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Purification and Characterization of 2,4-Dichlorophenoxyacetate/α-Ketoglutarate Dioxygenase*

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The *Alcaligenes eutrophus* 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenase, encoded by the *tfdA* gene of plasmid pJP4, is an Fe(II)-dependent enzyme that catalyzes the conversion of 2,4-dichlorophenoxyacetate to 2,4-dichlorophenol and glyoxylate concomitant with the decomposition of α-ketoglutarate to form succinate and carbon dioxide (Fukumori, F., and Hausinger, R. P. (1993) *J. Bacteriol.* 175, 2083–2086). Using recombinant *Escherichia coli* cells that overexpress the *tfdA* gene, the thermolabile enzyme (stable only up to 30 °C) was purified to apparent homogeneity (specific activity of 16.9 μmol of substrate converted min^{-1} mg of protein^{-1}) by a simple two-step procedure. The native protein has an apparent M_r of 50,000 ± 2,500, consistent with a homodimeric structure. Ferrous ion is absolutely required for activity and cannot be replaced by several other divalent cations tested. Ascorbic acid stimulates dioxygenase activity and reduces the rate of enzyme inactivation by a metal ion-mediated process. The enzyme exhibits maximum activity at pH 6.5–7, however, it is stable over a pH range of 6.5–11. Although capable of hydroxylating a wide range of phenoxyacetates and related compounds, the enzyme exhibits the greatest affinity (K_m 17.5 ± 1.0 μM) and highest catalytic efficiency for 2,4-dichlorophenoxyacetate. Similarly, α-ketoglutarate is the preferred co-substrate (K_m 3.20 ± 0.54 μM) for the enzyme, but it can utilize a range of other α-ketoacids with lower efficiency. Results from chemical modification studies are consistent with the presence of multiple essential histidine residues in the enzyme.

The biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D),[1] a broadleaf herbicide, has been studied as a model for microbial decomposition of chloroaromatic compounds (1–3). *Alcaligenes eutrophus* JMP134 is the most extensively studied 2,4-D degrader and the pathway found in this microbe is shown in Fig. 1. The genes responsible for the first five steps in this pathway (*tfdA*, *-B*, *-C*, *-D*, and *-E*) have been localized to the transmissible plasmid, pJP4 (4). The nucleotide sequences of *tfdCDE* (5) exhibit extensive similarity to the well studied *clcABD* and *tcbCDE* chlorocatechol degradation genes (reviewed in Ref. 3) and similarly provide for the catabolism of dichlorocatechol. The sequence of the *tfdB* gene (5), encoding 2,4-DCP hydroxylase, also exhibits similarity to other genes involved in phenol hydroxylation (*e.g.* see Ref. 6). In contrast to the downstream genes, however, the sequence of the *tfdA* gene (7) does not exhibit obvious evolutionary relationships to any other known genes. The *tfdA* gene product (TfdA) generally has been referred to as 2,4-D monooxygenase; however, we recently demonstrated that the enzyme carries out a very different type of reaction (8) that is mechanistically related to reactions catalyzed by a group of α-KG-dependent dioxygenases (9). The reaction requires α-KG and Fe(II), in addition to 2,4-D and oxygen, and produces carbon dioxide, succinate, 2,4-DCP, and glyoxylate (Fig. 2).

Here, we detail the purification and describe the catalytic properties of 2,4-D/α-KG dioxygenase. In addition, we examine the substrate ranges of the enzyme for other phenoxyacetates and α-ketoacids. Furthermore, we describe evidence consistent with the presence of multiple essential histidine residues in the enzyme.

EXPERIMENTAL PROCEDURES

Chemicals—Organic chemicals were obtained from Sigma or Aldrich and were of the highest purity available. α-[1-^{14}C]KG (1.8 MBq/mmol) was obtained from DuPont NEN.

Media, Organisms, and Plasmid—Modified LB medium (10 g of Tryptone, 5 g of yeast extract, and 5 g of NaCl per liter (pH 7.2)) supplemented with 100 μg/ml of ampicillin was used to grow the host strain *Escherichia coli* JM109 (10). *A. eutrophus* JMP134 (4) was grown at 30 °C in 1/10 PTYG medium (0.25 g of peptone, 0.25 g of Tryptone, 0.5 g of yeast extract, 0.5 g of glucose, 0.03 g of $MgSO_4$, and 0.0035 g of $CaCl_2$ per liter (pH 7.2)) supplemented with 2.5 mM 2,4-D. Plasmid pUS311 (8) that encodes the *tfdA* gene had been generated from pKJS31 (7).

Purification of TfdA—Unless stated otherwise, all purification steps were done at 4 °C. Cultures (2 liters) of *E. coli* JM109(pUS311) were grown overnight at 30 °C with moderate aeration. Cells were harvested by centrifugation (6,000 × *g*) for 20 min and suspended in 25 ml of 20 mM Tris, 1 mM EDTA, 0.4 mg/liter leupeptin (pH 7.2). The cell suspensions were stored at −20 °C until use. Suspended cells were adjusted to 1 mM phenylmethylsulfonyl fluoride and passed through a French pressure cell at 120 MPa. Cell extracts were obtained after clarification of disrupted cells by centrifugation at 100,000 × *g* for 30 min. To the extract was added 20 mM Tris, 1 mM EDTA, 0.1 mg/liter leupeptin (pH 7.2) (Buffer A) containing 3.4 M $(NH_4)_2SO_4$ and the fraction precipitating between 1.0 and 1.7 M was collected. The precipitate was dissolved in 20 ml of Buffer A and dialyzed against 4 liters of the same buffer for 16 h. The solution was then chromatographed on a Mono Q 10/10 column (Pharmacia LKB Biotechnology Inc.) in Buffer A and eluted with a 100 ml of linear salt gradient to 0.2 M NaCl at room temperature (about 20 °C). TfdA was found to elute at approximately 0.05 M NaCl.

Enzyme Assays—2,4-D/α-KG dioxygenase was assayed at 30 °C. The typical assay mixture (total volume of 1 ml) contained 1 mM α-KG, 50 μM ascorbate, 50 μM $(NH_4)_2Fe(SO_4)_2$, and 1 mM 2,4-D in 10 mM imidazole buffer (pH 6.75). Aliquots of the reaction mixture were quenched by the addition of EDTA. The concentration of released phenol derivative was

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[1] The abbreviations used are: 2,4-D, 2,4-dichlorophenoxyacetate; α-KG, α-ketoglutarate; 2,4-DCP, 2,4-dichlorophenol; DEP, diethylpyrocarbonate; TNBS, trinitrobenzenesulfonic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; 2,4,5-T, 2,4,5-trichlorophenoxyacetate; Bis-tris, bis(2-hydoxyethyl)iminotris(hydroxymethyl)methane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid.

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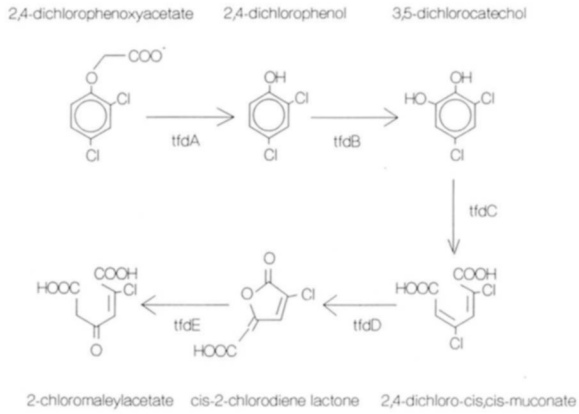

FIG. 1. **Pathway for degradation of 2,4-D in *A. eutrophus* JMP134.**

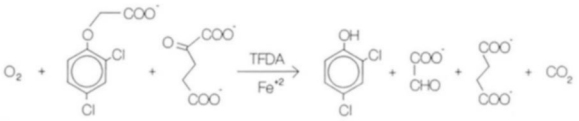

FIG. 2. **Overall reaction for 2,4-D/α-ketoglutarate dioxygenase.**

measured by a modification of the 4-aminoantipyrine method (11); to 1 ml of reaction mixture was added 100 µl of a pH 10 buffer solution (3.09 g of boric acid, 3.73 g of potassium chloride, and 44 ml of 1 N sodium hydroxide brought to 1 liter with distilled water), 10 µl of 2% 4-aminoantipyrine, and 10 µl of 8% potassium ferricyanide. The absorbance at 510 nm was determined after 20 min with a Gilford Response spectrophotometer. One unit of activity is defined as the amount of enzyme that forms 1 µmol of phenol derivative/min at 30 °C.

Examination of the kinetic parameters for 2,4-D and its analogues and for α-KG and its analogues involved holding the concentration of one substrate constant at 1 mM while varying the concentration of the substrate under consideration. For each of the phenoxyacetate derivatives the appropriate modified phenol compound was used as a standard in the spectrophotometric assay to account for their slightly different extinction coefficients. In contrast, 2,4-DCP served as the standard for kinetic evaluation of each of the α-ketoacids. Because of the extremely low K_m value for α-KG, special conditions were used with this substrate. Enzyme (0.1 µg) was incubated with α-[1-^{14}C]KG (45 kBq/mmol) under standard conditions for up to 1 min, the reaction was stopped by the addition of 1 N HCl, reaction mixtures were incubated for 1 h at room temperature with shaking to drive off the released carbon dioxide (α-KG is stable under the low pH conditions used in this experiment), and the remaining radioactivity in the solution was measured. Rates for α-KG utilization were calculated based on the released radioactivity. The capacity of the enzyme to react with several other non-phenoxyacetate substrates was assessed by monitoring spectroscopic changes or by high performance liquid chromatography (Hewlett-Packard HP-1050) methods using a C_{18} (octadecyl silane) reverse-phase column (4.0 × 250 mm) at a flow rate of 1.5 ml/min in various compositions of methanol, 0.1% H_3PO_4 in H_2O. For all compounds that served as substrate for the enzyme, apparent kinetic constants were obtained (12) from rates that were calculated by using linear regression analysis. Values of k_{cat} were based on a subunit M_r of 32,171 (7).

Analytical Methods—Protein concentration was measured (13) using bovine serum albumin as a standard. The native M_r of TfdA was estimated by gel filtration through a Superose 12 HR 10/30 column (Pharmacia; flow rate 15 ml/h) in 20 mM Tris, 1 mM EDTA, 100 mM NaCl (pH 7.2). The calibration proteins were thyroglobulin, M_r 670,000; bovine γ-globulin, M_r 158,000; chicken ovalbumin, M_r 44,000; equine myoglobin, M_r 17,000; and vitamin B-12, M_r 1,350 (Bio-Rad).

Gel Electrophoresis and Immunoblotting—Denaturing SDS-polyacrylamide gel electrophoresis was carried out on 12% polyacrylamide gels (14). Gels were stained with Coomassie Brilliant Blue. Standard proteins included phosphorylase *b*, M_r 92,500; bovine serum albumin, M_r 66,200; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,500; and lysozyme, M_r 14,400 (Bio-Rad).

Antibodies were generated against purified TfdA in a white New Zealand rabbit, and the IgG fraction was obtained from the serum (15). Immunoblots were prepared by blotting the proteins onto nitrocellulose, probing with anti-TfdA rabbit IgG, and developing with goat anti-rabbit IgG-alkaline phosphatase conjugates (16).

Chemical Modification Studies—DEP was freshly diluted with ethanol and its concentration was determined by using 10 mM imidazole (pH 7.0) based on the increase in absorbance at 240 nm (ϵ = 3000 M^{-1} cm^{-1}) (17). Reactions were carried out by incubating the enzyme (1.2 µM) with DEP and other additions as indicated in 10 mM MOPS buffer (pH 6.5) at room temperature (21–22 °C). Aliquots (25 µl) were withdrawn at various time intervals, the reactions were quenched with 755 µl of 13.3 mM imidazole buffer (pH 6.75), and residual activities were determined as in the standard procedure. Reactivation of the DEP-modified TfdA (treated for 30 min with 1 mM DEP) was examined after adjusting the solution to 10 mM imidazole and 0.5 M hydroxylamine (using neutralized reagent). TfdA activity at various times was assessed after removal of excess reagent by using a Bio-Rad 10 DG desalting column. Inactivation studies of the enzyme (15 µM) by various other reagents, dissolved in H_2O, were performed in 10 mM imidazole or 10 mM MOPS buffers (pH 6.75). Aliquots (2 µl) were withdrawn at various time intervals and residual activities were determined as described above.

RESULTS

Purification of TfdA—2,4-D/α-KG dioxygenase was purified to over 95% homogeneity from *E. coli* JM109(pUS311) with 56% yield by using the 2-step procedure summarized in Table I. Comparison of the isolated protein to cell extracts from the recombinant strain by denaturing gel electrophoresis (Fig. 3) demonstrated that the cells produced the *tfdA* gene product as more than 10% of the total protein. Gel filtration chromatography was used to estimate a M_r of 50,000 ± 2,500 for the native enzyme, consistent with a homodimeric structure (subunit M_r of 32,171; Ref. 7).

The presence of protease inhibitors during early stages of TfdA purification enhances the stability of the enzyme by pre-

TABLE I
Purification of 2,4-D / α-ketoglutarate dioxygenase

Purification step	Total protein	Total activity	Specific activity	Purification
	mg	*units (%)*	*units/mg*	*-fold*
Cell extracts	750	1690 (100)	2.2	1
Ammonium sulfate precipitation	225	1400 (83)	6.2	2.8
Mono Q	58	980 (58)	16.9	7.7

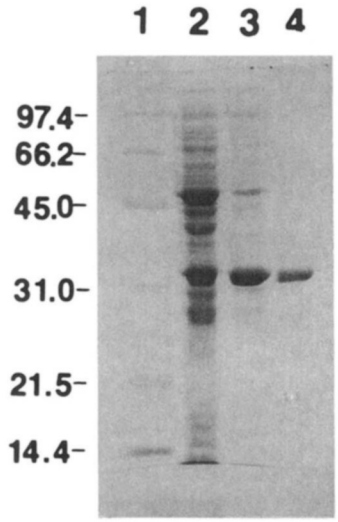

FIG. 3. **Purification of Alcaligenes eutrophus JMP134 2,4-D/α-KG dioxygenase from *E. coli* JM109(pUS311).** Protein samples were analyzed by denaturing gel electrophoresis on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue. *Lane 1*, molecular weight markers with the relative molecular mass values indicated in kilodaltons on the *left margin*; *lane 2*, supernatant of disrupted cells of *E. coli* JM109(pUS311); *lane 3*, fraction precipitated from 1–1.7 M ammonium sulfate; *lane 4*, fraction containing purified TfdA as eluted from MonoQ ion exchange column.

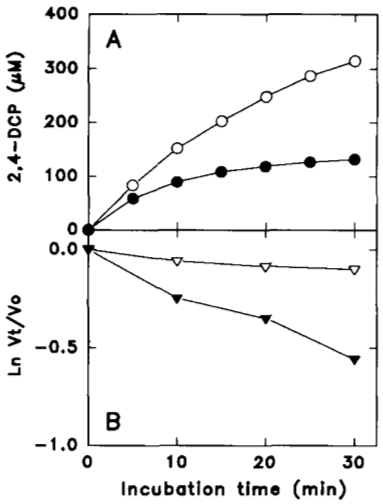

FIG. 4. **Time- and ferrous ion-dependent inactivation of 2,4-D/α-KG dioxygenase.** *A*, Progress curves of 2,4-DCP formation for TfdA (1 μg/ml) incubated in the standard assay with (○) and without (●) ascorbate. *B*, TfdA (2 μg/ml) was incubated at room temperature in 10 mM imidazole buffer (pH 6.75) with no additions (▽), and with 100 μM ferrous ion and 100 μM ascorbate (▼). Aliquots were withdrawn over time and assayed under standard conditions.

venting conversion of the subunit to an inactive TfdA fragment of apparent M_r 27,000. Whereas NH_2-terminal sequence analysis of the non-degraded subunit revealed the residues expected from DNA sequence analysis (S-V-V-A-N-; Ref. 7), the amino-terminal sequence of the proteolytic fragment of TfdA (F-L-Y-A-E-L-) was consistent with hydrolytic cleavage after arginine (residue 77) and loss of the NH_2-terminal fragment. By using anti-TfdA IgG in Western blot analysis of various samples, the conversion was found to occur after cell disruption rather than during the cell cultivation period. The same methods were used to demonstrate that the M_r of 32,000 form of the protein was present in cell extracts of *A. eutrophus* JMP134.

Maintenance of low temperatures during bacterial growth and enzyme isolation also is important for obtaining high levels of activity. As previously described (8), TfdA forms aggregates in *E. coli* JM109(pUS311) cells that are grown at 37 °C but remains soluble for cells grown at 30 °C. Indeed, we have observed that colonies grown at the higher temperature are turbid, whereas those grown at the more moderate temperature remain clear. Furthermore, the purified 2,4-D/α-KG dioxygenase was found to be very sensitive to thermal inactivation. At a protein concentration of 0.3 μM, TfdA remains stable only up to 30 °C and is completely inactivated within 5 min at 40 °C. In contrast, the purified enzyme was found to be stable at 4 °C for at least several weeks after purification.

Catalytic Properties of TfdA—As previously noted (8), activity of 2,4-D/α-KG dioxygenase requires ferrous ion and is enhanced by ascorbic acid. The ferrous ion requirement could not be met by other divalent metal ions including Co(II), Cu(II), Li(II), Mg(II), Mn(II), Ni(II), and Zn(II) when added as sulfate salts at a concentration of 100 μM. As shown by the time-dependent decrease in enzyme activity in Fig. 4*A*, however, ferrous ion alone is unable to sustain enzyme catalysis over long time periods. The rate of activity loss was greatly reduced (although not completely eliminated) by inclusion of ascorbic acid in the assay. The ascorbic acid serves to prevent inactivation rather than to reactivate the enzyme as shown by studies in which addition of ascorbate to partially active enzyme in the ferrous ion-alone treatment failed to restore full activity. Similarly, activity could not be recovered from inactive enzyme that was dialyzed against 1 mM EDTA and/or 1 mM dithiothreitol and then reassayed in the presence of ferrous ion plus ascorbate. Catalytic turnover of the enzyme was not required for inactivation as shown by the loss of activity during enzyme incubation with ferrous ion prior to addition of substrate (Fig. 4*B*). Although not completely characterized, the inactivation clearly results from a metal ion-mediated event as demonstrated by the retention of activity when the enzyme is stored in the absence of metal ions and the presence of EDTA. To minimize enzyme inactivation in the kinetic studies described below, reactions were initiated by addition of enzyme to assay mixtures and sufficiently short assay periods were used to calculate initial rates. The concentration dependences for ferrous ion and ascorbate on initial 2,4-D/α-KG dioxygenase rates are shown in Fig. 5.

The pH dependence of 2,4-D/α-KG dioxygenase was examined. The enzyme activity exhibits a narrow pH optimum of 6.5–7.0 (Fig. 6*A*). In contrast, TfdA is stable over a wide pH

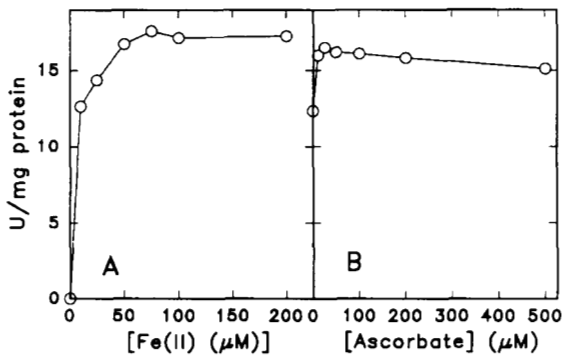

FIG. 5. **Effect of Fe(II) and ascorbic acid concentrations on the activity of 2,4-D/α-KG dioxygenase.** *A*, Fe(II) concentration dependence on 2,4-D/α-KG dioxygenase activity; *B*, ascorbic acid concentration dependence on enzyme activity.

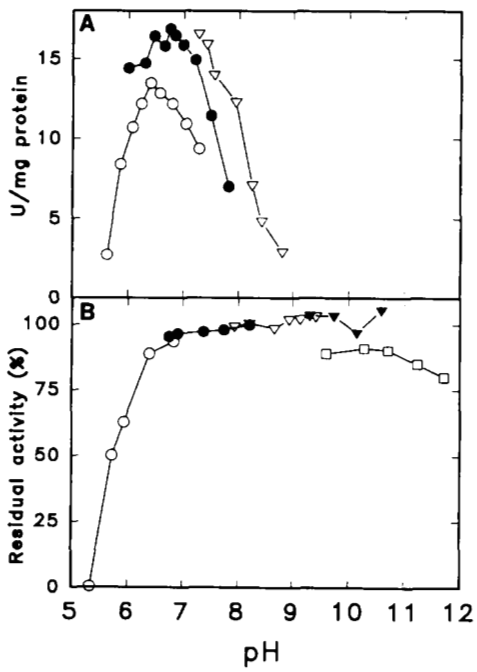

FIG. 6. **pH dependence of 2,4-D/α-KG dioxygenase.** *A*, enzyme activity. The enzyme assays were done in the following buffers at a concentration of 5 mM: Bis-Tris, pH 5.6–7.3 (○); imidazole, pH 6.0–7.6 (●); Tris, pH 7.3–8.8 (▽), all at 30 °C. *B*, enzyme stability. The residual enzyme activity after incubation at various pH values for 20 h at 4 °C was determined by using standard assay conditions. The enzyme was incubated in the following buffers at a concentration of 10 mM: MES, pH 5.3–6.8 (○); imidazole, pH 6.7–8.2 (●); Tris, pH 7.9–9.4 (▽); CHES, pH 9.3–10.6 (▼); CAPS, pH 9.6–11.7 (□).

TABLE II
Phenoxyacetate substrate specificity and kinetic parameters for 2,4-D / α-ketoglutarate dioxygenase

All experiments were performed at 30 °C in 10 mM imidazole buffer (pH 6.75) containing 1 mM α-KG, 50 μM ascorbate, 50 μM $(NH_4)_2Fe(SO_4)_2$, and the indicated substrates over the indicated concentration ranges.

Substrate (concentration range studied)	K_m	k_{cat}	k_{cat}/K_m
	μM	min^{-1}	$min^{-1}\cdot mM^{-1}$ (%)
Phenoxyacetate (200–1000 μM)	460 ± 23	443 ± 10	960 (3)
2-Chlorophenoxyacetate (40–200 μM)	110 ± 6.5	380 ± 11	3,450 (11)
4-Chlorophenoxyacetate (40–200 μM)	117 ± 6.2	595 ± 15	5,090 (17)
2,3-Dichlorophenoxyacetate (50–200 μM)	102 ± 8.5	288 ± 12	2,820 (9)
2,4-Dichlorophenoxyacetate (10–50 μM)	17.5 ± 1.0	529 ± 16	30,200 (100)
3,4-Dichlorophenoxyacetate (50–500 μM)	219 ± 7.6	307 ± 5	1,400 (5)
2,4,5-Trichlorophenoxyacetate (20–150 μM)	59.6 ± 3.4	96 ± 3	1,610 (5)
4-Chloro-2-methylphenoxyacetate (20–150 μM)	89.0 ± 8.7	233 ± 12	2,620 (9)
DL-2-Phenoxypropionate (0.5–5 mM)	1170 ± 120	5.1 ± 0.2	4.4 (–)
2-(2,4-Dichlorophenoxy)propionate (0.05–1 mM)	191 ± 6.7	61 ± 3	320 (1)
3-Phenoxypropionate (5–25 mM)	12900 ± 3200	3.2 ± 0.4	0.25 (–)

range (although it is totally inactivated by acidic conditions (pH < 6.5)) as shown by measuring the remaining activity after incubation in various pH buffers at 4 °C for 20 h (Fig. 6*B*).

Substrate Range of TfdA—In addition to 2,4-D, the enzyme catalyzes the α-KG-dependent release of the expected phenol derivatives from several other phenoxyacetates (Table II). The enzyme exhibits greatest affinity and catalytic efficiency toward 2,4-D, however, many ring-substituted compounds are reasonable substrates with similar catalytic rates but lower affinity. Non-halogenated phenoxyacetate possesses a larger K_m value than do the halogenated substrates. Similarly, the K_m value for 2-phenoxypropionate is substantially larger than that of 2-(2,4-dichlorophenoxy)propionate. The additional methyl group in the side chain of these two compounds, however, greatly decreases their k_{cat} values for hydroxylation, perhaps due to the change from a secondary to a tertiary carbon atom. Although it is a very poor substrate 3-phenoxypropionate is hydroxylated by the enzyme, demonstrating that the substrate binding site can accommodate one extra methylene carbon in the side chain. In contrast, TfdA exhibits no activity toward analogues in which (i) the phenoxy group is two carbons atoms more distant from the carboxyl group (4-(2,4-dichlorophenoxy)-butyrate), (ii) the carboxyl and phenoxy groups are located at adjacent positions on an aromatic ring (2-phenoxybenzoate), (iii) the acid group is reduced (2-phenoxyethanol), or (iv) the phenoxy oxygen is replaced by a methylene group (hydrocinnamic acid). Similarly, the enzyme exhibits insignificant activity toward methyl esters of phenoxyacetate and 4-chlorophenoxyacetate. Although trace amounts of product phenols were observed (<0.5%), they may have arisen from uncatalyzed hydrolysis of the substrate, with subsequent enzymatic action on the phenoxyacetates, as the amount of product did not depend on the enzyme concentration. Finally, because other investigators have characterized a mechanistically related 4-hydroxyphenylpyruvate dioxygenase (17–19), we tested whether this compound could serve as a substrate for TfdA. We found that 4-hydroxyphenylpyruvate is not utilized by this enzyme.

The K_m and k_{cat} values for several α-ketoacids were determined to examine the specificity of the enzyme for α-KG (Table III). Although the enzyme exhibits a broad α-ketoacid substrate range, the highest affinity and greatest catalytic rate were observed for α-KG. The non-α-ketoacid carboxyl group is not required for recognition by the enzyme, but the K_m values for the two substrates possessing a second acidic group are significantly increased and the catalytic rates are greatly reduced compared to the other substrates. Addition of an extra methylene group between the α-ketoacid group and the free carboxyl group, as in α-ketoadipate, leads to small changes in the kinetic constants: a 6.4-fold increase in K_m and a 55% decrease in k_{cat} compared to α-KG. In contrast, removal of one of the methylene groups, as in oxalacetate, led to an ineffective substrate. Furthermore, using 25 μg of enzyme and 50-min incubation times, β-ketoglutarate, malonate, succinate, and glutarate (all up to 1 mM concentrations) were found to be incapable of supporting 2,4-D hydroxylation. Finally, in the absence of 2,4-D, no decomposition of α-KG was observed.

TABLE III
α-Ketoacid substrate specificity and kinetic parameters for 2,4-D / α-ketoglutarate dioxygenase

Enzyme reactions were performed at 30 °C in 10 mM imidazole buffer (pH 6.75) containing 1 mM 2,4-D,50 μM ascorbate, 50 μM $(NH_4)_2Fe(SO_4)_2$, and the indicated substrates over the indicated ranges of concentration.

Substrate (concentration range studied)	K_m	k_{cat}	k_{cat}/K_m
	μM	min^{-1}	$min^{-1}\cdot mM^{-1}$ (%)
α-KG (1.5–10 μM)	3.20 ± 0.54	643 ± 44	2.0×10^5 (100)
α-Ketoadipate (10–100 μM)	20.6 ± 1.1	290 ± 5	14100 (7.05)
Pyruvate (500–2500 μM)	1020 ± 86	58 ± 2	60 (0.03)
α-Ketobutyrate (150–800 μM)	464 ± 61	89 ± 6	190 (0.10)
α-Ketovalerate (300–1250 μM)	607 ± 47	404 ± 14	660 (0.33)
α-Ketocaproate (200–1000 μM)	583 ± 50	158 ± 7	270 (0.14)
α-Ketoisovalerate (200–1000 μM)	745 ± 36	16 ± 1	20 (0.01)

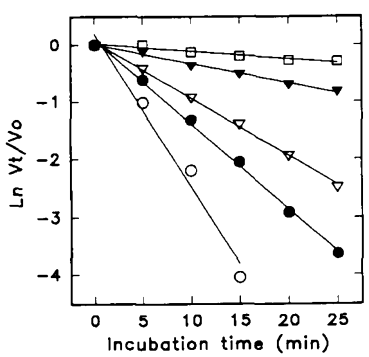

FIG. 7. **Kinetics of inactivation of 2,4-D/α-KG dioxygenase with DEP.** Pseudo-first-order plots for TfdA inactivation by DEP at concentrations of 0.13 mM (□), 0.27 mM (▼), 0.67 mM (▽), 1.0 mM (●) and 1.34 mM (○) in 10 mM MOPS buffer (pH 6.5, 21–22 °C). Ln (V_t/V_0) (where V_t is the velocity at time t and V_0 is the initial velocity) is shown as a function of time.

Chemical Modification of TfdA—DEP modification of TfdA in MOPS buffer (pH 6.75) resulted in the rapid loss of enzyme activity. A plot of the natural logarithm of remaining activity *versus* time at various concentrations of DEP exhibits pseudo first-order kinetics (Fig. 7). Replotting the apparent first-order

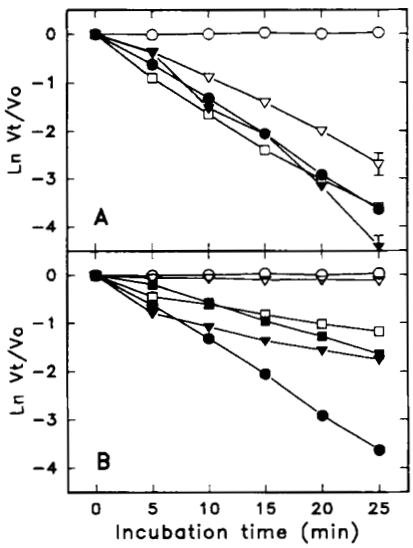

FIG. 8. **Substrate/ligand protection against DEP inactivation of 2,4-D/α-KG dioxygenase.** Residual activities are plotted against the incubation time. The enzyme was incubated with 1 mM DEP in 10 mM MOPS buffer (pH 6.5, 22 °C) in the presence of the indicated compounds. *A*, 1 mM 2,4-D (▽), 1 mM α-KG (▼), 0.5 mM Fe(II) and 0.5 mM ascorbate (□), and no addition (●). *B*, 1 mM 2,4-D, 0.5 mM Fe(II), and 0.5 mM ascorbate (▼), 1 mM α-KG, 0.5 mM of Fe(II), and 0.5 mM ascorbate (□), 1 mM 2,4-D and 1 mM α-KG (▽), 0.1 mM 2,4-D and 0.1 mM α-KG (■), and no addition (●). Activity was also measured in a control sample to which ethanol was added instead of DEP (○).

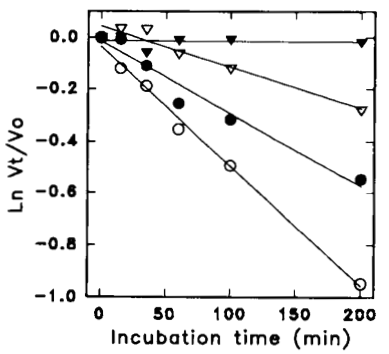

FIG. 9. **Kinetics of inactivation of 2,4-D/α-KG dioxygenase with TNBS.** Pseudo-first-order plots for inactivation of TfdA by TNBS at concentrations of 0 mM (▼), 0.5 mM (▽), 1 mM (●), and 2.5 mM (○) in 10 mM MOPS buffer (pH 6.75, 22 °C).

rate constants as a function of DEP concentration yields a straight line that provides a second-order rate constant of 6.14 M^{-1} s^{-1}. The ability of 2,4-D, α-KG, Fe(II) plus ascorbate, and combinations of these substances to protect the enzyme against DEP inactivation was examined. Whereas none of the individual compounds are able to significantly protect the enzyme from inactivation by DEP (Fig. 8*A*), the combinations of 2,4-D plus Fe(II) or α-KG with Fe(II) clearly decreases the inactivation rate (Fig. 8*B*). Furthermore, the combined presence of 2,4-D and α-KG is very effective in protecting the enzyme from inactivation by DEP.

Although commonly considered a histidine-selective reagent, DEP is known to react with tyrosine, lysine, and cysteine residues (17). We were unable to use the reported spectroscopic method (17) to determine whether a DEP-reactive tyrosine residue is present in TfdA because the DEP-modified protein forms a precipitate. This problem also prevented spectroscopic quantitation of the total DEP-reactive residues. Two approaches were used to assess whether the enzyme possesses a DEP-reactive lysine residue: hydroxylamine treatment of DEP-

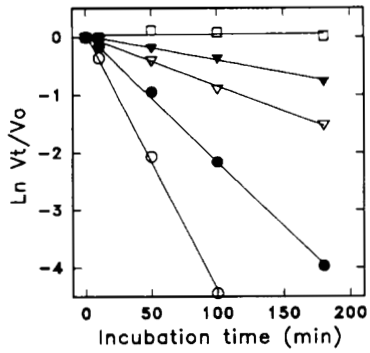

FIG. 10. **Kinetics of inactivation of 2,4-D/α-KG dioxygenase with EDC.** Pseudo-first-order plots for inactivation of TfdA by EDC at concentrations of 0 mM (□), 10 mM, (▼), 20 mM (▽), 50 mM (●), and 100 mM (○) in 10 mM MOPS buffer (pH 6.75, 22 °C).

modified protein and enzyme reactivity toward TNBS. Of the possible DEP-reactive residues usually only modified lysine or cysteine groups are stable to hydroxylamine treatment. Although we found that DEP-treated enzyme was incapable of being reactivated by 0.5 M hydroxylamine (not shown), it remains possible that one or more carbethoxyhistidine residues may be inaccessible to the reagent. The latter explanation is most consistent with our results using the lysine-selective reagent, TNBS. The enzyme was slowly inactivated by TNBS (Fig. 9), however, no protection from inactivation was observed when 1 mM α-KG, 1 mM 2,4-D, or 1 mM α-KG plus 1 mM 2,4-D was included in the reaction mixture. With regard to possible cysteine reactivity toward DEP, neither iodoacetamide (to 100 mM) nor *N*-ethylmaleimide (to 25 mM) inactivated the enzyme after 100 min of incubation. Similarly, 2,3-butanedione (to 100 mM) failed to inactivate the enzyme. In contrast, the carboxyl group-specific modifier, EDC, slowly inactivates the enzyme at high concentrations with pseudo-first-order kinetics (Fig. 10). A replot of the apparent rate constants *versus* EDC concentration is linear and allows calculation of the second-order rate constant of 0.018 M^{-1} s^{-1}. Analysis of substrate protection was impossible with this reagent since 2,4-D and α-KG both have carboxyl groups. These results are inconsistent with the presence of essential cysteine or arginine residues or of highly reactive lysine or carboxyl groups in the enzyme.

DISCUSSION

2,4-D/α-KG dioxygenase was purified to apparent homogeneity from recombinant *E. coli* cells by a simple two-step procedure and several properties of the enzyme were examined. This discussion will focus on comparison of its enzymic properties to that of other α-KG-dependent enzymes, the substrate specificity of the enzyme, and evidence consistent with the presence of multiple essential histidine residues in TfdA.

The specific requirements for α-KG and Fe(II), and the stimulation of activity by ascorbate, are typical characteristics of α-KG-dependent-dioxygenases (9). For example, prolyl 4-hydroxylase is relatively stable when assayed in the presence of ascorbate, whereas, it is rapidly inactivated in the absence of this reductant with 90% of the enzyme becoming inactive in approximately one minute corresponding to 15–30 turnovers (20, 21). In contrast, ascorbate-free 2,4-D/α-KG dioxygenase activity is much more stable with the enzyme carrying out approximately 4000 turnovers before becoming completely inactivated after 30–40 min. Inactivation of prolyl or lysyl hydroxylases is thought to involve a side reaction of these enzymes in which α-KG is decarboxylated in the absence of the peptide substrate leaving the metallocenter in an oxidized state that subsequently must be reduced by ascorbate (22, 23). TfdA,

however, does not appear to catalyze such an ascorbate-dependent decarboxylation of α-KG uncoupled from 2,4-D hydroxylation and enzyme inactivation occurs in the absence of either substrate. Rather than serving to reduce an oxidized enzyme-bound metal center, ascorbate appears to reduce the rate of some other metal ion-mediated enzyme inactivation process. The decrease in enzyme activity that is observed in assays containing both ferrous ion and ascorbate is not due to product inhibition as shown by the lack of any effect when 1 mM 2,4-DCP was included in assay mixtures containing various concentrations (10, 20, 50, and 100 μM) of 2,4-D.

Our results establish the broad substrate range for the hydroxylase reaction of the *tfdA*-encoded dioxygenase. Pieper *et al.* (24) reported that whole cells of *A. eutrophus* JMP134 that were deregulated for *tfdA* expression could degrade a series of 11 phenoxyacetates with chloro- and methyl-substitutions on the phenol ring (although only three of these compounds were growth substrates). Detailed kinetic studies of degradation rates, however, were impossible in their whole cell preparations because of substrate transport and other complications. Here, we were able to evaluate the kinetic constants for seven of the compounds examined by Pieper *et al.* (24) and four other substrates of TfdA. With regard to the observed substrate range of TfdA, it is important to emphasize that not all of these *in vitro* substrates are used by the *A. eutrophus* cells *in situ*. For example, we established that TfdA catalyzes conversion of 2,4,5-T to 2,4,5-trichlorophenol despite the reported inability of *A. eutrophus* JMP134 to catabolize it (25). These results suggest that either the strain can not transport 2,4,5-T or it lacks the ability to degrade 2,4,5-trichlorophenol. Whereas we were unable to detect utilization of 4-(2, 4-dichlorophenoxy)butyric acid by purified TfdA, a *Flavobacterium* species could degrade 4-(2,4-dichlorophenoxy)butyric acid as well as 2-(2,4-dichlorophenoxy)propionic acid, 2-(4-chloro-2-methylphenoxy)propionic acid, and 2,4-D (26). These results may indicate that the enzyme corresponding to TfdA in *Flavobacterium* has a different substrate range or that another enzyme is required; *e.g.* the four-carbon side chain of the substrate may be oxidized to remove two carbons and form 2,4-D. Similarly, we were unable to demonstrate utilization of the methyl ester of 2,4-D despite the known ability of numerous microorganisms to degrade this compound (27). It is likely that a separate enzyme synthesized by these microbes first hydrolyses the methyl ester to release 2,4-D that is subsequently metabolized by a TfdA-like enzyme. Examination of the kinetic values obtained for TfdA reveals that the rates of catalysis for the various substrates are generally quite similar with the exception of the sluggish rates observed with the propionates. Rather, the major differences in the catalytic efficiencies for these substrates involve their affinities for the enzyme. TfdA appears to prefer chlorinated substrates over the unsubstituted compounds. The position of the phenoxy groups at the C-2 carbon of an acid is very important for recognition, although one extra methylene group can be tolerated. The importance of the carboxyl group for substrate recognition is consistent with the presence of a positively charged residue in the 2,4-D binding site.

Although TfdA exhibits greatest efficiency with α-KG as the cosubstrate, many other α-ketoacids are able to be used and α-ketoadipate supports significant levels of hydroxylation. Similar utilization of α-ketoadipate has been reported in the reaction of some other α-KG-dependent dioxygenases such as prolyl 4-hydroxylase (28), but not in γ-butyrobetaine hydroxylase (29). The greatly enhanced affinity of α-ketoacids containing a second carboxyl group is consistent with the presence of a positively charged residue at the α-ketoacid binding site.

Chemical modification studies of TfdA were used to rule out the presence of essential and accessible cysteine or arginine residues and of highly reactive and essential lysine and carboxyl groups. In contrast, DEP rapidly inactivates the enzyme. Myllylä *et al.* (30) reported DEP-dependent inactivation of prolyl 4-hydroxylase and provided some evidence for substrate protection. In our more detailed analysis of substrate protection, neither 2,4-D, α-KG, nor Fe(II) alone was found to protect the enzyme from inactivation; yet, combinations of 2,4-D or α-KG with Fe(II) provide some protection, and a combination of 2,4-D and α-KG afforded nearly complete protection. These results are compatible with the presence of multiple essential histidine residues, as illustrated in Fig. 11, but do not rule out the possible DEP-reactivity of alternate residues. Consistent with the expected requirements for positive charges at the binding sites of 2,4-D and α-KG, we propose that essential histidine residues are present at the binding sites for both of these substrates. In addition, we propose that one or more additional histidine residues may be buried in the protein at the Fe(II) binding site, consistent with the known ability of histidine residues to function as metallocenter ligands. For example, spectroscopic analysis of isopenicillin N synthase, possibly a mechanistically related α-KG-dependent enzyme, suggested the presence of three histidine ligands to Fe(II) in that protein (31, 32). Binding of 2,4-D and α-KG would protect the histidine residues at the substrate binding sites and additionally may protect the Fe(II) ligands by steric constraints. Binding of either 2,4-D plus Fe(II) or α-KG and Fe(II) might lead to the observed reduced rate of inactivation. In contrast, addition of any one compound alone is unable to protect the three or more distinct sites of inactivation postulated in this model. Further studies clearly are required to assess the validity of this model.

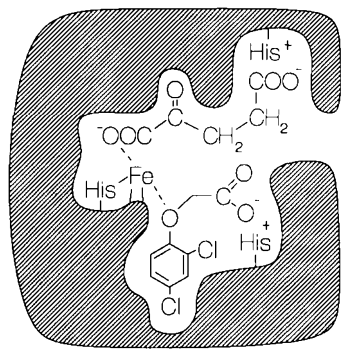

FIG. 11. **Model of the TfdA active site indicating potential roles of essential histidine residues.** The varied abilities of 2,4-D, α-KG, Fe(II), and mixtures of these compounds to protect against TfdA modification by DEP is interpretted in terms of a model that includes three distinct roles for histidine residues: assisting in the binding of 2,4-D, facilitating the binding of α-KG, and ligation of the metallocenter. The illustration is not meant to depict the Fe(II) coordination number or geometry, and the indicated Fe(II) coordination by the α-ketoacid group and the phenoxy oxygen is purely speculative at this time.

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