Cu^{2+}ES \quad (1)$$

$$Cu^{2+}ES \overset{k_3}{\rightleftharpoons} Cu^{2+}E_{red} + P \quad (2)$$

$$Cu^{2+}E_{red} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} Cu^{+}E^{*}_{red} \quad (3)$$

$$Cu^{+}E^{*}_{red} + O_2 \overset{k_6}{\rightleftharpoons} Cu^{2+}E + O_2^{2-} \quad (4)$$

[a] Adapted from ref 51. This reaction sequence applies to *one* subunit or active site (the native enzyme is a dimer): E, resting state; E_{red}, two-electron-reduced form; E^*_{red}, one-electron-reduced form; S, amine substrate; P, aldehyde product.

The data reported here are consistent with the view that the copper and quinone cofactor are in proximity, might be coordinated, and *certainly* interact, in ways that are plausibly (at least) related to the catalytic mechanism. Although the data that suggest such interactions are extensive, the structural bases for these interactions are uncertain. For example, the resonance Raman data in Figure 9 establish that copper removal does not produce drastic changes in the immediate environment of the derivatized cofactor, but the data do imply that more specific interactions may be present. Variable temperature MCD studies could, in principle, identify any Cu(II) LMCT transitions in amine oxidases and possibly provide convincing evidence for Cu(II)–quinone coordination. Vibrational assignments for the resonance Raman spectra would also be very helpful. Work along both of these lines is in progress.

What we wish to emphasize is that the data presented here definitively establish that electron transfer between reduced quinone and Cu(II) is possible, generating a semiquinone and Cu(I). Our proposal for the role of the exogeneous ligand is illustrated in Scheme I; an internal redox equilibrium is proposed, such that the redox partition can be altered to favor the semiquinone state by ligands that stabilize Cu(I). The observation that *tert*-butyl isocyanide can also induce radical formation argues against cyanide-specific chemistry.[51] It should be pointed out that Scheme I is almost certainly too simple to account for all the intermediates that have been detected in the reactions of substrate-reduced amine oxidases with cyanide in the absence or presence of O_2. Scheme I has obvious similarities with certain, previously proposed, reoxidation mechanisms, summarized in Scheme II.[52] A key point is that a semiquinone–Cu(I) intermediate could be thermodynamically disfavored, and thus not present in detectable amounts, but highly reactive and hence an intermediate in the reoxidation of substrate-reduced amine oxidases. Indeed, Cu(I) is usually very reactive toward O_2.[53] Evidence consistent with the presence of a bound O_2^- intermediate, generated by the reaction of O_2 with Cu(I), has been described in the literature.[41a,c] Specifically, various Cu(II) complexes that efficiently catalyze superoxide dismutation are also effective amine oxidase inhibitors, whereas similar metal complexes that do not react with superoxide also do not inhibit the enzymes. In addition, oxygen rapidly reoxidizes Cu(I) produced by stoichiometric di-

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thionite reduction.[54]

The rates for reduction of porcine plasma amine oxidase by substrate amines are much faster than the subsequent reoxidation rate; in contrast, both rates are slower but practically identical for the bovine plasma enzyme, whereas the plant amine oxidases display extremely fast rates of reaction with O_2.[46] If the reoxidation mechanism involves sequential single-electron-transfer steps, then the rate of such steps will be very sensitive to copper–quinone interactions. The distance and overlap between the Cu(II) $d_{x^2-y^2}$ orbital and the donor orbital on the reduced cofactor will be critical, for example. There may also be significanct Franck–Condon barriers to electron transfer, as the Cu(I) site appears to have a different structure from that of the Cu(II) site, both with respect to coordination number and geometry.[55] These factors could easily account for the rather large variations in reoxidation rates displayed by amine oxidases even though the active-site structure is remarkably conserved. In other words, the notable reactivity differences among amine oxidases may primarily reflect variations in the copper–quinone interactions among these enzymes. Possible differences in the Franck–Condon factors could be assessed by correlating Cu(I) site structures (deduced from EXAFS) with reactivity.

Summary

The data described in this paper establish the following:

(1) The Cu(II) site structure in *Arthrobacter* P1 methylamine oxidase is at least very similar to the Cu(II) site structures in eukaryotic amine oxidases.

(2) The organic cofactor in *Arthrobacter* P1 methylamine oxidase is identical with that found in other amine oxidases and is probably PQQ or a closely related compound. Moreover, the microenvironment of the quinone in methylamine oxidase is essentially the same as that in the plasma amine oxidases.

(3) Electron transfer between the reduced quinone and Cu(II) occurs in the presence of ligands that stabilize Cu(I), generating a semiquinone form of the *Arthrobacter* methylamine oxidase that is identical with the semiquinone forms previously generated in eukaryotic amine oxidases. Some of the unpaired spin density in the semiquinone must reside on the nitrogen derived from the substrate.

In addition, other new spectroscopic evidence for copper–quinone interactions has been presented.

Acknowledgment. We thank Dean Wilcox and Charles Dickinson for access to the EPR spectrometers at Dartmouth College and the University of Massachusetts. We also thank Judith Klinman and Peter Knowles for several helpful discussions. Research at Amherst College was supported by the NIH (Grant GM 27659 to D.M.D.); research at the Veterans Administration Medical Center and UCSF was supported by Program Project Grant HL-16251-16, the Veterans Administration, and a UCSF Academic Senate Grant (W.S.M.).

Registry No. Cu, 7440-50-8; NH_3, 7664-4-7; CN^-, 57-12-5; methylamine oxidase, 80891-30-1; phenylhydrazine, 100-63-0.

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Crystal and Molecular Structure of the Hexasaccharide Complex (*p*-Nitrophenyl α-maltohexaoside)$_2$·Ba(I$_3$)$_2$·27H$_2$O

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Abstract: *p*-Nitrophenyl α-maltohexaoside (I) [4-nitrophenyl penta[*O*-α-D-glucopyranosyl-(1→4)]-α-D-glucopyranoside], an amylose fragment with a blocked reducing end, crystallizes as the complex (I)$_2$·Ba(I$_3$)$_2$·27H$_2$O in space group $P2_12_12_1$ with a = 33.732 (13) Å, b = 29.212 (10) Å, c = 14.442 (4) Å, and Z = 4. On the basis of I0055 counter X-ray Cu Kα diffraction data, the structure was determined by Patterson, direct, and difference Fourier methods and least-squares refined to R = 0.097 and R_w = 0.092 for 7590 independent reflections with $F_o > 3\sigma(F_o)$. The triiodide ions are almost linear and are arranged in the c-direction as an infinite zigzag chain with interunit angles from 121.3° to 166.1°. The structures of the two molecules of I in the asymmetric unit resemble cleaved cyclodextrins distorted in the form of lock washers with left-handed screw sense; all 12 glucoses are in the $^4C_1$ chair form. Two lock washers in opposite directions wrap around two I_3^- ions to form a left-handed antiparallel double helix. It is stabilized by van der Waals interactions with the polyiodide chain, as observed with amylose and cyclodextrins, and by both intramolecular interresidue and intermolecular O(2)---O(3′) hydrogen bonds, several of which are mediated and augmented by water bridges. The glucoses in the center of the molecules are more regularly arranged than those at the ends. They were used to mathematically construct an amylose antiparallel double helix with 2 × 8 glucoses per turn with a pitch height of 18.64 Å. In the crystal structure, adjacent double-helical complexes related by 2_1 screw symmetry along c are arranged such that an "infinite", wavy double helix is formed. It is stabilized by stacking interactions between the *p*-nitrophenyl groups, by hydrogen-bonded water molecules serving as intermolecular bridges, by interactions between I_3^- units, and by coordination of Ba^{2+} to four different molecules of I. The ligands are arranged symmetrically around Ba^{2+} in the form of a capped square antiprism with two O(5) atoms occupying the caps and glucoses chelated pairwise with their O(2), O(3) and O(5), O(6) oxygens, respectively. All except one of the 27 water molecules in the asymmetric unit are in direct hydrogen-bonding contact with the double helix. There is a characteristic, systematic hydration scheme such that glucose atoms O(2), O(3) and/or O(5), O(6) chelate water molecules to form five-membered cyclic structures, similar to the chelation of the Ba^{2+}. This motif in glucose hydration is so systematic that it will probably occur in other heavily hydrated crystalline amylose fragments and, above all, in solution.

The crystallization of oligosaccharides is particularly difficult, probably because the molecules are very flexible and adopt a variety of conformations that are stabilized by hydrogen bonds of intra- and intermolecular type. Only a few single crystal structure analyses are reported of longer oligomers, with the tetramer stachyose the largest characterized molecule so far.[1,2]

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