Alternative Reactivity of an α-Ketoglutarate-Dependent Iron(II) Oxygenase: Enzyme Self-Hydroxylation

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α-Keto acid-dependent oxygenases, which constitute the largest class of mononuclear nonheme iron enzymes,[1] couple the oxidative decarboxylation of an α-keto acid to the oxidation of a target unactivated C−H bond on the primary substrate.[2] Recent crystallographic studies of CAS and DAOCS[3,4] show that the ferrous center is coordinated to a 2-His-1-carboxylate facial triad,[5] with bidentate coordination of the cosubstrate α-ketoglutarate (α-KG); the remaining coordination site is presumably for O_2. This report focuses on TfdA, a ferrous enzyme that catalyzes the first step in the biodegradation of the herbicide 2,4-dichlorophenoxyacetate (2,4-D).[6] We show that the Fe(II)TfdA(α-KG) complex reacts with O_2 even in the absence of 2,4-D to form a blue species. This novel product arises from the hydroxylation of an active site residue (Trp112) and provides insight into the nature of the reactive species involved in oxygen activation.

The addition of α-KG and Fe(II) to TfdA apo protein[6a] under Ar elicits a weak pink chromophore ($\lambda_{max} \sim 530$ nm, $\epsilon \sim 200$ M^{-1} cm^{-1}) that is associated with Fe(II)-to-(α-KG) charge-transfer transitions[6b,7] (Figure 1). Addition of the primary substrate 2,4-D and subsequent exposure to O_2 immediately result in the loss of the pink color and enzyme turnover (12.1−13.3 μmol/min/mg) producing 2,4-dichlorophenol, glyoxalate, succinate, and CO_2. In the absence of 2,4-D, however, Fe(II)TfdA(α-KG) reacts with O_2 over a 30-min period at 22 °C to elicit a blue chromophore ($\lambda_{max} \sim 580$ nm; $\epsilon \sim 1000$ M^{-1} cm^{-1}; Figure 1). EPR studies show that the blue species is a high-spin Fe(III) complex, with EPR features at $g = 7.4$, 5.8, 4.3, and 2.0 ($S = {}^5/_2$, and $E/D \sim 0.06$) (Figure S1, Supporting Information). Spin quantification shows that these signals reflect over 90% of the iron present,

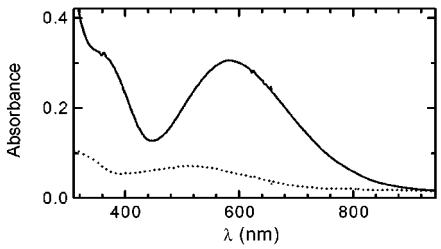

Figure 1. Visible spectrum of Fe(II)TfdA(α-KG) (0.3 mM in subunits in 25 mM Tris-HCl buffer, pH 8.0) (dotted trace) and that its air-oxidized blue product (solid trace).

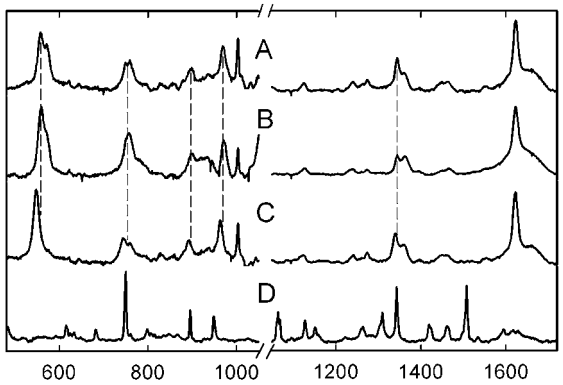

Figure 2. Resonance Raman spectra (λ_{ex} 568.2 nm, 22 °C) of blue TfdA (1.5 mM) prepared in $H_2{}^{16}O$ buffer with $^{16}O_2$ (A) in $H_2{}^{16}O$ buffer with $^{18}O_2$ (B) and in $H_2{}^{18}O$ buffer with $^{16}O_2$ (C) and the Raman spectrum of 5-hydroxyindole recorded at 77 K (D).

suggesting that the chromophore originates from ligand-to-iron(III) charge-transfer transitions.

Resonance Raman spectroscopy is a useful probe for ligand-to-metal charge transfer transitions, as ligand vibrations are selectively enhanced. The resonance Raman spectrum of the blue TfdA sample exhibits a set of peaks at 564, 750, 898, 970, 1240, 1274, 1344, and 1622 cm^{-1} (Figure 2A). These vibrations likely arise from a hydroxylated arene, but one which differs from the Tyr and catecholate residues found in previously characterized iron proteins (Table 1).[8,9] In general the features around 500−600 cm^{-1} are assigned to metal−ligand vibrations, while those above 1100 cm^{-1} are associated with deformations of the aromatic ring. Note, for example, the absence of vibrations at ca. 1170 and 1500 cm^{-1} in TfdA and the presence of features in the 800−1000 cm^{-1} region not found in the other proteins. The results for TfdA show that the modified residue does not arise from the hydroxylation of a Phe or Tyr residue. Comparison with model compounds indicates that the TfdA features most closely correspond to peaks observed for 5-hydroxyindole, suggesting the hydroxylation of a tryptophan residue in TfdA (Table 1).

The hydroxylated Trp residue was identified by nanoscale capillary LC/MS/MS analysis of the trypsin digested blue TfdA sample (Figure S2). Product ion spectra were searched against a theoretical TfdA digest in silico, and the doubly charged parent ions of two related peptides were observed: one at *m*/*z* 1295

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(3) Abbreviations used: 2,4-D, 2,4-dichlorophenoxyacetate; α-KG, α-ketoglutarate (or 2-oxoglutarate); CAS, clavaminic acid synthase; DAOCS, deacetoxycephalosporin C synthase; HPP, *p*-hydroxyphenylpyruvate; RNR, ribonucleotide reductase; TfdA, 2,4-D/α-KG dioxygenase.

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Table 1. Resonance Raman Features of Nonheme Iron Protein Chromophores

protein	resonance Raman shifts (cm^{-1})											
transferrin[8a,b]		570					1174	1288			1508	1613
uteroferrin[8a,c]		575		805	872		1168	1285			1503	1603
HPP diogenase[8d]		583		751	881		1170	1284			1502	1601
tyrosine hydroxylase-dopamine[9a]	527	604	635				1130	1271	1320	1426	1476	
RNR R2 Dopa-298[9b,12]	512	592	619				1143	1263	1319	1350	1475	1569
TfdA (^{16}O)		**564**		**750**	**898**	**970**	1240	1274	**1344**			1622
TfdA (^{18}O)		**549**		**746**	**892**	**964**	1240	1274	**1340**			1622
5-hydroxyindole			616	750	896	947	1226	1273	1344			

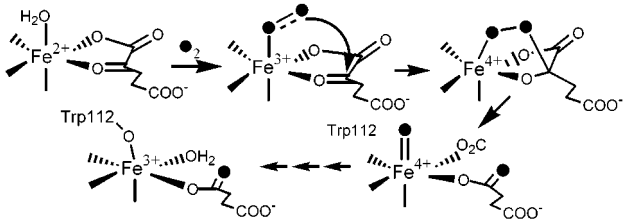

Figure 3. Proposed mechanism for the self-hydroxylation reaction.

corresponding to a fragment containing residues 102−124 and the other at *m*/*z* 1303 corresponding to the same peptide fragment but with a 16-dalton mass increase. Further MS/MS analysis identified the hydroxylated residue as HO-Trp112. Notably, this residue is adjacent to His113, a metal ligand.[6a]

Activity assays show that blue TfdA is inactive even in the presence of ascorbate and excess α-KG. However, treatment with the stronger reductant dithionite recovers 62 ± 12% of the activity. Furthermore, if dithionite reduction is followed by dialysis against EDTA for 6 h and against pH 8.0 Tris-HCl buffer (25 mM) overnight and then reconstitution with $Fe(NH_4)_2(SO_4)_2$, 85 ± 15% of the enzyme activity is regained, so the catalytically active site can be regenerated, presumably by displacing the HO-Trp ligand.

The observed hydroxylation of Trp112 occurs at the expense of α-KG, which is decarboxylated into succinate and CO_2 (Table S1) in an O_2-dependent reaction. The active oxidant for this class of enzymes has been proposed to be either an iron-peroxo or a high valent iron-oxo species derived from the oxidative decarboxylation of α-KG (Figure 3).[1,2] In the absence of the primary substrate, this oxidant attacks a nearby aromatic residue in the active site to afford the HO-Trp. Ligation to the iron center and subsequent autoxidation to the Fe(III) state generates the intense chromophore.

Isotope labeling experiments provide insight as to the source of the oxygen atom that is incorporated into Trp112. Blue TfdA generated with $^{18}O_2$ gives a resonance Raman spectrum identical to that of the sample prepared with $^{16}O_2$ (Figure 2B), while the sample prepared in $H_2{}^{18}O$ with $^{16}O_2$ exhibits a spectrum in which several of the features are downshifted (Figure 2C and Table 1). The feature at 564 cm^{-1} exhibits the largest downshift of 15 cm^{-1}, a change comparable to ^{18}O-downshifts of 8−13 cm^{-1} observed for the Fe−O−aryl vibrations of phenolate and catecholate complexes at ca. 600 cm^{-1}, when one ^{18}O label is introduced.[9a,10] These studies implicate an intermediate that allows water to become fully incorporated into OH-Trp in TfdA, and exclude the direct hydroxylation of Trp112 by the iron-peroxo species. Indeed some ^{18}O from $H_2{}^{18}O$ has been found to be incorporated into products of the reactions catalyzed by CAS, DAOCS, and α-ketoisocaproate oxygenase.[11] These ^{18}O incorporation results are consistent with two possible pathways: (a) a concerted oxygen-atom transfer to Trp112 from an Fe(IV)-oxo oxidant that is fully equilibrated with $H_2{}^{18}O$ prior to the transfer or (b) a stepwise mechanism involving one-electron oxidation of Trp112 by an iron-peroxo or iron-oxo species to form a Trp radical intermediate and then oxidative C−O bond formation with an Fe(III)−OH species that is fully equilibrated with solvent water, as proposed for the formation of Dopa-208 in F208Y RNR R2.[12]

Many α-KG-dependent dioxygenases are known to undergo uncoupling reactions and self-inactivate,[2a,b] but molecular details of the inactivation have not been available. Indeed the transformation of TfdA into a blue chromophore is the first instance of enzyme self-hydroxylation reported for this diverse class of α-keto acid-dependent enzymes. Such self-hydroxylation may also occur for another α-keto acid-dependent enzyme, HPP dioxygenase, which was reported to have a blue chromophore ($\lambda_{max} \sim 595$ nm) associated with a tyrosinate ligand.[8d] However, no tyrosine is coordinated to the Fe center in the recently reported crystal structure of the enzyme.[13] Based on the present study on TfdA, the apparent contradiction between the spectroscopic and crystallographic results for HPP dioxygenase may be resolved by suggesting the blue form results from an "uncoupled" self-hydroxylation of a phenylalanine residue in the active site.

Besides TfdA and HPP dioxygenase, other instances of enzyme self-hydroxylation in the course of oxygen activation have also been reported for mutant RNR R2 proteins[9b,14] and the pterin-dependent tyrosine hydroxylase.[15] These results lead us to consider the possibility that the "uncoupled" self-hydroxylation reaction may not be an uncommon side reaction of nonheme iron enzymes. For those that require an α-keto acid or a pterin, this side reaction may have biochemical significance and serve to protect the enzymes from a more destructive irreversible oxidation such as radical-induced peptide chain cleavage, which would lead to a permanent loss of the catalytic activity. In addition, the slower rate of the "uncoupled" reaction relative to the coupled reaction may afford us an opportunity to investigate yet unobserved intermediate steps in the mechanism of this fascinating class of oxygenases.

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Supporting Information Available: Figure S1 showing an EPR spectrum of the blue TfdA protein, Figure S2 showing mass spectrometric data confirming the presence of HO-Trp112, and Table S1 providing the data of the stoichiometry of α-KG consumption and succinate formation in the O_2-dependent reaction (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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