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Degradation of Chloroaromatics: Purification and Characterization of Maleylacetate Reductase from *Pseudomonas* sp. Strain B13

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Maleylacetate reductase of *Pseudomonas* sp. strain B13 was purified to homogeneity by chromatography on DEAE-cellulose, Butyl-Sepharose, Blue-Sepharose, and Sephacryl S100. The final preparation gave a single band by polyacrylamide gel electrophoresis under denaturing conditions and a single symmetrical peak by gel filtration under nondenaturing conditions. The subunit M_r value was 37,000 (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Estimation of the native M_r value by gel filtration gave a value of 74,000 with a Superose 6 column, indicating that the enzyme is dimeric. The pH and temperature optima were 5.4 and 50°C, respectively. The pI of the enzyme was estimated to be 7.0. The apparent K_m values for maleylacetate and NADH were 58 and 30 μM, respectively, and the maximum velocity was 832 U/mg of protein for maleylacetate. Maleylacetate and various substituted maleylacetates, such as 2-chloro- and 2-methylmaleylacetate, were reduced at significant rates. NADPH was also used as a cofactor instead of NADH with nearly the same V_{max} value, but its K_m value was estimated to be 77 μM. Reductase activity was inhibited by a range of thiol-blocking reagents. The absorption spectrum indicated that there was no bound cofactor or prosthetic group in the enzyme.

Microbial degradation of chlorinated aromatics via chlorocatechols as the key intermediates has been described (27). Further degradation proceeds through the modified *ortho*-cleavage pathway (Fig. 1), including ring cleavage by catechol 1,2-dioxygenase and lactonization of chloromuconates by chloromuconate cycloisomerase. The conversion of the dienelactones, products of the lactonization with accompanied chloride elimination from the 4- or 5-position, produces maleylacetates because of the action of the dienelactone hydrolase.

It has been shown for *Pseudomonas* sp. strain B13 that a maleylacetate reductase catalyzes the conversion of maleylacetate into 3-oxoadipate. In addition, the same enzyme converts 2-chloromaleylacetate, the metabolite in the degradation of 2,4-dichlorophenoxyacetate or 3,5-dichlorobenzoate, into 3-oxoadipate. During this conversion, maleylacetate was formed as an intermediate after chloride elimination (11).

In the present paper we describe the purification and characterization of this bifunctional enzyme of *Pseudomonas* sp. strain B13.

MATERIALS AND METHODS

Organism and culture conditions. *Pseudomonas* sp. strain B13 (DSM624) was grown at 30°C in 30 separate 0.6-liter cultures with mineral medium containing 3-chlorobenzoate (10 mM) as growth substrate (3). After the cultures had grown up to an optical density at 546 nm of 1.7, cells were harvested and stored at −20°C.

Preparation of cell extracts. Frozen cells were combined and suspended in buffer A (50 mM Tris/HCl, pH 7.0, containing 0.5 mM dithioerythritol [DTE]). Disruption took place at 4°C by one passage through a French pressure cell (140 MPa; Aminco, Silver Spring, Md.). Cell debris were removed by centrifugation at 100,000 × *g* for 60 min at 4°C.

Enzyme assays. Maleylacetate reductase was measured spectrophotometrically by the decrease of the cofactor NADH at 340 nm. The standard assay mixture contained (in 1 ml) 50 mM Tris/HCl buffer (pH 7.0), 0.4 μmol of maleylacetate, and 0.4 μmol of NADH. The reaction was initiated by adding 10 μl of diluted pure enzyme stock solution. In contrast to crude extract, the pure enzyme produced no nonspecific NADH oxidation. One enzyme unit is defined as 1 μmol of substrate converted per minute at 25°C.

Kinetic measurements. The K_m values of the maleylacetates were determined in a substrate concentration range of 0.5 to 2.0 of the respective K_m value. A concentration of 0.4 mM NADH was used as the cofactor.

The pH optimum was determined by the standard assay except that the buffer was replaced by 50 mM histidine/HCl buffer (pH 4.7 to 6.8), 50 mM K/Na-phosphate buffer (pH 6.0 to 8.0), or 50 mM Tris/HCl buffer (pH 7.0 to 8.5).

The temperature optimum was determined by the standard assay.

For testing the influence of inhibitors, a sample of diluted pure enzyme stock solution (0.05 μg) was preincubated for 10 min at 25°C in 50 mM Tris/HCl buffer, pH 7.0, in the presence of the reagent (see Table 2). The reaction was started by adding 0.4 μmol of NADH and maleylacetate to the mixture. Reactivation with dithiothreitol was tested in the mixture of enzyme and *p*-chloromercuribenzoate after incubation for 10 min.

Protein determination. Protein concentration was determined by the Bradford method (1), with crystalline bovine serum albumin as the standard. During the enzyme purification, the eluted protein was detected with a Pharmacia UV-1 monitor at 254 nm.

Enzyme purification. All enzyme purification procedures were carried out at 4°C.

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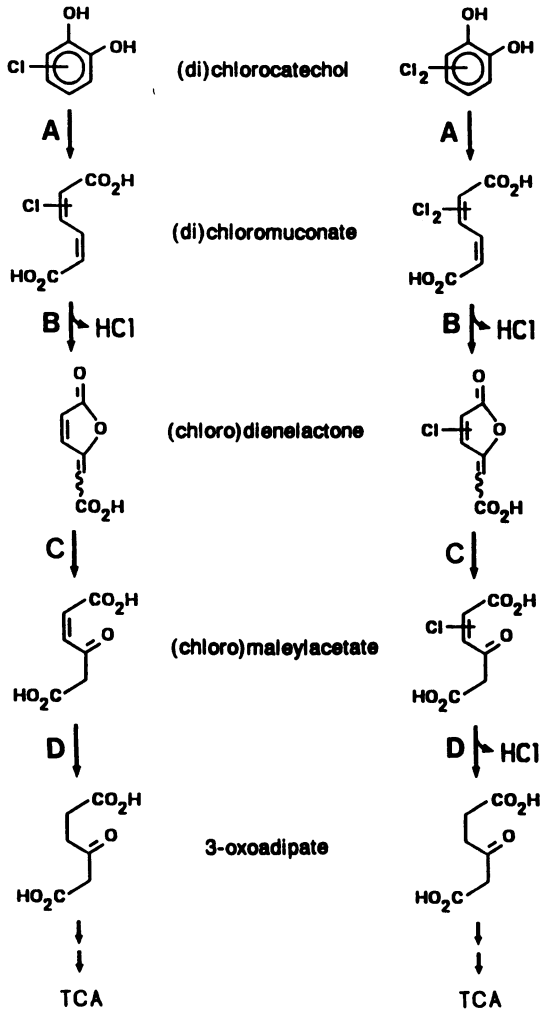

FIG. 1. Proposed pathways for the degradation of chloro- and dichlorocatechols as central sequences in the bacterial degradation of chloroaromatics. Key to enzymes: A, catechol 1,2-dioxygenase; B, muconate cycloisomerase; C, dienelactone hydrolase; D, maleylacetate reductase.

Step 1: chromatography on DE-52. Sampled crude extracts (160 ml) were applied at 60 ml/h to a DE-52 cellulose column (5 by 15 cm) which had been preequilibrated with buffer A (50 mM Tris/HCl, pH 7.0, containing 0.5 mM DTE). After the column was washed with 800 ml of buffer A at 150 ml/h, maleylacetate reductase was eluted with 4,000 ml of buffer A containing NaCl in a linear gradient from 0 to 1 M. Fractions of 25 ml were collected at a flow rate of 150 ml/h. Eight fractions (200 ml in total) containing the highest levels of activity were pooled.

Step 2: ammonium sulfate treatment and chromatography on Butyl-Sepharose CL-4B. Pooled fractions from step 1 were concentrated to 80 ml by ultrafiltration (ultrafiltration unit 8200, filter YM30; Amicon). A saturated aqueous solution of $(NH_4)_2SO_4$ (pH 7.0, containing 1 mM EDTA) was added with constant stirring to the concentrate to give 55% saturation. After 20 min the precipitate was removed by centrifugation at 8,000 × *g* for 20 min. The pellet was redissolved with buffer B (50 mM Tris/HCl, pH 7.0, containing 1 M $(NH_4)_2SO_4$, 1 mM EDTA, and 0.5 mM DTE) to a final volume of 30 ml. The solution was cleared by centrifugation at 8,000 × *g* for 15 min. The supernatant, containing reductase activity, was applied at 50 ml/h to a Butyl-Sepharose CL-4B gel column (2.6 by 19 cm), preequilibrated with buffer B. After the column was washed with 700 ml of buffer B, maleylacetate reductase was eluted with 600 ml of buffer A in a linear gradient of $(NH_4)_2SO_4$ from 1 to 0 M. Fractions of 5 ml were collected at a flow rate of 60 ml/h. Eleven fractions (55 ml in total) containing the highest levels of activity were pooled.

Step 3: chromatography on Blue-Sepharose CL-6B. The pooled fractions from step 2 were desalted by ultrafiltration and applied at 24 ml/h to a Blue Sepharose CL-6B column (2.6 by 35 cm) preequilibrated with buffer A. The column was washed extensively with the same buffer until no further protein eluted (ca. 800 ml). Then, the maleylacetate reductase was eluted with 1,200 ml of buffer A in a linear gradient of Tris/HCl from 0.05 to 1 M. Fractions of 4.4 ml were collected at a flow rate of 45 ml/h. Seventeen fractions (75 ml in total) containing the highest levels of activity were pooled.

Step 4: gel filtration on Sephacryl S100-HR. Combined fractions from step 3 were concentrated to 10 ml by ultrafiltration. The concentrate was applied to a Sephacryl S100 HR column (2.5 by 85 cm) that had previously been equilibrated with 100 mM Tris/HCl buffer, pH 7.0, containing 0.5 mM DTE. The flow rate was maintained at 24 ml/h, and fractions of 4 ml were collected. Three fractions (12 ml in total) containing the highest levels of activity were pooled.

Step 5: storage. The combined fractions from step 4 were concentrated to ca. 4 ml by ultrafiltration (see step 4). Simultaneously, elution buffer was replaced by storage buffer (100 mM Tris/HCl, pH 7.0, containing 50% [vol/vol] glycerol, 1 mM DTE, and 0.5 mM Na_2 EDTA). This pure enzyme stock solution was stored at −20°C.

Determination of M_r values. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the subunit M_r value and the purity of the maleylacetate reductase. It was performed by the method of Laemmli (15) on 1-mm-thick vertical slab gels (13.5 by 15.5 cm) containing 12.3% (wt/vol) acrylamide in the resolving gels. Electrophoresis was performed at a voltage of 200 V for 4 h, and the apparatus was cooled. Protein was detected by staining the gels with silver (16). The calibration proteins were bovine albumin (M_r, 66,000), chicken ovalbumin (M_r, 45,000), porcine stomach pepsin (M_r, 34,700), trypsinogen (M_r, 24,000), bovine β-lactoglobulin (M_r, 18,400), and chicken egg white lysozyme (M_r, 14,300) (Sigma Chemical Co., St. Louis, Mo.). The M_r value of the native protein was determined by means of gel filtration on a Superose 6 fast protein liquid chromatography column (1 by 30 cm) at a flow rate of 0.5 ml/min. The column buffer was 100 mM Tris/HCl buffer, pH 7.0. Horse ferritin (M_r, 450,000), bovine catalase (M_r, 240,000), rabbit aldolase (M_r, 158,000), bovine serum albumin (M_r, 68,000), chicken ovalbumin (M_r, 45,000), chymotrypsinogen A (M_r, 25,000), and horse cytochrome *c* (M_r, 12,500) were used as the reference proteins (Boehringer, Mannheim, Germany). Standards and samples were injected in 100-μl samples, and the proteins were detected by monitoring the eluate at 254 nm.

Isoelectric focusing. Isoelectric focusing was carried out on 3.8% (wt/vol) polyacrylamide gels containing 2% (vol/vol) ampholytes by the method of O'Farrell (19) with carrier ampholytes in a pH range of 3 to 10.

Absorption spectra. Spectra were recorded on a Shimadzu Recording Spectrophotometer UV-240. Kinetic measurements at a single wavelength were carried out on an

TABLE 1. Purification of the maleylacetate reductase from *Pseudomonas* sp. strain B13

Purification step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (units mg^{-1})	Recovery of activity (%)	Purification factor
Crude extract	160	3,200	12,352	3.9	100	1
DE-52, eluate	200	627	10,214	16.3	83	4.2
Butyl-Sepharose CL-4B, eluate	55	45	7,931	176.2	64	45.2
Blue-Sepharose CL-6B, eluate	75	7.9	4,482	567.3	36	145.5
Sephacryl S100-HR, eluate	12	4.8	2,798	582.9	23	149.5

UVIKON 820 spectrophotometer (Fa. Kontron, Eching, Germany).

Chemicals. Maleylacetate and substituted maleylacetates were prepared in situ by alkaline hydrolysis of the corresponding dienelactones (5). Dienelactones were prepared by Wittig reaction of substituted maleic anhydrides (12). All other chemicals were purchased commercially and were of the highest available purity.

RESULTS

Purification of maleylacetate reductase. Maleylacetate reductase was purified from 3-chlorobenzoate-grown *Pseudomonas* strain B13 cells. The results of a typical enzyme purification procedure are summarized in Table 1. Fig. 2 to 4 show the elution profiles of the enzyme with regard to the corresponding sort of chromatography. During purification the specific activity of the enzyme increased to 583 U/mg of protein, indicating a 150-fold purification with 23% recovery of activity.

In the first step the reductase eluted from the DE-52 anion-exchange column at ca. 0.15 M NaCl. The subsequent protein separation by hydrophobic interaction chromatography on Butyl-Sepharose is shown in Fig. 2. Enzyme activity was eluted at ca. 0.55 M $(NH_4)_2SO_4$. The protein elution profile for the following Blue-Sepharose chromatography step is illustrated in Fig. 3. Activity was eluted at ca. 0.23 M Tris/HCl. During the final gel filtration on Sephacryl S100-HR (Fig. 4) the protein was eluted as a single symmetrical protein peak corresponding to the enzyme activity. The purity of the maleylacetate reductase was determined by SDS-PAGE (Fig. 5). After staining with silver the gel showed only one protein band.

Stability. Purified enzyme retained 80% of its initial activity after storage at −20°C for 2 months in 100 mM Tris/HCl buffer (pH 7.0) containing 50% (vol/vol) glycerol, 1 mM DTE, and 0.5 mM EDTA.

M_r values and isoelectric point. The M_r value of the purified enzyme was determined by gel filtration to be approximately 74,000, as calculated from three independent determinations. M_r estimation of the subunits by SDS-PAGE yielded a single band with an M_r of 37,000. Therefore it can be assumed that the enzyme consists of two subunits of identical size. The isoelectric point of the purified enzyme was determined to be 7.0.

pH and temperature optima. The effect of the pH on the enzyme activity was observed in three different buffer systems over the range of 4.7 to 8.5. The optimum activity was determined to be at pH 5.4 in 50 mM histidine/HCl buffer (Fig. 6). Activity was drastically reduced at values above pH 5.3. The examination of temperature influence on the enzyme activity over the range of 4 to 60°C showed an optimum activity at 50°C in 50 mM Tris/HCl buffer, pH 7.0 (Fig. 7).

Cofactor requirements and catalytic properties. Purified maleylacetate reductase was colorless. The absorption spectrum showed a strong absorption at 217 nm and a weak absorption at 275 nm, while absorption in the visible range was absent.

The V_{max} values for maleylacetate, 2-chloromaleylacetate, and 2-methylmaleylacetate of the homogeneous en-

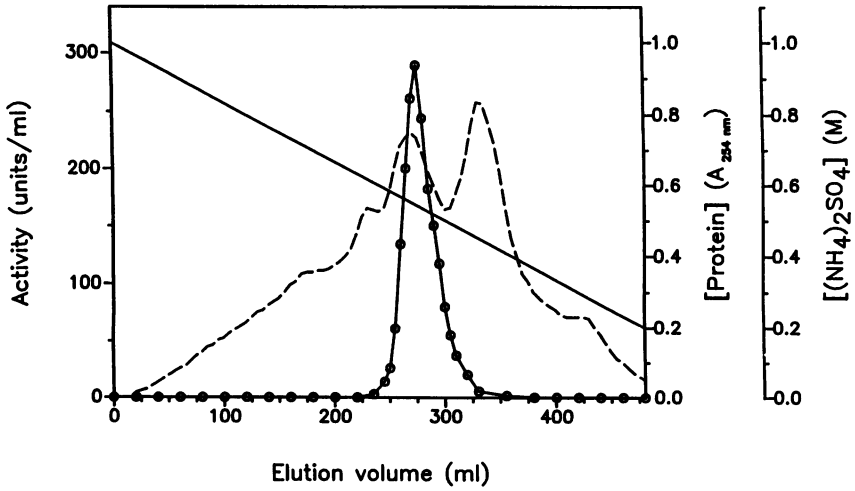

FIG. 2. Butyl-Sepharose CL-4B chromatography of maleylacetate reductase from *Pseudomonas* sp. strain B13. The enzyme was eluted with buffer A containing $(NH_4)_2SO_4$ in a linear gradient from 1 to 0 M (——). Fractions were assayed for reductase activity (○) and protein content (– – –).

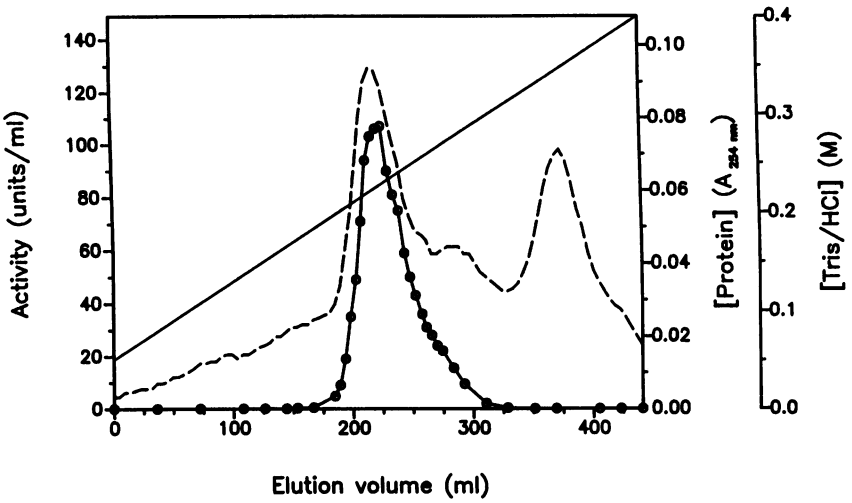

FIG. 3. Blue-Sepharose CL-6B chromatography of maleylacetate reductase from *Pseudomonas* sp. strain B13. The enzyme was eluted with buffer A containing Tris/HCl in a linear gradient from 0.05 to 1 M (——). Fractions were assayed for reductase activity (○) and protein content (- - -).

zyme were 832, 701, and 58 U/mg of protein, respectively. On the basis of the M_r value 37,000 of the subunits, the catalytic constants were 30,800 min^{-1} for maleylacetate, 25,950 min^{-1} for 2-chloromaleylacetate, and 2,150 min^{-1} for 2-methylmaleylacetate. K_m values were obtained from Hanes plots. The K_m values for maleylacetate and 2-chloro- and 2-methylmaleylacetate were found to be 58, 76, and 308 μM, respectively, at a constant concentration of 0.4 mM NADH. The K_m value for NADH was measured in the presence of 0.4 mM maleylacetate and was found to be 30 μM. When NADH was replaced by NADPH, a K_m value of 77 μM resulted. The relative maximum velocities with NADH and NADPH were nearly identical.

Inhibition and activation. The data of effects of various compounds on the activity of maleylacetate reductase are listed in Table 2. Chelating agents, such as EDTA or *o*-phenanthroline, had no effect on enzyme activity. The maleylacetate reductase showed no significant requirement for usual bivalent-metal ions since the activity of the enzyme remained unchanged upon addition of cyanide ions. Heavy-metal ions like Zn^{2+}, Cu^{2+}, Ag^{+}, and Hg^{2+} inhibited the enzyme almost completely, indicating the involvement of thiol groups. The heavy-metal derivative *p*-chloromercuribenzoate, which brings about highly specific modifications of cysteine side chains in enzymes, had a completely inhibitory effect at a concentration of 0.1 mM on the maleylacetate reductase. The activity could be protected from this inhibitor by the addition of an excess of dithiothreitol. Ions such as Fe^{2+}, Fe^{3+}, Co^{2+}, Mn^{2+}, and Mg^{2+} did not affect the maleylacetate reductase activity (data not shown).

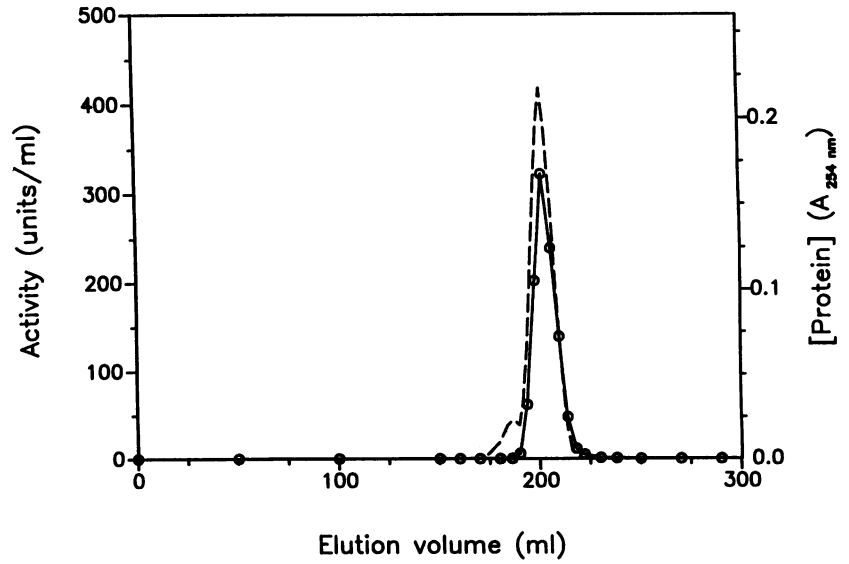

FIG. 4. Sephacryl S100-HR chromatography of maleylacetate reductase from *Pseudomonas* sp. strain B13. Fractions were assayed for maleylacetate reductase activity (○) and protein content (- - -).

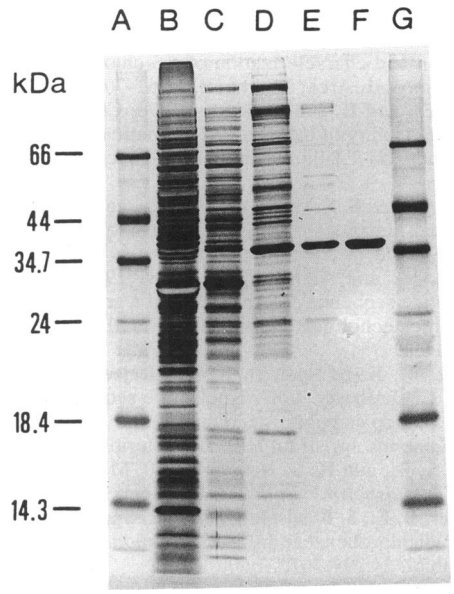

FIG. 5. Purification of maleylacetate reductase monitored by SDS-PAGE. Lanes: A and G, M_r markers (ca. 1 μg each); B, crude extract (20 μg of protein); C, DEAE elution pool (5 μg of protein); D, Butyl-Sepharose elution pool (3 μg of protein); E, Blue-Sepharose elution pool (0.6 μg of protein); F, Sephacryl gel filtration pool (1 μg of protein).

DISCUSSION

The enzymology of the degradative route for chlorocatechols, the modified *ortho*-cleavage pathway, has been investigated for various chloroaromatic-degrading strains: the 3-chlorobenzoate-utilizing *Pseudomonas* strain B13 and *Pseudomonas putida* AC858, the 2,4-D-utilizing *Alcaligenes eutrophus* JMP134, the 1,2-dichlorobenzene-utilizing *Pseudomonas* spp. P51 and JS100, the 1,4-dichlorobenzene-utilizing *Pseudomonas* spp. RHO1 and JS6 and *Alcaligenes* sp. A175, and the 3-chloroaniline-utilizing *Pseudomonas acidovorans* CA28. The data of the enzyme specificity indicate that the pathways are specialized for the conversion

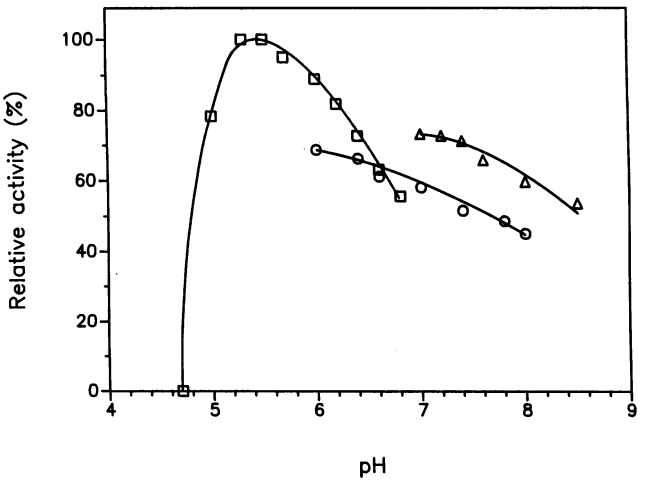

FIG. 6. Effect of pH on maleylacetate reductase activity. Activity was assayed in 50 mM histidine/HCl buffer (□), 50 mM Tris/HCl buffer (△) or 50 mM K/Na-phosphate buffer (○).

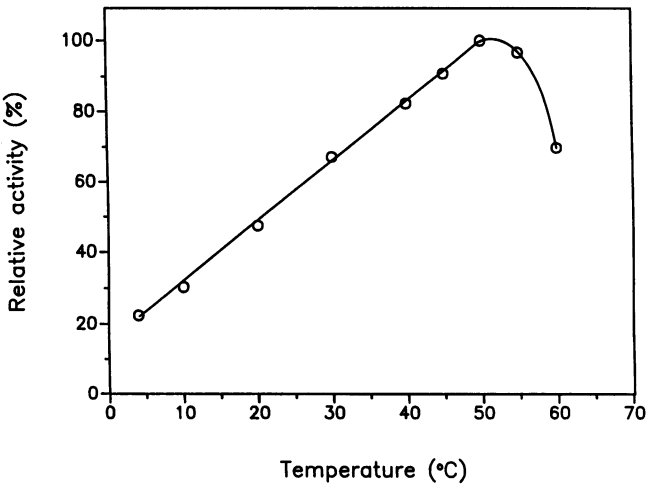

FIG. 7. Effect of temperature on maleylacetate reductase activity.

of the respective substrate (and its metabolites) which has been used as the enrichment compound. Therefore enzymes evolved for the handling of monosubstituted substrates use the monosubstituted compounds more rapidly than the dichlorinated ones, while those enzymes in 2,4-D or dichlorobenzene degradation prefer dichlorosubstrates with special preference for the isomers formed in the degradation.

The catechol 1,2-dioxygenase of *A. eutrophus* JMP134 shows the highest V_{max} values with disubstituted catechols, especially 3,5-dichlorocatechol, the metabolite from 2,4-D (25), whereas the corresponding enzyme from strain B13 shows a slight preference for monosubstituted compounds (4, 26). Nearly identical data on the specificity concerning the catechol 1,2-dioxygenase have been obtained for the strains B13 and CA28 enriched with a monochlorinated substrate (10). A preference for 3,4-dichlorocatechol, the metabolite from 1,2-dichlorobenzene, was reported for the strains P51 (33) and JS100 (9). In contrast, no preference has been observed with the catechol 1,2-dioxygenase of strains RHO1, A175, and JS6 for 3,6-dichlorocatechol, the metabolite from 1,4-dichlorobenzene (21, 31, 32).

A clear distinction was observed between the cycloisomerizing enzymes of strain B13 and JMP134. While strain B13 induced a chloromuconate cycloisomerase with prefer-

TABLE 2. Effect of inhibitors on the maleylacetate reductase activity

Addition to the assay mixture	Concn (mM)	Relative enzyme activity (%)
None		100
$AgNO_3$	1	0
$HgCl_2$	1	2
$CuSO_4$	1	0
$ZnCl_2$	1	0
NaCN	1	100
o-Phenanthroline	1	100
EDTA	1	103
Dithiothreitol	1	100
p-Chloromercuribenzoate	0.02	0
p-Chloromercuribenzoate + dithiothreitol	0.1	
	10	98

ence for monochloromuconates (30), 2,4-dichloromuconate is the preferential substrate for the dichloromuconate cycloisomerase of strain JMP134 (13).

With respect to the next degradative step, dienelactone hydrolase from B13 showed a considerably higher affinity for the *trans*-dienelactone (30), whereas the enzyme from strain JMP134 lacks this preference (29).

Biochemical and molecular genetic data, such as homogeneous enzyme preparations for crystallization and nucleotide sequences, are available for the enzymes mentioned in the chlorocatechol degradation (2, 6, 7, 13, 18, 24, 33) to allow research on the enzyme mechanisms and the evolution of these enzymes. The dienelactone hydrolase has been crystallized and a model based on X-ray data (20, 22, 23) and the nucleotide sequence (7) has been published.

Whereas a number of data are known for the initial enzymes of the modified *ortho*-cleavage pathway, only limited data exist on the enzyme functioning in the further degradation of maleylacetate and chlorinated analogs. The role of a maleylacetate reductase in the degradation pathway of chloro- and dichlorocatechols has recently been demonstrated for strains B13 (11) and JMP134 (34). The enzyme brings about the elimination of the chlorosubstituent from 2-chloromaleylacetate as well as the reduction of the intermediately formed maleylacetate by use of two molar equivalents of NADH to give 3-oxoadipate.

The results in this paper show that the maleylacetate reductase functioning in the degradation of chloroaromatics in *Pseudomonas* sp. strain B13 is remarkably similar to an enzyme from *Trichosporum cutaneum* involved in the degradation of resorcinol (8). Both reductases are NADH-dependent enzymes but also tolerate NADPH. The enzyme of strain B13 and the corresponding enzyme of the yeast show similar M_r values (74,000) and consist of two identical subunits. Similar sensitivity to thiol-blocking reagents, no requirement for bivalent-metal ions, and insensitivity towards chelating agents are other features common to both enzymes.

Since a variety of substituted maleylacetates produced from the respective dienelactones are now available (12), investigations to compare maleylacetate reductases from different bacterial origins are possible. The maleylacetate reductases of the chloroaromatics-degrading strains B13, JMP134, and RHO1 have been purified to homogeneity (references 17 and 28 and this paper), offering the chance of crystallization for X-ray analysis. The data on the molecular genetics—the gene of the maleylacetate reductase from a *Pseudomonas* sp., able to grow with L-tyrosine, has been cloned (14)—along with those of the enzyme specificity and of the X-ray analysis might allow the creation of a model of maleylacetate reductases and might help answer the questions of the origin and the natural function of the enzyme.

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REFERENCES

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
2. **Broderick, J. B., and T. V. O'Halloran.** 1991. Overproduction, purification, and characterization of chlorocatechol dioxygenase, a non-heme iron dioxygenase with broad substrate tolerance. Biochemistry **30**:7349–7358.
3. **Dorn, E., M. Hellwig, W. Reineke, and H.-J. Knackmuss.** 1974. Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. Arch. Microbiol. **99**:61–70.
4. **Dorn, E., and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. Biochem. J. **174**: 85–94.
5. **Evans, W. C., B. S. W. Smith, P. Moss, and H. N. Fernley.** 1971. Bacterial metabolism of 4-chlorophenoxyacetate. Biochem. J. **122**:509–517.
6. **Frantz, B., and A. M. Chakrabarty.** 1987. Organization and nucleotide sequence determination of a gene cluster involved in 3-chlorocatechol degradation. Proc. Natl. Acad. Sci. USA **84**: 4460–4464.
7. **Frantz, B., K.-L. Ngai, D. K. Chatterjee, L. N. Ornston, and A. M. Chakrabarty.** 1987. Nucleotide sequence and expression of *clcD*, a plasmid-borne dienelactone hydrolase gene from *Pseudomonas* sp. strain B13. J. Bacteriol. **169**:704–709.
8. **Gaal, A. B., and H. Y. Neujahr.** 1980. Maleylacetate reductase from *Trichosporon cutaneum*. Biochem. J. **185**:783–786.
9. **Haigler, B. E., S. F. Nishino, and J. C. Spain.** 1988. Degradation of 1,2-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **54**:294–301.
10. **Hinteregger, C., M. Loidl, and F. Streichsbier.** 1992. Characterization of isofunctional ring-cleaving enzymes in aniline and 3-chloroaniline degradation by *Pseudomonas acidovorans* CA28. FEMS Microbiol. Lett. **97**:261–266.
11. **Kaschabek, S. R., and W. Reineke.** 1992. Maleylacetate reductase of *Pseudomonas* sp. strain B13: dechlorination of chloromaleylacetates, metabolites in the degradation of chloroaromatic compounds. Arch. Microbiol. **158**:412–417.
12. **Kaschabek, S. R., and W. Reineke.** Unpublished data.
13. **Kuhm, A. E., M. Schlömann, H.-J. Knackmuss, and D. H. Pieper.** 1990. Purification and characterization of dichloromuconate cycloisomerase from *Alcaligenes eutrophus* JMP134. Biochem. J. **266**:877–883.
14. **Kukor, J. J., R. H. Olsen, and J.-S. Siak.** 1989. Recruitment of a chromosomally encoded maleylacetate reductase for degradation of 2,4-dichlorophenoxyacetic acid by plasmid pJP4. J. Bacteriol. **171**:3385–3390.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert.** 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science **211**:1437–1438.
17. **Müller, D., and W. Reineke.** Unpublished data.
18. **Ngai, K.-L., M. Schlömann, H.-J. Knackmuss, and L. N. Ornston.** 1987. Dienelactone hydrolase from *Pseudomonas* sp. strain B13. J. Bacteriol. **169**:699–703.
19. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis. J. Biol. Chem. **250**:4007–4021.
20. **Ollis, D. L., and K.-L. Ngai.** 1985. Crystallization and preliminary X-ray crystallographic data of dienelactone hydrolase from *Pseudomonas* sp. B13. J. Biol. Chem. **260**:9818–9819.
21. **Oltmanns, R. H., H. G. Rast, and W. Reineke.** 1988. Degradation of 1,4-dichlorobenzene by enriched and constructed bacteria. Appl. Microbiol. Biotechnol. **28**:609–616.
22. **Pathak, D., K.-L. Ngai, and D. Ollis.** 1988. X-ray crystallographic structure of dienelactone hydrolase at 2.8 Å. J. Mol. Biol. **204**:435–445.
23. **Pathak, D., and D. Ollis.** 1990. Refined structure of dienelactone hydrolase at 1.8 Å. J. Mol. Biol. **214**:497–525.
24. **Perkins, E. J., M. P. Gordon, O. Caceres, and P. F. Lurquin.** 1990. Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J. Bacteriol. **172**:2351–2359.
25. **Pieper, D. H., W. Reineke, K.-H. Engesser, and H.-J. Knackmuss.** 1988. Metabolism of 2,4-dichlorophenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP134. Arch. Microbiol. **150**:95–102.

26. **Reineke, W., and H.-J. Knackmuss.** 1980. Hybrid pathway for chlorobenzoate metabolism in *Pseudomonas* sp. B13 derivatives. J. Bacteriol. **142:**467–473.
27. **Reineke, W., and H.-J. Knackmuss.** 1988. Microbial degradation of haloaromatics. Annu. Rev. Microbiol. **42:**263–287.
28. **Schlömann, M.** 1993. Personal communication.
29. **Schlömann, M., D. H. Pieper, and H.-J. Knackmuss.** 1990. Enzymes of haloaromatics degradation: variations of *Alcaligenes* on a theme by *Pseudomonas*, p. 185–196. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.) *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
30. **Schmidt, E., and H.-J. Knackmuss.** 1980. Chemical structure and biodegradability of halogenated aromatic compounds. Conversion of chlorinated muconic acids into maleylacetic acid. Biochem. J. **192:**339–347.
31. **Schraa, G., M. L. Boone, M. S. M. Jetten, A. R. W. van Neerven, P. J. Colberg, and A. J. B. Zehnder.** 1986. Degradation of 1,4-dichlorobenzene by *Alcaligenes* sp. strain A175. Appl. Environ. Microbiol. **52:**1374–1381.
32. **Spain, J. C., and S. F. Nishino.** 1987. Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **53:**1010–1019.
33. **van der Meer, J. R., R. I. L. Eggen, A. J. B. Zehnder, and W. M. de Vos.** 1991. Sequence analysis of the *Pseudomonas* sp. strain P51 *tcb* gene cluster, which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates. J. Bacteriol. **173:**2425–2434.
34. **Vollmer, M. D., K. Stadler-Fritzsche, and M. Schlömann.** 1993. Conversion of 2-chloromaleylacetate in *Alcaligenes eutrophus* JMP134. Arch. Microbiol. **159:**182–188.