On the mechanistic level, however, the differences lie in the rate at which processes occur rather than in the nature of mechanistic steps. The mechanism of the accelerated hydrolysis of polyphosphates in the presence of either platinum(II) or cobalt(III) can be described by three steps: (1) coordination of a metal ion to the polyphosphate to reduce the nucleophilicity of the phosphate ligand; (2) coordination of a second metal ion having cis coordination positions available; (3) hydrolytic attack by the cis hydroxide ion on the polyphosphate chain. The strength of binding in the first step determines the stoichiometry necessary for the accelerated hydrolysis to be observed. Apparently the hard cobalt(III) forms a tight complex with the polyphosphate so that an excess concentration of metal ion must be present before step 2 can occur. The softer platinum(II) forms a weaker complex, leaving metal ions available for step 2 even in the presence of excess phosphate. The weaker binding also accounts for the possibility of Scheme II, in which aquation must occur before hydrolysis. The slower aquation in the case of cobalt(III) for a complex such as IX prevents bidentate pyrophosphate from being rapidly hydrolyzed by the presence of a second metal ion.

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Registry No. *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, 20115-64-4; $Pt(NH_3)_2H_2P_2O_7$, 98736-88-0; $H_4P_2O_7$, 2466-09-3; $H_5P_3O_{10}$, 10380-08-2.

Supplementary Material Available: Figure 4, ambient-temperature (≈25 °C) 36.3-MHz phosphorus-31 NMR spectrum of blue product isolated from the reaction of triphosphate ion with *cis*-diaquodiammineplatinum(II) at pH 3.0 (1 page). Ordering information is given on any current masthead page.

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Copper(II) Coordination Chemistry in Bovine Plasma Amine Oxidase: Azide and Thiocyanate Binding

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Azide and thiocyanate binding to the Cu(II) sites in bovine plasma amine oxidase have been investigated by absorption, CD, and EPR spectroscopy. Anion complexes of the resting enzyme display characteristic absorption bands: N_3^-, λ = 385 nm ($\Delta\epsilon$ = 3200 M^{-1} cm^{-1}); SCN^-, λ = 365 nm ($\Delta\epsilon$ = 2400 M^{-1} cm^{-1}). The energies and intensities of these bands are consistent with N_3^-/SCN^- → Cu(II) ligand-to-metal charge-transfer assignments, where the anions are coordinated within the equatorial plane in a tetragonal Cu(II) complex. EPR data support a tetragonal structure for the Cu(II)–anion complex and further indicate that the Cu(II) electronic ground state is not greatly perturbed by anion binding. Azide binding produces CD bands at 700 (d–d), 400 (LMCT), and 480 nm; this last result suggests that the absorption and CD bands displayed by the resting enzyme in the 400–500-nm range are due in part to Cu(II) electronic transitions. Azide and thiocyanate also bind to the substrate-reduced enzyme, producing LMCT transitions that are quite similar to those observed for anion complexes of the resting enzyme. Although simple titration curves are not obtained for anion binding in phosphate buffers, it is clear that the substrate-reduced amine oxidase has a lower anion affinity than the resting form. Taken together, the data establish that anion binding is a Cu(II) ligand-substitution reaction that does not lead to major structural changes in the Cu(II) site. Beef plasma amine oxidase is inhibited by N_3^- and SCN^- but not by F^-, Cl^-, or I^-. When O_2 is present in saturating concentrations, the anion inhibition pattern changes from mixed to uncompetitive, as the amine concentration increases. Thus anion binding to both the resting enzyme and at least one other enzyme form (probably a reduced species) decreases substrate oxidation rates.

Elucidating the active-site structure and mechanism of copper-containing amine oxidases is of considerable interest. These enzymes catalyze the two-electron oxidative deamination of primary amines to aldehydes.[1-4] Dioxygen serves as the electron acceptor, being reduced to hydrogen peroxide. The overall reaction is

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$$

A wide variety of mono-, di-, and polyamines can serve as substrates, depending on the enzyme source, e.g. fungi, plants, and mammals.[1-4] Copper-containing amine oxidases contain two Cu(II) ions per enzyme molecule, which is composed of two noncovalently bound subunits with a total molecular weight of ~180000. In addition, these enzymes contain another cofactor that reacts with carbonyl reagents and is reduced by substrates.[5] Copper is generally thought to participate in the reoxidation of the substrate-reduced enzyme.[1-4] No Cu(I) intermediates were detected by rapid-freeze EPR experiments during the reoxidation of benzylamine-reduced pig plasma amine oxidase.[6] This result, together with other data, led Knowles and co-workers to propose that Cu(II)-coordinated hydroxide acts as a nucleophile to assist in hydride transfer to O_2.[7] On the other hand, some results suggest that a Cu(II)/Cu(I) redox cycle is possible.[4,8] There are reasonable circumstances whereby a catalytically competent Cu(I) enzyme form would not have been detected under the conditions of the EPR experiment. Further complicating this picture are NMR relaxation results obtained with pig kidney diamine oxidase, which indicated that neither the substrate amino groups nor NH_3 binds near Cu(II).[9] Thus the exact mechanistic role of copper in amine oxidases is still somewhat obscure, and it has even been suggested that copper is not directly involved in catalysis.[9] Some structural information on the copper site is available. On the basis of EPR g values and the energies of the d–d bands, amine oxidase

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Cu(II) is tetragonal with primarily nitrogen/oxygen ligands.[1-4,10] H_2O is coordinated in an equatorial position and may also occupy an axial position.[7] Various EPR measurements are consistent with at least one or two coordinated nitrogen(s).[8,10-12] Recent EXAFS results suggest that three to four "imidazole-like" ligands are coordinated.[13] EPR and NMR relaxation data also indicate that the two Cu(II) ions in certain amine oxidases are magnetically inequivalent and may have somewhat different environments.[7,14]

It is now well established that investigating the effects of potential Cu(II) ligands on the spectroscopic and catalytic/functional properties of copper proteins can be very useful in evaluating possible roles for copper and in elucidating the active-site structure.[15-22] Previous work on the pig plasma and pig kidney amine oxidases established that anionic entering groups (i.e. N_3^-, SCN^-, CN^-) substitute equatorially and were effective enzyme inhibitors.[7,20,21] Hence the study of anion binding to amine oxidases can provide both structural and mechanistic information. Further, by monitoring any variations in the Cu(II) ligand-substitution chemistry in response to perturbations of the enzyme, one can assess protein influences on the intrinsic reactivity of the Cu(II) ion. Finally, the results from such experiments constitute a useful basis for comparing amine oxidases isolated from various sources. Although copper-containing amine oxidases have several structural and mechanistic properties in common, substantive differences have also been reported. The question of active site variability in these enzymes should be thoroughly investigated. Results from spectroscopic and steady-state kinetics experiments on beef plasma amine oxidase are presented in this paper; comparisons are also made to earlier results on pig kidney diamine oxidase and pig plasma amine oxidase. Some of the work has been briefly summarized previously.[21]

Experimental Section

Beef plasma amine oxidase was purified by a published method.[23] Enzyme preparations used in these experiments were homogeneous as judged by SDS gel electrophoresis with protein staining and displayed specific activities comparable to the best values in the literature. Benzylamine was obtained from Aldrich and distilled prior to use. *p*-Hydroxybenzylamine was synthesized as previously described[24] and recrystallized at frequent intervals. All other chemicals were the highest grade commercially available. Enzyme activity was assayed with either benzylamine[25] or *p*-hydroxybenzylamine.[24] One unit is defined as an absorbance change of 0.001 min^{-1} at 250 or 330 nm, respectively. The extinction coefficients are 12 500 M^{-1} cm^{-1} for benzaldehyde and 25 000 M^{-1} cm^{-1} for p-hydroxybenzaldehyde. Steady-state kinetics experiments were carried out with *p*-hydroxybenzylamine under the standard assay conditions because azide and thiocyanate absorb in the UV region. Kinetics results were analyzed in several ways, including Wilkinson's statistical treatment,[26] to insure that a consistent inhibition pattern emerged. Titrations were carried out as previously described.[20] For titrations with the substrate-reduced amine oxidase, the enzyme was first

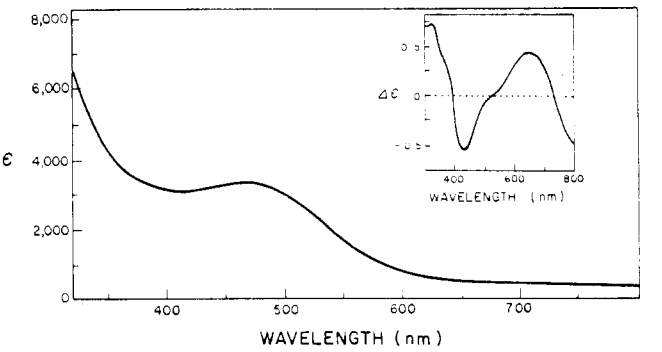

Figure 1. Absorption and CD spectra of resting beef plasma amine oxidase.

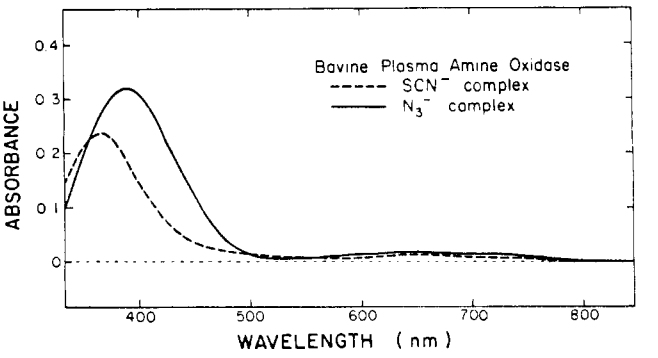

Figure 2. Difference absorption spectra of 0.05 mM beef plasma amine oxidase anion complexes.

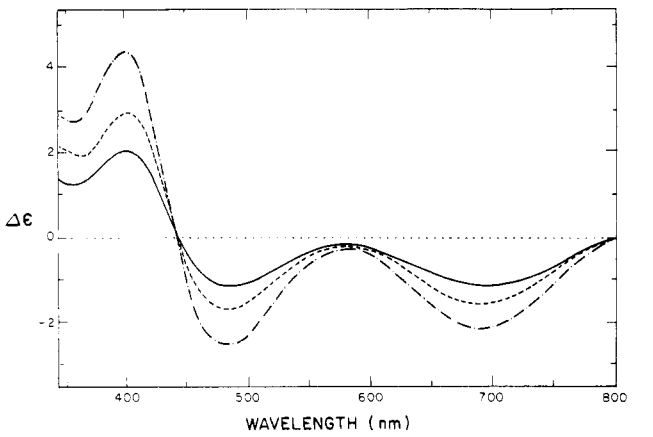

Figure 3. CD spectra obtained by digitally subtracting the resting amine oxidase spectrum from spectra of the enzyme in the presence of azide at various concentrations. Azide concentrations were (—) 25 mM, (--) 50 mM, and (···) 185 mM.

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made anaerobic by several cycles of gentle pumping and flushing with nitrogen in a serum-stoppered cuvette. Two–four equivalents of an oxygen-free benzylamine solution were then added by a gastight syringe. Enzyme reduction was monitored by following the bleaching of the 480-nm band. Reduction was considered complete when no further absorbance decreases were observed upon further benzylamine additions and the final spectrum was stable. Anion solutions were purged for several hours by argon and aliquots added via gastight syringe. All spectra were obtained in 0.1 M potassium phosphate buffer (pH 7.0 or 7.2) at room temperature unless otherwise noted. NaCl or KCl was used to maintain constant ionic strength within a series of rate measurements or during titrations. Extinction coefficients are quoted per mole of copper. All absorption spectral measurements were made with a Cary 219 spectrophotometer interfaced to an Apple II computer. CD spectra were obtained with a modified JASCO J-40A instrument fitted with a photoelastic modulator. Photomultiplier output is fed into a PAR lock-in amplifier set at the modulator frequency and then to a Bascom-Turner digital recorder. Base line subtraction and data processing were done digitally with the recorder. A Varian E-109 instrument was used to measure EPR spectra at low temperatures, utilizing an Air Products Heli-Trans system. Frequencies were measured with a PRD Electronics, Inc., frequency meter.

Results

Absorption and CD spectra of resting beef plasma amine oxidase are shown in Figure 1. These spectra are in good agreement with those published previously.[10,27,28] The principal absorption band at ~480 nm and the CD band at 425 nm are principally associated with the organic cofactor. CD bands at 660 and 800 nm have been assigned as Cu(II) ligand field transitions.[10] Azide or thiocyanate addition to the resting enzyme produces the difference absorption spectra shown in Figure 2. Intense new bands are evident at 385 ($\Delta\epsilon$ = 3200 M^{-1} cm^{-1}) and 365 nm ($\Delta\epsilon$ = 2400 M^{-1} cm^{-1}), which are characteristic of the azide and thiocyanate complexes of resting beef plasma amine oxidase, respectively. Such energies and intensities suggest $N_3^- \rightarrow$ Cu(II) and $SCN^- \rightarrow$ Cu(II) LMCT assignments for these bands. Theoretical considerations and comparisons to model compounds are consistent with equatorial coordination by the anions. Changes in the 600–800-nm region are slightly more pronounced upon N_3^- coordination compared to SCN^- coordination, indicating that the Cu(II) ligand field is perturbed more in the former case, as might be expected from the positions of these anions in the spectrochemical series relative to H_2O.[29] Anion binding also produces characteristic changes in the CD spectra (Figure 3). The positive CD band at 400 nm can be assigned to the $N_3^- \rightarrow$ Cu(II) charge-transfer transition. Very similar CD spectra are observed upon SCN^- binding except that the $SCN^- \rightarrow$ Cu(II) charge-transfer transition is at 360 nm. In keeping with the absorption spectral results, azide binding perturbs that part of the resting enzyme's CD spectrum associated with Cu(II) d–d transitions (~700 nm) to a greater extent than does thiocyanate binding. In addition, an intense new CD band is evident at 480 nm in the spectrum of the azide complex (Figure 3), and an analogous feature is observed near 500 nm upon SCN^- binding as well. Since these bands correlate with all the other spectroscopic indicators for anion coordination to Cu(II), they are logically assigned as Cu(II) electronic transitions. No spectral changes were observed upon I^-, Cl^-, or F^- addition to the resting enzyme. Complex titration curves were obtained with both N_3^- and SCN^- in 0.1 M potassium phosphate (pH 7.0) buffer. It is not possible to characterize the binding of either anion to the resting enzyme by a single equilibrium constant. Enzyme–phosphate interactions may be at least partially responsible for complex anion binding behavior since very clean titration curves are obtained in 5 mM Pipes, 0.1 M NaCl buffer (pH 7.0).[21b] In this context we note that amine oxidase activity has been reported to be sensitive to phosphate concentration.[27] Rough estimates (±50%) of the equilibrium constants for enzyme–anion complex formation can be obtained by monitoring the charge-transfer bands[17c] and are including in Table I along with spectroscopic data.

Table I

enzyme	N_3^- complex: λ_{max}, nm ($\Delta\epsilon$, M^{-1} cm^{-1})	K, M^{-1}	SCN^- complex: λ_{max}, nm ($\Delta\epsilon$, M^{-1} cm^{-1})	K
beef plasma amine oxidase (resting)	385 (3200)	42	365 (2400)	25
		3		1
beef plasma amine oxidase (reduced)	390 (4000)	50	360 (3000)	5
		<1		<1
pig kidney diamine oxidase[a]	410 (6300)	130	365 (3000)	55
pig plasma amine oxidase[b]	390 (5600)	40		

[a] From ref 20. [b] From ref 22.

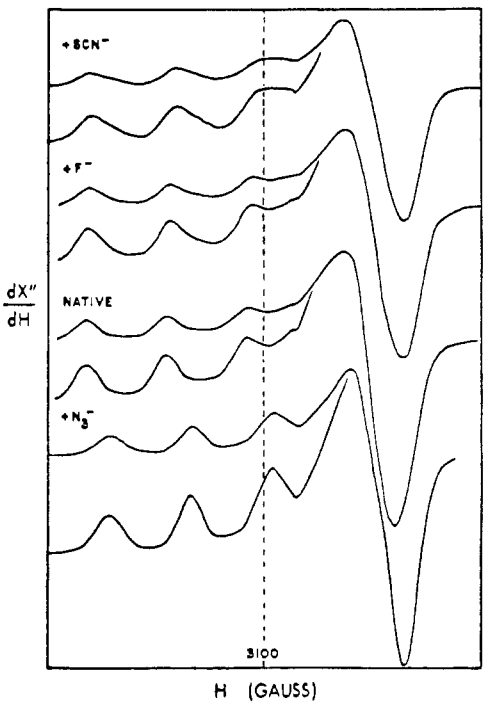

Figure 4. EPR spectra of beef plasma amine oxidase derivatives at 40 K. Microwave power was 2 mW, and the modulation amplitude was 10 G.

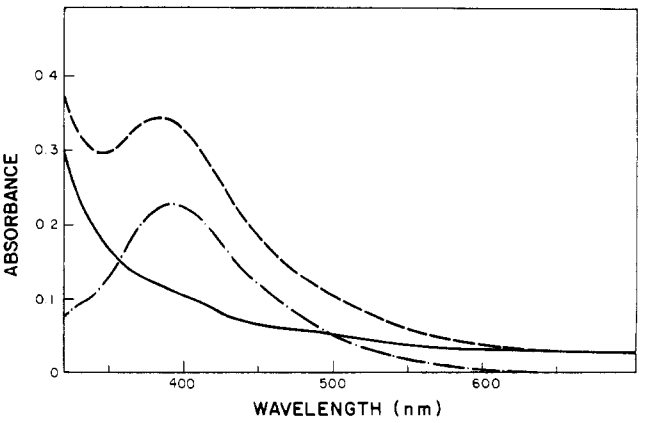

Figure 5. Azide binding to ≃0.03 mM substrate-reduced beef plasma amine oxidase (E_{red}): spectrum of E_{red} (–); spectrum of E_{red} + 600 mM N_3^- (--); difference spectrum (---).

Figure 4 displays the EPR spectra of resting beef plasma amine oxidase and its azide and thiocyanate complexes. Parameters estimated from these spectra are as follows: resting enzyme, $g_\perp$ = 2.060, $g_\parallel$ = 2.28, $A_\parallel$ = 155 G; thiocyanate complex, $g_\perp$ = 2.055, $g_\parallel$ = 2.255, $A_\parallel$ = 170 G; azide complex, $g_\perp$ = 2.050, $g_\parallel$ = 2.240,

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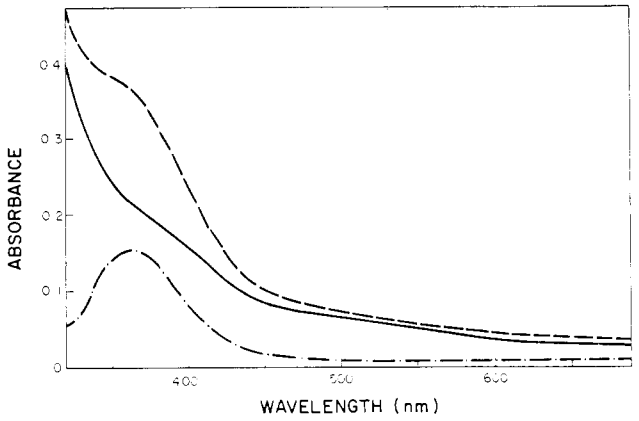

Figure 6. Thiocyanate binding to ≃0.04 mM substrate-reduced beef plasma amine oxidase (E_{red}): spectrum of E_{red} (—); spectrum of E_{red} + 700 mM SCN– (--); difference spectrum (···).

$A_{\parallel}$ = 160 G. The spectrum in the presence of 0.10 M fluoride (Figure 5) is identical with that of the resting enzyme. Although the g and A values are not based on simulations that allow for possible inequivalency between the two copper sites, the trend in g values (resting > SCN^- complex > N_3^- complex) is unambiguous. Similar changes have been previously observed in studies on azide and cyanide binding to the pig plasma amine oxidase.[7]

We have also examined anion binding to the substrate-reduced enzyme. Although the Cu(II) EPR spectrum is slightly perturbed by substrate reduction,[30] Cu(II) is not appreciably reduced to Cu(I). Under anaerobic conditions the substrate-reduced amine oxidase is stable; absorption spectral changes produced by azide and thiocyanate binding in these conditions are shown in Figures 5 and 6. The LMCT bands are clearly evident but are shifted slightly relative to those of the resting enzyme. Anion binding to the reduced enzyme in phosphate buffer is also complicated. Estimated equilibrium constants and the spectroscopic data are set out in Table I. All the characteristic spectroscopic properties of the resting enzyme were restored by dialysis following experiments with either the oxidized or substrate-reduced enzyme.

Both N_3^- and SCN^-, but not F^-, Cl^-, or I^-, are effective amine oxidase inhibitors. Steady-state kinetics experiments (O_2 saturating, amine substrate variable) of the inhibition of beef plasma amine oxidase by azide and thiocyanate in phosphate buffer are summarized in Table II. The inhibition patterns[31] were consistently observed in several independent experiments but reliable K_i (K_i') values could not be obtained owing to the complex anion-binding behavior of the amine oxidase in phosphate buffer. Both anions are mixed inhibitors at relatively low substrate concentrations but are uncompetitive inhibitors vs. *p*-hydroxybenzylamine at substrate concentrations in the 400–850 μM range. At higher *p*-hydroxybenzylamine concentrations, substrate inhibition is still apparent in the presence of the anions, a result that is inconsistent with previous suggestions that copper may be the second (inhibitory) substrate binding site.[9] Similar results are obtained with benzylamine as the substrate. Azide and thiocyanate inhibition is readily reversible via dialysis. The lack of inhibition by F^- is somewhat surprising given that it is an effective inhibitor of several other copper-containing oxidases.

Table II

enzyme	anion	inhibition	K_i (K_i'), mM
beef plasma amine oxidase	N_3^-	mixed → uncompetitive	~100
	SCN^-	mixed → uncompetitive	~150
pig kidney diamine oxidase[a]	N_3^-	mixed → uncompetitive	2.0
	SCN^-	uncompetitive	22.4
pig plasma amine oxidase[b]	N_3^-	uncompetitive	40
	SCN^-	no effect	

[a] From ref 20. [b] From ref 22.

Discussion

Tetragonal Cu(II) complexes display facile ligand-substitution chemistry.[29] Thus the rapid, reversible coordination of anions such as N_3^- and SCN^- by the Cu(II) ions in amine oxidases is quite reasonable. Furthermore it is clear that these anions are coordinated equatorially.[32,33] The EPR data are consistent with this picture. First, the $g_{\parallel} > g_{\perp} > 2.0$ pattern that is diagnostic for tetragonal Cu(II)[34] is not altered by anion binding. Second, the small shifts in the g values indicate that the Cu(II) electronic structure has not been greatly perturbed. Assuming for simplicity that the orbital reduction factors do not change upon ligand substitution, then the observed shifts in the g values can be accounted for by an increase in the relevant ligand field splittings of approximately 10–20%. Such effects are observed with azide and thiocyanate binding to $Cu(dien)^{2+}$: the d–d bands blue shift slightly and both $g_{\parallel}$ and $g_{\perp}$ for $Cu(dien)^{2+}$–X decrease in the order $X = H_2O > SCN^- > N_3^-$.[35] Anion binding to the amine oxidase produced a similar pattern of decreasing g values, resting enzyme > SCN^- complex > N_3^- complex, consistent with a nitrogen-donor ligand replacing an oxygen-donor ligand (probably H_2O).[36] NMR relaxation evidence has been presented for azide displacement of an equatorial H_2O from Cu(II) in pig plasma amine oxidase.[7] The key point is that all the spectroscopic data indicate that anion binding is a simple ligand-substitution reaction without substantial structural changes of the metal site.

Suzuki and co-workers have previously presented evidence for a Cu(II) electronic transition at ~480 nm. A band ($\Delta\epsilon$ = 600 M^{-1} cm^{-1}) was observed in the difference absorption spectrum between the native enzyme and a metal-depleted form that was assigned as a LMCT transition.[37] A weak shoulder (~500 nm) is evident in the CD spectrum of the resting enzyme (Figure 1), which may gain rotational strength as a consequence of anion binding. The CD band (Figure 3) with energy of ~20000 cm^{-1} is outside the ranges normally observed for d–d or imidazole (π) → Cu(II) LMCT transitions in tetragonal Cu(II) complexes with mixed imidazole N/O-donor ligands.[15b,38] Thus, a cofactor ↔ Cu(II) charge-transfer assignment is not unreasonable. Cofactor coordination to Cu(II) has been proposed;[39a] a considerable variety of results indicate that the cofactor and Cu(II) are in proximity and interact.[1-4,12,30,37,39] Recent EXAFS experiments are not inconsistent with cofactor coordination.[13] Clearly much more work is necessary (and warranted) before any detailed assignment can be made.

The estimated equilibrium constants for azide and thiocyanate binding to beef plasma amine oxidase (Table I) are in the range normally observed for coordination to tetragonal Cu(II).[32,33,40]

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It should be noted that the equilibrium constants will depend on pH, buffer composition, and ionic strength. Our conditions (Table I) were chosen to correspond with the conditions employed for anion binding and turnover kinetics experiments previously reported for bovine plasma and other amine oxidases. More than one equilibrium constant is required to fit the data for anion binding to the resting enzyme. This may indicate that the two copper ions have different affinities for anions, possibly as a result of differential phosphate effects. Other spectroscopic and chemical evidence consistent with inequivalent copper ions in various amine oxidases was obtained in phosphate-buffered solutions.[4,7,9] We are currently investigating amine oxidase–phosphate interactions in more detail.

Interestingly, substrate reduction perturbs the anion-binding properties of beef plasma amine oxidase. Small shifts and changes in the LMCT intensities are evident (Table I), indicative of only minor changes in the Cu(II) electronic structure. EPR data are also inconsistent with major structural rearrangements of the copper sites following substrate reduction. The overall affinity of beef plasma amine oxidase for anions is markedly reduced by reduction with benzylamine (Table I). A protein conformational change may be responsible, or the ligand displaced by exogenous ligands may become (or be replaced by) a poorer leaving group. In either case the data definitely suggest that the amine oxidation site and the Cu(II) site interact, which may be mechanistically significant.

All copper-containing amine oxidases seem to utilize a double-displacement or ping-pong mechanism.[1-4] The following mechanism for beef plasma amine oxidase has been suggested on the basis of kinetics studies and product analysis.[41,42]

$$E + RCH_2NH_2 \rightleftharpoons E{\cdot}RCH_2NH_2 \rightarrow E_{red} + RCHO$$

$$E_{red} \rightleftharpoons E_{red}' \rightarrow E + H_2O_2 + NH_3$$

There is general agreement on the anaerobic production of the aldehyde (which is essentially an irreversible step in the absence of excess aldehyde) with generation of a reduced enzyme species, the isomerization step, and late release of H_2O_2; however, the timing of NH_3 release remains controversial.[41-44] Initial velocity and product inhibition patterns implied that H_2O_2 and NH_3 releases were random.[41] The most recent work on the pig plasma amine oxidase indicates unambiguously that NH_3 release occurs in the second step with the same rate constant as H_2O_2 formation.[43] The switch from a mixed to an uncompetitive anion inhibition pattern as a function of substrate concentration is readily understandable. Mixed inhibition results from inhibitor binding to both the free enzyme and at least one other enzyme form, e.g. the enzyme-substrate complex, E_{red}, or E_{red}'.[45] High substrate concentrations prevent anion binding to the free enzyme, so an uncompetive inhibition pattern is then observed. We have directly confirmed spectroscopically that inhibitory anions do bind to E_{red} (E_{red}'); exogenous ligand binding to the enzyme–substrate complex is likely to be very similar. Slope and intercept replots were linear (data not shown), consistent with an inhibition mechanism involving ligand substitution.[46] Further support for this idea comes from the observation that noncoordinating anions (F^-, Cl^-, I^-) do not produce inhibition.

Some qualitative and quantitative differences are evident between the data described here and those obtained on other amine oxidases (Tables I and II). For example, the pig plasma enzyme apparently has no affinity for thiocyanate and only the beef plasma enzyme displays complex anion binding behavior in phosphate buffer. Still, the overall similarity among the resting amine oxidases and their reactions with anions is striking. Anion coordination produces analogous LMCT transitions in each case. Moreover similar changes in the EPR spectra of the plasma enzymes result, namely, a decrease in the g values with little change in $A_{\parallel}$. Clearly, all three amine oxidases display typical Cu(II) ligand-substitution chemistry. The data suggest that ligand substitution does not lead to major structural changes in the Cu(II) site, such as a change in overall geometry, for example. Thus it is quite plausible that inhibition by N_3^- and SCN^- results directly from the electronic and chemical changes accompanying coordination to Cu(II). If the present results are included, considerable evidence is now available that strongly supports a common mechanism for amine oxidases and an important role for copper in catalysis. Knowles and Yadav have suggested that equational anion coordination increases the pK_a for axially coordinated H_2O, thereby decreasing the concentration of the catalytically active hydroxide species.[7] Other results suggest that copper may directly mediate electron transfer between the reduced cofactor and O_2;[4,8,12,21,37,44,47] anion binding could perturb the copper sufficiently to substantially reduce the rate constant(s) for reoxidation. Similar mechanisms have been suggested to account for the inhibition of blue-copper oxidases produced by anion coordination of the tetragonal type 2 Cu(II), which is thought to mediate internal electron transfer.[16] Detailed spectroscopic and kinetics studies of the recently prepared single copper derivative of beef plasma amine oxidase[4] should be informative.

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Registry No. N_3, 14343-69-2; SCN, 302-04-5; Cu, 7440-50-8; amine oxidase, 9059-11-4.

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