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Maleylacetate Reductases in Chloroaromatic-Degrading Bacteria Using the Modified *ortho* Pathway: Comparison of Catalytic Properties

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The maleylacetate reductases from *Pseudomonas aeruginosa* RHO1 and *Alcaligenes eutrophus* JMP134 were tested for activity and affinity to various maleylacetates as well as dechlorinating properties. The dechlorinating activity and the k_{cat}/K_m values revealed high-level similarity of these reductases to that of *Pseudomonas* sp. strain B13.

Various chloroaromatics are degraded by bacteria via chlorocatechols as central intermediates. After several steps in the following modified *ortho* cleavage pathway, (chloro)maleylacetate is formed. The degradation of 2,4,6-trichloro- and pentachlorophenol as well as 2,4,5-trichlorophenoxyacetate, which involves (chlorinated) 1,2,4-trihydroxybenzene as the central intermediate, also gives rise to (chloro)maleylacetate.

In general, (chloro)maleylacetate is further converted to (chlorinated) 3-oxoadipate by a maleylacetate reductase (Fig. 1). In the present paper we compare maleylacetate reductases of bacteria, enriched with different chloroaromatic compounds, with respect to their substrate specificities.

Maleylacetate reductase was purified to homogeneity from 1,4-dichlorobenzene-grown cells of *Pseudomonas aeruginosa* RHO1, according to the method of Kaschabek and Reineke (5). During the purification the specific activity of the enzyme increased to 170 U/mg of protein, indicating a 74-fold purification with 13% recovery of activity. The M_r of the native protein was determined by gel filtration on Superose 6 to be approximately 74,000. M_r estimation of the subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis yielded a single band with a value of 37,000. The isoelectric point of the purified enzyme was determined by the method of O'Farrell to be 7.0. The optimum activity was determined to be at pH 5.8 in 50 mM histidine-HCl buffer. Activity was drastically reduced at pH values below 5.8. Examination of the temperature influence showed that in 50 mM Tris-HCl buffer, pH 7.0, the highest level of enzyme activity could be observed at 45°C. Furthermore, inhibitory experiments with chelating agents, cyanide ions, and *p*-chloromercuribenzoate showed that the maleylacetate reductase of strain RHO1 strongly resembles that of *Pseudomonas* sp. strain B13 (5).

In general, the enzymes of strain RHO1 and *Alcaligenes eutrophus* JMP134 showed broad substrate specificities (Table 1). Both reductases required 2 mol of NADH per molequivalent of 2-halogenated maleylacetates for the complete turnover of these substrates. In contrast, only 1 mol of NADH was consumed with substrates bearing no halogen substituent in position 2. The reduction of the 2-halogenated maleylacetates resulted in the elimination of 1 molequivalent of the corresponding halide, while halomaleylacetates missing a halogen substituent in position 2 showed no elimination of halide. These properties are shared with the enzyme of strain B13 (6). Overall, both enzymes revealed low-level activities with methylmaleylacetates. 3-Bromomaleylacetate was found to be the best substrate for maleylacetate reductases.

The enzymology of the modified *ortho* pathway for chlorocatechol degradation has been studied for several chloroaromatic-degrading strains. Mostly, the enzymes in these organisms were specialized for the conversion of their enrichment substrate. For example, enzymes such as chlorocatechol 1,2-dioxygenase, evolved for the turnover of monosubstituted substrates, use the monosubstituted compounds more rapidly than the dichlorinated ones, while the enzymes of dichloroaromatic degradation prefer dichlorinated substrates, especially the isomer formed in the degradation pathway (2–4, 9, 10, 12). Interestingly, no specialization has been found for the investigated maleylacetate reductases, although the strains have been enriched from quite different sources with different chloroaromatics: strain B13 was enriched with 3-chlorobenzoate from

TABLE 1. Kinetic data for the conversion of various maleylacetates with purified maleylacetate reductase from *P. aeruginosa* RHO1 and *A. eutrophus* JMP134[a]

Substrate	*P. aeruginosa* RHO1		*A. eutrophus* JMP134	
	K_m (μM)	k_{cat}[a] (min^{-1})	K_m (μM)	k_{cat}[a] (min^{-1})
Maleylacetate	65	33,700	50	8,120
5-Fluoro-maleylacetate	22	3,500	14	1,230
2-Chloro-maleylacetate	78	27,300	52	6,130
3-Chloro-maleylacetate	29	24,000	30	5,600
5-Chloro-maleylacetate	50	700	44	700
2,3-Dichloro-maleylacetate	170	11,400	ND	ND
2,5-Dichloro-maleylacetate	288	1,000	ND	ND
2-Bromo-maleylacetate	117	24,800	98	9,420
3-Bromo-maleylacetate	71	34,800	63	15,020
5-Bromo-maleylacetate	95	15,900	84	6,300
2-Methyl-maleylacetate	374	2,000	330	3,260
3-Methyl-maleylacetate	365	3,900	292	670
5-Methyl-maleylacetate	125	3,500	133	2,450

[a] k_{cat} values were calculated on the basis of 37,000 and 35,000 for the subunits for strains RHO1 and JMP134, respectively. Kinetic measurements were carried out as described previously (6). The K_m values for maleylacetate and 2-chloromaleylacetate correspond to those determined previously with the maleylacetate reductase of strain JMP134 (11). ND, not determined.

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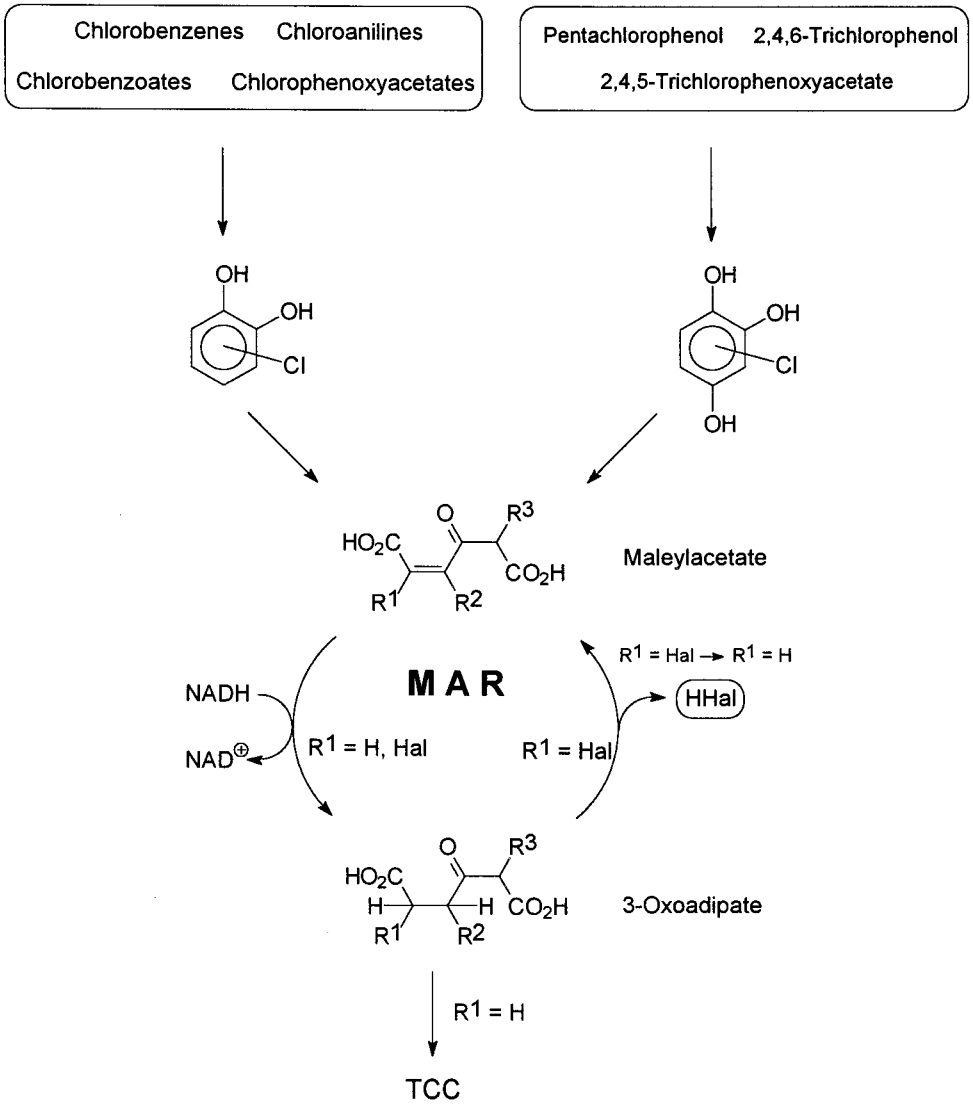

FIG. 1. Role of the enzyme maleylacetate reductase in bacterial degradation of chloroaromatics. R^1, R^2, and R^3 are hydrogen or halogen. In the case of 2-halogenated maleylacetates (R^1 = halogen), the cycle takes place twice. First, the substrate is reduced to the respective halogenated 3-oxoadipate, which is converted back into maleylacetate without a substituent in position 2. This step is accompanied by the elimination of halide. During the second passage, the reduction of maleylacetate takes place with the consumption of another mole of NADH to yield the respective 3-oxoadipate. Abbreviations: MAR, maleylacetate reductase; TCC, tricarboxylic acid cycle.

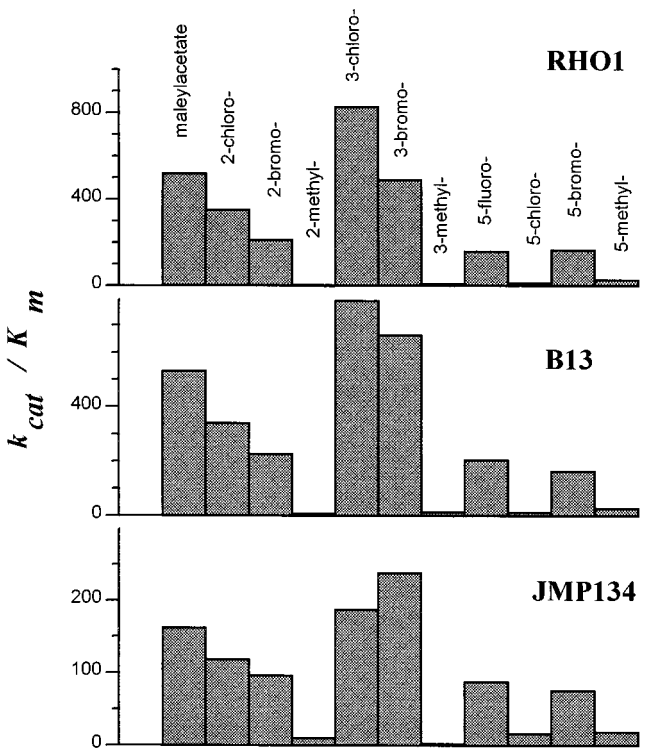

FIG. 2. Comparison of k_{cat}/K_m values for the maleylacetate reductases from *P. aeruginosa* RHO1, *Pseudomonas* sp. strain B13, and *A. eutrophus* JMP134. The data for the enzyme of strain B13 were obtained from reference 6.

wastewater in Göttingen, Germany (1); strain RHO1 was enriched with 1,4-dichlorobenzene from sediment of the Rhine River in Leverkusen, Germany (7); and strain JMP134 was enriched with 2,4-dichlorophenoxyacetate from soil in Australia (8). The kinetic measurements with maleylacetate and various substituted maleylacetates revealed similar substrate affinities and activities. Similarities were also observed with respect to the dehalogenating properties. A comparison of k_{cat}/K_m values (Fig. 2) clearly indicates that the maleylacetate reductases of the strains B13 and RHO1 are highly similar in their specificities. Overall, the values obtained with the enzyme of strain JMP134 are lower than those of the other ones.

In general, the high level of similarity among the three maleylacetate reductases illustrates that the different maleylacetates formed during the degradation of the different chloroaromatics did not reflect a significantly different selection pressure.

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