Coordination Chemistry of Copper-Containing Amine Oxidases: Nuclear Magnetic Relaxation Dispersion Studies of Copper Binding, Solvent-Water Exchange, Substrate and Inhibitor Binding, and Protein Aggregation

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Abstract: The magnetic field dependence of T_1^{-1} of solvent protons (nuclear magnetic relaxation dispersion (NMRD) profiles) was measured for solutions of the resting (oxidized) forms of amine oxidases from bovine plasma, porcine plasma, porcine kidney, and *Arthrobacter* P1. These amine oxidases may be divided into two classes on the basis of three factors: the paramagnetic contribution to the proton relaxation rate ($T_{1_p}^{-1}$), the temperature dependence of $T_{1_p}^{-1}$, and the effects of the Cu(II) ligand azide on $T_{1_p}^{-1}$. Specifically, the magnitudes of the paramagnetic contributions are consistent with the presence of at least one liganded water molecule in rapid exchange with bulk solvent water for both classes, but an additional exchangeable water ligand is present in the amine oxidases from porcine plasma and *Arthrobacter* P1. This water exchanges rapidly at 25 °C, but relatively slowly at 5 °C, and is probably displaced upon anion binding. Exogenous ligands, such as azide, are known to bind equatorially to tetragonal Cu(II) ions in all amine oxidases; nonetheless, azide binding only slightly decreases $T_{1_p}^{-1}$ of the bovine plasma and porcine kidney amine oxidases but significantly reduces $T_{1_p}^{-1}$ of the porcine plasma protein. This suggests that the additional water molecule is equatorial and contributes to the NMRD profiles of the porcine plasma and *Arthrobacter* P1 amine oxidases. Substrate has no effect on the NMRD profiles of the bovine plasma enzyme. NMRD measurements were also used to monitor copper incorporation into metal-depleted amine oxidases from porcine and bovine plasma. For both oxidases, the relaxivity ($T_{1_p}^{-1}$ per millimole per liter of protein), specific activity, and the intensity of the principal absorption band are approximately linear functions of the copper content. Close examination of the diamagnetic contributions to the profiles, which principally relate to the rotational relaxation rate of the large protein molecules, suggests that the plasma amine oxidases tend to associate under the conditions of the present experiments: 0.5 mM protein, pH 7.0, and ionic strength (μ) 0.1. Indeed our data show that NMRD measurements are a sensitive method for detecting self-association of these enzymes under common experimental conditions.

Introduction

Copper-containing amine oxidases are very widely distributed and are believed to play critical roles in the metabolism of biogenic primary amines. Amine oxidases have now been isolated from a variety of eukaryotic sources, including yeasts, plants, and mammalian plasma and tissue.[1-3] These enzymes were the first from eukaryotic organisms that were suggested to contain the novel coenzyme pyrroloquinoline quinone (PQQ), previously found only in methylotrophic bacteria.[4a] Recently, Klinman and co-workers have provided very strong evidence that 6-hydroxydopa is the cofactor in bovine plasma amine oxidase;[4b] the oxidized form of this cofactor is a quinone. The first copper-containing amine oxidase from a prokaryotic source to be isolated and characterized is *Arthrobacter* P1 methylamine oxidase.[5,6] All the copper-containing amine oxidases isolated to date (with one possible exception) contain two copper ions per protein molecule.[1-3] Generally, these enzymes are composed of two subunits that appear to be similar, if not identical, to a total molecular weight of 170000–190000. The quinone:protein stoichiometry is uncertain in some cases, but recent results are beginning to converge on a 2:1 ratio.[7,8] Hence, the active site in this class of amine oxidases is likely to be composed of one copper ion and one quinone. It should also be noted that the quinone cofactors in amine oxidases are covalently bound. A structural model for the Cu(II) site is displayed in Figure 1. The Cu(II) site structure was derived primarily from X-ray absorption[9,10] and pulsed EPR spectroscopy.[11] NMR[12] and fluorescence-quenching studies[13] on porcine plasma amine oxidase derivatized with substituted phenylhydrazines have placed the Cu(II) ions and the quinone groups within several angstroms of each other. Numerous independent experiments have established that the copper and quinone interact,[1-3,6,14-16] as expected from their proximity in the active site,

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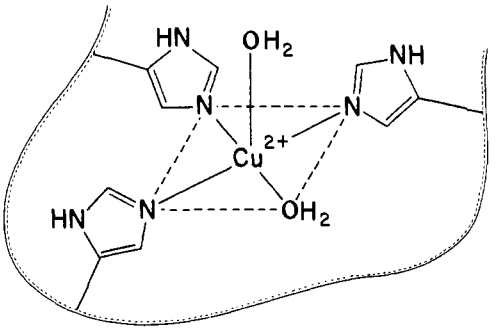

Figure 1. Model for the Cu(II) sites in copper-containing amine oxidases.

but the question of whether the cofactor is actually a copper ligand is unresolved. Both the copper ions and the quinones are absolutely required for activity.

The reaction catalyzed by copper-containing amine oxidases is schematically shown below:

$$E + RCH_2NH_2 \rightarrow E{:}RCH_2NH_2 \rightarrow E_{red} + RCHO \quad (1)$$

$$E_{red} + O_2 + H_2O \rightarrow E + NH_3 + H_2O_2 \quad (2)$$

Copper removal[1,2,7,17-20] or ligand substitution at enzyme-bound Cu(II)[1-3,21-27] greatly decreases the rate of step 2. In fact, the metal-depleted, substrate-reduced enzymes generally react with oxygen very slowly. Several potential Cu(II) ligands have been shown to be effective amine oxidase inhibitors. Azide, thiocyanate, cyanide, and ammonia are known to bind to an equatorial coordination position on the Cu(II)[3,21-28] and, at least for azide and cyanide, displace a solvent-derived ligand, either H_2O or OH^-. No major structural rearrangements in the copper site accompany ligand substitution. Therefore, it appears that labile coordination positions on the Cu(II) ions are important to catalysis. This inference is supported by the finding that the dissociation and inhibition constants for exogenous Cu(II) ligands are generally equal within the experimental uncertainties.[21-23,25-28]

Nuclear magnetic relaxation dispersion (NMRD) measurements, in which the solvent ^1H longitudinal (spin–lattice) relaxation rate (T_1^{-1}) is measured over a wide range of magnetic field, are well-established as a probe of macromolecule–solvent interactions, particularly when the macromolecule contains a paramagnetic metal ion.[29-32] Among other applications, NMRD experiments can be used to demonstrate the accessibility of a bound metal ion and, hence, the active site to exchanging solvent molecules. Recent theoretical advances have made it possible to quantitate the basic effects of Cu(II) on T_1^{-1}, which are particularly complex for $s = {}^1/_2$ ions, for which hyperfine interactions generally dominate the relaxation process.[30,31] In principle, both structural and dynamical information about Cu(II)–solvent interactions can be obtained from analysis of NMRD profiles, and previous work has shown that NMRD measurements are also an extremely useful technique for investigating metal ion binding to apoproteins.[32,33]

Herein we report extensive studies of the NMRD profiles of porcine plasma amine oxidase (PPAO) and bovine plasma amine oxidase (BPAO) as a function of the copper content of these proteins. Our interest was stimulated in part by reports in the literature that the two copper sites in each of these amine oxidases may have different physical and functional properties.[20,34] For example, the two copper sites in PPAO were shown to be inequivalent by Q-band EPR spectroscopy,[7,27] implying that the two sites may have different structures. Both EPR and NMRD are informative probes of paramagnetic species and the interactions between paramagnetic metal ions and their environment. Unlike Q-band EPR, NMRD measurements are readily conducted under conditions that are comparable to those used for measuring copper binding and enzymatic activity. Under our conditions, the relaxation rate (T_1^{-1}), the specific activity, and the intensity of the principal electronic absorption band are all linear functions of the Cu(II) content of both PPAO and BPAO.

We have also measured the NMRD profiles of the resting (oxidized) forms of porcine kidney amine oxidase (PKAO) and *Arthrobacter* P1 amine oxidase (APAO); in all cases, including BPAO and PPAO, the data are consistent with the presence of at least one coordinated water molecule that is in rapid exchange with solvent water on an NMRD time scale, $\leq 10^{-6}$ s. The data further indicate that amine oxidases may be divided into two classes that have water molecules with different solvent-exchange dynamics. Finally, the NMRD data provide clear evidence for the self-association of particularly the BPAO but also of the PPAO.

Experimental Procedures

Enzyme Preparation and Characterization. Amine oxidases from porcine plasma,[35] porcine kidney,[24] and bovine plasma[36,37] were purified by either literature procedures or minor modifications thereof. Methylamine oxidase from *Arthrobacter* P1 was purified by new methods to be published elsewhere.[38] All the enzymes were homogeneous as judged by sodium dodecyl sulfate (SDS) and gradient gel electrophoresis. The final specific activities were equal to or greater than the highest values previously reported. Gel electrophoresis was carried out with a Pharmacia Phastsystem under the following conditions: 8–25% polyacrylamide continuous-gradient gels; 200–300 ng of sample applied per well; staining with 0.1% PhastGel Blue R. Discontinuous buffer systems, supplied by Pharmacia, were used for all gels. Enzyme activity was measured with benzylamine or *p*-[(dimethylamino)methyl]benzylamine as described for the following enzymes: PPAO,[35] BPAO,[39] and PKAO.[40] Methylamine oxidase was assayed with benzylamine under the conditions of the bovine plasma enzyme assay. Substrates were recrystallized or distilled prior to use. A previously described method was used to synthesize *p*-[(dimethylamino)methyl]benzylamine.[41] Protein concentration

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was estimated with either the A_{280} and the appropriate extinction coefficient or the Bradford assay.[42] These methods were in good agreement for all the enzymes.

The precise methods used to remove and reincorporate copper may be critical, so we will describe our procedures in some detail. Metal-depleted BPAO was prepared by dializing a sample (approximately 0.05 mM) of the dithionite-reduced form against 10 mM cyanide in 0.1 M potassium phosphate buffer (pH 7.0, 1000× sample volume) for 20 h under anaerobic conditions with three changes of buffer. The sample was then dialyzed extensively against the same buffer to remove cyanide. The appropriate amounts of copper(II) nitrate (atomic absorption standard) were added to aliquots of the dilute apoprotein to give the desired Cu:protein stoichiometries, and these were allowed to equilibrate for 3–20 h; 3 h was the minimum time necessary to obtain a stable specific activity and visible absorption spectrum. Any unbound copper was removed by dialysis against metal-free buffer (with a minimum of two changes) prior to concentration for the NMRD experiments. A slightly different procedure was used to prepare metal-depleted PPAO. The dithionite-reduced enzyme was dialyzed anaerobically against 10 mM cyanide in 0.05 M Pipes buffer (pH 7.0) for 20 h; the majority of the cyanide was removed by gel filtration over G-25, followed by extensive dialysis against Pipes buffer. This procedure routinely removed >90% of the copper. Samples containing <5% copper have been prepared when the duration of the dialysis against cyanide was extended. Copper reconstitution was also carried out in Pipes buffer. At pH 7.0, activity is recovered over a period of 6 days following the addition of copper to metal-depleted PPAO.[7] Therefore, the activities of all the NMRD samples were carefully monitored, and a sample was used only after stable activity had been attained. Copper content, specific activity, and the visible absorption spectrum of each sample were measured prior to and immediately following the NMRD experiments. The copper content of all NMRD samples was determined by flame atomic absorption with a Perkin-Elmer 2380 spectrometer. Extreme care was taken to minimize contamination by trace metal ions. Buffers were prepared from distilled, deionized water and passed over Chelex columns. Glassware was thoroughly acid washed with a 1:1 mixture of concentrated nitric and sulfuric acids.

NMRD Measurements. The methodology for obtaining and analyzing NMRD data is well-documented in the literature.[30-33,43] Briefly, T_1^{-1} NMRD profiles were measured over the field range 0.01–50-MHz proton Larmor frequency, corresponding to 0.000 24–1.2 T, with an automated field-cycling relaxometer.[43] The sample tube is surrounded by circulating Freon, allowing measurements in the range −10 to +35 °C, with the temperature regulated to within ±0.1 °C to prevent temperature fluctuations from contributing to the uncertainty of the data, which is limited by noise to ~0.5%. For each value of T_1^{-1} reported, 23 pairs of data points at a given field were fit to a single exponential. All profiles for samples containing substoichiometric copper were measured at least twice to check consistency and reproducibility. Typical conditions were the following: 0.1–0.5 mM protein (0.2–1.0 mM copper), and Pipes or potassium phosphate buffer (0.05 M, pH 7.0).

Data Reduction. Solvent relaxation by paramagnetic Cu(II) ions bound to macromolecules has been extensively studied, and the basic theory is now reasonably well understood.[29-31] Equations 3–6 summarize the contributions to the relaxation rate that should be considered here for relaxation by Cu(II) ions in proteins.

$$T_1^{-1} = T_{1_w}^{-1} + T_{1_p}^{-1} + T_{1_d}^{-1} \quad (3)$$

$$T_{1_p}^{-1} = \frac{n[M]}{55.5} \sum_i \frac{1}{T_{M_i} + \tau_{M_i}} \quad (4)$$

$$R = (T_1^{-1} - T_{1_w}^{-1})/[M] \quad (5)$$

$$R_p = (T_1^{-1} - T_{1_d}^{-1} - T_{1_w}^{-1})/[M] \quad (6)$$

Here T_1^{-1} is the observed field-dependent and temperature-dependent relaxation rate, which can be regarded as the sum of a small buffer background $T_{1_w}^{-1}$, a diamagnetic contribution $T_{1_d}^{-1}$, which (ideally) would be obtained from the protein with the paramagnetic Cu(II) ions reduced to diamagnetic Cu(I), and $T_{1_p}^{-1}$, a paramagnetic contribution that is dominated by solvent exchange from the inner coordination spheres of the paramagnetic ions. The summation is over all the coordinated water molecules on a single Cu(II) ion (assuming all the n ions per protein are identical). τ_{M_i} is the lifetime for exchange of the ith coordinated water, and T_M is its proton longitudinal relaxation time when coordinated. R, called the "relaxivity", is the total relaxation rate induced per millimole per liter of protein ([M]), and R_p is its paramagnetic component.

The phenomenology of $T_{1_d}^{-1}$ is well established[44,45] as is a reasonable theory for $T_{1_p}^{-1}$ of Cu(II)-containing proteins.[29-31] However, it is sufficient for the present purposes to realize that $T_{1_d}^{-1}$ dominates at lower field and $T_{1_p}^{-1}$ dominates at higher fields; the major conclusions are based firmly on the phenomenology.

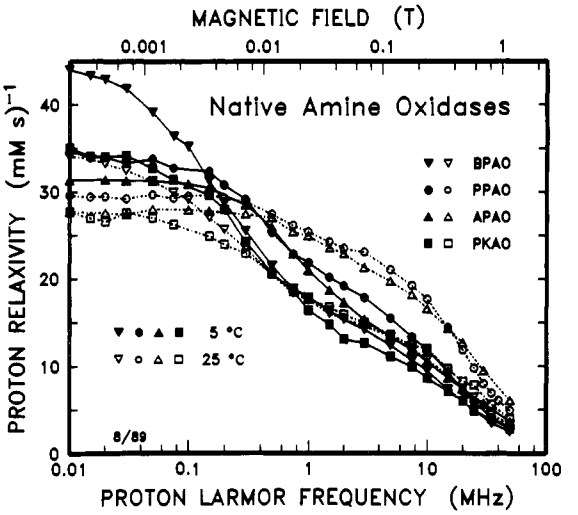

Figure 2. NMRD profiles (total relaxivities) of native amine oxidases in either 50 mM KPO_4 (pH 7.0) (BPAO, APAO, and PKAO) or 50 mM Pipes + 50 mM NaCl (pH 7.0) (PPAO). Relaxivities are plotted per millimole per liter of protein. Key: BPAO, 5 °C (▼), 25 °C (▽); PPAO, 5 °C (●), 25 °C (○); APAO, 5 °C (▲), 25 °C (△); PKAO, 5 °C (■), 25 °C (□).

Table I. Effect of Azide on NMRD of Amine Oxidases

enzyme	azide concn[a]	relaxivity[b]	relaxivity[c]
BPAO	0	43.6	13.3
	10	44.6	13.2
	50	43.1	13.3
	100	41.3	12.9
	200	38.4	12.7
	400	35.4	12.1
PKAO	0	34.2	11.6
	3	34.5	10.9
	10	34.6	10.8
	160	31.3	10.4
PPAO	0	34.8	16.2
	10	32.0	12.9
	20	31.6	12.6
	40	31.3	12.3
	80	30.8	11.9
	200	29.8	11.3

[a] Millimoles per liter. [b] 5 °C, 0.02 MHz. [c] 5 °C, 5 MHz.

Results

Relaxivity of the Resting Enzymes. The NMRD profiles for several native amine oxidases, at 5 and 25 °C, are shown in Figure 2; they involve, as shown in eqs 3 and 5, the sum of the diamagnetic contribution $T_{1_d}^{-1}$ of the protein and the paramagnetic contribution $T_{1_p}^{-1}$ of Cu(II) ions to the observed relaxation rates. It is clear from the magnitudes of the relaxivities at high fields (corresponding to >1 MHz), where $T_{1_p}^{-1}$ dominates, and from their different dependence on temperature, that these amine oxidases can be divided into two classes: BPAO and PKAO in one and PPAO and APAO in the other. Specifically, the relaxivities of BPAO and PKAO are relatively lower and not very temperature-dependent, whereas the relaxivities of APAO and PPAO are

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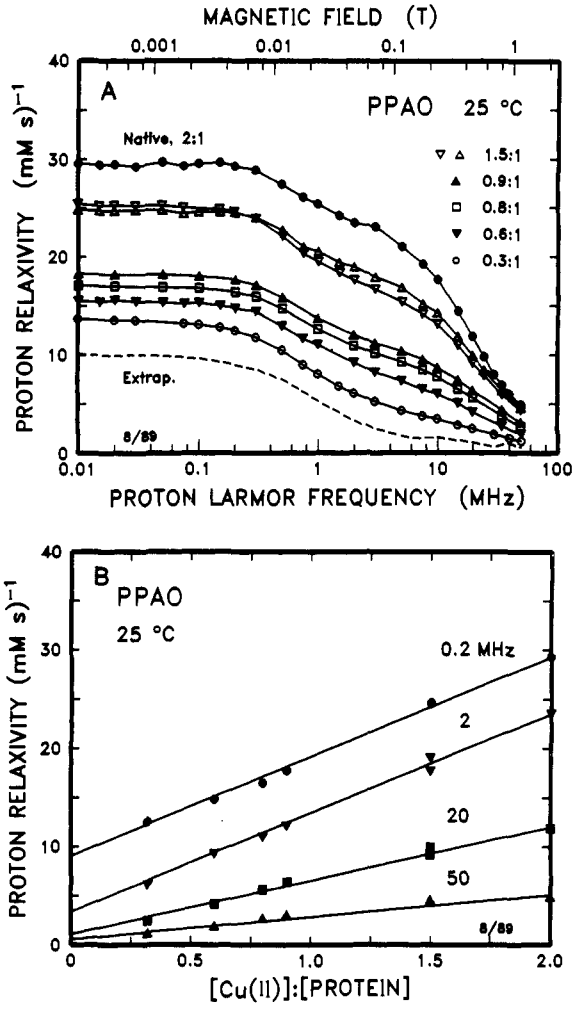

Figure 3. (A) NMRD profiles (total relaxivities) of PPAO at 25 °C for different Cu(II):protein ratios. Dotted line represents the NMRD profile derived by extrapolation of the relaxivities at each field for varying copper loadings to zero copper. Relaxivities are plotted per millimole per liter of protein; buffer is 50 mM Pipes + 50 mM NaCl (pH 7.0). (B) Total relaxivity of PPAO at 0.2-, 2-, 20-, and 50-MHz proton Larmor frequencies and 25 °C as a function of the Cu(II):protein ratio.

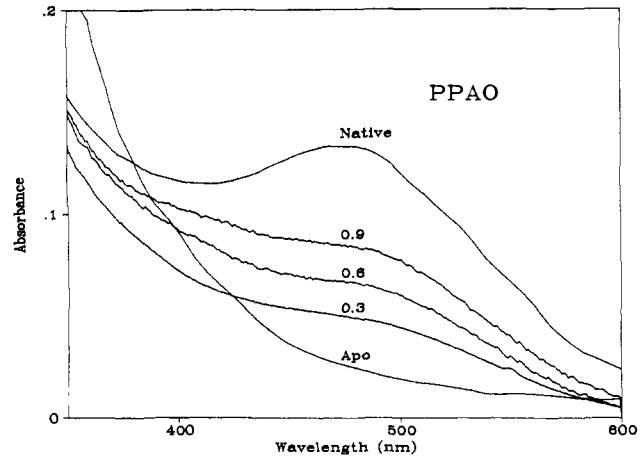

Figure 4. Visible absorbance spectra of PPAO at several Cu(II):protein ratios. [Protein] = 0.05 mM in 50 mM KPO_4 (pH 7.0). Note: The actual copper content of the apo sample is 3% (0.06:1 = Cu(II):protein). NMRD samples (in Pipes buffer) were diluted with phosphate buffer for these measurements.

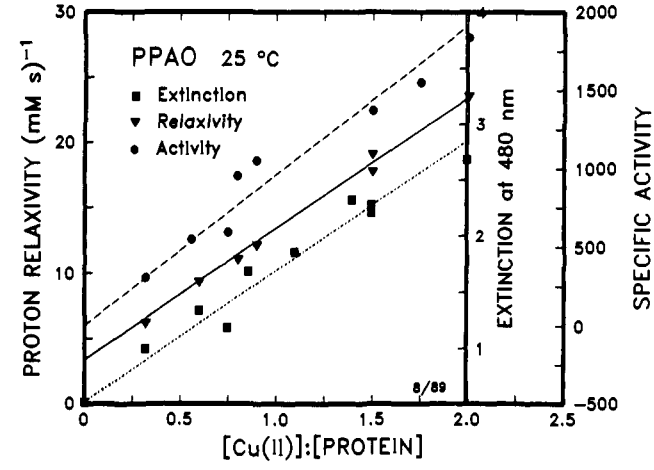

Figure 5. Visible absorbance at 480 nm (■) total relaxivity at 2 MHz (▼), and specific activity (●) of PPAO as a function of Cu(II):protein ratio. Note that the absorbance spectra and the relaxivity were measured at 25 °C, but the activity was measured 37 °C. The units of specific activity are those described in ref 35. Extinction at 480 nm is reported (mM^{-1} cm^{-1}).

much higher at 25 °C but comparable to those of BPAO and PKAO at 5 °C. The NMRD profile of the substrate-reduced (E_{red}, eq 2) form of BPAO is identical with that of the resting enzyme.

Effects of Azide Binding. Azide is an effective inhibitor of copper-containing amine oxidases and is known to coordinate equatorially to Cu(II) in these enzymes, with a dissociation constant ≅20 mM, displacing water or hydroxide.[3,21-28] The effects of azide on the 5 °C relaxivities of the native BPAO, PPAO, and PKAO are summarized in Table I. At moderate concentrations (less than 100 mM), azide has very little effect on the relaxivity of PKAO and BPAO; at high concentrations, some decrease in the magnitude is apparent. (When $[N_3^-]$ = 100 mM, the enzymes are at least 80% complexed by azide.[21-27]) Hence, we conclude that azide binding to an equatorial coordination position does not significantly perturb the interaction of the Cu(II) ions of PKAO and BPAO with solvent water. Therefore, the rapidly exchanging, coordinated water molecules in these enzymes are probably not equatorial.

Azide perturbs the NMRD of PPAO to a greater extent than that of the other amine oxidases examined, as indicated in Table I. This result is consistent with a previous report, which suggested that, at 25 °C, azide could displace at most approximately 50% of the coordinated water that contributed to solvent relaxation via rapid exchange.[27] Our NMRD measurements were made at 5 °C, where previous results indicate that the binding of anions is stronger,[3] but where, as noted below, the contribution from at least one of the exchangeable waters is much reduced. When temperature effects are taken into account, the data for PPAO in Table I are consistent with the previous results.[27]

Correlation of T_{1p}^{-1}, Activity, and Absorption with Cu(II) Content. Figure 3A displays the NMRD profiles of PPAO samples with varying Cu(II) content. Figure 3B shows the relaxivity at fixed fields as a function of the Cu(II):protein ratio. It is evident that the relaxivity is well-described as a linear function of the copper:protein stoichiometry. Clearly the two copper sites in the enzyme are not distinguishable by NMRD. Because it proved difficult to prepare a completely metal depleted sample of PPAO, the T_{1p}^{-1} profile in Figure 3A was constructed by extrapolating the relaxivity at each field (cf. Figure 3B) to zero copper.

The procedure for copper removal from amine oxidases involves prior reduction of the enzyme with dithionite. Consequently, copper removal concurrently bleaches the characteristic absorption spectrum of the native amine oxidases because the reduced form of the quinone cofactor absorbs at higher energy than does the oxidized form.[46] Copper removal also abolishes enzymatic ac-

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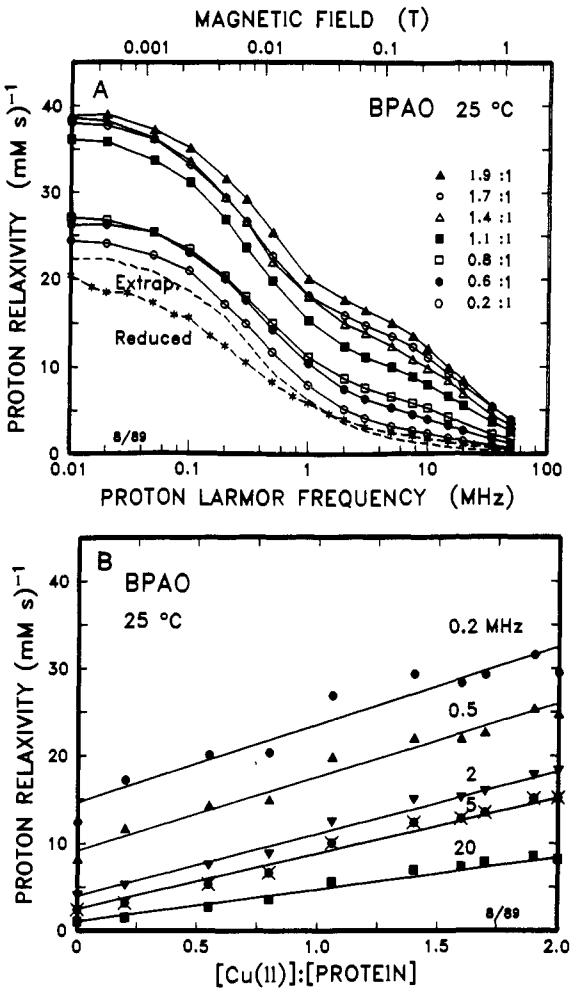

Figure 6. (A) NMRD profiles (total relaxivities) of BPAO at 25 °C for different Cu(II):protein ratios. Also shown are the NMRD profiles of reduced BPAO (*) and that derived by extrapolation of the relaxivities at each field for varying copper loadings to zero copper (---). Relaxivities are plotted per millimole per liter of protein; buffer is 50 mM KPO_4 (pH 7.0). (B) Total relaxivity of BPAO at 0.2-, 0.5-, 2-, 5-, and 20-MHz proton Larmor frequencies and 25 °C as a function of the Cu(II):protein ratio. Note: The data at 0 Cu(II):protein are from reduced BPAO.

tivity. Reconstitution of the metal-depleted protein with Cu(II) restores the absorption spectrum (Figure 4) and the activity of amine oxidases. Figure 5 summarizes the dependence of relaxivity, visible absorption spectrum, and specific activity on the Cu(II) content of PPAO. Within the precision of the data, these three quantities are linear functions of the copper:protein stoichiometry. Note that the increase in relaxivity is a measure of the interaction of protein-bound Cu(II) ions with solvent, whereas the increase in the absorbance at 480 nm reflects oxidation of the cofactor, which is ostensibly catalyzed by copper. As noted earlier, both copper and the cofactor are required for activity. Reconstitution restored full activity to PPAO.

Data analogous to Figures 3A, B and 5 are shown for BPAO in Figures 6A,B and 7. In this case, the diamagnetic contribution to the total relaxivity of the native enzyme could be determined from measurements of the reduced Cu(I) form of the native enzyme; both NMRD profiles (native and reduced) are included in Figure 6A. An apoprotein NMRD profile, obtained when the relaxivities at each field (cf. Figure 6B) were extrapolated to zero copper, is also shown in Figure 6A. Significant differences are apparent in the 0.01–0.5-MHz region between the extrapolated profile and the Cu(I) form, in part explaining the differences in the relaxivity at low fields between the native (Figure 2) and reconstituted (1.9:1) BPAO.

Indeed, variations in the low-field region of approximately this magnitude were observed among the profiles of reduced samples

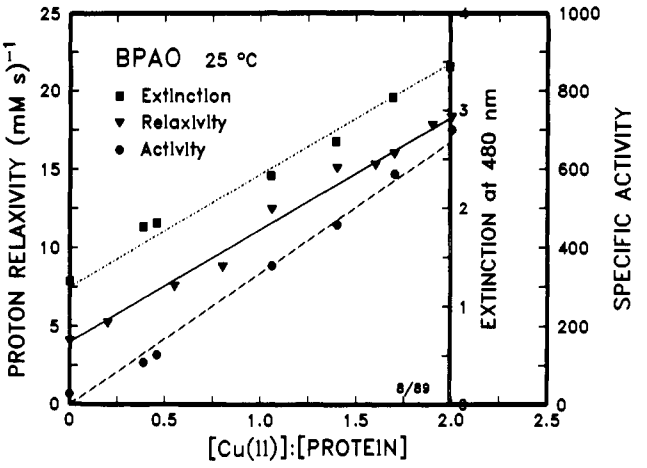

Figure 7. Visible absorbance at 480 nm (■), total relaxivity at 2 MHz (▼), and specific activity (●) of BPAO as a function of the Cu(II):protein ratio. All measurements were made at 25 °C. Extinction at 480 nm is reported (mM^{-1} cm^{-1}).

that had been partially reconstituted with copper (data not shown). This result suggests that $T_{1_d}^{-1}$ of BPAO depends on the sample history (including the exact reduction conditions) and copper content. The most likely source of the observed variation is different degrees of oligomerization. Nevertheless, the increase in relaxivity as copper binds to the metal-depleted protein, the recovery of the visible absorption band (assignable to the oxidized quinone cofactor), and the specific activity can all be described reasonably well by a linear dependence on the copper:protein stoichiometry (Figures 6B and 7). Note, however, that the specific activity of the reconstituted enzyme containing two Cu(II) ions per protein molecule is only about 70% of that of the native enzyme. Our methods for copper removal and reconstitution generally gave 70–80% recovery of specific activity, even though the absorption spectrum and copper content of the reconstituted enzyme were identical with that of the native enzyme. It is possible that the procedures used to remove copper and then reconstitute the protein produced some minor modification of the enzyme or of a fraction of the active sites. The correlation between copper content and enzyme activity is a complicated issue and may be related to the question of the number of reactive quinone groups in the enzyme as isolated.

Diamagnetic and Paramagnetic Contributions of Amine Oxidases. Figure 8A presents the diamagnetic contributions $T_{1_d}^{-1}$ to the NMRD relaxivity profiles of native amine oxidases shown in Figure 2 at 5 and 25 °C measured for the reduced Cu(I) forms of the enzymes. The dotted and solid lines represent the apo profiles at 25 °C, derived when the relaxivities of PPAO (Figure 3B) and BPAO (Figure 6B), respectively, at each field were extrapolated to zero Cu(II) content. This latter procedure was the only one used for determining $T_{1_d}^{-1}$ for PPAO, as it proved difficult to prepare the Cu(I) form of the enzyme. For BPAO, the diamagnetic NMRD profile of the enzyme with the Cu(II) ion reduced to the Cu(I) is not identical with that obtained by extrapolation of the relaxivities to zero copper content. It appears that both diamagnetic forms (apo and reduced) of BPAO self-associate considerably under the conditions of the NMRD experiments, as judged from the much higher low-field relaxivities than those measured for PPAO (Figure 8A). For the concentrations used in these experiments, the effect of protein concentration on the relaxivity in the absence of association is negligible.[45]

Figure 8B shows $T_{1_p}^{-1}$ of native amine oxidase solutions of Figure 2 at 5 and 25 °C derived by subtraction of $T_{1_d}^{-1}$ (reduced profiles for BPAO and APAO, extrapolated profile for PPAO) of Figure 8A from the total relaxivities of Figure 2. The two classes of amine oxidases, though readily distinguishable in Figure 2, can now be characterized more precisely by the differences in both the magnitude and temperature dependence of $T_{1_p}^{-1}$. Identical conclusions would be reached if the extrapolated $T_{1_d}^{-1}$ profile was used for BPAO.

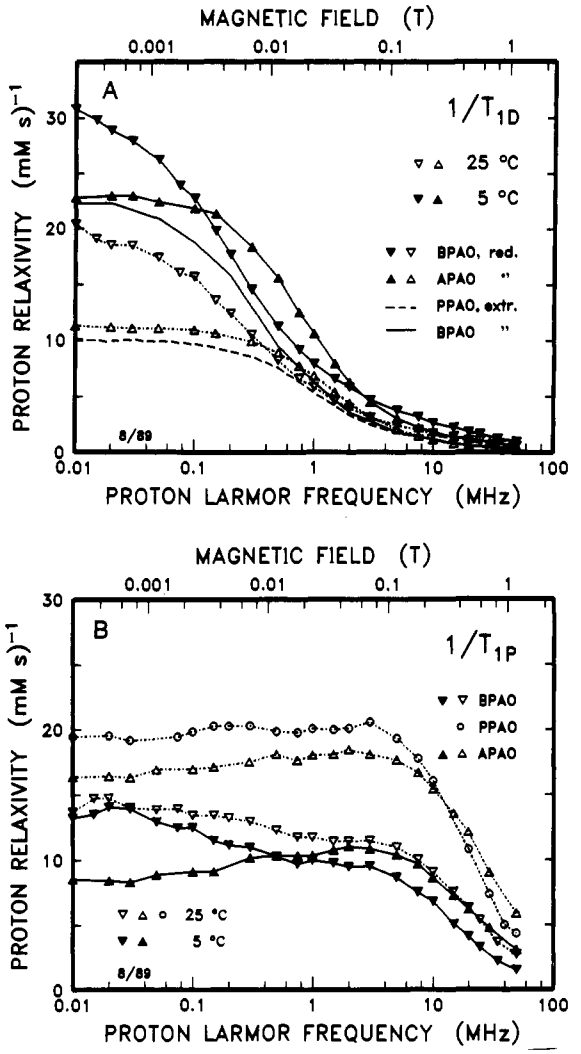

Figure 8. (A) Diamagnetic contributions T_{1d}^{-1} to the total relaxivities of amine oxidases at 5 and 25 °C per millimole per liter of protein. Key: reduced BPAO, 5°C (▼), 25 °C (▽); reduced APAO, 5 °C (▲), 25 °C (△); extrapolated PPAO, 25 °C (---); extrapolated BPAO, 25 °C (—). (B) Paramagnetic contributions T_{1p}^{-1} to the total relaxivities of amine oxidases at 5 and 25 °C per millimole per liter of protein. Key: BPAO, 5 °C (▼), 25 °C (▽); PPAO, 25 °C (○); APAO, 5 °C (▲), 25 °C, (△). Data were derived by subtraction of the diamagnetic contributions T_{1d}^{-1} of Figure 10 from the total relaxivities of Figure 2. Where available, data of reduced native enzymes were used for T_{1d}^{-1}.

At 25 °C, T_{1p}^{-1} of PPAO and APAO is about twice as large as that of BPAO, suggesting perhaps that the former may result from two coordinated water molecules in fast exchange, while the latter has only one such water ligand. At 5 °C, T_{1p}^{-1} of both classes of amine oxidases behaves approximately the same and would correspond to only one fast-exchanging water molecule. However, a difference of 2 in magnitude of T_{1p}^{-1} could also result from other factors, as considered in the Discussion.

Figure 9 compares the paramagnetic contribution T_{1p}^{-1} of amine oxidases and other Cu(II)-containing proteins (SOD[47,48] and Cu transferrin).[30]

Discussion

The NMRD profiles for amine oxidases from bovine plasma and porcine kidney, on the one hand, and porcine plasma and bacterial *Arthrobacter* P1, on the other, establish that the interactions of the Cu(II) sites with solvent water fall into two

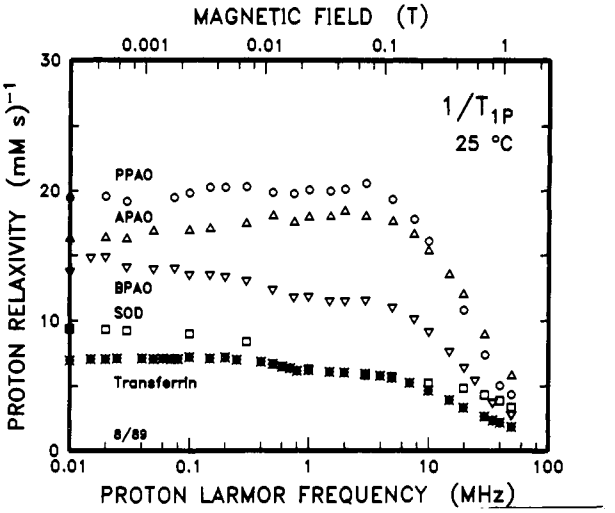

Figure 9. Comparison of the paramagnetic contributions T_{1p}^{-1} of PPAO, APAO, and BPAO to that of Cu_2Zn_2SOD (from ref 48) and Cu transferrin (from ref 52) at 25 °C per millimole per liter of protein.

distinct classes, on the basis of the magnitude of the relaxivity (particularly at high fields), its temperature dependence, and the effects of exogenous ligands. Indeed, these results are (to our knowledge) the first to document definitively significant variations in the reactivity of the copper sites among amine oxidases. As will be seen, this result is not necessarily inconsistent with a large amount of data, which suggest that the copper site structure in amine oxidases has been highly conserved. The coordination chemistry of Cu(II) in amine oxidases has been investigated extensively.[3] Comparable ligand substitution reactivities are observed, which may be understood as reflecting the presence of an equatorially coordinated water (or possibly OH^-) that acts as the leaving group in a ligand-substitution reaction. Given the insensitivity of the NMRD profiles of BPAO and PKAO to ligand substitution, if the equatorial group is water, it must exchange too slowly to contribute to T_{1p}^{-1}. Alternatively, the equatorial ligand might be hydroxide.[10] Therefore, another water ligand must also be present, one that exchanges rapidly on the NMRD time scale and is not displaced by anions. The number, or type, of solvent-derived ligands and the associated solvent-exchange dynamics must be different in PPAO and APAO, compared with BPAO and PKAO. Barker et al.[27] suggested that exchange of equatorial and axial water contributed equally to solvent relaxation in PPAO and that azide (or other exogenous ligands) displaced only the equatorial water. This picture is consistent with our results if the equatorial water is taken to be a water molecule with a τ_M such that its contribution to T_{1p}^{-1} has a large activation energy (>10 kcal/mol) so that its contribution is seen at 25 but not at 5 °C.

When the several profiles in Figure 9 are compared, more can be said from a qualitative analysis of the phenomenology than from an examination of the values of the parameters derived from a fit to a theory, which—for all its numerical complexity—remains oversimplified. Additionally, the limited reproducibility of the data in the low-field region makes a fit premature. Cu(II) transferrin (with HCO_3^-) is though to have an OH^- ligand on the Cu(II), with a second-sphere hydrogen-bonded water in rapid exchange with solvent.[30] A single proton of this water is at a distance from the Cu(II) ion comparable to that of the two protons of the axial waters of the Cu(II) ions of SOD.[47-49] The latter have been shown to be well-described by theory, with an axial water in the standard geometry (3.4–3.6 Å between the paramagnetic center and proton).[31,48]

The BPAO and SOD data are also similar; the larger magnitude of the former correlates with the lower field at which the BPAO profile begins to decrease compared with that of SOD. A

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(48) Bertini, I.; Banci, L.; Brown, R. D., III; Koenig, S. H.; Luchinat, C. *Inorg. Chem.* **1988**, *27*, 951-953.
(49) Tainer, J. A.; Getzoff, E. D.; Beem, K. M.; Richardson, J. S.; Richardson, D. C. *J. Mol. Biol.* **1982**, *160*, 181-217.

quantitative fit of theory to both profiles would be consistent with a single axial water molecule in the same geometry in both BPAO and SOD, with either the water of the BPAO exchanging more slowly or the relaxation time of its Cu(II) ions somewhat longer. From Figure 8B, the profiles of BPAO and APAO are similar at 5 °C, indicating that both amine oxidase classes have analogous axial waters. What is different is that both PPAO and APAO (Figure 8B) have another contribution to the relaxivity at 25 °C that is absent at 5 °C in these proteins and absent at all temperatures in the other class of amine oxidases. We suggest that this additional contribution arises from an equatorial water with a relatively large activation energy for exchange (~10 kcal/mol) and $\tau_M > 10$ μs at 5 °C.

Pulsed EPR (electron spin echo envelope modulation, ESEEM) studies have provided independent and direct evidence for coordinated water (or OH^-) in amine oxidase Cu(II) sites.[11] In addition, azide and cyanide were shown to displace an equatorial water (or OH^-) in BPAO and PKAO; however, other Cu(II)–water proton (deuteron) interactions persist in the anion complexes.[11] Analysis of the residual deuteron ESEEM suggests that axial D_2O (H_2O) remains coordinated in the anion complexes. In the ESEEM context, an axial water ligand is defined as one for which the interactions of the protons with the unpaired electron on the Cu(II) ion are weak, owing to the longer distance and smaller contact contribution to the hyperfine coupling, i.e., off the plane of the electronic wave function. The existence of such a water ligand is also suggested by the relatively minor effects that anions have on the NMRD profiles of BPAO and PKAO: If solvent relaxation is dominated by exchange of axially coordinated water, which is not displaced by exogenous ligands, then the NMRD profile may not be greatly affected by equatorial ligand substitution. Should exogenous ligands displace the axial water, the NMRD profile is expected to change significantly. Thus, the NMRD data, when considered together with the ligand-substitution chemistry and the ESEEM results, indicate that the Cu(II) ions in amine oxidases contain both equatorially and "axially" coordinated water, as idealized in Figure 1, with the equatorial waters being more labile in the PPAO and APAO class of proteins.

ESEEM data on the one-copper derivative of BPAO suggest that phenylhydrazine binding causes the displacement of second coordination shell or ambient water.[50] Similar results have been obtained on the benzylamine-reduced enzyme.[51] Yet benzylamine has no effect on the NMRD profile of substrate-reduced BPAO under anaerobic conditions (data not shown), even though ≥90% of the copper remains Cu(II). We conclude that, whatever the basis for the effects of substrates or carbonyl reagents upon the Cu(II) sites in BPAO, the solvent-exchange rates and accessibility of the Cu(II) ions are not affected. Barker et al. previously observed that amphetamine sulfate, a competitive inhibitor against benzylamine binding to PPAO, had no effect on $T_{1_p}^{-1}$ of this enzyme.[27]

The relationships among the copper content, the degree of equivalence of the two copper sites, and the activities of amine oxidases have been a matter for some debate. Let us first consider the situation with regard to copper content and activity. A report[17,33] that a fully active, one-copper derivative of BPAO could be prepared has not proved reproducible.[50] All recent correlations between copper content and activity, including our results, show that only preparations with a copper:protein stoichiometry of 2:1 display the catalytic activity of the homogenous, freshly isolated enzyme. This result does not necessarily require equivalent copper sites, nor does it imply that the copper ions have identical functions, or even that both are involved in the enzyme reaction. The properties of the active site in PPAO can be discussed in this context. Simulations of the Q-band EPR spectrum consistently indicate that the two Cu(II) ions in the resting enzyme are inequivalent.[7,27] One site is axial at 35 GHz, but the other appears rhombic. When copper is added to the metal-depleted protein, the Cu(II) ions are equally distributed between the two sites at all copper:protein stoichiometries.[7] However, the two copper sites in PPAO cannot be distinghished by NMRD measurements. Knowles and co-workers have recently provided evidence that PPAO does contain two quinones per protein molecule but that only one reacts rapidly with carbonyl reagents and (presumably) substrates.[7] This reactivity difference may represent an extreme case of half-of-the-sites reactivity, or there may be a fundamental structural asymmetry in the active sites such that one site is unreactive. It is possible that one active site is modified during purification; if so, the changes in the affected active site would have to be very reproducible from one preparation to another. Further purification of PPAO by HPLC[8] produces only small (<10%) increases in the specific activity, suggesting that the enzyme does not contain significant amounts of inactivated protein.[51b] In our view, the most plausible interpretation of current data on PPAO is as follows: Either the two copper sites in PPAO have equal affinities for Cu(II), or alternatively copper binding by the apoprotein could be highly cooperative, with metal–ion binding an "all-or-none" process. The enzyme probably also contains two quinone molecules, only one of which reacts readily in enzyme samples purified by the standard procedures.[1,2,7] Remarkably, aerobic incubation of copper-depleted forms of PPAO for 7 days (at room temperature) does not lead to complete reoxidation of the quinone. Clearly the reduced cofactor, in the absence of its associated copper, is very resistant to aerial oxidation. When copper binds to either site in the apoprotein, the quinone at that site is oxidized. Hence, both the oxidized-quinone absorption and enzymatic activity would correlate directly with copper content; full activity would be restored when the native copper content is achieved.

There are reports in the literature that copper ions in the two sites of other amine oxidases display intrinsic differences in either their spectroscopic properties or their reactivity. For example, carbonyl reagents are claimed to perturb the EPR spectrum of only 50% of the copper in BPAO,[34] despite the fact that a single Cu(II) spectrum is seen in the resting enzyme with 35-GHz EPR[7] and that the ESEEM spectra of the native and one-copper forms of the bovine plasma enzyme are identical (within the signal to noise ratio).[11,50] Recent work on the quinone cofactor content of BPAO may help resolve this inconsistency. Klinman and co-workers have provided compelling evidence that the fully active enzyme contains two quinones per protein molecule;[8] stoichiometries less than 2 indicate that partially inactivated enzyme is present in the preparation. Results with the carbonyl reagent 2-hydrazinopyridine are also consistent with two reactive carbonyl groups (i.e., one per copper site) in the *native* BPAO.[14] Hence, one possible explanation for apparent differences in the behavior of the copper ions in certain preparations of BPAO is that the coppers are signaling differences in the reactivity of the quinones, it being well-established that Cu(II) ions are perturbed by reaction of carbonyl reagents.[1-3]

Our results do not support models that invoke nonlinear relationships between copper content and other key properties of amine oxidases. One should be aware that these experiments are not particularly straightforward to perform. Metal-depleted amine oxidases efficiently scavenge adventitious copper, and there are sizable variations among amine oxidases in the rates at which the properties of the native enzyme are recovered following reconstitution. It is clear, nonetheless, that the correlation between copper content and activity that we observe is inconsistent with a previous report that BPAO containing 1 equiv of copper displays essentially no activity.[20] Note that the procedures used here for metal removal and reconstitution are not identical with those used by these workers,[20] although they are similar. Variations in the percentage of active quinone cofactor in the freshly isolated enzymes may also complicate comparisons between experiments done in different laboratories. Samples containing between 0 and 2

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(51) (a) McCracken, J.; Peisach, J.; Dooley, D. M. Unpublished observations. (b) Knowles, P. F. Unpublished observations.
(52) Koenig, S. H.; Brown, R. D., III. In *The Coordination Chemistry of Metalloenzymes*; Bertini, I.; Drago, R. S., Luchinat, C., Eds.; Reidel: Dordrecht, 1983; pp 19–33, Figure 2.

equiv of copper display reproducible activities under our conditions. Moreover, the relaxivity and absorbance are also well-described as linear functions of the copper content. Collectively, our data on BPAO are consistently interpreted in terms of equivalent copper sites that either bind copper independently or bind it in a highly cooperative fashion.[8] If recent determinations[8,14] are correct, each copper is associated with a quinone.

Finally, close examination of the apoprotein NMRD profiles in Figure 8A suggests that metal-depleted BPAO and PPAO are not monomeric under the conditions of the NMRD experiments. Molecular weight estimates from the data and Figure 2 of ref 44 are as follows: porcine enzyme, 500 000 Da; bovine enzyme, 900 000 Da. Assuming a molecular weight of 190 000 for the native, monomeric enzymes,[1,2] the NMRD values correspond to oligomers of two or three monomers for the porcine enzyme and of about five monomers for the bovine enzyme. Although there is considerable uncertainty in these estimates, the NMRD data strongly indicate that at least the copper-depleted enzymes associate under conditions that are similar to those used for many spectroscopic experiments, i.e., protein concentrations of a few tenths of a millimole per liter. The oligomerization is probably concentration-dependent, as analytical ultracentrifugation studies on oxidized PPAO,[27] dithionite-reduced PPAO,[51b] and oxidized BPAO[53] at protein concentrations <0.05 mM revealed no evidence of a high molecular weight species.[54] We emphasize that the reconstitution of BPAO and PPAO with copper was carried out at low protein concentrations, i.e., under conditions where protein association should not be a factor. The NMRD results suggest that dilute protein concentrations may be preferable for the reconstitution of metal-depleted metalloenzymes; this is certainly the case for the plasma amine oxidases. Given the high paramagnetic relaxivities observed for the resting enzymes, self-association of the resting amine oxidases, if it occurs, does not appear to decrease the solvent accessibility of the Cu(II) sites.

Summary

NMRD profiles, considered together with previous studies of the copper site structure and reactivity of amine oxidases, provide strong support for multiple water ligands to Cu(II) in these enzymes. However, amine oxidases may differ in the rates of exchange of the coordinated water molecules with solvent water. A single rapidly exchanging water molecule mediates the paramagnetic contribution to solvent relaxation by BPAO and PKAO, and anions that are known to displace equatorially coordinated water from the Cu(II) ions produce only minor effects on the NMRD profiles of these enzymes. Hence, the rapidly exchanging water ligands in BPAO and PKAO are probably not equatorial; the equatorial waters must exchange slowly. The enzymes from porcine plasma and *Arthrobacter* P1 display an additional relaxation process at 25 °C, which we suggest involves rapid exchange of an equatorial water ligand; this additional relaxation process is much less evident at 5 °C, suggesting a relatively large activation energy. The NMRD profiles of BPAO, PPAO, PKAO, and APAO are quantitatively very similar at low temperature (5 °C), suggesting that the Cu(II) sites in all these enzymes contain a nonequatorial water ligand and an additional, solvent-derived ligand (either H_2O or OH^-) that is equatorial. The proteins divide into two classes characterized by the rate of exchange of this equatorial ligand. This conclusion is consistent with previous suggestions[3,9] that the Cu(II) site structure in amine oxidases has been conserved.

The relaxivity, absorption spectrum, and specific activity are linearly correlated with the copper content of both BPAO and PPAO. Our data on the porcine enzyme can be readily interpreted in terms of nearly equivalent, independent copper sites, each associated with a quinone molecule, but equally well in terms of cooperative Cu(II) binding to inequivalent sites. Either PPAO displays pronounced negative cooperativity in its reactions with substrates or carbonyl reagents or one of the quinones is simply unreactive. In contrast, the data on BPAO are fully consistent with a model involving equivalent, independent active sites containing one quinone and one Cu(II) ion in the resting state. Our data do not support previous suggestions that the copper sites in BPAO are distinguishable with regard to structure or function. One possible source for the reported variations in the properties of the bovine enzyme may be the recently discovered heterogeneity in the content of the reactive quinone.[8]

Finally, the NMRD profiles indicate that metal-depleted forms of BPAO and PPAO oligomerize under the conditions of the NMRD experiments.

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Registry No. AO, 9059-11-4; Cu, 7440-50-8; methylamine oxidase, 80891-30-1; azide, 14343-69-2.

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(54) Light-scattering measurements also show no evidence for oligomerization of resting BPAO at these concentrations.

Polymerization Mechanisms of Propellanes

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Abstract: The polymerization mechanism of [1.1.1]propellanes is studied with the concept of atomic valence numbers. SINDO1 calculations were performed on the dimerization of [1.1.1]propellane and its reaction with donor- and acceptor-substituted ethylenes. An analysis of the configuration interaction (CI) wave functions in terms of newly developed partitioning scheme allows one to classify the reactivity in diradical and zwitterionic mechanisms. An explanation is given for the copolymerization of acceptor-substituted olefins with propellanes that is in agreement with recent experimental results.

Introduction

Several years ago, we described a concept of valence with atomic, bond, and molecular valence numbers on the self-consistent field (SCF)[1] and configuration interaction (CI)[2] level. This concept was used to define diradicals and zwitterions.[3] A diradical was defined as a species where two atoms have atomic valence numbers reduced by 1 unit compared with the standard values of the atoms. These standard values are 4 for carbon, 3 for

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