# Substrate Binding to the α-Ketoglutarate-Dependent Non-Heme Iron Enzyme Clavaminate Synthase 2: Coupling Mechanism of Oxidative Decarboxylation and Hydroxylation

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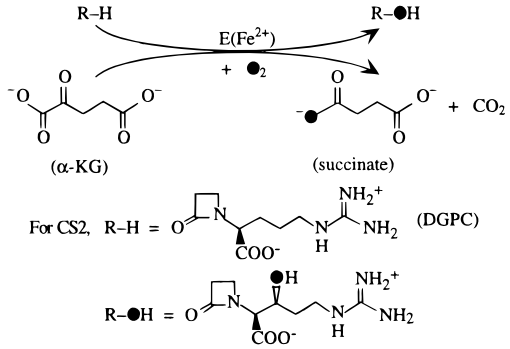
Clavaminate synthase 2 (CS2) belongs to the largest class of mononuclear non-heme iron enzymes, which require α-ketoglutarate (α-KG) as a cosubstrate.[1] Most members of this class catalyze a coupled reaction of the hydroxylation of an unactivated C−H bond in a substrate and the oxidative decarboxylation of the cosubstrate α-KG, leading to succinate and $CO_2$.[2,3] During the reaction, one atom of dioxygen is incorporated as the hydroxyl group of the product and the other into the carboxylate group of succinate (Scheme 1). CS2 catalyzes the hydroxylation of deoxyguanidinoproclavaminic acid (DGPC) to guanidinoproclavaminic acid.[4] It can also catalyze the oxidative cyclization of proclavaminic acid and the subsequent desaturation of the intermediate to clavaminic acid, which is metabolized into the potent *β*-lactamase inhibitor clavulanic acid.[1,5] Our previous studies on the ferrous active site and its interaction with α-KG using circular dichroism (CD), magnetic circular dichroism (MCD), and variable-temperature and variable-field (VTVH) MCD spectroscopies have demonstrated that resting CS2 contains a six-coordinate ferrous center and that α-KG binds directly to $Fe^{2+}$ ion in a bidentate mode.[6] Here we communicate the effects of interaction of the DGPC substrate with the CS2/$Fe^{2+}$ active site in the absence and presence of α-KG. Our results show that, only when both substrate DGPC and cosubstrate α-KG are present, the ferrous site is converted into a five-coordinate species which can react with dioxygen to form an active oxygen intermediate. This provides significant insight into how the oxidative decarboxylation of α-KG is coupled to the hydroxylation of the substrate.

Recombinant CS2 was purified according to published procedures[7] and modifications.[6] The substrate DGPC was synthesized as previously described[8] and added in microliter quantities to the enzyme sample from anaerobic stock solutions in MOPS buffer, pD 7.0. Samples for MCD spectroscopy contained 55−65% (v/v) of degassed glycerol-$d_3$ as a nonpurturbing (see below), low-temperature glassing agent. Near-IR (600−2000 nm) and UV/vis (300−850 nm) CD titrations and near-IR MCD spectroscopies were performed as described.[6]

**Scheme 1**




A high-spin $Fe^{2+}$ ion produces d→d transitions in the near-IR region with a characteristic energy splitting pattern which reflects the coordination geometry and can be directly probed by CD and MCD spectroscopies.[9] Six-coordinate distorted octahedral $Fe^{2+}$ sites exhibit two transitions at ∼10 000 $cm^{-1}$, split by ∼2000 $cm^{-1}$; five-coordinate sites exhibit transitions at ∼10 000 and ∼5000 $cm^{-1}$; and distorted tetrahedral four-coordinate sites exhibit transitions only in the 4000−7000-$cm^{-1}$ region.[10]

Addition of DGPC (up to 15 equiv) to CS2/$Fe^{2+}$ without α-KG causes no change to the weak near-IR CD bands (two transitions at 8370 and 10 740 $cm^{-1}$, see Supporting Information), which were assigned as d→d transitions of the $Fe^{2+}$ ion in the resting CS2/$Fe^{2+}$.[6] Therefore, in the absence of α-KG, either DGPC substrate does not bind to the enzyme, or its binding does not perturb the $Fe^{2+}$ site. Figure 1 shows the near-IR and UV/vis (inset) CD titration of CS2/$Fe^{2+}$ with DGPC in the presence of α-KG. Before DGPC was added, the CS2/$Fe^{2+}$/α-KG complex showed a characteristic CD spectrum: an unsymmetric negative near-IR band centered around 9000 $cm^{-1}$, which is resolved into two $Fe^{2+}$ d→d transitions at 8650 and 10 400 $cm^{-1}$; a broad visible band from 16 000 to 26 000 $cm^{-1}$, and an intense UV band at ∼28 500 $cm^{-1}$ assigned as an $Fe^{2+}$ 3d to α-KG $\pi^*$ metal-to-ligand charge transfer (MLCT) transition and an α-KG n→$\pi^*$ transition, respectively. [6] When DGPC was added, a new low-energy near-IR band at ∼5000 $cm^{-1}$ developed with a concomitant intensity decrease and energy increase of the high-energy near-IR band, Figure 1. These changes saturate between 1.0 and 1.5 equiv of DGPC relative to CS2/$Fe^{2+}$/α-KG complex ($K_B$ > 5000 $M^{-1}$, consistent with steady-state kinetics[4]). The new CS2/$Fe^{2+}$/α-KG/DGPC species had two bands in the near-IR CD spectrum, one ∼5000 and the other ∼9200 $cm^{-1}$, consistent with a five-coordinate $Fe^{2+}$ site. Figure 1 inset shows the corresponding CD titrations in the UV/vis region. During the titration, the broad MLCT band (∼21 000 $cm^{-1}$) increased in intensity while the α-KG n→$\pi^*$ band (28 500 $cm^{-1}$) decreased in intensity. MLCT intensity requires direct orbital overlap, thus α-KG binding to the $Fe^{2+}$. From model studies,[11] the MLCT spectrum of the CS2/$Fe^{2+}$/α-KG complex requires that α-KG binds in a bidentate mode.[6] The fact that these transitions remain at virtually the same energy with substrate binding[12] demonstrates that, in the CS2/$Fe^{2+}$/α-KG/DGPC complex, α-KG remained bound to $Fe^{2+}$ in a bidentate mode. Addition of 55% (v/v) glycerol-$d_3$ did not alter the above CD spectrum.

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(12) Note that the absorbance and MCD intensities of both of the MLCT and n→$\pi^*$ transition also do not change (data not shown), indicating that the CD intensity changes are associated with a site conformation change upon substrate binding.

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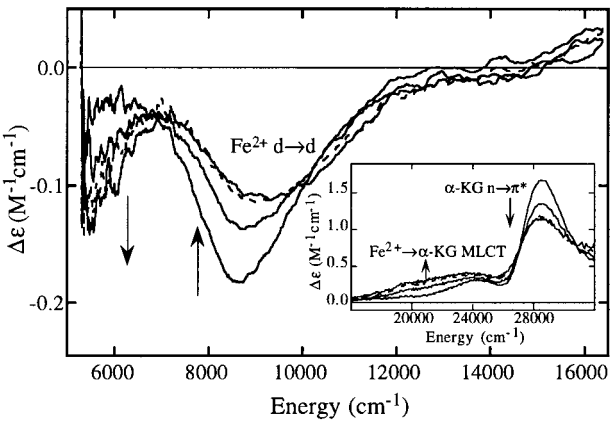



**Figure 1.** Near-IR CD titration studies of DGPC substrate binding with CS2/$Fe^{2+}$/α-KG complex at 4 °C. Amount of DGPC added is 0, 0.5, 1.0 (dashed line), and 1.5 equiv relative to CS2/$Fe^{2+}$/α-KG. Inset: Corresponding CD titration spectra in the UV/vis region. Note: Near-IR CD data have been smoothed in order to more clearly show the low-energy band at ~5000 $cm^{-1}$, which is not in its entirety due to a solvent cutoff at ~1900 nm (~5260 $cm^{-1}$).

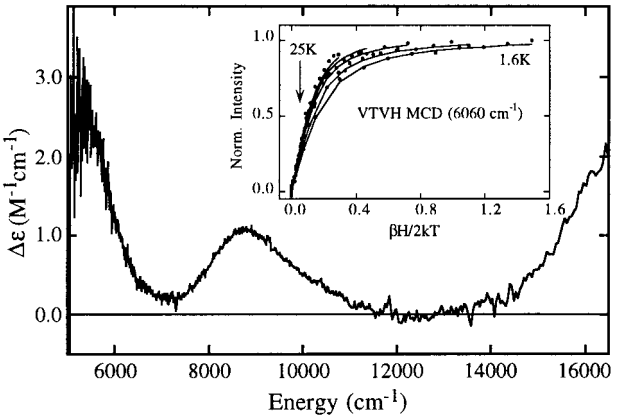
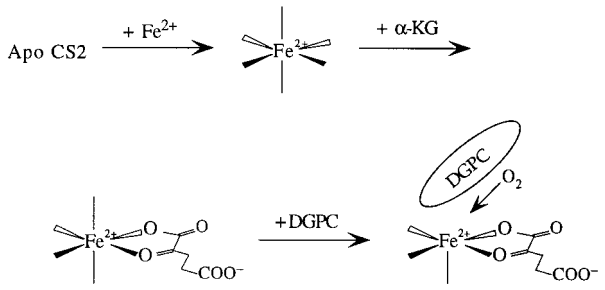



**Figure 2.** Near-IR MCD spectrum for CS2/$Fe^{2+}$/α-KG/DGPC complex at 5 K and 7 T. Inset: Normalized VTVH MCD data (symbols) for a series of fixed temperatures (1.6, 2, 3, 5, 8, 15, and 25 K) and the best fit (lines, see text) for the CS2/$Fe^{2+}$/α-KG/DGPC complex recorded at 6060 $cm^{-1}$.

Figure 2 shows the near-IR MCD (5 K, 7 T) spectrum of this species. The spectrum contains two peaks at ~5200 and 8800 $cm^{-1}$, giving $\Delta^5E_g = 3600$ $cm^{-1}$. The low-temperature near-IR MCD is consistent with the CD studies with only a small temperature shift of the higher energy band to ~400 $cm^{-1}$ lower energy. The VTVH MCD data (symbols) observed for CS2/$Fe^{2+}$/α-KG/DGPC complex at 6060 $cm^{-1}$ and the best fit (lines) to the data are shown in the inset of Figure 2. The VTVH MCD data cannot be fit with a negative zero-field splitting (ZFS) non-Kramers doublet model, while the fit using a positive ZFS model gives reasonable results.[9] The ground-state spin Hamiltonian parameters obtained from the fit are $g_{||} = 9.2 \pm 0.2$ and $\delta = 2.9 \pm 0.2$ $cm^{-1}$, corresponding to $D = +8.5$ $cm^{-1}$ and $|E/D| = 0.20$. Thus, the octahedral $^5T_{2g}$ ground-state splitting parameters are $\Delta \equiv d_{xz,yz} - d_{xy} = 850 \pm 100$ $cm^{-1}$ and $|V| \equiv d_{yz} - d_{xz} = 450 \pm 100$ $cm^{-1}$ (see ref 9 for details of analysis). Therefore, from the near-IR CD, MCD, and VTVH MCD studies, the active site of the CS2/$Fe^{2+}$/α-KG/DGPC complex is described as a five-coordinate, strong axial, square-pyramidal ferrous site.

The above results lead to the CS2 catalytic mechanism for substrate hydroxylation in Scheme 2. After $Fe^{2+}$ and α-KG binding, DGPC substrate binds to the active site in the proximity of the $Fe^{2+}$ ion, and the interaction of DGPC with the protein matrix results in the dissociation of an $Fe^{2+}$ ligand, forming a five-coordinate site with an approximate square-pyramidal geometry. In the literature for various α-KG-dependent enzymes,[13−15] the binding of substrate is usually considered to follow the binding of dioxygen based on the observation of uncoupled oxidative decarboxylation of α-KG in the absence of substrate, which leads to enzyme deactivation. This order is not consistent with the slow rate of the uncoupled reaction (at most, several percent of the coupled reaction).[13−15] Alternately, from the CD and MCD studies, the binding of substrate forms a five-coordinate species, which has an open coordination position on $Fe^{2+}$ for rapid $O_2$ reaction in the mechanism for coupled hydroxylation. Furthermore, the conversion of the $Fe^{2+}$ site to a five-coordinate species only in the presence of both substrate (DGPC) and cosubstrate (α-KG) provides a mechanism through which the oxidative decarboxylation of cosubstrate and the hydroxylation of substrate are coupled. Since the coupled hydroxylation is a critical characteristic of most members of α-KG-dependent non-heme enzymes, it is reasonable to consider the molecular mechanism in Scheme 2 to be general for this class. The conversion from a six- to a five-coordinate $Fe^{2+}$ center upon substrate binding has been observed in other mononuclear non-heme enzymes (phthalate dioxygenase,[16] pterin-dependent phenylalanine hydroxylase,[17] and isopenicillin N synthase[18]) as well as in the binuclear non-heme enzymes (stearoyl-ACP $\Delta^9$-desaturase[19]). There appears to be a general mechanistic strategy present for a number of non-heme iron enzymes where the ferrous site is six-coordinate and relatively unreactive with $O_2$, while upon substrate (and cosubstrate) binding an open coordination position becomes available on the $Fe^{2+}$ for reaction with $O_2$, which generates a highly active oxygen intermediate for direct attack at the unactivated substrate.

**Scheme 2**

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**Supporting Information Available:** Near-IR CD spectra of the CS2/$Fe^{2+}$ complex in the absence and in the presence of substrate DGPC, showing no spectral change (1 page, print/PDF). See any current masthead page for ordering information and Web access instructions.

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