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The purification and characterisation of 4-chlorobenzoate:CoA ligase and 4-chlorobenzoyl CoA dehalogenase from *Arthrobacter* sp. strain TM-1

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Abstract

4-Chlorobenzoate:CoA ligase, the first enzyme in the pathway for 4-chlorobenzoate dissimilation, has been partially purified from *Arthrobacter* sp. strain TM-1, by sequential ammonium sulphate precipitation and chromatography on DEAE-Sepharose and Sephacryl S-200. The enzyme, a homodimer of subunit molecular mass approximately 56 kD, is dependent on Mg^{2+}-ATP and coenzyme A, and produces 4-chlorobenzoyl CoA and AMP. Besides Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+} and Zn^{2+} are also stimulatory, but not Ca^{2+}. Maximal activity is exhibited at pH 7.0 and 25 °C. The ligase demonstrates broad specificity towards other halobenzoates, with 4-chlorobenzoate as best substrate. The apparent Michaelis constants (K_m) of the enzyme for 4-chlorobenzoate, CoA and ATP were determined as 3.5, 30 and 238 μM respectively. 4-Chlorobenzoyl CoA dehalogenase, the second enzyme, has been purified to homogeneity by sequential column chromatography on hydroxyapatite, DEAE-Sepharose and Sephacryl S-200. It is a homotetramer of 33 kD subunits with an isoelectric point of 6.4. At pH 7.5 and 30 °C, K_m and k_{cat} for 4-CBCoA are 9 μM and 1 s^{-1} respectively. The optimum pH is 7.5, and maximal enzymic activity occurs at 45 °C. The properties of this enzyme are compared with those of the 4-chlorobenzoyl CoA dehalogenases from *Arthrobacter* sp. strain 4-CB1 and *Pseudomonas* sp. strain CBS-3, which differ variously in their N-terminal amino acid sequences, optimal pH values, pI values and/or temperatures of maximal activity.

Abbreviations: 4-CB – 4-chlorobenzoate; 4-CBA – 4-chlorobenzoic acid; 4-CBCoA – 4-chlorobenzoyl CoA; 4-HB – 4-hydroxybenzoate; 4-HBA – 4-hydroxybenzoic acid; 4-HBCoA – 4-hydroxybenzoyl CoA; PCB – polychlorinated biphenyl

Introduction

Halogenated aromatic compounds are widely distributed in the environment, and many of them are resistant to microbial degradation. Halogenated benzoic acids have been used as models to study their biodegradation. They are used as herbicides (e.g. 2,3,6-trichlorobenzoic acid), and occur as partial degradation products of other xenobiotics such as polychlorinated biphenyls (PCBs; Hützinger & Veerkamp 1981; Peyton 1984).

4-Chlorobenzoate (4-CB) is a breakdown product of PCBs (Ahmed & Focht 1973) but it is also employed as a herbicide (Sheets et al. 1968). 4-CB degradation can occur aerobically in two ways: one proceeding via 4-chlorocatechol, with elimination of the chlorine atom after *ortho*-ring cleavage, when the product, 3-chloro-*cis*, *cis*-muconate, undergoes lactonisation. This route has been observed in *Pseudo-*

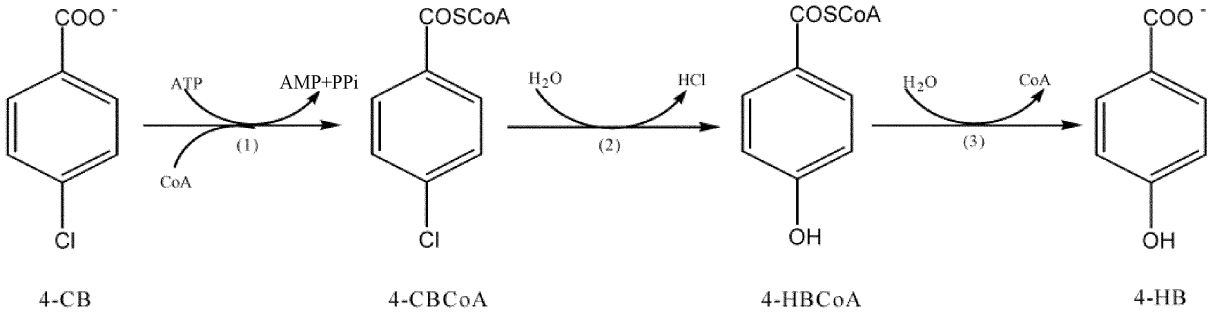

Figure 1. The conversion of 4-chlorobenzoate to 4-hydroxybenzoate by *Arthrobacter* sp. strain TM-1. The three steps are catalysed by 4-CB:CoA ligase (1), 4-CBCoA dehalogenase (2) and 4-HBCoA thioesterase (3).

monas aeruginosa strain 3 mT (Ajithkumar & Kunhi 2000), but was also shown to proceed in derivative strains of *Pseudomonas* B13 containing the TOL plasmid and expressing toluate 1,2-dioxygenase; and in *Pseudomonas* strain WR912 (Hartmann et al. 1979; Reinecke & Knackmuss 1979, 1980; Reineke et al. 1982).

The second route is followed in several micro-organisms such as *Nocardia* and *Pseudomonas* (Klages & Lingens 1979, 1980), *Arthrobacter globiformis* (Zaitsev & Karasevich 1981a, b), *Arthrobacter* sp. strain TM-1 (Marks et al. 1984a), *Pseudomonas* sp. strain CBS-3 (Müller et al. 1984, 1988), *Alcaligenes denitrificans* NTB-1 (van den Tweel et al. 1986), *Arthrobacter* sp. strain SB8 (Shimao et al. 1989), and *Pseudomonas* sp. strain DJ-12 (Chae & Kim 1997; Chae et al. 1999). Here, dechlorination of 4-CB constitutes the initial reaction sequence (Figure 1), proceeding via 4-chlorobenzoyl CoA (4-CBCoA) and 4-hydroxybenzoyl CoA (4-HBCoA) to yield 4-hydroxybenzoate (4-HB); hence three enzymes are involved.

The present report describes the partial purification of 4-CB:CoA ligase and complete purification of 4-CBCoA dehalogenase from *Arthrobacter* sp. strain TM-1 (Marks et al. 1984a), and their characterisation and comparison, for the first time, with the corresponding enzymes from other micro-organisms. Strain TM-1, which was originally isolated from sewage sludge by enrichment culture on 4-CB, converts 4-CB to 4-HB before further dissimilation via the *ortho*-ring fission pathway for protocatechuate (Marks et al. 1984a). Experiments with ^{18}O indicated that the hydroxy group of 4-HB is derived from water and not molecular oxygen (Marks et al. 1984b). Divalent manganese ions and ATP were shown to be required for the dechlorination (Marks et al. 1984a; Smith 1991).

Materials and methods

Chemicals and enzymes

Unless otherwise stated, all chemicals of both general and analytical reagent grade were obtained from Fisher Ltd (Loughborough, UK), BDH Ltd. (Poole, UK) or Sigma-Aldrich Chemical Co. (Poole, UK). Coomassie Blue dye reagent was from Bio-Rad laboratories (Watford, UK). Enzymes and protein markers were purchased from Sigma-Aldrich Chemical Co. 4-CBCoA and 4-HBCoA were synthesised by the methods of Merkel et al. (1989). The identity of synthetic 4-CBCoA was confirmed by mass spectrometry, which gave a prominent peak at m/z = 905, corresponding with the molecular ion $[M-1]^+$. 4-HBCoA was recovered 92% pure; mass spectrometry gave a prominent peak at m/z 887 ($[M-1]^+$), confirming the identity of the product.

Organism and growth conditions

Arthrobacter sp. strain TM-1 was grown and maintained at 25 °C on tryptone soya agar plates and subcultured every 30 days. Batch cultures (10 L) were grown in minimal-salts medium (Evans et al. 1970) containing 2 mM 4-CB as sole carbon source, in a fermenter at 25 °C, with stirring and 30% pO_2 saturation. The fermenter was inoculated with 1 L of an exponentially-growing culture. 4-CB consumption was monitored spectrophotometrically, and sequential additions thereof were made during growth. The average yield was 4 g (wet weight) of cells per litre of culture fluid, which were stored at −25 °C until use.

Enzyme assays

Ligase activity was usually measured at 25 °C in 50 mM phosphate, pH 7.0, containing 2 mM DTT, us-

ing a coupled enzyme assay consisting of myokinase, pyruvate kinase and lactate dehydrogenase (Chang et al. 1992). NADH oxidation was monitored spectrophotometrically at 340 nm. The disappearance of 1 μmol NADH is equal to the disappearance of an equimolar amount of 4-CB. Assay solutions (1 ml) contained 0.25 mM 4-CB, 0.26 mM CoA, 0.5 mM ATP, 0.5 mM $MgSO_4$, 5 mM KCl, 3 mM PEP, 11 units myokinase, 9 units pyruvate kinase, 9 units lactate dehydrogenase, 0.2 mM NADH, and 15 μg enzyme, added to initiate the reaction. One enzyme unit is defined as the amount required to convert 1 μmol of 4-CB into 4-CBCoA per minute at pH 7.0 and 25 °C.

4-CBCoA dehalogenase was assayed spectrophotometrically using 0.5 ml quartz cuvettes. Phosphate buffer (50 mM), pH 7.5, containing 2 mM DTT, and 5 μl of 10 mM 4-CBCoA were equilibrated at 30 °C. Reactions were initiated by addition of 7.5 μg enzyme, and the increase in absorption at 300 nm due to 4-HBCoA formation (ϵ_{300nm} for 4-HBCoA = 8200 M^{-1} cm^{-1}; Chang et al. 1992) was monitored over time. One enzyme unit is defined as the amount required to convert 1 μmol of 4-CBCoA into 4-HBCoA per minute at pH 7.5 and 30 °C.

Protein estimation

Protein was determined by the method of Bradford (1976), using bovine gamma-globulin as the standard. Buffers identical with those containing the protein samples were used in preparing blanks.

Preparation of crude cell-free extracts

4-CB-grown cells (10 g) were suspended in 10 ml of 20 mM potassium phosphate buffer, pH 7.0, at 4 °C, and disrupted by three passages through an X-press (Biox AB, Sweden) at −25 °C. On thawing, 5 ml phosphate buffer containing DNase (Type I, BDH Ltd.) was added to the suspension, which was subsequently centrifuged at 40,000 × g and 4 °C for 30 min in a Model L8-70 ultracentrifuge (Beckman Instruments, High Wycombe, Bucks., UK) to remove unbroken cells and cell debris. The supernatant was removed and either used immediately or stored at −25 °C until required.

Purification of 4-CB:CoA ligase

Cell-free extract (10 ml) prepared from the previous step was treated with ammonium sulphate. The fraction precipitating between 40 and 70% (w/v) saturation was collected by ultracentrifugation at 10,000 × g for 30 min. The protein pellet was dialysed at 4 °C for 24 h against three changes of 1 L of 20 mM phosphate buffer, pH 7.0, containing 2 mM DTT, and loaded onto a column (2.5 × 40 cm) of DEAE-Sepharose CL-6B equilibrated against 50 mM phosphate buffer, pH 7.0, containing 2 mM DTT. Elution was carried out with 100 ml of the equilibration buffer at 60 ml h^{-1} to remove unbound protein, followed by a gradient (0–0.5 M KCl in 50 mM phosphate buffer, pH 7.0). The final step was achieved by gel filtration on a Sephacryl S-200HR column (1.6 × 65 cm) eluted at 60 ml h^{-1} with 0.1 M phosphate buffer, pH 7.0, containing 2 mM DTT.

Purification of 4-CBCoA dehalogenase

Crude cell-free extract was applied to a column (5.2 cm i.d. × 4.1 cm) of Hydroxyapatite HT (Bio-Rad Laboratories Ltd., Watford, UK), equilibrated with 20 mM potassium phosphate buffer, pH 7.0, at a flow rate of 2.0 ml min^{-1}, then eluted with a linear gradient of 20 to 400 mM potassium phosphate, pH 7.0 (500 ml). Fractions containing 4-CBCoA dehalogenase were collected and concentrated in an Amicon concentration cell (30 kD cutoff membrane), then applied at a flow rate of 1.0 ml min^{-1} to a DEAE Sepharose CL-6B column (2.5 × 9.2 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 0.1 M KCl and 2 mM DTT, and eluted using a KCl gradient (0.1 to 0.5 M; 500 ml). The final step was achieved by gel filtration on a Sephacryl S-200HR column, as described above.

Determination of subunit and holoenzyme molecular masses

Subunit molecular masses of the enzymes were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% (w/v) stacking gel and 12% (w/v) separating gel, and a standard buffer system (Laemmli, 1970). Samples and standards (Dalton VIIL Molecular Weight Mixture; Sigma) were denatured at 100 °C for 5 min in buffered SDS containing 2-mercaptoethanol. The molecular weight of the native enzyme was determined by gel filtration on a Sephacryl S-200HR column as above, calibrated with the following standards (3 mg each): Blue Dextran; ferritin (440 kD); catalase (232 kD); L-lactate dehydrogenase (136 kD); bovine serum albumin (66 kD) and ovalbumin (45 kD).

Isoelectric focusing gel electrophoresis

IEF was run over a gradient of pH 3-10 using 10 × IEF cathode (20 mM lysine, 20 mM arginine, pH 10.7) and 10 × IEF anode (7 mM phosphoric acid, pH 2.1) buffers. Markers were β-lactoglobulin A (pI 5.20); carbonic anhydrase B (bovine; 5.85, and human; 6.55); myoglobin – basic band (7.35), and lentil lectin – acidic band (8.15). Samples were loaded in 80% glycerol onto a pre-cast gel (pH 5–8) in a Bio-Rad mini-Protean II cell assembly, and submitted to electrophoresis at 100 V for 1 h, followed by 250 V for 1 h and finally 500 V for 0.5 h; then stained with Crocein Scarlet/Coomassie Blue R-250 for 45 min and destained overnight according to standard methods.

Cofactor requirements for 4-CB:CoA ligase

Cell-free extract was loaded onto a Sephadex G-25 column and eluted with phosphate buffer, pH 7.0, at 4 °C to remove endogenous cofactors. Assay mixtures comprised eluted cell-free extract (final protein concentration, 7.5 mg ml^{-1}) diluted in 50 mM phosphate buffer containing 10 mM 4-CB; total volume of 0.5 ml. Reactions were initiated by the addition of the following cofactors in various combinations: 10 mM ATP, 10 mM CoA, and 10 mM $MgSO_4$, $MnSO_4$ or $ZnSO_4$. After 10 min at 25 °C, the reactions were terminated by addition of 20 μl of 2 M H_2SO_4, which did not affect the stability or recovery of any of the analytes. ATP and AMP levels were measured using a Shimadzu LC-4A HPLC on a LiChroSpher 100 C18 reverse phase column (125 × 4 mm; bead size 5 μm), eluted with 50 mM potassium phosphate buffer, pH 5.5, at a flow rate of 1.5 ml min^{-1}. Detection was by measurement of A_{254nm} of the eluate, using authentic ATP and AMP as standards for comparison of R_f values and quantitation.

4-CB consumption was determined by HPLC. Methanol (100 μl) was added to solubilise 4-CBA after acidification, and the mixture was centrifuged at 13,000 × g for 10 min. The supernatant was submitted to HPLC to detect 4-CB consumption. The mobile phase was methanol/water/acetic acid (60:40:1, by vol). Samples (20 μl) were injected onto a column (125 × 5 mm) of LiChroSpher 100 RP-18 (particle size, 5 μm; Merck) at a flow rate of 2.0 ml/min. Detection was by measurement of A_{254nm}.

Effect of pH on enzyme activity

The optimum pH for 4-CB:CoA ligase activity was determined at 25 °C in 0.1 M potassium phosphate (pH 5.5 to 8.5). The optimum pH for 4-CBCoA dehalogenase activity was determined at 30 °C from measurements in 0.1 M potassium phosphate (pH 5.2 to 8.0), Clark and Lubs (pH 8.1 to 10.1) and 0.05 M Tris-HCl (pH 6.5 to 8.1) buffers.

Effect of temperature on enzyme activity

Maximal activity of 4-CB:CoA ligase over the range 10 to 45 °C was determined in 50 mM potassium phosphate buffer containing 2 mM DTT, pH 7.0. Maximal activity of 4-CBCoA dehalogenase over the range 15 to 60 °C was determined in 50 mM potassium phosphate buffer containing 2 mM DTT, pH 7.5.

Metal ion requirements

Initial velocities of partially-purified 4-CB:CoA ligase were measured at 25 °C in the presence or absence of 0.5 mM divalent metal ions added as their sulphates or the chloride (Ca^{2+}). One-ml reaction mixtures contained the components stipulated for the enzyme-linked assay of 4-CB:CoA ligase (see above), made to volume with 50 mM phosphate buffer, pH 7.0, containing 2 mM DTT.

Substrate specificity of 4-CB:CoA ligase

4-CB:CoA ligase activity towards benzoate, and 4-nitro-, 4-bromo-, 4-iodo- and 4-fluorobenzoate (0.25 mM) was measured at 25 °C using 1-ml reaction mixtures containing the components stipulated above for the enzyme-linked assay of 4-CB:CoA ligase, all at the same concentrations except for CoA, ATP and $MgSO_4$, which were added at 0.8, 5 and 5 mM respectively.

Stability of 4-CBCoA dehalogenase

The effect of DTT on 4-CBCoA dehalogenase activity and its stability in storage at +4, −25 and −196 °C was measured in the presence of 1–5 mM DTT.

Stoichiometries

The conversion of 4-CB to 4-HB by crude extracts containing the ligase, dehalogenase and thioesterase was followed using HPLC. After incubating mixtures

(1 ml) at 25 °C for 15 min, reactions were terminated by addition of 20 μl of 2 M H_2SO_4, as described above for 4-CB assay. The acidified, methanol-treated, centrifuged supernatant was submitted in 20 μl samples to HPLC on a column (125 × 5 mm) of LiChroSpher 100 RP-18 and run at a flow rate of 2.0 ml/min to detect 4-CB consumption as above, or 4-HB formation. The mobile phase was methanol/water/acetic acid (as above for 4-CB; 30:70:1, by vol. for 4-HB). Detection was by measurement of A_{254nm}.

For measurement of the stoichiometric relationship between 4-CB and 4-CBCoA, the reaction mixture (200 μl) contained 100 mM potassium phosphate buffer, pH 7.2, 2 mM DTT, 10 mM ATP, 0–5 mM CoA, 5 mM $MgSO_4$ and partially-purified 4-CB:CoA ligase. After equilibration at 25 °C, the reaction was initiated by the addition of 4-CB (final concentration 1 mM). Reactions were terminated after 1 or 40 min by adjustment to pH 2 (2 M H_2SO_4), and analysis was performed by HPLC using a LiChrospher 100 RP-18 column (Merck; bead size 5 μm; 125 × 4 mm). The mobile phase was 50 mM phosphate, pH 5.5/*iso*-propanol (90:10, by vol), at a flow rate of 2 ml min^{-1}. Using the uv detector set at 254 nm, identities were confirmed by comparison of R_f values with those of authentic 4-CBA and 4-CBCoA.

To determine the stoichiometric relationship between 4-CBCoA and 4-HBCoA, the reaction mixture contained 7.5 μg 4-CBCoA dehalogenase in 50 mM potassium phosphate buffer, pH 7.5, containing 2 mM DTT. After equilibration at 30 °C, the reaction was initiated by addition of 50 nmol 4-CBCoA. The reaction was terminated at 1 or 15 min by adjustment to pH 2 (2 M H_2SO_4), and the products were analysed by HPLC on a LiChrospher 100 RP-18 column as above, using authentic 4-CBCoA and 4-HBCoA as standards.

Determination of kinetic parameters

Using the coupled enzyme assay procedure described above, the apparent Michaelis constant (K_m) of the ligase for 4-CB was determined from reaction rates measured over the concentration range 10–70 μM 4-CB in the presence of 0.8 mM CoA, 5 mM ATP, and 5 mM Mg^{2+}. K_m for CoA was determined from initial reaction rates measured over the range 26.3–105.2 μM CoA in the presence of 0.2 mM 4-CB, 5 mM ATP, and 5 mM Mg^{2+}, and K_m for ATP was derived from rates measured between 50 and 300 μM ATP in the presence of 0.25 mM 4-CB, 0.8 mM CoA, and 5 mM Mg^{2+}. All measurements were carried out at 25 °C in 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM DTT.

The K_m value for 4-CBCoA dehalogenase was determined from initial rates measured at 30 °C over a range 5–40 μM 4-CBCoA in 50 mM phosphate buffer, pH 7.5, containing 2 mM DTT, in 1 ml volumes. Reactions were initiated by the addition of 0.057 μmol enzyme. The value for k_{cat}, based on the subunit molecular mass, was determined from V_{max} derived from a Lineweaver–Burk plot and the molar quantity of purified enzyme present, determined using the Bradford method.

N-terminal sequencing

Purified 4-CBCoA dehalogenase, submitted to electrophoresis by SDS-PAGE, was recovered by electrotransfer onto Problott (PVDF) membrane (Perkin-Elmer Corp., Beaconsfield, UK), dried in air and presented for N-terminal amino acid sequencing using a Procise Sequencer (Applied Biosystems) at the Department of Biochemistry, University of Cambridge. The purified enzyme was subjected to 22 cycles of Edman degradation.

Results

4-CB:CoA ligase

Purification

From cell-free extract containing 90 mg protein, 13 mg of partially purified 4-CB:CoA ligase was recovered, which eluted at approximately 0.29 M KCl from DEAE-Sepharose. The results of a typical purification procedure are summarized in Table 1 and Figure 2A. Although the enzyme was free of 4-CBCoA dehalogenase, it was found to be in mixture with 4-hydroxybenzoate hydroxylase, which appears to have a similar subunit size and pI (see below) in this organism.

Physicochemical properties

The subunit size of the ligase was estimated by SDS-PAGE to be 56 kD (Figure 2A). The molecular mass of the native enzyme, estimated from the elution volume from Sephacryl S-200, was 112 kD; hence the enzyme is a dimer. The pI was 5.8.

Table 1. Purification of 4-CB:CoA ligase and 4-CBCoA dehalogenase from *Arthrobacter* sp. strain TM-1

Step	Protein (mg)	Activity (units)	Sp. Act. (unit/mg)	Yield (%)	Purification (*x*-fold)
I. Purification of 4-CB:CoA ligase					
Cell-free extract	90	18.9	0.21	100	
Ammonium sulphate (40–70%)	59	17.1	0.29	90	1.4
DEAE-Sepharose CL-6B	24	15.6	0.65	83	3.0
Sephacryl-200HR	13	10.3	0.86	55	4.1
II. Purification of 4-CBCoA dehalogenase					
Cell-free extract	84.3	25.8	0.31	100	
HT-Hydroxyapatite	8.0	19.6	2.45	76	8.0
DEAE-Sepharose CL-6B	5.5	15.5	2.82	60	9.1
Sephacryl-200HR	3.1	11.2	3.61	43	11.6

Enzyme was extracted from 3.2 g of wet cell paste.
One unit of 4-CB:CoA ligase is defined as the amount required to convert 1 μmol of 4-CB into 4-CBCoA per minute at 25 °C; one unit of 4-CBCoA dehalogenase is defined as the amount required to produce 1 μmol of 4-HBCoA per minute at pH 7.5 and 30 °C.

Cofactors

Ligase activity was dependent on ATP, CoA and Mg^{2+}. Four other divalent cations (Mn^{2+}, Co^{2+}, Zn^{2+} and Fe^{2+}) were stimulatory but less so than Mg^{2+}; their relative effects are summarised in Table 2. Mn^{2+}, notably, was the ion originally identified as necessary for activity in cell extracts of strain TM-1 (Marks et al. 1984a). Ca^{2+} was inactive.

pH optimum and temperature effects

The optimum pH for the ligase in potassium phosphate buffer was 7.0 (Figure 3), and maximal activity was observed at 25 °C (Figure 4).

Specificity

A range of 4-substituted benzoates was investigated as potential substrates (Table 3). The ligase was active towards benzoate and all the 4-halobenzoates, but not 4-nitrobenzoate. Maximal activity was shown towards 4-CB and least towards benzoate.

Stoichiometry and kinetics

The ligase converted 4-CB to 4-CBCoA in 98% yield (Table 4), and AMP was detected as a co-product of the reaction in approximately stoichiometric proportion to the amount of ATP consumed. The K_m values for 4-CB, CoA and ATP at pH 7.0 and 25 °C, determined from Lineweaver–Burk plots, were 3.5, 30 and 238 μM respectively.

Table 2. Comparison of metal ion requirements of 4-CB:CoA ligases from *Arthrobacter* sp. strain TM-1 and from *Pseudomonas* sp. strain CBS-3

Metal ion	*Arthrobacter* TM-1[a] Relative velocity (%)	*Pseudomonas* CBS-3[b] Relative velocity (%)
Mg^{2+}	100	100
Mn^{2+}	71	87
Co^{2+}	59	77
Fe^{2+}	26	70
Zn^{2+}	24	23
Ca^{2+}	0	ND[c]
Ni^{2+}	ND[c]	11

Initial velocities of partially-purified 4-CB:CoA ligase were measured at 25 °C in the presence or absence of 0.5 mM divalent metal ions added as their sulphates, or the chloride (Ca^{2+}). One-ml reaction mixtures contained the components stipulated for the enzyme-linked assay of 4-CB:CoA ligase (see Methods), made to volume with 50 mM phosphate buffer, pH 7.0, containing 2 mM DTT.
[a] Data from this study. The reaction was performed in phosphate buffer, pH 7.0.
[b] Data from Löffler et al. (1992). 4-CB:CoA ligase was isolated from *Pseudomonas* sp. strain CBS-3; the reaction was performed in Tris/HCl buffer, pH 8.4.
[c] ND = not determined.

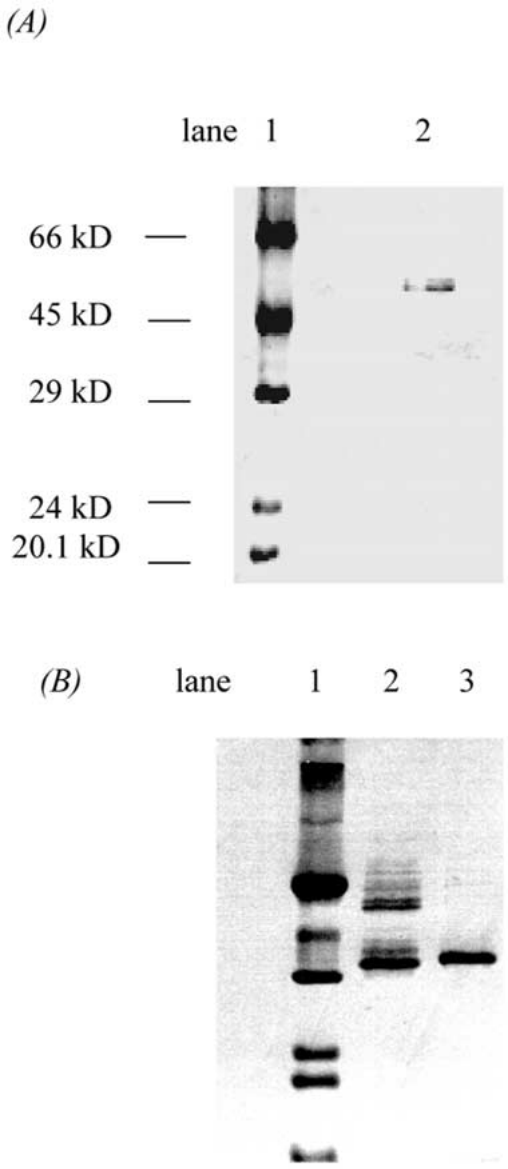

Figure 2. SDS-PAGE analysis of 4-CB:CoA ligase and 4-CBCoA dehalogenase purified from cell-free extract of *Arthrobacter* sp. strain TM-1. (A) Ligase. Lane 1, standard protein markers (bovine serum albumin, 66 kD; ovalbumin, 45 kD; carbonic anhydrase, 29 kD; trypsinogen, 24 kD; trypsin inhibitor, 20.1 kD;): lane 2, partially purified 4-CB:CoA ligase. (B) Dehalogenase. Lane 1: Protein markers (as above, and additionally lysozyme, 14.4 kD); lane 2: crude extract; lane 3; purified 4-CBCoA dehalogenase.

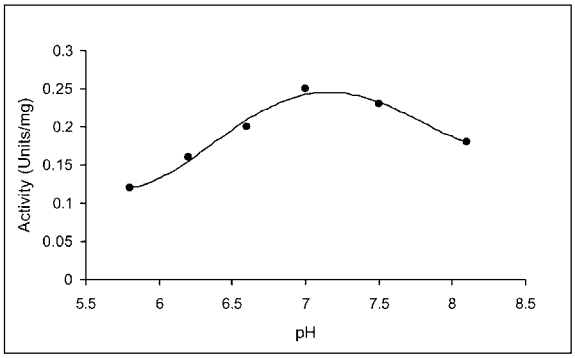

Figure 3. Effect of pH on 4-CB:CoA ligase activity. Plot of 4-CB:CoA ligase activity versus pH. One-ml portions of the reaction mixture, containing 0.25 mM 4-CB, 0.26 mM CoA, 0.5 mM ATP, 0.5 mM $MgSO_4$, 5 mM KCl, 3 mM PEP, 11 units myokinase, 9 units pyruvate kinase and 9 units lactate dehydrogenase in 50 mM phosphate buffer, pH 5.2 to 8.0, were equilibrated at 25 °C prior to the assay. Reactions were initiated by addition of 0.2 μmol NADH and 15 μg enzyme, and NADH oxidation was monitored spectrophotometrically at 340 nm. Blanks contained no NADH. The disappearance of 1 μmol of NADH is equivalent to the consumption of an equimolar amount of 4-CB; 1 unit of enzyme is defined as the amount required to convert 1 μmol of 4-CB to 4-CBCoA per minute at 25 °C.

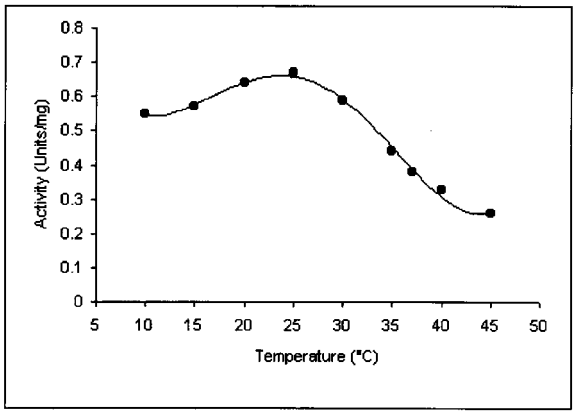

Figure 4. Effect of temperature on 4-CB:CoA ligase activity. Plot of 4-CB:CoA ligase activity versus temperature. Reaction mixtures (1.0 ml), containing the components listed under Figure 3 in 50 mM phosphate buffer, pH 7.0, were incubated at temperatures ranging from 10 to 45 °C. Reactions were initiated by addition of 0.2 μmol NADH and 15 μg enzyme, and were monitored as described under Figure 3.

4-CBCoA dehalogenase

Purification

From 3.2 g wet weight of cells, 3.1 mg of pure 4-CBCoA dehalogenase was isolated: a yield of approx. 1 mg of dehalogenase per gram of cell paste. Pure dehalogenase was obtained by sequential chromatography on hydroxyapatite, DEAE-Sepharose and

Table 3. Comparison of the substrate specificity of 4-CB:CoA ligases from *Arthrobacter* sp. strain TM-1 and *Pseudomonas* sp. strain CBS-3

Substrate	*Arthrobacter* TM-1[a] relative velocity (%)	*Pseudomonas* CBS-3[b] relative velocity (%)
4-Chlorobenzoate	100	100
4-Bromobenzoate	93	105
4-Iodobenzoate	73	90
4-Fluorobenzoate	67	36
4-Methylbenzoate	ND[d]	22
4-Methoxybenzoate	ND[d]	9
4-Nitrobenzoate	0[c]	7
Benzoate	62	5

4-CB:CoA ligase activity towards benzoate and the substituted benzoates (0.25 mM) was measured at 25 °C in phosphate buffer, pH 7.0, using 1-ml reaction mixtures containing the components at concentrations stipulated for the enzyme-linked assay of 4-CB:CoA ligase (see Methods), except that the CoA, ATP and $MgSO_4$ concentrations were 0.8, 5 and 5 mM respectively.

[a] Data from this study.

[b] Data from Löffler et al. (1992). 4-CB:CoA ligase was isolated from *Pseudomonas* sp. strain CBS-3, and the reaction was performed in Tris/HCl buffer, pH 8.4.

[c] Data from Smith (1991). The reaction was performed in phosphate buffer, pH 7.0.

[d] ND = not determined.

Table 4. Stoichiometric relationship between 4-CB consumption and 4-CBCoA production by 4-CB:CoA ligase

4-CB supplied (nmol)	CoA supplied (nmol)	4-CB consumed (nmol)	4-CBCoA produced (nmol)
0	500	0	0
100	0	0	0
100 (1 min)	500	8.8	8.6
100 (40 min)	500	43.2	42.4

The reaction mixture (200 μl) contained 100 mM potassium phosphate buffer, pH 7.2, 2 mM DTT, 10 mM ATP, 0–5 mM CoA, 5 mM $MgSO_4$ and partially-purified 4-CB:CoA ligase. The reaction (at 25 °C) was initiated by addition of 4-CB (final concentration, 1 mM), and terminated after 1 or 40 min by pH adjustment to 2 with 2 M H_2SO_4. Analysis was performed by HPLC using a LiChrospher 100 RP-18 column, as specified in Methods. Identities were confirmed by comparison of R_f values with those of authentic 4-CBA and 4-CBCoA.

Sephacryl S-200. Homogeneous dehalogenase eluted from Sephacryl-200HR with a specific activity of 3.61 units (mg of protein)$^{-1}$ (see Table 1).

Physicochemical properties

The subunit size of the dehalogenase was estimated by SDS-PAGE to be 33 kD, and the molecular mass of the native enzyme, estimated by chromatography on Sephacryl S-200, was 131 kD; hence the enzyme is a tetramer.

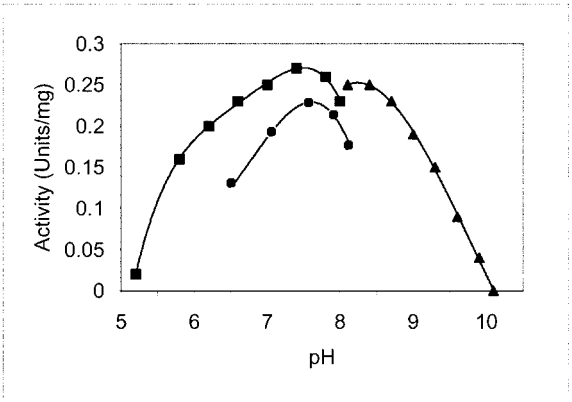

Figure 5. Effect of pH on 4-CBCoA dehalogenase activity. Plot of 4-CBCoA dehalogenase activity versus pH. Buffers used for the determination of optimum pH, 50 mM phosphate (■; pH 5.2 to 8.0); 50 mM Tris (●; pH 6.5 to 8.1), and 50 mM Clark & Lubs (▲; pH 8.1 to 10.1), containing 2 mM DTT and 5 μl of 10 mM 4-CB-CoA, were equilibrated at 30 °C. Reactions, initiated by addition of 7.5 μg enzyme, were monitored spectrophotometrically at 300 nm for 4-HBCoA formation over the initial 5 min period during incubation at 30 °C. Blanks contained no substrate. One enzyme unit is defined as the amount required to convert 1 μmol of 4-CBCoA into 4-HBCoA per minute at pH 7.5 and 30 °C.

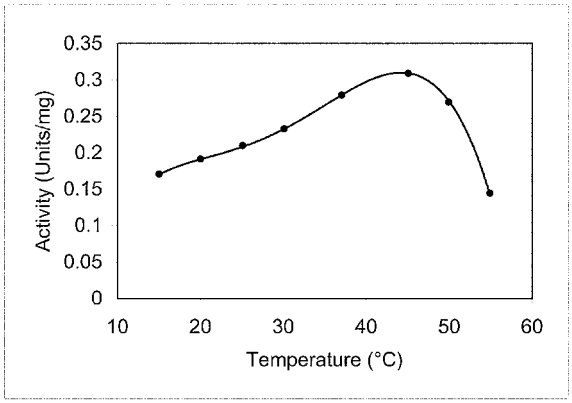

Figure 6. Effect of temperature on 4-CBCoA dehalogenase activity. Plot of 4-CBCoA dehalogenase activity versus temperature. Enzyme activity was determined, as described in Figure 5, by assay of the 4-HBCoA produced over the initial 5 min period during incubation in 50 mM phosphate buffer, pH 7.5, containing 2 mM DTT, at different temperatures.

pH optimum and temperature effects

The optimum pH for dehalogenase activity in potassium phosphate buffer was 7.5 (Figure 5), and maximal activity was observed at 45 °C (Figure 6). Isoelectric focusing of the dehalogenase yielded a pI value of 6.42.

Table 5. Stoichiometric relationship between 4-CBCoA consumption and 4-HBCoA production by 4-CBCoA dehalogenase

4-CBCoA supplied (nmol)	4-CBCoA consumed (nmol)	4-HBCoA produced (nmol)
50[a]	0	0
50 (1 min)	23.2	22.8
50 (15 min)	47.5	47.1

The reaction mixture comprised 7.5 μg 4-CBCoA dehalogenase in 50 mM potassium phosphate buffer, pH 7.5, containing 2 mM DTT. The reaction, at 30 °C, was initiated by addition of 50 nmol 4-CBCoA, and terminated at 1 or 15 min by adjustment to pH 2 with 2 M H_2SO_4. Products were analysed by HPLC on a LiChrospher 100 RP-18 column as detailed in Methods, using authentic 4-CBCoA and 4-HBCoA as standards.
[a] No enzyme added.

Effect of DTT

Maintenance of dehalogenase in the intact state after purification was critically dependent on the presence of DTT. The enzyme was increasingly stimulated by addition of DTT up to 2 mM, beyond which there was no further activation. In the absence of DTT, activity was rapidly lost at 4 °C and −25 °C, and storage in liquid nitrogen (−196 °C) caused a 30% loss; but 2 mM DTT preserved the enzyme for 2 weeks at 4 °C and indefinitely at −25 °C and −196 °C.

Stoichiometry and kinetics

4-CBCoA was converted by the dehalogenase to 4-HBCoA in 99% yield (Table 5). The values for k_{cat} and the apparent K_m at pH 7.5 and 30 °C were found from a Lineweaver–Burk plot to be 1 ± 0.02 s^{-1} and 9 ± 2 μM respectively.

Sequencing

Edman degradation of the N-terminus of the dehalogenase revealed the sequence shown in Table 6 for the first 20 amino acids.

Discussion

4-CB:CoA ligase, the first enzyme in the conversion of 4-CB to 4-HB, was purified from *Arthrobacter* strain TM-1 using ammonium sulphate precipitation, anion exchange chromatography and gel filtration (Table 1). SDS-PAGE analysis showed that the subunit molecular mass of the enzyme was 56 kD, similar to that of the ligase from *Pseudomonas* strain CBS-3 (Löffler et al. 1992). However, analysis of the first twenty N-terminal amino acids of the enzyme revealed the sequence shown in Table 7, which is not that of 4-CB:CoA ligase (GenBank accession number: AF042490) but bears some similarity (the residues given in bold) to the N-terminal residues of the 4-hydroxybenzoate hydroxylase from *Pseudomonas* strain CBS-3 (Seibold et al. 1996; Table 7). Both ligase and hydroxylase activities were detected in the enzyme preparation from strain TM-1, which suggests that the N-terminus detected is that of the hydroxylase. No data on the structure of the gene for *Arthrobacter* 4-hydroxybenzoate hydroxylase is available for comparison, so whether the sequence above accurately represents the hydroxylase N-terminus is not clear. The complete sequence of the 4-chlorobenzoate dehalogenase operon of *Arthrobacter* strain TM-1 has been determined by Gartemann et al. (1998; Genbank accession number: AF042490), who regarded bases 9586 to 9734 as comprising a gene for 4-hydroxybenzoate hydroxylase, on the basis of some similarity to the corresponding gene in *Pseudomonas* CBS-3 (Seibold et al. 1996; Genbank accession number: X74827). The translation of the first 60 bases is shown in Table 7. This peptide, only 49 residues long, is unlikely to produce a functional enzyme, and bears little structural similarity at the N-terminus to the sequence presented here for the presumed sample of ligase.

Because the hydroxylase and ligase of strain TM-1 appear to share similar molecular masses (approximately 57 kD) and pI values (5.8), it was not possible to separate them using methods that exploit differences between these values.

ATP consumption, measured in crude cell-free extracts (data not shown), occurred during the ligase reaction, with approximately stoichiometric production of AMP and 4-CBCoA. ATP, CoA and a divalent cation were all essential for ligase activity. The present investigation confirms previous work by Marks (1984a, b) and Smith (1991), who showed that dialysis or desalting of cell-free extracts from strain TM-1 by gel filtration caused a total loss of enzyme activity, which was restored by ATP and Mn^{2+}. Löffler et al. (1991, 1992) reported that 4-CB:CoA ligase activity from *Pseudomonas* strain CBS-3 was likewise restored by supplementation with ATP, CoA and Mg^{2+}; Mn^{2+}, Co^{2+}, Fe^{2+} and Zn^{2+} were also effective metal ion cofactors. Chang et al. (1992) reported that the metal ion requirement of a recombinant form of the 4-CB:CoA ligase from *Pseudomonas* CBS-3 produced in *Escherichia coli* was satisfied with Mg^{2+},

Table 6. Comparison of N-terminal amino acid sequences of 4-CBCoA dehalogenase from different organisms

Organism	Amino acid sequences																				
Arthrobacter TM-1[a]			S	S	N	S	D	H	H	I	S	V	E	H	T	D	G	V	A	T	I	R
Arthrobacter TM-1[b]		M	S	S	N	S	D	H	H	I	S	V	E	H	T	D	G	V	A	T	I	R
Arthrobacter SU[c]		M	S	S	N	S	D	H	H	I	S	V	E	H	T	D	G	V	A	T	I	R
Arthrobacter 4-CB1[d]	A	V	S	S	N	S	D	H	H	I	S	V	E	H	T	D	G	V	A	T	I	R
Pseudomonas CBS3[e]	M	T	E	A	I	G	H	R	V	E	D	G	V	A	E	I	T	K	L	P		
Pseudomonas DJ-12[f]	M	Y	E	A	I	G	H	R	V	Q	D	G	V	A	E	I	T	I	N	L		

[a] Data from this study.
[b] Data from Gartemann et al. (1998; GenBank Accession number: AF042490).
[c] Data from Schmitz et al. (1992).
[d] Data from Crooks and Copley (1994).
[e] Data from purified 4-CBCoA dehalogenase (Löffler et al. 1995).
[f] Data from Chae et al. (1999).

Table 7. Comparison of the N-terminal amino acid sequences of some 4-hydroxybenzoate hydroxylases and a 4-CB:CoA ligase from various organisms with the sequence obtained from the ligase of TM-1

Organism	Amino acid sequence																			
Ligase sample																				
Arthrobacter TM-1[a]	T	R	**T**	P	I	A	**T**	E	**V**	A	**I**	M	**G**	**A**	**G**	**P**	**A**	**G**	**L**	M
Hydroxylases																				
Pseudomonas CBS-3[b]	M	K	**T**	V	T	R	**T**	Q	**V**	G	**I**	I	**G**	**A**	**G**	**P**	**A**	**G**	**L**	L
Arthrobacter TM-1[c]	T	R	T	Q	V	G	I	V	G	G	G	P	A	G	L	M	L	S	H	L
Azotobacter chroococcum[d]	M	K	T	Q	V	A	I	I	G	A	G	P	A	G	L	L	L	G	Q	L
Ligase																				
Arthrobacter TM-1[c]	M	R	T	A	F	E	L	V	A	W	S	A	H	R	Q	P	G	A	V	A

[a] Data from this study.
[b] Data from Seibold et al. (1996; GenBank Accession number: X74827).
[c] Data from the dehalogenase operon, from Gartemann et al. (1998; GenBank Accession number: AF042490).
[d] Data from Quinn et al. (2001; GenBank Accession number: AF019891).

Mn^{2+} or Co^{2+} (K_m and k_{cat} values obtained in the presence of the different metal ions were very similar); but not with Ca^{2+} or Zn^{2+}.

The ligase of strain TM-1 thioesterifies all four 4-halobenzoates, and benzoate itself. Smith (1991) demonstrated that crude cell-free extract of TM-1 converted all the 4-halobenzoates to 4-hydroxybenzoate, but did not act on 2- or 3-halobenzoates, or on the more highly substituted compounds. Purified 4-CB:CoA ligase from *Pseudomonas* sp. CBS-3 acted on the 4-halobenzoates, 4-methyl-, 4-methoxy- and 4-nitrobenzoate, and benzoate, but not on benzoates substituted at C(2) and/or C(3), C(5) with chloro-, fluoro-, iodo-, amino-, nitro- or hydroxyl groups (Löffler et al. 1992). Chang et al. (1992) showed that the recombinant 4-CB:CoA ligase from CBS-3 was not active with aliphatic carboxylic acids, including palmitate, caproate, laurate and butyrate; thus it is distinct from the fatty acid:CoA ligases. Likewise, 4-coumarate, phenylacetate, 4-hydroxy- and 2,3-dihydroxybenzoate, and 2-aminobenzoate were not substrates.

4-CBCoA dehalogenase, the second enzyme in the conversion of 4-CB to 4-HB, was purified 11.6-fold to homogeneity using hydroxyapatite (which proved essential in separating the enzyme from the ligase), anion exchange and gel filtration chromatography, with 43% recovery (Table 1). Maintenance of activity after purification was critically dependent on the presence of DTT, as has been observed for the corresponding enzymes of other organisms (Crooks & Copley 1994; Löffler et al. 1995). Gel filtration gave a molecular mass of 131 kD for the intact dehalogenase whilst SDS-PAGE gave 33 kD for the subunit; thus the enzyme is a tetramer of identical subunits. The subunit is therefore the same size as that of the en-

Table 8. A comparison of the physical and biochemical properties of the 4-CB:CoA ligases from *Arthrobacter* strain TM-1 and *Pseudomonas* strain CBS-3

Characteristic	*Arthrobacter* TM-1 (this study)	*Pseudomonas* CBS-3[a]	*Pseudomonas* CBS-3[b] (recombinant *E. coli.*)
Optimum pH	7.0	8.4	7.0
Optimum temperature	25 °C	35 °C	25 °C
Stoichiometry			
4-CB : 4-CBCoA	1 : 0.98	ND[c]	ND[c]
Molecular size (kD)			
Native/subunit	112/56	115/57	114/57
Cofactors	CoA/ATP/Mg^{2+}	CoA/ATP/Mg^{2+}	CoA/ATP/Mg^{2+}
K_m values			
4-CB (μM)	3.5	50–65	9
CoA (μM)	30	110–190	80
ATP (mM)	0.238	2.4–3.5	0.1

[a] Data from Löffler et al. (1992) for 4-CB:CoA ligase isolated from Pseudomonas sp. strain CBS-3.
[b] Data from Chang et al. (1992) for *Pseudomonas* 4-CB:CoA ligase isolated from an *E. coli* clone.
[c] ND = not determined.

Table 9. A comparison of the physical and biochemical properties of the 4-CBCoA dehalogenases from *Arthrobacter* and *Pseudomonas*

Characteristic	*Arthrobacter* TM-1[a]	*Arthrobacter* 4-CB1[b]	*Pseudomonas* CBS-3[c]
Optimum pH	7.5	8.0	10.0
Optimum temperature	45 °C	ND[d]	60 °C
Molecular size			
Native (kD)	131	116	120
Subunit (kD)	33	33	31
K_m (μM)	8.9–9.2	34 ± 3	2.4–2.7
k_{cat} (s^{-1})	1.0	1.30 ± 0.05	5
Isoelectric point (pI)	6.42	6.1	6.7
Required addition of DTT (mM)	2	2	1
Reaction Stoichiometry	1 : 0.99	ND[d]	ND[d]

[a] Data from this study.
[b] Data from Crooks and Copley (1994), derived using purified 4-CBCoA dehalogenase.
[c] Data from Löffler et al. (1995), derived using purified 4-CBCoA dehalogenase.
[d] ND = not determined.

zyme from *Arthrobacter* sp. strain CB-1 (Crooks & Copley, 1994), but differs from the dehalogenase subunit (30 kD) of *Pseudomonas* sp. strain CBS-3 (Löffler et al. 1995).

4-CBCoA has been identified in strain TM-1 as the product accumulating in equimolar proportion from the action of 4-CB:CoA ligase on 4-CB, and as the substrate for the dehalogenase, yielding stoichiometric quantities of 4-HBCoA. Löffler & Müller (1991) identified 4-CBCoA as a key intermediate in the dehalogenation of 4-CB by *Pseudomonas* CBS-3; hence closely similar enzyme systems for 4-CB dechlorination occur in *Arthrobacter* and *Pseudomonas*, members of two distinct bacterial families.

Comparison of the first 20 N-terminal amino-acid residues of 4-CBCoA dehalogenases from different bacteria (Table 6) revealed that the sequence of the enzyme from strain TM-1 is identical (except for the absence of an initial methionine residue) with the deduced sequences of the enzymes from *Arthrobacter* SU and another clone of *Arthrobacter* TM-1: the dehalogenase from *Arthrobacter* sp. 4-CB1 bears

an additional alanine and a valine residue at the N-terminus. The corresponding region of the enzyme from *Pseudomonas* sp. CBS-3 differs considerably.

Comparisons of the physical and biochemical properties of the ligase and dehalogenase from *Arthrobacter* strain TM-1 with those of *Arthrobacter* sp. 4-CB1 and *Pseudomonas* sp. CBS-3 are given in Tables 8 and 9, which reveal, in particular, differences between the enzymes from the two *Arthrobacter* strains.

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