Spectroscopic and Kinetics Studies of the Inhibition of Pig Kidney Diamine Oxidase by Anions*

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The role of copper in pig kidney diamine oxidase has been probed by examining the effects of potential Cu(II) ligands on the spectroscopic and catalytic properties of the enzyme. In the presence of azide and thiocyanate, new absorption bands are evident at 410 nm ($\epsilon = 6300$ M$^{-1}$ cm$^{-1}$) and 365 nm ($\epsilon = 3000$ M$^{-1}$ cm$^{-1}$), respectively. These bands are assigned as ligand-to-metal charge-transfer transitions, $N_3^-$/SCN$^- \rightarrow$ Cu(II). One anion/Cu(II) is coordinated in an equatorial position. Anion binding can be completely reversed by dialysis. The equilibrium constants for diamine oxidase-anion complex formation are 134 M$^{-1}$ ($N_3^-$) and 55 M$^{-1}$ (SCN$^-$). Azide and thiocyanate are linear uncompetitive inhibitors with respect to the amine substrate when O$_2$ is present at saturating concentrations. Taken together, the data are consistent with a functional role for Cu(II) in diamine oxidase catalysis.

Pig kidney diamine oxidase catalyzes the oxidative deamination of primary amine groups in several diamines, with the concomitant reduction of oxygen to hydrogen peroxide (1, 2). The enzyme has a molecular weight of 172,000 and contains two Cu(II) ions, presumably one in each subunit (3). In addition, the enzyme contains another cofactor that is sensitive to carbonyl reagents and is reduced by substrates (1, 2). The role of the Cu(II) ions in diamine oxidase is obscure. EPR experiments have conclusively demonstrated that substrates do not reduce enzyme-bound Cu(II), although substrate binding does perturb the Cu(II) EPR spectrum (4). NMR relaxation measurements indicated that neither the reactive end of the substrate, nor the product NH$_3$ bind near the Cu(II) (5). Since participation by coordinated H$_2$O in the hydrolysis of any intermediate seems ruled out by this result, and O$_2$ did not appear to compete with H$_2$O for a Cu(II) coordination site, it was suggested that copper is not directly involved in the catalytic process (6). Rather, Cu(II) was proposed to be the inhibitory binding site (occupied at high substrate concentrations), a suggestion first advanced by Zeller (6). In contrast, considerable evidence has been presented for the participation of Cu(II) in reactions catalyzed by pig plasma amine oxidase (7, 8).

A simple and straightforward approach to this problem is to examine the effects of potential Cu(II) ligands on diamine oxidase. If Cu(II) is the inhibitory binding site, then exogenous ligand coordination should prevent substrate inhibition. Activation of histamine oxidation by diamine oxidase in the presence of diethyldithiocarbamate (9) has been cited as evidence that Cu(II) functions in this manner (5). Diethyldithiocarbamate can also be used to remove copper from the enzyme, producing an inactive apoprotein (1, 2, 10), a result that is consistent with either a structural or catalytic role for copper. On the other hand, if Cu(II) does have a catalytic role, then exogenous ligand coordination should produce inhibition. Both azide and thiocyanate are known to coordinate to and inhibit other copper-containing oxidases (2) and amine oxidases (11, 12) without displacing protein-derived Cu(II) ligands; azide has been shown to be a diamine oxidase inhibitor (2), but its inhibitory mechanism has not previously been investigated. These anions are monodentate ligands with respect to a single copper ion, so complications arising from metal removal can be avoided. We, therefore, decided to investigate the interactions of azide and thiocyanate with diamine oxidase.

**EXPERIMENTAL PROCEDURES**

Pig kidney diamine oxidase was purified by modifications of methods in the literature (3, 13). The enzyme was electrophoretically pure and displayed a specific activity of 1.44 units mg$^{-1}$. Activity was measured using p-dimethylaminomethylbenzylamine as the substrate (1, 2). The role of Cu(II) ions in diamine oxidase is obscure. EPR experiments have conclusively demonstrated that substrates do not reduce enzyme-bound Cu(II), although substrate binding does perturb the Cu(II) EPR spectrum (4). NMR relaxation measurements indicated that neither the reactive end of the substrate, nor the product NH$_3$ bind near the Cu(II) (5). Since participation by coordinated H$_2$O in the hydrolysis of any intermediate seems ruled out by this result, and O$_2$ did not appear to compete with H$_2$O for a Cu(II) coordination site, it was suggested that copper is not directly involved in the catalytic process (6). Rather, Cu(II) was proposed to be the inhibitory binding site (occupied at high substrate concentrations), a suggestion first advanced by Zeller (6). In contrast, considerable evidence has been presented for the participation of Cu(II) in reactions catalyzed by pig plasma amine oxidase (7, 8).

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**RESULTS AND DISCUSSION**

Spectra of resting diamine oxidase and its $N_3^-$ and SCN$^-$ complexes are displayed in Fig. 1. Intense new bands are evident at 410 nm ($\epsilon = 6300$ M$^{-1}$ cm$^{-1}$) and 365 nm ($\epsilon = 3000$ M$^{-1}$ cm$^{-1}$) in the presence of $N_3^-$ and SCN$^-$, respectively. The position and intensity of these bands indicate that they are ligand-to-metal charge-transfer transitions, $N_3^-$/SCN$^- \rightarrow$ Cu(II). EPR spectra are entirely consistent with a tetragonal coordination geometry in resting diamine oxidase (4). Comparison of the data in Fig. 1 to results on Cu(II) complexes (16, 17) further indicates that $N_3^-$ and SCN$^-$ are equatorially coordinated to a tetragonal Cu(II) center. The requirement

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* This research was supported by the National Institutes of Health Grant GM27659 and by a William and Flora Hewlett Foundation Grant of Research Corporation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Anion Binding to Diamine Oxidase

for equatorial coordination may be understood in the following way. Tetragonal Cu(II) complexes have four ligands with relatively short bond lengths, that are (at least) approximately planar; axial ligands (when present) are at considerably longer distances. In such a complex, the Cu(II) d_{x^2-y^2} orbital contains the unpaired electron and is located within the equatorial plane, giving a 2B ground state. Energies and especially intensities of charge-transfer transitions depend critically on overlap between the donor and acceptor orbitals; an ε > 1000 M⁻¹ cm⁻¹ requires good overlap and therefore equatorial coordination. Anions have been shown to bind to galactose oxidase (18) and pig plasma amine oxidase (12) in an equitorial position.

In addition to the ligand-to-metal charge-transfer band at 410 nm, a feature is observed ~ 650 nm (ε ~ 300 M⁻¹ cm⁻¹) in the N₃⁻ spectrum, a region where the Cu(II) d-d transitions are likely to occur, indicating that the ligand field around Cu(II) has been perturbed. No such feature is evident in the SCN⁻ spectra, consistent with the positions of the anions, relative to H₂O, in the spectrochemical series (19). Slopes of the plots from optical titration experiments (Fig. 2) are equal to −K⁻¹, where K is an equilibrium constant for enzyme-anion complex formation (15). We find K for N₃⁻ and SCN⁻ to be 134 and 55 M⁻¹, respectively. These values are not typical for N₃⁻/SCN⁻ coordination to tetragonal Cu(II) centers in proteins (17). It has been inferred from variable frequency proton relaxation measurements that the coppers have different electronic relaxation times and hence different chemical environments (20). However, there is no evidence for a second binding constant in our titration data, which suggests that the two copper ions in diamine oxidase are equivalent with respect to anion coordination. Anion binding by only one of the two copper ions would require unreasonably large extinction coefficients. It should be noted that anion binding can be completely reversed via dialysis.

N₃⁻ and SCN⁻ are effective diamine oxidase inhibitors at concentrations where the predominant enzyme form is the anion complex. Also, substrate inhibition (at [95] > 6K_pro) was still evident. Reciprocal plots of the steady state inhibition data for N₃⁻ and SCN⁻ are given in Figs. 3 and 4. In most cases, comparison of these plots distinguishes the various possible inhibition patterns (21). By this criterion both anions are uncompetitive inhibitors with respect to p-dimethylamino-nomethylbenzylamine when O₂ is present at saturating concentrations. Intercept replots (Figs. 5 and 6) are linear, indicating that anion binding leads to dead-end complexes; that is, the enzyme-anion complex is completely inactive. Linear replots are also consistent with an inhibition mechanism involving ligand substitution at the metal center, as opposed to metal removal (22). The basic features of the diamine oxidase mechanism (23) are summarized in Scheme 1.

Thus, uncompetitive inhibition versus p-dimethylamino-nomethylbenzylamine can result from inhibitor binding to either the enzyme-substrate complex or E_total (or both), according to Cleland's (24) or Hearon's (25) rules. Apparent dissociation constants from kinetics data, K[N₃⁻] = 2.0 ± 1.0 mM, K[SCN⁻] = 22.4 ± 0.8 mM, are of the same order as the reciprocals of the binding constants, K⁻¹(N₃⁻) = 7.5 mM, K⁻¹(SCN⁻) = 18.2 mM. This strongly supports the idea that inhibition by N₃⁻ and SCN⁻ is a consequence of complex formation. Further, substantial deviations from tetragonal

FIG. 1. Absorption spectra of 0.023 M diamine oxidase and its azide and thiocyanate complexes at 20°C, pH 7.2. -- , diamine oxidase; --- , N₃⁻ complex; ----, SCN⁻ complex.

FIG. 2. Determination of the binding constants for anion coordination to diamine oxidase at 20°C, pH 7.2. Enzyme concentration was approximately 0.02 mM.
Anion Binding to Diamine Oxidase

leaving group in the ligand substitution reactions is H₂O, which has been identified as a Cu(II) ligand by proton relaxation measurements (5).

Our results are consistent with a functional role for the Cu(II) ions in diamine oxidase. It has been suggested that copper is involved in the reoxidation of the substrate-reduced enzyme by dioxygen (1, 6, 12). In this context, three attractive mechanisms for the observed inhibition are: 1) anion coordination sufficiently alters the electronic and/or geometric structure of the Cu(II) site such that it is no longer functional, 2) O₂ binding is prevented by coordinated anions, and 3) that coordinated H₂O (or OH⁻) is involved in the hydrolysis of an enzyme-bound intermediate and is displaced by N₃⁻ or SCN⁻ (8). Since the NMR relaxation experiments cited previously were performed only on the resting enzyme, mechanisms 2 and 3 are permitted if structural and/or electronic changes accompany enzyme reduction by substrates. Evidence for such changes has been presented for pea seedling diamine oxidase (26). Experiments designed to test these possible inhibition mechanisms and further probe Cu(II) functions in amine oxidases are in progress.

REFERENCES


symmetry in the Cu(II) site upon reduction by substrates is inconsistent with the EPR data (4), so exogenous ligand binding to Cu(II) in ES and E_red is expected to be very similar to ligand-binding to the resting enzyme. The most likely
Anion Binding to Diamine Oxidase

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