Site-directed Mutagenesis of 2,4-Dichlorophenoxyacetic Acid/α-Ketoglutarate Dioxygenase

IDENTIFICATION OF RESIDUES INVOLVED IN METALLOCENTER FORMATION AND SUBSTRATE BINDING*

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2,4-Dichlorophenoxyacetic acid (2,4-D)/α-ketoglutarate (α-KG) dioxygenase (TfdA) is an Fe(II)-dependent enzyme that catalyzes the first step in degradation of the herbicide 2,4-D. The active site structures of a small number of enzymes within the α-KG-dependent dioxygenase superfamily have been characterized and shown to have a similar HXXD₅₀₋₇₀HX₁₀ᵦRX₅₁/RK₁₀₅ arrangement of residues that make up the binding sites for Fe(II) and α-KG. TfdA does not have obvious homology to the dioxygenases containing the above motif but is related in sequence to eight other enzymes in the superfamily that form a distinct consensus sequence (HX(D/E)X₁₁₈₋₁₂₇ HX₁₁₀ᵦRX/RK). Variants of TfdA were created to examine the roles of putative metal-binding residues and the functions of the other seven histidines in this protein. The H167A, H200A, H213A, H245A, and H262A forms of TfdA formed inclusion bodies when overproduced in Escherichia coli DH5α; however, these proteins were soluble when fused to the maltose-binding protein (MBP). MBP-TfdA exhibited kinetic parameters similar to the native enzyme. The H8A and H235A variants were catalytically similar to wild-type TfdA. MBP-H213A and H216A TfdA have elevated $K_m$ values for 2,4-D, and the former showed a decreased $k_{cat}$, suggesting these residues may affect substrate binding or catalysis. The H113A, D115A, MBP-H167A, MBP-H200A, MBP-H245A and MBP-H262A variants of TfdA were inactive. Gel filtration analysis revealed that the latter two proteins were highly aggregated. The remaining four inactive variants were examined in their Cu(II)-substituted forms by EPR and electron spin-echo envelope modulation (ESEEM) spectroscopic methods. Changes in EPR spectra upon addition of substrates indicated that copper was present at the active site in the H113A and D115A variants. ESEEM analysis revealed that two histidines are bound equatorially to the copper in the D115A and MBP-H167A TfdA variants. The experimental data and sequence analysis lead us to conclude that His-113, Asp-115, and His-262 are likely metal ligands in TfdA and that His-213 may aid in catalysis or binding of 2,4-D.

2,4-Dichlorophenoxyacetic acid (2,4-D)³/α-ketoglutarate (α-KG) dioxygenase (TfdA) is an Fe(II)- and α-KG-dependent enzyme that catalyzes the first step in degradation of the herbicide 2,4-D. This enzyme couples the oxidative decarboxylation of α-KG to the hydroxylation of a side chain carbon atom. The resultant hemiacetal spontaneously decomposes to form 2,4-dichlorophenol and glyoxalate (1). Mechanistically, TfdA resembles numerous other α-KG-dependent dioxygenases from plants, animals, fungi, and bacteria that catalyze similar hydroxylation reactions at unactivated carbon centers (2, 3).

Members of the α-KG-dependent dioxygenase superfamily are not closely related by their sequences but rather appear to fall into one of three groups of related enzymes or fall into a fourth group of unrelated sequences (4). The best studied α-KG-dependent hydroxylases, including prolyl and lysyl hydroxylase (5) and flavanone hydroxylase (3, 6), have an HXXD₅₀₋₇₀HX₁₀ᵦRX₅₁/RK₁₀₅ motif in common (7, 8), and this motif is present in the more than 20 enzymes, defined here as Group I, within the α-KG-dependent dioxygenase superfamily (8, 9). Site-directed mutagenesis studies have confirmed the importance of these residues for activity (8, 10–13), and the crystal structures of two Group I enzymes, isopenicillin N synthase (IPNS) and deacetoxycephalosporin C synthase (DAOCS), indicate that these residues comprise the metallocenter and α-KG-binding site (14–17). TfdA is not closely related in sequence to the Group I α-KG-dependent dioxygenases described above but is clearly homologous (25–30% identity) to Escherichia coli taurine/α-KG dioxygenase (Taud) (18) and sulfonate/α-KG dioxygenase from Saccharomyces cerevisiae (19). Furthermore, PSI-BLAST analyses (20) find additional rela-

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1 The abbreviations used are: 2,4-D, 2,4-dichlorophenoxyacetic acid; α-KG, α-ketoglutarate; TfdA, 2,4-D/α-KG dioxygenase; TauD, taurine/α-KG dioxygenase; cw-EPR, continuous wave electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; IPNS, isopenicillin N synthase; DAOCS, deacetoxycephalosporin synthase; MBP, maltose-binding protein; FT, Fourier transform; MOPS, 4-morpholinepropanesulfonic acid.
tionships to γ-butyro betaine hydroxylase and clavamine synthase. Alignment of these Group II sequences indicates the conservation of two histidines and one aspartate (His-113, His-262, and Asp 115 in TfdA) as well as an invariant arginine that may be analogous to the α-KG-binding arginine in DAOCS and related enzymes (Table I). A third set of enzyme sequences (Group III) from members of the α-KG-dependent dioxygenase superfamily, including phytanoyl-CoA hydroxylase and proline hydroxylase, exhibit the presence of a third related motif despite the lack of overall sequence similarity to Group I or Group II enzymes.

In this study, we used site-directed mutagenesis methods to examine the roles of potential metal-binding residues in the above motif (His-113, His-262, and Asp-115) and the remaining seven histidines in TfdA. Previously published work showed that TfdA was inactivated by diethylpyrocarbonate, a histidine-selective reagent, and provided evidence consistent with the presence of multiple histidines in the active site (21). Spectroscopic studies of TfdA showed the presence of two equatorially bound imidazole nitrogens as ligands to the active site metal and indicated that one imidazole ligand may be displaced or shifted to an axial position upon substrate binding (22–24). Based on analyses of different TfdA variants, we identify several likely metal ligands and provide evidence that another one or two histidines may aid in substrate binding.

**EXPERIMENTAL PROCEDURES**

**Recombinant Plasmids—**All plasmids were constructed from pUS311 (21), a pUC19 derivative that contains the *Rhodobacter sphaeroides* JMP134 tfdA gene (Fig. 1). The H8A, D115A, H213A, H216A, H235A, and H262A TfdA variants were created by direct mutation of tfdA in pUS311 by the Stratagene Quickchange System (Stratagene, La Jolla, CA). All mutagenic primers are listed in Table II. Two alternative approaches were used to construct the three remaining variants. Plasmids encoding H133A and H167A TfdA variants were created by CLONTECH mutagenesis of pXHtfdA, a pUC19 plasmid containing the 5′-tfdA-HindIII fragment of the tfdA gene (Fig. 1). To create the complete gene containing the indicated mutations, the Xbal-HindII fragment was cloned into pHKtfdA, which contains the 3′ end of the tfdA gene. pHKtfdA was constructed in two steps. First, the 1.4-kilobase pair XbaI-SauI fragment from pUS311, containing the complete tfdA gene, was cloned into pBC KS (Stratagene) cut with XbaI and XhoI to give the complete pBCtfdA. This step had the benefit of eliminating a HindII site that interfered with further cloning steps. The 727-base pair internal HindII box of pBCtfdA was cut with XhoI and XbaI to give complete H200A tfdA gene. The identity of all final constructs was confirmed by sequence analysis. To insert the genes encoding H167A and H262A variants of TfdA into a plasmid that would allow for isopropyl-1-thio-β-D-galactopyranoside-controlled expression, the XhoI-SauI fragments from the corresponding plasmids described above were cloned into pET22a (Novagen) prepared with the same enzymes.

To create the metal-binding protein (MBP)-TfdA fusion proteins, the wild-type tfdA gene was amplified from pUS311 with TfdA-MBPF and TfdA-MBPR primers (Table II) to create an XbaI site directly upstream of the GTC start codon of the tfdA gene and the HindIII restriction site 54 base pairs downstream of the stop codon. The polynucleotide chain reaction product was cloned directly into the pGEM-T vector according to the manufacturer’s instructions (Promega, Madison, WI). The XbaI-HindIII fragment was isolated from the resulting plasmid and cloned into the pMAL-c2 vector that had been digested with the same enzymes. The identity of the newly created *malE*-tfdA gene fusion was confirmed by sequencing. Substitution of the mutation-containing internal NruI fragment for the same fragment of the wild-type *malE*-tfdA gene created MBP-fusion forms of altered TfdAs. First, pMAL-tfdA was digested with NruI, and the vector fragment was purified and ligated to create pMAL-tfdAA/NruI. The resultant plasmid was linearized with NruI and dephosphorylated with calf intestine alkaline phosphatase prior to ligation with the NruI fragments isolated from the previously described mutant genes. Constructs were confirmed by restriction analysis.

**Protein Purification—**H8A, H113A, D115A, H216A, H235A, and wild-type TfdA proteins were purified from *E. coli* DH5α cells carrying pUS311 and its mutated derivatives according to a previously described protocol (21). In addition, the non-mutated enzyme and the TfdA variants H167A, H200A, H213A, H245A, and H262A were purified as MBP-TfdA fusion proteins from *E. coli* DH5α by the protocol described in the pMAL Protein Fusion and Purification System Manual (New England Biolabs, Beverly, MA).

**Analysis of Kinetic Parameters—**Specific activities of the wild-type and variant TfdA proteins were determined by previously described spectrophotometric assay (21). The typical assay mixture contained 1 mM 2,4-D, 1 mM α-KG, 100 mM (NH₄)₂Fe(SO₄)₂, and 100 mM ascorbic acid in 10 mM MOPS buffer (pH 6.75) at 30 °C. The reactions were quenched by the addition of EDTA to a concentration of 5 mM. 2,4-Dichlorophenol was quantified by reaction with 4-aminonitripyrrole followed by measurement of the absorbance at 510 nm. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of dichlorophenol/min. Protein concentrations were determined using...
the Bio-Rad Protein Assay with bovine serum albumin as a standard. For calculation of the \( k_{\text{cat}} \) values, the TfdA variants were assumed to have \( M_\text{T} = 31,600 \) and the MBP-TfdA variants were assigned \( M_\text{T} = 74,500 \).

The low \( K_m \) values for \( \alpha\)-KG (\( \sim 2-6 \mu \text{M} \) for the wild-type enzyme) precluded use of the 4-aminoantipyrine assay for accurate determination of this value. The alternative method used to measure the \( K_m \) values for \( \alpha\)-KG quantified the amount of \( ^{14}\text{C}\)CO\(_2\) liberated from \( \alpha\)-KG during the course of the reaction (21).

Native Protein Analysis by Gel Filtration—Size exclusion chromatography was used to estimate the native molecular weights of TfdA, MBP-TfdA, and mutant proteins. The proteins were chromatographed on a Superose 6 gel filtration column (1.0 × 30 cm, Amersham Pharmacia Biotech) in 20 mM Tris buffer (pH 7.5), 1 mM EDTA, and 200 mM NaCl at a flow rate of 0.2 ml/min. The elution volumes were compared with those for gel filtration standards (Bio-Rad) including thyroglobulin, myoglobin, bovine gamma globulin, chicken ovalbumin, 14-kDa ribonuclease, and vitamin B\(_12\), 3.5-kDa.

Spectroscopic Analysis—Proteins for electron paramagnetic resonance (EPR) and electron spin–echo envelope modulation (ESEEM) spectroscopic analyses were exchanged into 25 mM MOPS (pH 6.75) by repeated concentration and dilution in Centricon 30 (Amicon) centrifugal concentrators. The final subunit concentration was 0.5 mM for the non-fusion forms of TfdA and 0.4 mM for the MBP-TfdA proteins. CuCl\(_2\) was added to a concentration of 450 and 350 \( \mu \text{M} \), respectively. Buffered solutions of Cu(II) and 2,4-D were added for final concentrations of 5 mM. Glycerol was present at 40% in all samples.

X-band EPR spectra were obtained at 77 K on a Bruker ESP-300E spectrometer. ESEEM data were collected on a home-built spectrometer; the microwave bridge of this instrument has been previously described in detail (25). Data collection and analyses were controlled by a Power Computing model 200 Power PC using software written with LabView version 5.01 (National Instruments). Electron spin echoes were digitized, averaged, and integrated by a Tektronix model 620B digital oscilloscope interfaced to the spectrometer computer via an IEEE-488 bus. Two four-channel delay and gate generators (Stanford Research Systems model DGS35), a Bruker BH-15 magnetic field controller, and a Hewlett-Packard model 8656B radiofrequency synthesizer were also interfaced using IEEE-488 protocol. Data were collected using a reflection cavity that employed a folded microstrip resonator (26). A three-pulse stimulated echo sequence (90°-90°-90°-T-90°) was used.

ESEEM spectra were generated by Fourier transformation of the time domain data using dead time reconstruction (27). Simulations of the experimental data were performed on a Sun SparcIIf work station. Simulation programs were written in FORTRAN and based on the density matrix formalism developed by Mims (28). Software for the frequency analysis of the experimental and simulated data was written in Matlab (Mathworks, Natick, MA).

Sequence Comparisons—Related sequences were initially detected by BLAST (29) and PSI-BLAST (30) analyses. Alignments were generated using the CLUSTAL algorithm (30), and the figure was prepared using Genceed (31).

**RESULTS**

**Production of the Mutant TfdAs**—Initially, all of the mutant genes were expressed from their pUC19-based plasmids except for those encoding H113A, H167A, and H200A TfdA, which were in pBC KS-derived plasmids. By using the standard protocol to produce soluble, wild-type TfdA (growth at 30°C to early stationary phase), only the H8A, H113A, D115A, H216A, and H235A variants existed as soluble proteins. All of the other TfdA variants were present as inclusion bodies even when grown at lower temperatures (22°C), in M9 minimal medium, or in LB broth containing 660 mM sorbitol and 2.5 mM betaine (32). In addition, isopropyl-\( \beta\)-d-thiogalactopyranoside-controlled production of H1167A and H262A proteins from mutant genes cloned into pET23a did not yield soluble samples even when the harvested cell pellets were suspended in buffer containing 20% glycerol to limit protein aggregation.

To overcome the solubility problems for the five TfdA variants, MBP-TfdA fusion proteins were created. Wild-type TfdA and the MBP-TfdA fusion protein had essentially identical \( k_{\text{cat}} \) and very similar \( K_m \) values for \( \alpha\)-KG and 2,4-D (Table III). A slight increase in the apparent \( K_d \) for Fe(II) may reflect some metal binding capacity of MBP. Since the presence of the fusion protein did not appear to greatly affect the kinetic parameters of wild-type enzyme, similar fusion proteins were created for the H167A, H200A, H213A, H245A, and H262A TfdA variants.

**Kinetic Analyses of Altered TfdAs**—Results from kinetic analyses of the four active mutant proteins are summarized in Table III. H8A TfdA was soluble and active but was rapidly proteolyzed to an inactive form. By electrophoretic comparisons, the cleavage site appeared to be the same as in wild-type TfdA (between Arg-77 and Phe-78) (21). The rate of proteolysis of H8A TfdA was enhanced compared with that seen for the wild-type enzyme despite the presence of EDTA and protease inhibitors in the purification buffer. Because purified H8A TfdA was more than 75% degraded, the catalytic rate constant was calculated with the estimated amount of intact enzyme. These calculations indicate rates and \( K_m \) values similar to those for the wild-type enzyme. Similarly, the kinetic parameters for H235A TfdA were comparable to the native enzyme. In contrast, two variants exhibited differences from wild-type enzyme in their kinetic parameters. The H213A MBP-TfdA variant exhibited a 20-fold reduction in \( k_{\text{cat}} \) and a 10-fold increase in \( K_m \) for 2,4-D. In addition, H216A TfdA had a modest (2.5-fold) increase in the \( K_m \) for 2,4-D and no change in catalytic rate. The other kinetic parameters for H213A MBP-TfdA and H216A TfdA (\( K_m \) for \( \alpha\)-KG and \( K_d \) for ferrous ion) did not differ significantly from the wild-type values. Six soluble TfdA variants (H113A, D115A, MBP-H167A, MBP-H200A, MBP-H245A, and MBP-H262A) exhibited no activity even when assayed with elevated substrate and cofactor concentrations (10 mM \( \alpha\)-KG, 5 mM 2,4-D, and 250 \( \mu \text{M} \) Fe(II)).

**Evaluation of the Structural Consequences of the Mutations**—To assess whether the inactive mutant proteins assumed conformations similar to the wild-type enzyme, their apparent molecular weights were estimated by gel filtration analysis. The observed size of wild-type TfdA was found to be 51 kDa by comparison to protein standards, suggesting that TfdA forms a compact dimer or an elongated monomer. The elution volume for both H113A and D115A corresponded exactly to wild-type TfdA indicating that these proteins are not significantly altered in their quaternary structure. MBP-TfdA eluted both in the void volume (approximately 25% of the protein) and at a position corresponding to 216 kDa (roughly 75% of the protein), suggesting that MBP-TfdA forms at least a dimer. Because each MBP-TfdA subunit is comprised of two domains separated by a 13-amino acid linker, the resultant protein may migrate with a larger apparent molecular weight. MBP-H167A and MBP-H200A samples demonstrated the same two-peak profile as MBP-TfdA but with larger proportions eluting in the void volume. MBP-H245A and MBP-H262A proteins were soluble; however, gel filtration analysis indicated the presence of only highly aggregated material eluting in the void volume. Because these mutant proteins exhibited aberrations in their folding properties, the catalytic role of His-245 and His-262, if any, could not be assessed.
EPR Spectroscopic Characterization of Variants with Altered Metal Sites—The metallocenter properties for selected TfdA variants were probed by EPR spectroscopy. To circumvent the problems that arise in EPR measurements of integer spin paramagnetic centers, Fe(II) was substituted with cupric ion. Although the Cu(II) form of TfdA is inactive, Cu(II) binds competitively with respect to Fe(II) (\(K_a = 1–3 \times 10^5\)), and copper-substituted TfdA has been used previously to study the metal coordination environment of this enzyme in the presence and absence of substrates (22–24). Spectral parameters of wild-type Cu(II)-TfdA, Cu(II)-TfdA + α-KG, and Cu(II)-TfdA + α-KG + 2,4-D (Fig. 2A and Table IV), agreed well with those reported previously (23, 24). Earlier studies of copper-substituted wild-type TfdA indicated that the metal is bound in a type 2 environment with a mixture of O and N ligands in the equatorial plane. Upon addition of α-KG and 2,4-D to the enzyme, the spectral parameters are altered to a more rhombic signal with accompanying resolution of ligand hyperfine coupling (Fig. 2A). These results suggest that binding of the co-substrates to the enzyme leads to a better defined copper site with α-KG binding directly to the metallocenter (23, 24). The small \(A_g\) (less than 14 mT) for the α-KG- and 2,4-D-bound sample indicates a significant distortion from planarity.

The four inactive mutant forms of TfdA with quaternary structures similar to the corresponding wild-type protein (H113A, D115A, MBP-H167A, and MBP-H200A) were analyzed by EPR spectroscopy to assess the metal coordination environments. No significant differences between the spectra of MBP-TfdA and the non-fusion wild-type TfdA were observed (data not shown). EPR spectra of the copper-substituted samples, in all cases, showed contributions from multiple copper sites indicating a mixture of copper centers, most likely resulting from copper binding in multiple conformations or at sites other than the active site. The presence of alternative copper-binding sites is not surprising in an enzyme with nine histidines.

The EPR spectra for Cu-H113A TfdA alone and in the presence of α-KG and 2,4-D (Fig. 2B and Table IV) differ significantly from spectra of wild-type enzyme and show modest changes in Cu(II) \(g\) values and hyperfine tensor principal values upon substrate additions. The broadening of the EPR signal in the \(g\) region upon addition of α-KG again suggests a mixture of copper site conformations. Thus, it appears that alteration of His-113 significantly affects the metal binding properties for TfdA such that copper is no longer constrained to a single active site configuration.

The EPR spectrum of D115A-TfdA (Fig. 2C) has parameters similar to those observed in the copper-substituted wild-type protein (Table IV), although with less resolution of \(A_g\). Addition of α-KG (in the presence or absence of 2,4-D) has a dramatic effect on the appearance of the D115A data, enhancing resolution of the Cu(II) hyperfine peaks at \(g_{22}\) and the ligand hyperfine structure in the \(g_{22}\) region (Fig. 2C). The appearance of these superhyperfine interactions may result from a subtle shift in the orientation of the principal \(g\) tensor such that the imidazole ligands occupy an increasingly equatorial position (33). These results are consistent with the formation of a tighter or more regular copper-binding site upon addition of the co-substrate.

The EPR spectrum for MBP-H167A TfdA (Fig. 2D), like that for the H113A variant, is poorly resolved, probably due to binding of copper in multiple configurations instead of formation of one major conformation. Additionally, few significant changes are seen upon addition of either α-KG or 2,4-D. The MBP-H200A EPR spectrum (Fig. 2E) is better resolved than that of H113A or MBP-H167A and clearly shows a second set of resonances of lower amplitude with parameters identical to those observed for the copper-substituted wild-type enzyme (Table IV). The presence of this wild-type signal suggests that His-200 is most likely not a copper-binding ligand in TfdA. As found for H113A and MBP-H167A, addition of α-KG and/or 2,4-D has little effect on the spectrum. ESEEM Spectroscopic Characterization of Variants with Altered Metal Sites—Pulsed EPR (ESEEM) spectroscopy has proven useful for determining the number of histidines bound to copper in proteins. This approach has been previously used to determine that copper-substituted wild-type TfdA binds cop-
per in a site with two histidyl residues directly coordinated to the metal. As previously reported (23), three-pulse ESEEM spectra collected in the 

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\begin{array}{ccc}
\text{TABLE IV} \\
\text{Summary of the EPR spectral parameters for} \\
\text{Cu(II)-substituted TfdA variants} \\
\hline
\text{Species} & g_i & A_i & g_z \\
\text{Wild-type TfdA} & 2.34 & 16.3 & 2.07 \\
\text{Wild-type TfdA/α-KG} & 2.36 & 15.1 & 2.07 \\
\text{Wild-type TfdA/α-KG/2,4-D} & 2.38 & 12.0 & 2.09 \\
\text{H113A} & 2.30 & 16.6 & 2.07 \\
\text{H113A/α-KG} & 2.33 & 15.8 & 2.07 \\
\text{H113A/α-KG/2,4-D} & 2.35 & 14.0 & 2.08 \\
\text{D115A} & 2.30 & 17.0 & 2.06 \\
\text{D115A/α-KG} & 2.30 & 16.2 & 2.06 \\
\text{D115A/α-KG/2,4-D} & 2.34 & 16.8 & 2.06 \\
\text{MBP-H167A} & 2.30 & 17.1 & 2.07 \\
\text{MBP-H167A/α-KG/2,4-D} & 2.30 & 17.1 & 2.07 \\
\text{MBP-H200A} & 2.30 & 17.1 & 2.07 \\
\text{MBP-H200A/α-KG/2,4-D} & 2.34 & 16.1 & 2.07 \\
\end{array}
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features at 0.7, 1.5, and 4.0 MHz that are indicative of histidyl imidazole equatorially coordinated to Cu(II) (36). The spectra obtained for D115A and MBP-H167A variants are shown in Figs. 5B and 6B (solid lines), respectively. The resolution in these spectra is poor when compared with similar data obtained for rigid Cu(II) proteins or model complexes (39, 40). In Fig. 5A, the normalized three-pulse ESEEM data for D115A TfdA (solid line) are shown with the computer-simulated data.
for one (dashed line) and two (dotted line) coordinated histidyl ligands obtained using spin Hamiltonian parameters typical for the remote nitrogen of imidazole equatorially bound to Cu(II). To compare the amplitudes of the ESEEM simulations with those obtained experimentally, the simulated ESEEM patterns of Fig. 5A were multiplied by background decay functions characterized by an e^{-1} time of 600 ns. This decay time constant is a factor of 6 faster than that used to make the comparison between simulation and experiment for wild-type Cu(TfdA) in Fig. 3. Unfortunately, the severity of this decay impairs our ability to distinguish whether 1 or 2 nitrogen nuclei are giving rise to the ESEEM of D115A based on modulation depths.

The Fourier transforms of the experimental and simulated ESEEM functions of Fig. 5A are shown in Fig. 5B. Although the combination lines are weaker in the data for D115A (solid line) than for wild-type TfdA, the combination line at −3.0 MHz is observed, and its frequency is predicted nicely by the 2-His simulation. The broader line widths of the FT spectra found for D115A TfdA, and the subsequent difficulty in observing the combination lines most likely reflects a distribution of 14N superhyperfine couplings for the Cu(II) sites of the variant. To account for the observed line widths and relative peak amplitudes in our simulations, it was necessary to average several calculated data sets obtained with 14N isotropic hyperfine coupling constants that ranged from 1.6 to 1.8 MHz. This assumption of a distribution of hyperfine couplings results in better agreement between experiment and theory for our ESEEM simulations and is in accord with the poor resolution observed in the CW-EPR spectrum of Cu(II)-D115A TfdA in the absence of substrates.

A parallel analysis was undertaken for the MBP-H167A data, and the results are provided in Fig. 6. The dash and dot patterns of Fig. 6A are simulation results for one and two equatorially bound histidyl ligands, respectively. The broadness of the lines at 0.6 and 0.9 MHz results in these frequencies being unresolved in the experimental data. This effect could be simulated using a higher asymmetry parameter, \( \eta \), for the nuclear quadrupole interaction but not without significant deficiencies in the prediction of the double quantum peak at 4 MHz. A more satisfactory method of accounting for this coalescence in the simulations was through the averaging of calculation results obtained using a distribution of isotropic hyperfine coupling constants, as in the simulations for D115A. The simulations in Fig. 6 are the result of averaging five such data sets where the 14N isotopic hyperfine coupling ranged from 1.6 to 1.9 MHz. This method resulted in the broadening of the low frequency peaks to the point where they began to overlap, without reducing the magnitude of the double quantum feature at 4 MHz. As in the analysis of D115A, different background decay functions were applied to these simulations to provide the best match to the modulation intensities for the initial 1.5 \( \mu s \) of the ESEEM data (solid line). The background decay required to reduce the initial amplitudes of both one and two imidazole simulations was a factor of 10 faster than that used to simulate wild-type Cu(II)-TfdA ESEEM. This problem, combined with the lack of resolved combination peaks in the data, makes it difficult to determine the number of bound histidines. Although the modulation intensities are best accounted for by the one-histidine model, the features of the ESEEM spectrum are better simulated assuming two coupled nitrogens. The ESEEM patterns associated with the H113A and MBP-H200A mutants were too shallow to be consistent with single histidyl coordination, and it is likely that these mutant proteins have multiple Cu(II)-binding sites, some with equatorially coordinated histidine ligands(s) and some without.

The Cu(II)-substituted forms of H113A, D115A, MBP-H167A, and MBP-H200A were also analyzed by ESEEM spec-

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**Fig. 5.** Spectral simulation of ESEEM data for D115A-Cu-TfdA. Experimental (solid line), computer-simulated 1-His (dashed line), and simulated 2-His (dotted line) time domain spectra (A) and the corresponding Fourier-transformed data (B) for copper-substituted D115A TfdA are shown. Magnetic field = 3050 G, \( \tau = 230 \) ns, \( T = 50 \) ns G, \( \nu = 8.8 \) GHz, 4.2 K, \( g_N = 0.40347, e^2Q = 1.55 \) MHz, \( \eta = 0.87, A_{xx} = 1.6 \) MHz, \( A_{yy} = 1.9 \) MHz, \( A_{zz} = 2.2 \) MHz. The simulated data were treated with an exponential decay function to give \( \gamma_0 = (y_0 - 0.05)\exp((\tau + T/600)^{0.5}) - 0.05 \).

**Fig. 6.** Spectral simulation of ESEEM data for MBP-H167A-Cu-TfdA. Experimental (solid line), computer-simulated 1-His (dashed line), and 2-His (dotted line) time domain spectra (A) and the corresponding Fourier-transformed data (B) are shown. Magnetic field = 3100 G, \( \tau = 300 \) ns, \( T = 40 \) ns, \( \nu = 8.8 \) GHz, 4.2 K, \( g_N = 0.40347, e^2Q = 1.60 \) MHz, \( \eta = 0.82, A_{xx} = 1.55 \) MHz, \( A_{yy} = 1.9 \) MHz, \( A_{zz} = 2.0 \) MHz. The simulated data were treated with an exponential decay function to give \( \gamma_0 = (y_0 - 0.03)\exp((\tau + T/350)^{0.5}) - 0.03 \).
troscopy in the presence of α-KG and 2,4-D. Only slight variations in the line shape of the higher frequency peak at ~4.0 MHz were observed for the three histidine mutants. This result contrasts sharply with those for copper-substituted wild-type TfdA where addition of the co-substrates resulted in data that suggested a shift from two equatorially bound histidyl ligands to only one. The lack of similar changes in the ESEEM of these mutants upon addition of the co-substrates supports the conclusion that alteration of these key histidyl residues significantly affects the metal binding properties of the TfdA enzyme.

Three-pulse ESEEM spectra for D115A TfdA in the presence of α-KG and α-KG + 2,4-D (Fig. 7) show that addition of co-substrates leads to improved resolution in the ESEEM spectra without significant changes in the amount of unmodulated intensity. These results mirror the improved resolution observed in the cw-EPR spectra. For the ternary complex with both co-substrates bound to the D115A variant, all three combination lines from the electron spin manifold that gives rise to the intense low frequency peaks are resolved (Fig. 7B). Furthermore, the cw-EPR spectra of D115A treated with co-substrates show well resolved ligand hyperfine structure at $g_z$.

TfdA. Together, these data indicate that Asp-115 is very likely a metal-binding residue and may also be involved in formation of the proper catalytic conformation. Because these observations pertain to the Cu(II) form of TfdA, it is difficult to draw conclusions about the specific electronic role of Asp-115 in the native Fe(II) TfdA based on these observations.

The identity of the second metal-binding histidine, predicted by various spectroscopic studies, is less clear. Spectral simulations of His-167 are consistent with the presence of two imidazole nitrogens per copper in this protein. Furthermore, His-167 is replaced with an arginine in the TfdA sequence encoded by pIJB in Burkholderia cepacia (GenBank accession number AAB47567). Thus, even though this residue most closely matches the spacing observed in the Group I sequences, it is unlikely that His-167 is the third metal ligand to the TfdA iron metallocenter. EPR and ESEEM analyses of H200A TfdA suggest that His-200 is important for creation of the metallocenter. Although His-200 is conserved among the enzymes with high identities to TfdA, such as TauD from E. coli and sulfonate/α-KG dioxygenase from yeast, a corresponding histidine is not found in more distantly related Group II enzymes such as TauD from E. coli and sulfonate/α-KG dioxygenase from yeast, a corresponding histidine is not found in more distantly related Group II enzymes such as TauD from E. coli and sulfonate/α-KG dioxygenase.

**DISCUSSION**

EPR, ESEEM, and extended x-ray absorption fine structure spectroscopies of both the Fe(II)- and Cu(II)-forms of wild-type TfdA had previously indicated that the resting enzyme has two imidazoles and a mixture of bound nitrogens and oxygens as ligands to the metal (22–24). ESEEM and x-ray absorption data indicated that both imidazole ligands are retained upon addition of α-KG, whereas addition of 2,4-D leads to the displacement or $g_\text{tensor}$ reorientation of a histidine out of the equatorial plane (22–24). Our present results confirm the importance of multiple histidines and one aspartate for TfdA activity and allow us to assign functions to selected residues.

Mutation of His-113 or Asp-115 to alanine both eliminates activity and alters the structure of Cu(II)-metallocenter site. Both variants show EPR spectra consistent with at least two distinct Cu(II)-binding sites. Substrate binding perturbs these spectra giving rise to better resolution of $g_z$ features associated with each metal site suggesting that the copper is likely present at the same binding site as in the wild-type enzyme. His-113 and Asp-115 comprise the HX(D/E) motif that is strictly conserved in enzymes related to TfdA (Fig. 8) and more distinctly related α-KG-dependent dioxygenases (Table I) (7). Site-directed mutagenesis studies analogous to those described here demonstrated the importance of this motif in a number of Group I enzymes including aspartyl, lysyl, prolyl, and flavinone hydroxylases (8, 10–13). Furthermore, crystal structures of IPNS and DAOCS established these residues as ligands to the Group I metal centers. Our studies support the proposal that residues in the HX(D/E) motif are ligands to the metal in TfdA and, most likely, other Group II enzymes.

Cu(II)-D115A TfdA ESEEM and EPR spectra (particularly for the case where substrates are bound) indicate the presence of two imidazole ligands at one of the Cu(II) sites. The intensity of the non-modulated echo signal was greater for this variant, as compared with that found for the wild-type enzyme, providing evidence for another copper binding environment that lacks equatorially bound histidines. The absence of the aspartate ligand alters the chemistry that occurs at the active site upon addition of the co-substrates. The geometry of the Cu(II) site shows less dispersion when α-KG and 2,4-D are bound as evidenced by the increased resolution in the EPR spectrum and ESEEM pattern. These observations are consistent with those made for the wild-type Cu(II) TfdA; however, the structural rearrangement triggered by formation of the Cu(II)-α-KG-2,4-D ternary complex in wild-type TfdA is not observed for D115A TfdA. Together, these data indicate that Asp-115 is very likely a metal-binding residue and may also be involved in formation of the proper catalytic conformation. Because these observations pertain to the Cu(II) form of TfdA, it is difficult to draw conclusions about the specific electronic role of Asp-115 in the native Fe(II) TfdA based on these observations.

The identity of the second metal-binding histidine, predicted by various spectroscopic studies, is less clear. Spectral simulations of α-KG and 2,4-D were bound as evidenced by the increased resolution in the EPR spectrum and ESEEM pattern. These observations are consistent with those made for the wild-type Cu(II) TfdA; however, the structural rearrangement triggered by formation of the Cu(II)-α-KG-2,4-D ternary complex in wild-type TfdA is not observed for D115A TfdA. Together, these data indicate that Asp-115 is very likely a metal-binding residue and may also be involved in formation of the proper catalytic conformation. Because these observations pertain to the Cu(II) form of TfdA, it is difficult to draw conclusions about the specific electronic role of Asp-115 in the native Fe(II) TfdA based on these observations.

The identity of the second metal-binding histidine, predicted by various spectroscopic studies, is less clear. Spectral simulations of H167A are consistent with the presence of two imidazole nitrogens per copper in this protein. Furthermore, His-167 is replaced with an arginine in the TfdA sequence encoded by pIJB in Burkholderia cepacia (GenBank accession number AAB47567). Thus, even though this residue most closely matches the spacing observed in the Group I sequences, it is unlikely that His-167 is the third metal ligand to the TfdA iron metallocenter. EPR and ESEEM analyses of H200A TfdA suggest that His-200 is important for creation of the metallocenter. Although His-200 is conserved among the enzymes with high identities to TfdA, such as TauD from E. coli and sulfonate/α-KG dioxygenase from yeast, a corresponding histidine is not found in more distantly related Group II enzymes such as γ-butyrobetaine hydroxylase or clavamine synthase (Fig. 8). Mutational analysis of prollyl 4-hydroxylase (10) provided evidence for a non-ligand histidine at the active site that is important for α-KG binding and for controlling the decarboxylation of α-KG in the absence of substrate. Perhaps His-200 serves a similar function in TfdA, providing an explanation for the changes in active site structure upon its alteration. Sequence comparisons of all Group II enzymes, including several putative dioxygenases with overall similarity to TfdA, reveal that His-262 in TfdA is the only other invariant histidine (Fig.
FIG. 8. Sequence comparison of TfdA and other Group II α-KG-dependent dioxygenases. The accession numbers and sequence identifiers are shown in parentheses for the following Group II sequences: R. eutropha 2,4-D/α-KG dioxygenase (TfdA, M16730); E. coli taurine/α-KG dioxygenase (TauD, P37610); putative α-KG dioxygenase from sulfate-ester transport gene cluster in Pseudomonas putida (AtsK, AF126201); S. cerevisiae sulfonate/α-KG dioxygenase (YSD, Q12358); Erwinia carotovora carbapenem biosynthesis enzyme C (CarC, AAD38231); Pseudomonas aeruginosa pyoverdine biosynthesis enzyme (PvcB, AF002222); Pseudomonas syringae SyrI, syringomycin biosynthesis enzyme I (SyrB, AAB63253); Pseudomonas sp. g-butyrobetaine hydroxylase (GBBH, P80193); Caenorhabditis elegans g-butyrobetaine hydroxylase (GBBH_CE, Z66523); Streptomyces clavuligerus clavaminate synthase 1 and 2 (CS1, A44241; CS2, L06214). Highly conserved residues (black boxes) and residues conserved in more than 75% of the sequences (dark gray boxes) are highlighted. Amino acid numbers for each sequence are shown at the right of each line; mutated TfdA residues are numbered above.
family (Group I, indicated by the inability of phenoxyethanol to serve as a
ole nitrogen and the substrate carboxylate group; however, 2,4-D binding by electrostatic interactions between the imidaz-
that His-213 (perhaps also assisted by His-216) facilitates
onstrated a decreased catalytic rate. These data may indicate
in the model. Mutation of His-213 affects both substrate bind-
gesting and the rate of catalysis and therefore is shown to be
present near the active site to indicate potential substrate interactions, but the precise role of the residue is still to be
defined. In the absence of a crystal structure, the site-directed mutagenesis, metallocenter spectroscopy, and kinetic analysis studies described here provide the most complete working model of the TfdA active site. This model begins to define the
ecessary elements for catalysis in Group II α-KG-dependent dioxygenases and finds similarities between these enzymes and other well studied representatives of this superfamily.

Note Added in Proof—The recently reported structure of clavaminate synthase (45) confirms our proposed roles for residues in the Hx/D/E\textsubscript{X}_5\textsubscript{H\textsubscript{X}}_1\textsubscript{O\textsubscript{2}}(R/K) motif for the Group II α-KG-dependent dioxygen-

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Site-directed Mutagenesis of 2,4-Dichlorophenoxyacetic Acid/α-Ketoglutarate Dioxygenase: IDENTIFICATION OF RESIDUES INVOLVED IN METALLOCENTER FORMATION AND SUBSTRATE BINDING
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