Chemical structure and biodegradability of halogenated aromatic compounds

Conversion of chlorinated muconic acids into maleoylactic acid

Eberhard SCHMIDT* and Hans-Joachim KNACKMUSST†

*Institut für Mikrobiologie der Universität und der Gesellschaft für Strahlen- und Umweltforschung Göttingen, Grisebachstrasse 8, D-3400 Göttingen, Federal Republic of Germany, and

†Lehrstuhl für Chemische Mikrobiologie der Gesamthochschule, Wuppertal, Federal Republic of Germany

(Received 25 January 1980/Accepted 2 July 1980)

1. An enzyme for the cycloisomerization of 2- and 3-chloro-*cis,cis*-muconic acid was isolated from 3-chlorobenzoate-grown cells of *Pseudomonas* sp. B13. It was named muconate cycloisomerase II, because it could clearly be differentiated by its K_m and V_{max} values from an ordinary muconate cycloisomerase, which functioned in benzoate catabolism and exhibited low activity with the chlorinated substrates. 2-Chloro-*cis,cis*-muconic acid was converted into *trans*- and 3-chloro-*cis,cis*-muconic acid into *cis*-4-carboxymethylenebut-2-en-4-olide together with dehalogenation. 2. An enzyme was isolated from chlorobenzoate-grown cells, which converted the 4-carboxymethylenebut-2-en-4-olides into maleoylactic acid.

Degradation of halogenated aromatic compounds by aromatic-degrading bacteria appears to be critical for several reasons. First, ring cleavage of halogenated catechols and cycloisomerization of halomuconic acids are crucial catabolic steps because normal *ortho*-pyrocatechases (catechol 1,2-dioxygenases, EC 1.13.11.1) and cycloisomerases (EC 5.5.1.1) exhibit very low activities for the halogenated substrates (Dorn & Knackmuss, 1978a,b). *Pseudomonas* sp. B13, which had been isolated by continuous enrichment on benzoate and 3-chlorobenzoate (Dorn *et al.*, 1974), induces an additional pyrocatechase with altered substrate specificity (pyrocatechase II) during growth with the chlorinated substrate (Dorn & Knackmuss, 1978a). In addition high activities for chlorinated muconic acids were found in 3-chlorobenzoate-grown cells of this organism. Therefore another isoenzyme for the cycloisomerization of chloromuconic acids was suggested. Corresponding cycloisomerizing activities were also postulated in other haloaromatic-utilizing bacteria (Evans *et al.*, 1971b; Gaunt & Evans, 1971; Sharpee *et al.*, 1973). The purity of these enzyme preparations and their substrates did not allow detailed analysis of the reaction products and mechanism of cycloisomerization. The present paper describes chloride-eliminating cycloiso-

merization and further degradation of 2- and 3-chloro-*cis,cis*-muconic acid by enzyme preparations from 3-chlorobenzoate-grown cells of *Pseudomonas* sp. B13.

Experimental

Chemicals

Muconic acids were prepared as described by Schmidt *et al.* (1980). Reagents used in buffers and growth media were of analytical grade and obtained from Merck, Darmstadt, Germany. Biochemicals were purchased from C. F. Boehringer, Mannheim, Germany. DEAE-cellulose (DE-52) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K., and DEAE-Sephadex A-50 and Sephadex G-200 (fine grade) were from Pharmacia, Uppsala, Sweden. Aquacide 1-A was received from Calbiochem, San Diego, CA, U.S.A.

Organism

Cells of *Pseudomonas* sp. B13 (parent strain 426 of the Deutsche Sammlung von Mikroorganismen, Göttingen, Germany) were grown at 30°C in a medium described previously (Dorn *et al.*, 1974).

Large-scale growth of biomass

For the production of large cell mass, *Pseudomonas* sp. B13 was grown on 26 mM-3-chlorobenzoate in a 10-litre fermenter up to an absorption

† Present address and address for reprint requests: Institut für Mikrobiologie der Universität Göttingen, D-3400 Göttingen, Federal Republic of Germany.

of $A_{546} = 3$. Cells were harvested by centrifugation at 20 000 g for 10 min.

Preparation of cell extract

Freshly harvested cells were suspended in 50 mM-Tris/HCl buffer, pH 7, containing 2 mM-MnSO₄. Portions (30 ml) of this suspension were supplemented with about 3 mg of deoxyribonuclease and disrupted in a French press (Aminco, Silver Springs, MD, U.S.A.) at 80 kPa. Cell debris was removed by centrifugation at 10 000 g for 30 min. The supernatant was centrifuged at 100 000 g for 60 min (Omikron model; Heraeus-Christ, Osterode, Germany). The supernatant was used as cell extract and the pellet was discarded. During the purification procedures, protein concentrations were determined as described by Bradford (1976). This method was verified by use of the procedure of Beisenherz *et al.* (1953). Bovine serum albumin (Cohn fraction V) was used throughout as protein standard.

Chromatography and DEAE-cellulose, DEAE-Sephadex and Sephadex G-200 gel filtration

Precycled DEAE-cellulose and preswollen Sephadex were used. All chromatography procedures were performed at 4°C.

Concentration of cycloisomerase II by Aquacide

The pooled fractions from DEAE-cellulose chromatography containing cycloisomerase II activity were concentrated at 4°C by use of a dialysis bag (Union Carbide Corporation, Chicago, IL, U.S.A.) powdered with Aquacide 1-A (Calbiochem, San Diego, CA, U.S.A.). The protein solution was slightly agitated on a rotation shaker.

Concentration of 4-carboxymethylenebut-2-en-4-olide hydrolase by use of a Diaflow chamber

The pooled fractions of 4-carboxymethylenebut-2-en-4-olide hydrolase from DEAE-cellulose chromatography were transferred into a Diaflow chamber type 202 (Amicon, Lexington, MA, U.S.A.) equipped with a PM-10 filter (Amicon). The continuously stirred protein solution (43 ml) was concentrated at 4°C to one-fifth the original volume within 4 h, when 304 000 Pa of N₂ was applied.

Enzyme assays

All enzyme activities were determined by the use of spectrophotometric methods in silica cuvettes with 10 mm path length in a Zeiss DMR 10 recording spectrophotometer at 25°C. One enzyme unit is defined as the amount that catalyses the formation of 1 µmol of product or the disappearance of 1 µmol of substrate per min, under assay conditions.

Muconate cycloisomerases [4-carboxymethyl-4-

hydroxyisocrotonolactone lyase (decyclizing), EC 5.5.1.1]. Assays with *cis,cis*-muconic acid and the substituted analogues as substrates were carried out by the method of Ornston (1966b). Cycloisomerase I was measured by use of *cis,cis*-muconic acid and cycloisomerase II by use of 2-chloro-*cis,cis*-muconic acid as substrate. Assays with chlorinated muconic acids as substrates were supplemented with purified 4-carboxymethylenebut-2-en-4-olide hydrolase. To avoid accumulation of products absorbing at 260 nm, cycloisomerase activity was tested in the presence of a 10-fold excess of the auxiliary enzyme. When enzymically prepared stock solutions of substituted muconic acids (Schmidt *et al.*, 1980) were used as substrates, the content of the *cis,cis*-isomer was determined as follows. Cycloisomerase II and 4-carboxymethylenebut-2-en-4-olide hydrolase were added to the enzymically prepared muconic acids in 33 mM-Tris/HCl, pH 8, containing 1 mM-MnCl₂. (The auxiliary hydrolase is dispensable when methyl-*cis,cis*-muconic acids were assayed.) When the turnover was complete, the difference in A_{260} was measured against a reference containing the same components except that cycloisomerase II was deactivated by pretreatment with EDTA (27 mM) for 10 min. The amounts of substituted *cis,cis*-muconic acids were calculated on the basis of the molar absorption coefficients (ϵ) given by Dorn & Knackmuss (1978b).

4-Carboxymethylenebut-2-en-4-olide hydrolase. Activity of 4-carboxymethylenebut-2-en-4-olide hydrolase was measured spectrophotometrically at 280 nm in a 3 ml cuvette containing 100 µmol of phosphate buffer, pH 6.5, and 0.3 µmol of *trans*-4-carboxymethylenebut-2-en-4-olide as substrate. Its molar absorption coefficient was found to be $\epsilon_{280} = 15\,625 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (see below).

4-Carboxymethylbut-3-en-4-olide hydrolase (EC 3.1.1.24). 4-Carboxymethylbut-3-en-4-olide hydrolase was determined by the procedure of Ornston (1966a), except that the reaction was carried out at pH 6.5. 4-Carboxymethylbut-2-en-4-olide was prepared by acid treatment of *cis,cis*-muconic acid by the method of Elvidge *et al.* (1950). The isolated compound exhibited a melting point of 109°C [111°C was found by Elvidge *et al.* (1950)]. The auxiliary enzyme for the transformation of 4-carboxymethylbut-2-en-4-olide to 4-carboxymethylbut-3-en-4-olide was prepared by treating crude extracts from cells of *Ps. putida* A 3.13 (DSM 291) at 55°C for 30 min (Ornston, 1966b).

Analysis of kinetic data

The initial-velocity data were examined graphically in double-reciprocal plots. Michaelis constants (K_m) and inhibitor constants (K_i) were calculated from values of appropriate intercepts by the method of Lineweaver & Burk (1934).

Analytical methods

Experimental conditions for n.m.r., u.v. and i.r. spectroscopy, mass spectrometry and high-pressure liquid chromatography were as described in the preceding paper (Schmidt *et al.*, 1980).

Results

Two muconate cycloisomerases

Dorn & Knackmuss (1978a) observed high activities for substituted muconic acids only in 3-chlorobenzoate-grown cells of *Pseudomonas* sp. B13. Conversely in benzoate-grown cells of this strain high activity was found only for unsubstituted muconic acid. Further indication of the presence of two different cycloisomerases was obtained from heat treatment of the crude extracts: when those from benzoate-grown cells were treated for 10 min at 60°C, 68% of the original muconate cycloisomerase activity had been retained. In contrast under the same conditions only 4% of the activity from 3-chlorobenzoate-grown cells was recovered. Significantly the relative activities for muconic acid and substituted muconic acids did not change in both extracts during heat treatment. Presumably a heat-stable cycloisomerase was induced during growth with benzoate and a heat-labile enzyme during growth with 3-chlorobenzoate. According to the designation of the two isoenzymes for ring cleavage of catechol and chlorocatechols by this organism (Dorn & Knackmuss, 1978a) we name the two corresponding isoenzymes for the subsequent catabolic step cycloisomerase I and cycloisomerase II. The former enzyme functions in benzoate catabolism, whereas cycloisomerase II is required in the

3-chlorobenzoate pathway exhibiting high activity for chlorinated muconic acids.

The stabilities of both cycloisomerase activities in crude extracts were compared under different storage conditions. Compared with cycloisomerase II (see Table 1) the isoenzyme I was generally more stable. No loss of cycloisomerase I activity was noticed within 30 h at 6°C, when 50 mM-Tris/HCl buffer, pH 7, was supplemented with 2 mM-MnCl₂. These conditions were used for most of the purification procedures and for determination of cycloisomerase activities in crude extracts of *Pseudomonas* sp. B13 and strains with analogous catabolic sequences (Reineke & Knackmuss, 1979).

Purification of muconate cycloisomerase II

DEAE-cellulose chromatography of crude cell extract. Cells of *Pseudomonas* sp. B13 were harvested during exponential growth with 3-chlorobenzoate. The crude cell extract (see the Experimental section) was used in ion-exchange chromatography without prior treatment. A total of 69 ml of crude cell extract was applied to a DEAE-cellulose column (2.5 cm × 20 cm) previously equilibrated with 50 mM-Tris/HCl buffer, pH 7, containing 2 mM-MnSO₄. Subsequently the enzymes were eluted with a linear gradient generated with 400 ml of the same buffer in the mixing chamber and 400 ml of buffer containing 0.2 M-NaCl in the reservoir. The eluate was collected in portions (7 ml in 10 min). Fractions 16–26 containing the bulk of the activity of 4-carboxymethylenebut-2-en-4-olide hydrolase were combined and stored at -18°C for a separate purification procedure (see below). Fractions 40–56 (120 ml) containing 81% of the cycloisomerase II were pooled. Enrichment of

Table 1. *Stability of cycloisomerase II in crude extracts under different storage conditions*

Cells of *Pseudomonas* sp. B13 were harvested during exponential growth on 3-chlorobenzoate, suspended in 20 mM-Tris/HCl buffer, pH 8 and disrupted in a French press. After centrifugation (100 000 g for 60 min) this stock solution was diluted 1:10 with the actual buffer solution. Abbreviation used: Hepes, 4-(2-hydroxymethyl)-1-piperazine-ethanesulphonic acid.

Buffer	Concentration (mM)	pH	Additions	Temperature (°C)	Activity after storage for 30 h (%) (zero time = 100%)
Tris	100	6	—	6	49
Tris	100	7	—	6	50
Tris	100	8	—	6	39
Tris	50	8	—	6	47
Tris	50	7	—	6	55
Tris	50	7	—	25	47
Tris	50	7	—	-16	1
Tris	50	7	Bovine serum albumin (10 mg/ml)	6	3
Tris	50	7	0.02% Azide	6	56
Tris	50	8	2-Mercaptoethanol (10 mM)	6	31
Tris	50	7	Dithioerythrol (1 mM)	6	50
Tris	50	7	MnCl ₂ (2 mM)	6	64
Hepes	50	7	MnCl ₂ (2 mM)	6	67

cycloisomerase II and its separation from 4-carboxymethylenebut-2-en-4-olide hydrolase are summarized in Tables 2 and 3.

Sephadex G-200 gel filtration of muconate cycloisomerase II. To improve the efficiency of subsequent Sephadex G-200 gel filtration the pooled fractions of cycloisomerase II were concentrated to one-third the original volume. Primary treatment by $(\text{NH}_4)_2\text{SO}_4$ precipitation or concentration by use of a Diaflow chamber resulted in a considerable loss of activity (70% or 50–60%). On the other hand the use of Aquacide turned out to be an appropriate method. The original volume (120 ml) was decreased to 36 ml within 4 h and 85–95% of the original activity was recovered. The resulting protein solution was applied to a column (5 cm $\times$ 16 cm) of Sephadex G-200 that had been equilibrated with 50 mM-Tris/HCl buffer, pH 7, supplemented with 2 mM-MnSO₄. The gel filtration was carried out with the same buffer and a constant hydrostatic pressure of a 15 cm water column. Fractions (4.2 ml) were collected. A relatively short column was used to complete the whole procedure within 3.5 h. Cycloisomerase II had been eluted from the column before 4-carboxymethylenebut-2-en-4-olide hydrolase. The fractions of cycloisomerase II that were now free from NaCl were directly applied to a DEAE-Sephadex column.

DEAE-Sephadex chromatography of muconate cycloisomerase II. For further purification a DEAE-Sephadex column (2.5 cm $\times$ 15 cm) was equilibrated with 50 mM-Tris/HCl buffer, pH 7, containing 2 mM-MnSO₄. The protein was eluted with a linear gradient generated with 200 ml of the same buffer in the mixing chamber and 200 ml of buffer containing 0.3 M-NaCl in the reservoir. A relatively high yield (48%) of cycloisomerase II was obtained when the

whole purification procedure was completed within 48 h. For identification of cycloisomerization products preparative amounts of chloromuconic acids had to be converted and consequently larger amounts of cycloisomerase II were required. After DEAE-Sephadex chromatography the relative specific activities of cycloisomerase II and 4-carboxymethylenebut-2-en-4-olide hydrolase were about 400:1 (see Table 3).

Gel filtration of crude extract from benzoate-grown cells

To compare cycloisomerization products of chloromuconic acids by cycloisomerases I and II the former activity had to be separated from hydrolase activities in benzoate-grown cells. Crude cell extract from exponentially grown cells was prepared as described in the Experimental section. For complete separation of cycloisomerase I from hydrolase activities only a small volume of crude extract (3 ml, equivalent to 21 mg of protein) could be applied to a Sephadex G-200 column (2.5 cm $\times$ 30 cm). Cycloisomerase I was clearly separated from hydrolase activities, whereas those for 4-carboxymethylenebut-2-en-4-olide and 4-carboxymethylbut-3-en-4-olide appeared in the same fractions.

Binding and turnover of substituted muconic acids by muconate cycloisomerases I and II

Apparent K_m values and maximum cycloisomerization velocities of substituted *cis,cis*-muconic acids were determined as described in the Experimental section. Substituted *cis,cis*-muconic acids were prepared from substituted catechols by pyrocatechase II from *Pseudomonas* sp. B13 as described by Schmidt *et al.* (1980). These enzymically prepared solutions contained protein and 2-mer-

Table 2. Summary of the purification experiments with cycloisomerase II from *Pseudomonas* sp. B13

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	69	4002	714	0.178	100
DEAE-cellulose column	120	546	575	1.053	81
Aquacide	36	468	513	1.096	72
Sephadex G-200 column	89	205	382	1.863	53
DEAE-Sephadex column	63	121	342	2.826	48

Table 3. Efficiency of separation of cycloisomerase II from 4-carboxymethylenebut-2-en-4-olide hydrolase

	Cycloisomerase II (units/ml)	Hydrolase (units/ml)	Ratio of cycloisomerase and hydrolase
Crude extract	10.3	97.4	0.10
DEAE-cellulose column	4.8	0.75	6.4
Sephadex G-200 column	4.3	0.04	107.5
DEAE-Sephadex column	5.43	0.014	387.9

Table 4. Apparent K_m values and maximum velocities of *cis,cis*-muconic acid and substituted *cis,cis*-muconic acids for cycloisomerases I and II from *Pseudomonas* sp. B13

The kinetic constants were determined by use of double-reciprocal plots ($1/[S]$ against $1/v$). *cis,cis*-Muconic acid was dissolved in 100 mM-Tris/HCl, pH 8. The substituted muconic acids were prepared as described in the preceding paper (Schmidt *et al.*, 1980) and directly used for enzymic tests. V_{max} values correspond to the concentrations of muconic acids extrapolated to infinity.

Substrate	Cycloisomerase I		Cycloisomerase II	
	K_m (μ M)	V_{max} (%)	K_m (μ M)	V_{max} (%)
<i>cis,cis</i> -Muconic acid	55.8	100	92.8	100
2-Methyl- <i>cis,cis</i> -muconic acid	105.0	90	30.9	276
3-Methyl- <i>cis,cis</i> -muconic acid	18.0	11.7	13.4	232
2-Chloro- <i>cis,cis</i> -muconic acid	415.0	0.8	60.1	180
3-Chloro- <i>cis,cis</i> -muconic acid	134.8	7.1	128.6	201

captoethanol, which might interfere with the cycloisomerase reaction. Compared with the K_m value of 60μ M from enzymically prepared 2-chloro-*cis,cis*-muconic acid, a somewhat lower value (40μ M) was measured with a fresh solution of the crystalline compound (Schmidt *et al.*, 1980). Therefore the data for K_m and V_{max} in Table 4 have to be taken as approximate values, because these with the exception of *cis,cis*-muconic acid were obtained from enzymically prepared stock solutions of substituted muconic acids. Rapid spontaneous cycloisomerization did not allow the isolation of analytically pure 3-substituted muconic acids, whereas 2-chloro-*cis,cis*-muconic acid could be isolated as a pure compound (Schmidt *et al.*, 1980). Since cycloisomerase I exhibited very low activity with 2-chloro-*cis,cis*-muconic acid, this substrate was used for enzymic tests of cycloisomerase II in crude cell extracts of *Pseudomonas* sp. B13 and its construction strains (Reineke & Knackmuss, 1979).

Cycloisomerization of 2-chloro-*cis,cis*-muconic acid (1, Scheme 1)

For enzymic cycloisomerization of 2-chloro-*cis,cis*-muconic acid the following conditions were used. In 20 ml of a 50 mM-Tris/HCl buffer, pH 7, 20 mg of $MnSO_4$ and 8.8 mg of crystalline 2-chloro-*cis,cis*-muconic acid (equivalent to 2.5 mM) were dissolved. The reaction was started by the addition of 3 units of cycloisomerase II and followed by high-pressure liquid chromatography. Any decrease in pH during the reaction was counteracted by an addition of NaOH (0.1 M).

Within 20 min 2-chloro-*cis,cis*-muconic acid was almost quantitatively converted into *trans*-4-carboxymethylenebut-2-en-4-olide (3, Scheme 1). Turnover of 2-chloro-*cis,cis*-muconic acid by cycloisomerase I was very slow (see Table 4), but the analysis of the reaction mixture by high-pressure liquid chromatography revealed the same metabolite. Under the described conditions of reverse-phase

high-pressure liquid chromatography (Schmidt *et al.*, 1980) *trans*-4-carboxymethylenebut-2-en-4-olide exhibited a retention time of 4.2 min, whereas the *cis* isomer appeared after 8.2 min.

Cycloisomerization of 3-chloro-*cis,cis*-muconic acid (2, Scheme 1)

3-Chloro-*cis,cis*-muconic acid was isolated as its disodium salt by the method described by MacDonald *et al.* (1954) for 3-carboxy-*cis,cis*-muconic acid. The product also contained small amounts of *cis*-4-carboxymethylenebut-2-en-4-olide (approx. 0.5% by high-pressure liquid chromatography) and presumably NaCl. Since 3-chloro-*cis,cis*-muconic acid rapidly cycloisomerizes under acidic conditions (Schmidt *et al.*, 1980) the concentration of this compound in Tris/HCl could not be quantified directly by reverse-phase high-pressure liquid chromatography. Therefore part of the solution was incubated at pH 2.5 for 20 min. The sum of the concentrations of the resulting *cis*- and *trans*-4-carboxymethylenebut-2-en-4-olide were determined chromatographically and corresponded to the initial concentration of 3-chloromuconic acid.

Turnover of 3-chloro-*cis,cis*-muconic acid by cycloisomerase II was assayed under the same conditions as described for 2-chloro-*cis,cis*-muconic acid. After 7 min, 3-chloro-*cis,cis*-muconic acid was converted quantitatively into *cis*-4-carboxymethylenebut-2-en-4-olide (4, Scheme 1). A stoichiometric amount of the same product is produced from 3-chloro-*cis,cis*-muconic acid by cycloisomerase I.

Isolation and identification of products

For the isolation of *cis*- and *trans*-4-carboxymethylenebut-2-en-4-olide (4 and 3, Scheme 1) as enzymic cycloisomerization products, 500 ml of the acidified solution (pH 2) was concentrated at 20°C to one-tenth the original volume and extracted repeatedly with ethyl acetate (500 ml). The *trans* isomer crystallized spontaneously from ethyl ace-

tate after cooling (-10°C). The *cis* isomer was re-crystallized from dichloroethane. Both products were identical with the compounds described by Schreiber *et al.* (1980) by comparison of u.v., mass and n.m.r. spectra. The maxima of u.v. absorption were 276 nm ($\epsilon = 17200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) for the *cis* isomer and 277 nm ($\epsilon = 17550 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) for the *trans* isomer in 33 mM-phosphate buffer, pH 6.5.

Muconic acid and monomethylmuconic acids were converted by cycloisomerase I or cycloisomerase II into carboxymethylbut-2-en-4-olides. The products were identified by use of high-pressure liquid chromatography. Additionally the products from monomethylmuconic acids were identified by i.r. spectroscopy by comparison with authentic compounds (Knackmuss *et al.*, 1976).

Biological inertness of 4-carboxymethyl-2-chlorobut-2-en-4-olide

Chloro-substituted 4-carboxymethylbutenolides could conceivably be intermediates during cycloisomerization of chloro-*cis,cis*-muconic acids to 4-carboxymethylenebut-2-en-4-olides. 4-Carboxymethyl-2-chlorobut-2-en-4-olide was isolated when 2-chloro-*cis,cis*-muconic acid was treated with H_2SO_4 (Schmidt *et al.*, 1980). However this compound (in 50 mM-Tris/HCl buffer, pH 7) was not further converted by crude extract from 3-chlorobenzoate-grown cells of *Pseudomonas* sp. B13. Since 2-chloro-*cis,cis*-muconic acid was metabolized under the same conditions without accumulation of 4-carboxymethyl-2-chlorobut-2-en-4-olide, this butenolide cannot be a metabolite in the 3-chlorobenzoate pathway.

Purification of 4-carboxymethylenebut-2-en-4-olide hydrolase

Fractions containing 4-carboxymethylenebut-2-en-4-olide hydrolase were obtained by DEAE-cellulose chromatography from crude cell extract of *Pseudomonas* sp. B13 grown on 3-chlorobenzoate (see above). To improve the efficiency of the subsequent gel filtration, the protein solution was concentrated in a Diaflow chamber (see the Experimental section) to one-fifth of the original volume

(43 ml). Denatured protein was removed from the concentrated protein solution by centrifugation at 10000g. The supernatant, which contained 477.2 units/ml, was applied to a Sephadex G-200 column (2.5 cm $\times$ 30.5 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.

The gel filtration was carried out with the same buffer and a constant hydrostatic pressure of a 15 cm water column. Fractions (3.4 ml) were collected. Fractions containing 4-carboxymethylenebut-2-en-4-olide hydrolase were pooled and added to a DEAE-Sephadex column (2.5 cm $\times$ 36 cm). The column was eluted with a linear NaCl gradient generated with 50 mM-Tris/HCl buffer (500 ml), pH 7, in the mixing chamber and the same buffer (500 ml) in the reservoir containing 0.25 M-NaCl. Fractions (6 ml) were collected. In the eluate only one protein peak appeared, which coincided with the activity of the hydrolase. Table 5 summarizes the purification of 4-carboxymethylenebut-2-en-4-olide hydrolase. The apparent K_m value of the hydrolase was 15 μM for *trans*-4-carboxymethylenebut-2-en-4-olide and 400 μM for the *cis* isomer. At substrate saturation the maximum velocities were almost identical for both substrates.

Separation of hydrolase activities with 4-carboxymethylbut-3-en-4-olide and with 4-carboxymethylenebut-2-en-4-olide as substrate

Crude cell extracts of *Pseudomonas* sp. B13 exhibited activity for 4-carboxymethylbut-3-en-4-olide and for 4-carboxymethylenebut-2-en-4-olide after growth on 3-chlorobenzoate as well as on benzoate. Crude cell extract from cells grown on 3-chlorobenzoate exhibited 2.9 units/mg for *trans*-4-carboxymethylenebut-2-en-4-olide and 0.5 unit/mg for 4-carboxymethylbut-3-en-4-olide. In benzoate-grown cells the corresponding activities were 0.1 unit/mg and 0.6 unit/mg. By DEAE-cellulose chromatography of a crude extract of cells grown on benzoate, two discernible hydrolase activities were separated.

Crude cell extract (3 ml, equivalent to 36 mg of protein) from cells harvested during exponential growth on benzoate was layered on a DEAE-cellulose column (2.5 cm $\times$ 14 cm), previously equili-

Table 5. Purification of 4-carboxymethylenebut-2-en-4-olide hydrolase from 3-chlorobenzoate-grown cells of *Pseudomonas* sp. B13

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	30	1837	4831	2.63	100
DEAE-Cellulose column	43	104.2	3824	36.7	79
Diaflow chamber	8	99.7	3818	38.3	79
Sephadex G-200 column	28	19.6	2093	106.8	43
DEAE-Sephadex column	51	5.1	1048	205.5	22

brated with 50 mM-Tris/HCl buffer, pH 7. Subsequently the enzymes were eluted with a linear gradient generated with 150 ml of the same buffer in the mixing chamber and 150 ml of buffer containing 0.2 M-NaCl in the reservoir. The eluate was collected in portions (5.5 ml). The activities for 4-carboxymethylenebut-2-en-4-olide and 4-carboxymethylbut-3-en-4-olide were clearly separated. The two hydrolases exhibited no cross activities and no competitive inhibition between the two substrates.

Conversion of 4-carboxymethylenebut-2-en-4-olide to maleoylacetic acid

Both the *cis* and *trans* isomers of 4-carboxymethylenebut-2-en-4-olide (2 mM in 50 mM-Tris/HCl buffer, pH 7) were converted into the same product by 4-carboxymethylenebut-2-en-4-olide hydrolase. A single peak was observed at 205 nm by means of high-pressure liquid chromatography. The same product appeared when the pH of the stock solutions of the butenolides were adjusted to 12 by addition of concentrated NaOH. The yield after chemical conversion was insignificantly lower. Tiedje *et al.* (1969) noted that 4-carboxymethylenebut-2-en-4-olide (presumably *cis*-configured) was converted into maleoylacetic acid by hydrolase from *Arthrobacter* sp. as well as by addition of NaOH. The authors isolated the maleoylacetic acid by continuous diethyl ether extraction from an acidified solution. Presumably a mixture of maleoylacetic acid and acetylacrylic acid was isolated, because the latter is formed from maleoylacetic acid at pH 2 as reported by Chapman & Ribbons (1976). In fact our product from hydrolase treatment was slowly converted into a second product under acidic conditions (pH 2). For isolation of the original hydrolysis product the enzymic preparation was cooled to 0°C, acidified to pH 2 and extracted several times with cold diethyl ether as fast as possible (15 min). After evaporation of the diethyl ether a colourless oil was retained. Two products were detected by high-pressure liquid chromatography: the original hydrolysis product and a second compound with longer retention time. Since decarboxylation during extraction under acidic conditions could not be avoided completely, the decarboxylation product was isolated instead of the original product. The oily residue from extraction was treated with concentrated formic acid. After complete conversion formic acid was evaporated and the crystalline residue was re-crystallized from diethyl ether/light petroleum. Melting point, u.v.-absorption maximum and the mass of the compound corresponded to the values given by Chapman & Ribbons (1976) for *trans*-acetylacrylic acid.

Discussion

Conversion of 3-chloromuconic acid into maleoylacetate has been postulated as part of the degrada-

tive pathway of different halogenated aromatic compounds (Bollag *et al.*, 1968; Tiedje *et al.*, 1969; Evans *et al.*, 1971a). However the individual reaction steps of the fate of chloromuconic acids, particularly of the 2-isomer, have not yet been studied.

Different muconate cycloisomerases with discernible substrate specificities were induced during growth of *Pseudomonas* sp. B13 with benzoate or 3-chlorobenzoate as substrate. Both enzymes catalyse the same type of reaction, so that chlorinated muconic acids were converted into the same products by cycloisomerase I or cycloisomerase II. However, the isoenzymes differ significantly in their relative maximum cycloisomerization velocities with differently substituted muconic acids as substrates. With unsubstituted muconic acid as reference substrate (100%), the $V_{\max}$ values of cycloisomerase I with 2- and 3-chloromuconic acid as substrates were only 1 and 7%. In contrast the corresponding maximum velocities of cycloisomerase II were found to be 180 and 200%, which correspond to the catalytic function in 3-chlorobenzoate catabolism.

Since the relative maximum cycloisomerization velocities of 3-methyl- and 3-chloro-muconic acid by cycloisomerase I do not differ significantly, this reaction seems to be governed by the size rather than by the electronegativity of the substituent in the 3-position. In contrast cycloisomerase II converted 3-chloro- as well as 3-methyl-muconic with 200% of the maximum velocity with reference to unsubstituted muconic acid (100%). This indicates a relaxed stereospecificity of this enzyme.

The electronic influence of substituents in the 2-position of muconic acid appears to be much more critical. Whereas 2-methylmuconic acid was converted with high velocities by both isoenzymes, considerably decreased turnover rates were observed, particularly with cycloisomerase I, when the corresponding halo-substituted substrates are cycloisomerized. According to the high electronegativity of fluorine the reaction velocity with 2-fluoro-*cis*,*cis*-muconic acid is zero for both enzymes. On the other hand 3-fluoromuconic acid is readily cycloisomerized even by cycloisomerase I, as manifested by the ability of *Pseudomonas* sp. B13 to utilize 4-fluorobenzoate as growth substrate (Schreiber *et al.*, 1980).

Considering the fact that the same reaction products, namely 4-carboxymethylenebut-2-en-4-olides (3 and 4, Scheme 1) and chloride, are formed during cycloisomerization of chloromuconic acids by both isoenzymes, the actual catalytic function of cycloisomerase II during catabolism of the halo-aromatic ring is the cycloisomerization reaction. Therefore dehalogenation and regeneration of the diene system must be regarded as a spontaneous

secondary reaction of the cycloisomerization of halomuconic acids. Correspondingly cycloisomerase II functions exclusively as a cycloisomerizing enzyme when muconic acid or methylmuconic acids are used as substrates since hydrogen and methyl, unlike chlorine, cannot be eliminated as anions. 4-Carboxymethylbut-2-en-4-olides instead of 4-carboxymethylenebut-2-en-4-olides are the reaction products in this case.

For the cycloisomerization of 3-chloro-*cis,cis*-muconic acid (2, Scheme 1) we propose 4-carboxymethyl-4-chlorobut-2-en-4-olide as an intermediate, which spontaneously generates *cis*-4-carboxymethylenebut-2-en-4-olide (4, Scheme 1) by anti-elimination of hydrogen chloride. Interestingly the *cis* isomer (4) is also the main product (90% compared with only 10% of *trans* isomer) of the acid-catalysed cycloisomerization of 3-chloromuconic acid (Schmidt *et al.*, 1980). 4-Carboxymethyl-4-fluorobut-2-en-4-olide has been postulated as a metabolite of 4-fluorobenzoate (Harper & Blakley, 1971), which would seemingly fit the proposed cycloisomerization mechanism for 3-fluoromuconic acid. Unfortunately these authors did not report u.v. data, which would establish the absence of a diene chromophore. Moreover 3-fluoro-*cis,cis*-muconic acid was directly converted into *cis*- and *trans*-4-carboxymethylenebut-2-en-4-olide under acidic conditions (Schmidt *et al.*, 1980). These unfavourable conditions were described by Harper & Blakley (1971) for the isolation of the postulated 4-carboxymethyl-4-fluorobut-2-en-4-olide.

Cycloisomerization of 2-chloro-*cis,cis*-muconic acid (1, Scheme 1) under highly acidic conditions yielded 4-carboxymethyl-2-chlorobut-2-en-4-olide (Schmidt *et al.*, 1980). This compound is not further converted by crude extracts or cycloisomerase II and is probably not an intermediate in the biological reaction. The identification of *trans*-4-carboxymethylenebut-2-en-4-olide (3) as the only reaction product from enzymic cycloisomerization of 2-chloro-*cis,cis*-muconic acid (1) suggests 4-carboxychloromethylbut-2-en-4-olide as an intermediate, which again could release HCl spontaneously by anti-elimination.

Enzyme activities for the hydrolysis of 4-carboxymethylbut-3-en-4-olide and 4-carboxymethylenebut-2-en-4-olides were induced in benzoate as well as in 3-chlorobenzoate-utilizing cells of *Pseudomonas* sp. B13. Nevertheless the catalysis of these highly analogous reactions must be assigned to different enzymes, one functioning exclusively in benzoate, the other in 3-chlorobenzoate catabolism. Although nearly the same specific activities of 4-carboxymethylbut-3-en-4-olide hydrolase were induced during growth with both substrates, this enzyme is inactive with 4-carboxymethylenebut-

Scheme 1. Proposed catabolic route of 3-chlorobenzoic acid to maleoylacetic acid

2-en-4-olides and consequently useless in 3-chlorobenzoate catabolism. Conversely 4-carboxymethylenebut-2-en-4-olide hydrolase is highly specific for 4-carboxymethylenebut-2-en-4-olides. Consistent with its inefficiency in the benzoate pathway it is induced with significantly lower activity during growth with this substrate. In the 3-chlorobenzoate pathway it acts equally well on *cis*- and *trans*-4-carboxymethylenebut-2-en-4-olide generating the same product, namely maleoylacetate. Apparently the 3-chlorobenzoate pathway, which diverges

through non-selective initial dioxygenation into a 3- and a 4-chlorocatechol branch, converges again by the action of the hydrolase according to Scheme 1.

References

- Beisenherz, G., Boltze, H. J., Bücher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E. & Pfeleiderer, G. (1953) *Z. Naturforsch. Teil B* **8**, 555–577
- Bollag, J.-M., Briggs, G. C., Dawson, J. E. & Alexander, M. (1968) *J. Agric. Food Chem.* **16**, 829–833
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Chapman, P. J. & Ribbons, D. W. (1976) *J. Bacteriol.* **125**, 985–998
- Dorn, E. & Knackmuss, H.-J. (1978a) *Biochem. J.* **174**, 73–84
- Dorn, E. & Knackmuss, H.-J. (1978b) *Biochem. J.* **174**, 85–94
- Dorn, E., Hellwig, M., Reineke, W. & Knackmuss, H.-J. (1974) *Arch. Microbiol.* **99**, 61–70
- Elvidge, J. A., Linstead, R. P., Orkin, B. A., Sims, P., Baer, H. & Pattison, D. B. (1950) *J. Chem. Soc.* 2228–2235
- Evans, W. C., Smith, B. S. W., Moss, P. & Fernley, H. N. (1971a) *Biochem. J.* **122**, 509–517
- Evans, W. C., Smith, B. S. W., Fernley, H. N. & Davies, J. I. (1971b) *Biochem. J.* **122**, 543–551
- Gaunt, J. K. & Evans, W. C. (1971) *Biochem. J.* **122**, 533–542
- Harper, D. B. & Blakley, E. R. (1971) *Can. J. Microbiol.* **17**, 1015–1023
- Knackmuss, H.-J., Hellwig, M., Lackner, H. & Otting, W. (1976) *Eur. J. Appl. Microbiol.* **2**, 267–276
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
- MacDonald, D. L., Stanier, R. Y. & Ingraham, J. L. (1954) *J. Biol. Chem.* **210**, 809–820
- Ornston, L. N. (1966a) *J. Biol. Chem.* **241**, 3787–3795
- Ornston, L. N. (1966b) *J. Biol. Chem.* **241**, 3795–3799
- Reineke, W. & Knackmuss, H.-J. (1979) *Nature (London)* **277**, 385–386
- Schmidt, E., Remberg, G. & Knackmuss, H.-J. (1980) *Biochem. J.* **192**, 331–337
- Schreiber, A., Hellwig, M., Dorn, E., Reineke, W. & Knackmuss, H.-J. (1980) *Appl. Environ. Microbiol.* **39**, 58–67
- Sharpee, K. W., Duxbury, J. M. & Alexander, M. (1973) *Appl. Microbiol.* **26**, 445–447
- Tiedje, J. M., Duxbury, J. M., Alexander, M. & Dawson, J. E. (1969) *J. Agric. Food Chem.* **17**, 1021–1026