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Conversion of 2-chloromaleylacetate in *Alcaligenes eutrophus* JMP134

Martin Dominik Vollmer, Karin Stadler-Fritzsche, Michael Schlömann

Institut für Mikrobiologie, Universität Stuttgart, Azenbergstrasse 18, W-7000 Stuttgart 1, Federal Republic of Germany

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Abstract. 2,4-Dichlorophenoxyacetate (2,4-D) in *Alcaligenes eutrophus* JMP134 (pJP4) is degraded via 2-chloromaleylacetate as an intermediate. The latter compound was found to be reduced by NADH in a maleylacetate reductase catalyzed reaction. Maleylacetate and chloride were formed as products of 2-chloromaleylacetate reduction, the former being funnelled into the 3-oxoadipate pathway by a second reductive step. There was no indication for an involvement of a pJP4-encoded enzyme in either the reduction or the dechlorination reaction.

Key words: 2,4-Dichlorophenoxyacetate – Maleylacetate reductase – Chloride elimination – Maleylacetate – Chloromaleylacetate – *Alcaligenes eutrophus* – pJP4

Many mono- and dichlorosubstituted aromatic compounds, such as chlorobenzoates, chlorophenols, chlorophenoxyacetates, chlorobenzenes and others, are degraded by bacteria via a modification of the widely distributed 3-oxoadipate pathway (Fig. 1; for recent reviews see: Reineke and Knackmuss 1988; Häggblom 1990; Neilson 1990; Chaudhry and Chapalamadugu 1991; Engesser and Fischer 1991). The "modified *ortho* cleavage pathway" degrades chlorocatechols, generated by initial conversion of the growth substrate, to maleylacetate, in the case of monochlorocatechols, or to a chloromaleylacetate, in the case of dichlorosubstituted substrates (Bollag et al. 1968; Tiedje et al. 1969; Evans et al. 1971a, b; Schmidt and Knackmuss 1980; Spain and Nishino 1987; Haigler et al. 1988). The enzymes involved in these reactions are usually plasmid encoded (Pemberton et al. 1979; Chatterjee et al. 1981; van der Meer et al. 1991a) and differ from those of the catechol branch of the 3-oxoadipate pathway in having an altered substrate specificity (Dorn and Knackmuss 1978; Schmidt and Knackmuss 1980; Spain and Nishino 1987; Pieper et al. 1988; Gorlatov et al. 1989; van der Meer et al. 1991b). Chloride is eliminated during or directly after the cycloisomerization reaction (Fig. 1) resulting in an additional carbon-carbon double bond in the lactonic product compared to the 3-oxoadipate pathway.

As originally shown for *Pseudomonas* sp. B13, maleylacetate is funnelled into the pathway for unsubstituted

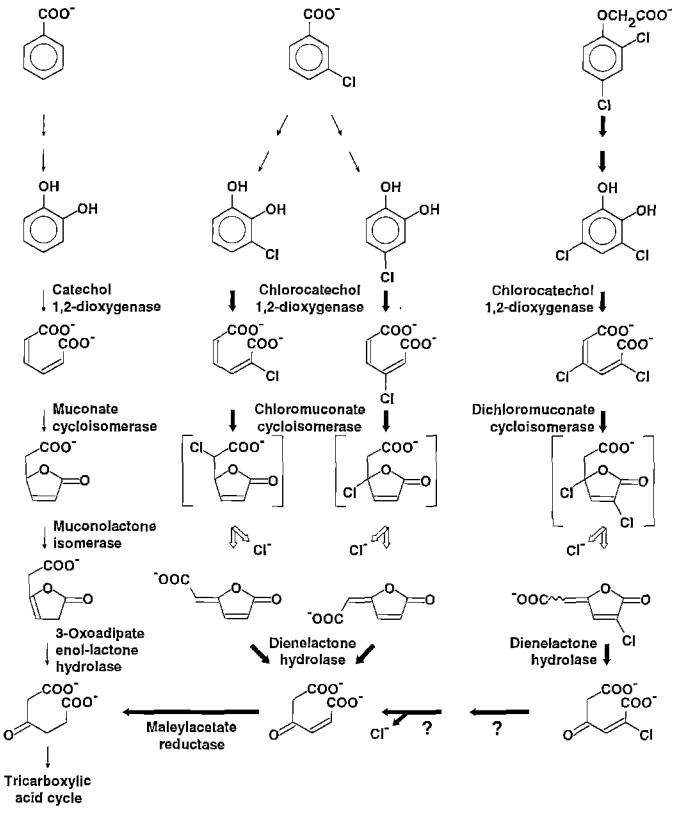

Fig. 1. Degradative pathways for benzoate, 3-chlorobenzoate, and 2,4-dichlorophenoxyacetate. Enzymes of the ordinary 3-oxoadipate pathway are shown as *light arrows*, those with a specific function in chloroaromatics degradation are represented by *heavy arrows*. *Open arrows* indicate reactions assumed to be non-enzymatic or gratuitously enzyme-catalyzed. The lactones in brackets are formal reaction intermediates, but do not necessarily imply a mechanism

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetate

Correspondence to: M. Schlömann

catechol by its reduction to 3-oxoadipate (Reineke 1984). This reaction is catalyzed by maleylacetate reductase (EC 1.3.1.32), an enzyme previously shown to be involved in the bacterial degradation of resorcinol (Chapman and Ribbons 1976).

In contrast, the metabolic fate of chloromaleylacetates formed in the degradation of dichloroaromatics remained less clear. Generally it is agreed upon that 2-chloromaleylacetate, an intermediate of 2,4-dichlorophenoxyacetate (2,4-D) degradation, is reduced to 2-chloro-4-oxoadipate. This compound was originally proposed to be further converted by *Arthrobacter* sp. via chlorosuccinate which was thought to be first dehalogenated and then reduced to succinate (Fig. 2 A; Duxbury et al. 1970). Later Chapman (1979) suggested that chloride might be already eliminated from 2-chloro-4-oxoadipate, thus giving rise to maleylacetate which then would be converted to 3-oxoadipate in a second reductive reaction (Fig. 2B). Chapman did not give any experimental evidence for his suggestion, and it remained unclear, whether or not the two reductive reactions were catalyzed by the same enzyme and whether the dehalogenation was thought to be spontaneous or enzyme catalyzed. Recently the mechanism postulated by Chapman was substantiated by Kaschabek (1990) who showed that a preparation of partially purified maleylacetate reductase from *Pseudomonas* sp. B13 converted 2-chloromaleylacetate to 3-oxoadipate. In the course of this reaction 2 mol of NADH were consumed per mol of 2-chloromaleylacetate, and maleylacetate was detected as an intermediate.

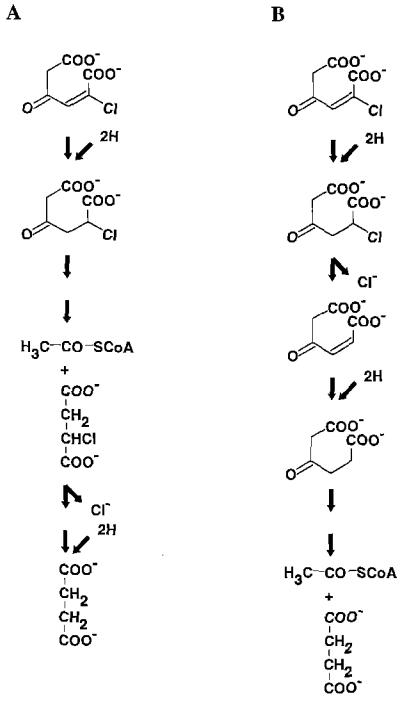

Fig. 2A, B. Illustration of the different mechanisms proposed for the further conversion and dehalogenation of the 2,4-D degradation intermediate 2-chloromaleylacetate. **A** Degradation via chlorosuccinate proposed for *Arthrobacter* sp. (Duxbury et al. 1970). **B** Degradation via chloride elimination from 2-chloro-4-oxoadipate to give maleylacetate as suggested by Chapman (1979)

In the course of the genetic analysis of the 2,4-D-degradative pathway in *Alcaligenes eutrophus* JMP134 (pJP4), Don et al. (1985) identified a gene *(tfdF)* which was necessary for growth with 2,4-D, but not for 3-chlorobenzoate utilization. The authors could not ascribe a metabolic function to this gene, and their hypothesis that TfdF might be a chlorodienelactone isomerase (Schwien et al. 1988) was recently refuted (Pieper et al. 1991). Van der Meer et al. (1991a, b) identified a gene *(tcbF)* homologous to *tfdF* on plasmid pP51. They observed that it is not involved in the degradative steps between 3,4-dichlorocatechol and 5-chloromaleylacetate, but also could not reveal its function. It therefore was to be investigated whether TfdF might play a role in the conversion of chloromaleylacetate.

In this paper we provide evidence that also in *A. eutrophus* JMP134 (pJP4) 2-chloromaleylacetate is reduced by maleylacetate reductase which is presumedly chromosomally encoded. The maleylacetate formed as a product of this reaction is further reduced with consumption of a second NADH. There was no indication that a pJP4-encoded enzyme (like TfdF) would be necessary for the observed chloride elimination. Some of the results were previously presented in a preliminary form (Schlömann et al. 1990a; Vollmer and Schlömann 1992).

Materials and methods

Organisms and culture conditions

Alcaligenes eutrophus JMP134 has been isolated on the basis of being able to grow with 2,4-D as sole source of carbon and energy (Pemberton et al. 1979). Strain JMP222 is a derivative of JMP134 cured of pJP4. The strains were grown in the mineral medium described by Dorn et al. (1974), the buffer concentration being increased twofold (Hartmann et al. 1979). Unless mentioned otherwise strain JMP134 was raised with 4 or 5 mM 2,4-D, JMP222 with 2 mM 4-fluorobenzoate. All cultures were grown aerobically at 30 °C, liquid cultures in baffled Erlenmeyer flasks on a rotary shaker. Stock cultures were maintained on agar plates with the appropriate carbon source (2 mM). To obtain biomass for large scale protein purification *A. eutrophus* JMP134 was grown in a 250 l fermenter using repeated additions of 5 mM 2,4-D and a mineral medium as described previously for *A. eutrophus* 335 (Schlömann et al. 1990c).

Preparation of cell-free extracts

Cells were harvested by centrifugation during exponential growth and, unless mentioned otherwise, they were resuspended in 50 mM Tris/H_2SO_4 pH 7.5. The cells were disrupted using an Aminco French press and the cell debris was removed by centrifugation at 100000 × *g* for at least 30 min at 4 °C. Cell extracts were used the same day. The biomass from the 250 l fermenter (556 g wet weight) was harvested by continuous flow centrifugation and was stored frozen until used. The extract was prepared from a 250 g portion, which was thawed and resuspended in 50 mM Tris/HCl pH 7.5 containing 2 mM $MnSO_4$ and 0.1 mM dithiothreitol. The extract was treated with desoxyribonuclease I and cleared by centrifugation at 100000 × *g* for 30 min at 4 °C and subsequent filtration.

Enzyme assays

Activities of chlorocatechol 1,2-dioxygenase (EC 1.13.11.1), dienelactone hydrolase (EC 3.1.1.45), and maleylacetate reductase (EC 1.3.1.32) were measured as described previously (Schlömann et al. 1990b) using 3,5-dichlorocatechol, trans-dienelactone (trans-4-carboxymethylenebut-2-en-4-olide), and maleylacetate plus NADH, respectively, as substrates. Dichloromuconate cycloisomerase (EC 5.5.1.7) was assayed by the method of Kuhm et al. (1990). One enzyme activity unit is defined to catalyze the conversion of 1 µmol of substrate per minute. Protein was measured by the Bradford procedure (Bradford 1976).

Protein chromatography

For analytical anion exchange chromatography 1 ml of the respective cell-free extract (5 mg of protein), prepared in 50 mM Tris/HCl pH 7 + 4 mM $MnSO_4$, was diluted tenfold in elution buffer and applied to a Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden). Protein was eluted by a NaCl gradient (0 to 0.3 M) in 50 mM Tris/HCl pH 7.5 at room temperature. A flow rate of 1 ml/min and a fraction volume of 0.5 ml were used.

For the large scale purification the cell-free extract of *A. eutrophus* JMP134 (400 ml containing 23.6 g of protein) was first cleaned and partially desalted on a Pharmacia Sephadex G25 Superfine column (BP 113/15, bed length 14.5 cm). The eluent was 20 mM Tris/HCl pH 7.5 containing 2 mM $MnSO_4$ + 0.1 mM dithiothreitol. The extract was then chromatographed an a Pharmacia Q Sepharose Fast Flow column (BP113/30, bed length 14.5 cm, volume 1.45 l) using the same buffer with a two step NaCl gradient from 0 to 0.5 M over 15 l and from 0.5 to 2 M over 0.5 l. The chromatography was performed at room temperature at a flow rate of 100 ml/min. Fractions (250 ml each) containing maleylacetate reductase were pooled and concentrated on an Amicon PM10 ultrafiltration membrane. Further purification steps included: firstly a gel filtration on a Pharmacia Sephacryl S-200 HR column (XK 26/100, bed length 85 cm) with 50 mM Tris/HCl pH 7.5 + 100 mM NaCl + 0.1 mM dithiothreitol as elution buffer, secondly an affinity chromatography on Pharmacia Red Sepharose CL-6B (C 16/20, bed length 11 cm) with 20 mM Tris/HCl pH 7.5 + 0.5 mM dithiothreitol and a NaCl gradient from 0 to 1.5 M for elution, and finally a second anion exchange chromatography on Mono Q HR 5/5 with a 0 to 0.5 M NaCl gradient in 50 mM Tris/HCl pH 7.5 + 0.5 mM dithiothreitol.

Analytical methods

The concentrations of 2-chloromaleylacetate and maleylacetate were determined by reversed-phase high pressure liquid chromatography (HPLC) with a Grom SIL 100 C8 column (250 mm length, 4.6 mm internal diameter; Grom, Herrenberg, FRG). The mobile phase was either an aqueous solution of 20% (v/v) methanol and 0.1% (w/v) H_3PO_4 or an aqueous solution of 3% (v/v) methanol with 0.8% (w/v) H_3PO_4 adjusted to pH 3.0 with 10 N NaOH. The flow rate was 1 ml/min, and detection was performed at 210 nm. Typical retention volumes in the former case were: Maleylacetic acid 4.59 ml, 2-chloromaleylacetic acid 5.95 ml, NAD^+ 3.91 ml, and NADH 3.67 ml. The other mobile phase resulted in the following typical retention volumes: Maleylacetic acid 4.34 ml, 2-chloromaleylacetic acid 4.75 ml, NAD^+ 5.50 ml, and NADH 6.70 ml.

NADH concentrations were measured photometrically at 340 nm in a Kontron Uvikon 810P spectrophotometer. Chloride ion concentrations were determined with an ion selective combination electrode (model 94-17B, Orion, Boston, USA) which was calibrated with NaCl (0.1–10 mM) in 50 mM Tris/H_2SO_4 pH 7.5 before each measurement.

Chemicals

Solutions containing 2-chloromaleylacetate were prepared from 3,5-dichlorocatechol using cell-free extracts of 2,4-D-grown cells of strain JMP134 in 50 mM Tris/H_2SO_4 pH 7.5 (in absence of NADH). After the turnover was finished, protein was removed by ultrafiltration through Amicon Centricon 10 microconcentrators. Maleylacetate and 2-chloromaleylacetate for the chloride-release experiment were obtained by alkaline hydrolysis with NaOH of cis-dienelactone and 2-chloro-cis-dienelactone, respectively. 2-Chloromaleylacetate formation was controlled by HPLC. Specifically it was confirmed that chemically and enzymatically produced 2-chloromaleylacetate were chromatographically identical. All solutions of maleylacetate und 2-chloromaleylacetate were used the same day. 3,5-Dichlorocatechol was available from a previous synthesis (Dorn and Knackmuss 1978). Cis-dienelactone was generously provided by G. Ashley (Evanston, USA) and 2-chloro-cis-dienelactone by S. Kaschabek and W. Reineke (Wuppertal, FRG). 3-Oxoadipic acid was purchased from Aldrich.

Results

Coelution of maleylacetate and 2-chloromaleylacetate reducing activities

As a first step in an enrichment of maleylacetate reductase a cell-free extract of 2,4-D-grown cells of *Alcaligenes eutrophus* JMP134 (pJP4) was submitted to an anion exchange chromatography (Fig. 3). Maleylacetate and 2-chloromaleylacetate reducing activities coeluted from the column, while the other enzymes tested (chlorocatechol 1,2-dioxygenase, dichloromuconate cycloisomerase, and dienelactone hydrolase) were clearly separated from the reductase activities. Even after further purification of maleylacetate reductase by gel filtration chromatography, Red Sepharose affinity chromatography, and Mono Q anion exchange chromatography, the enzyme preparation was still active with 2-chloromaleylacetate. This suggests that in *A. eutrophus* JMP134 2-chloromaleylacetate is reduced by maleylacetate re-

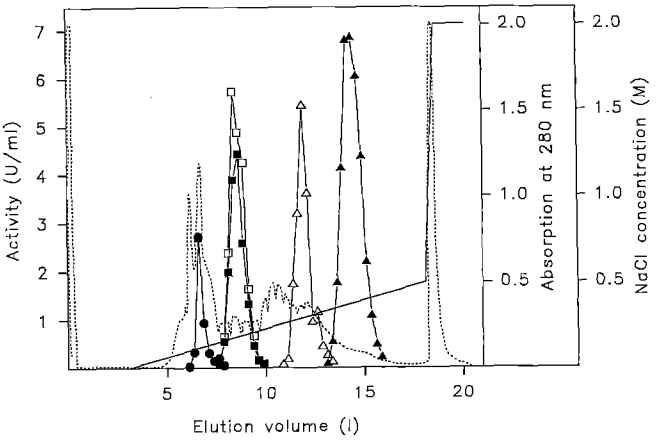

Fig. 3. Coelution of maleylacetate and 2-chloromaleylacetate reductase activities of *Alcaligenes eutrophus* JMP134 during anion exchange chromatography. An extract of 2,4-D-grown cells was applied to a Q Sepharose Fast Flow column and eluted with a NaCl gradient *(solid line)*. Fractions were assayed for the activity of maleylacetate reductase (□), 2-chloromaleylacetate reductase (■), chlorocatechol 1,2-dioxygenase (△), dichloromuconate cycloisomerase (▲), and dienelactone hydrolase (●; activity scaled down by a factor of 10 for this plot). The protein content of the eluate was monitored continuously as absorption at 280 nm *(dotted line)*

ductase. However, since homogeneity of the enzyme was not achieved, additional proof was required for the above inference.

Reciprocal inhibition of maleylacetate and 2-chloromaleylacetate turnover

If maleylacetate and 2-chloromaleylacetate are converted by the same enzyme, then there should be a competition for the binding site on the enzyme and they should inhibit each others turnover. Therefore reaction mixtures containing cell-free extract of *A. eutrophus* JMP134 and the two substrates in varying amounts were analyzed by HPLC (Fig. 4). In the absence of 2-chloromaleylacetate with maleylacetate as substrate the reaction proceeded fast (Fig. 4A). With 2-chloromaleylacetate as sole substrate the velocity of the turnover in the beginning was almost as fast as the reaction with maleylacetate (Fig. 4B). During the reaction, however, maleylacetate accumulated as intermediate and the turnover of 2-chloromaleylacetate slowed down. When 2-chloromaleylacetate and maleylacetate were present at the same time, both were converted slower than when supplied as single substrate (Fig. 4C and D). A higher ratio of maleylacetate to 2-chloromaleylacetate resulted in an even slower turnover of 2-chloromaleylacetate (Fig. 4D).

Analytical anion exchange chromatography

It had previously been shown that *A. eutrophus* JMP222, a derivative of strain JMP134 cured of plasmid pJP4, induces a maleylacetate reductase activity during growth with 4-fluorobenzoate (Schlömann et al. 1990b). When extracts of 4-fluorobenzoate-grown cells of *A. eutrophus* JMP222 and of 4-fluorobenzoate- or 2,4-D-grown cells of JMP134 were analyzed by anion exchange chromatography under the same conditions, maleylacetate reductase activities in all three cases occurred at the same elution volume (Fig. 5), indicating that probably the same enzyme was expressed in these extracts.

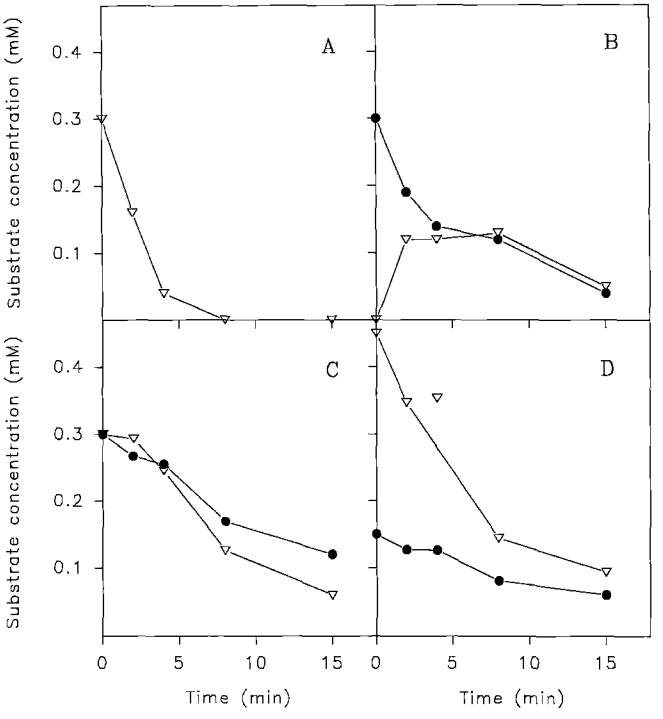

Fig. 4A–D. Reciprocal inhibition of 2-chloromaleylacetate (●) and maleylacetate (▽) turnover by maleylacetate reductase from *Alcaligenes eutrophus* JMP134. **A** and **B** show the turnover of maleylacetate and 2-chloromaleylacetate, respectively, supplied as single substrates in a concentration of 0.3 mM. In **C** the course of the reaction is depicted for the simultaneous presence of 0.3 mM maleylacetate and 0.3 mM 2-chloromaleylacetate. In **D** the initial concentrations were 0.15 mM 2-chloromaleylacetate and 0.45 mM maleylacetate. In addition to the substrates the reaction mixtures (1 ml) contained: 50 mM Tris/H_2SO_4 pH 7.5, 0.9 mM NADH, and cell-free extract corresponding to a maleylacetate reductase activity of 0.01 U. To allow for time for HPLC analyses and to avoid possible artifacts resulting from storage of samples, for each time point a separate reaction was run for the respective period and analyzed immediately

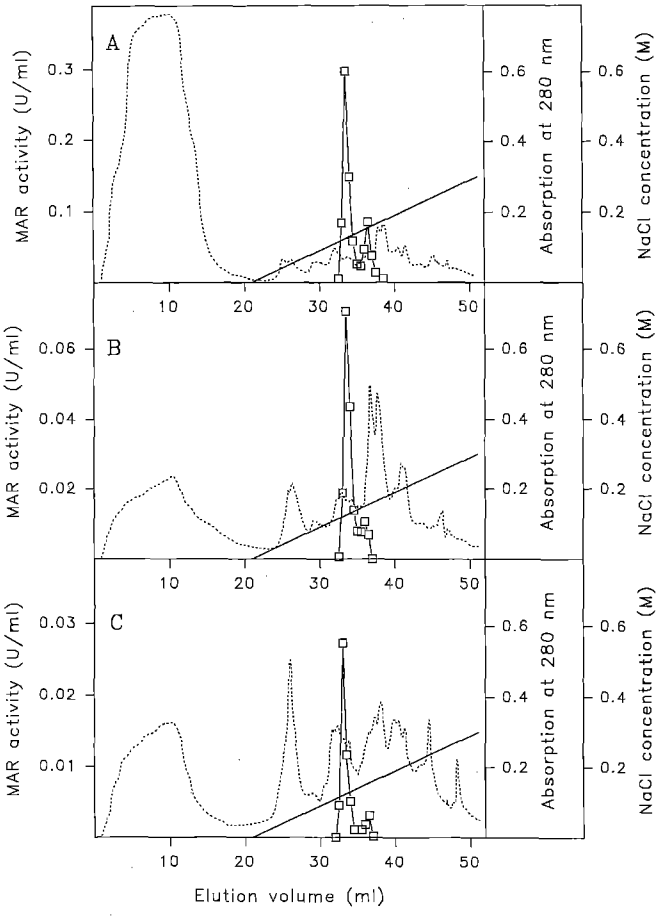

Fig. 5A–C. Analytical anion exchange chromatography of maleylacetate reductase (MAR) activities (□) from 4-fluorobenzoate-grown cells of *Alcaligenes eutrophus* JMP222 **(A)**, 4-fluorobenzoate-grown cells of *A. eutrophus* JMP134 **(B)**, and from 2,4-D-grown cells of *A. eutrophus* JMP134 **(C)**. The proteins were eluted by a NaCl gradient *(solid line)*. The protein content of the eluate was monitored continuously as absorption at 280 nm *(dotted line)*

Conversion of 2-chloromaleylacetate in the presence and absence of pJP4-encoded enzymes

The occurrence of maleylacetate reductase in 4-fluorobenzoate-grown cells of *A. eutrophus* JMP222 offered an easy way to investigate the conversion of 2-chloromaleylacetate in the presence and absence of pJP4-encoded enzymes by using extracts of 2,4-D-grown cells of strain JMP134 on one hand and of 4-fluorobenzoate-grown cells of strain JMP222 on the other. If TfdF or another pJP4-encoded enzyme would be involved in the dechlorination of 2-chloro-4-oxoadipate, the turnover of 2-chloromaleylacetate by JMP222 extracts should cease at the level of 2-chloro-4-oxoadipate. An indication for this would be a reduced consumption of NADH with 2-chloromaleylacetate as substrate, and in addition chloride should not be released.

Figure 6 shows that with 2-chloromaleylacetate as substrate cell-free extracts of both strains, JMP134 (Fig. 6A) as well as JMP222 (Fig. 6B), oxidized two molecules of NADH per molecule of substrate in a fast reaction. If maleylacetate was used as substrate only one NADH was consumed in both cases. Overnight an additional NADH was oxidized with maleylacetate as well as with 2-chloromaleylacetate. This seems to be due to the reduction of the resulting 3-oxoadipate, since this compound, when added as the only substrate, also caused a slow NADH oxidation. The oxidation of the latter NADH could be prevented, if coenzyme A and succinyl CoA were supplied as cosubstrates (data not shown).

Chloride was completely released in the presence as well as in the absence of pJP4-encoded enzymes (Fig. 7), and there was no evidence for a faster chloride release with JMP134 as compared to JMP222 extracts. HPLC analyses of 2-chloromaleylacetate conversion revealed the temporary occurrence of maleylacetate. With extracts from both strains this compound could be detected within the first minutes after start of the reaction (Fig. 8). Maleylacetate disappeared simultaneously with the oxidation of the second NADH.

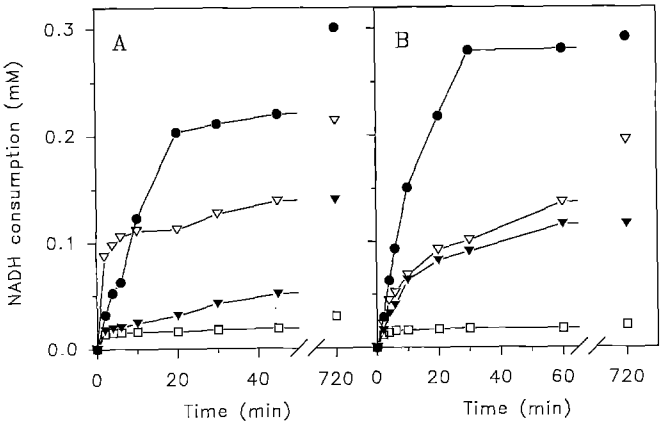

Fig. 6A, B. NADH consumption during turnover of 2-chloromaleylacetate (●), maleylacetate (▽), 3-oxoadipate (▼), and without substrate (□), with cell-free extracts of *Alcaligenes eutrophus* JMP134 **(A)** and *A. eutrophus* JMP222 **(B)**. The incubation mixtures (1 ml) contained 0.1 mM substrate, 0.4 mM NADH, 50 mM Tris/H_2SO_4 pH 7.5, and the respective extract corresponding to a maleylacetate reductase activity of 0.0026 U

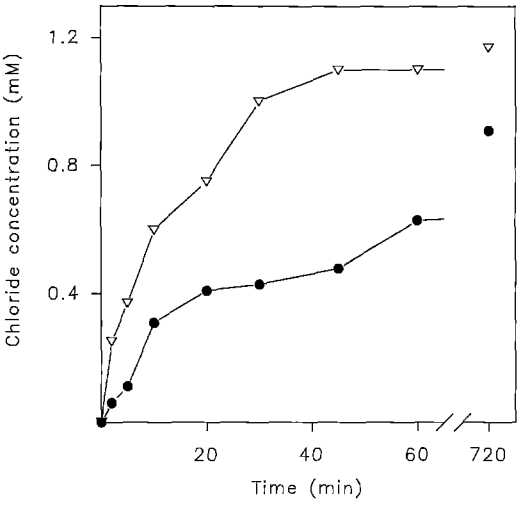

Fig. 7. Chloride release during turnover of 2-chloromaleylacetate by cell-free extracts of *Alcaligenes eutrophus* JMP134 (●) and *A. eutrophus* JMP222 (▽). The concentration of free chloride was determined with a chloride sensitive electrode. The incubation mixture (3 ml) contained 1 mM 2-chloromaleylacetate, 4 mM NADH, 50 mM Tris/H_2SO_4 pH 7.5, and the respective extract corresponding to a maleylacetate reductase activity of 0.015 U

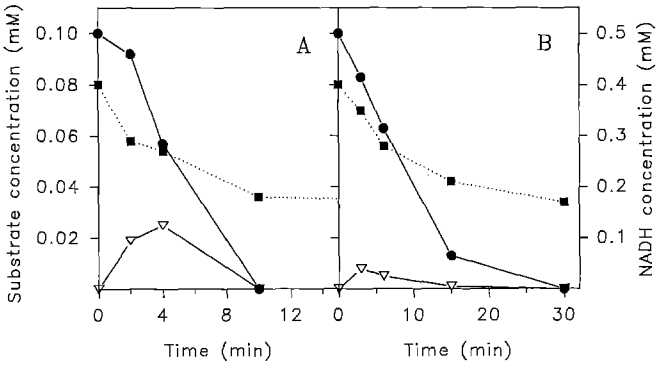

Fig. 8A, B. Turnover of 2-chloromaleylacetate (●) with cell-free extracts of *Alcaligenes eutrophus* JMP134 **(A)** and *A. eutrophus* JMP222 **(B)**. Maleylacetate (▽) was formed as an intermediate of the reaction. The dotted lines show the consumption of NADH (■) during the turnover. The reaction mixtures (1 ml) contained 0.1 mM 2-chloromaleylacetate, 0.4 mM NADH, 50 mM Tris/H_2SO_4 pH 7.5, and the respective extract corresponding to a maleylacetate reductase activity of 0.075 U. Concentrations were determined by HPLC, those of NADH in addition by photometric measurements

Discussion

2,4-Dichlorophenoxyacetate has long been known to be degraded by bacteria via 2-chloromaleylacetate as an intermediate (Tiedje et al. 1969; Evans et al. 1971b). This apparently is also true for *Alcaligenes eutrophus* JMP134, since conversion of 3,5-dichlorocatechol by cell-free extracts of 2,4-D-grown cells of this strain yields the same product as hydrolysis of authentic 2-chlorodienelactone (see Materials and methods).

The different models offered for the further metabolism of 2-chloromaleylacetate agree in proposing a reduction as the next step, formally yielding 2-chloro-4-

oxoadipate as the product (Fig. 2; Duxbury et al. 1970; Chapman 1979). Until now, however, the enzymology of this reaction has not yet been adequately addressed. *A. eutrophus* JMP134 has been shown to induce maleylacetate reductase during growth with 2,4-D, while the enzyme is absent from fructose- or succinate-grown cells (Pieper et al. 1988; Schlömann et al. 1990b). In the presence of 2,4-D maleylacetate reductase is also induced in *Pseudomonas* sp. PKO1 harboring the pJP4-derivative pRO103 (Kukor et al. 1989). These observations suggest that maleylacetate reductase plays a role in 2,4-D degradation. But since for example chlorocatechols and chloromuconates are converted by different enzymes than catechol and muconate, the question has to be raised whether 2-chloromaleylacetate is converted by maleylacetate reductase or by a special 2-chloromaleylacetate reducing enzyme.

Two results presented in this manuscript suggest that the first possibility is indeed correct. Maleylacetate and 2-chloromaleylacetate reducing activities coeluted during anion exchange chromatography (Fig. 3) and were also not separated during subsequent purification steps of maleylacetate reductase. In addition it was shown that maleylacetate inhibits 2-chloromaleylacetate turnover and vice versa, implying that both compounds bind to the same enzyme. Thus *A. eutrophus* JMP134 apparently does not need a special enzyme for 2-chloromaleylacetate reduction, since the compound is reduced by the normal maleylacetate reductase. Considering that 2-chloro-4-oxoadipate, the immediate product of 2-chloromaleylacetate reduction, gives rise to maleylacetate (see below; Fig. 2B), maleylacetate reductase in 2,4-D degradation seems to have a dual function, 2-chloromaleylacetate reduction as well as maleylacetate reduction. Essentially the same conclusion was drawn for the catabolism of 3,5-dichlorocatechol by *Pseudomonas* sp. B13 (Kaschabek 1990; Kaschabek and Reineke 1992). These authors showed that partially purified maleylacetate reductase of strain B13 in addition to maleylacetate also converted 2-chloro- and 2-methylmaleylacetate.

Because of their failure to identify a maleylacetate reductase defective mutant during Tn5 mutagenesis of *A. eutrophus* JMP134, Don et al. (1985) assumed that this enzyme might be specified by a chromosomal gene. This corresponds to the chromosomal location of a maleylacetate reductase gene in the nonfluorescent *Pseudomonas* sp. strain PKO1 (Kukor et al. 1989). Our observation that *A. eutrophus* JMP134 and its cured derivative strain JMP222 express maleylacetate reductase activities with the same elution volume (Fig. 5) confirms the conclusion of Don et al. (1985) that this enzyme should be encoded on the chromosome (or on a megaplasmid identified in both strains by Friedrich et al. (1983)).

As mentioned above, for the further metabolism of the product arising from 2-chloromaleylacetate reduction two models have been proposed (Fig. 2). One assumes a degradation via chlorosuccinate (Fig. 2A) and has been suggested for 2,4-D metabolism by *Arthrobacter* sp. (Duxbury et al. 1970) and more recently for 3,5-dichlorosalicylate degradation by *Pseudomonas* sp. strain JWS (Schindowski et al. 1991). According to the other model, put forth by Chapman (1979), chloride is eliminated from 2-chloro-4-oxoadipate (Fig. 2B). This mechanism was experimentally supported by the investigation of 2-chloromaleylacetate reduction in *Pseudomonas* sp. B13 (Kaschabek 1990; Kaschabek and Reineke 1992). It also seems to play a role in the conversion of 2,5-dichloromaleylacetate in the 1,2,4-trichlorobenzene degrading strain *Pseudomonas* sp. PS12 (Sander et al. 1991). The results of our experiments on 2-chloromaleylacetate metabolism were consistent with the model proposed by Chapman (1979) (Fig. 2B). Especially the occurrence of maleylacetate as a metabolite clearly conflicts with the alternative mechanism (Fig. 2A). In addition to the oxidation of 2 mol of NADH per mol of 2-chloromaleylacetate, as predicted by Chapman's model, a third mol of NADH was oxidized in a considerably slower reaction (Fig. 6). This is probably due to a reduction of 3-oxoadipate to 3-hydroxyadipate, as indicated by the slow oxidation of one mol of NADH per mol of 3-oxoadipate. Since in the presence of succinyl-CoA and CoA only 2 mol of NADH were oxidized per mol of 2-chloromaleylacetate, the slow reaction can be considered to be an unphysiological artifact. The overlap of several reductive reactions makes quantitation difficult and may explain different ratios of NADH oxidized mentioned in a preliminary report (Schlömann et al. 1990a).

The immediate appearance of maleylacetate and chloride in the course of 2-chloromaleylacetate reduction indicates that they are not artifacts of a slow decomposition of the primary product 2-chloro-4-oxoadipate. It rather can be concluded that the chloride elimination is either a relatively fast spontaneous, non-enzymatic reaction or a reaction gratuitously catalyzed by maleylacetate reductase (assuming that JMP222 does not have a special 2-chloro-4-oxoadipate dechlorinating enzyme).

It should be noted that with respect to 2-chloromaleylacetate metabolism there was no significant difference between JMP134 and JMP222. Thus pJP4-encoded enzymes are apparently not involved in these reactions, and the function of TfdF in 2,4-D degradation is still to be elucidated.

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