Metal Coordination Environment of a Cu(II)-Substituted α-Keto Acid-Dependent Dioxygenase That Degrades the Herbicide 2,4-D

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Received December 30, 1996

α-Keto acid-dependent enzymes utilize a mononuclear nonheme iron center to activate dioxygen for a diverse range of metabolic processes involving the oxidation of an unactivated C−H bond.[1] One member of this class, the (2,4-dichlorophenoxy)acetate/α-ketoglutarate dioxygenase (TfdA) encoded on the pJP4 plasmid of *Alcaligenes eutrophus* JMP134 catalyzes the conversion of the herbicide (2,4-dichlorophenoxy)acetate (2,4-D) to 2,4-dichlorophenol and glyoxylate, coupled with the oxidative decarboxylation of α-ketoglutarate (α-KG) to succinate and CO_2, as shown in Scheme 1.[2] Previously, we have cloned and expressed the *tfdA* gene in *Escherichia coli* and purified the 64 kDa homodimeric protein to homogeneity.[3] Chemical modification studies indicate that multiple histidines, but no cysteines, are necessary for activity; otherwise there is scant information on the active site.[3] In this paper, we describe EPR and ESEEM studies of Cu(II)-substituted TfdA that provide the first insights into the metal coordination environment of the activated complex of an α-keto acid-dependent enzyme.

Steady-state kinetic analysis of TfdA demonstrates that Cu(II) is a very good competitive inhibitor versus Fe(II) (K_i = 0.1−0.3 μM), strongly implying that Cu(II) occupies the same binding site. Indeed, its K_i, which is 100-fold lower than the concentration of Fe(II) required for half-maximal activity (10−30 μM),[3] suggests tighter binding by Cu(II). Thus, spectroscopic analysis of the Cu-substituted enzyme is likely to reflect important features of the native Fe(II) enzyme.

Figure 1 shows X-band EPR spectra of Cu(II)-substituted TfdA (Cu-TfdA) alone and in the presence of the cofactor α-KG and the substrate 2,4-D. The EPR spectrum of Cu-TfdA has $g_{\|}$ (2.29) > $g_{\perp}$ (2.07) and $A_{\|}$ = 16.1 mK (Figure 1A), indicating that the Cu(II) center resides in a type 2 environment with a mixture of N and O ligands in the equatorial plane.[4] These EPR parameters are altered upon addition of the cofactor α-KG (Figure 1B; $g_{\perp}$ = 2.07, $g_{\|}$ = 2.36, $A_{\|}$ = 14.9 mK); however, the addition of the substrate 2,4-D does not affect the Cu-TfdA spectrum unless α-KG is also present (Figure 1C). The ternary Cu-TfdA/α-KG/2,4-D complex exhibits a more rhombic EPR signal with $g_{\|}$ = 2.37 and a significantly decreased $A_{\|}$ value of 12.0 mK. Very similar EPR spectra are observed when either of the α-KG analogs, pyruvate (K_m = 1.02 mM,[3] $g_{\|}$ = 2.36, $A_{\|}$ = 12.8 mK) or glutarate (K_i = 0.45 mM, $g_{\|}$ = 2.37, $A_{\|}$ = 12.5 mK) is used in forming the ternary complex. These comparisons show that only the 1-carboxylate of the α-KG cofactor is crucial for producing the distinctive EPR features of the ternary complex. For type 2 Cu(II) centers, these high $g_{\|}$ values (>2.35) are associated with an oxygen-rich environment,[4] while these small $A_{\|}$ values (<14.0 mK) indicate a significant distortion from planarity.[5,6] Comparable EPR parameters have been observed for Cu-substituted enolase complexed with 2-phosphoglycerate[7] and Cu-substituted ribulose-1,5-bisphosphate carboxylase/oxygenase complexed with 2-carboxyarabinitol bisphosphate.[8] The crystal structures of both of these complexes[9,10] show metal centers with distorted carboxylate-rich 6O ligand environments, which rationalize their unusually sharp signals (∼1 mT fwhm), high $g_{\|}$ values (∼2.4), and small $A_{\|}$ values (∼12 mK).[7,8] The similarly small $A_{\|}$ value of the Cu-TfdA/α-KG/

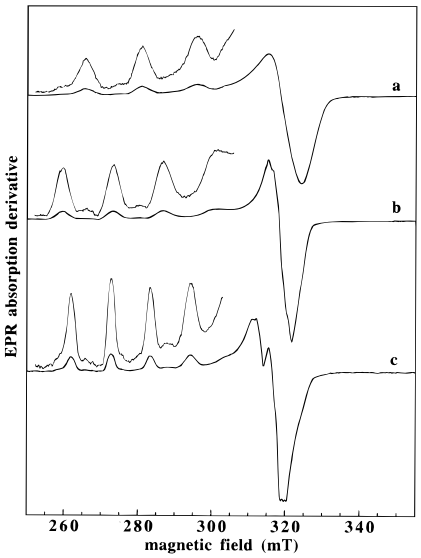
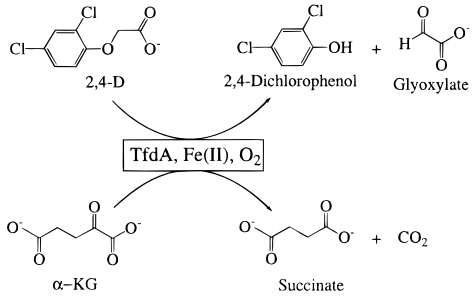


Figure 1. EPR spectra of (A) Cu-TfdA ($g_{\perp}$ = 2.07, $g_{\|}$ = 2.29, $A_{\|}$ = 16.1 mK, 4.2 mT fwhm at 273 mT), (B) Cu-TfdA + 5 mM α-KG ($g_{\perp}$ = 2.07, $g_{\|}$ = 2.36, $A_{\|}$ = 14.9 mK, 4.2 mT fwhm at 273 mT), and (C) Cu-TfdA + 5 mM α-KG + 5 mM 2,4-D ($g_{\perp}$ = 2.08, $g_{\|}$ = 2.37, $A_{\|}$ = 12.0 mK, 2.2 mT fwhm at 273 mT). Insets show $g_{\|}$ with intensity magnified 5 times. Spin quantitation indicates that all of the added Cu is bound in all three cases. Samples were prepared by slow addition of $CuCl_2$ (450 μM in H_2O) to apo-TfdA (500 μM subunit concentration in 25 mM MOPS buffer, pH 6.9) followed by addition of substrate solution (50 mM substrate in 25 mM MOPS buffer, pH 6.9). Samples were then inserted in 0.3 mm i.d. quartz tubes and frozen by slow immersion in liquid N_2. EPR spectra were obtained at 20 K as single 4 min scans from 220 to 380 mT using 0.20 mW power at 9.23 GHz, 1.0 mT modulation amplitude.

Scheme 1



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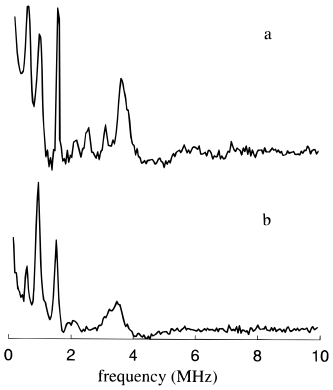

Figure 2. Three-pulse ESEEM data of (A) Cu-TfdA + 5 mM α-KG and (B) Cu-TfdA + 5 mM α-KG + 5 mM 2,4-D. Samples were prepared as described in Figure 1. Conditions for these measurements: (A) microwave frequency 8.98 GHz, magnetic field strength 280 mT, τ value 375 ns, number of scans averaged 1; (B) microwave frequency 8.92 GHz, magnetic field strength 275 mT, τ value 380 ns, number of scans averaged 4. Conditions common to both measurements were sample temperature (4.2 K), microwave pulse power (32 W), pulse width (20 μs, fwhm), and each time point (represents the average of 34 events).

2,4-D complex indicates a comparably distorted environment; however, its somewhat lower $g_{||}$ and broader signals (2.2 mT fwhm) suggest the presence of an endogenous nitrogen ligand.

ESEEM experiments have proven useful for establishing the presence of imidazole ligands strongly bound to a Cu center.[11] Three-pulse ESEEM spectra of Cu-TfdA complexes all exhibit sharp peaks at 0.6, 0.9, and 1.5 MHz together with a broader feature at 3.5 MHz, typical of the distal nitrogen of imidazole bound equatorially to Cu (Figure 2).[11] In addition, the spectra of Cu-TfdA (not shown) and Cu-TfdA/α-KG (Figure 2A) show weak "combination" peaks at 2.1, 2.5, and 3.1 MHz indicative of the presence of at least two imidazole ligands in this complex.[12] The ESEEM spectrum of the ternary complex (Figure 2B) shows a substantial decrease in modulation intensity and a lack of the "combination" peaks observed for the Cu-TfdA and Cu-TfdA/α-KG samples. This finding shows that the binding of 2,4-D has altered the hyperfine coupling to one of the histidyl imidazole ligands. While this might indicate displacement of one of the bound histidines, there is also a change in the relative intensities of the "fundamental" ESEEM peaks at 0.6, 0.9, and 1.5 MHz, which suggests a shift in the relative orientation of the *g*-tensor principal axis system. Hence, the formation of the ternary complex may redefine the tetragonal axis so that one of the histidyl imidazole ligands is now coordinated in an axial position and contributes minimally to the spectrum.

Figure 3 shows our working model for the metal coordination sphere in Cu-TfdA: a facial array of three endogenous protein ligands together with three coordination sites available for exogenous ligands. The endogenous His ligands in the equatorial plane are deduced from the ESEEM data, consistent with chemical modification studies of Fe-TfdA.[3] The axial Asp ligand, not yet corroborated by experiment, is proposed on the basis of its appearance in the TfdA sequence at a position (D115) between two His residues (H113 and H167) that fits well into the His-X-Asp-$X_{(53-57)}$-His motif that is common to α-keto acid-dependent dioxygenases and isopenicillin N synthase (IPNS).[13] Such a facial triad of 1 carboxylate and 2 His ligands binds the metal centers in IPNS[14] and 2,3-dihydroxybiphenyl 1,2-dioxygenase (an extradiol cleaving catechol dioxygenase),[15] and is emerging as the minimal coordinative scaffolding for mononuclear nonheme iron(II) centers that activate oxygen.[1a]

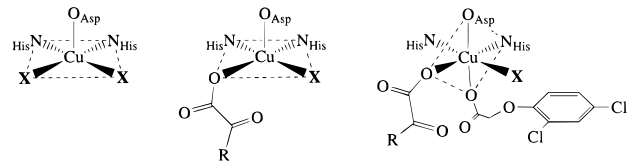

Figure 3. Working model for the metal coordination sphere in Cu-TfdA. The X marks coordination sites proposed to be occupied by solvent and/or endogenous ligands which are available for binding by exogenous ligands. The dashed lines define the equatorial plane of the Cu(II) center which gives rise to the observed EPR and ESEEM spectral features. This equatorial plane is proposed to reorient by 90° upon formation of the ternary complex.

As found for IPNS and extradiol-cleaving catechol dioxygenases,[1a,16,17] we propose that the remaining three coordination sites for Cu-TfdA are occupied by solvent (and/or other endogenous ligands) and are displaced upon binding α-KG, 2,4-D, and other exogenous ligands.[18] The highly distinctive spectral properties of the ternary complex suggest coordination of the carboxylates of both α-KG and 2,4-D affording a Cu equatorial ligand set consisting of a His residue and three carboxylates. This ligand set, which includes the putative Asp ligand, is proposed to result from a reorientation of the equatorial plane upon formation of the ternary complex (Figure 3) and poises the metal center to bind O_2 at the remaining sixth coordination site during catalysis. The fact that only the combination of cofactor and substrate elicits the characteristic ternary complex EPR signal is consistent with the tight coupling between the oxidative decarboxylation of α-KG and the cleavage of 2,4-D during catalysis.[2] It also suggests the existence of a synergistic interaction that very likely activates the native Fe(II) site for dioxygen binding as proposed in the enzyme mechanism.[19,20] These results thus provide the first direct glimpse of the metal coordination environment of an α-keto acid-dependent enzyme as it goes through its catalytic cycle; the insights we have obtained lay the foundation for future spectroscopic studies of the iron enzyme.

Acknowledgment. This research was supported by NIH grants GM 33162 (L.Q.) and GM 54065 (J.M.), NSF Science and Technology Center Grant BIR 9120006 (R.P.H.), and the Michigan State University Agricultural Experiment Station (R.P.H.). We also thank Professor Stenbjorn Styring for providing timely insights on the interpretation of the EPR spectra.

JA964449X

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