

Dehalogenation of 4-chlorobenzoate

Characterisation of 4-chlorobenzoyl-coenzyme A dehalogenase from *Pseudomonas* sp. CBS3

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Abstract

Pseudomonas sp. CBS3 is capable of growing with 4-chlorobenzoate as sole source of carbon and energy. The removal of the chlorine of 4-chlorobenzoate is performed in the first degradation step by an enzyme system consisting of three proteins. A 4-halobenzoate-coenzyme A ligase activates 4-chlorobenzoate in a coenzyme A, ATP and Mg²⁺ dependent reaction to 4-chlorobenzoyl-coenzyme A. This thioester intermediate is dehalogenated by the 4-chlorobenzoyl-coenzyme A dehalogenase. Finally coenzyme A is split off by a 4-hydroxybenzoyl-CoA thioesterase to form 4-hydroxybenzoate. The involved 4-chlorobenzoyl-coenzyme A dehalogenase was purified to apparent homogeneity by a five-step purification procedure. The native enzyme had an apparent molecular mass of 120,000 and was composed of four identical polypeptide subunits of 31 kDa. The enzyme displayed an isoelectric point of 6.7. The maximal initial rate of catalysis was achieved at pH 10 at 60 °C. The apparent K_m value for 4-chlorobenzoyl-coenzyme A was 2.4–2.7 μM . V_{max} was $1.1 \times 10^{-7} \text{ M sec}^{-1}$ (2.2 $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ of protein). The NH₂-terminal amino acid sequence was determined. All 4-halobenzoyl-coenzyme A thioesters, except 4-fluorobenzoyl-coenzyme A, were dehalogenated by the 4-chlorobenzoyl-CoA dehalogenase.

Abbreviations: CBA – chlorobenzoate, CoA – coenzyme A, HBA – hydroxybenzoate, DTT – dithiothreitol, HPLC – high performance liquid chromatography, PAGE – polyacrylamide gel electrophoresis

Introduction

4-Chlorobenzoate was found as intermediate in the degradation of polychlorinated biphenyls (Abramowicz 1990) and of the herbicide bidisin (Köcher et al. 1976). Furthermore, 4-CBA was chosen as a model substance to study the degradation of halogenated aromatic compounds due to its low toxicity and good water solubility.

Several bacterial species, among them *Pseudomonas* sp. CBS3, are capable of using 4-CBA as sole source of carbon and energy. In the cases of *Pseudomonas* sp. CBS3 (Löffler et al. 1991; Löffler & Müller 1991; Scholten et al. 1991; Chang et al. 1992)

and *Acinetobacter* sp. 4-CB1 (Copley & Crooks 1992; Crooks & Copley 1993), 4-chlorobenzoyl-CoA and 4-hydroxybenzoyl-CoA were found as intermediates in the dehalogenating reaction as shown in Fig. 1. Groenewegen et al. (1992) found 4-chlorobenzoyl-CoA as intermediate in the degradation of 4-CBA by the coryneform bacterium NBT-1.

The genes for the three proteins of the 4-CBA dehalogenase from *Pseudomonas* sp. CBS3, namely 4-halobenzoate-CoA ligase, 4-chlorobenzoyl-CoA dehalogenase and 4-hydroxybenzoyl-CoA thioesterase, have been cloned in *E. coli* (Savard et al. 1986; Elsner et al. 1991; Scholten et al. 1991). Chang et al. (1992) succeeded in separately cloning and over-

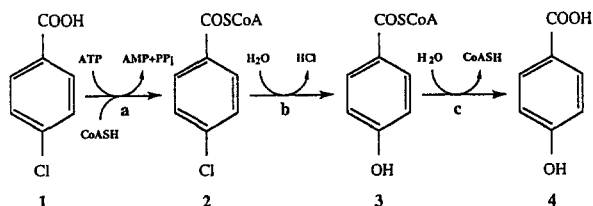


Fig. 1. The reaction catalysed by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3. The three steps are catalysed by 4-chlorobenzoate-CoA ligase (a), 4-chlorobenzoyl-CoA dehalogenase (b) and 4-hydroxybenzoyl-CoA thioesterase (c). (1) 4-chlorobenzoate; (2) 4-chlorobenzoyl-CoA; (3) 4-hydroxybenzoyl-CoA; (4) 4-hydroxybenzoate.

expressing the three genes in *E. coli* and Babbitt et al. (1992) determined the sequence of the three genes. The proteins encoded by the overexpressed genes were characterised from extracts of the different *E. coli* clones (Elsner et al. 1991; Chang et al. 1992). From the original strain *Pseudomonas* sp. CBS3, containing the 4-chlorobenzoate-CoA ligase, the 4-chlorobenzoyl-CoA dehalogenase and the 4-hydroxybenzoyl-CoA thioesterase, only the 4-chlorobenzoate-CoA ligase was studied in more detail so far (Löffler et al. 1992). This paper deals with the purification and characterisation of the hydrolytic aryl-dehalogenating enzyme 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp. CBS3.

Materials and methods

Organism and growth conditions. The isolation, characterisation and cultivation of *Pseudomonas* sp. CBS3 has been reported (Klages & Lingens 1980; Löffler et al. 1992). Cells were harvested at the end of the exponential growth phase. *Pseudomonas* sp. CBS3 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) under the number DSM 6613.

Chemicals. Chemicals were purchased from Merck (Darmstadt, Germany), Fluka (Neu-Ulm, Germany) and Sigma (Heidelberg, Germany). Materials for chromatography were obtained from Pharmacia (Freiburg, Germany) and Sigma. 4-Chlorobenzoyl-CoA was synthesised enzymatically with the purified 4-chlorobenzoate-CoA ligase (Löffler et al. 1992). 0.6 mg of the purified 4-CBA-CoA ligase were immo-

bilised on 1.5 ml phenylbutylamine-Eupergit (Röhm, Weiterstadt, Germany) in a 10 ml column using 50 mM Tricine-NaOH buffer, pH 7.1. When no 4-CBA-CoA ligase activity in the supernatant was measurable, 150 μ mol ATP, 150 μ mol MgCl₂, 100 μ mol CoA and 100 μ mol 4-CBA were added to a final volume of 6 ml. A continuous flow (0.25 ml min⁻¹) through the gel matrix was maintained using a peristaltic pump. The reaction product 4-chlorobenzoyl-CoA was purified from the supernatant by preparative HPLC (Löffler & Müller 1991). 4-Fluoro-, 4-bromo- and 4-iodobenzoyl-CoA were synthesised with the soluble 4-CBA-CoA ligase (10 μ g) in a total volume of 600 μ l. ATP, Mg²⁺, CoA and the 4-halobenzoate (10 mM each) were added and incubated at 30 °C. After 5 hours the reaction was stopped by acidification with 4 M HCl. The thioesters were purified with HPLC and used as putative substrates for the 4-chlorobenzoyl-CoA dehalogenase.

Preparation of cell-free crude extracts. 50 g frozen cells (wet weight) were thawed and suspended in 50 ml 50 mM potassium phosphate buffer (pH 7.5). The suspension was subjected to ultrasonic treatment for 10 min in 1-sec bursts at an output of 80 W with an ultrasonic disintegrator (Branson sonifier 450). The cell debris was removed by centrifugation at 45,000 \times g and 4 °C for 60 min. The precipitate was washed once with 25 ml 50 mM potassium phosphate buffer (pH 7.5). The combined supernatants were designated as crude cell extract and contained 30–35 mg of protein ml⁻¹.

Enzyme assays

1. 4-Chlorobenzoyl-CoA dehalogenase. The dehalogenation of 4-chlorobenzoyl-CoA was followed by measuring the disappearance of the substrate or the formation of products with HPLC at 258 nm as described (Löffler & Müller 1991). 4-Hydroxybenzoyl-CoA inhibited the dehalogenase significantly. To avoid this product inhibition, 4-hydroxybenzoyl-CoA thioesterase was added to ensure that the intermediately formed 4-hydroxybenzoyl-CoA was converted to 4-HBA and CoA. The reaction mixture (0.2 ml) contained 200 mM potassium phosphate buffer, pH 7.5, 0.1 mM 4-chlorobenzoyl-CoA, an appropriate amount of 4-hydroxybenzoyl-CoA thioesterase and the protein fraction (0.5–1.2 U ml⁻¹). The assay mixture was allowed to equilibrate at 30 °C and then the reaction was started by the addition

of the substrate. The reaction was stopped after 2 to 15 min through the addition of 5 μ l 4 M HCl.

One unit is defined as the amount of enzyme which dehalogenates 1 μ mol of 4-chlorobenzoyl-CoA per min under these conditions.

II. 4-Hydroxybenzoyl-CoA thioesterase. 4-Hydroxybenzoyl-CoA was synthesised enzymatically with the purified 4-chlorobenzoyl-CoA dehalogenase from 4-chlorobenzoyl-CoA. The 4-hydroxybenzoyl-CoA thioesterase hydrolysed 4-hydroxybenzoyl-CoA and the formation of 4-HBA and CoA was followed by HPLC.

In general, enzyme activities were determined in a range in which product formation versus protein concentration and incubation time was linear.

Purification of 4-chlorobenzoyl-CoA dehalogenase

All purification steps were carried out under aerobic conditions at room temperature, unless otherwise indicated.

Heat treatment. The crude cell extract was incubated at 55 °C in a water bath for 5 min. The precipitated protein was removed by centrifugation (45,000 \times g, 4 °C, 20 min) and washed once with 25 ml of 20 mM potassium phosphate buffer, pH 7.0. The clear supernatants were combined and concentrated by ultrafiltration (cut off 30 kDa).

Hydroxyapatite chromatography. Hydroxyapatite was prepared as described (Atkinson et al. 1973). The soluble protein fraction was loaded onto a hydroxyapatite column (diameter 2.5 cm; volume 90 ml), previously equilibrated with 20 mM potassium phosphate buffer, pH 7.0, at a flow rate of 1.5 ml min⁻¹. Adsorbed proteins were eluted in a 600 ml linear gradient of 20 to 300 mM potassium phosphate buffer, pH 7.0. Fractions of 11 ml were collected.

Q-sepharose chromatography. The fractions containing 4-chlorobenzoyl-CoA dehalogenase activity from the hydroxyapatite column were pooled, concentrated by ultrafiltration (cut off 30 kDa). The concentrated extract was applied at a flow rate of 0.7 ml min⁻¹ onto a Q-Sepharose column (diameter 2.5 cm; volume 35 ml) which had been equilibrated with 20 mM Tris-HCl buffer, 1 mM dithiothreitol (DTT), pH 7.8. The dehalogenase was eluted in a 500 ml linear

NaCl gradient (80 to 280 mM NaCl) and fractions of 10 ml were collected.

Gel filtration chromatography. The fractions containing dehalogenase activity from the Q-Sepharose column were pooled and concentrated by ultrafiltration (cut off 30 kDa). Portions of 1 ml were passed through a Sephadex G 150 sf column (diameter 1.5 cm; volume 170 ml) at a flow rate of 3.2 ml h⁻¹. The column was previously equilibrated with 25 mM potassium phosphate buffer, 150 mM NaCl, 0.2 mM Triton X-100, 1 mM DTT, pH 7.8. Fractions of 2 ml were assayed for dehalogenating activity. For determination of the native molecular mass, a Superdex Hiload 200 16/60 column (diameter 1.6 cm; volume 124 ml), a Sephadex G 150 sf column (diameter 1.5 cm; volume 170 ml) and a TSK G 3000 sw column (7.5 \times 60 mm) were used. The columns were calibrated with the following molecular mass standards: ferritin, 440 kDa; catalase, 240 kDa; aldolase, 160 kDa; γ -globulin, 150 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsin, 25.8 kDa.

Mono Q chromatography. The final purification step was performed with a Mono Q HR 5/5 column equilibrated with 20 mM Tris-HCl, 0.2 mM Triton X-100, 100 mM NaCl, 1 mM DTT, pH 8.0, with a fast protein liquid chromatography system (Pharmacia). The dehalogenase containing fractions from the gel filtration column were pooled and rinsed with the same buffer in an ultrafiltration cell (cut off 10 kDa). Portions of 1 ml were applied onto the Mono Q column which was run at a flow rate of 1 ml min⁻¹. Proteins were eluted with a step and linear gradient of sodium chloride (percentage of 1 M NaCl in volume of buffer: 15%, 20 ml; 15–25%, 28 ml). Fractions of 1 ml were collected and tested for dehalogenating activity.

Protein estimation. Protein concentrations were determined by the method of Lowry et al. (1953). Ovalbumin was used as the standard. Buffers identical to those containing the protein samples were used as blanks.

Determination of the NH₂-terminal amino acid sequence

The NH₂-terminal amino acid sequence of the dehalogenase was obtained with protein bands blotted from SDS-PAGE gels onto Immobilon membranes. Semidry blotting was performed as recommended by Pharmacia

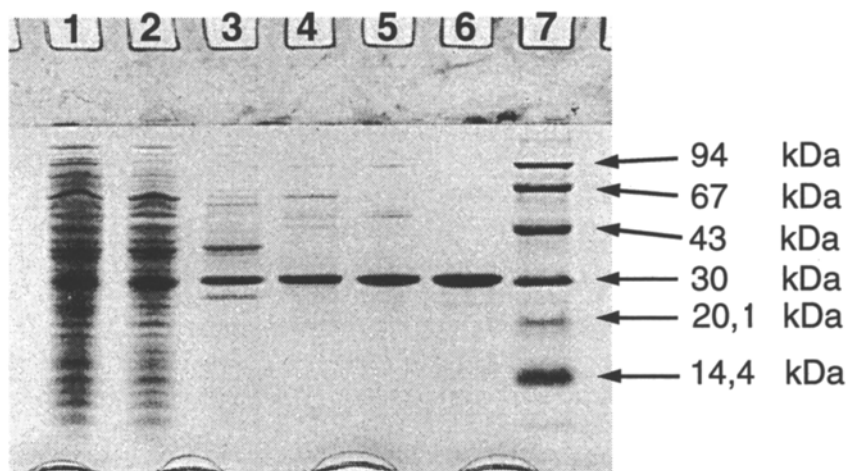


Fig. 2. SDS-PAGE of the pooled fractions of the successive purification steps. Lanes: 1, crude cell extract; 2, extract after heat treatment; 3, after hydroxyapatite; 4, after Q-Sepharose; 5, after Sephadex G 150 sf; 6, after Mono Q; 7, molecular mass standards, containing phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000) and α -lactalbumin (14,400). Proteins were stained with Coomassie Brilliant Blue R-250.

(bulletin SD RE-072, Pharmacia LKB, Freiburg, Germany). Proteins were subjected to automated Edman degradation in a 471A gas-phase protein sequencer (Applied Biosystems, Weiterstadt, Germany).

Gel electrophoresis. In SDS-PAGE the discontinuous buffer system described by Schägger & von Jagow (1987) was used. For molecular mass estimation the Low Molecular Weight Calibration Kit and the GelImage System from Pharmacia were used. Native PAGE was performed as described (Weightman & Slater 1980; Hames & Rickwood 1990). For isoelectric focusing precast gels from Pharmacia and Serva were used (pH range 3.5–9.5). IEF was done as described in the manufacturer's recommendations. The pH-gradient was followed using the Broad pI Calibration Kit (pH 3–10) from Pharmacia and with a surface pH-electrode.

Results

Dehalogenating activity in cell-free extracts. The dechlorination of 4-chlorobenzoyl-CoA in cell-free extracts of *Pseudomonas* sp. CBS3 was only detectable when cells were grown aerobically in mineral salts medium with 4-CBA. Other inducers were 4-iodobenzoate and 4-bromobenzoate. No conversion of 4-chlorobenzoyl-CoA to 4-hydroxybenzoyl-CoA

was observed in cell-free extracts when the cells were grown in mineral salts medium with glucose or a complex medium without 4-chlorobenzoate, 4-bromobenzoate or 4-iodobenzoate. This indicated that the 4-chlorobenzoyl-CoA dehalogenase was not expressed constitutively but was induced in the presence of these 4-halobenzoates as was confirmed for the 4-chlorobenzoate-CoA ligase (Löffler et al. 1992) and the 4-hydroxybenzoyl-CoA thioesterase (Löffler & Müller, unpublished results). 4-Fluorobenzoate was not used as a growth substrate and inhibited the growth of *Pseudomonas* sp. CBS3 with the other 4-halobenzoates.

Cell-free crude extracts converted 4-chlorobenzoyl-CoA to 4-HBA. The next degradation step was dependent on NAD(P)H and lead to 3,4-dihydroxybenzoate (manuscript in preparation). The further degradation followed the well known β -keto adipate pathway (Stanier & Ornston 1973).

Purification of 4-chlorobenzoyl-CoA dehalogenase. The results of the purification procedure described in Materials and Methods are summarised in Table 1. The 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp. CBS3 was purified about 16-fold with an average yield of 19 mg per 50 g of cells (wet weight). The five-step purification procedure resulted in a homogeneous enzyme preparation (Fig. 2, 4 and 5). The purified 4-chlorobenzoyl-CoA dehalogenase

Table 1. Purification protocol for 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp. CBS3. Starting material: 50 g of cell (wet weight), grown with 4-chlorobenzoate.

Purification step	Volume [ml]	Protein [mg/ml]	Total protein [mg]	Total activity [U]	Specific activity [U/mg]	Purification [n fold]	Recovery [%]
Crude extract	105	39,5	4148	588	0,14	1	100
Temperature-precipitation	109	21,3	2322	523	0,23	1,6	89
Hydroxyapatite	230	1,95	449	334	0,75	5,4	57
Q-Sepharose	130	1	130	150	1,15	8,2	26
Sephadex G 150 sf	48	1,05	50	93	1,94	13,9	16
Mono Q	160	0,12	19	45	2,25	16	8

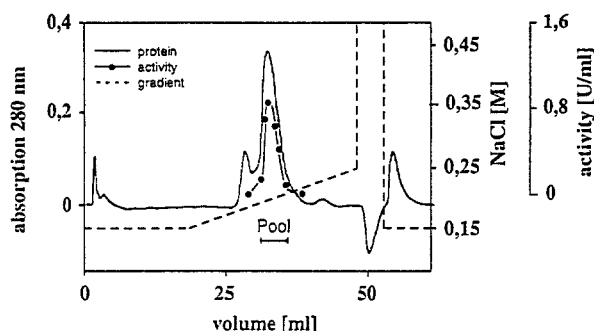


Fig. 3. Elution profile from a Mono Q 5/5 column (the pooled fractions showing dehalogenating activity from the gel filtration step were applied onto the column). Proteins were eluted in a step-and linear gradient of NaCl (—). The 4-chlorobenzoyl-CoA dehalogenase activity was associated with the major protein peak.

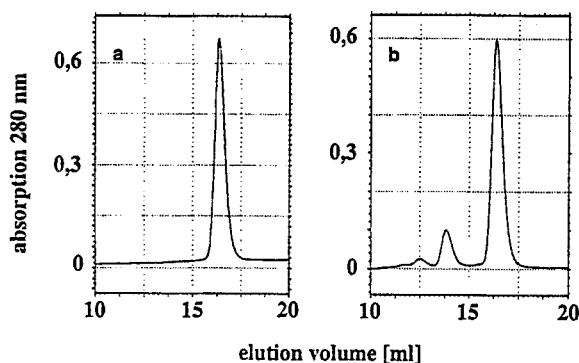


Fig. 4. In size exclusion chromatography experiments on TSK G 3000 sw the purified 4-chlorobenzoyl-CoA-dehalogenase eluted in a single peak if the buffer contained 2 mM DTT (a); without DTT additional protein peaks showing dehalogenating activity were obtained (b).

catalysed the dehalogenation of 4-chlorobenzoyl-CoA to 4-hydroxybenzoyl-CoA. For the gel filtration step on Sephadex G 150 sf the addition of DTT (1 mM) and Triton X-100 (0.2 mM) to the buffer was essential. If no detergent was added to the elution buffer, it was not possible to correlate the dehalogenating activity to a distinct protein peak, probably due to a hydrophobic character of the enzyme. The omission of DTT resulted in two peaks both showing dehalogenating activity. One peak revealed a K_{av} -value of 0.365 indicating a molecular mass of about 100,000. The other protein peak eluted near the void volume (K_{av} -value of 0.245) indicating a molecular mass of more than 200,000. In the presence of DTT only one peak correlated to a molecular mass of 100,000 was found. The same phenomenon was observed in native gradient gel electrophoresis experiments (see below). For

all adsorption chromatographic steps the addition of DTT (1 mM) and Triton X-100 (0.2 mM) had no significant effect on the resolution. However, the volume of fractions containing dehalogenating activity from the Q-Sepharose column could be decreased by about 20% through the addition of 1 mM DTT.

The last chromatographic purification step was performed with a Mono Q 5/5 column. The 4-chlorobenzoyl-CoA dehalogenase was eluted at around 190 mM chloride. An elution profile is shown in Fig. 3. To obtain a good purification not more than 1 ml of enzyme solution (2–3 mg of protein) was applied onto the column per run.

Physicochemical properties of the enzyme. In size exclusion chromatography experiments the dehaloge-

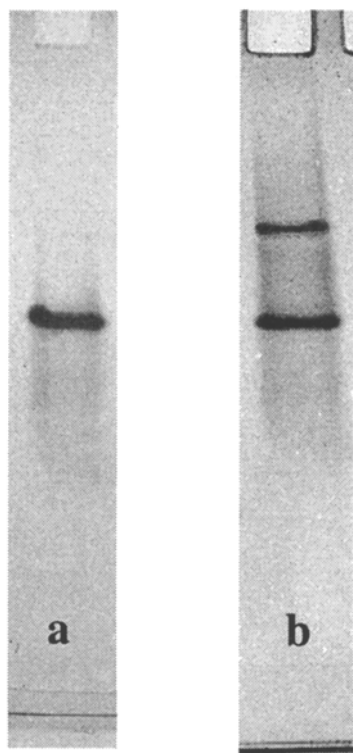


Fig. 5. Native gradient gel polyacrylamide electrophoresis of the purified 4-chlorobenzoyl-CoA dehalogenase (about 30 μ g of protein for each lane). a: in the presence of DTT (1 mM); b: without DTT

nase displayed a molecular mass of 100,000 (Sephadex G 150 sf), 120,000 (Superdex 200) and 125,000 (TSK G 3000 sw). When a homogenous enzyme preparation was applied, the protein profile was unaffected by the addition of Triton X-100 to the running buffer. However, when DTT was omitted an altered elution profile as shown in Fig. 4 was obtained (see above, purification on Sephadex G 150 sf). The dehalogenating activity correlated with this altered protein profile. Using a TSK G 3000 sw column the first peak (a) eluted in the void volume. The second peak (b) and the major peak (c) could be attributed to molecular masses of 280,000 and 125,000, respectively. The fractions of all three peaks revealed no differences in the values for the specific activity. These data suggest that the 4-chlorobenzoyl-CoA dehalogenase was active in tetrameric, octameric and higher multimeric forms.

In native PAGE (uniform 10%, pH 8.8, 8.3, 7.5, and 5 – 22.5%, pH 8.3, gradient gels) a single band could be detected after staining for protein when the buffer contained at least 1 mM DTT. Without DTT an additional band with a molecular mass of about 300,000, estimated according to the method described by Hedrick & Smith (1968), was visible (Fig. 5).

Under denaturing conditions (SDS-PAGE) the purified enzyme migrated as a single band and had an estimated molecular mass of 31,000 ($\pm 1,000$). The same result was obtained under reducing (DTT, 2-mercaptoethanol) or non reducing conditions, indicating the absence of disulphide bonds between the subunits. Analysis of the NH_2 -terminus revealed that the native dehalogenase was composed of identical subunits in an α_4 - (or higher oligomeric) structure. The NH_2 -terminal amino acid sequence was Met-Tyr-Glu-Ala-Ile-Gly-His-Arg-Val-Glu-Asp-Gly-Val-Ala-Glu-Ile-Thr-Ile-Lys-Leu-Pro-?-His-?-Asn-Ala-Leu-Ser-Val-Lys-Ala-Met-Gln-Glu-Val-Thr-Asp--Ala-Leu-Asn- (? = no definite identification possible, but should be Arg according to the DNA sequence published by Babbitt et al.)

Isoelectric focusing of the native enzyme revealed one single band at pH 6.7 when stained for protein. However, this result could only be obtained when the purified enzyme was applied above its pI. Applying the enzyme at more than 0.5 pH units below its pI resulted in protein precipitation.

The absorption spectrum of the purified enzyme (up to 14 mg ml⁻¹) showed no significant absorption above 300 nm. Dialysis against ethylenediaminetetraacetate containing buffer (20 mM) or addition of ethylenediaminetetraacetate (up to 10 mM) to the enzyme assay system had no effect on the dehalogenating activity. Extensive dialysis for 10 days with repeated buffer exchange at 4 °C did also not cause a loss in activity. Therefore a metal requirement of the enzyme is unlikely although a strongly protein-bound metal ion can not be excluded.

Impure preparations of the enzyme could be stored at -20 °C without loss of activity for several months. However, a pure enzyme preparation was completely inactive after a single freeze and thawing cycle. After freezing and thawing a white precipitate was visible which consisted of the 4-chlorobenzoyl-CoA dehalogenase, as confirmed by SDS-PAGE. The storage of pure filter-sterilised (0.2 μ m) enzyme solutions was carried out at +4 °C. Under these conditions the dehalogenase was stable for at least a month. Alternatively, the enzyme solution (1 mg ml⁻¹ in 100 μ l aliquots) was frozen in liquid nitrogen and stored at -80 °C. Thawing was performed in a water bath (35 °C). Using this method the denaturation could be minimised.

Catalytic properties. The 4-chlorobenzoyl-CoA dehalogenase converted 4-chloro-, 4-bromo- and 4-iodobenzoyl-CoA to 4-hydroxybenzoyl-CoA. 4-

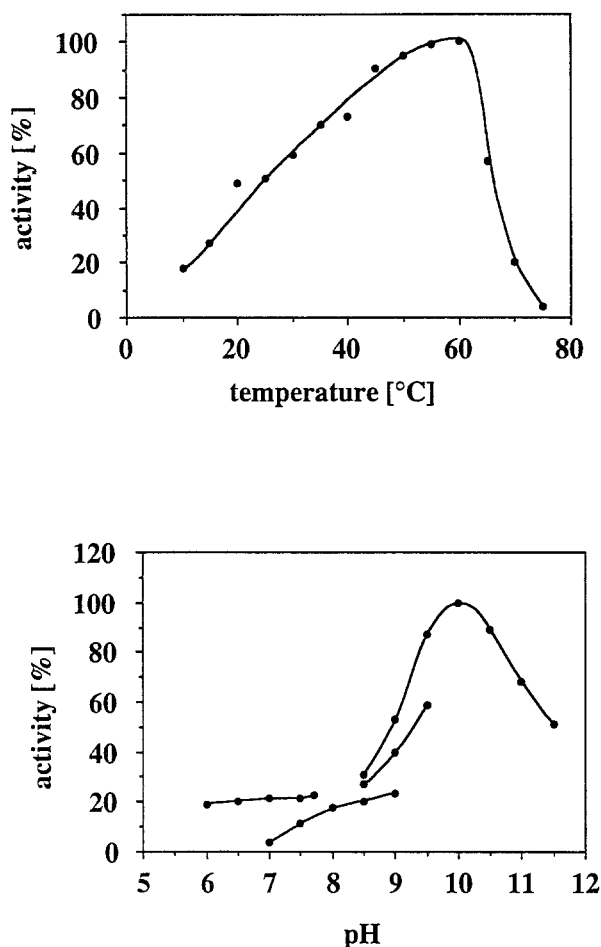


Fig. 6. Temperature- and pH optimum for the maximal initial rate of catalysis of the 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp. CBS3. 200 mM potassium phosphate buffer, pH 7.5, was used for the determination of the temperature optimum. For the determination of the pH optimum the following buffers (200 mM each) were used: potassium phosphate (pH 6.0–7.7), Tris-HCl (pH 7.0–9.0), diethanolamine-HCl (pH 8.5–9.5) and glycine-NaOH (pH 8.0–11.5). The dehalogenating activity was determined as described in Material and Methods with the exception that the 4-hydroxybenzoyl-CoA thioesterase was omitted from the test system. The disappearance of 4-chlorobenzoyl-CoA and the formation of 4-hydroxybenzoyl-CoA was followed by HPLC.

Fluorobenzoyl-CoA was not dehalogenated by this enzyme. The pH optimum for the maximal initial rate of catalysis was 10.0 (Fig. 6). Residual activities at pH 9.0 and pH 11.5 were 50%. The dehalogenase was stable over a pH-range from 6.5 to 11. After a 60 min preincubation at pH 6 and 11.5, 90% of the initial activity was measured whereas a 10 min incubation at pH 4.5 or 12 caused a total loss of activity.

The temperature optimum for the maximal initial rate of catalysis was 60 °C (Fig. 6). The residual

dehalogenating activities at 30 °C and 65 °C were 60%. The enzyme activity was unaffected by a 15 min preincubation at 50 °C. After a 15 min preincubation at 60 °C about 70% of the initial activity was found whereas a 15 min preincubation at 65 °C caused a total loss of activity.

The effects of different additives on the dehalogenase activity were studied. Monovalent cations (Na^+ , K^+ and Li^+ , up to 10 mM) did not cause any effect whereas the enzyme was inactivated by Ag^+ (1 mM). Several divalent cations (Cu^{2+} , Zn^{2+} , Mn^{2+} , Co^{2+} , Fe^{2+} and Ni^{2+} (5 mM each) inhibited the enzyme to a certain extent (100%, 36%, 32%, 22%, 20% and 16%, respectively). Sulfhydryl group blocking agents like 5,5'-dithio-bis(2-nitrobenzoate) inhibited the enzyme (1 mM, 22% inhibition; 5 mM, 61%; 10 mM, 80%). A 120 min preincubation of the dehalogenase with 1 mM 5,5'-dithio-bis(2-nitrobenzoate) caused a total loss of activity. Sodium azide (up to 10 mM) did not affect the enzyme activity. On the other hand, 1 mM SDS caused a 80% inhibition, at higher concentrations (2 mM and more) no dehalogenating activity was detectable. A Michaelis-Menten type saturation kinetic was obtained with 4-chlorobenzoyl-CoA as the substrate. The apparent kinetic constants for the purified enzyme were calculated from Eadie-Hofstee plots. The K_m for 4-chlorobenzoyl-CoA was 2.4–2.7 μM (measured in potassium phosphate buffer, pH 7.5). V_{max} was determined to be $1.1 \times 10^{-7} \text{ M sec}^{-1}$. The value for k_{cat} , the turnover number, was in the range of 5 s^{-1} based on the assumption that one catalytic centre existed per subunit.

Discussion

Several dehalogenases involved in the dehalogenation of aliphatic compounds have been reported (Hardman 1990). In contrast, not much information is available about aryl-dehalogenating enzymes. The 4-chlorophenylacetate-3,4-dioxygenase from *Pseudomonas* sp. CBS3 (Markus et al. 1986; Schweizer et al. 1987), the 2-chlorobenzoate-1,2-dioxygenase from *Pseudomonas cepacia* (Fetzner et al. 1992), the pentachlorophenol-4-monooxygenase from *Flavobacterium* sp. ATCC 39723 (Xun & Orser 1991) and the tetrachloro-p-hydrochinon dehalogenase from *Flavobacterium* sp. ATCC 39723 (Xun et al. 1992) have been purified and characterised. The first three enzymes catalyse an oxidative dehalogenation whereas the latter enzyme performs a reductive dehalogenation.

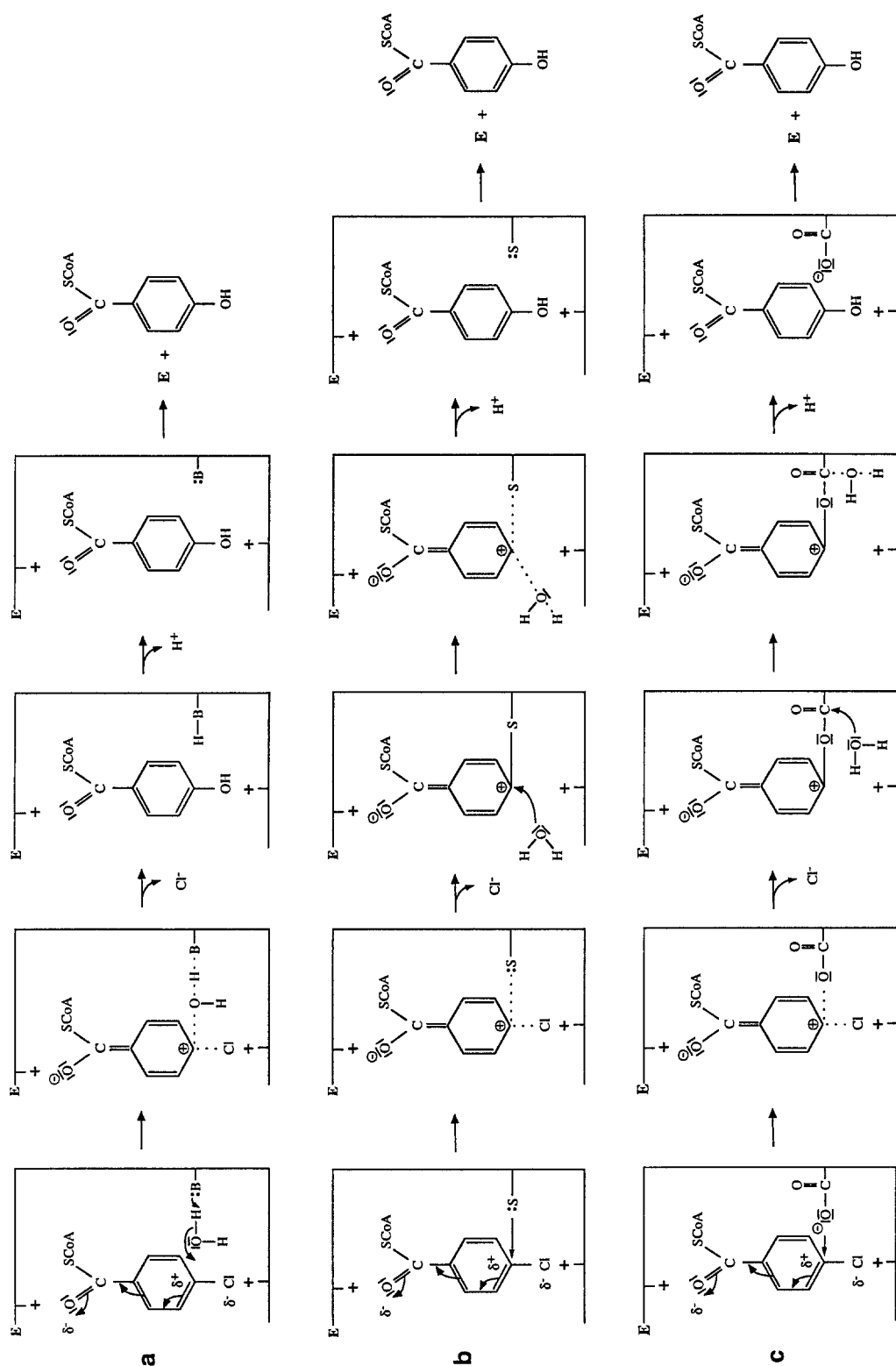


Fig. 7 Possible mechanisms of the dehalogenation of 4-chlorobenzoyl-CoA by the 4-chlorobenzoyl-CoA dehalogenase (E): (a) attack of an activated water molecule on the activated carbon atom C4; (b) attack of an enzyme bound sulfhydryl group on the activated carbon atom C4 and the intermediate formation of an ester; (c) attack of an enzyme bound carboxylate group on the activated carbon atom C4 and the intermediate formation of an ester.

In contrast, the 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp. CBS3 catalyse a hydrolytic aryl dehalogenation (Müller et al. 1984). The three proteins of the 4-chlorobenzoate dehalogenase have been characterised from their different *E. coli* clones (Chang et al. 1992). However, the purification and characterisation of the 4-chlorobenzoyl-CoA dehalogenase from the original strain *Pseudomonas* sp. CBS3 has not been described.

In SDS-PAGE the 4-chlorobenzoyl-CoA dehalogenase was visible as a major band in crude extracts (see Fig. 2). The other two enzymes involved in the conversion of 4-CBA to 4-HBA, e.g. 4-chlorobenzoate-CoA ligase and 4-hydroxybenzoyl-CoA thioesterase were not visible in crude extracts (Löffler et al. 1992). The necessity for different amounts of these enzymes in crude extracts could be explained by comparing their specificity constants (k_{cat}/K_m). The k_{cat}/K_m value for the dehalogenase was two orders of magnitude lower than the respective values for the ligase (Löffler et al. 1992) and the thioesterase (Chang et al. 1992). The lower catalytic activity of the 4-chlorobenzoyl-CoA dehalogenase was compensated with a higher enzyme content in the cell.

The data on the catalytic properties of the 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp. CBS3 were comparable to the data obtained by Chang et al. (1992) from an *E. coli* clone harbouring the gene encoding for the 4-chlorobenzoyl-CoA dehalogenase. However, the value for the specific activity of the purified 4-chlorobenzoyl-CoA dehalogenase determined in this work exceeded the value determined by Chang et al. (1992) about three fold. Although the experimental conditions were slightly different in these two studies, this discrepancy is most likely due to the different purities of the enzyme preparation.

Size exclusion chromatography showed that the dehalogenase was active in a tetrameric form or higher multimeric forms. This phenomenon was also observed in the case of pentachlorophenol-4-monooxygenase from *Flavobacterium* sp. ATCC 39723 (Xun & Orser 1991). This aggregation could be avoided by the addition of NaCl. In the case of the 4-chlorobenzoyl-CoA dehalogenase the addition of NaCl (up to 500 mM) had no effect on the aggregation. The formation of these multimeric forms could be prevented by the addition of at least 1 mM DTT. This suggested the participation of sulfhydryl groups in the formation of these multimeric forms. Each polypeptide chain contained five cysteine residues (deduced

from the sequence data from Babbitt et al. 1992) which might be involved in the disulphide bond formation.

4-Chlorobenzoyl-CoA is an activated molecule in comparison to 4-chlorobenzoate. In the thioester the ortho- and para-positions are activated for a nucleophilic attack due to the low tendency of a thioester for π -bond formation. The decreased resonance stabilisation results in a pronounced electrophilicity of the C = O group in a thioester. A possible stabilisation of a positively charged (activated) C₄-atom involving two positively charged amino acid residues at the active site of the enzyme is shown in Fig. 7.

The elimination of the chlorine substituent could proceed by the following mechanisms:

- The nucleophilic substitution of the chlorine is performed by the attack of an activated water molecule on the positively charged C₄-atom (general base catalysis) (Fig. 7a).
- The substitution of the chlorine is initiated through the nucleophilic attack of an enzyme bound sulfhydryl group followed by the hydrolysis of the thioether by a nucleophilic attack of an (activated) water molecule on the C₄-atom of the substrate (Fig. 7b).
- The chlorine substituent is eliminated as a result of a nucleophilic attack of an enzyme bound carboxylate followed by the hydrolysis of the ester bound through (activated) water (Fig. 7c).

All three possibilities explain the labelling of the para-hydroxyl group and of one oxygen atom in the carboxylate group when the reaction was performed in H₂¹⁸O (Müller et al. 1984). The intermediate formation of an enzyme bound ester leads in the first cycle to a 4-HBA molecule with an unlabelled carboxylate group but with a labelled carboxylate group at the enzyme. However, all further conversions would lead to a product with a labelled para-hydroxy group and a labelled carboxylate group.

The first mechanism follows a general base catalysis in which the electrophilic C₄-atom is attacked by a hydroxyl ion acting as the nucleophile. At high concentrations of hydroxyl ions a dehalogenation without the dehalogenating enzyme should occur. However, the formation of 4-hydroxybenzoyl-CoA under alkaline conditions (up to pH 11) without enzyme could not be observed (Löffler & Müller, unpublished results).

The sensitivity of the dehalogenase against sulfhydryl blocking agents could be a hint for the second mechanism. However, the stability of thioethers against hydrolysis, which would be formed intermediately in such a reaction, does not favour this mechanism

(Little & Williams 1971). Therefore the most probable mechanism seems to be the attack of a carboxylate group on the positively charged C₄-atom (Fig. 7c). Recently, such a hydrolytic dehalogenation mechanism involving an aspartate residue as the nucleophile was elucidated in detail in the case of 1,2-dichloroethane dehalogenase from *Xanthobacter autotrophicus* GJ10 (Verschuere et al. 1993).

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