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Involvement of Two α-Ketoglutarate-Dependent Dioxygenases in Enantioselective Degradation of (*R*)- and (*S*)-Mecoprop by *Sphingomonas herbicidovorans* MH

KATHRIN NICKEL, MARC J.-F. SUTER, AND HANS-PETER E. KOHLER*

Swiss Federal Institute for Environmental Science and Technology (EAWAG), CH-8600 Dübendorf, Switzerland

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Cell extracts of *Sphingomonas herbicidovorans* MH grown on (*R*)-mecoprop contained an enzyme activity that selectively converted (*R*)-mecoprop to 4-chloro-2-methylphenol, whereas extracts of cells grown on (*S*)-mecoprop contained an enzyme activity selective for the *S* enantiomer. Both reactions were dependent on α-ketoglutarate and ferrous ions. Besides 4-chloro-2-methylphenol, pyruvate and succinate were detected as products of the reactions. Labeling experiments with $^{18}O_2$ revealed that both enzyme activities catalyzed a dioxygenation reaction. One of the oxygen atoms of pyruvate and one of the oxygen atoms of succinate were derived from molecular oxygen. Analysis of cell extracts obtained from cells grown on different substrates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that growth on (*R*)-mecoprop and (*S*)-mecoprop caused the appearance of prominent protein bands at 34 and 32 kDa, respectively. Both protein bands were present when cells grew on the racemic mixture. The results demonstrate that *S. herbicidovorans* initiated the degradation of each enantiomer of mecoprop by a specific α-ketoglutarate-dependent dioxygenase. By comparing conversion rates of various phenoxy herbicides, we confirmed that the two enzyme activities were distinct from that of TfdA, which catalyzes the first step in the degradation of 2,4-dichlorophenoxyacetic acid in *Ralstonia eutropha* JMP134.

Substituted phenoxyalkanoic acid herbicides are widely used in weed control. 2,4-Dichlorophenoxyacetic acid (2,4-D) and the chiral compounds (*R*,*S*)-2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop) and (*R*,*S*)-2-(2,4-dichlorophenoxy)propionic acid (dichlorprop) are members of this class of herbicides. The herbicidal activities of the last two compounds are exclusively associated with the *R* enantiomers. Today, the pure *R* enantiomers of mecoprop and of dichlorprop are commercially available as mecoprop-P and dichlorprop-P, respectively. Yet many commercial formulations contain the racemic mixtures and not the pure *R* enantiomers (27). In order to understand the environmental fate of the chiral compound mecoprop, it is important to study the degradation of the enantiomers independently, as has been recently emphasized (1, 2, 9, 22, 29).

Studies concerning the fate and the persistence of phenoxyalkanoic acids in soil revealed that degradation of these herbicides is caused mainly by microorganisms (17, 23). The breakdown of 4-chloro-2-methylphenoxyacetic acid (MCPA) and 2,4-D starts with the removal of the alkanoic acid side chain to yield the corresponding phenol (17). Consequently, a great deal of research effort was put into unravelling the subsequent degradation of 2,4-dichlorophenol and 4-chloro-2-methylphenol, the metabolites of 2,4-D and MCPA, respectively (10). In soil, the microbial metabolism of mecoprop leads to the appearance of 4-chloro-2-methylphenol (24). This metabolite is also formed in a mixed bacterial culture with mecoprop as the sole source of carbon and energy (14). So far, only two pure bacterial strains have been isolated for their ability to degrade mecoprop and dichlorprop (12, 26). One of them, *Alcaligenes denitrificans*, degrades only the *R* enantiomer of mecoprop (26), whereas the other, *Sphingomonas herbicidovorans* MH (formerly *Flavobacterium* sp. strain MH), completely degrades both enantiomers of mecoprop in an enantioselective manner (29). The pathway for the degradation of 2,4-dichlorophenol in *S. herbicidovorans* MH seems to correspond to the one in 2,4-D-grown *Ralstonia eutropha* JMP134, i.e., via *ortho* cleavage of the aromatic ring (12, 19).

In contrast to the ample knowledge of the biochemistry of the degradation of the phenolic metabolites, little is known of the biochemistry of the initial steps in the degradation of phenoxyalkanoic acid herbicides. Recently, it has been shown that *R. eutropha* JMP134 does not initiate metabolism of 2,4-D by a monooxygenase but by an α-ketoglutarate-dependent dioxygenase which is encoded by the *tfdA* gene of plasmid pJP4 (7). The enzyme was purified from *Escherichia coli* cells that overexpressed the *tfdA* gene, and its properties were described (8). In contrast, the initial attack on 2,4,5-trichlorophenoxyacetic acid in *Burkholderia cepacia* AC1100 is catalyzed by an oxygenase, TftAB, which shows homology to two multicomponent dioxygenases involved in benzoate and toluate degradation and is not dependent on α-ketoglutarate (4, 5, 28). Horvath et al. (12) deduced from their studies that a side-chain-cleaving activity must be crucial for the ability to degrade dichlorprop and mecoprop, but they could not detect the initial enzyme activity of the degradation pathways in cell extracts of *S. herbicidovorans* MH.

We recently described the ability of *S. herbicidovorans* MH to use both enantiomers of mecoprop as the sole source of carbon and energy (29). We now report that *S. herbicidovorans* MH initiates degradation of mecoprop by two α-ketoglutarate-dependent dioxygenase activities: one that is specific for (*R*)-mecoprop and one that is specific for (*S*)-mecoprop.

* Corresponding author. Mailing address: Department of Microbiology, EAWAG, Ueberlandstrasse 133, CH-8600 Dübendorf, Switzerland. Phone: 41 1 823 5521. Fax: 41 1 823 5547. E-mail: kohler@eawag.ch.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. herbicidovorans* MH (DSM 11019) (29) was grown on mineral salts medium with different phenoxyalkanoic acids as the carbon and energy sources. The mineral salts medium consisted of 20 mM phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 6.5), $(NH_4)_2SO_4$ (0.24 g/liter), $MgSO_4 \cdot 7H_2O$ (0.15 g/liter), $Ca(NO_3)_2 \cdot 4H_2O$ (2 mg/liter), and a trace element solution (0.4 ml/liter) that contained the following compounds: $FeSO_4 \cdot 7H_2O$ (2.50 g/liter), $MnSO_4 \cdot H_2O$ (0.75 g/liter), $ZnSO_4 \cdot 7H_2O$ (1.30 g/liter), $CuSO_4 \cdot 5H_2O$ (0.25 g/liter), $Co(NO_3)_2 \cdot 6H_2O$ (0.30 g/liter), $Na_2MoO_4 \cdot 2H_2O$ (0.15 g/liter), $NiSO_4 \cdot 7H_2O$ (0.01 g/liter), H_3BO_3 (0.10 g/liter), and H_2SO_4 (5.0 ml/liter). Carbon sources were supplied at 200 mg/liter. After being autoclaved, the medium was supplemented with peptone (40 mg/liter; Biolife, Milano, Italy) and with a vitamin solution (1 ml/liter containing pyridoxin-HCl [0.1 g/liter], biotin [20 mg/liter], folic acid [20 mg/liter], thiamine-HCl [50 mg/liter], riboflavin [50 mg/liter], nicotinic acid [50 mg/liter], Ca-pantothenate [50 mg/liter], 4-aminobenzoic acid [50 mg/liter], nicotinamide [50 mg/liter], and vitamine B_{12} [50 mg/liter]). Strain MH was maintained for several months at 4°C in liquid mineral salts medium with 2,4-D or racemic mecoprop as the carbon source, as well as on nutrient broth agar plates. For long-term storage at −80°C, cells grown on nutrient broth, which consisted of Standard I nutrient broth (5 g/liter; Merck, Darmstadt, Germany) in 20 mM phosphate buffer (pH 6.5), were incubated with 15% (vol/vol) glycerol for 2 h at room temperature prior to being frozen. *R. eutropha* JMP134(pJP4) was cultivated like *S. herbicidovorans* MH with 2,4-D (200 mg/liter) as the sole carbon and energy source.

Precultures (50 ml) were grown in mineral salts medium with the appropriate carbon source in 250-ml Erlenmeyer flasks at 25°C on a rotary shaker (100 rpm). Cells used for preparing cell extracts were cultivated in 2-liter Erlenmeyer flasks equipped with magnetic stirring bars in 800 ml of mineral salts medium with the appropriate carbon source. Cells were grown at room temperature, with stirring being held constant at 370 rpm.

Chemicals. Racemic mecoprop (98%) was obtained from Rhône-Poulenc, Paris, France. The pure (*S*)-mecoprop and (*R*)-mecoprop were prepared as described in reference 29. (*R*)-Dichlorprop and (*S*)-dichlorprop were kindly provided by C. Bolliger and C. Zipper, Swiss Federal Institute for Environmental Science and Technology (3). 2,4-D, 4-(4-chloro-2-methylphenoxy)butyric acid (MCPB), and 4-chloro-2-methylphenol were obtained from Aldrich-Chemie, Steinheim, Germany. MCPA as well as high-performance liquid chromatography (HPLC)-grade methanol was bought from Fluka Chemicals, Buchs, Switzerland. $^{18}O_2$ was obtained from Eurisotop, Centre d'Etudes de Saclay, Gif-sur-Yvette, France. L-Lactate dehydrogenase from rabbit muscle was bought from Boehringer Mannheim, Rotkreuz, Switzerland. All other chemicals were purchased from Merck and Fluka Chemicals.

Preparation of cell extracts. At the end of the exponential growth phase, cells were harvested by centrifugation (15 min at 7,500 × *g*) at 4°C and washed with an excess of mineral salts medium. One gram of wet cells was resuspended in 3 ml of a 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$, 1 mM dithiothreitol, and 3 μg of DNase I. This cell suspension was passed three times through a French press (Hypramag, Zurich, Switzerland) at 1 MPa. After DNA digestion for 1 h at 4°C, the crude extract was centrifuged at 16,000 × *g* to remove cellular particles and membranes. The protein contents of cell extracts varied from 8 to 15 mg/ml. Protein was determined with the Bio-Rad protein assay kit (Bio-Rad, Munich, Germany). Cell extracts were stored for several months at −20°C without significant loss of enzyme activities. In cell extracts stored at 4°C for 1 day, the enzyme activities decreased.

Enzyme assays. The enzyme assays were performed in 50 mM imidazole buffer (pH 6.75) at 23°C. The assay mixture (total volume, 0.5 ml), if not stated otherwise, contained 1 mM ascorbate, 100 μM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ (the stock solution was acidified to a pH of 2), 1 mM α-ketoglutarate, and 1 mM (*R*)-, (*S*)-, or racemic mecoprop. The assays were started by the addition of 50 μl of cell extract, and during the incubation the reaction mixture was gently stirred in order to assure oxygen saturation. Samples were taken at the appropriate time intervals and boiled for 2 min to stop the reaction. Denatured protein was removed by centrifugation at 16,000 × *g* for 10 min. Standards of reference substances were treated in the same manner.

Labeling with $^{18}O_2$. Incorporation of ^{18}O into the products of the enzyme reactions was measured in order to confirm the dioxygenation reactions. The enzyme assays in the presence of $^{18}O_2$ were carried out in 14-ml serum flasks, which were closed with gas-tight rubber septa and equipped with small magnetic stirring bars. The incubation mixture (total volume, 0.5 ml) consisted of 1 mM ascorbate, 100 μM ferrous ions, 1 mM α-ketoglutarate, 1 mM NADH, 50 μl of cell extract, 1.4 U of L-lactate dehydrogenase, and 50 mM 1,3-bis[tris-(hydroxymethyl)-methylamino]propane (pH 6.75). A 1:1 mixture of $^{18}O_2$ and $^{16}O_2$ was obtained by displacing 2.6 ml of $^{18}O_2$ into the head space of the flask with a gas-tight syringe (Dynatech, Baton Rouge, La.). After addition of the appropriate enantiomer of mecoprop (1 mM), the reaction mixture was gently stirred for 20 min. The reaction was stopped, and the reaction mixture treated as described above. The metabolites were subsequently analyzed by HPLC-mass spectrometry (MS). Analogous experiments with $^{16}O_2$ were carried out with open vials.

Analytical procedures. (i) HPLC. Quantitation of the mecoprop enantiomers and of 4-chloro-2-methylphenol was achieved by HPLC with a Nucleodex-α-PM column (200 by 4 mm; Macherey-Nagel, Düren, Germany) under isocratic conditions. The mobile phase consisted of a 70/30 (vol/vol) mixture of methanol and 50 mM NaH_2PO_4 (pH 3.0), and the flow rate was 0.7 ml/min. Phenoxyalkanoic acids and the substituted phenols were detected with a photodiode array detector (Waters-Millipore, Milford, Mass.) at a wavelength of 230 nm. By this method, detection limits of 4 μM for the mecoprop enantiomers and 11 μM for 4-chloro-2-methylphenol could be achieved. Typical retention times were 7.0, 8.3, and 13.0 min for (*R*)-mecoprop, (*S*)-mecoprop, and 4-chloro-2-methylphenol, respectively.

(ii) Colorimetric assay for phenolic compounds. Alternatively, phenolic compounds formed during the enzyme assay were determined by the method of King et al. (15), except that the absorption of the resulting red antipyrine dye was measured at its specific maximum of 510 nm. This method was valid for 4-chloro-2-methylphenol as well as for 2,4-dichlorophenol in the range of 1 to 50 μM. If necessary, samples were diluted with 50 mM imidazole buffer (pH 6.75) prior to incubation with the dye reagents.

(iii) GC-MS. Gas chromatography (GC)-MS analysis of metabolites and their trimethylsilyl (TMS) derivatives was performed as described elsewhere (16).

(iv) HPLC-MS. Pyruvate, lactate, and succinate were analyzed by HPLC-MS. The samples were applied to a Nucleosil-C_{18} column (250 by 4 mm; Macherey-Nagel). Reversed-phase chromatography was performed isocratically with H_2SO_4 (pH 2.65) as the mobile phase at a flow rate of 0.5 ml/min and at 10°C by means of an HP1100 series HPLC system (Hewlett-Packard, Urdorf, Switzerland). Typical retention times for pyruvate, lactate and succinate were 6.5, 8.0, and 20.0 min, respectively. The HPLC was coupled to an MS (Micromass, Manchester, Great Britain) equipped with an electrospray ion source. Mass spectra were acquired with the negative ion mode, a needle voltage of 3.5 kV, a cone voltage of 30 V, nitrogen as the nebulizer and drying gas, and an ion source temperature of 150°C. The mass range of 50 to 500 Da was scanned once per second.

Electrophoresis and molecular weight determinations. Proteins of the crude cell extracts were separated under denaturing and reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Slab gels (100 by 60 by 0.75 mm) were prepared with 12.5% acrylamide. A mixture of the following proteins (molecular weights in parentheses; Pharmacia Biotech, Uppsala, Sweden) served as a molecular weight marker: phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). Gels were stained with Coomassie brilliant blue G-250. The destaining solution consisted of 30% methanol, 1.5% acetic acid, and 68.5% water.

TABLE 1. Dependence on cofactors and on supplements of the initial conversion reactions of cell extracts from (*R*)- and (*S*)-mecoprop-grown *S. herbicidovorans* MH during incubation with racemic mecoprop

Cofactor(s) and supplement(s)	% Conversion of (*R*)-mecoprop by crude cell extract of (*R*)-mecoprop-grown cells[a]	% Conversion of (*S*)-mecoprop by crude cell extract of (*S*)-mecoprop-grown cells[a]
Fe^{2+}, ascorbate, α-keto-glutarate, L-cysteine, catalase, O_2	100 (65.5)	100 (68.0)
−Fe^{2+}[b]	10	33
−Ascorbate[b]	56	26
−α-Ketoglutarate[b]	0	0
−L-Cysteine[b]	114	101
−Catalase[b]	113	100
−Active protein[b,c]	0	0

[a] Specific activity in nanomoles of substrate consumed per milligram of protein per minute is given in parentheses.
[b] Complete assay minus the specified component.
[c] Proteins were denatured by boiling the cell extract prior to the assay.

RESULTS

Mecoprop transformation with cell extracts. Cell extracts of (*S*)-mecoprop-grown cells of *S. herbicidovorans* MH effected the selective transformation of (*S*)-mecoprop to 4-chloro-2-methylphenol upon incubation with a racemic mixture of mecoprop (Table 1). On the other hand, cell extracts of (*R*)-mecoprop-grown cells contained an enzyme activity that specifically transformed the *R* enantiomer. The product of this reaction was also 4-chloro-2-methylphenol. In both cases, disappearance of the substrate was coupled to the concomitant

stoichiometric formation of the phenolic product. Both reactions strictly required α-ketoglutarate, and the rates of conversion were dependent on ferrous ions and L-ascorbic acid. Omission of these two components from the reaction mixture led to a decrease of the reaction rates, whereas omission of L-cysteine and catalase did not (Table 1). Addition of NADH and NADPH to the reaction mixture did not have a significant effect on the conversion rates. Boiling of the cell extracts for 2 min completely abolished the activities. Decreases in the turnover rates observed during the reactions were probably due to the oxidation of the ferrous ions in the reaction mixture. Addition of fresh ferrous ion solution to the reaction mixture at 5-min intervals guaranteed a constant conversion rate for at least 20 min. Optimal assay conditions were as follows: 50 mM imidazole buffer (pH 6.75), 0.1 mM ferrous ions, 1 mM L-ascorbic acid, 1 mM α-ketoglutarate, and 1 mM substrate. The enzyme activities were lost at pH values of 6.5 and lower.

Identification of the reaction products. The products of the reactions catalyzed by the two enzyme activities were identical. In both instances, the oxidative cleavage of the ether bond yielded 4-chloro-2-methylphenol and pyruvate. 4-Chloro-2-methylphenol was identified by HPLC. It cochromatographed with an authentic standard, and its UV spectrum (diode array) was identical to that of the standard. Additionally, the compound was identified by GC-MS of its TMS derivative. Its mass spectrum was identical to that of the TMS derivative of the authentic standard. Both TMS derivatives had identical retention times on the GC column under the conditions employed. Pyruvate was identified by HPLC-MS. It cochromatographed with authentic pyruvate and produced an identical mass spectrum. The cosubstrate α-ketoglutarate was oxidatively decarboxylated to succinate and presumably CO_2 during the course of the reactions. We did not measure CO_2, but succinate was identified by GC-MS. Its TMS derivative cochromatographed with the TMS derivative of authentic succinate, and both compounds produced identical mass spectra. We also identified succinate by HPLC-MS. The retention time and the mass spectrum of authentic succinate and those of the metabolite produced by the enzyme reaction were identical.

Incorporation of ^{18}O into succinate and pyruvate. In order to confirm the dioxygenation reaction for the formation of 4-chloro-2-methylphenol and pyruvate from (*R*)- and (*S*)-mecoprop by the two α-ketoglutarate-dependent dioxygenase activities, the incubations were carried out in the presence of $^{18}O_2$. As the oxygen atom of the keto group of pyruvate readily exchanges with water by the reversible formation of pyruvate hydrate (25), we fixed the incorporated ^{18}O label by the lactate dehydrogenase-catalyzed reduction of pyruvate to lactate (18). The *R*-enantiomer-specific and the *S*-enantiomer-specific activities produced identical products with identical isotope patterns. Results are shown only for the transformation of (*R*)-mecoprop by the *R*-enantiomer-specific enzyme activity (Fig. 1). 4-Chloro-2-methylphenol was isolated, derivatized, and analyzed by GC-MS. Lactate and succinate were directly analyzed by HPLC-MS. Figure 1 shows the mass spectra (segment around the signal of the molecular ion) of lactic acid (Fig. 1A and D), succinic acid (Fig. 1B and E), and the TMS derivative of 4-chloro-2-methylphenol (Fig. 1C and F) from incubations with $^{16}O_2$ from air (Fig. 1A to C) and with a one-to-one mixture of $^{16}O_2$ and $^{18}O_2$ (Fig. 1D to F). The isotope patterns unequivocally show that one atom of molecular oxygen was incorporated into lactate and succinate but that none was incorporated into 4-chloro-2-methylphenol.

Transformation of related substrates. Cell extracts from *S. herbicidovorans* MH grown on different substrates were examined for their ability to transform mecoprop and other phenoxyalkanoic acid herbicides to the corresponding phenols. The activities of the cell extracts were highly dependent on the growth substrate (Table 2). Growth on the *R* enantiomer of mecoprop effected high activity in the extract for the transformation of the *R* enantiomer of mecoprop and of the *R* enantiomer of dichlorprop. When the cells grew with (*S*)-mecoprop as the carbon and energy source, the prevalent enzyme activity in the extract was specific for the *S* enantiomers of those two herbicides. Cell extract of cells grown on complex medium also contained an enzyme activity that preferentially turned over the *S* enantiomers of mecoprop and dichlorprop. Cell extract of cells grown on 2,4-D exhibited an activity for the *S* enantiomers and did not appear to contain an activity specific for 2,4-D. This is in contrast to cell extract of *R. eutropha* JMP134 grown on 2,4-D. This extract acted on 2,4-D, MCPA, and (*S*)-dichlorprop but not on (*S*)-mecoprop. These data indicate that the two α-ketoglutarate-dependent dioxygenase activities in *S. herbicidovorans* MH are distinct from the activity of TfdA, the α-ketoglutarate-dependent dioxygenase that initiates the degradation of 2,4-D in *R. eutropha* JMP134. All cell extracts of *S. herbicidovorans* MH converted MCPA. Activities for

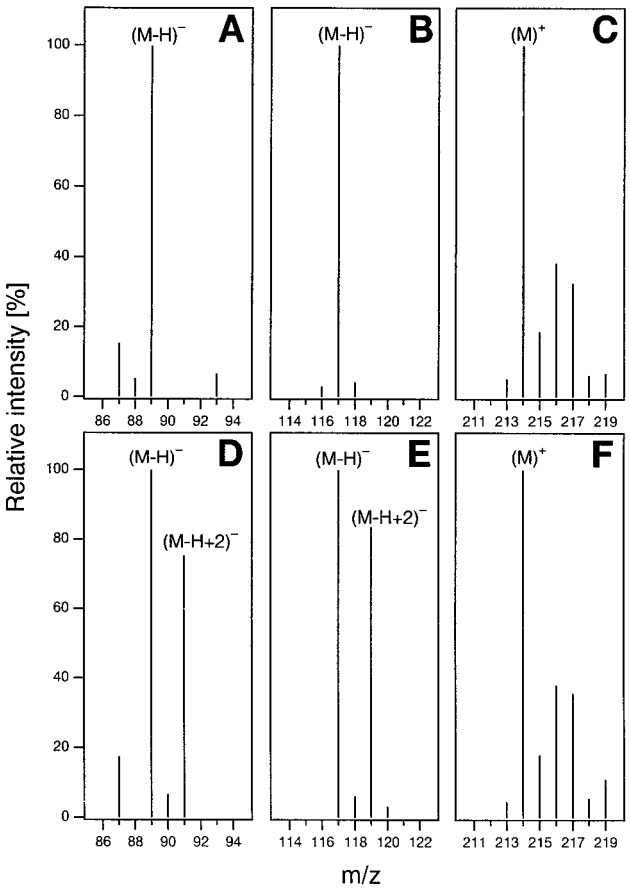

FIG. 1. Mass spectral analysis of the products from (*R*)-mecoprop and α-ketoglutarate by cell extracts of (*R*)-mecoprop-grown cells of *S. herbicidovorans* MH in the presence of $^{18}O_2$. The assay mixture contained ascorbate (1 mM), ferrous ions (100 μM), α-ketoglutarate (1 mM), NADH (1 mM), (*R*)-mecoprop (1 mM), and L-lactate dehydrogenase (1.4 U). L-Lactate dehydrogenase effected the quick conversion of the direct reaction product pyruvate to lactate necessary for trapping the ^{18}O label. Segments of the mass spectra of lactic acid (A and D), succinic acid (B and E), and the TMS derivative of 4-chloro-2-methylphenol (C and F) from incubations with $^{16}O_2$ (A to C) and with a 1:1 mixture of $^{18}O_2$ and $^{16}O_2$ (D to F) are shown.

TABLE 2. Enzyme activities in cell extracts of *S. herbicidovorans* MH grown on different substrates and in cell extracts of *R. eutropha* JMP134 grown on 2,4-D

Assay substrate	% Enzyme activity[a] in cell extracts of:				
	S. herbicidovorans MH grown on:				*R. eutropha* JMP134 grown on 2,4-D
	(*R*)-Mecoprop	(*S*)-Mecoprop	CM	2,4-D	
(*R*)-Mecoprop	100 (19.3)	19	20	41	0
(*S*)-Mecoprop	26	100 (25.1)	100 (42.9)	100 (24.2)	2
(*R*)-Dichlorprop	84	11	10	16	0
(*S*)-Dichlorprop	13	106	97	56	25
MCPA	10	39	29	24	73
MCPB	5	4	2	9	0
2,4-D	11	15	10	15	100 (120.7)

[a] Specific activities in nanomoles of phenolic compound produced per milligram of protein per minute are given in parentheses. CM, complex medium.

MCPA were highest in those cell extracts that were active with the *S* enantiomer of mecoprop.

Protein patterns of different cell extracts on SDS-polyacrylamide gels. The protein compositions of crude extracts of cells grown on different substrates were compared after separation of the proteins by SDS-PAGE. The cell extract from (*S*)-mecoprop-grown cells contained a large amount of a protein with a molecular mass of 32 kDa, while the predominant protein in cell extract from (*R*)-mecoprop-grown cells had a molecular mass of 34 kDa (Fig. 2). When a racemic mixture of mecoprop served as the growth substrate, both proteins were present in approximately equal amounts. A 32-kDa protein was present in cell extract from cells grown on complex medium as well as on 2,4-D. Cell extract of *R. eutropha* JMP134 showed a prominent protein band of 32 kDa on the SDS gel, as was described for TfdA (8).

DISCUSSION

S. herbicidovorans MH is able to use both enantiomers of the herbicide mecoprop as the sole source of carbon and energy. However, the enantiomers are selectively degraded, with the *S* enantiomer disappearing much faster from the culture medium than the *R* enantiomer when strain MH grows on the racemic mixture (29). The cause of the selective metabolism of the two enantiomers of mecoprop may be the presence of enantioselective enzyme activities, the presence of a specific transport system for each enantiomer, or both. The results presented in this study reveal the existence of an enantioselective initial step in the degradation of each enantiomer.

The enzyme activity of cell extracts obtained from cells grown on the pure enantiomers selectively acted on the growth enantiomer. Both enzyme activities depended on α-ketoglutarate and ferrous ions, and for both, the first intermediates were the achiral compounds 4-chloro-2-methylphenol and pyruvate. Succinate was produced from α-ketoglutarate during the course of both reactions. Experiments with $^{18}O_2$ confirmed that both enzyme activities catalyzed a dioxygenation reaction. The appearance of the ^{18}O label in pyruvate and not in phenol suggests that the oxidative cleavage of the ether bond proceeded via hydroxylation at the C-2 carbon of the propionic acid side chain. The resulting unstable intermediate could decompose to 4-chloro-2-methylphenol and pyruvate. A similar process was proposed for the oxidation of diethyl ether by the toluene 2-monooxygenase enzyme system (13). This suggestion is also consistent with a general reaction mechanism for α-ketoglutarate-dependent dioxygenases which is thought to start with the formation of a ferryl oxidant (Fe^{IV}═O) by the binding of oxygen to the iron cofactor, a process that is linked to the oxidative decarboxylation of α-ketoglutarate to succinate (6, 11, 20, 21). We therefore propose a degradation scheme based on two distinct α-ketoglutarate-dependent dioxygenases for the initial transformation of (*R*)- and (*S*)-mecoprop to 4-chloro-2-methylphenol and pyruvate (Fig. 3) by *S. herbicidovorans* MH. Both enzymes seem to share their overall chemistries with TfdA, the enzyme catalyzing the first step of the degradation of 2,4-D by *R. eutropha* JMP134 (7, 8), but not their substrate specificities (Table 2). Whereas TfdA preferentially transforms 2,4-D, the enzyme activities present in cell extracts of *S. herbicidovorans* MH specifically turned over either the *R* or the *S* enantiomers of mecoprop and of dichlorprop. They showed only small activities with 2,4-D as the substrate (Table 2).

Nevertheless, degradation of other phenoxyalkanoic acid herbicides might be effected by these enzyme activities. After growth of *S. herbicidovorans* MH on 2,4-D, we observed the presence of (*S*)-mecoprop-degrading activity in the cell extract (Table 2 and Fig. 2). Strain MH grew as fast on 2,4-D as it grew on (*S*)-mecoprop, although in such cell extracts the activity for 2,4-D was only 15% of that for (*S*)-mecoprop. A reasonable explanation for these findings is the existence of specific transport systems. We also found that the enzyme activity for the conversion of (*S*)-mecoprop was present in *S. herbicidovorans* MH when the cells grew on complex medium (Table 2 and Fig. 2) and on succinate (data not shown). This indicates that the enzyme activity specific for the *S* enantiomer is constitutively expressed. As experiments with washed-cell suspensions showed that cells grown on complex medium did not take up oxygen when they were incubated with (*S*)-mecoprop (29), we suggest the existence of an inducible transport system for (*S*)-mecoprop. After growth on (*R*)-mecoprop, the prevailing enzyme activity is specific for the *R* enantiomer (Table 2). Figure 2 clearly shows that in such cell extracts no prominent protein band that corresponds to the *S*-enantiomer-specific enzyme activity is present. We therefore conclude that the otherwise constitutively expressed *S*-enantiomer-specific enzyme activity was repressed under these conditions. A closer examination of the induction and repression processes will be necessary to

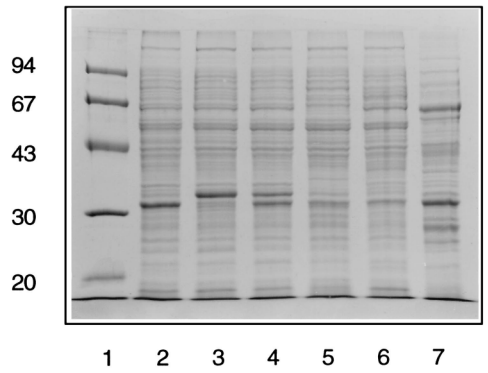

FIG. 2. SDS-PAGE of cell extracts from *S. herbicidovorans* MH grown with various growth substrates and of cell extract from *R. eutropha* JMP134 grown on 2,4-D. Lane 1, molecular mass markers (subunit sizes are given in kilodaltons on the left); lanes 2 to 6, cell extracts of *S. herbicidovorans* MH grown with (*S*)-mecoprop (lane 2), (*R*)-mecoprop (lane 3), racemic mecoprop (lane 4), complex medium (lane 5), and 2,4-D (lane 6); lane 7, cell extract of *R. eutropha* JMP134 grown on 2,4-D. Ten micrograms of protein per lane was applied.

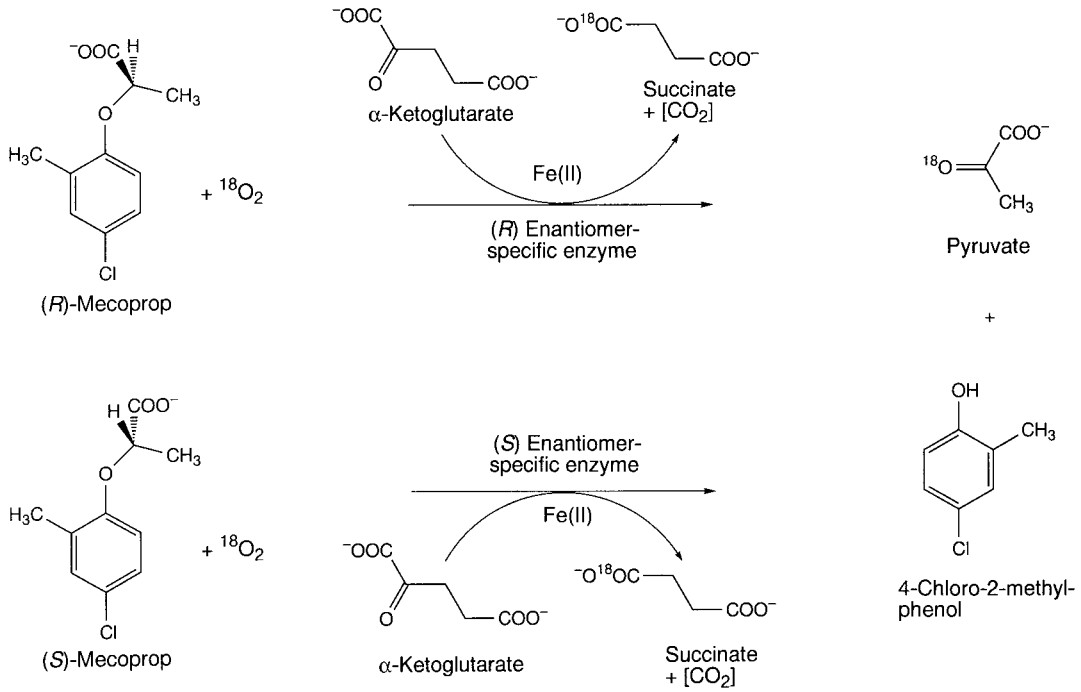

FIG. 3. Proposed scheme for the initial steps in the degradation of both enantiomers of mecoprop in *S. herbicidovorans* MH.

better understand how strain MH regulates degradation of (*R*)- and (*S*)-mecoprop.

Although the results of the experiments with cell extracts grown on different carbon sources need confirmation by additional experiments with pure enzymes, the data presented in Table 2 comprise some interesting aspects of the substrate specificities of the three proposed enzyme activities. TfdA-containing cell extract of *R. eutropha* JMP134 showed highest activity with 2,4-D followed by MCPA (73%) and (*S*)-dichlorprop (25%). This sequence is in agreement with data for purified TfdA, for which the k_{cat} values for MCPA and (*R*,*S*)-dichlorprop were 44 and 12%, respectively, of that for 2,4-D (8). However, (*S*)-mecoprop was a poor substrate of TfdA and (*R*)-dichlorprop and (*R*)-mecoprop were not substrates of TfdA at all (Table 2). Therefore, substrate specificity of TfdA seems to depend on two parts of the substrate molecule: on the aromatic moiety and on the substitution and configuration of the C-2 carbon atom of the alkanoic acid side chain. TfdA apparently accepted only 2-phenoxy-propionic acid derivatives with an *S* configuration at the C-2 carbon of the side chain. It would be very interesting to examine the kinetic parameters of purified TfdA with the pure dichlorprop enantiomers. The two mecoprop-degrading enzyme activities of *S. herbicidovorans* MH were highly active with the phenoxypropionic acid derivatives but also acted on 2,4-D (11 to 15%), MCPA (10 to 39%), and MCPB (4 to 5%). Each enzyme activity strongly depended on the substitution and the configuration at the C-2 carbon of the alkanoic acid side chain. The substitution on the aromatic part of the molecule did not seem to play such an important role in substrate specificity.

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