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Evidence of cytochrome P450-catalyzed cleavage of the ether bond of phenoxybutyrate herbicides in *Rhodococcus erythropolis* K2-3

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Abstract

Bacterial strain *Rhodococcus erythropolis* K2-3 can cleave the ether bond of the phenoxybutyrate herbicides, i.e., 4-(2,4-dichlorophenoxy)butyrate (2,4-DB) and 4-(4-chloro-2-methylphenoxy)butyrate (MCPB), by an enzyme system that is constitutively expressed. The enzyme(s) involved were investigated in this study. The rate of disappearance of 2,4-DB determined in a whole cell assay amounted to 0.6 mmol/h · $g_{dry\,mass}$. Carbon monoxide difference spectra of dithionite-reduced whole cells and crude cell extracts suggested that strain K2-3 contains a soluble cytochrome P450 (P450), named $P450_{PB-1}$. The addition of various phenoxybutyrate substrates to crude cell extracts resulted in typical difference spectra following the type I pattern of substrate binding with P450. The rate of 2,4-DB cleavage was reduced by inhibitors of P450: 5 mM metyrapone and carbon monoxide at a CO/O_2 ratio of 10 reduced the activity by about 20%, and 70%, respectively. The ether cleaving activity completely disappeared after disruption of the cells and could not be detected in crude extracts. To elucidate the enzymatic basis of this reaction, P450 was partially purified. With the resulting enzyme preparation, 2,4-DB cleavage activity was re-established, becoming measurable after the addition of either phenazine methosulfate or ferredoxin and ferredoxin/NADP oxidoreductase from spinach. We detected no activities attributable to α-ketoglutarate-dependent dioxygenase or NAD(P)H-dependent monooxygenase. These results collectively indicate that cleavage of the ether bond of phenoxybutyrate herbicides is catalyzed by P450-mediated activity in this strain. One of the products derived from this reaction is dichlorophenol, and comparative chromatographic analyses suggest that the other product is a C4-carbonic acid, most likely succinic semialdehyde/succinate.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetate; 2,4-DB – 4-(2,4-dichlorophenoxy)butyrate; DCP – 2,4-dichlorophenol; MCPB – 4-(4-chloro-2-methylphenoxy)butyrate; OD – optical density; P450 – cytochrome P450; PMS – phenazine methosulfate

Introduction

A spectrum of phenoxyalkanoates used as herbicides are usually substituted in the 2- and 4-positions of the aromatic ring, resulting in either 2,4-dichloro- or 4-chloro-2-methyl-derivatives (Hassall 1990). Microorganisms that are capable of degrading these compounds do not essentially discriminate between these two classes of derivatives. However, selectivity is observed with respect to the alkanoic acid moiety. A large spectrum of strains can only degrade phenoxyacetate derivatives such as 2,4-dichlorophenoxyacetate (2,4-D) or 4-chloro-2-methyl-phenoxyacetate (MCPA) in a productive manner. These bacteria are unable to effectively attack the structurally similar phenoxypropionate and phenoxybutyrate derivatives (Pieper et al. 1988; Häggblom et al. 1992; Hoffmann et al. 1996; Fulthorpe et al. 1996). A limited range of microorganisms has been found which can productively degrade a wider spectrum of

compounds, including at least both phenoxyacetates and racemic phenoxypropionates (Zipper et al. 1996; Ehrig et al. 1997; Müller & Babel 1999; Smejkal et al. 2001; Mai et al. 2001). The enzymatic mechanism whereby the ether bond is cleaved was the same so far determined being catalyzed by an α-ketoglutarate-dependent dioxygenase with both the phenoxyacetate herbicides (Fukumori & Hausinger 1993a) and the phenoxypropionate herbicides (Zipper et al. 1996; Nickel et al. 1997; Müller & Babel 1999; Müller et al. 2001). Only one case is known in which cleavage was attributed to a monooxygenase reaction (Xun & Wagnon 1995). With regard to phenoxybutyrate herbicide degradation, however, only few strains are known which attack these compounds (Macrae et al. 1963; Horvath et al. 1990; Mai et al. 2001), and the enzymatic reaction responsible for catalyzing the initial step of the metabolic pathways leading to productive use of these compounds has not been described at all.

We have isolated Gram-positive strains of *Rhodococcus erythropolis* and *Aureobacterium* sp. which are capable of cleaving the ether bond of these herbicides, i.e., 4-(2,4-dichlorophenoxy)butyrate (2,4-DB) and 4-(4-chloro-2-methyl-phenoxy)butyrate (MCPB), while excreting the phenolic moiety into the medium as a dead-end product (Mertingk et al. 1998). However, the mechanism of this step remained obscure. The aim of the present investigation was to elucidate the enzymatic basis of this reaction.

Materials and methods

Growth media and cultivation

The strains were routinely cultivated aerobically in 200 ml batches on a rotary shaker at 30 °C using a complex medium containing 3 g/l peptone, 3 g/l yeast extract and 10 mM fructose, pH 8.5, or in minimal medium I with 15 mM xylitol or 10 mM glucose and 0.1 mg/l thiamine · HCl, pH 8.5. Minimal medium I was composed of (in mg/l): NH_4Cl (761), KH_2PO_4 (340), K_2HPO_4 (435), $CaCl_2{\cdot}2H_2O$ (3.671), $MgSO_4{\cdot}7H_2O$ (71.2), $FeSO_4{\cdot}7H_2O$ (4.98), $ZnSO_4{\cdot}7H_2O$ (0.440), $MnSO_4{\cdot}H_2O$ (0.615) and $Na_2MoO_4{\cdot}2H_2O$ (0.252).

To obtain material for the purification of cytochrome P450, *R. erythropolis* K2-3 was cultivated continuously on 20 mM glucose in a Biostat MD laboratory bioreactor (B. Braun, Melsungen, Germany) at a working volume of 1.3 l and a dilution rate of D = 0.08 h^{-1}, at 30 °C and pH 8.5 using minimal medium II composed of (in mg/l): NH_4Cl (761), KH_2PO_4 (340), K_2HPO_4 (435), $CaCl_2{\cdot}6H_2O$ (13.76), $MgSO_4{\cdot}7H_2O$ (142.4), $FeSO_4{\cdot}7H_2O$ (9.96), $ZnSO_4O$ (0.88), $MnSO_4{\cdot}H_2O$ (1.23) and $Na_2MoO_4{\cdot}2H_2O$ (0.504). It also contained 0.13 ml/l silicone antifoam emulsion (30%, Serva, Heidelberg, Germany) and 0.2 mg/l thiamine · HCl. Cells were harvested daily by centrifugation (22,000 g) from effluent suspension (ice-cooled, collecting biomass quantities of about 1 g dry mass), washed and resuspended in a small volume of 20 mM Tris/HCl buffer, pH 7.5 + 40 mM $MgSO_4$ (buffer A), and kept frozen at −20 °C until further use.

Resting cell assays

Rate of cleavage of 2,4-DB

Cells grown on the various media under batch or continuous conditions were harvested by centrifugation (22,000 g), washed and resuspended in minimal medium I, pH 8.5, to give a final biomass concentration corresponding to an OD_{700} of 0.5. After addition of 2,4-DB (dissolved in a double molar concentration of NaOH) the suspension was incubated at 30 °C on a rotary shaker and the disappearance of 2,4-DB and the corresponding formation of DCP was followed by analyzing aliquots of cell-free supernatants by HPLC.

Oxygen consumption rate

Cells were separated from culture broth by centrifugation, washed with 0.1 M phosphate buffer pH 7.5 and resuspended to give an OD_{700} of 30. Oxygen consumption was measured in an Oxygraph (Cyclobios Paar, Innsbruck, Austria) in a volume of 2 ml using 0.1 M phosphate buffer pH 7.5, which was saturated with gas (a mixture of either oxygen and carbon monoxide or oxygen and nitrogen, in various ratios). A quantity of 50 μl of the cell suspension was added, and after attaining a linear rate of endogenous respiration, the reaction was started by adding 2,4-DB to a final concentration of 2 mM. Activities were calculated from the linear part of the decline in oxygen concentration. The extent of inhibition of 2,4-DB oxidation by carbon monoxide was calculated by comparison to assay mixtures containing corresponding concentrations of nitrogen instead of CO.

Preparation of crude cell extracts

Cells from both batch and continuous cultures were washed and resuspended in 20 mM Tris/HCl, pH 7.5

+ 40 mM $MgSO_4$ (buffer A), 0.1 M Tris/HCl buffer, pH 7.5 + 0.5 mM dithiothreitol + 5 mM ascorbic acid + 5 mM α-ketoglutarate + 25 mM $MgSO_4$ or 0.01 M imidazole/HCl buffer, pH 6.75. They were then disrupted or lysed using a French press, lysozyme treatment or bead beating. Disruption using a French press (Aminco, Silver Spring, MD, USA) was performed by four passages through the cell at a pressure of 140 MPa. Cell lysis by lysozyme was performed by incubating 600 μl of a cell suspension (OD_{700} = 10) for 10 min in an ice bath in the presence of 3 mg of the enzyme. Disruption with mini beads of different sizes (diameters: 0.1–0.25 mm, 0.25– 0.5 mm or 0.5–0.75 mm) was performed with an MM2 model mill (Retsch, Haan, Germany) for 10–60 min (4 °C) at maximum frequency. The last of these techniques was used for disrupting the cells under anaerobic conditions, for which suspensions were flushed with nitrogen in an anaerobic box. Crude cell extracts were obtained by centrifuging the disrupted preparations for 15–30 min at 22,000 g at 4 °C. When used for CO difference spectrum measurements, substrate binding studies and enzyme purification, an additional ultracentrifugation step (Optima TLX Ultracentrifuge, Beckman, Fullerton, USA) was included for 1 h at 150,000 g (4 °C).

Permeabilization

Cells were washed twice with 50 mM triethanolamine/HCl buffer, pH 7.3 and resuspended to give an OD_{700} of about 1.9. Then, 2–200 mg of a nisin preparation (2.5% nisin contained in denatured milk protein and NaCl; Sigma, St. Louis, USA) was added to 5 ml of this suspension and incubated for 30 min at 30 °C. Suspensions of permeabilized cells were used directly for enzymatic investigations without further treatment.

Carbon monoxide (CO) difference spectra

Cell suspensions or protein solutions were reduced by adding several grains of sodium dithionite. Samples were dispensed into two cuvettes and a baseline spectrum was spectrophotometrically recorded (Hitachi U-2000, Tokyo, Japan) in the range of 400–500 nm. One cuvette was then gently gassed with carbon monoxide for 30 sec, after which a difference spectrum was immediately monitored in the same spectral range and baseline-corrected.

Substrate-binding spectra

Equal volumes of crude cell extracts were placed into two cuvettes and a baseline spectrum was recorded in the range of 340–500 nm (Hitachi U-2000). Various potential effectors were added in different concentrations to one cuvette while equal volumes of pure solvent were added to the other. After mixing, the difference spectrum was recorded. Substrate binding was quantified by the differences in absorption at the minima and maxima of the spectra.

Purification of cytochrome P450

When purifying cytochrome P450, all steps were carried out at 15 °C. Three buffers were used: Buffer A (20 mM Tris/HCl + 40 mM $MgSO_4$, pH 7.5), Buffer B (buffer A + 1 M NaCl, pH 7.5), and Buffer C (Buffer A + 1 M $(NH_4)_2SO_4$, pH 7.5). The P450 contents of the fractions were determined by the CO difference spectral method, using the differential coefficients of adsorption at 446 nm and 490 nm as derived under reduced conditions for $P450_{cam}$ of *Pseudomonas putida* (Gunsalus & Wagner 1978): $\Delta\epsilon^{446-490}$ = 92.8 cm^{-1} mM^{-1}. Protein was measured according to Bradford (1976) using a commercial assay kit (Merck; Darmstadt, Germany) with bovine serum albumin as a standard.

Step 1. Disruption: crude cell extracts were obtained by passing cells, resuspended in buffer A, through a French press at a concentration of about 40 g dry mass/l.

Step 2. Protamine sulfate treatment: crude cell extract was treated for 15 min with 0.1% protamine sulfate in an ice bath and supernatant was obtained by centrifugation for 15 min at 22,000 g at 4 °C.

Step 3. Ion exchange chromatography on Source 30Q (column 25/10) (Pharmacia, Uppsala, Sweden) with a linearly increasing NaCl gradient using buffers A and B in a total volume of 180 ml; flow rate 2 ml/min, fractions of 6 ml.

Step 4. Ammonium sulfate precipitation: addition to 1 M, followed by incubation in an ice bath for 30 min, collection of supernatant by centrifugation for 15 min at 22,000 g at 4 °C.

Step 5. Hydrophobic interaction chromatography on butylsepharose (column 25/10) (Pharmacia) with a linearly decreasing ammonium sulfate gradient using buffers C and A in a total volume of 180 ml; flow rate 2 ml/min, fractions of 6 ml.

Step 6. Concentration in a Vivaspin 20ml concentrator (Sartorius, Göttingen, Germany), exclusion

limit 30 kDa; centrifugation for 45 min at 6,000 g at 4 °C, swing-out rotor (Hermle K380, Gosheim, Germany).

Step 7. Gel permeation chromatography on Superdex 200 prep grade (column 10/100) (Pharmacia); flow rate 0.5 ml/min with buffer A, fractions of 6 ml.

Step 8. Concentration using a Vivaspin 20 ml concentrator (Sartorius), exclusion limit 30 kDa; centrifugation for 20 min at 6,000 g at 4 °C, swing-out rotor (Hermle).

Step 9. Ion exchange chromatography by FPLC on MonoQ (column 5/5) (Pharmacia) with a linearly increasing NaCl gradient using buffers A and B in a total volume of 30 ml; flow rate 1 ml/min, fractions of 1 ml.

Purified proteins were electrophoretically separated by SDS PAGE in 4–12% pre-cast polyacrylamide gels using a PowerEase 500 unit. The proteins in the Novex Mark 12^{TM} Wide Range Standard mixture were used to provide molecular weight markers. Sample buffer contained 20% glycerol (v/v), 4% SDS (w/v) and 0.05% (w/v) bromophenol blue in 125 mM Tris/HCl, pH 6.8. The running buffer contained 2.9 g Tris base, 14.4 g glycine and 1 g SDS per liter. Electrophoresis was run at 125 V. Gels were stained with a SilverXpress kit following the manufacturer's protocol. All equipment, gels and kits were supplied by Novex (San Diego, USA).

Measurement of enzyme activity

α-Ketoglutarate-dependent dioxygenase

Ether cleaving dioxygenase activity was measured according to Fukumori & Hausinger (1993b) by determining the phenolic intermediates liberated in this enzymatic reaction after their reaction with 4-aminoantipyrine. The standard assay for the enzyme reaction contained 1 mM herbicide (sodium salt), 1 mM α-ketoglutarate, 1 mM ascorbic acid and 10 μM ammonium iron(II) sulfate in 10 mM imidazole/HCl buffer (pH 6.75). The reaction was performed at 30 °C and started by adding the enzyme preparation. After appropriate times, usually within an interval of 10 min, samples (up to 5) were taken and the enzyme reaction was stopped by adding 100 μl of 20 mM EDTA to 1 ml of the reaction mixture. The phenolic products were determined by adding 100 μl of borate buffer pH 10 (3.09 g H_3BO_4; 3.73 g KCl; 44 ml NaOH ad. 1 l), 10 μl 2% (w/v) 4-aminoantipyrin and 10 μl 8% (w/v) potassium hexacyanoferrate(III). After incubation for 5 min at 30 °C, the absorbance of the samples was measured at 510 nm using a U-2000 spectrophotometer (Hitachi; Tokyo, Japan).

NAD(P)H-dependent monooxygenase activity

Measurement was performed in a similar manner as for α-ketoglutarate-dependent dioxygenase activity, except that the assay contained 0.5 mM NAD(P)H and α-ketoglutarate was omitted. Optional 10 μM FAD was added.

Cytochrome P450-dependent activity

Method A: Crude cell extracts with a protein content of 10.7 g/l in 10 mM Tris/HCl, pH 7.5, were separated from low molecular weight components by a single passage through a HiPrep Sephadex G25-fine desalting column (26/10) (Pharmacia), exclusion limit 25 kDa. Chromatography was performed at a flow rate of 5 ml/min using the buffer mentioned above. Fractions containing P450 were concentrated and washed with the same buffer using a VivaSpin 20 ml concentrator (Sartorius; Göttingen, Germany) at 6,000 g and 4 °C in a swing-out rotor (Hermle K380), yielding conditioned crude cell extracts (protein content, 12.1 g/l). The assay mixture contained 385.5 μl conditioned crude cell extract, 2.0 mM NADPH, 26.25 μg ferredoxin from spinach (Sigma), 0.5 U ferredoxin/NADP oxidoreductase from spinach (Sigma) and 1.25 mM 2,4-DB, giving a total volume of 0.5 ml. The reaction was started by adding substrate. Samples were taken from a compartment in this assay chamber that was separated from the rest of the chamber by a semi-permeable membrane (dialysis tube) and contained initially 350 μl of 10 mM Tris/HCl buffer, pH 7.5. This was used to avoid interference by high protein concentrations in the following steps of product analysis.

Method B (according to Eble & Dawson 1984, with modifications): This method was used to measure activity with enriched enzyme preparations. The assay mixture contained, in 3 ml of buffer A: 10 mM NADH or NADPH, 0.6 mM 2,4-DB, 0.04 mM phenazine methosulfate (PMS), and 100 μl enzyme (containing 185 μM P450). The reaction was started by adding PMS. The assay was gently gassed with oxygen during incubation in the dark. Samples taken were used directly for analytical purposes without further preparation.

In both cases 2,4-DB and DCP were detected by HPLC, whereas the other reaction products were measured using ion chromatography with conductometric detection (see below).

Isocitrate dehydrogenase

Isocitrate dehydrogenase assay mixtures contained 50 μl crude cell extract, 10 mM $MnSO_4 \cdot H_2O$, 1 mM NADP, and 2.5 mM isocitrate in 2 ml of triethanolamine/HCl buffer, pH 7.3. The reaction was started by adding isocitrate, and the formation of NADPH was monitored at 340 nm (Hitachi U-2000).

When applied to permeabilized cells, the concentration of NADP was doubled, and NADPH formation was monitored after removing cells by passing portions from the assay mixture through a 0.2 μm membrane.

Succinic semialdehyde dehydrogenase

The succinic semialdehyde dehydrogenase activity was measured according to Lütke-Eversloh & Steinbüchel (1999). The assay mixtures contained 1 mM NADP, 80 μl crude cell extract, 0.4 mM succinic semialdehyde and buffer A to a final volume of 1 ml. The reaction was started by adding succinic semialdehyde. NADPH formation was recorded spectrophotometrically at 340 nm over a period of 10 min.

Determination of reaction products

After the reactions had been terminated, the phenolic moiety was determined by HPLC according to Oh & Tuovinen (1990) or using a colorimetric assay as described by Fukumori & Hausinger (1993b).

The second reaction product, potentially a C4-carbonic acid, was sought after the P450-catalyzed reaction had proceeded in the presence of either PMS or ferredoxin and ferredoxin/NADP oxidoreductase. The reactions in portions taken from assay mixtures were stopped by freezing them in dry ice, and the reaction products were analyzed by ion chromatography (Dionex, Sunnyvale, CA, USA) using an IonPak AS11 250 $\times$ 4 column and conductometric detection. Analytes were eluted with a 0.5 to 38.3 mM KOH gradient over 15.5 min after 2.5 min of isocratic 0.5 mM KOH at a flow rate of 2 ml/min.

Determination of biomass concentration

The biomass concentration was determined by measuring the optical density at 700 nm (OD_{700}) and by weighting washed pellets of cells after drying to constant weight at 105 °C. OD_{700} is related to dry mass by a factor of about 0.3.

Results

2,4-DB cleaving activity in complex cell systems and disrupted cell preparations

Resting cells of *Rhodococcus erythropolis* K2-3 grown on complex medium were able to degrade 2,4-DB immediately without any lag phase. The reaction rate, as measured by the disappearance of this substrate, was about 0.6 mmol/h $\cdot$ $g_{\text{dry mass}}$ under these conditions. DCP was liberated to the medium at a corresponding rate, demonstrating it to be a dead-end product in this strain (Mertingk et al. 1998). In contrast, disruption of the cells by passage through a French press, use of mini beads, or lysozyme treatment, resulted in the complete loss of this activity, after which neither the disappearance of 2,4-DB nor the formation of DCP was detected in the respective assays. Furthermore, neither the presence of proteinase inhibitors nor the use of nisin to permeabilize cells stabilized activity. (The efficiency of the permeabilization was assessed by measuring the activity of isocitrate dehydrogenase, which became measurable after treatment of cells with nisin, the optimum level being around 1 g/l of nisin). Attempts to measure α-ketoglutarate-dependent dioxygenase activity and NAD(P)H-dependent monooxygenase activity with the specified assays were not successful with either crude cell extracts or permeabilized cells. (The α-ketoglutarate-dependent assay was shown to be functional by admixing crude cell extracts of *Delftia acidovorans* MC1, which expresses the required enzyme activities, with crude cell extracts of strain K2-3).

Role of Cytochrome P450

Treatment of whole cells and crude cell extracts of *R. erythropolis* K2-3 with carbon monoxide resulted in carbon monoxide difference spectra as shown in Figure 1. Clearly, there are maxima at 448 nm, which are taken as indication for the presence of P450 in this strain. This cytochrome proved to be soluble, as preparations with the particulate fractions obtained after ultracentrifugation of the crude cell extract did not give the characteristic absorption peaks. Degradation of 2,4-DB was investigated after incubation with metyrapone, which is known to be a general inhibitor of P450. In the presence of 5 mM the degradation rate was decreased by 15–20%. In contrast, application of 0.1 mM 1-aminobenzotriazole, an inhibitor of certain

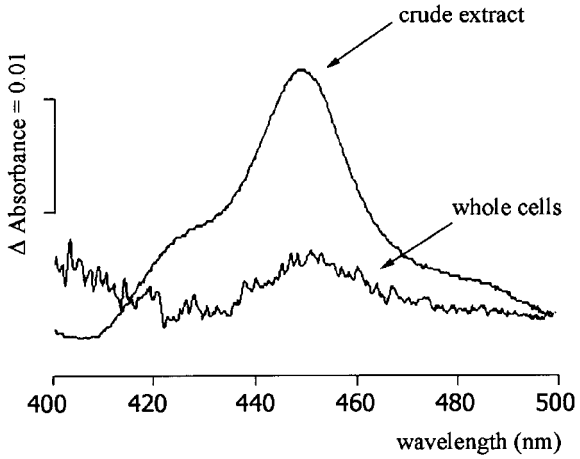

Figure 1. Carbon monoxide difference spectra of dithionite-reduced samples with whole cells (OD_{700} = 3.0, buffer A) and cell extracts (protein = 2.1 g/l, buffer A) of *R. erythropolis* K2-3 grown on xylitol.

P450 enzymes, had no effect. However, strong inhibition was observed in the presence of carbon monoxide: the oxygen consumption rate related to 2,4-DB utilization by whole cells was decreased by 35% at a CO/O_2 ratio of five and by 70% at a CO/O_2 ratio of 10.

Substrate difference spectra in the presence of 2,4-DB and MCPB had a maximum at around 385–390 nm and a minimum at 420 nm (Figure 2). This follows the characteristic type I pattern for molecules that interact with P450 enzymes and is related to the formation of enzyme-substrate complexes. The application of 4-phenoxybutyrate resulted in the same pattern (not shown). Double reciprocal plots of substrate concentration versus differences in absorption of the respective maximum and minimum resulted in Ks-values of 2.52 μM and 2.47 μM, respectively, with 2,4-DB and MCPB. Difference spectra obtained in the presence of the inhibitor metyrapone showed a significant minimum at 388 nm and a slight maximum at 425 nm, thus following the type II pattern (not shown). The application of 2-(2,4-dichlorophenoxy)propionate, DCP, guaiacol, veratrole, ethoxyphenol or p-anisic acid was accompanied by a shift in the difference spectrum, but the resulting spectra did not correspond to known patterns.

CO difference and substrate binding spectra were also determined with crude cell extracts derived from other Gram-positive strains that are capable of cleaving the ether bonds of phenoxybutyrate herbicides, namely strain K2-12 of *Rhodococcus erythropolis* and strains K2-10, K2-16 and K2-17 of *Aureobacterium*

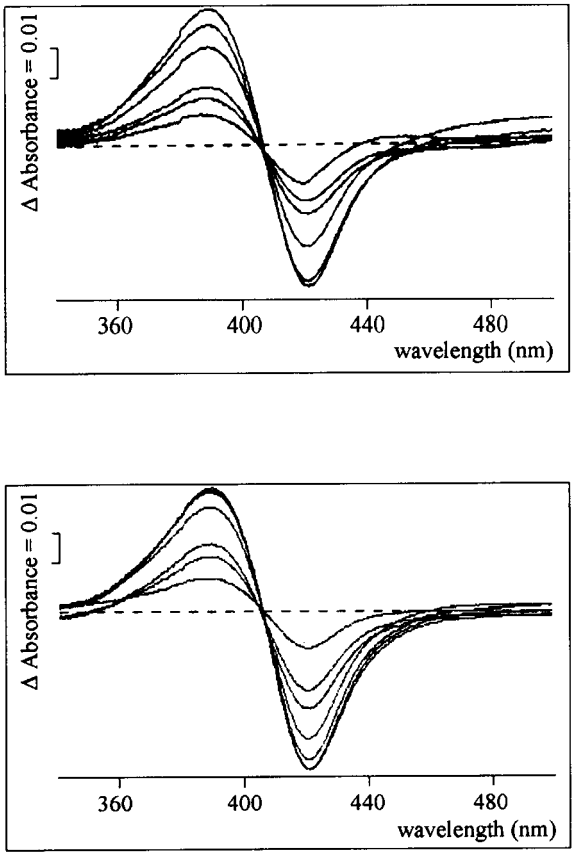

Figure 2. Difference spectra for cell extracts of *R. erythropolis* K2-3 obtained with various concentrations of 2,4-DB (upper diagram; 3.33 g/l protein, 0.485 μM P450) and MCPB (bottom; 3.56 g/l protein, 0.636 μM P450). The substrate concentrations tested were 1, 2, 3, 13, 63 and 163 μM.

sp. (cf. Mertingk et al. 1998). These showed the same characteristics as observed with strain K2-3 (not shown).

Reaction and cleavage products

In order to obtain direct evidence of the involvement of P450 in the cleavage of phenoxybutyrates, the enzyme was partially purified, following the protocol shown in Table 1, and enrichment by a factor of 60 was achieved. SDS-PAGE analysis indicated that two proteins increased markedly in relative abundance following this procedure (not shown). One of them corresponded to a mass of about 45 kDa, which is in the range typically found for P450 from various sources (Guengerich et al. 1991). The other protein was about twice as large. Besides these, impurities remained. However, as enzyme activity became measurable with this preparation, no further attempts were

Table 1. Purification of cytochrome P450 from *Rhodococcus erythropolis* K2-3

Purification Step	Volume (ml)	Protein (mg)	P450 (μmol)	Recovery (%)	Purification (-fold)
1	81	750	0.089	100	1
2	73	830	0.095	100	1
3	17	176	0.095	100	4.5
6	2	23.6	0.047	49.5	17
8	1.1	7.0	0.040	42.4	48
9	0.85	2.6	0.018	18.9	60

The purification steps correspond to the order given in Materials and methods.

made to improve the purification at this stage of the investigation.

The application of this enzyme preparation resulted in the cleavage of 2,4-DB as shown in Figure 3. In the presence of PMS and NAD(P)H, maximum turnover rates of around 0.17 mol product formed (mol P450 $\cdot$ min)$^{-1}$ were determined (corresponding to 2.3 mU/mg protein). After about 100 min, corresponding to a turnover (in total) of about 100 μM of substrate, the reaction ceased and could not be reactivated by, for instance, supplying an additional quantity of NAD(P)H. The reaction was accompanied by the liberation of DCP, one of the two expected reaction products. However, less DCP was detected than 2,4-DB disappeared in this assay. Although not investigated in detail, aeration of the assay may have contributed to this effect.

Identification of the second product proved to be more difficult. For successful detection of this compound, PMS had to be replaced by ferredoxin + ferredoxin/NADP oxidoreductase from spinach, because of the low substrate turnover and strong interference in chromatographic separation when using PMS. The reaction proceeded at a higher rate with the spinach components and came to an end within 1.75 h at a substrate turnover of 370 μM. The sensitivity of the analytical assay was further increased by separating reaction components (i.e., the proteins) and the reaction products from each other by inserting a semipermeable membrane (dialysis tube) between them. Chromatograms obtained after incubation in the presence and absence of 2,4-DB are shown in Figure 4. A clear peak emerged at 7.15 min in the assay with 2,4-DB. This peak coincided with succinate, as shown by the addition of a succinate standard to these samples, at a concentration that increased the peak height by about 20%. This is a strong indication that a C4 carbonic acid is the second substrate. Following a pu-

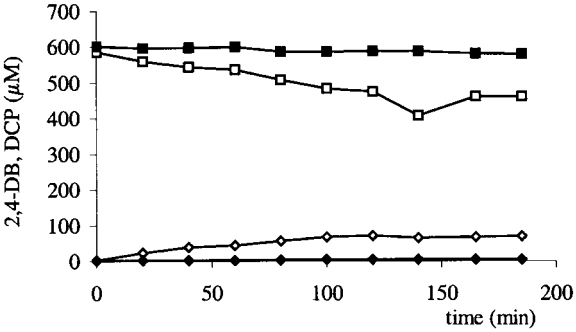

Figure 3. Cleavage of 2,4-DB by enriched P450 in the presence of PMS. Results of assays are shown with enzyme preparation plus 2,4-DB (□) resulting DCP (◇); and assays lacking enzyme preparation with 2,4-DB (■), DCP (♦).

tative mechanism in this cleavage reaction, this is most probably succinic semialdehyde. However, attempts to measure this compound directly failed. This may be attributed to the fact that cell extracts contained a soluble succinic semialdehyde dehydrogenase that is constitutively expressed and leads to the conversion of the compound to succinate. The specific activity of succinic semialdehyde dehydrogenase amounted to 0.026 U/mg in crude extracts of glucose-grown cells.

Discussion

Cleavage of the ether bond of phenoxyalkanoate herbicides by a P450-dependent reaction is described here for the first time. We detected no evidence in *R. erythropolis* K2-3 for the activity of other enzymes reported to be involved in the degradation of phenoxyalkanoate herbicides in some cases, i.e., α-ketoglutarate-dependent dioxygenases (Fukumori & Hausinger 1993a; Nickel et al. 1997; Müller & Babel 1999; Müller et al. 2001) and a monooxygenase (Xun & Wagnon 1995). Evidence for this step being catalyzed by P450 was obtained from binding studies with 4-(2,4-dichlorophenoxy)butyrate (2,4-DB), 4-(4-chloro-2-methylphenoxy)butyrate (MCPB) and 4-phenoxybutyrate, which generated type I pattern spectrograms. This pattern results from a shift of electrons of ferrous ions from a low to a high spin state and is one of the steps involved in the catalytic cycle of these enzymes (Jefcoate 1978; Guengerich 1990; Sariaslani 1991). Thus, it is typically found with compounds that can be converted catalytically, i.e., "proper substrates" (Sariaslani 1991). Most likely this also applies to other strains of *R. erythropolis* as well as strains of *Aureobacterium* isolated from contaminated building rubble

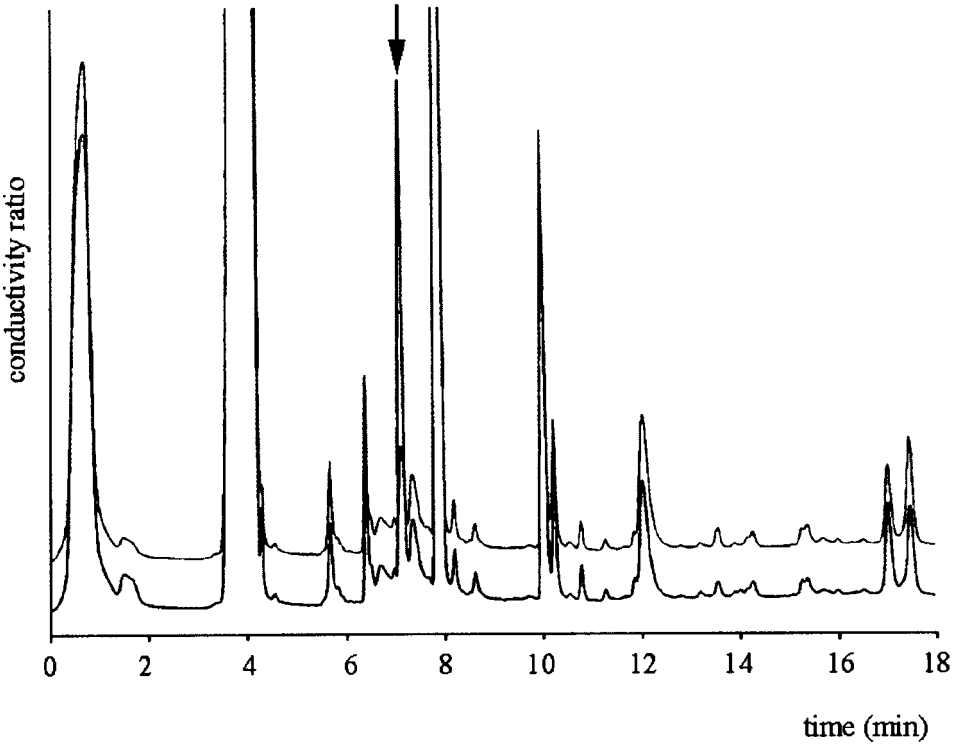

Figure 4. Ion chromatogram of the P450 assay after the cleavage of 2,4-DB by P450 in the presence of spinach ferredoxin and spinach ferredoxin/NADP oxidoreductase (lower line) and of the same assay without 2,4-DB (upper line). The arrow indicates the position of the peak corresponding to succinic acid (according to its retention time).

(Mertingk et al. 1998) that have proved to be able to cleave phenoxybutyrate herbicides: binding studies with their respective substrates revealed type I patterns similar to those observed with strain K2-3.

Binding constants of P450 of strain K2-3 for 2,4-DB and MCPB were almost identical (and in the same order as found for alkoxy derivatives with P450 from other species, cf. Dardas et al. 1985; Karlson et al. 1993). This indicates that substituents in the aromatic structure are likely to be discriminated to a lesser degree with the enzyme of strain K2-3, but that substrate specificity is primarily governed by the alkanoate moiety. The latter conclusion is based on the fact that incubating the enzyme in the presence of 2,4-dichlorophenoxyacetate or 2-(2,4-dichlorophenoxy)propionate did not result in type I patterns, in accordance with the finding that these substrates were not cleaved by this strain (Mertingk et al. 1998). The cleavage reaction was inhibited by carbon monoxide and metyrapone (Eble & Dawson 1984). Binding of the latter compound resulted in a type II spectrum as found with other P450 enzymes (Dardas et al. 1985; Sutherland 1986). By contrast, 1-aminobenzotriazol, also known to be an inhibitor of certain P450 enzymes (Ortiz de Montellano & Mathews 1981), had no effect in the case of strain K2-3.

P450-dependent oxidations have been shown to be versatile reactions that detoxify many xenobiotic compounds in various organisms (Omura 1999), including bacteria, which are known to be able to attack a wide range of substrates by these reactions (Munro & Lindsay 1996). With respect to ether bond cleavage, this applies to aryl-alkyl compounds such as isovanillate (Broadbent & Cartwright 1971), guaiacol and veratrole (Sterjiades et al. 1982; Sutherland 1986). The oxidation of ethoxy compounds, for instance, appears to require the induction of P450 in strains of *Streptomyces griseus* and *Rhodococcus rhodochrous* (Trower et al. 1989; Karlson et al. 1993). An interesting result was observed in a strain of *Acinetobacter* sp. engineered for heterologous expression of the P450 system from *Streptomyces griseus*. This construct showed activity for the oxidation of a range of structurally diverse compounds, including 2,4-D (Lamb et al. 2000).

The enzyme of strain K2-3 was not measurable in crude cell extracts, even in permeabilized cells. This is a pattern often observed in P450-dependent reactions. The enzyme system typically found in bacteria consists of three components: NAD(P)/ferredoxin oxidoreductase, ferredoxin and cytochrome P450 (Guengerich 1990; Peterson & Lu 1991). An exception is the enzyme from *Bacillus megaterium*, which unifies all components of electron transfer and catalysis within a single protein, $P450_{BM-3}$ (Narhi & Fulco 1986). Cell disruption may lead to the inactivation of one of the components. The enzyme system from *Nocardia* sp., for instance, is known to be extremely sensitive to oxygen (Cartwright et al. 1971).

There was no detectable reaction with cell-free extracts of strain K2-3 obtained under a nitrogen atmosphere. However, ether-cleaving reactions with 2,4-DB as the substrate became measurable after adding artificial electron carriers such as PMS or foreign natural electron transmitters like the reductase/ferredoxin couple from spinach. In the first case, the specific turnover rates amounted to 0.17 min^{-1}. This is lower, by about an order of magnitude, than the values of 1.5 min^{-1} found by Eble & Dawson (1984) in a system optimized for measuring $P450_{cam}$ and 5 min^{-1} in the case of $P450_{RR1}$ (Eltis et al. 1993). Addition of putidaredoxin in the $P450_{cam}$ assay resulted in a significant increase in the measured rate, to 30 min^{-1}, indicating that the application of PMS alone may be insufficient to support an adequate electron flow. Addition of the specific electron transfer components of a given P450 system, i.e., the appropriate oxidoreductase and ferredoxin, was also shown to be essential for high specific rates: up to 2040 min^{-1} in the case of $P450_{cam}$ according to measurements of NADPH consumption. This is consistent with our results, since substrate turnover was significantly increased in our assays by applying ferredoxin and ferredoxin reductase to an assay mixture with crude cell extract. The specific turnover rate exceeded that obtained by application of PMS alone by a factor of at least ten. However, the figures we produced are not regarded as 'true' specific rates for the P450 reaction(s), as the principal intention was to optimize the assays for detection of the products rather than to quantify the rates precisely.

Consequently, it would be of interest to identify the specific molecules that interact with $P450_{PB-1}$ *in vivo*. Isolation and purification is a prerequisite in this case for reconstituting a system that is active in ether cleavage. Moreover, the specificity of the electron donor could be established. This would help to elucidate the benefits that *R. erythropolis* K2-3 derives from cleaving phenoxybutyrates.

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