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Bacterial degradation of 3-chloroacrylic acid and the characterization of *cis*- and *trans*-specific dehalogenases

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Abstract

A coryneform bacterium that is able to utilize *cis*- and *trans*-3-chloroacrylic acid as sole carbon source for growth was isolated from freshwater sediment. The organism was found to produce two inducible dehalogenases, one specific for the *cis*- and the other for *trans*-3-chloroacrylic acid. Both dehalogenases were purified to homogeneity from cells induced for dehalogenase synthesis with 3-chlorocrotonic acid. The enzymes produced muconic acid semialdehyde (3-oxopropionic acid) from their respective 3-chloroacrylic acid substrate. No other substrates were found. The *cis*-3-chloroacrylic acid dehalogenase consisted of two polypeptide chains of a molecular weight 16.2 kDa. *Trans*-3-chloroacrylic acid dehalogenase was a protein with subunits of 7.4 and 8.7 kDa. The subunit and amino acid compositions and the N-terminal amino acid sequences of the enzymes indicate that they are not closely related.

Introduction

The bacterial utilization of halogenated compounds requires that microorganisms are able to produce enzymes that can cleave or labilize carbon-halogen bonds. With saturated aliphatic compounds, dehalogenation usually proceeds by enzyme-catalyzed nucleophilic substitution reactions, of which three types are known (Janssen et al. 1989). Substitution with glutathione takes place in the glutathion transferase mediated bacterial decomposition of dichloromethane (Kohler-Staub & Leisinger 1985), an activity that also occurs in higher organisms for *gem*-dihalogenated alkanes (Anders & Pohl 1985). Direct replacement of halogen substituents by water has been found in organisms degrading 2-halocarboxylic acids (Goldman et al. 1968) and haloalkanes (Keuning et al. 1985). Vicinal haloalcohols can be dehalogenated by nucleophilic substitution with the neighboring hydroxyl group (Castro & Bartnicki 1968; van den Wijngaard et al. 1991).

A limitation of these hydrolytic enzymes is their lack of activity with compounds possessing halogen substituents on unsaturated carbon atoms, which is probably the result of the inherent insensitivity of vinyl halides toward nucleophilic displacement reactions. However, conversion of vinylic compounds under aerobic conditions may proceed by monooxygenases that catalyze electrophilic insertion of activated oxygen. This reaction is responsible for the cometabolic degradation of chlorinated ethylenes by methanotrophic bacteria (Oldenhuis et al. 1989; Fox et al. 1990).

As an alternative, halogenated unsaturated compounds could possibly be converted by addition of water to the carbon-carbon double bond. If an unstable *geminal* haloalcohol is the product, this

would yield dehalogenation by chemical decomposition, leaving a keto or carbonyl compound. This reaction could take place in 3-chloroallylalcohol utilizing bacteria after oxidation of the substrate to the carboxylic acid (Belser & Castro 1971) and in organisms growing on 3-chlorobutyric acid after conversion of this compound to its coenzyme A derivative (Kohler-Staub & Kohler 1989). The dehalogenation of these and other β-substituted chlorocarboxylic acids thus is still poorly understood. Studies of Bollag & Alexander (1971) and the reports cited above have not identified the dehalogenating enzyme, and it is unknown what range of compounds can be converted and how this is related to the mechanism of dehalogenation.

We have studied the characteristics of the dehalogenating enzymes in a bacterium metabolizing 3-chloroacrylic acid (3CAA). This compound was proposed to be formed as an intermediate during the degradation of the nematocide 1,3-dichloropropene (Belser & Castro 1971; van Dijk 1974; Roberts & Stoydin 1976). Unlike with saturated 2-chlorocarboxylic acids (Goldman et al. 1968; Motosugi & Soda 1983), there are no reports on the biochemical properties of the enzymes involved in the dehalogenation of saturated or unsaturated 3-chlorocarboxylic acids. Here, we describe the presence of two novel isomer-selective dehalogenating enzymes in a coryneform bacterium that utilizes 3-chloroacrylic acids as sole carbon source. Both the *cis*- and *trans*-specific enzymes were purified and characterized.

Materials and methods

Growth conditions

The medium (MMY) used in all experiments contained per l 5.37 g of $Na_2HPO_4.12H_2O$, 1.36 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, 5 ml of metals solution (Janssen et al. 1985) and 10 mg of yeast extract. Carbon sources were added at 5 mM or as indicated. Stock solutions of carbon sources were sterilized separately over a 0.2 μm filter or by autoclaving. Organic solvents that were tested as carbon source were found to be sterile.

With 3CAA and gaseous or volatile substrates, growth was carried out in closed flasks filled to one-fifth of their volume with medium. The growth temperature was 30° C and the flasks were incubated under rotary shaking. Cells were harvested by centrifugation at 16,000 g and 4° C.

Cultures of 10 l used for the purification of the 3CAA dehalogenases were grown on 1.1% citrate in MMY medium to which extra $(NH_4)_2SO_4$ (1.5 g/l), $MgSO_4$ (0.2 g/l), metals solution (15 ml/l), and yeast extract (50 mg/l) were added. Cultivation was carried out in a Braun Biostat E fermenter at pH 7.0, 30° C, and 70% oxygen saturation. Prior to harvesting, the culture was induced for 12 h with 2 mM *trans*-3-chlorocrotonic acid, which is a gratuitous inducer (see 'Results'). Addition of 1 mM *cis*-3CAA 2 h before harvesting led to rapid chloride production in the fermenter, showing that dehalogenase activity was induced. The cells were harvested by continuous centrifugation with a Sharples TIP.

Growth was followed turbidimetrically at 450 nm. Chloride liberation during growth was determined after removing the cells by centrifugation with a colorimetric assay (Bergmann & Sanik 1957). Degradation of *cis*- and *trans*-3CAA in cultures was followed by gas chromatography.

Isolation of a 3-chloroacrylic acid degrading organism

An organism was isolated from freshwater sediment by batch enrichment under aerobic conditions at 30° C in MMY medium with 5 mM *trans*-3CAA as carbon source. A pure culture, designated strain FG41, was obtained by repeated streaking on MMY agar containing 5 mM *trans*-3CAA and on nutrient broth agar plates. The organism was maintained on 0.8% nutrient broth agar.

Several metabolic traits of strain FG41 were tested as described by Gerhardt et al. (1981). Taxonomic characterization of the organism was also performed by NCIMB (Aberdeen, Scotland, UK).

Assays

Cis-3CAA and *trans*-3CAA were analyzed by gas chromatography. Samples taken from cultures were immediately centrifuged (5 min at 16,000 g) to remove cells. To 0.5 ml of supernatant, 0.5 ml of 10 mM 3-chloropropionic acid was added as a standard. After addition of 2 ml of methanol and 0.2 ml of 25% H_2SO_4, the mixture was incubated at 100° C for 2 hours. The methylesters were extracted with 3 ml hexane, and the organic layer was analyzed by split injection of 1 μl in a gas chromatograph equipped with a CPsil5-CB column (25 m × 0.22 mm). The oven temperature was held at 35° C with an injector temperature of 300° C and a flame ionization detector (FID) temperature of 300° C. Acrylic acid and crotonic acid were derivatized similarly and analyzed on a CPwax52CB column (25 m × 0.21 mm) at 45° C for 3 min followed by a temperature increase to 200° C at 10° C/min.

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Dehalogenase activities were determined with *cis*-3CAA or *trans*-3CAA as a substrate. A suitable amount of enzyme (10 to 50 mU) in 20 to 50 μl of 10 mM Tris.SO_4 buffer, pH 7.5, containing 1 mM of EDTA and 1 mM of β-mercaptoethanol (TEM buffer) was incubated with 2.5 ml of 5 mM substrate in 50 mM of Tris.SO_4 buffer, pH 7.5 (substrate solution). Activities were determined by measuring chloride concentrations of four 0.5 ml samples removed at different timepoints within 30 min. The chloride concentrations were determined by using a spectrophotometric assay with mercuric thiocyanate and ferric ammonium sulfate, as described by Bergmann & Sanik (1957). One unit of activity was defined as the amount of enzyme that catalyzed the formation of 1 μmole of chloride per min. Some assays (e.g. when glutathione was present) were performed with a chloride electrode.

The colorimetric production assay was also used for determining Michaelis Menten kinetic constants. Initial rates of chloride production were determined at 8 different substrate concentrations between 0.3 and 5 mM, and K_m and V_{max} values were determined by fitting to the Michaelis Menten equation by non-linear regression or the Lineweaver Burke plot.

Dehalogenase activities were also measured by determining the production of muconic acid semialdehyde (MSA) with a colorimetric assay specific for β-carbonyl carboxylic acids (Walker 1954; Hayashi 1961). Incubations were carried out as above, but with enzyme preparations dialyzed against 100 mM phosphate buffer, pH 7.5, and substrate also dissolved in phosphate buffer rather than in Tris, since it was expected that amines could catalyze decarboxylation of the rather unstable MSA. Samples of 20 μl were taken from incubation mixtures (30° C) at different timepoints and added to icecold 80 μl phosphate buffer and 20 μl 20% trichloroacetic acid to stop the reaction and inactivate the enzyme. After neutralization with 80 μl 1 M sodium acetate, pH 5.8, MSA was converted to its formazan with diazotized p-nitroaniline and determined colorimetrically at 440 nm as described (Hayaishi et al. 1961; Walker 1954). Using the reported relation between MSA concentration and absorbance (Hayaishi et al. 1961), we found values for MSA concentrations that were higher than theoretically possible, which may be caused by the lability of the synthesized MSA used for calibration. Therefore, A_{440} readings instead of concentrations are given. Control experiments indicated that in this assay the following compounds reacted negative: *cis*- and *trans*-3-chloroacrylic acid, acetaldehyde, carbon dioxide, and acrylic acid. Oxaloacetic acid reacted positive.

Acetaldehyde and MSA production from 3-CAA was tested in closed tubes (total volume 14 ml) at 30° C containing 3 ml 20 mM substrate in 50 mM Tris.SO_4 buffer (pH 7.5). Enzyme (300 mU) was added and 200 μl samples from the gas phase were taken at different timepoints and analysed by gas chromatography, using split injection on a CPwax52-CB column (25 m × 0.25 mm; 50° C injector temperature 55° C). The injector temperature of 55° C was chosen to prevent decomposition of possible unstable products, such as MSA. Analysis of MSA was also attempted by injecting the aqueous phase on a Poraplot Q

column (25 m × 0.25 mm; 80° C; injector temperature 80° C).

Hydratase activity for crotonic acid and acrylic acid was tested by following substrate removal in incubation mixtures containing 5 mM substrate in 2.5 ml 50 mM Tris.SO_4, pH 7.5, and 50 mU of enzyme (for 3CAA). Samples of 0.5 ml were withdrawn at different timepoints, and analyzed by gas chromatography after conversion of carboxylic acids to methylesters as described above for 3CAA's.

Preparation of extracts and enzyme purification

Extracts Cells were harvested by centrifugation and washed with TEM buffer. Washed cells were resuspended in 2–4 volumes of icecold TEM buffer and disrupted at 4° C by 4 passages through a French pressure cell (Aminco) at 15,000 lb/inch^2. This was found to give much more efficient lysis of the cells than sonication. Unbroken cells and debris were removed by centrifugation for 20 min at 16,000 × g. All further operations were carried out at 0–4° C and all buffers contained 1 mM EDTA and 1 mM β-mercaptoethanol to avoid inactivation of the enzymes.

(I) Ammonium sulfate fractionation. The extract was diluted to 5 mg of protein per ml and fractionated by stepwise addition of solid $(NH_4)_2SO_4$ to 50, 60, and 82.5% saturation. After each step the precipitate was collected by centrifugation at 15,000 × g for 20 min, redissolved in TEM buffer and assayed for activity. The 60 to 82.5% fraction contained highest dehalogenase activity for both 3CAA isomers and was dialyzed overnight against TEM buffer.

(II) DEAE-cellulose chromatography. The dialysate was applied to a DEAE-cellulose column (2 by 28 cm) equilibrated with TEM buffer. After washing the column with 30 ml of TEM, elution was carried out with a 0 to 1 M linear gradient of ammonium sulfate in TEM (total volume 400 ml, flow rate 28 ml/h, fraction volume 7.5 ml). Activity for *cis*-3CAA eluted at an ammonium sulfate concentration of 0.11 to 0.25 M, and activity for *trans*-3CAA eluted at a concentration of 0.25 to 0.39 M. Fractions containing activity for *cis*-3CAA and *trans*-3CAA were pooled separately and dialyzed overnight against 4 mM potassium phosphate buffer (pH 6.8) containing EDTA and β-mercaptoethanol (PEM buffer). Further purification of the dehalogenases was carried out separately.

(III) Hydroxylapatite chromatography. The dialysates were applied to hydroxylapatite columns (1.1 by 13 cm) equilibrated with 4 mM PEM (pH 6.8). The columns were washed with 15 ml of PEM buffer (pH 7.0), and elution was carried out with a linear gradient of 4 to 200 mM phosphate buffer, pH 7.0 (total volume 250 ml, flow rate 11 ml/h, fraction volume 5.4 ml). Activity for *cis*-3CAA eluted at a phosphate concentration of 10 to 22 mM and activity for *trans*-3CAA at 14 to 20 mM. Active fractions were pooled and dialyzed overnight against TEM buffer.

(IV) Mono Q FPLC. Of both dialyzed enzyme solutions, 1 mg amounts were further purified by FPLC as the final purification step. Enzyme was applied to a Mono Q FPLC column (5 by 0.5 cm) at room temperature and the column was washed with 4 ml of 50 mM TEM buffer. Elution was carried out with a linear gradient of 0 to 30 mM of ammonium sulfate in 50 mM TEM buffer (0.8 MPa; total volume 30 ml, fraction volume 1 ml). The *cis*-3CAA dehalogenase eluted at an ammonium sulfate concentration of 13 to 14 mM and *trans*-3CAA dehalogenase at 22 to 24 mM ammonium sulfate.

Molecular weight determinations

The molecular weight of the native enzymes was estimated by gel filtration on a FPLC Superose 12 column. The column was used with 4 mM PEM buffer containing 100 mM ammonium sulfate and calibrated with alcohol dehydrogenase (molecular weight 80,000 Da), bovine serum albumin (68,000), ovalbumin (45,000), trypsin inhibitor (20,100) and cytochrome c (12,300).

The molecular weight of the denatured enzymes was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). The gels contained 15% acrylamide and were stained with Coomassie brilliant blue G-250. Transferrin (Molecular weight 78,000 Da), albumin (66,300), ovalbumin (45,000), carbonic anhydrase (30,000), myoglobin (17,200), cytochrome c (12,300) and insulin (6,000) were used as reference proteins.

Amino acid analysis and N-terminal amino acid sequence determination

Determination of N-terminal amino acid sequences and amino acid compositions was performed by Eurosequence BV (Groningen, The Netherlands). For this, the enzymes were dialyzed against triethanolamine-acetic acid buffer (pH 7.0). The proteins were hydrolysed with 5.7 N HCl for two hours at 166° C and analyzed on HPLC (Model 120A, ABI). N-terminal amino acid sequences were determined by Edman degradation with an automatic sequenator (Model 477A, Applied Biosystems).

Sequences were analyzed with the PCGENE package (Genofit, Geneva) and the program FASTP and compared with sequences in the SWISS-PROT protein database (EMBL, Heidelberg), release 11.

The sequences of the subunits of the *trans*-3CAA dehalogenase were determined after separating 150 μg of protein by SDS-PAGE and electro-blotting the peptides on a PVDF-membrane (Immobilon-P, Millipore) using a 25 mM Tris/192 mM glycine buffer containing 15% methanol as transfer buffer (pH 8.3).

Materials and chemicals

Columns for gas chromatography were obtained from Chrompack, Middelburg, The Netherlands. Calibration proteins were obtained from Sigma or Boehringer. Yeast extract was from Becton Dickenson Microbiology Systems. All organic compounds were purchased from Janssen Chimica, Beerse, Belgium, except *trans*-3-chlorocrotonic acid which was prepared during a synthetic organic chemistry course at this university and estimated to be at least 98% pure by NMR spectroscopy. All other chemicals were of analytical grade.

Results

Isolation and characterization of strain FG41

Enrichment of a 3-chloroacrylic acid (3CAA) degrading bacterium was carried out with *trans*-3CAA as the growth substrate and freshwater sediment from a channel near a chemical production plant as the inoculum. From a positive enrichment, a pure culture was obtained by streaking on nutrient agar and by testing colonies by replica plating. One positive organism was strain FG41, which appeared to be able to grow on both isomers of 3-chloroacrylic acid, both in liquid culture and on plates. The organism could be maintained by biweekly transfer on nutrient agar plates without loss of its 3CAA degradation capacity.

Strain FG41 exhibited cream-colored, opaque, round, regular, entire, shiny, and convex formed colonies on BHI agar. The organism grew at 30 and 37° C but not at 45° C. It was identified as a gram-positive, non-motile coryneform bacterium. The range of compounds that supported growth was tested by replica plating. Growth was possible with *cis*-3CAA, *trans*-3CAA, crotonic acid, ethanol, citrate, pyruvate and lactate. No growth was observed on glucose, *trans*-3-chlorocrotonic acid, 3-chloropropionic acid, 2-chloropropionic acid, chloroacetic acid, 3-chloroallyl alcohol, 3-chloropropanol, 1,3-dichloropropane, *cis*- and *trans*-1,3-dichloropropene, acrylic acid, acetaldehyde and methanol.

Degradation of cis-*3CAA and* trans-*3CAA in batch cultures*

Growth with *cis*-3CAA and *trans*-3CAA as substrates was followed in batch cultures (Fig. 1a and b). Almost immediately after inoculation, growth and simultaneous chloride production and deple-

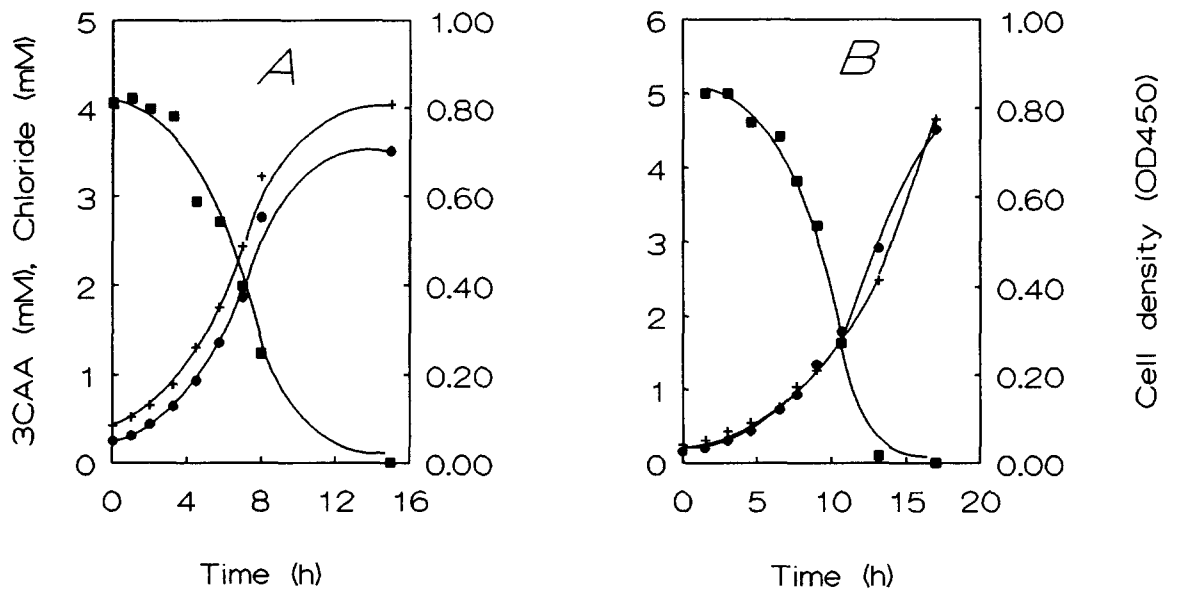

Fig. 1. Growth of strain FG41 in batch cultures on 5 mM *cis*-3CAA (*A*) and 5 mM *trans*-3CAA (*B*). Growth was measured turbidimetrically at 450 nm (+). The chloride levels (●) and 3CAA concentrations (■) were followed.

tion of the substrate started. Growth was exponential with both 3CAA's and generation times were 2.8 h and 3.8 h for the *cis*- and *trans*-isomer, respectively. Essentially complete dechlorination occurred.

Dehalogenation in crude extracts

The presence of dehalogenating activity in cell free extracts was tested with extract prepared from cells grown on *trans*-3CAA to the late exponential phase of growth. It was found that extracts from cells grown in the presence of *trans*-3CAA contained an activity that readily liberated inorganic chloride from both *cis*- and *trans*-3CAA (Table 1). This dehalogenase did not require glutathione, NADPH, NADH, or metal ions for activity.

The regulation of the dehalogenases was studied by measuring dehalogenating activities in extracts prepared from cells grown on citrate medium to which 1 mM of the potentially inducing compounds was added 3 h before harvesting. It appeared that the dehalogenase activities were not constitutively present but could be induced by *cis*-3CAA, *trans*-3CAA and *trans*-3-chlorocrotonic acid (Table 1) but not by crotonic acid or acrylic acid. In all cases, the ratio between the *cis*- and *trans*-3CAA dehalogenase activities was 0.25–0.5.

A culture grown on 10 mM citrate in the presence of 1 mM *trans*-3-chlorocrotonic acid was subsequently tested for the presence of chloride in the culture fluid. Since no chloride could be detected, *trans*-3-chlorocrotonic acid could be used as a gra-

Table 1. Induction of 3CAA dehalogenases in strain FG41 grown on citrate.

Inducer (1 mM)	Specific activities[a] (U/mg of protein)	
	cis-3CAA	*trans*-3CAA
none	< 0.01	< 0.01
cis-3CAA	0.27	1.14
trans-3CAA	0.26	1.08
trans-3-chlorocrotonic acid	0.14	0.53

[a] Specific activities of 3CAA dehalogenases in crude extracts of cells grown on 20 mM citrate induced for 3 hours with the inducer indicated.

tuitous inducer for the synthesis of both dehalogenases.

Purification of the 3CAA dehalogenases

The purification scheme for the 3CAA dehalogenases of strain FG41 is summarized in Table 2. The elution profile of the DEAE-cellulose column (Fig. 2) showed that the *cis*- and *trans*-specific dehalogenases were separated and thus were different enzymes. From a 10 l culture, grown to a density of 1.7 g dry weight per l, an amount of 9 mg of each the *cis*- and *trans*-specific 3CAA dehalogenase was obtained after hydroxylapatite chromatography. Only a small amount of the enzyme preparations was further purified by FPLC. Figure 3 shows a SDS-polyacrylamide gel with protein samples of all different purification steps. The *cis*-3CAA dehalogenase was purified 24-fold and *trans*-3CAA dehalogenase was purified 29-fold. This implies that each of the dehalogenases represented about 4% of the total cellular protein.

After filter sterilization, the enzymes were stored in TEM buffer for several months at 4° C with no significant loss of activity.

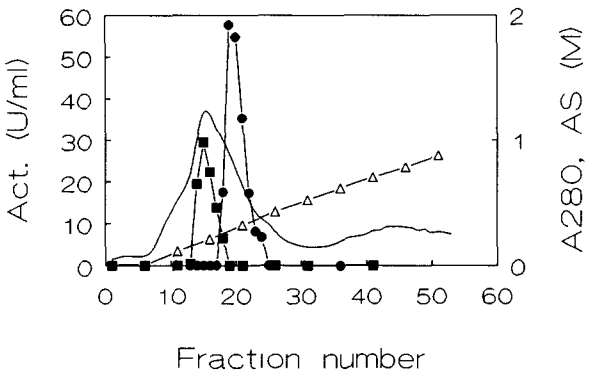

Fig. 2. Separation of *trans*-3CAA dehalogenase and *cis*-3CAA dehalogenase by DEAE-cellulose chromatography. Fractions were assayed for protein at 280 nm (——); ammonium sulfate concentration (△); *cis*-3CAA dehalogenase (■); and *trans*-3CAA dehalogenase activity (●).

Amino acid analysis and N-terminal amino acid sequence determination

The molecular weight of *cis*-3CAA dehalogenase was estimated by gel filtration to be 38 kDa. Since the band obtained with SDS-PAGE represents a polypeptide with an estimated molecular weight of 16.2 kDa, the *cis*-3CAA dehalogenase is probably a homodimeric protein.

After gel filtration, the molecular weight of *trans*-3CAA dehalogenase was estimated to be

Table 2. Purification of *cis*- and *trans*-3-chloroacrylic acid dehalogenases.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg of protein)	Yield (%)	Purification factor
trans-3CAA dehalogenase					
Crude extract	1310	1260	0.98	100	1.0
Ammonium sulfate	109	399	3.7	32	3.7
DEAE-cellulose	32	373	12	30	12
Hydroxylapatite	8.6	218	26	17	26
Mono Q FPLC[a]	–	–	28	–	29
cis-3CAA dehalogenase					
Crude extract	1310	638	0.49	100	1.0
Ammonium sulfate	109	211	1.9	33	3.9
DEAE cellulose	38	159	4.1	19	8.5
Hydroxylapatite	9.9	95	9.6	15	20
Mono Q FPLC[a]	–	–	12	–	24

[a] Only a part of the enzyme solutions was purified by Mono Q FPLC.

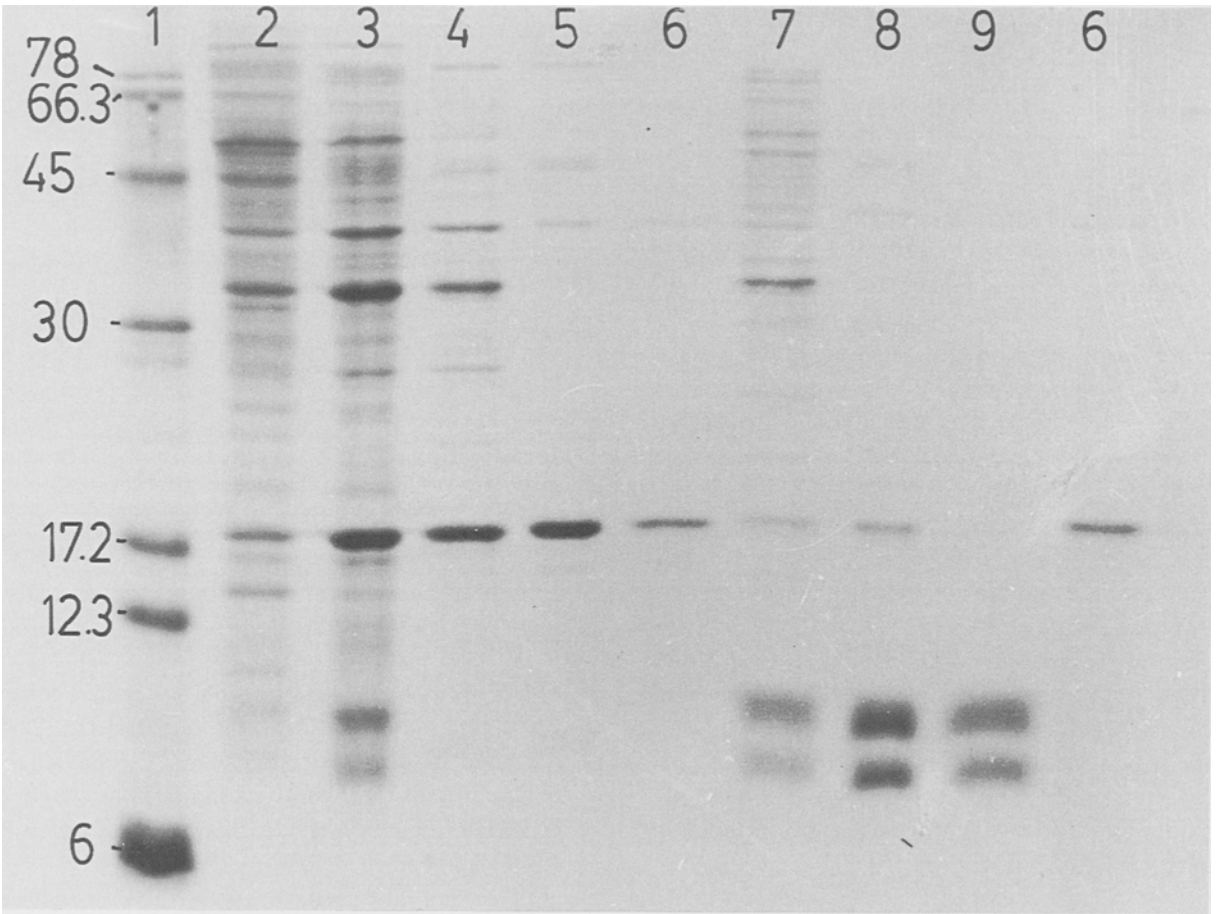

Fig. 3. SDS-polyacrylamide gel of samples of different purification steps: (1), reference proteins; (2), crude extract; (3), 60 to 80% ammonium sulfate precipitate; (4), *cis*-3CAA dehalogenase after DEAE-cellulose chromatography; (5), idem, after hydroxylapatite chromatography; (6), 15 μg of purified *cis*-3CAA dehalogenase; (7), *trans*-3CAA dehalogenase fraction after DEAE-cellulose chromatography; (8), idem, after hydroxylapatite chromatography; (9), 15 μg of purified *trans*-3CAA dehalogenase.

50 kDa. Upon SDS-PAGE, two bands were observed with similar staining intensities and estimated molecular weight of 8.7 and 7.4 kDa.

In order to investigate whether the enzymes were biochemically related, the amino acid composition and N-terminal amino acid sequences were determined. The amino acid compositions of the enzymes (Table 3) are similar, but *cis*-3CAA dehalogenase contained 8, while *trans*-3CAA dehalogenase contained no tyrosine residues. Data of absorption at 280 nm (not shown) indicate that *trans*-3CAA dehalogenase also contained no tryptophan.

The following N-terminal sequence of the *cis*-3CAA dehalogenase was obtained:

1 Pro-Val-Tyr-Met-Val-Tyr-Val-Ser-Gln-Asp-
11 Arg-Leu-Thr-Pro-Ser-Ala-Lys-His-Ala-Val-
21 Ala-Lys-Ala-Ile-Thr-Asp-Ala-His-Arg-Gly-
31 Leu-Thr-Gly-Thr-Gln-His-Phe-Leu-Ala-Gln-
41 Val-Asn-Phe-Gln-Glu-Gln-Pro-Ala-

For the 8.7 kDa subunit of *trans*-3CAA dehalogenase the obtained sequence was:

1 Ser-Ile-Ile-Ser- ? -Asp-Met-Arg-Glu-Gly-
11 Arg-Thr-Asp-Asp-Gln-Lys-Arg-Ala-Leu-Ser-
21 Gly- ? -Leu-Ile-Glu-Ala-Val- ? -Asn-Val-

31 Thr-Gly-Glu-Pro-

This will represent about 40% of the whole sequence of the 8.7 kDa subunit. The N-terminal sequence of the 7.4 kDa subunit was:

1 Pro-Phe-Ile-Glu-Ile-Arg-Leu-Pro-Lys-Pro-
11 Leu-

Computer alignment of the N-terminal sequences of the *cis*- and *trans*-3CAA dehalogenases did not result in any detectable homology. The N-terminal sequences of the two subunits of the *trans*-3CAA dehalogenase also where completely different from each other.

Enzymatic activities of the 3CAA dehalogenases

The identity of the products formed from 3CAA's by the dehalogenases was investigated. Gas chromatographic analysis of incubations (water and headspace) containing enzyme and 5 mM 3-chloroacrylic acids showed the presence of acetaldehyde. No malonic acid semialdehyde (MSA) was detected on different GC columns. This could indicate that the dehalogenating enzymes also catalyzed decarboxylation. However, β-carbonyl carboxylic acids such as MSA rapidly undergo non-enzymatic decarboxylation (Jencks 1987), which could occur during incubation or analysis. Therefore, a colorimetric assay was used for MSA, and it appeared that with both the *cis*- and the *trans*-specific dehalogenase the production of chloride from 3CAA was accompanied by MSA formation (Fig. 4). With the *cis* and *trans* enzymes, the same amount of MSA was produced per amount of chloride liberated.

Both dehalogenase were completely isomer-selective. Neither of the two dehalogenases showed detectable dehalogenase or hydratase activity (< 1 mU/mg of protein) with 3-chloropropionic acid, 2-chloropropionic acid, chloroacetic acid, crotonic acid, allylchloride, 3-chloroallylalcohol, or *cis*- and *trans*-1,3-dichloropropene. The *cis* dehalogenase did not convert *trans*-3CAA and the *trans* dehalogenase showed no detectable activity with *cis*-3CAA.

Both enzymes showed optimum activity at pH 7.5 to 8.0 in Tris-SO_4 buffers.

Dechlorination of *cis*-3CAA with the *cis* dehalogenase followed Michaelis Menten kinetics. The K_m value was 1.1 mM and the V_{max} 34 mU/mg of purified enzyme, corresponding to a turnover number of 20 s^{-1}.

The rate of dehalogenation of *trans*-3CAA by the *trans*-specific dehalogenase was also dependent on substrate concentration according to Michaelis Menten kinetics. A K_m of 1.4 mM and a V_{max} of 19 mU per mg of pure protein were determined. This corresponds to a turnover number of 16 s^{-1}, based on a molecular weight of 50 kDa.

The activity of both the *cis*-3CAA dehalogenase and the *trans*-3CAA dehalogenase was completely lost if $HgCl_2$ (0.1 mM) was added prior to incubation. Both enzymes were not significantly inhibited by the thiol reagents iodoacetamide (1 mM), p-

Table 3. Amino acid composition of the 3CAA dehalogenases.

Amino acid	*cis*-3CAA dehalogenase		*trans*-3CAA dehalogenase	
	Content (mole%)	No. of residues[ab]	Content (mole%)	No. of residues[c]
Asx	10.3	30	14.1	60
Glx	17.4	50	17.9	76
Ser	5.1	14	5.5	23
His	5.5	16	5.1	22
Gly	3.6	10	3.1	13
Thr	5.5	16	5.1	22
Ala	9.7	28	8.2	34
Arg	6.7	20	6.1	26
Tyr	2.4	8	0.1	0
Cys	1.4	4	0.7	3
Val	9.1	26	7.5	32
Met	2.9	8	2.7	11
Phe	5.6	16	4.3	18
Ile	2.9	8	6.6	28
Leu	6.7	20	9.1	38
Lys	2.4	6	1.9	8
Pro	2.3	6	2.0	8

[a] Tryptophan was not determined which might lead to an overestimation of other residues.
[b] Values were calculated on the basis of a molecular weight of 32,400 with two identical monomers.
[c] Values were calculated on the basis of a molecular weight of 48,300.

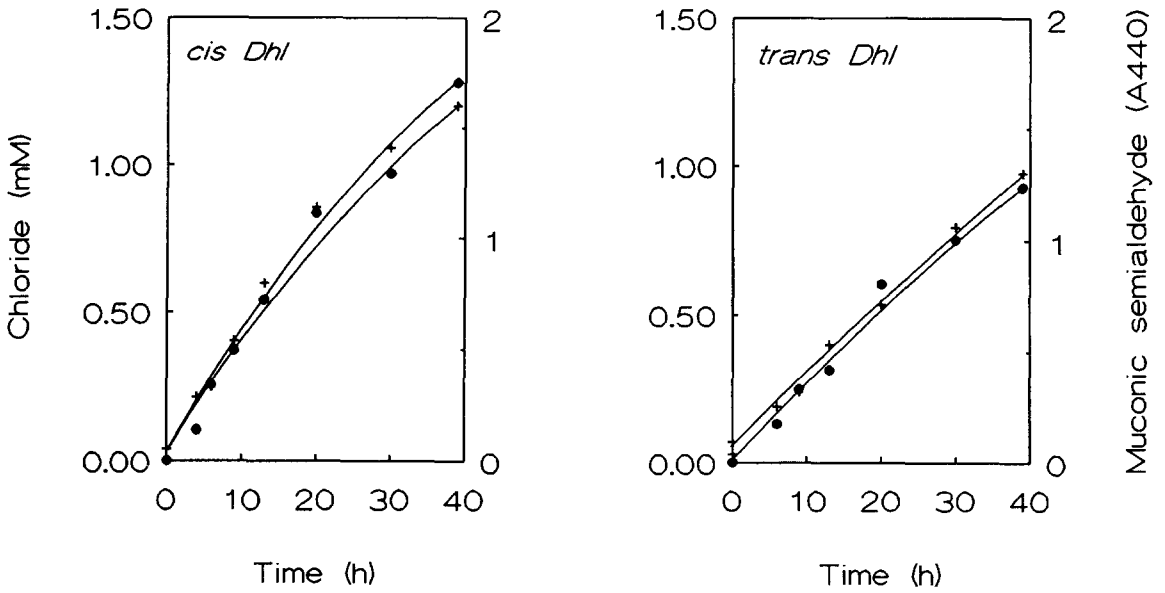

Fig. 4. Chloride liberation and muconic acid semialdehyde formation during incubation of *cis*-3CAA dehalogenase and *trans*-3CAA dehalogenase with their respective substrates (5 mM). Symbols. (●), chloride; (+), muconic acid semialdehyde (MSA).

chloromercurybenzoate (1 μM) and N-ethylmaleimide (1 mM).

Some substrate analogs were tested for inhibition. Neither of the enzymes was inhibited by crotonic acid (20 mM), *trans*-3-chlorocrotonic acid (20 mM), or the 3-chloroacrylic acid isomer that was not a substrate. The *cis* dehalogenase was not inhibited by acrylic acid, but this compound strongly inhibited dehalogenation of *trans*-3CAA by the *trans* specific enzyme. At 0.1 mM acrylic acid and 5 mM *trans*-3CAA, the enzyme activity was reduced to 32% of the control.

Discussion

The results presented in this paper show that the coryneform bacterium strain FG41 is able to use *cis*- and *trans*-3CAA as sole carbon and energy source for growth and that dehalogenation of the 3CAA's is catalyzed by two different dehalogenases. *Trans*-3CAA dehalogenase only showed activity for *trans*-3CAA and *cis*-3CAA dehalogenase was completely specific for *cis*-3CAA. The dehalogenases were found to liberate inorganic chloride from the 3-chloroacrylic acids, and hydroxymuconic acid semialdehyde was formed by both enzymes. This compound is known to be rather unstable and may, like other β-carbonyl carboxylic acids, undergo decarboxylation leading to acetaldehyde. Therefore, derivatization of the carboxyl group to the corresponding formazan was used for detection (Hayaishi et al. 1961) rather than gas chromatography or derivatization of the aldehyde function to a phenylhydrazone. With a chloroallyl alcohol degrading bacterium, MSA also has been described as a product of dehalogenation of 3-CAA (Belser & Castro 1971), suggesting that a similar dehalogenase was present in that organism.

The route of malonic acid semialdehyde assimilation has been studied previously. It has been described as an intermediate in the bacterial utilization of β-alanine (Hayashi et al. 1961; Waters & Venables 1986) and propynoic acid (Yamada & Jakoby 1959). In these cases, MSA was found to be further converted by an acetyl CoA and NAD dependent malonic acid semialdehyde oxidative decarboxylase to acetyl CoA, with reduction of the NAD (Hayashi 1961; Waters & Venables 1986).

The dehalogenases were very selective, and with all other compounds that were tested, no activity could be detected. Hydration of acrylic acid did not

occur, indicating that a halogen on the β-position is necessary. The lack of activity with 3-chloroallyl alcohol indicates that the compound must have a carboxyl group to be a substrate. The absence of cross-activity with the *cis*- and *trans*-isomers and the lack of activity of both enzymes for *trans*-3-chlorocrotonic acid, crotonic acid and acrylic acid might be due to steric reasons, which would be in agreement with the observation that *cis*- and *trans*-3CAA do not inhibit *trans*- and *cis*-dehalogenase, respectively. Acrylic acid inhibites *cis*- but not *trans*-3CAA dehalogenase, again suggesting different ways of substrate binding between the two enzymes. Thiol groups are probably not involved in the active center since thiol reagents do not inhibit the dehalogenases.

The two dehalogenases appeared to be present in similar relative amounts after induction with either *cis*- or *trans*-3CAA or with 3-chlorocrotonic acid. This could mean that the enzymes are controlled by the same regulatory protein. The two enzymes appeared not to be closely related, however, as indicated by differences in subunit size and composition, amino acid composition, and N-terminal amino acid sequences. The *cis*-3CAA dehalogenase is probably an α_2 protein of 16 kDa subunits, while the *trans*-specific enzyme has could be a hexameric protein ($\alpha_3\beta_3$) with 8.7 and 7.4 kDa subunits.

The results suggest that the dehalogenation of 3CAA's proceeds by hydration of the carbon-carbon double bond, yielding an unstable intermediate from which HCl is eliminated and MSA is produced. Thus, hydration of unsaturated carboxylic acids with β-chlorine substituents is a possible way of dehalogenation, and *cis*- and *trans*-specific dehalogenases can be involved. Direct proof that dehalogenation is mechanistically a hydration reaction is still lacking. The non-halogenated compounds acrylic and crotonic acid, which would lead to stable hydrated products, were not converted by either of the dehalogenases. This indicates that the enzymes are not hydratases that, just as a result of a lack of substrate specificity, cause dehalogenation in case a chlorine is present on the β-carbon of the substrate. The selective induction by chlorinated substrates and the observation that acrylic acid strongly inhibits one of the dehalogenases point to dehalogenation being the real function of the enzymes, and also to a role of the chlorine substituent in the actual catalytic reaction of the *cis*-3CAA specific enzyme.

Alignment (FASTX) of the N-terminal sequences of the dehalogenase peptides with the EMBL protein library resulted in a some similarity between the *cis*-dehalogenase and 3-dehydroshikimate dehydratase (EC 4.2.1.-) (Rutledge 1984) as the highest scoring protein, with 35.4% identity (similarity 41.6%) in a 64 amino acid overlap. Although it is tempting to speculate about similarities between hydratases and a dehydratase, it is doubtful whether this is really significant, since this score is not high, does not involve the N-terminus of the dehydratase, and the total amino acid composition and properties are very different. Thus, the activities of the dehalogenases, their biochemical characteristics and sequence data do not point to an evolutionary relationship of with hydratases, such as the enoyl CoA hydratase involved in β-oxidation or fumarase, or with other sequenced proteins. Interesting questions about the evolutionary origin of these enzymes, which specifically act on halogenated compounds that are not normally present in the environment, thus remain unanswered, as for other bacterial dehalogenases.

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