JOURNAL OF BACTERIOLOGY, Jan. 1995, p. 320–325 Vol. 177, No. 2
0021-9193/95/$04.00+0

Maleylacetate Reductase of *Pseudomonas* sp. Strain B13: Specificity of Substrate Conversion and Halide Elimination

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Received 9 September 1994/Accepted 4 November 1994

Maleylacetate reductase (EC 1.3.1.32) plays a major role in the degradation of chloroaromatic compounds by channelling maleylacetate and some chlorinated derivatives into the 3-oxoadipate pathway. Several substituted maleylacetates were prepared in situ by alkaline or enzymatic hydrolysis of dienelactones as the precursor. The conversion of these methyl-, chloro-, fluoro-, and bromo-substituted maleylacetates by maleylacetate reductase from 3-chlorobenzoate-grown cells of *Pseudomonas* sp. strain B13 was studied. Two moles of NADH per mole of substrate was consumed for the conversion of maleylacetates which contain a halogen substituent in the 2 position. In contrast, only 1 mol of NADH was necessary to convert 1 mol of substrates without a halogen substituent in the 2 position. The conversion of 2-fluoro-, 2-chloro-, 2,3-dichloro-, 2,5-dichloro-, 2,3,5-trichloro-, 2-bromo-, 2,3-dibromo-, 2,5-dibromo-, 2-bromo-5-chloro-, 2-chloro-3-methyl-, and 2-chloro-5-methylmaleylacetate was accompanied by the elimination of halide from the 2 position and the temporary occurrence of the corresponding dehalogenated maleylacetate as an intermediate consuming the second mole equivalent of NADH. The properties of the halogen substituents influenced the affinity to the enzyme in the following manner. K_m values increased with increasing van der Waals radii and with decreasing electronegativity of the halogen substituents (i.e., low steric hindrance and high electronegativity positively influenced the binding). The K_m values obtained with 2-methyl-, 3-methyl-, and 5-methylmaleylacetate showed that a methyl substituent negatively affected the affinity in the following order: 2 position ≥ 3 position >> 5 position. A reaction mechanism explaining the exclusive elimination of halogen substituents from the 2 position is proposed.

Widespread use of chlorinated organic compounds presents persistent problems in the environment, particularly because many of these are resistant to biological degradation. A number of these are chlorinated aromatics. Aerobic bacteria use some chloroaromatics as sources of carbon and energy, whereby chlorinated catechols are the central metabolites in the degradation. The chlorocatechol degradation pathway starts with *ortho* ring cleavage caused by catechol 1,2-dioxygenase giving rise to chloro-*cis*,*cis*-muconates (6). The cycloisomerization of the ring cleavage products by chloromuconate cycloisomerase is coupled to chloride elimination leading to (chloro)dienelactones (13). (Chloro)maleylacetates, the compounds resulting from the hydrolysis of the dienelactones by dienelactone hydrolase (13), are further converted to (chloro)-3-oxoadipates by maleylacetate reductase. Thereby, the elimination of chlorine is observed from 2-chloromaleylacetate (8, 17). Maleylacetate reductases have been purified from *Pseudomonas* sp. strain B13 (9), a 3-chlorobenzoate-degrading strain (5), *Alcaligenes eutrophus* JMP134 (15), a 2,4-dichlorophenoxyacetic acid-degrading strain (12), and the yeast *Trichosporon cutaneum* (7) grown with resorcinol.

In this article we characterize the substrate specificity of the enzyme from strain B13 with special emphasis on the influence of type and position of substituents (i.e., fluoro, chloro, or bromo) on the elimination of halogen substituents.

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MATERIALS AND METHODS

Strain, culture conditions, and preparation of cell extracts. The media and methods for cultivation of *Pseudomonas* sp. strain B13 as well as the preparation of cell extracts have been described previously (5, 9).

Purification of maleylacetate reductase from strain B13. The maleylacetate reductase of *Pseudomonas* sp. strain B13 was purified by chromatography on DEAE-cellulose, Butyl-Sepharose, Blue-Sepharose, and Sephacryl S100 as described previously (9). The purity of the enzyme was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Enzyme assay. Maleylacetate reductase was measured spectrophotometrically by the decrease of the cofactor NADH at 340 nm (9).

Analysis of kinetic data. K_m and V_{max} values were calculated by linear regression to the Michaelis-Menten equation, by using the transformation of Hanes, in a substrate concentration range from 0.3 to 2.0 of the respective K_m value. A concentration of 0.4 mM NADH was used as the cofactor. k_{cat} values were calculated on the basis of the M_r value of 37,000 for the subunits.

Estimation of protein concentrations. Protein concentrations were determined by the method of Bradford (2).

Analytical methods. High-performance liquid chromatography (HPLC) of substrates and metabolites was conducted as described previously (8). Halide ions were detected on an ion chromatograph (Qic Analyser; Dionex, Sunnyvale, Calif.) on an Ionpac AS4A column with a 1.8 mM Na_2CO_3–1.7 mM $NaHCO_3$ eluent.

Chemical synthesis of dienelactones and maleylacetates. A general outline for the different strategies of preparing dienelactones and maleylacetates is given in Fig. 1 and 2.

(i) 5-Oxo-2(5*H*)-furanylideneacetic acids (dienelactones). The Wittig reaction between stabilized phosphoranes of the type $(Ph)_3P{=}CRCO_2{}^tBu$ and mono- or disubstituted maleic anhydrides was used as an efficient method for the synthesis of dienelactones (11). Although a broad spectrum of different substituted dienelactones was obtained, 3-chloro- and 3-bromodienelactone were not available by this method. These derivatives were yielded either by acidification of dinatrium *cis*,*cis*-3,4-dichloromuconate or by peracetic acid oxidation of the corresponding 4,5-dihalocatechol (10). Dienelactone and 22 substituted derivatives are available now.

(ii) *cis*-Hex-2-ene-4-oxo-1,6-dicarboxylic acids (maleylacetates). The corresponding maleylacetates were prepared in situ by alkaline or enzymatic hydrolysis.

With respect to the different stabilities of the maleylacetates, the following variations in the preparation procedure were necessary (Table 1). One milliliter

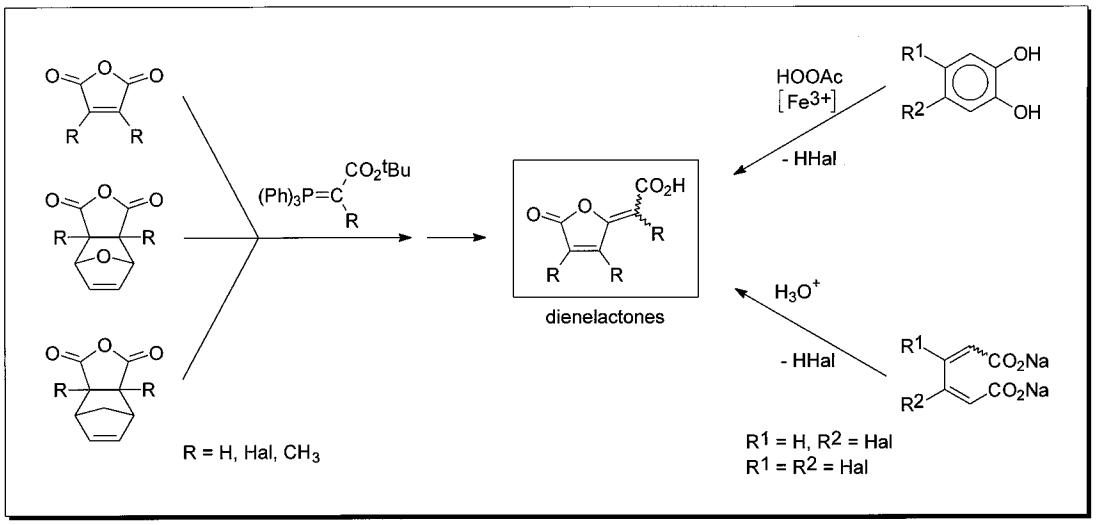

FIG. 1. Strategies for the synthesis of dienelactones (10, 11).

of a freshly prepared dienelactone solution (5 mM) was rapidly mixed with the recommended amount of 2 N NaOH. After the first incubation most of the resulting maleylacetates were ready for enzyme assays. To stabilize 5-chlorinated and 5-brominated maleylacetates, acidification with H_3PO_4 (85%) was necessary. After a second incubation period these derivatives showed a constant concentration for 2 to 4 h. Alternatively, maleylacetates can be prepared by an enzyme assay which contains (in 985 μl) 50 μmol of Tris-HCl buffer (pH 7.5) and 0.05 to 0.2 μmol of dienelactone. To this assay, 5 to 10 μl (approximately 0.5 to 1 μg) of the *A. eutrophus* JMP134 dienelactone hydrolase was added. The complete conversion of dienelactone was followed up by the decrease of A_{300}. The activity of the maleylacetate reductase was then measured by adding 0.40 μmol of NADH and 10 μl of the *Pseudomonas* sp. strain B13 pure reductase. Because of the extreme instability of 2,3,5-trichloro- and 2,3-dimethyl-5-chlorodienelactone, no pure standard could be obtained for any maleylacetate derivative.

Chemicals. All other chemicals were purchased commercially and were of the highest available purity.

RESULTS

In situ preparation of maleylacetates. Maleylacetates are the keto form in a pH-dependent equilibrium of 3-hydroxy-2,4-hexadienedioic acids (Fig. 2). Because of being highly unstable, none of these tautomeric compounds has been isolated until now, and therefore they have to be prepared in situ. Dienelactones are stable precursor compounds since they contain the *cis* configuration of the carbon-carbon double bond which is necessary for a substrate of maleylacetate reductase. Two different procedures are available for the cleavage of the ester in dienelactones: (i) chemical hydrolysis under alkaline conditions and (ii) enzymatic conversion using dienelactone hydrolases.

Both procedures lead to identical products, as has been shown by detecting the UV spectra. Besides the decrease in the

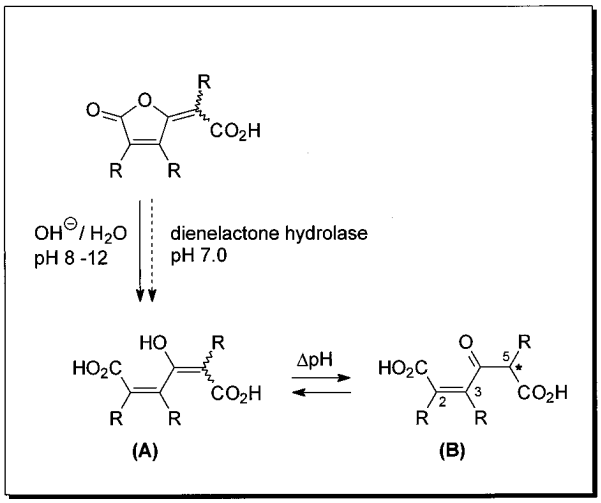

FIG. 2. Preparation of maleylacetates from dienelactones. (A) Enol form; (B) keto form.

TABLE 1. In situ preparation of maleylacetates by alkaline hydrolysis

Dienelactone[a]	Incubation with 2 N NaOH (μl [min])	Incubation (min) with 6 μl of H_3PO_4 (85%)	Stability (h)[b]
Dienelactone[c]	7.5 [15]		8
2-Fluoro-[c]	7.5 [0.5]		1/60
3-Fluoro-[c,d]			1/60
5-Fluoro-[c]	6.5 [15]		8
2-Chloro-[c]	6.5 [15]		8
3-Chloro-[c]	7.5 [0.5]		1/60
5-Chloro-[c]	7.5 [3]	15	4
2,3-Dichloro-[c]	7.5 [5]		1
2,5-Dichloro-[c]	7.5 [0.5]	15	4
2-Bromo-[c]	7.5 [0.5]		1/60
3-Bromo-[c]	7.5 [0.5]		1/60
5-Bromo-[c,d]			1/60
2,3-Dibromo-[c]	6.5 [0]		1/120
2,5-Dibromo-[c]	5 [2]	180	2
2-Bromo-5-chloro-[c]	7.5 [0.5]	15	4
2-Methyl-[c]	7.5 [15]		8
3-Methyl-[c]	7.5 [15]		8
5-Methyl-	15 [30]		8
2,3-Dimethyl-	15 [30]		8
2,3,5-Trimethyl-	15 [30]		8
2-Chloro-5-methyl-	11.5 [1]		1/60
2-Chloro-3-methyl-	11.5 [1]		1/60
2-Methyl-3-chloro-	11.5 [1]		1/60

[a] One milliliter (5 mM).
[b] Period during which the solution is suitable for the reductase assay.
[c] Can be converted at a sufficient rate with the dienelactone hydrolase of strain JMP134.
[d] Converted by dienelactone hydrolase.

TABLE 2. Conversion of maleylacetates with purified maleylacetate reductase from *Pseudomonas* sp. strain B13[a]

Maleylacetate	NADH consumption[b]	Halide formation[b]	Intermediate formation[c]	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($1/\mu M \cdot min$)	Relative V_{max} (%)
Maleylacetate	1.03			58	30,800	531	100
2-Fluoro-	2.00	0.94	Maleylacetate	ND[d]	ND	ND	ND
3-Fluoro-	0.98	<0.05		4.3	1,220	284	4
5-Fluoro-	1.03	<0.05		20	4,070	204	13
2-Chloro-	1.93	0.98	Maleylacetate	76	25,950	341	84
3-Chloro-	0.99	<0.05		28	22,090	789	72
5-Chloro-	1.02	<0.05		50	630	12.6	2
2,3-Dichloro-	1.83	0.94	3-Chloro-	190	19,350	102	63
2,5-Dichloro-	1.96	0.93	5-Chloro-	279	930	3.3	3
2,3,5-Trichloro-	1.40	ND	ND	ND	ND	ND	ND
2-Bromo-	1.92	0.99	Maleylacetate	113	25,680	227	83
3-Bromo-	0.91	0.08		65	43,200	665	140
5-Bromo-	1.05	0.12		90	14,800	164	48
2,3-Dibromo-	1.93	1.29	ND	347	5,880	16.9	19
2,5-Dibromo-	1.96	1.22	5-Bromo-	ND	ND	ND	ND
2-Methyl-	1.02			308	2,150	7.0	7
3-Methyl-	1.04			272	3,770	13.9	12
5-Methyl-	0.96			110	3,070	27.9	10
2-Chloro-3-methyl-	1.93	0.92	3-Methyl-	>500	<3,700		<12
2-Chloro-5-methyl-	1.73	0.79	5-Methyl-	248	3,920	15.8	13
2-Methyl-3-chloro-	0.97	<0.05		>500	<3,700		<12
2-Bromo-5-chloro-	1.96	0.99[e]	5-Chloro-	519	1,180	2.3	4

[a] No reaction was observed for 2,3-dimethyl-, 2,3,5-trimethyl-, or 2,3-dimethyl-5-chloromaleylacetate.
[b] Mole equivalents per mole of substrate.
[c] Maleylacetate detected during enzymatic turnover.
[d] ND, not determined.
[e] Bromide.

substrate concentration, which was shown by the decrease of the $A_{280-320}$, an increase of the extinction in the range of 250 to 260 nm was observed. This absorption corresponds to that for the enol form (form A in Fig. 2), which is thermodynamically favored under physiological conditions. In the HPLC analysis using slightly acid solvents only $A_{<210}$ occurred, indicating the presence of the keto form (form B in Fig. 2).

Since the maleylacetates prepared in situ and some of the precursors, the dienelactones, showed a very diverse stability in aqueous solution, various modifications of the general procedure had to be established.

Substrates such as maleylacetate, 5-fluoro-, 2-chloro-, 2-methyl-, 3-methyl-, 5-methyl-, 2,3-dimethyl-, and 2,3,5-trimethylmaleylacetate were stable in solution without a decrease in the concentration over a period of 8 h. They were synthesized by adding NaOH to an aqueous solution of the corresponding dienelactone. In contrast, the concentrations of substrates such as 3-chloro-, 2-bromo-, 3-bromo-, 2,3-dibromo-, 2-methyl-3-chloro-, 2-chloro-3-methyl-, and 2-chloro-5-methylmaleylacetate changed drastically within minutes so that a preparation of the corresponding maleylacetate was necessary for each enzyme assay.

Maleylacetates bearing chlorine or bromine substituents in the 5 position showed a remarkable property. Although the UV spectrum of a solution did not change after preparation and further storage, the rate measured with maleylacetate reductase drastically decreased. The V_{max} value determined with a 5-chloromaleylacetate solution changed from 600 U per mg of protein within 5 h to a constant value of 17 U per mg of protein. However, it made no difference whether enzymatic or alkaline hydrolysis was used. The change was feasible within 15 min by slight acidification of the solution. An analogous observation was made during the preparation of 5-bromomaleylacetate from 5-bromodienelactone. This change took place much more slowly, and the acid-catalyzed reaction was finished after a few hours. Since the cleavage of 5-bromodienelactone required high pH values, nucleophilic substitution by the OH^- ions led to the formation of a by-product, probably 5-hydroxymaleylacetate, with a yield of about 10%. Therefore, enzyme-catalyzed ester hydrolysis was used to prepare 5-bromomaleylacetate. 3-Fluoromaleylacetate, which was highly unstable under alkaline conditions, forming products of unknown structure, was produced in the same way.

2,5-Dibromomaleylacetate was prepared at lower pH values so that the formation of hydroxymaleylacetates could be avoided. Together with 2,5-dichloromaleylacetate, this compound isomerized in the same manner as described for the 5-monohalo derivatives.

Additional problems arose with 2-fluoro-, 2,3-dichloro-, 2,3,5-trichloro-, and all 2,5-dihalogenated dienelactones. These dienelactones showed high instability in aqueous solution. In the case of 2-fluoromaleylacetate, the necessity of starting with an absolutely fresh prepared solution of 2-fluorodienelactone together with the extreme instability of 2-fluoromaleylacetate prevents measurement of K_m values.

Stoichiometry of the enzymatic reduction of maleylacetates. Stoichiometric parameters like NADH consumption and halide formation, the appearance of intermediates during reduction, and kinetic data (K_m and V_{max}) are summarized in Table 2.

(i) NADH consumption. Total NADH consumption was determined under standard assay conditions by varying the substrate concentration in the range of 0.05 to 0.4 mM. The enzyme catalyzed the consumption of 2 mol of NADH per eq of 2-halogenated maleylacetates (2-fluoro-, 2-chloro-, 2,3-dichloro-, 2,5-dichloro-, 2,3,5-trichloro-, 2-bromo-, 2,3-dibromo-, 2,5-dibromo-, 2-bromo-5-chloro-, 2-chloro-3-methyl-, and 2-chloro-5-methylmaleylacetate).

In contrast, only 1 mol of NADH was consumed with substrates bearing no halogen substituent in the 2 position.

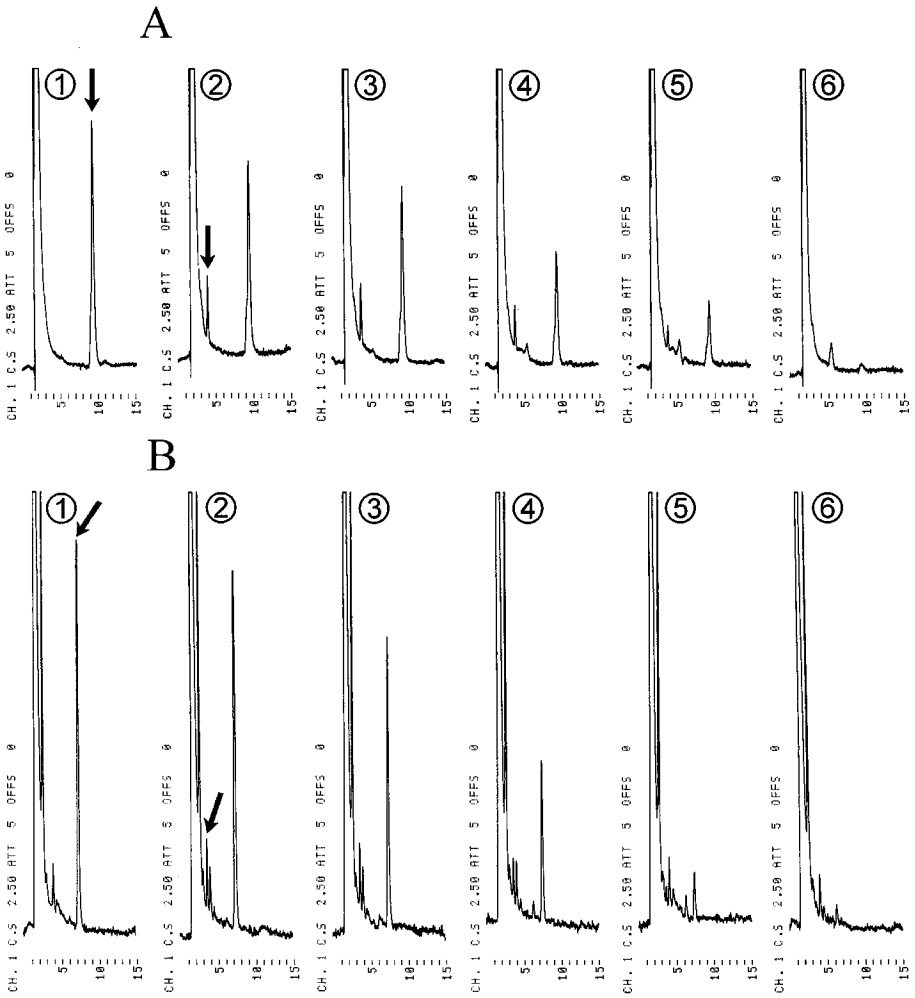

FIG. 3. (A) Detection of 3-chloromaleylacetate as an intermediate during the reduction of 2,3-dichloromaleylacetate by the maleylacetate reductase of strain B13. The assay mixtures (1 ml) contained 2,3-dichloromaleylacetate (100 μM), NADH (300 μM), and purified reductase (0.25 μg). NADH consumption was determined by the decrease in A_{340}. The reaction was stopped at various periods (NADH consumption of 0, 25, 50, 100, 150, and 200 μM), and the assay mixture was immediately analyzed by HPLC (chromatograms 1 to 6, respectively). The retention times were 3.9 and 9.5 min for 3-chloro- and 2,3-dichloromaleylacetate, respectively (arrows). (B) Detection of 5-chloromaleylacetate as an intermediate during the reduction of 2,5-dichloromaleylacetate by the maleylacetate reductase of strain B13. The assay mixtures (1 ml) contained 2,5-dichloromaleylacetate (100 μM), NADH (300 μM), and purified reductase (0.5 μg). NADH consumption was determined by the decrease in A_{340}. The reaction was stopped at various periods (NADH consumption of 0, 40, 80, 120, 160, and 200 μM), and the assay mixture was immediately analyzed by HPLC (chromatograms 1 to 6, respectively). The retention times were 3.7 and 7.6 min for 5-chloro- and 2,5-dichloromaleylacetate, respectively (arrows).

2,3-Dimethyl-, 2,3-dimethyl-5-chloro-, and 2,3,5-trimethylmaleylacetate showed no enzymatic turnover. The corresponding dienelactones were not converted by dienelactone hydrolase of *A. eutrophus* JMP134.

(ii) Halide elimination. Halide elimination was determined under modified standard assay conditions, by using phosphate buffer and varying the substrate concentration in the range of 0.05 to 0.4 mM. The turnover of the 2-halogenated derivatives 2-fluoro-, 2-chloro-, 2-bromo-, 2-chloro-3-methyl-, and 2-chloro-5-methylmaleylacetate as well as all 2,3- and 2,5-dihalogenated derivatives resulted in the elimination of 1 mole equivalent of the corresponding halide. With 2-bromo-5-chloromaleylacetate, the elimination of bromide was observed. In contrast, halomaleylacetates missing a halogen substituent in the 2 position such as 3-fluoro-, 5-fluoro-, 3-chloro-, 5-chloro-, 3-bromo-, 5-bromo-, and 2-methyl-3-chloromaleylacetate showed no elimination of halide during reduction with maleylacetate reductase.

Because of its high instability no exact values were obtained for 2,3,5-trichloromaleylacetate.

(iii) Intermediates during enzymatic reduction of halogenated maleylacetates. To detect intermediates during dehalogenation, standard enzyme assays containing the corresponding 2-halomaleylacetate and NADH were incubated with maleylacetate reductase. The NADH consumption was controlled by the decrease of A_{340}. After different times the reaction was quenched by the addition of 3 μl of H_3PO_4 (85%) to the enzyme assay, which was immediately followed by HPLC analysis (Fig. 3).

All 2-halomaleylacetates except 2,3-dibromomaleylacetate showed the appearance of the corresponding maleylacetate, missing the halogen in the 2 position.

During the reduction of 3-chloro- and 2-methyl-3-chloromaleylacetate the appearance of maleylacetate or 2-methylmaleylacetate, respectively, was not observed.

(iv) Kinetic parameters (K_m and V_{max}). For kinetic measure-

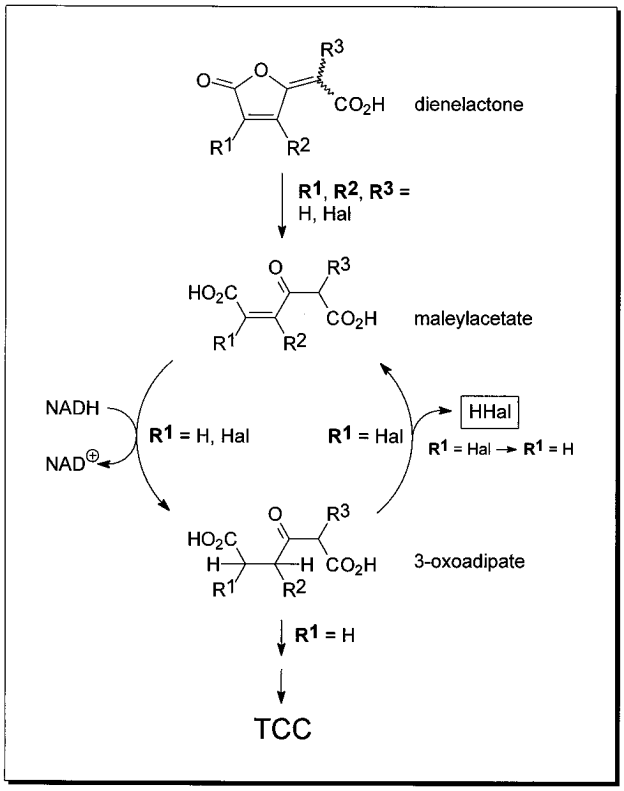

FIG. 4. Degradation of various dienelactones via the corresponding maleylacetates to the 3-oxoadipates and cycling with halogen-substituted maleylacetates including elimination of substituents in the 2 position. TCC, tricarboxylic acid cycle.

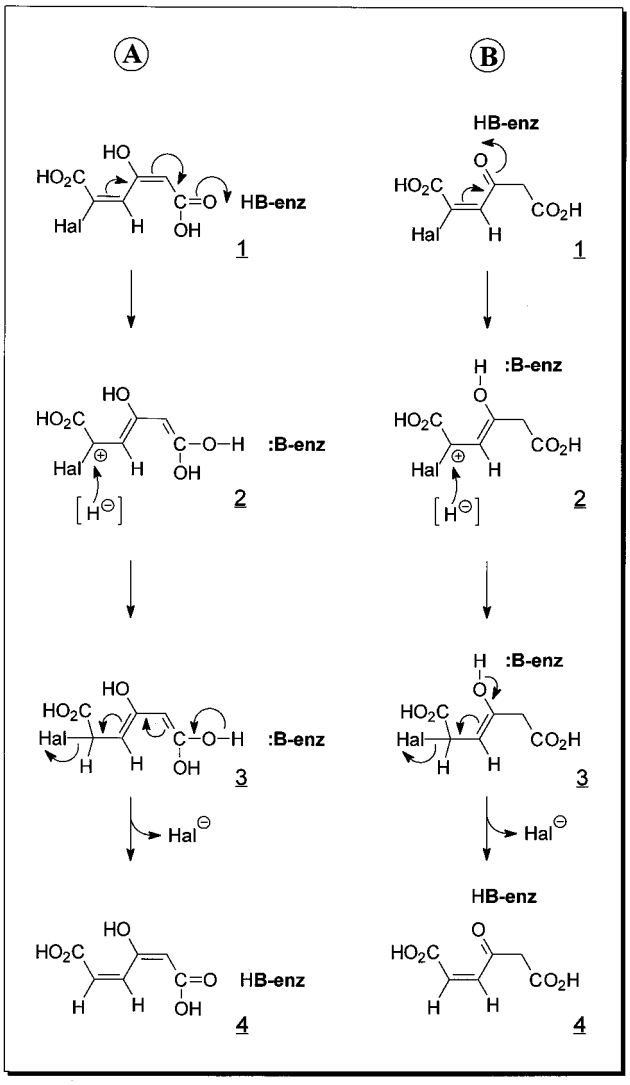

FIG. 5. Proposed reaction mechanism for the elimination of halogen substituents from the 2 position of hydroxymuconate (A) or maleylacetate (B).

ments NADH was used as the cofactor at a concentration of 0.4 mM. The substrate concentration ranged from 0.3 to 2.0 of the respective K_m value. A summary of all determinations is given in Table 2. K_m values decreased for a special halogen substituent according to the following pattern: 2 position > 5 position > 3 position. The following order of the K_m values was observed for a particular position: bromo > chloro > fluoro. For methyl-substituted maleylacetates K_m values decreased according to the following substitution pattern: 2 position ≥ 3 position >> 5 position.

DISCUSSION

This article describes an enzyme which reduces the carbon-carbon double bond in maleylacetate, a metabolite within the *ortho* cleavage pathway of chlorocatechol.

The substrate range of the maleylacetate reductase of strain B13 was shown to be remarkably broad, since various halogen- and methyl-substituted derivatives were also converted. Only against di- and trimethylated maleylacetates did the enzyme show no activity.

Although the enzyme has been called maleylacetate reductase in the literature (1, 3, 4, 7, 8, 17), Seibert et al. (15) pointed out that the real substrate of maleylacetate reductase might not be maleylacetate in the strict sense, since at neutral and alkaline pH values the keto form of maleylacetate was not present in significant amounts and the enol form appeared to dominate in the equilibrium. Therefore, hydroxymuconate instead of maleylacetate was thought to be the substrate. Which type of isomer, *cis*,*cis*-hydroxymuconate or the *trans*,*cis* form, is functioning as the substrate is not known yet. The failure of the enzyme to reduce fumarylacetate (8) suggests that the *cis* geometry of the double bond, which will be reduced during the reaction of maleylacetate or hydroxymuconate, is necessary for a compound to be a substrate.

Since the enzyme is completely inactivated in the presence of sulfhydryl-blocking reagents such as *p*-chloromercuribenzoate and heavy metal ions like Zn^{2+}, Cu^{2+}, Ag^{+}, and Hg^{2+}, the enzyme seems to contain thiol groups, which are essential for activity. In addition, it is unlikely that bivalent metal ions function in the catalysis, since complexing agents did not inhibit the enzyme activity (9).

Combining previous results with the observations described in this article, a proposed pathway for the reduction of substituted maleylacetates by the maleylacetate reductase from *Pseudomonas* sp. strain B13 is shown in Fig. 4.

(i) Nonsubstituted maleylacetate, the monomethylmaleylacetates, and halogenated maleylacetates, missing a halogen substituent in the 2 position, were reduced by use of 1 mole equivalent of NADH. In contrast, the quantitative reduction of halomaleylacetates, containing a halogen substituent in the 2

position, took place with the consumption of 2 mole equivalent of NADH.

(ii) Only substrates halogenated in the 2 position showed a loss of 1 mole equivalent of halide.

(iii) During reactions characterized by the consumption of 2 mol of NADH and the loss of halide, with two exceptions, the corresponding maleylacetate, dehalogenated in the 2 position, was obtained. The absence of 3-bromomaleylacetate during the reduction of 2,3-dibromomaleylacetate could be explained by its remarkable high turnover rate. Whether 3,5-dichloromaleylacetate occurred as the intermediate from 2,3,5-trichloromaleylacetate could not be verified, because this compound was not available as a standard.

In an analogy to a reaction mechanism suggested for an enzyme (EC 1.3.1.31) reducing the carbon-carbon double bond of α,β-unsaturated carboxylate anions of the methylbutenoate or cinnamate type (16), which also eliminate halide from the double bond (14), we propose that the following mechanism could play a role in the enzyme-catalyzed dehalogenation of maleylacetates when the substrate is used in the form of hydroxymuconate (Fig. 5A [the numbering of carbon has been made uniform for hydroxymuconate and maleylacetate to facilitate the discussion]).

A proton-mediated substrate activation, which has also been postulated for the saturation of double bonds by steroid reductases (18, 19), occurs by initial protonation of one of the oxygen atoms of the carboxy group of hydroxymuconate, leading to a positively charged carbon-2 as depicted by the mesomeric form 2 in Fig. 5. This species takes up a hydride from NADH and stabilizes itself by elimination of HHal, forming back the double bond when 2-halohydroxymuconates are the substrates. When hydroxymuconate is the substrate, stabilization occurs by the addition of a proton so that 3-oxoadipate is the product.

In an alternative mechanism the reaction starts with the proton-mediated activation of the keto group of maleylacetate (Fig. 5B). This protonation of the Michael system seems to be more likely than the protonation of the carboxylic group, as in the scheme in Fig. 5A, which needs a highly acid group in the enzyme. The uptake of a hydride at the carbonium ion will be followed by the elimination of HHal as in the other mechanism. Besides the higher reactivity of the keto group against protonation, another observation favors the latter mechanism with maleylacetate as the substrate: the pH optimum of the maleylacetate reductase from *Pseudomonas* sp. strain B13 was 5.4. This could be explained by the higher concentration of the tautomeric maleylacetate at this pH value. However, further experimental evidence is necessary to decide if chloride is eliminated spontaneously from 2-chlorooxoadipates because of their instability or if this process is integrated in the reaction of the enzyme.

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

We thank Detlef Müller for valuable discussions and critical reading of the manuscript. We are indebted to Michael Schlömann, University of Stuttgart, for providing the purified dienelactone hydrolase of *A. eutrophus* JMP134 and for stimulating discussions on the modified *ortho* cleavage pathway.

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