Spectroscopic Studies of Pig Kidney Diamine Oxidase–Anion Complexes

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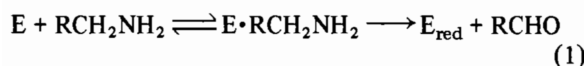
Abstract

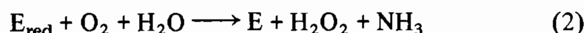
Complexes of pig kidney diamine oxidase with azide, thiocyanate, and cyanide have been characterized by EPR and circular dichroism spectroscopy. Cu(II) d–d bands are observed in the CD spectrum of the resting enzyme at ~800 nm (12 500 cm^{-1}) and 575 nm (17 400 cm^{-1}). Anion binding produces characteristic changes in the CD spectra. $N_3^-/SCN^- \rightarrow$ Cu(II) ligand-to-metal charge-transfer transitions are located at 390 nm (25 600 cm^{-1}) and 365 nm (27 400 cm^{-1}), respectively. In addition, an intense new band grew in at ~500 nm (20 000 cm^{-1}) when azide or thiocyanate were added, which may be assigned as a Cu(II) electronic transition that gains rotational strength in the anion complex. EPR spectra established that the Cu(II)–anion complexes are tetragonal; however, the magnitudes of the anion-induced shifts in the EPR parameters were consistent with substantial perturbations of the Cu(II) electronic ground state in the thiocyanate and cyanide complexes. Prominent superhyperfine splitting was apparent in the EPR spectra of the diamine oxidase complexes with thiocyanate and cyanide. The superhyperfine structure is (at least) partially attributable to endogenous Cu(II) ligands, probably from imidazole.

Introduction

The study of ligand binding to copper-containing amine oxidases can provide both structural and mechanistic information. Several anions are known to bind equatorially to Cu(II) in amine oxidases, possibly displacing a coordinated water molecule [1–6]. Copper-containing amine oxidases use a double-displacement mechanism, schematically illustrated below [7–9].



$$E + RCH_2NH_2 \rightleftharpoons E\cdot RCH_2NH_2 \longrightarrow E_{red} + RCHO \quad (1)$$



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

Ligand substitution at enzyme-bound Cu(II) has been shown to decrease the rates of some steps leading to E_{red} as well as the subsequent oxidation rate of E_{red}. As ligand substitution does not produce major structural changes in the Cu(II) sites [1–6], it is likely that inhibition by exogenous ligands results directly from the physical and chemical perturbations accompanying coordination. Pig kidney diamine oxidase contains two cupric ions per enzyme molecule [7–9]; the coppers have different electronic relaxation times and thus slightly different environments [10]. Nevertheless, azide and thiocyanate binding are each well-characterized by a single equilibrium constant [4]. The extinction coefficients of the $N_3^-/SCN^- \rightarrow$ Cu(II) LMCT** transitions are most consistent with anion binding to both Cu(II) ions [4]. Azide and thiocyanate are linear uncompetitive inhibitors with respect to the amine substrate at saturating O_2 concentrations [4].

Relatively little structural information on the copper sites in the pig kidney diamine oxidase is available. The enzyme's EPR parameters are typical for tetragonal Cu(II) with nitrogen/oxygen ligands [11]. Recent X-ray absorption spectroscopic data suggest that the Cu(II) sites in pig kidney diamine oxidase are electronically similar to $[Cu(imidazole)_4]^{2+}$†. Although substrates apparently do not bind to or significantly reduce the Cu(II), its EPR spectrum is perturbed [11, 12]. An organic cofactor is also present and is reduced by substrates. Some experiments indicate that the cofactor and Cu(II) interact [11–19]; cofactor coordination to Cu(II) has been proposed [19]. Recent work has tentatively identified the cofactor as pyrolloquinolinequinone (methoxatin) [20, 21]. Pyridoxal phosphate has also been proposed [7–9]. We have previously characterized azide and thiocyanate binding to pig kidney diamine oxidase by absorption spectroscopy and steady-state kinetic measurements [4]. Work on other amine oxidases demonstrates that additional structural details can be obtained

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**Abbreviations used: LMCT, ligand-to-metal charge-transfer; A^s, superhyperfine splitting.

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by circular dichroism (CD) and EPR spectroscopy [1–3, 6, 22]. This paper describes the results of such experiments on diamine oxidase.

Experimental

Pig kidney diamine oxidase was purified by modifications of published procedures [23, 24], as described below. Steps (a) to (d) were carried out as described by Kluetz and Schmidt [24].

(a) Homogenization
(b) Heat denaturation
(c) Ammonium sulfate fractionation
(d) DEAE-cellulose chromatography
(e) Sephadex DEAE A-50 chromatography.

Active fractions from the DEAE-cellulose column were pooled, concentrated, and diluted with cold distilled-deionized water, to the conductivity of 0.015 M potassium phosphate buffer (pH 7.2). The solution was loaded onto a 4 × 20 cm column of DEAE-sephadex A-50 equilibrated with the buffer above. Diamine oxidase was eluted with a linear 0.015–0.15 M gradient of potassium phosphate buffer (pH 7.2).

(f) Hydroxylapatite chromatography. Pooled fractions from (e) were applied to a 2.5 × 30 cm column of hydroxylapatite, equilibrated with 0.015 M buffer. The column was washed with several column volumes of 0.1 M potassium phosphate buffer/0.2 M ammonium sulfate (pH 7.2). Elution was accomplished with 0.1 M potassium phosphate buffer/0.5 M ammonium sulfate (pH 7.2). The active fractions were pooled and concentrated to 5 ml.

(g) Sephacryl S-200 chromatography. The concentrated pool from (f) was loaded into a 1.6 × 100 cm column of Sephacryl S-200 and eluted with 0.015 M buffer. The final sample was dialyzed against 0.1 M potassium phosphate buffer (pH 7.2) and stored at −70 °C.

Enzyme activity was measured using *p*-dimethylaminomethylbenzylamine [24, 25]. Absorption spectra were obtained with a Cary 219 spectrophotometer. Concentrated NaN_3 or KSCN solutions were prepared immediately before anion binding experiments. A modified JASCO J-40A was used to measure CD spectra (2.0 nm resolution). Baseline corrections and difference spectra were generated digitally with a Bascom–Turner recorder/signal processor. A Varian E-9 instrument was used for EPR spectroscopy. Enzyme concentrations (in 0.1 M potassium phosphate buffer, pH 7.2) were ~0.07 mM for CD experiments and ~0.86 mM for EPR experiments. Enzyme solutions were extensively dialyzed against Chelex-treated buffers to remove adventitious metal ions prior to EPR measurements. Other pertinent experimental details are given in the figure legends. Extinction coefficients are quoted per mol of copper.

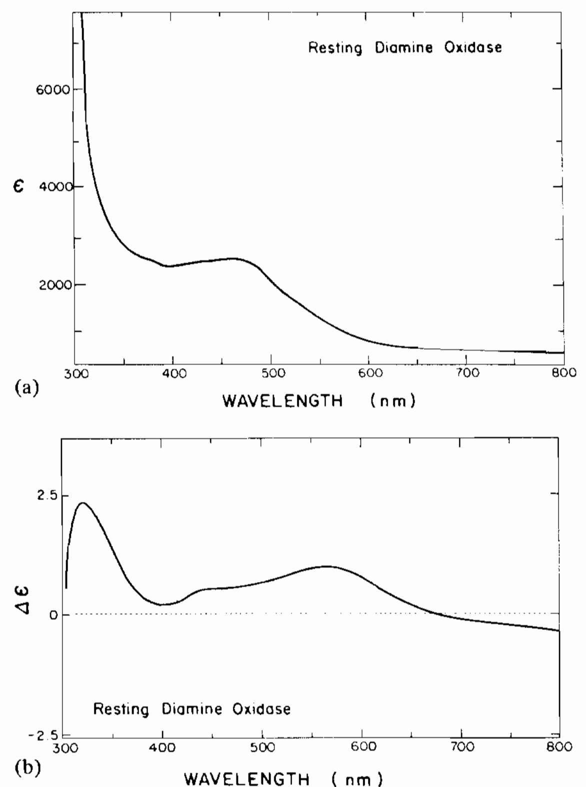

Fig. 1. (a) Absorption spectrum of resting pig kidney diamine oxidase. (b) CD spectrum of resting pig kidney diamine oxidase.

Results and Discussion

The absorption and CD spectra of resting diamine oxidase are shown in Fig. 1. Note that the 410 nm absorption feature, evident to some extent in most published pig kidney diamine oxidase spectra, is absent. Absorption spectra of the azide and thiocyanate complexes of this enzyme preparation essentially are identical to those previously reported [4]. Several bands are resolved in the CD spectrum, including two features at ~800 nm (12 500 cm^{-1}) and 575 nm (17 400 cm^{-1}) that can be confidently assigned as Cu(II) d–d transitions. For comparison, beef plasma amine oxidase displays Cu(II) d–d transitions at 800 nm and 650 nm (15 400 cm^{-1}) [6, 22]. These energies are within the range normally observed for ligand-field transitions of tetragonal Cu(II) complexes with nitrogen/oxygen donor ligands [26–28]. The shoulder centered at ~450 nm (22 200 cm^{-1}) and the 325 (30 770 cm^{-1}) band may be assigned as either charge-transfer transitions or to the organic cofactor. A positive CD band in the 360–325 nm region is observed in the spectra of all the copper-containing amine oxidases examined to date [6, 22, 29], but only the beef plasma enzyme displays an intense negative band at 425 nm [6, 22].

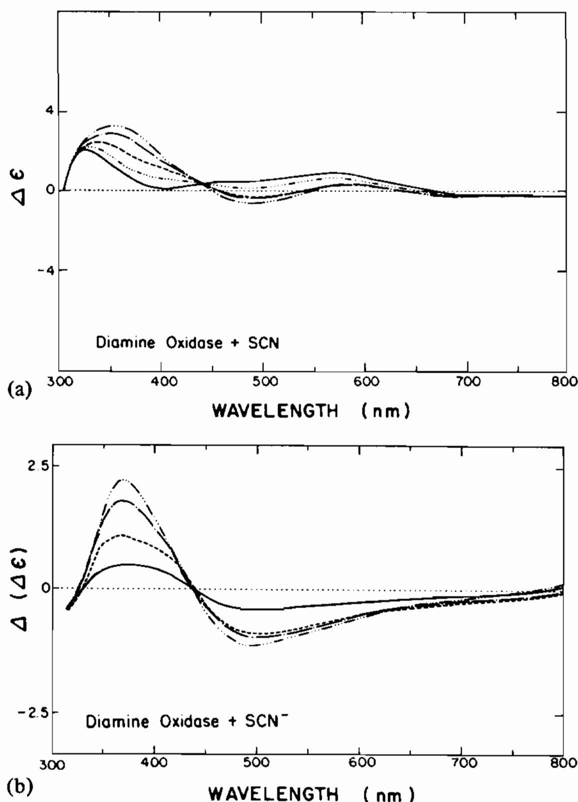

Fig. 2. (a) CD spectral changes produced by thiocyanate binding to pig kidney diamine oxidase. [SCN^-] = 35.5 mM (–·····–); 140 mM (-------); 270 mM (–·–·–); 395mM (–···–); resting (———). (b) Difference CD spectra generated by digitally subtracting the resting enzyme's spectrum from the spectra in (a). [SCN^-] = 35.5 mM (———); 140 mM (-------); 270 mM (–·–·–); 395 mM (–···–).

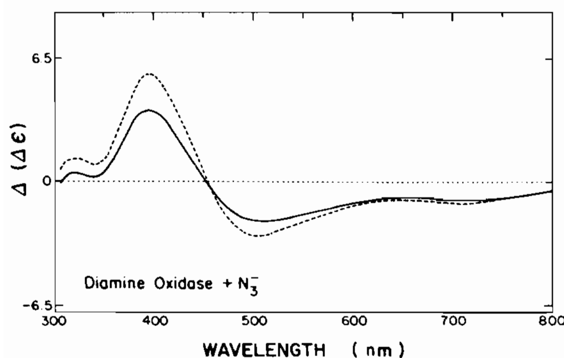

Fig. 3. Difference CD spectra produced by azide binding to resting pig kidney diamine oxidase. [N_3^-] = 14 mM (———); 83 mM (-------).

Anion binding perturbs the CD spectrum of resting diamine oxidase (Figs. 2 and 3). At the highest anion concentration employed, >90% of the enzyme is complexed in both cases. Isosbestic points are evident at 440 nm in the SCN^- titration (Fig. 2a) and at 455 nm in the N_3^- titration (not shown). In analogy to the absorption spectral results, the bands at 390 nm (25600 cm^{-1}) and 365 nm (27400 cm^{-1}) are assigned as $N_3^-/SCN^- \rightarrow$ Cu(II) LMCT transitions. An intense new band occurs at ~500 nm in the difference CD spectra of the azide and thiocyanate complexes of diamine oxidase. Beef plasma amine oxidase complexes with these anions also display intense negative bands near 500 nm [6]. We have previously argued that a cofactor ⟷ Cu(II) charge-transfer assignment for this band is plausible and that ligand substitution increases its rotational strength [6].

The EPR spectrum of resting diamine oxidase prepared by our procedure (Fig. 4) is in good agreement with published spectra [11], with $g_\perp = 2.061$, $g_\parallel = 2.286$, $A_\parallel = 0.0173$ cm^{-1}. EPR spectra of three diamine oxidase–anion complexes are shown in Figs. 5–7. Parameters estimated from these spectra are: (N_3^-), $g_\perp = 2.058$, $g_\parallel = 2.224$, $A_\parallel = 0.0156$ cm^{-1}; (SCN^-) $g_\perp = 2.045$, $g_\parallel = 2.210$, $A_\parallel = 0.0190$ cm^{-1}; (CN^-) $g_\perp = 2.036$, $g_\parallel = 2.195$, $A_\parallel = 0.0171$ cm^{-1}. Although we believe these values are reasonably accurate, they are not based on simulations that allow for slightly inequivalent copper sites. The

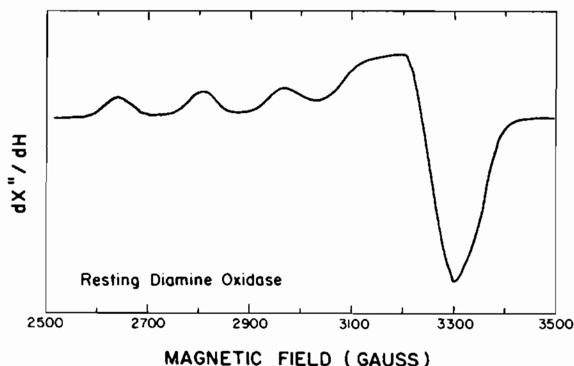

Fig. 4. EPR spectrum of resting pig kidney diamine oxidase at 77 K. Experimental conditions: frequency = 9.25 GHz; power = 15 mW; modulation amplitude = 5.0 gauss.

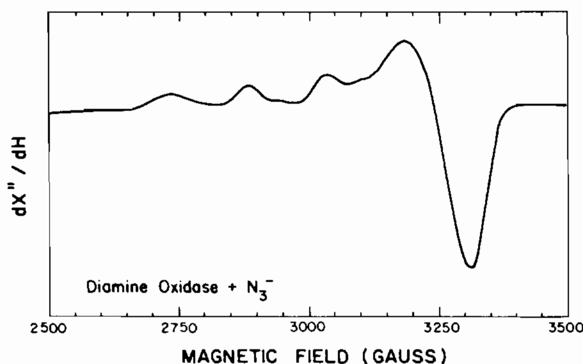

Fig. 5. EPR spectrum of the diamine oxidase–azide complex at 77 K. [N_3^-] = 100 mM. Experimental conditions: frequency = 9.25 GHz; power = 15 mW; modulation amplitude = 5.0 gauss.

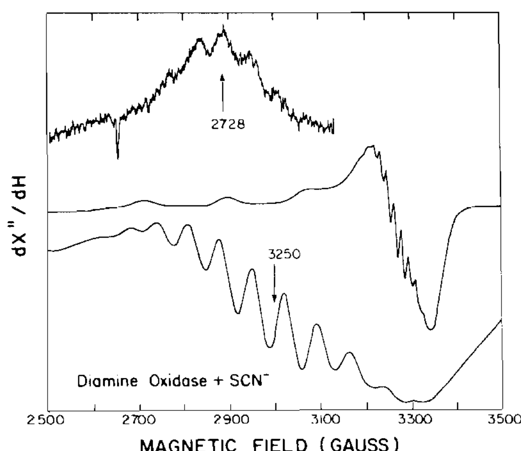

Fig. 6. EPR spectra of the diamine oxidase–thiocyanate complex at 77 K. $[SCN^-]$ = 98 mM. Frequency = 9.25 GHz, modulation amplitude = 5.0 gauss for all spectra. Top: expansion of the lowest field hyperfine line, power = 30 mW. Middle: full spectrum, power = 15 mW. Bottom: expansion of the $g_\perp$ region, power = 15 mW.

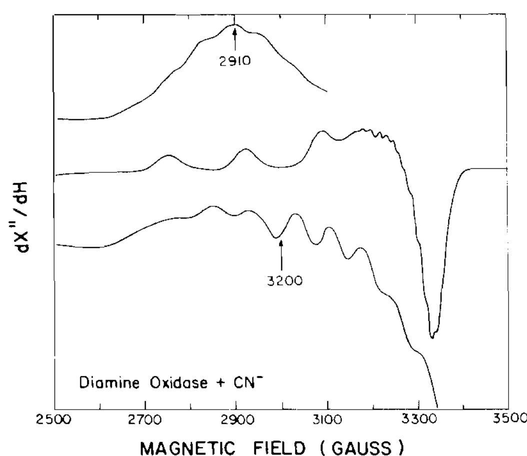

Fig. 7. EPR spectra of the diamine oxidase–cyanide complex at 77 K. $[CN^-]$ = 26 mM. Frequency = 9.25 GHz, modulation amplitude = 5.0 gauss for all spectra. Top: expansion of the second hyperfine line, power = 20 mW. Middle: full spectrum, power = 15 mW. Bottom: expansion of $g_\perp$ region, power = 20 mW.

$g_\parallel > g_\perp > 2.0$ pattern, indicative of tetragonal Cu(II), is maintained in the anion complexes. Further, both $g_\parallel$ and $g_\perp$ decrease upon anion binding, which has been suggested to implicate an oxygen-donor ligand as the leaving group [30, 31]. No systematic variation is apparent in the $A_\parallel$ values, which are functions of orbital mixing and covalency, and thus may depend on the structure of the enzyme–anion complex in a complicated way. Changes in the g values may be analyzed by reference to the equations for tetragonal Cu(II) given below [32]:

$$g_\parallel = 2 - \frac{8k_\parallel \lambda^o}{\Delta_2} \tag{3}$$

$$g = 2 - \frac{2k_\perp \lambda^o}{\Delta_3} \tag{4}$$

where $k_\parallel$ and $k_\perp$ are the orbital reduction factors, λ^o is the free ion spin–orbit coupling factor (-828 cm^{-1}), and Δ_2 and Δ_3 are defined in Fig. 8. A 20–30% decrease in $k_\parallel/\Delta_2$ and a 5–40% decrease in $k_\perp/\Delta_3$ is required to fit the data. In contrast to the results for beef plasma amine oxidase (where the corresponding shifts are much smaller) [6], anion-induced changes in the diamine oxidase g values probably cannot be ascribed solely to increases in Δ_2 and Δ_3. The larger differences in the g values between the resting enzyme and the SCN^- complex, as compared to the N_3^- complex, also indicate that the covalency of the copper sites is perturbed by anion binding.

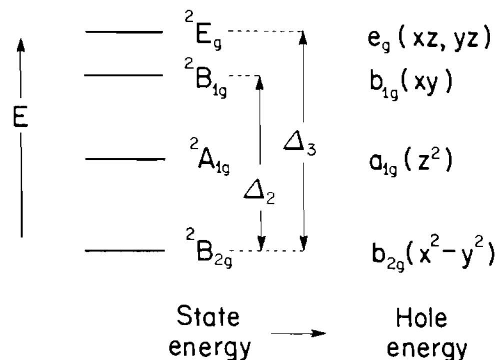

Fig. 8. Orbital and state energy-level diagram for tetragonal Cu(II) complexes assuming D_{4h} symmetry.

Superhyperfine splitting in the $g_\perp$ region has previously been detected for the resting and substrate-reduced forms of pig kidney diamine oxidase [11]. The superhyperfine structure in these two forms is quite weak and is clearly observable only in second-derivative format. Weak superhyperfine features are also present in the diamine oxidase–N_3^- complex (not shown) but pronounced superhyperfine coupling was observed in the EPR spectra of the SCN^- and CN^- complexes (Figs. 6 and 7). In both complexes $A_\perp^s$ = 14.5 gauss, $A_\parallel^s$ = 11 gauss; based on its magnitude and pattern, the superhyperfine splitting is attributable to equatorially coordinated nitrogen-donor ligands. Although the superhyperfine structure is not as well-resolved in the cyanide complex, its observation establishes that at least part of the superhyperfine coupling must arise from protein ligands, probably imidazole(s). Preliminary experiments suggest that cyanide also attacks the organic cofactor*. Since nucleophilic addition to the co-

*M. A. McGuirl and D. M. Dooley, unpublished results.

factor by substrates and carbonyl reagents perturbs the Cu(II) EPR spectrum, some of the differences between the resting enzyme and the CN^--complex may arise from longer-range effects, but these are likely to be negligible compared to the changes that accompany ligand substitution.

Overall, beef plasma, pig plasma and pig kidney amine oxidases display remarkably similar coordination chemistry [1–6]. The structures of the various anion complexes are probably also similar. To be sure, the effective Cu(II) ligand-fields may differ slightly, and ligand-substitution perturbs the pig kidney enzyme's Cu(II) sites to a greater extent. Additionally, phosphate interferes with anion binding to beef plasma amine oxidase [6] but does not affect some other amine oxidases [1–4]. Nevertheless, the recently proposed model for the copper sites in beef plasma amine oxidase [33] should be a reasonable model for the copper sites in the other amine oxidases. It now seems likely that copper is directly involved in amine oxidase catalysis [1–9, 12, 15, 34–36] despite some earlier doubts [37, 38]. Ligand-substitution produces comparable inhibition of each enzyme, suggesting a common mechanistic role for copper in amine oxidases. Currently, the most complete mechanistic data are from studies on the pig plasma enzyme [7–9, 35, 36, 39–41] and our understanding of other amine oxidase mechanisms is not as good. Further structural and mechanistic work is necessary, but two possible functions for copper have been proposed: (1) Cu(II)-coordinated hydroxide nucleophilically assists hydride transfer from the reduced organic cofactor to O_2 [36]; (2) Copper directly mediates electron-transfer from the reduced cofactor to O_2 [12, 15, 17, 18, 42, 43]. Inhibition via ligand-substitution is consistent with either mechanism.

Acknowledgements

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