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CD and MCD Studies of the Non-Heme Ferrous Active Site in (4-Hydroxyphenyl)pyruvate Dioxygenase: Correlation between Oxygen Activation in the Extradiol and α-KG-Dependent Dioxygenases

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The α-ketoglutarate (α-KG)-dependent dioxygenases comprise one extensive class of non-heme iron enzymes which require Fe^{II}, α-KG, and dioxygen for catalysis.[1] Involved in the tyrosine catabolism pathway, (4-hydroxyphenyl)pyruvate dioxygenase (HPPD) is an α-keto acid-dependent dioxygenase which catalyzes the conversion of (4-hydroxyphenyl)pyruvate (HPP) to homogentisate in a reaction involving decarboxylation, substituent migration, and aromatic oxygenation.[2] HPPD is unusual among the α-KG dioxygenases as it incorporates both atoms of dioxygen into a single substrate as the α-keto acid is supplied by the pyruvate substituent of HPP. This parallels another class of mononuclear non-heme iron enzymes, the extradiol dioxygenases, where the substrate also supplies the extra electrons required for O_2 activation. Interestingly, crystallographic studies of ferric HPPD have shown that it has a tertiary structure similar to that of the extradiol dioxygenase 1,2-dihydroxybiphenyl dioxygenase (1,2-DHBD), suggesting that HPPD may structurally relate the α-KG-dependent dioxygenases to the extradiol dioxygenases.[3] In the current study, circular dichroism (CD) and magnetic circular dichroism (MCD) have been employed to directly probe the resting Fe^{II} site of HPPD and its interaction with HPP substrate. The results provide insight into the mechanism of oxygen binding and activation by HPPD and its active-site structural relationship to both other α-KG-dependent dioxygenases (e.g., clavaminate synthase, CS2) and to the extradiol class of mononuclear non-heme iron enzymes.

Near-IR (NIR) CD spectroscopy has been used to probe Fe^{II} and HPP binding to HPPD (Figure 1). While the 278 K CD spectrum of apoHPPD is featureless, EPR and absorption of apoHPPD (Figure S1) indicate the presence of a <3% Fe^{III}-tyrosinate impurity due to self-hydroxylation as previously observed for HPPD.[4] Addition of 0.9 equiv of Fe^{II} to apoHPPD results in two broad features at ∼8500 and ∼10500 cm^{-1} which correspond to ligand field transitions of the ferrous site. Addition of 5 equiv HPP to resting HPPD/Fe^{II} results in a new ferrous spectrum with a negative feature at ∼9000 cm^{-1} and a positive feature at ∼11500 cm^{-1}. The spectrum does not change upon further addition of HPP, indicating complete conversion to the substrate-bound form. Addition of up to 50 equiv of α-KG to HPPD/Fe^{II} does not change the CD (or MCD) spectra (Figures S2 and S3), indicating no direct coordination of α-KG to Fe^{II}.

The 1.8 K, 7 T NIR MCD spectrum of resting HPPD/Fe^{II} (Figure 2B) contains positive ligand field features at <5000 and ∼8900 cm^{-1} and a negative feature at ∼11000 cm^{-1}. The additional positive feature at >15000 cm^{-1} is higher in energy than that observed for ferrous d→d transitions and is attributed to the Fe^{III}-tyrosinate impurity. Since ligand field theory dictates that a single Fe^{II} site can have no more than two d→d transitions, the presence of multiple features in the MCD spectrum of resting HPPD/Fe^{II} indicates the presence of a mixture of ferrous species. The positive band at <5000 cm^{-1} requires the presence of a 5C species as one component of the mixture, which should also contribute a signal

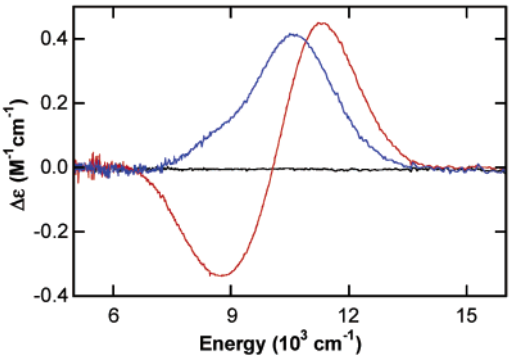

Figure 1. NIR CD spectra at 278 K of apoHPPD (black), apoHPPD + 0.9 equiv Fe^{II} (blue, Δε ×2) and HPPD/Fe^{II} + 5 equiv HPP (red).

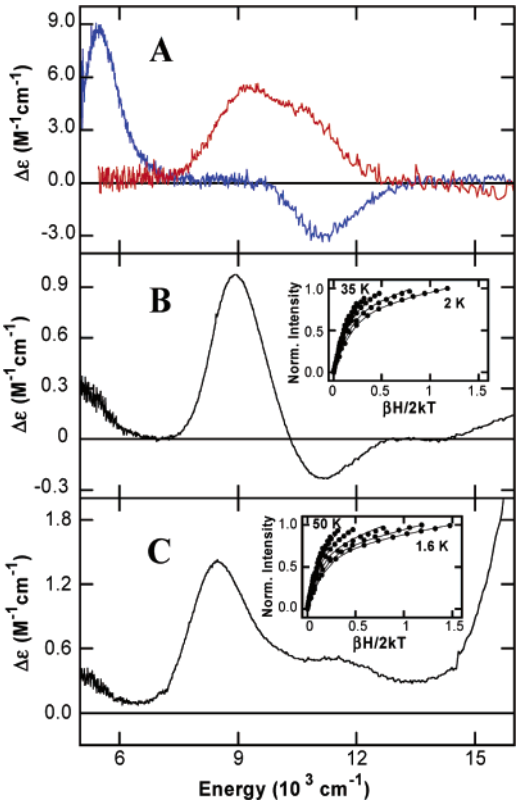

Figure 2. MCD spectra at 1.8 K and 7 T of (A) CS2/Fe^{II} (Δε ×35, red) and DHBD/Fe^{II} (blue), (B) HPPD/Fe^{II}, and (C) HPPD/Fe^{II}/HPP. (Insets show VTVH MCD data (symbols) and their best fit (lines) for HPPD/Fe^{II} collected at 8888 cm^{-1} and HPPD/Fe^{II}/HPP collected at 8503 cm^{-1}.

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to the ~11000 cm^{-1} region.[5] The ~8900 cm^{-1} band (and an additional unresolved high-energy transition in the ~10500 cm^{-1} region) corresponds to a 6C component. This is supported by an Fe K-Edge XAS pre-edge analysis of HPPD/Fe^{II} which indicates a predominately 6C ferrous site (unpublished results). Thus, resting HPPD is a mixture of spectroscopically distinct 5C and 6C species. VTVH MCD was used to probe the ground-state splittings of the resting ferrous site corresponding to the 6C band at ~8900 cm^{-1} (inset of Figure 2B). The saturation magnetization behavior is well-described by a negative ZFS non-Kramers doublet model with ground-state spin Hamiltonian parameters of $\delta = 2.6 \pm 0.2$ cm^{-1} and $g_{||} = 9.0 \pm 0.4$.[6] The value of δ for HPPD/Fe^{II} is much smaller than that found for the 6C site in CS2/Fe^{II} ($\delta = 4.5 \pm 0.15$ cm^{-1}).[7] These values of δ and $g_{||}$ give $\Delta = -300 \pm 150$ cm^{-1} with $|V/2\Delta| \approx 0.12$ for the dπ orbital splitting where Δ is energy difference between the d_{xy} and $d_{xz,yz}$ orbitals. $|V/2\Delta|$ defines the rhombicity and is far from the rhombic limit (0.33, which is usually typical for 6C sites), likely due to the presence of a weak water ligand.

The 1.8 K, 7 T NIR MCD spectrum of the substrate-bound complex, HPPD/Fe^{II}/HPP, (Figure 2C) contains ligand field features at <5000, ~8500, and ~11500 cm^{-1} and a charge-transfer feature (CT) at >15000 cm^{-1}, indicating HPP coordinates to Fe^{II}. As for resting HPPD/Fe^{II}, the presence of more than two ligand field transitions indicates a mixture of Fe^{II} species for HPPD/Fe^{II}/HPP, with the band at <5000 cm^{-1} requiring the presence of a 5C species. The presence of at least two bands in the 8−10000 cm^{-1} region indicates a 6C species is also present which must be the dominant component based on the low intensity of the <5000 cm^{-1} band and Fe K-pre-edge XAS (unpublished results). The saturation magnetization behavior for the 6C band at ~8500 cm^{-1} (inset of Figure 2C) is fitted as a ZFS non-Kramers doublet with ground-state spin Hamiltonian parameters of $\delta = 2.4 \pm 0.2$ cm^{-1} and $g_{||} = 8.9 \pm 0.3$, corresponding to $\Delta = -1000 \pm 200$ cm^{-1} and $|V/2\Delta| \approx 0.25$. The 1.8 K, 7 T UV/vis MCD spectrum (Figure S4) and ligand field splitting parameters of HPPD/Fe^{II}/HPP are nearly identical to those reported for CS2/Fe^{II}/α-KG (Fe^{2+}→ α-KG CT) and, from past correlations to model complexes, indicate bidentate binding of the α-keto acid moiety of HPP to Fe^{II}.[7,8]

Previous studies of mononuclear non-heme iron enzymes have shown two general approaches to oxygen activation: (1) a 6C resting Fe^{II} site which goes 5C upon substrate (and cofactor where required) binding (e.g., CS2,[7,8]) or (2) a 5C resting site which remains 5C upon substrate binding (e.g., 1,2-DHBD[9]). In both cases, the open coordination position provides a site for the oxygen reaction. However, the one-electron reduction of O_2 is unfavorable due its low redox potential and the formation of a ferric superoxide intermediate with a weak $Fe^{3+}-O_2^-$ bond.[9] The α-KG-dependent dioxygenases circumvent this problem by having the α-KG cofactor which binds to Fe^{II}, and when O_2 binds, the Fe^{II} and α-KG together can supply the three electrons necessary to generate a ferryl intermediate, while the extradiol dioxygenases utilize substrates (catecholates) which themselves supply all the necessary electrons. HPPD bridges these two approaches since the HPP substrate contains an internal α-keto acid moiety which supplies the additional electrons required for dioxygen reduction.

The MCD spectrum of resting HPPD is very similar to a combination of the spectra for resting CS2 (6C)[7] and resting 1,2-DHBD (5C)[9] (Figure 2A), in contrast to the 4C ferric active site observed crystallographically. Whereas 1,2-DHBD is purely 5C due to the steric interaction of a tyrosine residue near the iron site and the lack of an internal hydrogen bond from the monodentate carboxylate residue to a water ligand[9] (as found for 6C CS2 and phenylalanine hydroxylase), the orientation of the mondentate glutamate residue in HPPD also appears to preclude an internal H-bond and contributes to the generation of a 5C site.[3] In HPPD, the bulky tyrosine residue is replaced by a more distant, conserved phenyalanine, resulting in a mixture of two Fe^{II} active-site structures in HPPD as required by the above CD/MCD results, a behavior intermediate between the purely 5C site of 1,2-DHBD and the purely 6C site of CS2, While a purely 6C resting site is necessary for α-KG dioxygenases to minimize O_2 activation with bound α-KG cofactor in the absence of substrate, the 5C component of resting HPPD is not prone to uncoupled reaction since it is incapable of binding the α-KG cofactor.

Binding of HPP to resting HPPD results in a mixture of distinct 5C and 6C iron active sites. The metal-to-ligand CT transitions show the α-keto acid moiety binds to the 6C component while the higher-energy 5C feature of HPPD/Fe^{II}/HPP (Figure 2b) in the ~11000 cm^{-1} region changes dramatically from that in HPPD/Fe^{II}, indicating that HPP is also bound in the 5C component. However, the inability of this interaction to increase the amount of 5C present (likely due to conformational restrictions of having substrate and cofactor covalently linked) suggests the resting site must have a 5C component to maintain the open coordination position upon substrate binding. Unlike the resting enzyme, the 5C component present in substrate-bound HPPD would be highly reactive toward O_2 as all the necessary reducing equivalents for O_2 activation are present, thus enabling catalysis. The reactivity of the 5C component in HPPD/Fe^{II}/HPP is supported by ongoing studies of a series of substrate analogues in which lack of reactivity with phenylpyruvate (PPA) correlates with elimination of the low-energy 5C band in the MCD upon PPA binding.[10] If the 6C component of HPPD/Fe^{II}/HPP is sufficiently activated (i.e. having a weak H_2O ligand), it could also contribute to O_2 activation.

In summary, we have presented herein the first study to directly probe the resting Fe^{II} active site of HPPD and its interaction with substrate. Our CD/MCD results support a resting ferrous active site and approach to oxygen activation by HPPD which bridges those of the α-KG-dependent and extradiol dioxygenases.

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Supporting Information Available: NIR CD/MCD spectra of FeHPPD + α-KG, UV/vis MCD of FeHPPD + HPP and EPR and UV/vis absorption spectra of apoHPPD. This material is available free of charge via the Internet at http://pubs.acs.org.

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