Isolation and Characterization of the Three Polypeptide Components of 4-Chlorobenzoate Dehalogenase from *Pseudomonas* sp. Strain CBS-3†

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ABSTRACT: The three genes encoding the 4-chlorobenzene dehalogenase polypeptides were excised from a *Pseudomonas* sp. CBS-3 DNA fragment and separately cloned and expressed in *Escherichia coli*. The three enzymes were purified from the respective subclones by using an ammonium sulfate precipitation step followed by one or two column chromatographic steps. The 4-chlorobenzoate:coenzyme A ligase was found to be a homodimer (57-kDa subunit size), to require Mg^{2+} (Co^{2+} and Mn^{2+} are also activators) for activity, and to turn over MgATP (K_m = 100 μM), coenzyme A (K_m = 80 μM), and 4-chlorobenzoate (K_m = 9 μM) at a rate of 30 s^{-1} at pH 7.5 and 25 °C. Benzoate, 4-bromobenzoate, 4-iodobenzoate, and 4-methylbenzoate were shown to be alternate substrates while 4-hydroxybenzoate, 4-aminobenzoate, 2-aminobenzoate, 2,3-dihydroxybenzoate, 4-coumarate, palmate, laurate, caproate, butyrate, and phenylacetate were not substrate active. The 4-chlorobenzoate-coenzyme A dehalogenase was found to be a homotetramer (30 kDa subunit size) to have a K_m = 15 μM and k_{cat} = 0.3 s^{-1} at pH 7.5 and 25 °C and to be catalytically inactive toward hydration of crotonyl-CoA, α-methylcrotonyl-CoA, and β-methylcrotonyl-CoA. The 4-hydroxybenzoate-coenzyme A thioesterase was shown to be a homotetramer (16 kDa subunit size), to have a K_m = 5 μM and k_{cat} = 7 s^{-1} at pH 7.5 and 25 °C, and to also catalyze the hydrolyses of benzoyl-coenzyme A and 4-chlorobenzoate-coenzyme A. Acetyl-coenzyme A, hexanoyl-coenzyme A, and palmitoyl-coenzyme A were not hydrolyzed by the thioesterase.

Halogenated hydrocarbons having widespread use as industrial and agricultural agents constitute a particularly formidable class of environmental pollutants. Biodegradation of these compounds by microorganisms offers a promising approach to the detoxification of contaminated areas [for recent reviews, see Abramowicz (1990) and Commandeur and Parsons (1990)]. In recent years, a number of strains of soil-dwelling bacteria have been isolated which are able to catabolize a variety of halogenated hydrocarbons. One such strain, *Pseudomonas* sp. CBS-3, was isolated by requiring growth on 4-chlorobenzoate (4-CBA)[1] as the sole source of carbon (Keil et al., 1981). The 4-CBA is metabolized in this bacterium first by conversion to 4-hydroxybenzoate (4-HBA) and then to the metabolite, 3,4-dihydroxybenzoate.

Attempts to purify the 4-CBA dehalogenase from *Pseudomonas* sp. CBS-3 (Thiele et al., 1987) as well as from alternate host strains, *Norcardia* sp. (Klages & Lingens, 1979), *Alcaligenes* sp. NTP-1 (van den Tweel et al., 1986), and *Arthrobacter* sp. (Müller et al., 1988; Ruisinger et al., 1976), had been made, but with limited success. In 1986, the cloning of a 9.5-kB chromosomal DNA fragment from *Pseudomonas* sp. strain CBS-3 carrying the dehalogenase genes into *E. coli* using the cosmid vector pPSA 843 was reported (Savard et al., 1986). Transfer of the hybrid cosmid to the 4-CBA dehalogenase-minus strain *Pseudomonas putida* KT2440 conferred on this strain the ability to dehalogenate 4-CBA and to grow on it as the sole source of carbon. Further work resulted in the circumscription of the genes of the 4-CBA dehalogenase and to the identification of the protein products through selective gene expression (Scholten et al., 1991). The 4-CBA dehalogenase was found to consist of 57-, 30-, and 16-kDa polypeptide components. Fractionation of these polypeptides allowed the identification of 4-CBA:CoA ligase, 4-CBA-CoA dehalogenase, and 4-HBA-CoA thioesterase activities and, consequently, led to the proposal of the 4-CBA degradation pathway shown in Scheme I (Scholten et al., 1991). The fractionation of the ligase and dehalogenase activities from cellular extracts of the parent strain *Pseudomonas* sp. CBS-3 have also been recently reported (Elsner et al., 1991; Löffler & Müller, 1991).

In this paper, we report first on the purification of the three 4-CBA dehalogenase enzyme components from constructed *Escherichia coli* subclones and then on the physical and kinetic properties of the purified enzymes.

Materials and Methods

General. E. coli JM101 was a gift from John Gerlt (Department of Chemistry, University of Maryland), and plasmids pGP1-2 and pT7.5 and the bacterial strain *E. coli* K38 were a gift from Stanley Tabor (Department of Biological Chemistry, Harvard Medical School). The culture media used in these studies were Luria–Bertani (LB) broth and Noble agar. Restriction enzymes, pUC18, and T4 DNA ligase were purchased from U.S. Biochemical Corp. The 1-kb DNA ladder, X-Gal, isopropyl β-*D*-thiogalactopyranoside (IPTG), and normal and low-melting-point (LMP) agar were obtained from Bethesda Research Labs. Geneclean II was purchased from BIO 101 Inc. Enzymes, cofactors, and other chemicals were obtained from Sigma Chemical Co. DEAE-cellulose, hy-

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[1] Abbreviations: 4-CBA, 4-chlorobenzoate, 4-HBA; 4-hydroxybenzoate; ATP, adenosine 5′-triphosphate; CoA, coenzyme A; NADH, dihydronicotinamide adenine dinucleotide; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; NADP, nicotinamide adenine dinucleotide phosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside; LMP, low-melting-point; LB, Luria–Bertani; DTT, dithiothreitol; PEP, phosphoenolpyruvate.

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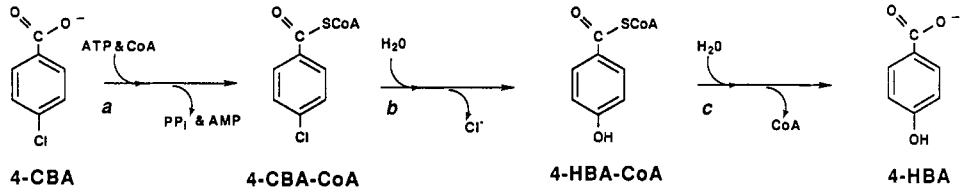
Scheme I: Reaction Steps of the Dehalogenation of 4-CBA in *Pseudomonas* sp. CBS-3 Catalyzed by (a) 4-CBA:CoA Ligase, (b) 4-CBA-CoA Dehalogenase, (c) 4-HBA-CoA Thioesterase



droxyapatite, and Sephadex G-200 were purchased from Whatman, Bio-Rad, and Pharmacia, respectively. The protein molecular weight standards were purchased from Pharmacia. PAGE analyses were performed on a BRL Model V-16 vertical slab gel unit by using the method of Laemmli (1970). Gels were run in Tris–glycine–SDS buffer (3 g/L Tris base, 14.4 g/L glycine, 1 g/L SDS). Protein samples were mixed with an equal volume of protein gel loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue, 5% 2-mercaptoethanol), heated in a boiling water bath for 4 min, and then loaded into an SDS–polyacrylamide gel. Agarose gel electrophoresis was performed upon a BRL Model H-5 electrophoresis unit. Agarose gels containing 0.5 μg/mL ethidium bromide were prepared by using standard methods (Sambrook et al., 1989). Gels were run in 1× Tris–acetate–EDTA buffer diluted from 50× stock solution (242 g/L Tris base, 57.1 mL/L glacial acetic acid, 100 mL/L 0.5 M EDTA). Samples were mixed with one-fifth volume of 6× agarose gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) before electrophoresis. 4-CBA-CoA and 4-HBA-CoA were synthesized according to the methods of Mieyal et al. (1974) and Merkel et al. (1989), respectively.

Construction of the SmaI–SalI–pT7.5 Subclone Encoding the 30-kDa Polypeptide, the NruI–NheI–pUC18 Subclone Encoding the 57-kDa Polypeptide, and the NheI–SphI–pUC18 Subclone Encoding the 16-kDa Polypeptide. Minipreparations of the pT7.5 plasmid carrying the 4.5-kb DNA insert encoding the dehalogenase genes (Scholten et al., 1991) were obtained from transformed *E. coli* JM101 cells by using the alkaline lysis method (Sambrook et al., 1989). The first ORF encoding the 4-CBA:CoA ligase was excised from the 4.5-kb DNA-containing pT7.5 plasmid with *Sma*I and *Sal*I, purified on a 1.5% LMP agarose gel, and then ligated to *Sma*I–*Sal*I-digested plasmid pT7.5. The ligation mixture was used to transform competent (Sambrook et al., 1989) *E. coli* K38 cells. The procedures used in subcloning the *Nru*I–*Nhe*I fragment from the 4.5-kb DNA fragment were the same as those described above except that pUC18 and *E. coli* JM101 were used as the vector and the host cell, respectively. *Nru*I and *Nhe*I were used to excise the second ORF from the 4.5-kb insert DNA, and *Sma*I and *Xba*I were used in cutting the pUC18 plasmid. The same procedures were used to construct the *Nhe*I–*Sph*I–pUC18 subclone except that *Nhe*I and *Sph*I were used to excise the third ORF from the 4.5-kb insert and that *Xba*I and *Sph*I were used to cut the pUC18 plasmid.

Enzyme Assays. Enzyme assays were performed at 25 °C, and the assay buffer used was 50 mM Hepes (pH 7.5) containing 1 mM DTT. The 4-CBA:CoA ligase activity was measured by using a coupled enzyme assay consisting of myokinase, pyruvate kinase, and lactate dehydrogenase. The oxidation of NADH was monitored spectrophotometrically at 340 nm ($\Delta\epsilon$ = 6220 M^{-1} cm^{-1}). The 1-mL assay solution contained 0.25 mM 4-CBA, 0.26 mM CoA, 0.5 mM MgATP, 0.2 mM NADH, 3 mM phosphoenolpyruvate (PEP), 5 mM KCl, myokinase (11 units), pyruvate kinase (9 units), and lactate dehydrogenase (9 units). An alternate 4-CBA:CoA ligase assay used coupled the 4-CBA-CoA formation to the 4-CBA-CoA dehalogenase catalyzed formation of 4-HBA-CoA (see below). The 4-CBA-CoA dehalogenase catalyzed formation of 4-HBA-CoA was measured by using a direct spectrophotometric assay wherein the increase in the absorptivity of the 1-mL reaction solution resulting from the conversion of 4-CBA-CoA (0.2 mM) to 4-HBA-CoA was monitored at 300 nm ($\Delta\epsilon$ = 8200 M^{-1} cm^{-1}). The 4-HBA-CoA thioesterase activity was measured by using a coupled enzyme system where 4-hydroxybenzoate hydroxylase converts 4-HBA to 3,4-dihydroxybenzoate with the concomitant consumption of NADPH. The decrease in the absorbance at 340 nm ($\Delta\epsilon$ = 6200 M^{-1} cm^{-1}) was monitored. The 1-mL assay solution contained 84 μM 4-HBA-CoA, 200 μM NADPH, 200 μM FAD, and 4-HBA hydroxylase (1 unit).

Purification of the 4-CBA:CoA Ligase. A single colony of *E. coli* JM101 containing the plasmid *Nru*I–*Nhe*I–pUC18 was used to inoculate 6 mL of LB medium containing 75 μg/mL ampicillin. The 6-mL overnight culture was then used to inoculate 6 L of fresh LB medium, and the culture was grown at 37 °C on a gyratory shaker. After 6 h (at the mid-log phase of cell growth), IPTG was added to a final concentration of 1 mM in the 6-L culture, which was then incubated at 37 °C for another 16 h. The cells were then harvested by centrifugation at 5000*g* for 15 min. Approximately 5 g of cells was obtained per liter of culture. Thirty grams of cells was suspended in 300 mL of buffer (50 mM Hepes, pH 7.5, 1 mM DTT). The cell suspension was passed through a French press at 16000 psi and then centrifuged at 8000*g* for 20 min. Ammonium sulfate was added to the supernatant to give a final concentration of 50% (w/v). The precipitate formed was collected by centrifugation at 8000*g* for 20 min. The protein pellet was dialyzed for 9 h against three changes of 2 L of pH 7.5 buffer (50 mM Hepes, 1 mM DTT). The dialysate was loaded onto a 2.5 × 40 cm DEAE-cellulose column which had been equilibrated with 50 mM Hepes (pH 7.5) containing 1 mM DTT. The column was first eluted with 240 mL of the equilibration buffer and then with 1 L of a 0–0.5 M KCl linear gradient in 50 mM Hepes (pH 7.5) containing 1 mM DTT. The fractions containing 4-CBA:CoA ligase activity eluted at approximately 0.17 M KCl.

Purification of the 4-CBA-CoA Dehalogenase. A single colony of *E. coli* K38 cells transformed with the plasma *Sma*I–*Sal*I–pT7.5 was used to inoculate 5 mL of LB medium containing 50 μg/mL ampicillin and kanamycin. The culture was incubated at 30 °C overnight and then transferred to a 6-L flask containing 3 L of fresh LB medium. The culture was incubated at 30 °C with shaking overnight, and then the cells were harvested, resuspended in 150 mL of fresh LB medium, and incubated at 42 °C for 45 min, then at 30 °C for another 45 min, and then harvested by centrifugation. Fifteen grams of cells was resuspended in 150 mL of suspension buffer. The subsequent steps taken to obtain the dialysate of the 40–70% ammonium sulfate induced protein precipitate and the procedures employed for the DEAE-cel-

lulose column chromatography were the same as those described above for the purification of the 4-CBA:CoA ligase. The 4-CBA-CoA dehalogenase eluted from the DEAE-cellulose column at approximately 0.25 M KCl. The active fractions were collected, concentrated to 6 mL, and then loaded onto a 2.5 × 85 cm Sephadex G-200 column. The column was eluted with 50 mM Hepes (pH 7.5) containing 1 mM DTT.

Purification of the 4-HBA-CoA Thioesterase. A single colony of *E. coli* JM101 cells containing the plasmid *Nhe*I–*Sph*I–pUC18 was used to inoculate 6 mL of LB medium containing 75 μg/mL ampicillin. The subsequent steps taken to obtain the dialysate of the 0–60% ammonium sulfate induced protein precipitate were similar to those described previously as were the procedures used for the DEAE-cellulose column chromatography. The DEAE-cellulose column fractions containing 4-HBA-CoA thioesterase activity (eluted at 0.25 M KCl) were combined and loaded onto a 2.5 × 35 cm hydroxyapatite column equilibrated with 50 mM Hepes (pH 7.5) containing 1 mM DTT. The column was first eluted with 240 mL of equilibration buffer and then with 1 L of a 0.0–0.5 M K_2HPO_4 linear gradient in 50 mM Hepes (pH 7.5) and 1 mM DTT. The fractions containing the 4-HBA-CoA thioesterase eluted at approximately 0.3 M K_2HPO_4.

Protein Molecular Weight Determination. The molecular weights of native proteins were determined by size-exclusion chromatography on a Blue Dextran 2000 calibrated Sephadex G-200 column [2.5 × 94 cm; 50 mM Hepes (pH 7.5), 0.1 M NaCl, and 1 mM DTT as eluant]. The molecular weights were estimated from the plot of K_{av} vs log MW of protein molecular weight standards, which were ovalbumin, bovine serum albumin, aldolase, catalase, and ferritin (MW = 43000, 67000, 158000, 232000, and 440000, respectively). K_{av} values were calculated using the equation $K_{av} = V_e - V_0/V_t - V_0$ where V_e is the elution volume of the protein, V_0 is the elution volume of Blue Dextran 2000, and V_t is the total gel bed volume (Davison, 1968). The molecular weights of the protein subunits were determined by SDS–PAGE using a 12% separating gel and 3% stacking gel. The subunit molecular weights were estimated from the plot of log MW vs R_f of protein molecular weight standards, which were phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme (MW = 97000, 66000, 43000, 31000, 21000, and 14000, respectively).

Determination of Metal Ion Requirements. Initial velocities of each of the three enzymic reactions were measured at 25 °C in the presence or in the absence (as the control) of a divalent metal ion at fixed concentration (0.5 mM). Metal ions were added as their chloride salts ($MgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$, and $ZnCl_2$) except for Fe^{2+}, which was added as its sulfate salt ($FeSO_4$). The 1-mL reaction mixtures (50 mM Hepes, 1 mM DTT; pH 7.5) used contained 0.25 mM 4-CBA, 0.26 mM CoA, 1 mM ATP, 4-CBA:CoA ligase (0.05 unit), and 4-CBA-CoA dehalogenase (1 unit) or 0.2 mM 4-CBA-CoA and 4-CBA-CoA dehalogenase (0.1 unit) or 100 μM 4-HBA-CoA, 200 μM NADPH, 200 μM FAD, 4-HBA hydroxylase (1 unit), and 4-HBA-CoA thioesterase (0.05 unit).

K_m and k_{cat} Measurement. The kinetic parameters K_m and V_{max} of each enzyme were determined from the initial velocity data measured using reaction solutions buffered at 25 °C with 50 mM Hepes and 1 mM DTT (pH 7.5) or 50 mM Tris-HCl (pH 8.5). The initial velocities of 4-CBA:CoA ligase (0.032 μM) catalyzed conversion of 4-CBA to 4-CBA-CoA were measured as a function of 4-CBA concentration (10–125 μM) [with CoA (0.8 mM), ATP (5 mM), and Mg^{2+} (7 mM) concentrations held constant] or as a function of CoA concentration (32–400 μM) [with 4-CBA (0.25 mM), ATP (5 mM), and Mg^{2+} (7 mM) concentrations held constant] or as a function of ATP concentration (0.1–1.0 mM) [with 4-CBA (0.25 mM), CoA (0.8 mM), and Mg^{2+} (7 mM) concentrations held constant]. The initial velocities of the dehalogenase catalyzed reactions were measured as a function of 4-CBA-CoA concentration (6-60 μM) using 1-mL reaction mixtures containing 0.85 μM 4-CBA-CoA dehalogenase in buffer. The initial velocities of 4-HBA-CoA thioesterase catalyzed reactions were measured as a function of 4-HBA-CoA concentration (2–20 μM) using 1-mL reaction mixtures containing 200 μM NADPH, 200 μM FAD, 4 units of 4-HBA hydroxylase, and 0.16 μM 4-HBA-CoA thioesterase in buffer. The initial velocity data were analyzed using eq 1 and the

$$V_0 = \frac{V_m[A]}{[A] + K_m} \quad (1)$$

fortran program of Cleland (1979). In eq 1, V_0 = initial velocity, V_m = maximal velocity, [A] = substrate concentration, and K_m = Michaelis constant. The k_{cat} values were then determined from the V_{max} values and total enzyme concentration used in the reaction according to the equation $k_{cat} = V_{max}/[E]$ where [E] was determined by using the Lowry method (Lowry et al., 1951) and was based on the subunit molecular weight.

Determination of Enzyme Substrate Specificity. The catalytic activity of the 4-CBA:CoA ligase toward benzoate (0.2–4.0 mM), 4-bromobenzoate (10–100 μM), 4-iodobenzoate (10–100 μM), 4-methylbenzoate (50–1000 μM), 4-hydroxybenzoate (1 mM), 4-aminobenzoate (1 mM), 2-aminobenzoate (1 mM), 2,3-dihydroxybenzoate (1 mM), 4-coumarate (1 mM), palmitic acid (1 mM), lauric acid (1 mM), caproic acid (1 mM), butyric acid (1 mM), or phenylacetic acid (1 mM) was measured using 1-mL assay solutions containing 0.8 mM CoA, 5 mM ATP, 7 mM $MgCl_2$, 0.25 mM NADH, 3 mM PEP, 5 mM KCl, myokinase (20 units), pyruvate kinase (18 units), lactate dehydrogenase (16 units), and 4-CBA:CoA ligase (up to 0.5 unit) in 50 mM Hepes (pH 7.5) at 25 °C. The hydratase activity of the 4-CBA-CoA dehalogenase toward crotonyl-CoA, α-methylcrotonyl-CoA, or β-methylcrotonyl-CoA was measured by using a coupled assay in which DL-β-hydroxyacyl-CoA formed by hydration of the corresponding 2-enoyl-CoA was oxidized in the presence of NAD and β-hydroxyacyl-CoA dehydrogenase. The formation of NADH was recorded spectrophotometrically at 340 nm. The 1-mL assay solutions contained 1.3 mM 2-enoyl-CoA, 0.5 mM NAD, β-hydroxyacyl-CoA dehydrogenase (10 units), and 4-CBA-CoA dehalogenase (67 μM; 1.6 units) in 50 mM Hepes (pH 7.5) and 1 mM DTT at 25 °C. The thioesterase activity of the 4-HBA-CoA thioesterase toward benzoyl-CoA (50–500 μM), 4-CBA-CoA (20–200 μM), acetyl-CoA (500 μM), hexanoyl-CoA (500 μM), or palmitoyl-CoA (500 μM) was measured by using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as a coupling reagent. The liberation of 5-thio-2-nitrobenzoate from DTNB (0.2 mM) by reaction with CoA was monitored spectrophotometrically at 412 nm ($\Delta\epsilon$ = 13600 M^{-1} cm^{-1}). The 1-mL assay mixtures contained 2.4 μM (1 unit) 4-HBA-CoA thioesterase in 50 mM Hepes (pH 7.5) and 1 mM EDTA at 25 °C.

Results and Discussion

Dehalogenase Subcloning and Protein Purification. The three genes encoding the 4-CBA dehalogenase polypeptides were excised from 4.5-kb *Pseudomonas* sp. DNA fragment (Scholten et al., 1991) for separate cloning and expression in *E. coli*. A *Sma*I–*Sal*I–pT7.5 subclone encoding the 4-CBA-

Table I: Purification of 4-CBA:CoA Ligase, 4-CBA-CoA Dehalogenase, and 4-HBA-CoA Thioesterase from *E. coli* Subclones

step	total protein (mg)	total[a] act. (units)	sp act. (units/mg)	% recovery	purification (*x*-fold)
I. Purification of 4-CBA:CoA Ligase from the *Nru*I–Nhe*I*–pUC18 Subclone					
extract from 30 g of cells	24.8×10^3	2800	1×10^{-1}	100	1
ammonium sulfate (0–50%)	5.2×10^3	1600	3×10^{-1}	60	3
DEAE-cellulose	60	1500	30	60	3×10^2
II. Purification of 4-CBA-CoA Dehalogenase from the *Sma*I–*Sal*I–pT7.5 Subclone					
extract from 14.5 g of cells	11.9×10^3	530	4×10^{-2}	100	1
ammonium sulfate (40–70%)	1.7×10^3	330	2×10^{-1}	60	5
DEAE-cellulose	560	250	4×10^{-1}	50	10
sephadex G-200	250	160	7×10^{-1}	30	16
III. Purification of 4-HBA-CoA Thioesterase from the *NHe*I–*Seh*I–pUC18 Subclone					
extract from 27 g of cells	21.8×10^3	4500	2×10^{-1}	100	1
ammonium sulfate (0–60%)	10.4×10^3	2500	2×10^{-1}	60	1
DEAE-cellulose	2.8×10^3	1500	6×10^{-1}	30	3
hydroxyapatite	90	1280	15	30	70

[a] One unit of the enzyme activity was defined as the amount of the enzyme required to produce 1 μmol of 4-CBA-CoA/min, 1 μmol of 4-HBA-CoA/min, or 1 μmol of 4-HBA/min at pH 7.5 and 25 °C.

CoA dehalogenase was constructed by insertion of the 1565-bp *Sma*I–*Sal*I DNA piece (carrying the first ORF on the 4.5-kb fragment) into the polylinker region of the expression plasmid pT7.5. Transformation of *E. coli* K38 cells with the recombinant plasmid *Sma*I–*Sal*I–pT7.5 and the coplasmid PGP1-2 placed the expression of the dehalogenase gene under the control of the T7 RNA polymerase/promoter system. A *Nru*I–*Nhe*I–pUC18 subclone encoding the 4-CBA:CoA ligase was constructed by ligation of the 2040-bp *Nru*I/*Nhe*I fragment (carrying the second ORF on the 4.5-kb fragment) with *Sma*I–*Xba*I-linearized pUC18. A *Nhe*I–*Sph*I–pUC18 subclone encoding the 4-HBA-CoA thioesterase was created by ligating the 661-bp *Nhe*I/*Sph*I DNA fragment (carrying the third ORF on the 4.5-kb fragment) with *Xba*I and *Sph*I treated pUC18. Transformation of *E. coli* JM101 cells with the *Nru*I–*Nhe*I–pUC18 or *Nhe*I–*Sph*I–pUC18 recombinant plasmids placed the expression of the two respective dehalogenase genes under the control of the *lac* promoter.

The steps used in the purification of each of the three dehalogenase polypeptides are summarized in Table I. Each purification procedure employed an initial ammonium sulfate precipitation step followed by DEAE-cellulose column chromatography of the dialyzed protein fraction. In the case of the 4-CBA:CoA ligase, these two steps were sufficient to obtain homogeneous protein which migrated as a single band on SDS–PAGE gels (see Figure 1) and showed no further fractionation on a Sephadex G-200 column. Pure 4-CBA-CoA dehalogenase was obtained (on the basis of SDS–PAGE analysis; Figure 1) by chromatography on a Sephadex G-200 gel filtration column following the DEAE-cellulose column chromatographic step. Hydroxyapatite column chromatography following the DEAE-cellulose column chromatography proved to be the most effective means of producing pure (on the basis of SDS–PAGE analysis; Figure 1) 4-HBA-CoA thioesterase. The yields of the three proteins purified from the respective *E. coli* clones are roughly 2 mg of 4-CBA:CoA ligase/g of cell paste, 17 mg of 4-CBA-CoA dehalogenase/g of cell paste, and 3 mg of 4-HBA-CoA thioesterase/g of cell paste.

Enzyme Molecular Weight Determinations. The molecular weights of the purified 4-CBA:CoA ligase, 4-CBA-CoA dehalogenase, and 4-HBA-CoA thioesterase were determined by using standard gel filtration techniques to be 114 000, 122 000, and 66 000, respectively. On SDS–PAGE gels, these proteins migrated as single bands corresponding to molecular weights of 57 000, 31 000, and 16 000, respectively (Figure 1). These subunit weights agree well with the protein molecular

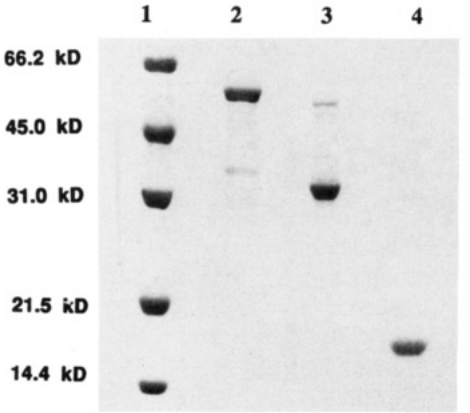


FIGURE 1: SDS–PAGE gel of the purified 4-CBA dehalogenase components: lane 1, protein molecular weight markers; lane 2, 4-CBA:CoA ligase; lane 3, 4-CBA-CoA dehalogenase; lane 4, 4-HBA-CoA thioesterase.

weights determined from the gene sequences (Babbitt et al., 1992) which are 57 156, 29 847, and 16 107, respectively. Hence, native 4-CBA:CoA ligase is a homodimer, native 4-CBA-CoA dehalogenase is a homotetramer, and native 4-HBA-CoA is a homotetramer.[2]

Benzoate:CoA and aminobenzoate:CoA ligases have been recently isolated from *Rhodopseudomonas palustris* (Geissler et al., 1988) and *Pseudomonas* sp. (Altenschmidt et al., 1991). SDS–PAGE analysis of each of these enzymes revealed subunit weights of ~60 000 in agreement with the 57 000 subunit weight of the *Pseudomonas* sp. CBS-3 4-CBA:CoA ligase. The *R. palustris* benzoate:CoA ligase is a monomer as are the two 2-aminobenzoate:CoA ligases of the *Pseudomonas* sp. strain. On the other hand, the *Pseudomonas* sp. benzoate:CoA ligase is, like the 4-CBA:CoA ligase of *Pseudomonas* sp. CBS-3, a homodimer.

Metal Ion Requirements. The metal ion requirements of the three enzymes of the 4-CBA → 4-HBA pathway were determined at pH 7.5, 25 °C, by using initial velocity techniques. The catalytic activities of the 4-CBA-CoA dehalogenase and 4-HBA-CoA thioestease were not affected by

[2] In our original communication (Scholten et al., 1991), we reported the isolation by DEAE-cellulose column chromatography of an $\alpha\beta$ heterodimer consisting of the 57-kDa and 30-kDa dehalogenase polypeptides. Careful chromatographic fractionation of the 57-kDa and 30-kDa dehalogenase components in this study has allowed us to conclude that they do not exist as a complex as originally thought, but rather they just happen to closely migrate on DEAE-cellulose columns.

Table II: Kinetic Constants of 4-CBA:CoA Ligase, 4-CBA-CoA Dehalogenase, and 4-HBA-CoA Thioesterase Determined at 25 °C in 50 mM K⁺Hepes/1 mM DTT (pH 7.5) (See Materials and Methods for Details)

substrate	K_m (μM)	k_{cat} (s^{-1})
I. Kinetic Constants of 4-CBA:CoA Ligase		
4-CBA[a]	8.50 ± 0.09	29 ± 1
CoA[b]	70 ± 6 (320 ± 40)[d]	35 ± 1 (32 ± 2)[d]
M(ATP)[c]	104 ± 5 (Mg^{2+})	24.2 ± 0.4
	43 ± 2 (Mn^{2+})	24.2 ± 0.2
	59 ± 3 (Co^{2+})	33 ± 4
4-bromobenzoate[a]	15 ± 1	25.6 ± 0.8
4-iodobenzoate[a]	17 ± 6	14 ± 2
benzoate[a]	700 ± 100	7.6 ± 0.6
4-methylbenzoate[a]	130 ± 20	38 ± 4
II. Kinetic Constants of 4-CBA-CoA Dehalogenase		
4-CBA-CoA	15 ± 1 (9.2 ± 0.5)[d]	0.26 ± 0.01 (0.409 ± 0.006)[d]
III. Kinetic Constants of 4-HBA-CoA Thioesterase		
4-HBA-CoA	4.5 ± 0.2 (2.8 ± 0.9)[d]	6.8 ± 0.1 (12 ± 1)
benzoyl-CoA	200 ± 10	0.26 ± 0.03
4-CBA-CoA	190 ± 20	0.123 ± 0.008

[a] Measured in the presence of 0.8 mM CoA, 5 mM ATP, and 7 mM Mg^{2+}. [b] Measured in the presence of 0.25 mM 4-CBA, 5 mM ATP, and 7 mM Mg^{2+}. [c] Measured in the presence of 0.8 mM CoA, 0.25 mM 4-CBA, and 0.5 mM uncomplexed divalent (M) metal ion. [d] Measured using 50 mM Tris-HCl, pH 8.5, as buffer.

exhaustive dialysis of the respective proteins against EDTA (10 mM) containing buffer or by the addition of divalent metal ion (Mg^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, Co^{2+}, or Zn^{2+}) to assay mixtures. Hence, no evidence for metal ion assisted catalysis could be found with either enzyme. These findings, however, do not exclude the possibility that one or both of these enzymes contain a metal ion cofactor, which may be so tightly bound that it cannot be removed by the protein purification steps or by EDTA treatment.

The 4-CBA:CoA ligase, on the other hand, was found to require a divalent metal ion cofactor for catalysis. This requirement is satisfied with Mg^{2+}, Mn^{2+}, or Co^{2+} but not with Ca^{2+} or Zn^{2+}. The Mg^{2+}, Mn^{2+}, and Co^{2+} activation of the ligase was examined by measuring the K_m for the metal–ATP complex and the turnover rate of the enzyme saturated with substrates and metal ion. The K_m and k_{cat} values obtained using the three different metal ions as cofactor are, as illustrated in Table II, very similar in magnitude.

Kinetic Constants. Initial velocity techniques were used to determine the k_{cat} and K_m values of the 4-CBA:CoA ligase, the 4-CBA-CoA dehalogenase, and the 4-HBA-CoA thioesterase at pH 7.5 and pH 8.5. The results obtained are presented in Table II. The k_{cat} and K_m values measured at physiological pH and at alkaline pH were, with the exception of the K_m for CoA, comparable in magnitude. The K_m values (obtained for the physiological substrates) fall between 5 μM and 100 μM, and the k_{cat} values fall between 0.3 and 30 turnovers per second. The 4-CBA-CoA dehalogenase catalyzes the rate-limiting step of the three-step 4-CBA → 4-HBA reaction pathway (k_{cat}/K_m = 1.7 × 10^4 M^{-1} s^{-1} at pH 7.5). Consequently, thioesterase discrimination between 4-CBA-CoA and 4-HBA-CoA is essential in order to avoid a futile cycle in which 4-CBA-CoA, formed at the expense of ATP, is hydrolyzed by the thioesterase before it is dehalogenated. We found, as reported in Table II, that 4-CBA-CoA is indeed hydrolyzed by the thioesterase. However, the 26-fold higher k_{cat}/K_m value of the dehalogenase for 4-CBA-CoA vs that of the thioesterase (=6.5 × 10^2 M^{-1} s^{-1} at pH 7.5) suggests that the 4-CBA-CoA formed by the ligase in vivo will partition to the dehalogenase.

Substrate Specificity. The substrate specificities of the three enzymes were briefly examined to screen alternate physiological roles and to gauge capacity for substrate discrimination. Examination of potential alternate substrates for the 4-CBA:CoA ligase showed that it is not active with aliphatic carboxylic acids including palmitate, caproate, laurate, and butyrate. It is thus catalytically distinct from the fatty acid:CoA ligases. Likewise, the substrates for 4-coumarate:CoA ligase, phenylacetate:CoA ligase, 4-hydroxybenzoate:CoA ligase, 2,3-dihydroxybenzoate:CoA ligase, and 2-aminobenzoate:CoA ligase are not substrates for the 4-CBA:CoA ligase.

To probe the extent to which the 4-CBA:CoA ligase active site is tailored for 4-CBA binding and catalysis, the substrate activities of a series of benzoate derivatives were compared. The results obtained (see Table II) support the following descending order of reactivity: 4-CBA (k_{cat}/K_m = 340 × 10^4 M^{-1} s^{-1}) > 4-bromobenzoate (k_{cat}/K_m = 170 × 10^4 M^{-1} s^{-1}) > 4-iodobenzoate (k_{cat}/K_m = 82 × 10^4 M^{-1} s^{-1}) > 4-methylbenzoate (k_{cat}/K_m = 29 × 10^4 M^{-1} s^{-1}) > benzoate (k_{cat}/K_m = 1 × 10^4 M^{-1} s^{-1}). 4–Aminobenzoate and 4-HBA, which were also tested, are not substrates. From these data, it is clear that the 4-chloro substituent plays an important role in substrate recognition (perhaps by binding to a small hydrophobic pocket on the enzyme) and that the 4-CBA:CoA ligase has specifically evolved to carry out 4-CBA thioesterification.

Using a similar approach, the 4-HBA-CoA thioesterase was shown to be inactive with aliphatic acyl-CoA thioesters including palmitoyl-CoA, hexanoyl-CoA, and acetyl-CoA, and, thus, to be catalytically distinct from the thioesterases involved in fatty acid metabolism. The relative reactivity of benzoyl-CoA derivatives toward thioesterase catalysis (see Table II) was found to be 4-HBA-CoA (k_{cat}/K_m = 1.5 × 10^6 M^{-1} s^{-1}) ≫ benzoyl-CoA (k_{cat}/K_m = 13 × 10^2 M^{-1} s^{-1}) ≈ 4-CBA-CoA (k_{cat}/K_m = 6.4 × 10^2 M^{-1} s^{-1}). These results show the thioesterase to be highly discriminating and to recognize the 4-HBA-CoA substrate, in part, through interaction with the 4-OH substituent.

Substrate specificity tests with the 4-CBA-CoA dehalogenase were used to screen the substrates of the 2-enoyl-CoA hydratases. Crotonyl-CoA, α-methylcrotonyl-CoA, and β-methylcrotonyl-CoA were shown to be inactive as substrates, and, thus, the 4-CBA-CoA dehalogenase was shown to be distinct from its closest catalytic analogue, the 2-enoyl-CoA hydratases.

Conclusions. The separate cloning and expression of the three genes encoding 4-CBA dehalogenase activity in *Pseudomonas* sp. CBS-3 demonstrates that the encoded polypeptides function as independent catalysts in a three-step, 4-CBA dehalogenating reaction pathway. Each of the three enzymes exhibits stringent substrate specificity suggesting that they have each evolved for the singular purpose of functioning in the degradation of 4-CBA. The parallel which exists between the chemical sequence of the 4-CBA degradative pathway and portions of the β-oxidation pathways operative in fatty acid and amino acid catabolism suggests that the 4-CBA:CoA ligase, 4-CBA-CoA dehalogenase, and 4-HBA-CoA thioesterase might have origins in one of these metabolic pathways. The sequence homology reported in the preceding paper (Babbitt et al., 1992) between the 4-CBA:CoA ligase and the acyl:CoA ligases, 4-coumarate:CoA ligase, fatty acid:CoA ligase, and acetyl:CoA ligase and between the 4-

CBA-CoA dehalogenase and several specimens of 2-enoyl-CoA hydratase of the fatty acid β oxidation pathway lend support to this idea.

As a final note, we compare the catalytic efficiencies of these so-called "retooled" enzymes to their probable contemporary counterparts. The 4-HBA-CoA thioesterase is, as far as we are aware, the first of its kind to be characterized. Its modest turnover rate (14 s^{-1} at pH 8.5), however, compares well with those reported for the long chain (3–30 s^{-1}) and medium chain (0.05 s^{-1}) fatty acyl-CoA thioesterases (Smith, 1981; Yabusalo & Ballou, 1981). The k_{cat} value observed for the 4-CBA:CoA ligase (30 s^{-1}), on the other hand, is lower than those reported for the bacterial benzoate-CoA ligases and 2-aminobenzoate-CoA ligases (20–400 s^{-1}) (Altenschmidt et al., 1991; Geissler et al., 1988). The most notable disparity in catalytic efficiency is found in comparing the 4-CBA-CoA dehalogenase (0.3 s^{-1}) to 2-enoyl-CoA hydratases (50–5000 s^{-1}) (Waterson & Hill, 1972; Yang et al., 1985; Fong & Schulz, 1981). The slow rate of catalysis displayed by the dehalogenase may reflect the high energy barrier often associated with nucleophilic substitution reactions of aryl halides (Miller, 1970).

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