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Purification and Characterization of Two Enantioselective α-Ketoglutarate-Dependent Dioxygenases, RdpA and SdpA, from *Sphingomonas herbicidovorans* MH

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α-Ketoglutarate-dependent (*R*)-dichlorprop dioxygenase (RdpA) and α-ketoglutarate-dependent (*S*)-dichlorprop dioxygenase (SdpA), which are involved in the degradation of phenoxyalkanoic acid herbicides in *Sphingomonas herbicidovorans* MH, were expressed and purified as His_6-tagged fusion proteins from *Escherichia coli* BL21(DE3)(pLysS). RdpA and SdpA belong to subgroup II of the α-ketoglutarate-dependent dioxygenases and share the specific motif $HXDX_{24}TX_{131}HX_{10}R$. Amino acids His-111, Asp-113, and His-270 and amino acids His-102, Asp-104, and His 257 comprise the 2-His-1-carboxylate facial triads and were predicted to be involved in iron binding in RdpA and SdpA, respectively. RdpA exclusively transformed the (*R*) enantiomers of mecoprop [2-(4-chloro-2-methylphenoxy)propanoic acid] and dichlorprop [2-(2,4-dichlorophenoxy)propanoic acid], whereas SdpA was specific for the (*S*) enantiomers. The apparent K_m values were 99 μM for (*R*)-mecoprop, 164 μM for (*R*)-dichlorprop, and 3 μM for α-ketoglutarate for RdpA and 132 μM for (*S*)-mecoprop, 495 μM for (*S*)-dichlorprop, and 20 μM for α-ketoglutarate for SdpA. Both enzymes had high apparent K_m values for oxygen; these values were 159 μM for SdpA and >230 μM for RdpA, whose activity was linearly dependent on oxygen at the concentration range measured. Both enzymes had narrow cosubstrate specificity; only 2-oxoadipate was able to replace α-ketoglutarate, and the rates were substantially diminished. Ferrous iron was necessary for activity of the enzymes, and other divalent cations could not replace it. Although the results of growth experiments suggest that strain MH harbors a specific 2,4-dichlorophenoxyacetic acid-converting enzyme, *tfdA*-, *tfdA*α-, or *cadAB*-like genes were not discovered in a screening analysis in which heterologous hybridization and PCR were used.

Phenoxyalkanoic acid herbicides are widely used to control broadleaf weeds in agriculture, lawns, and industry. These compounds are systemic postemergence herbicides that act as synthetic auxins (1, 2, 28). Well-known representatives are mecoprop [2-(*R*,*S*)-2-methyl-4-chlorophenoxypropanoic acid], dichlorprop [2-(*R*,*S*)-2,4-dichlorophenoxypropanoic acid], and 2,4-D (2,4-dichlorophenoxyacetic acid). Mecoprop and dichlorprop are chiral molecules that have one stereogenic center and therefore exist as two enantiomers. In the 1950s, it was shown that only the (*R*) enantiomers had herbicidal effects (29). However, both enantiomers were and still are applied as racemic mixtures, resulting in the introduction of large amounts of unwanted chemicals into the environment (10, 50, 51). *Sphingomonas herbicidovorans* MH, an *Alphaproteobacterium*, is able to grow on both enantiomers of mecoprop and dichlorprop, as well as on 2,4-D, as sole carbon and energy sources (21, 24, 54). In batch cultures, this organism degrades the two enantiomers of mecoprop and dichlorprop sequentially, and the (*S*) enantiomers are degraded first (25, 32).

In the first reaction, both mecoprop and dichlorprop are enantioselectively converted by *S. herbicidovorans* MH to achiral phenols and pyruvate (Fig. 1). Nickel et al. (35) showed in experiments with cell extracts that two distinct enzymes are involved. These enzymes are highly specific for the corresponding enantiomers and belong to the family of α-ketoglutarate-dependent dioxygenases. The α-ketoglutarate-dependent dioxygenases are a group of enzymes which are classified on the basis of their biochemical characteristics. They are nonheme iron-dependent dioxygenases that require both oxygen and α-ketoglutarate as substrates. For many of these dioxygenases ascorbate has been used as a reducing agent. α-Ketoglutarate-dependent dioxygenases catalyze a wide range of oxidative processes, such as hydroxylations, epoxidations, desaturations, ring formations, and expansion reactions (7, 17, 27, 38, 44). Despite the diversity of their primary sequences, all of these dioxygenases have a 2-His-1-carboxylate facial triad at the catalytic center (19) and also common amino acid motifs, on the basis of which they are classified into three subgroups (20). Relevant for the work described here is subgroup II, and the representatives of this subgroup have the motif $HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R$. More prominent members of this subgroup are taurine dioxygenase (TauD) from *Escherichia coli*, which is involved in taurine utilization (11), and 2,4-D dioxygenase (TfdA) from *Cupriviadus necator* (formerly *Ralstonia eutropha*) JMP134(pJP4). TfdA is involved in 2,4-D degradation and catalyzes the oxidative cleavage of the ether bond in 2,4-D, yielding 2,4-dichlorophenol (15, 16, 18).

Recently, several genes coding for the enzymes involved in phenoxypropanoic acid herbicide degradation in *S. herbi-*

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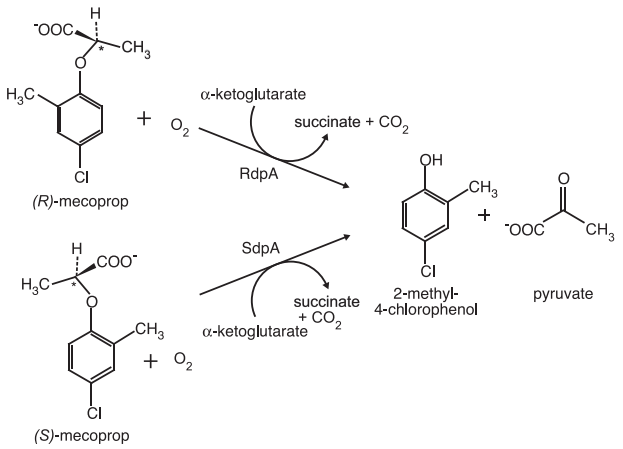

FIG. 1. Initial step in the degradation of chiral phenoxypropanoic acids in *S. herbicidovorans* MH catalyzed by the α-ketoglutarate-dependent (*R*)-dichlorprop dioxygenase (RdpA) and the α-ketoglutarate-dependent (*S*)-dichlorprop dioxygenase (SdpA).

cidovorans MH (31) and *Delftia acidovorans* MC1 (43) were identified and isolated. The genes coding for two α-ketoglutarate-dependent dioxygenases designated RdpA and SdpA were suggested to be responsible for the initial steps in the degradation of (*R*)- and (*S*)-mecoprop, respectively. The deduced amino acid sequence of RdpA from *S. herbicidovorans* MH is 30% identical to that of TfdA and 100% identical to that of RdpA from *D. acidovorans* MC1. The deduced amino acid sequence of SdpA from *S. herbicidovorans* MH exhibits only 60% identity to that of SdpA from *D. acidovorans* MC1 and about 30% identity to that of RdpA (30, 31, 43, 48).

In this study, we expressed and purified RdpA and SdpA from *S. herbicidovorans* MH as His_6-tagged fusion proteins. By measuring enzyme activities with a novel coupled enzyme assay, we verified that RdpA and SdpA are α-ketoglutarate-dependent dioxygenases belonging to subgroup II. We also characterized the kinetic behavior of the enzymes with various substrates and cosubstrates. In addition, we determined the substrate and cosubstrate specificities and obtained clear evidence that the two enzymes have opposite enantioselectivities.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* DH5α was used as a host for cloning experiments, and *E. coli* BL21(DE3)(pLysS) was used as a host for protein expression studies with pET-15b-based constructs (Novagen, Darmstadt, Germany). The *E. coli* strains were grown at 30°C or 37°C in Luria-Bertani medium (42). Ampicillin and chloramphenicol were added at final concentrations of 50 μg/ml and 25 μg/ml, respectively. Solid media were prepared by addition of 1.5% (wt/vol) agar.

Standard molecular techniques. Cloning and digestion were done by using established procedures (4, 42). Restriction enzymes and other DNA-modifying enzymes were purchased from Promega (Wallisellen, Switzerland) and Fermentas (Nunningen, Switzerland). Plasmids and cosmids were isolated by the boiling miniprep method or the alkaline lysis method described by Sambrook et al. (42) or by using an E.Z.N.A. plasmid miniprep kit II (Peqlab Biotechnologies GmbH, Baden-Dättwil, Switzerland) as suggested by the manufacturer. Purification of DNA fragments from agarose gel was carried out by using a MinElute gel extraction kit (QIAGEN AG, Basel, Switzerland) according to the protocol of the supplier.

Construction of N-terminal His_6-tagged recombinant enzyme expression plasmids. Expression plasmids pMec15 and pMec19 were constructed by reamplification by PCR of the *rdpA* and *sdpA* genes from pMec10 and pMec16, respectively, (31) with the following primers: 5′-CGC TCA TAT GCA TGC TGC ACT-3′ and 5′-AGC GGG GAT CCG CGT CGC C-3′ for *rdpA* and 5′-CAG GAG GAT TCA TAT GTC A-3′ and 5′-GCC AGC TGG ATC CGC CGA TGA-3′ for *sdpA*. In this way, an NdeI restriction site at the translational start and a BamHI restriction site after the stop codon were introduced (underlined nucleotides). The PCR-amplified fragments were first cloned into pGEM-T Easy (Promega), generating pMec14 and pMec18, which was verified by DNA sequencing. The plasmids were then digested with NdeI and BamHI, and the *rdpA* or *sdpA* inserts were recovered, purified, and ligated into the same sites of vector pET-15b. After transformation, this yielded plasmids pMec15 and pMec19.

Expression and purification of recombinant RdpA and SdpA. *E. coli* BL21-(DE3)(pLysS) harboring pMec15 or pMec19 was grown in Erlenmeyer flasks containing Luria-Bertani medium supplemented with ampicillin and chloramphenicol at 30°C with constant shaking. To express the recombinant proteins, cultures were grown until an optical density at 546 nm of 0.4 to 0.6 was reached, after which isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. The culture was incubated for another 3 h at 30°C, and the cells were harvested by centrifugation at 7,500 × *g* for 30 min at 4°C. The pellet was stored at −20°C until it was used. For preparation of cell extracts, 1 g (wet weight) of cells was resuspended in 3 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8.0), and the cells were broken by one passage through a French press at 1 MPa. The lysate was centrifuged for 45 min at 16,000 × *g* at 4°C, and aliquots were stored at −20°C.

His_6-tagged RdpA was purified by a one-step procedure. Up to 1 mg of protein was loaded on a 2-ml Ni-nitrilotriacetic acid (NTA) Superflow agarose column (QIAGEN) at a flow rate of 0.5 ml/min. Unbound protein was removed stepwise by washing with five bed volumes (~10 ml) of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole; pH 8.0). After the initial washing step, wash buffers containing 50 mM imidazole and then 100 mM imidazole were applied. RdpA was then eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole; pH 8.0) at a flow rate of 0.8 ml/min and collected in 1-ml fractions. Fractions were analyzed for α-ketoglutarate-dependent dioxygenase activity; active fractions were pooled and desalted with 20 mM Tris-HCl (pH 7.5) with PD-10 columns (Amersham Biosciences, Dübendorf, Switzerland). The purified RdpA solution was stored at 4°C.

Recombinant SdpA was purified by a two-step procedure. Unless indicated otherwise, all steps were carried out at 4°C. Up to 1 mg protein was loaded on a 5-ml Superflow agarose column (QIAGEN) at a flow rate of 0.3 ml/min. Unbound protein was removed stepwise with 5 column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl) containing 20 mM imidazole (pH 8) and 5 column volumes of wash buffer containing 50 mM imidazole (pH adjusted to 8). SdpA was then eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 200 mM imidazole; pH adjusted to 8) at a flow rate of 0.6 ml/min. One milliliter fractions were collected and analyzed, and active fractions were pooled and desalted with 20 mM Tris-HCl (pH 7.5) using PD-10 columns (Amersham Biosciences, Dübendorf, Switzerland) at room temperature. The SdpA solution was then loaded on a Bioscale Q2 column (Bio-Rad) equilibrated in the same buffer. The enzyme was eluted with a linear gradient from 20 mM Tris-HCl (pH 7.5) to 20 mM Tris-HCl (pH 7.5)–600 mM NaCl in 6 column volumes. SdpA was found to elute at approximately 220 mM NaCl. The fractions that exhibited activity were pooled and stored at 4°C.

Thrombin protease (Amersham Biosciences) was used to cleave the N-terminal His_6 tags of RdpA and SdpA. The N-terminal His_6 tag of approximately 10 μg enzyme was completely cleaved by 0.5 U thrombin protease within 2 h at room temperature.

Enzyme activity assays. (i) Coupled continuous assay. For purification and kinetic measurements of phenoxypropanoic acids, a coupled continuous assay with L-lactate dehydrogenase from rabbit muscle (Sigma-Aldrich, Buchs, Switzerland) as a reporter enzyme was developed. RdpA and SdpA stoichiometrically convert (substituted) phenoxypropanoic acids to the corresponding phenols and pyruvate. Pyruvate is converted by L-lactate dehydrogenase to lactate with concomitant consumption of NADH. NADH consumption was monitored spectrophotometrically by determining the decrease in absorbance at 340 nm. An extinction coefficient of 6.3 mM^{-1} cm^{-1} was used to calculate activities. The standard 0.5-ml assay mixture contained 50 mM imidazole (pH 6.75), 1 mM substrate, 1 mM α-ketoglutarate, 100 μM $(NH_4)_2Fe(SO_4)_2$, 1 mM ascorbic acid, 0.32 mM NADH, 1.2 U of L-lactate dehydrogenase, and 0.05 μg of purified RdpA or SdpA. Activity assays were done at 30°C unless indicated otherwise and were started by addition of the substrate. NADH consumption was monitored for 30 s, and the linear portion of the curve was used to calculate the activity. The substrate-specific oxidation rates were corrected for nonspecific NADH oxidation in the absence of substrate (endogenous rate). Specific activities were expressed as turnover rate (min^{-1}).

TABLE 1. Purification of α-ketoglutarate-dependent (*R*)-dichlorprop dioxygenase and α-ketoglutarate-dependent (*S*)-dichlorprop dioxygenase

Fraction	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (min^{-1})	Recovery (%)	Purification (*n*-fold)
α-Ketoglutarate-dependent (*R*)-dichlorprop dioxygenase						
Cell extract	10	133.1	30.9	7	100	1
Ni-NTA Superflow	7	1.8	7	133	22.7	19
α-Ketoglutarate-dependent (*S*)-dichlorprop dioxygenase						
Cell extract	3.5	42.57	18.48	14	100	1
Ni-NTA Superflow	5	1.05	7.71	247	41.70	17.1
BioScale Q2	3	0.71	6.34	299	34.32	20.7

(ii) Discontinuous assay. Experiments with alternative substrates were performed using a discontinuous assay by measuring the formation of the product by high-performance liquid chromatography. The standard assay mixture contained 50 mM imidazole (pH 6.75), 1 mM substrate, 1 mM α-ketoglutarate, 100 μM $(NH_4)_2Fe(SO_4)_2$, and 1 mM ascorbic acid. The assay mixtures were incubated at 30°C for 1 min and were inactivated in a boiling water bath for 2 min. Negative controls without enzyme were treated in the same manner. The products were analyzed on a Nucleodex α-PM column (200 by 4 mm; Macherey-Nagel, Düren, Germany) as described previously (35). Specific activity was defined as the amount of phenol derivative (μmol) that was formed per min per mg of protein. Oxidation rates were corrected for nonspecific formation of the product in the absence of the enzyme.

Oxygen electrode measurements. Kinetic measurements with oxygen were obtained with a Rank Brothers oxygen electrode at 30°C. The standard 1-ml assay mixture contained 50 mM imidazole (pH 6.75), 2 mM substrate, 2 mM α-ketoglutarate, 100 μM $(NH_4)_2Fe(SO_4)_2$, and 1 mM ascorbic acid. The assays were started by addition of the substrate, and the O_2 concentration was monitored at 2-s intervals for 15 min. An assay without substrate showed that the amount of background O_2 consumed was small and could be neglected for calculation of activities. Specific activity was defined as the amount of oxygen (μmol) reduced per min per mg of protein in the presence of a substrate and expressed as turnover rate (min^{-1}).

Kinetic measurements. The kinetic parameters K_m and k_{cat} were calculated using IgorPro software (WaveMetrics Inc., Lake Oswego, OR). The method used for calculation was based on a weighted nonlinear regression analysis of the Michaelis-Menten model. To calculate K_m and k_{cat} of RdpA with respect to oxygen, we used the first derivative (i.e., the velocity, expressed in μmol min^{-1}) of the oxygen concentration monitored with time.

Determination of the protein concentration and SDS-PAGE. Protein concentrations were determined by the Bradford method (6) with the Bio-Rad reagent dye concentrate reagent by following the instructions of the manufacturer (Bio-Rad, Reinach, Switzerland). Bovine serum albumin fraction V (Sigma-Aldrich) was used as a reference protein. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out with 12% acrylamide slab gels by using established procedures (26).

Molecular weight determination. Molecular weights of purified enzymes were determined by using a Superdex 200 preparation grade HiLoad 16/60 size exclusion column (Amersham Biosciences). Approximately 0.25 mg of protein was loaded on the HiLoad column and eluted at a flow rate of 0.2 ml/min with 50 mM Na_2HPO_4-KH_2PO_4 buffer (pH 7) containing 150 mM NaCl. A calibration curve was produced with the gel filtration standard from Bio-Rad containing bovine thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobulin (17 kDa), and vitamin B_{12} (1.35 kDa). Blue dextran was used to determine the void volume.

Screening for additional α-ketoglutarate-dependent phenoxyalkanoic acid dioxygenases. To screen for *tfdA*- and *tfdA*α-like genes, we amplified DNA by PCR performed with the *tfdA*-derived primers tfdAfor (5′-TGG CAI AGC GAC AGI TCC TTT-3′), tfdArev (5′-CIG CGG TTG TCC CAI ATC IC-3′), tfdAfor2 (5′-ACG GAG TTC TG[C/T] GA[C/T] AT-3′), and tfdArev2 (5′-AAC GCA GCG [G/A]T[G/T] ATC CCA-3′) (46) in all possible combinations and with chromosomal DNA of *S. herbicidovorans* MH. The oligonucleotides used for PCR were obtained from Microsynth GmbH (Balgach, Switzerland). As a positive control, total DNA of *C. necator* JMP134(pJP4) was used.

For Southern hybridization, total DNA of *S. herbicidovorans* MH fractionated on an agarose gel was blotted on a Hybond-XL membrane (Amersham Biosciences, Dübendorf, Switzerland) with a vacuum blotter (VacuGene XL; Amersham Biosciences) by the procedure described by Ravatn et al. (40). Total DNA was hybridized with a 0.7-kb EcoRI fragment from pCBA101 (0.7-kb EcoRI fragment with *tfdA* in pGEM7; van der Meer, unpublished) and with a 1.1-kb SalI fragment of pBHS1 harboring part of *cadAB* (23). The fragments were radioactively labeled with [α-^{32}P]dATP using a Random Primed DNA labeling kit or with digoxigenin-dUTP using a DIG High Prime II DNA labeling and detection starter kit according to the protocol of the manufacturer (Roche Applied Science, Rotkreuz, Switzerland). Hybridization experiments were performed in SDS/bovine serum albumin hybridization buffer (0.5 M sodium phosphate [pH 7], 7% sodium dodecyl sulfate, 1% bovine serum albumin) at 55°C for 16 h. The membranes were washed twice with 5× SSC containing 1 mM EDTA for 2 min at room temperature and twice with 2× SSC containing 0.1% SDS for 30 min at 55°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). When digoxigenin-labeled probes were used, immunological detection was carried out by using the supplier's protocol (Roche Applied Science).

Sequencing. DNA sequencing was performed with double-stranded DNA templates by using a Thermo Sequenase primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences) and M13 primers (5). Sequencing reaction mixtures were analyzed with an automated DNA sequencer (model 4200 IR^2; LI-COR Inc., Lincoln, NE). The primers used for sequencing were labeled at the 5′ end with IRD-700 or IRD-800 and were purchased from MWG Biotech AG (Ebersberg, Germany). Sequencing data were analyzed with DNASTAR software (DNASTAR Inc., Madison, WI) and were compared to the databases by BLAST searches (www.ncbi.nlm.nih.gov/BLAST, (45)).

Chemicals. Phenoxyalkanoic acids were purchased from Riedel-de Haën (Buchs, Switzerland) (PESTANAL; purity, 99%) or Sigma-Aldrich (purity, 97 to 99%). (*S*)-Mecoprop and (*S*)-dichlorprop were synthesized by C. Zipper (52). α-Ketoglutarate and other (di)carboxy acids were purchased from Sigma-Aldrich. All other chemicals were obtained from Fluka (Buchs, Switzerland) or from Merck AG (Dietikon, Switzerland).

RESULTS

Expression and purification of recombinant RdpA and SdpA. The *rdpA* and *sdpA* genes were expressed as N-terminal His_6-tagged fusion proteins in *E. coli* BL21(DE3)(pLysS) harboring pMec15 and pMec19, respectively. Expression resulted in high levels of recombinant RdpA and SdpA in the soluble fraction that accounted for approximately 16% and 10%, respectively, of the total protein as determined by measuring the band intensities of SDS-PAGE gels. A decrease in the incubation temperature during expression in *E. coli* did not increase the amount of the His_6-tagged enzymes in the cell extract. His_6-tagged RdpA was purified about 16-fold to apparent homogeneity by Ni-NTA affinity chromatography, whereas His_6-tagged SdpA was purified to apparent homogeneity by a two-step procedure (Table 1 and Fig. 2). The calculated molecular masses of the subunits of RdpA and SdpA were 34.9 kDa and 33.7 kDa, respectively, which was consistent with the sizes estimated by SDS-PAGE (approximately 36 kDa and 32 kDa, respectively).

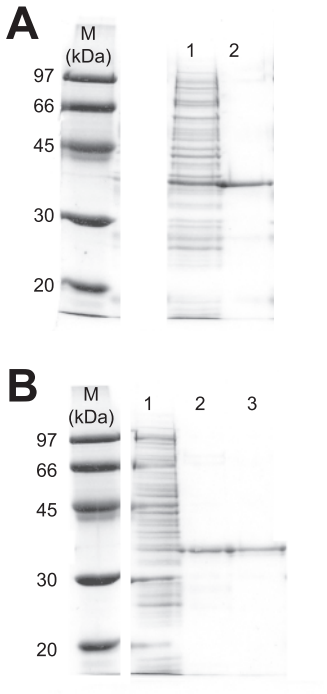

FIG. 2. Purification of RdpA (A) and SdpA (B) from *S. herbicidovorans* MH expressed as His_6-tagged fusion proteins. Protein samples were analyzed by denaturing gel electrophoresis on a 12% polyacrylamide gel and were stained with Coomassie brilliant blue. Lane M contained molecular mass markers. (A) Lane 1, extract of induced cells; lane 2, apparently pure RdpA (after Ni-NTA Superflow chromatography). (B) Lane 1, extract of induced cells; lane 2, partially purified SdpA (after Ni-NTA Superflow chromatography); lane 3, apparently pure SdpA (after ion-exchange chromatography).

The molecular weights of the native proteins were determined by size exclusion chromatography after the His_6 tag was cleaved by thrombin treatment. A molecular mass of 104 ± 7 kDa was obtained for native RdpA. This is consistent with a homotrimeric structure based on the calculated subunit molecular mass, 33.2 kDa. Native SdpA had a molecular mass of 32 kDa, a strong indication that the structure is monomeric.

The fusion proteins were treated with thrombin protease to cleave the His_6 tag, and the enzyme activities of the native and the His_6-tagged proteins were determined. In both cases, the His_6-tagged and native enzymes were equally active, showing that the His_6 tag did not affect activity. Therefore, all further experiments were carried out with the His_6-tagged recombinant proteins. Below, RdpA and SdpA refer to the to the His_6-tagged enzymes, unless indicated otherwise.

RdpA and SdpA are α-ketoglutarate-dependent dioxygenases that catalyze enantioselective reactions. RdpA and SdpA have been classified as α-ketoglutarate-dependent dioxygenases based on their amino acid sequences (31). This classification was verified by enzyme assays. RdpA and SdpA activities were completely absent in the absence of α-ketoglutarate, were strongly reduced in assays without ferrous iron, and were slightly reduced in the absence of ascorbate (Table 2).

The results of three different assays, a coupled continuous assay, a discontinuous assay, and an O_2 measurement assay, showed that there was stoichiometric product formation and substrate consumption. The specific activity of RdpA ranged from 2 to 4 U/mg protein. Determinations of the specific activity of RdpA were reproducible within single purified RdpA batches but could vary between independently induced *E. coli* cultures by a factor of two.

RdpA converted only the (*R*) enantiomers of phenoxypropanoic acids, and no activity was observed with the (*S*) enantiomers. The enantioselectivity was exactly the opposite for SdpA, which converted only the (*S*) enantiomers. In contrast to RdpA, SdpA was slightly inhibited in the presence of the nonsubstrate enantiomer (data not shown).

Physicochemical characterization. RdpA was stable at 4°C for up to 8 weeks and at room temperature for up to 7 days, but it was inactivated by several freezing and thawing cycles. Therefore, RdpA was stored at 4°C. Optimum activity was observed at pH 6.5 (Fig. 3A). Temperature dependence was also studied, but no optimum could be determined (Fig. 3B). The experiments were carried out with two independently purified RdpA batches. To avoid possible aberrant results due to inactivation of L-lactate dehydrogenase in the coupled continuous assay, specific activities were determined once by the coupled continuous assay and then by the discontinuous assay. RdpA activity was stable over a relatively wide temperature range, 20 to 55°C. Slightly higher activity was observed at 10°C and 20°C, but only in the discontinuous assay. However, the general trends for the two types of enzyme assays were similar and revealed no Arrhenius-like behavior.

SdpA was stable at room temperature for several hours and in 3 days lost less than 10% of its activity when it was stored at 4°C. Storage on ice and freeze-thaw cycles reduced the activity to about 80% of the original activity. More consistent specific activities were obtained when SdpA was prewarmed at room temperature prior to enzymatic assays. The pH optimum was pH 6.5 (Fig. 3C). In contrast to the RdpA activity, the SdpA activity had an optimum temperature between 35° and 40°C (Fig. 3D).

Substrate, cosubstrate, and cofactor specificities. Substrate specificities were determined by the coupled continuous assay for phenoxypropanoic acids and by the discontinuous assay for all other substrates. We examined the influence of the alkanoic acid group attached by an ether bond to the phenoxy ring by determining the activities with phenoxyacetic, phenoxypropanoic, and phenoxybutyric acids as substrates. The effects of different substituents, such as hydroxy, methyl, and chloride

TABLE 2. Dependence of α-ketoglutarate-dependent (*R*)- and (*S*)-dichlorprop dioxygenase activities on substrate, cosubstrate, and cofactors

Assay mixture	RdpA		SdpA	
	Activity (%)	Sp act (min^{-1})	Activity (%)	Sp act (min^{-1})
Complete	100	118.8	100	236.2
Complete without:				
(R)-Mecoprop	0	0	ND[a]	ND
(S)-Mecoprop	ND	ND	0	0
α-Ketoglutarate	0	0	0	0
Fe(II)	17.6	21.0	8.2	19.1
Ascorbate	85.3	101.4	37.8	89.2

[a] ND, not determined.

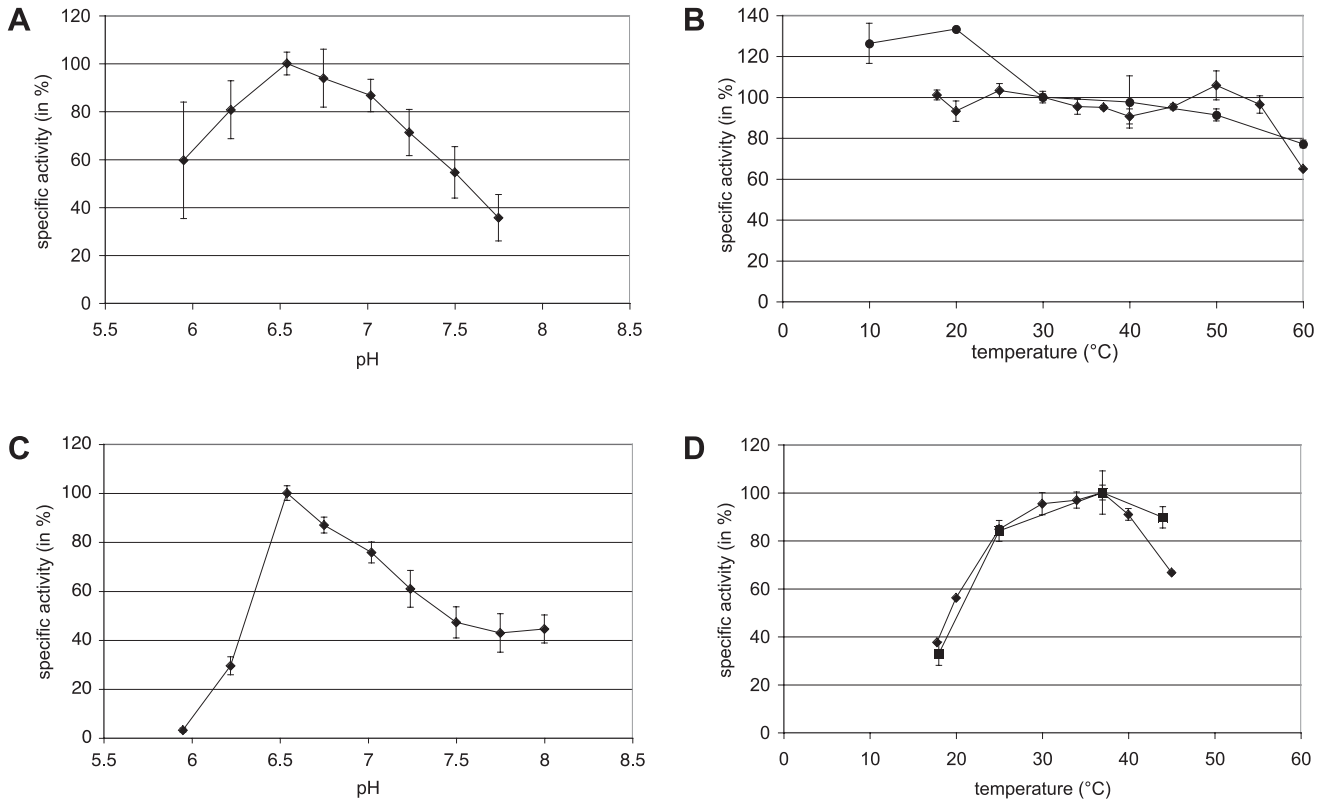

FIG. 3. Dependence of RdpA and SdpA on pH (A and C, respectively) and temperature (B and D, respectively). (A) The symbols indicate the means of six measurements. Enzyme activity was determined by the coupled continuous assay. (B) Symbols: ◆, data obtained with the discontinuous assay; ●, data obtained with the coupled continuous assay. The experiments were performed with two independently purified RdpA samples. (C) The symbols indicate the means of six measurements for two independent experiments. Enzyme activity was determined by the coupled continuous assay. (D) Symbols: ■, data obtained with the discontinuous assay; ◆, data obtained with the coupled continuous assay. The experiments were performed with two independently purified SdpA samples.

groups on the aromatic ring, were investigated as well. RdpA converted phenoxypropanoic acids and none of the phenoxyacetic or phenoxybutyric acids except 3-methyl-5-chlorophenoxyacetic acid (MCPA). 2-(*R*,*S*)-4-Chlorophenoxypropanoic acid was turned over at the highest rate, followed by dichlorprop and mecoprop. Interestingly, the hydroxylated phenoxypropanoic acids and the unsubstituted phenoxypropanoic acids were converted (Table 3). SdpA exhibited the highest turnover rates with substituted phenoxypropanoic acids, and mecoprop was transformed fastest (Table 3). In contrast to RdpA, SdpA was able to convert MCPA and 2,4-D at low but measurable rates.

We also tested whether several carboxylic and dicarboxylic acids could replace α-ketoglutarate as a cofactor. Since the reporter enzyme in the coupled continuous assay, L-lactate dehydrogenase, showed activity with some cosubstrates, in this case the RdpA and SdpA activities were determined by the discontinuous assay. RdpA and SdpA were quite specific for α-ketoglutarate, with specific activities of 132.8 min^{-1} and 226.1 min^{-1}, respectively, and exhibited only low levels of activity in the presence of 2-oxoadipate (1.9% and 5%, respectively). There was no activity with any of the other carboxylic acids tested (oxaloacetate, 2-oxobutyrate, and 2-oxovalerate).

In the absence of ferrous iron, the activities of the two enzymes were substantially reduced (Table 2). The activity was independent of the iron salt, and other divalent cations could not substitute for Fe(II) (Table 4). Some of the divalent cations, including Co(II), Mg(II), Mn(II), and Ni(II), slightly inhibited enzyme activity.

Kinetic analysis. The apparent kinetic constants are shown in Table 5. For RdpA, Michaelis-Menten kinetics was observed for all substrates except oxygen; the reaction rate was linearly dependent on the oxygen concentration up to a concentration of about 1.0 mM. Therefore, apparent K_m and k_{cat} values could not be determined. However, it was possible to calculate a specificity constant (k_{cat}/K_m) for oxygen, which was 0.64 μM^{-1} min^{-1}. For the phenoxypropanoic acid substrates, RdpA exhibited the lowest K_m value and highest specificity for (*R*)-mecoprop, whereas the k_{cat} was highest for (*R*,*S*)-4-chlorophenoxypropanoic acid. The apparent K_m value for α-ketoglutarate was 2.8 μM; this value is below the lowest α-ketoglutarate concentration for which activity could be measured, but the data fit a Michaelis-Menten curve well, and according to the goodness of fit of the nonlinear regression analysis the value was reliable. SdpA exhibited Michaelis-Menten kinetics with all substrates and cosubstrates tested. Mecoprop was a better substrate than dichlorprop; i.e., the apparent K_m value was lower and the catalytic efficiency was higher. SdpA had an apparent K_m of 19.6 μM for α-ketoglutarate and an apparent K_m of 159 μM for oxygen.

TABLE 3. Substrate specificities of α-ketoglutarate-dependent (*R*)- and (*S*)-dichlorprop dioxygenases[a]

Substrate	RdpA		SdpA	
	Activity (%) (mean ± SD)	Sp act (min^{-1})	Activity (%) (mean ± SD)	Sp act (min^{-1})
(*R*,*S*)-Mecoprop	100	108.3/140.9[b]	100	274.8/209.7[b]
(*R*,*S*)-Dichlorprop	115.5 ± 3.6	125.8	65.3 ± 9.7	170.4
2-(*R*,*S*)-Phenoxypropanoic acid	39.7 ± 7.6	41.9	11.6 ± 2.6	31.5
2-(*R*,*S*)-3-Chlorophenoxypropanoic acid	14.8 ± 2.0	17.5	33.2 ± 2.5	96.6
2-(*R*,*S*)-4-Chlorophenoxypropanoic acid	152.1 ± 9.0	171.3	66.1 ± 3.2	198.6
2-(*R*,*S*)-2,4,5-Trichlorophenoxypropanoic acid	49.4 ± 4.3	55.9	71.5 ± 6.6	217.7
2-(*R*,*S*)-4-Hydroxyphenoxypropanoic acid	51.3 ± 7.3	55.9	19.6 ± 3.2	60.4
2,4-D[c,d]	<2	<3.5	5.6 ± 5.2	11.1
MCPA[c]	7.7 ± 4.3	10.5	20.3 ± 6.2	41.9
2,4-Dichlorophenoxybutyric acid[c,e]	<2	<3.5	<1	<3.4
4-Chloro-2-methylphenoxybutyric acid[c]	<2	<3.5	2.3 ± 1.2	4.7

[a] The substrate range was determined with RdpA and SdpA purified from two independent batches, and the enzyme activity of each batch was measured three times with all substrates.
[b] Activity determined by the coupled continuous assay/activity determined by the discontinuous assay.
[c] Enzyme activity was determined by the discontinuous assay.
[d] Only one SdpA batch showed slight activity with 2,4-D. The means might be overestimates.
[e] In one experiment, very low activity was observed. However, the 2,4-dichlorophenol concentration was below the detection limit (1 μM), and no increase in the amount of 2,4-dichlorophenol converted per unit of time was observed when the amount of RdpA in the assay was increased. Therefore, the activity was defined as <2%.

Screening for other 2,4-D-degrading genes. We screened the total DNA of *S. herbicidovorans* MH for genes encoding a TfdA-like dioxygenase and the aromatic ring-hydroxylating dioxygenase CadAB by performing hybridization and PCR experiments. Hybridization experiments with both *tfdA*- and *cadAB*-derived probes did not give positive results, and no *tfdA*-like gene could be amplified with the PCR primers derived from the conserved regions of the *tfdA* and *tfdA*α genes.

DISCUSSION

RdpA and SdpA are α-ketoglutarate-dependent dioxygenases. α-Ketoglutarate-dependent dioxygenases are grouped according to their cosubstrate and cofactor requirements. Enzyme activity measurements demonstrated that RdpA and SdpA belong to the α-ketoglutarate-dependent dioxygenase enzyme family, since their activities were strictly dependent on oxygen and α-ketoglutarate. Without ascorbate the activities were reduced to about 85% and 39%, respectively, of the highest activities. Ascorbate is not formally needed in the reaction, and it is generally thought to maintain Fe(II) in the reduced state. Two other α-ketoglutarate-dependent dioxygenases, prolyl 4-hydroxylase and lysyl hydroxylase, are almost completely inactivated within 1 min in the absence of ascorbate (9, 33, 34, 39). The inactivation is thought to involve a side reaction in which α-ketoglutarate is decarboxylated. This reaction is coupled to the oxidation of Fe(II) to Fe(III). Ascorbate reduces the ferric iron, thereby reactivating the enzyme. TfdA, on the other hand, is not as sensitive to the absence of ascorbate and is inactivated only after 30 to 40 min (16). The reductions in RdpA and SdpA activities in the absence of ascorbate indicated that α-ketoglutarate decarboxylation side reactions took place, although the inactivation was not as fast as the inactivation of the peptide hydroxylases.

Alignment of RdpA and SdpA with other α-ketoglutarate-dependent dioxygenases revealed conservation of the 2-His-1-carboxylate facial triad in RdpA and SdpA, as well as several other conserved amino acid residues (Fig. 4). RdpA and SdpA have the metal binding motif of subgroup II of the α-ketoglutarate-dependent dioxygenase family, which is $HXDX_{24}TX_{131}$

TABLE 4. Cofactor specificities of α-ketoglutarate-dependent (*R*)- and (*S*)-dichlorprop dioxygenases

Cofactor	RdpA		SdpA	
	Activity (%)	Sp act (min^{-1})	Activity (%)	Sp act (min^{-1})
$Fe(NH_4)_2(SO_4)_2$	100	97.9	100	243.2
$Fe(SO_4)$	97.5	94.4	92.8	229.1
$CaCl_2$	15.9	14.0	0	0
$CoCl_2$	0	0	0	0
$MgCl_2$	9.4	10.5	0	0
$MnCl_2$	0	0	0	0
$NiCl_2$	0	0	0	0

TABLE 5. Kinetic parameters of α-ketoglutarate-dependent (*R*)-dichlorprop dioxygenase and α-ketoglutarate-dependent (*S*)-dichlorprop dioxygenase

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
α-Ketoglutarate-dependent (*R*)-dichlorprop dioxygenase			
(*R*)-Mecoprop (10–2,000 μM)	98.8 ± 6.4	114.2	1.16
(*R*)-Dichlorprop (50–2,000 μM)	163.7 ± 13.5	170.1	1.04
(*R*,*S*)-4-Chlorophenoxypropanoic acid (120–2,000 μM)	262.4 ± 10.1	241.9	0.92
O_2 (0.02–1.0 mM)	ND[a]	ND	0.64
α-Ketoglutarate (7–1,900 μM)	2.8 ± 0.8	135.6	48.82
α-Ketoglutarate-dependent (*S*)-dichlorprop dioxygenase			
(*S*)-Mecoprop (50–2,000 μM)	132 ± 17.7	303.9	2.3
(*S*)-Dichlorprop (120–2,000 μM)	494.8 ± 43.9	284.6	0.58
O_2 (0.02–0.2 mM)	159.32	116[b]	0.75
α-Ketoglutarate (8–1,000 μM)	19.6 ± 2.5	229.7	11.72

[a] ND, not determined.
[b] The kinetic values for O_2 were determined with material from two different SdpA purification batches, both of which were less active than the purification batches used for other kinetic experiments. However, the stoichiometry of the reaction was verified with material from a third purification batch (data not shown).

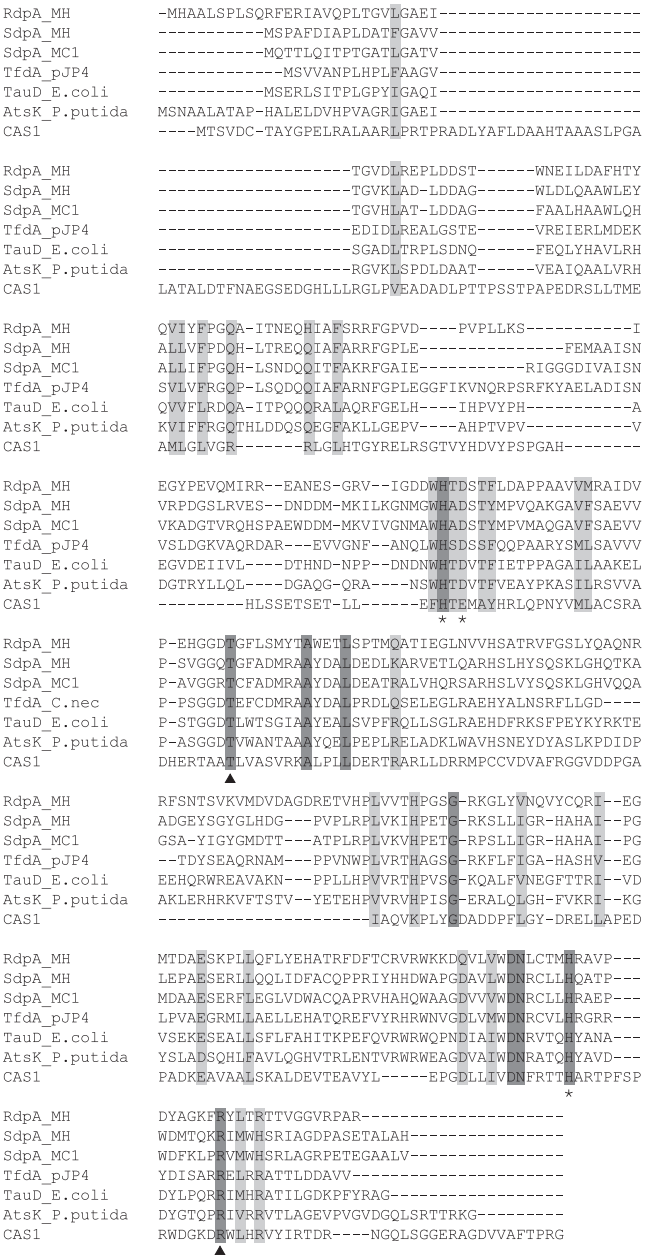

FIG. 4. Alignment of amino acid sequences of RdpA and SdpA from strain MH and other group II α-ketoglutarate-dependent dioxygenases. The alignment was performed with the T-Coffee software (http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi). TfdA_pJP4, 2,4-D dioxygenase from *C. necator* JMP134(pJP4) (accession no. AAA21983); RdpA_MH, (*R*)-dichlorprop dioxygenase from *S. herbicidovorans* MH (accession no. CAF32811); SdpA_MH, (*S*)-dichlorprop dioxygenase from *S. herbicidovorans* MH (accession no. CAF32813); SdpA_MC1, (*S*)-dichlorprop dioxygenase from *D. acidovorans* MC1 (accession no. AAM90963); TauD_E. coli, taurine dioxygenase from *E. coli* (accession no. AAC73471); AtsK_P. putida, alkylsulfate dioxygenase from *P. putida* (accession no. AAD31784); CAS1, clavaminate synthase from *Streptomyces clavuligeres* (accession no. A44241). Highly conserved residues (dark gray boxes) and conserved residues (light gray boxes) are highlighted. Asterisks indicate the conserved or predicted Fe(II)-binding residues, and triangles indicate the α-ketoglutarate binding residues (12, 36).

$HX_{10}R$. On the basis of the crystal structure of TauD, a structural model of TfdA, and mutagenesis studies (12, 20, 36), we concluded that His-111–Asp-113–His-270 and His-102–Asp-104–His-257 comprise the facial triads of RdpA and SdpA, respectively, which are responsible for iron binding. Residues Thr-138 and Arg-281 of RdpA and residues Thr-129 and Arg-268 of SdpA might act as α-ketoglutarate binding sites.

A comparison of kinetic data for RdpA from *S. herbicidovorans* MH with kinetic data for RdpA from *D. acidovorans* MC1 (48) revealed certain differences. Although the amino acid sequences of the enzymes are identical, the reported K_m values of the phenoxypropanoic acid derivatives for RdpA from *D. acidovorans* MC1 are less than the K_m values that we report here by a factor of about 3. The differences might be explained by the fact that different methods were used to obtain the kinetic data. In our hands, the method based on 2-aminoantipyridine (13–15) that was used for determination of the kinetic parameters of RdpA from *D. acidovorans* MC1 (48) never gave reproducible results for kinetic measurements at low substrate concentrations because of uncontrolled formation of background color. Nonspecific background activity might lead to overestimation of enzyme velocity at low substrate concentrations, which in turn might lead to underestimation of K_m values. Our problem with the aminoantipyrine method was the main reason for developing the continuous coupled enzyme assay described in Materials and Methods. With this assay, we were able to monitor the enzyme reaction continuously and to determine true initial velocities.

To our knowledge, the RdpA proteins from *S. herbicidovorans* MH and *D. acidovorans* MC1 are the only α-ketoglutarate-dependent dioxygenases with a homotrimeric structure (47). Most enzymes of this class are monomers or homodimers, such as SdpA and TfdA; tetramers, such as the alkylsulfatase from *Pseudomonas putida*, are also known (11, 16, 47).

RdpA from *S. herbicidovorans* was surprisingly insensitive to an increase in temperature (Fig. 3B). No Arrhenius-like activity profile was observed, and RdpA was found to be stable at temperatures up to 55°C. This finding correlates with the findings for an α-ketoglutarate-dependent 2,4-D dioxygenase from *Burkholderia cepacia* 2a, which also showed an unexpectedly small increase in activity with increasing temperature and at 10°C still exhibited 77% of the activity observed at 25°C (37). In contrast, TfdA from *C. necator* JMP134(pJP4) exhibits more typical temperature-dependent activity; the activity increases with higher temperatures up to 30°C, and the enzyme is completely inactivated at 40°C within 5 min (16).

RdpA and SdpA catalyze enantioselective reactions. RdpA converted only the (*R*) enantiomers of phenoxypropanoic acid herbicides, and SdpA converted only the (*S*) enantiomers. This confirmed the results of previous experiments with *S. herbicidovorans* cell extracts in which the existence of two distinct α-ketoglutarate-dependent dioxygenases in strain MH was proposed (35). We firmly concluded that *S. herbicidovorans* MH harbors two distinct enzymes with opposite enantioselectivities. Enantioselective conversion is more widespread in α-ketoglutarate-dependent dioxygenases. Also, RdpA from *D. acidovorans* MC1 did not significantly convert (*S*) enantiomers of phenoxypropanoic acids (48). Some α-ketoglutarate-dependent 2,4-D dioxygenases are also capable of enantioselective conversion of mecoprop and/or dichlorprop. For example, TfdA from *C. necator* JMP134(pJP4) and TfdA from *Burkhold-*

eria cepacia RASC cleave only the ether bond of (*S*)-dichlorprop, whereas TfdA from *Alcaligenes denitrificans* converts only the (*R*) enantiomer (41). This difference is surprising, given the high degree of sequence conservation among these three TfdA dioxygenases. None of the TfdA dioxygenases, however, is able to convert both enantiomers of phenoxypropanoic acids at significant catalytic rates. To oxidize both enantiomers, two distinct dioxygenases are required in strains such as *S. herbicidovorans* MH or *D. acidovorans* MC1 (31, 35, 47, 48). Kinetic investigations with site-directed variants of the enzymes demonstrated that not just one but many residues in RdpA and SdpA play a structural role in directing enantiospecificity (T. A. Müller, M. I. Zavodsky, M. Feig, L. A. Kuhn, and R. P. Hausinger, submitted for publication).

Substrate and cosubstrate specificities. RdpA exhibited narrow substrate specificity with respect to the alkyl moiety in the alkanoic acid side chain; only phenoxypropanoic acids were converted, and 2,4-D and phenoxybutyric acids were not converted. This is in contrast to SdpA, which showed measurable activity with 2,4-D. It is also in contrast to RdpA from *D. acidovorans* MC1, whose activity with 2,4-D was reported to be 14% of its activity with (*R*)-dichlorprop (48). This is yet another difference between the two enzymes for which there is not a satisfactory explanation at the moment. Neither RdpA nor TfdA from *C. necator* converted phenoxybutyric acids (16, 48). These results indicate that the active sites of RdpA and of TfdA are specific with regard to the alkanoic acid substituent and are indifferent with regard to the substituents of the aromatic ring. Additionally, RdpA was not inhibited by the (*S*) enantiomers of phenoxypropanoic acids, which indicates that there is no binding of these enantiomers at the active site.

α-Ketoglutarate-dependent dioxygenases have different cosubstrate specificities. AtsK, an alkylsulfatase from *P. putida* S-313, and TfdA, for instance, exhibit broad cosubstrate ranges, and many oxo acids substitute for α-ketoglutarate (16, 22). TauD and the inorganic α-ketoglutarate-dependent hypophosphite dioxygenase HtxA exhibited stricter specificities, and other than α-ketoglutarate, they used only 2-oxoadipate as a cosubstrate, albeit with reduced activities in the range from 4 to 23% (11, 16, 49). RdpA and SdpA showed the same cosubstrate specificities as these two enzymes, but the remaining activities with 2-oxoadipate were only 2% and 5%, respectively. The adaptation of RdpA and SdpA for α-ketoglutarate is apparent from the low apparent K_m values for α-ketoglutarate (3 to 20 μM).

A linear relationship between the O_2 concentration (0 to 1.0 mM) and the reaction rate was observed; hence, only the value for the apparent second-order rate constant k_{cat}/K_m that can be calculated from this relationship is shown in Table 5. Although the oxygen dependence of SdpA followed Michelis-Menten kinetics, the apparent K_m value (0.16 mM O_2) was also quite high. Compared to other α-ketoglutarate-dependent dioxygenases, RdpA and SdpA have unusually high apparent K_m values for oxygen. Apparent K_m values of 40 to 50 μM O_2 were observed for lysyl hydroxylase and an α-ketoglutarate-dependent 4-hydroxylase from *Catharanthus roseus* which is involved in vindoline biosynthesis (8, 39). Flavonol dioxygenase exhibits even a lower apparent K_m value, 5.26 μM (3). The activities of RdpA and SdpA are therefore highly dependent on the oxygen concentration in laboratory assays.

Existence of another dioxygenase involved in phenoxyalkanoic acid degradation in strain MH. RdpA did not convert 2,4-D, and SdpA only converted this compound at low rates. However, *S. herbicidovorans* MH does grow on 2,4-D, and it grows at a higher rate than it grows on mecoprop and dichlorprop (53). Therefore, another phenoxyalkanoic acid-converting enzyme, which is specific for 2,4-D, must be present in *S. herbicidovorans* MH. Our results of hybridization and PCR experiments suggest that *S. herbicidovorans* MH did not harbor a gene sufficiently similar to the *tfdA*, *tfdA*α, or *cadAB* genes to allow detection. Therefore, the nature of this additional enzyme in *S. herbicidovorans* MH remains unclear.

In conclusion, we describe the characteristics of two α-ketoglutarate-dependent dioxygenases, RdpA and SdpA, from *S. herbicidovorans* MH that catalyze enantioselective ether cleavage of phenoxypropanoic acids to form achiral phenols. These two dioxygenases are a good example of an enzyme pair in which each enzyme converts only one enantiomer of a chiral substrate.

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