Evidence for Nucleophilic Catalysis in the Aromatic Substitution Reaction Catalyzed by (4-Chlorobenzoyl)coenzyme A Dehalogenase†

Guang Yang, Po-Huang Liang, and Debra Dunaway-Mariano*

Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742

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ABSTRACT: (4-Chlorobenzoyl)coenzyme A dehalogenase catalyzes the hydrolytic dehalogenation of (4-chlorobenzoyl)coenzyme A (4-CBA-CoA) to (4-hydroxybenzoyl)coenzyme A (4-HBA-CoA). Rapid-quench techniques were used in conjunction with [^{14}C]-4-CBA-CoA to test for the formation of a covalent enzyme intermediate during catalysis. The rate of [^{14}C]-4-CBA-CoA (37 μM) consumption in the presence of a 2-fold excess of dehalogenase (75 μM) was determined to proceed at k = 6.5 s^{-1}, coincident with the formation of an enzyme intermediate containing covalently bound radiolabel. The radiolabeled enzyme reached a maximum level at 100 ms, corresponding to 27% of the starting [^{14}C]-4-CBA-CoA, before declining. The kinetics of formation and consumption of the radiolabeled enzyme observed during turnover are consistent with its intermediacy in the overall reaction. A single turnover reaction carried out in 98% ^{18}O-enriched water produced 4-HBA-CoA with 73–75% ^{16}O and 27–25% ^{18}O at the benzoyl ring C(4)-OH. In contrast, a multiple turnover reaction carried out in 93% $H_2{}^{18}O$ produced 4-HBA-CoA labeled at the C(4)-OH with 89% ^{18}O and 11% ^{16}O. These results were interpreted as evidence for formation of an aryl enzyme intermediate during 4-CBA-CoA hydrolytic dechlorination in the dehalogenase active site.

(4-Chlorobenzoyl)coenzyme A dehalogenase catalyzes the hydrolytic dehalogenation of (4-chlorobenzoyl)coenzyme A (4-CBA-CoA)[1] to (4-hydroxybenzoyl)coenzyme A (4-HBA-CoA). This enzyme has been discovered in a number of soil-dwelling bacterial strains where it functions in a 4-CBA to 4-HBA converting pathway (Scheme 1) along with 4-CBA: CoA ligase and 4-HBA-CoA thioesterase [for a recent review, see Dunaway-Mariano and Babbitt (1994)]. The 4-CBA pathway connects to the *ortho* cleavage pathway (and hence to the β-keto adipate pathway), thereby forming a route for the complete mineralization of this aromatic compound. While 4-CBA is not known to be a natural product, it is generated *in situo* as a byproduct of the oxidative biodegradation of 4-chlorobiphenyl, a ubiquitous, synthetic pollutant (Higson, 1992; Abramowicz, 1990; Commandeur & Parsons, 1990). Thus, the 4-CBA-CoA dehalogenase may be relatively new on the evolution scale, the product of recent gene retooling triggered by the presence of 4-CBA.

Insight into the mechanism of environmentally induced gene retooling might be gained from the lineage of the restructured gene. For this purpose, we have searched for the ancestors of the 4-CBA-CoA dehalogenase. We discovered that this enzyme shares significant structural similarity with 2-enoyl-CoA hydratase and Δ^3-*cis*,Δ^2-*trans*-enoyl-CoA isomerase (two enzymes of the fatty acid β-oxidation pathway) and with dihydroxynaphthoate synthase (an enzyme of the menaquinone (Vitamin K_2) pathway) (Babbitt et al., 1992; Dunaway-Mariano & Babbitt, 1994). A common feature of these enzymes seems to be electrophilic catalysis leading to polarization of the C=O of the CoA thioester substituent (Babbitt et al., 1992; Dunaway-Mariano & Babbitt, 1994). In addition to this particular group of enzymes we have found, as we describe in this paper, the *Pseudomonas* sp. strain CBS3 4-CBA-CoA dehalogenase is mechanistically (if not structurally) related to two enzymes which catalyze hydrolytic reactions at saturated carbon, namely, epoxide hydrolase and 2-haloalkane dehalogenase. Just recently, these two enzymes have been shown to use a novel form of covalent catalysis wherein an active site aspartate side chain adds to the electrophilic carbon to form an alkyl intermediate (Verschueren et al., 1993; Lacourciere & Armstrong, 1993, 1994). Hydrolysis occurs at the alkyl carbonyl, thereby transferring the aspartate oxygen atom to the alcohol product. In the text which follows, we provide evidence, derived from rapid-quench and ^{18}O-labeling experiments, that the *Pseudomonas* sp. strain CBS3 4-CBA-CoA dehalogenase reaction proceeds *via* an aryl intermediate.

EXPERIMENTAL SECTION

General. Scintillation counting was carried out with a Beckman LS 5801 scintillation counter. Gas chromatography–mass spectrometric (GC-MS) data were obtained by using HP5988A and HP5890 spectrometers with a 12.5 m DB-1 (0.2 mm i.d.) capillary column. HPLC separations were carried out with a Beckman HPLC instrument equipped with an ultrasphere 4.6 mm × 25 cm ODS-C_{18} reversed-phase column monitored at 260 nm and eluted (1.0 mL/min) with an acetonitrile/ammonium acetate gradient. Enzyme spectrophotometric assays were carried out as previously described (Liang et al., 1993). Methylation reactions were carried out using MNNG (1-methyl-3-nitro-1-nitrosoguanidine) diazomethane generator from Aldrich. 4-CBA-CoA and 4-HBA-CoA were synthesized according to the methods of Mieyal et al. (1974) and Merkel et al. (1989), respectively, and purified as previously described (Liang et al., 1993). 4-CBA-CoA dehalogenase was prepared according to the procedure of Chang et al. (1992). A Centricon-10 concentrator from Amicon was used to remove the dehalogenase from the reaction solutions containing ^{18}O-enriched water (95+%) (purchased

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* To whom correspondence should be addressed.

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[1] Abbreviations: 4-CBA, 4-chlorobenzoate; CoA, coenzyme A; 4-CBA-CoA, (4-chlorobenzoyl)coenzyme A; 4-HBA, 4-hydroxybenzoate; 4-HBA-CoA, (4-hydroxybenzoyl)coenzyme A; ATP, adenosine 5′-triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine.

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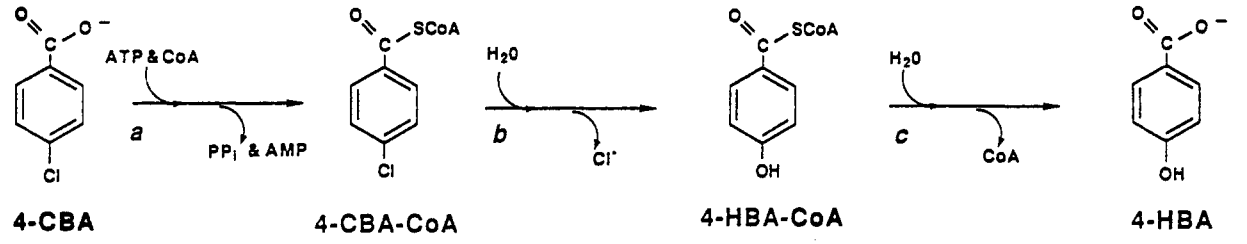
Scheme 1: 4-CBA to 4-HBA Converting Pathway of *Pseudomonas* sp. Strain CBS3[a]



[a] The reaction is catalyzed by (a) 4-CBA:CoA ligase, (b) 4-CBA-CoA dehalogenase, and (c) 4-HBA-CoA thioesterase (Scholten et al., 1991).

from EG & G Mound Applied Technologies). Methyl 4-methoxybenzoate (99%) was purchased from Aldrich, and [^{14}C]-4-CBA was purchased from California Bionuclear Corp.

Synthesis of [^{14}C]-4-CBA-CoA from [^{14}C]-4-CBA. One and one-half millimolar [^{14}C]-4-CBA (6.22 mCi/mmol), 1.5 mM ATP, 2.5 mM $MgCl_2$, 1.5 mM CoA, 1 unit of 4-CBA:CoA ligase, and 5 units of yeast inorganic pyrophosphatase (Sigma Co.) were incubated in a total volume of 450 μL. After 2 h at 25 °C, the reaction was quenched with 200 μL of CCl_4 and the mixture vortexed vigorously to precipitate the protein. The supernatant containing [^{14}C]-4-CBA-CoA was collected. The yield of [^{14}C]-4-CBA-CoA was judged to be close to 93%, based on the ratio of radioactivity which coeluted with the 4-CBA and 4-CBA-CoA standards from an HPLC reversed-phase column.

Rapid-Quench Studies. Rapid-quench experiments were carried out at 25 °C using a rapid-quench instrument from KinTek Instruments equipped with a thermostatically controlled circulator. The reactions were initiated by mixing 41 μL of 151 μM dehalogenase in 50 mM K$^+$Hepes/1mM DTT (specific activity = 2.0 unit/mg) with 41 μL of 75 μM [^{14}C]-4-CBA-CoA (specific activity = 6.22 mCi/mmol) in the same buffer and then quenched after a specified period of time with 153 μL of 0.1 N HCl. Control reactions were run under the same conditions except that the 41 μL of [^{14}C]-4-CBA-CoA was added after, not before, the acid quench. To measure the amount of radiolabeled enzyme present, the quenched reaction samples were diluted with 0.8 mL of 0.1 N HCl in a Centricon-10 concentrator (Amicon Co.). The Centricon-10 concentrator was centrifuged at 3000*g* for 20 min to remove radiolabeled substrate and product from the protein. The protein concentrate was diluted again with 0.8 mL of 0.1 N HCl and centrifuged. This procedure was repeated eight times (at which point less than 500 cpm remained in the protein sample of the control experiment). The radioactivity remaining in the protein sample was determined by scintillation counting.

To measure the amount of [^{14}C]-4-CBA-CoA and [^{14}C]-4-HBA-CoA in quenched reaction samples, the protein was first precipitated by vortexing the solution with 150 μL of CCl_4 and then pelleted by centrifuging this mixture. The resulting supernatant was mixed with unlabeled 4-CBA-CoA and 4-HBA-CoA and separated on an HPLC reversed-phase column (monitored at 260 nm). The [^{14}C]-4-CBA-CoA- and [^{14}C]-4-HBA-CoA-containing fractions were assayed by scintillation counting. The rate data were analyzed by computer fitting to the first-order rate equation $[P]_t = [P]_{max} \times [1 - \exp(-kt)]$.

Single Turnover of 4-CBA-CoA with 4-CBA-CoA Dehalogenase in $H_2{}^{18}O$. A 1 mL solution of 14.3 mg of 4-CBA-CoA dehalogenase in 50 mM K$^+$Hepes (pH 7.5) was lyophilized to a powder. To this powder was added 1 mL of 98% $H_2{}^{18}O$. After a 30 min incubation period at 25 °C, 0.105 mg of solid 4-CBA-CoA was added to yield a solution approximately 480 μM in enzyme active sites and 114 μM in substrate. After 5 min at 25 °C, 50 μL (11 units) of a buffered 4-HBA-CoA thioesterase solution ($H_2{}^{16}O$) was added to the reaction mixture. Following 15 min of further incubation at 25 °C, the protein was removed from the reaction solution using a Centricon-10 concentrator. The 4-HBA-containing solution was then acidified with 1 N HCl to pH 1 and extracted four times with 1 mL portions of ethyl acetate. The ethyl acetate extracts were combined and dried over anhydrous sodium sulfate and then concentrated to a solid *in vacuo*. The solid was dissolved in 0.5 mL of methanol and added to 2.5 mL of anhydrous ethyl ether in a MNNG diazomethane generator. The methylation reaction was initiated by the addition of 0.6 mL of 5 N sodium hydroxide to 133 mg of MNNG precursor. After approximately 15 h at 25 °C, the methanol–ether solution (which had evaporated to approximately 0.5 mL) was subjected to analysis by GC-MS. The methyl 4-methoxybenzoate product was identified by comparison with the authentic compound. GC-MS (MeOH–Et_2O) t_R = 6.13 min, *m/e* (amu) 166/168/170 (M*), 135/137/139 (M* – OCH_3), 107/109 (M* – $COOCH_3$), 92/94 (M* – $COOCH_3$ – CH_3).

Multiple Turnover of 4-CBA-CoA by 4-CBA-CoA Dehalogenase in $H_2{}^{18}O$. Solid 4-CBA-CoA (1.5 mg) was dissolved in 548 μL of $H_2{}^{18}O$ (95%). The reaction was initiated by the addition of 40 μL of 4-CBA-CoA dehalogenase (in buffered $H_2{}^{16}O$) (1.15 mg/mL; specific activity = 1.5 unit/mg) to give 2.76 mM 4-CBA-CoA and 2.6 μM dehalogenase in 5 mM K$^+$Hepes (pH 7) (93% $H_2{}^{18}O$). After 2 h at 25 °C, 50 μL (13 units) of a buffered 4-HBA-CoA thioesterase solution ($H_2{}^{16}O$) was added and the resulting mixture was incubated for an additional 2 h. The 4-HBA product was derivatized and analyzed as described above.

RESULTS

In a previous study, rapid-quench techniques were used in conjunction with radiolabeled 4-CBA-CoA to measure the time course for a single turnover on the dehalogenase and, hence, the rate constant for catalysis (Liang et al., 1993). The k_{cat} = 2.4 s^{-1} obtained was small enough to suggest that transient kinetic techniques might be suitable to test for possible intermediates along the reaction pathway. Using stopped-flow absorption techniques, we have observed that the enzyme–substrate complex proceeds through at least one long lived, spectrally distinct intermediate along the pathway to the enzyme–product complex (Liang, Scholten, and Dunaway-Mariano, unpublished results). In the present study, rapid-quench techniques were used to determine whether a covalent enzyme intermediate might be involved in this reaction. As described below, evidence for such an intermediate was found and, subsequently, ^{18}O-labeling experiments were carried out which allowed us to identify this intermediate as an aryl enzyme.

Rapid-Quench Studies of the 4-CBA-CoA Dehalogenase Reaction. To test for a covalent enzyme intermediate, [^{14}C]-

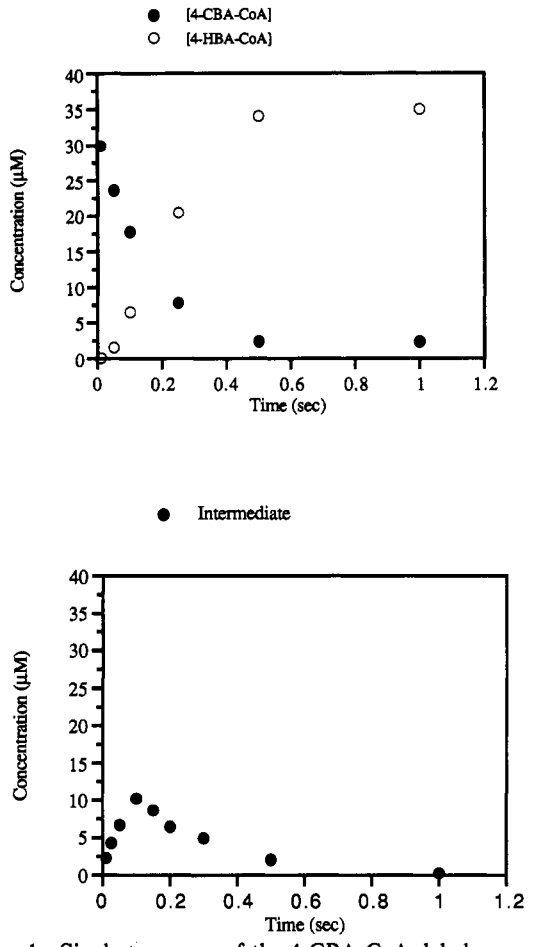


FIGURE 1: Single turnovers of the 4-CBA-CoA dehalogenase (75 μM) with [^{14}C]-4-CBA-CoA (37 μM) in 50 mM K$^+$Hepes at pH 7.5. (top) Analysis of the 4-CBA-CoA and 4-HBA-CoA contents of the reaction at varying conversion and (bottom) analysis of the amount of radiolabeled enzyme present at varying conversion.

4-CBA-CoA (prepared from [^{14}C]-4-CBA and CoA: see methods) was reacted with a 2-fold excess of buffered 4-CBA-CoA dehalogenase in a rapid-quench apparatus. The reaction was quenched at varying conversion with acid. In one experiment, the protein was precipitated by vortexing the quenched samples with CCl_4 and then removed by centrifuging the samples. The [^{14}C]-4-CBA-CoA and [^{14}C]-4-HBA-CoA present in the resulting solutions were separated by HPLC and quantitated by liquid scintillation counting. The time course for [^{14}C]-4-CBA-CoA consumption (k = 6.5 s^{-1}) and [^{14}C]-4-HBA-CoA formation (k = 2.9 s^{-1}) is shown in Figure 1, top. Note the slight lag apparent in the [^{14}C]-4-HBA-CoA progress curve, suggestive of the formation of a long lived intermediate.

In the parallel experiment, the acid-quenched samples were centrifuged in a Centricon-10 vial to remove substrate and product from the enzyme. The enzyme was subjected to exhaustive dilution and centrifuging to insure that all radioactivity which was not covalently attached to the enzyme has been removed. The protein remaining was then assayed for radioactivity by liquid scintillation counting. The amount of radioactivity found to be associated with the protein fractions generated from the quenched reaction samples is plotted in Figure 1, bottom. Note that within the first time point (10 ms), radiolabeled enzyme is present and it reaches (at 100 ms) a maximum level which corresponds to 27% of the starting [^{14}C]-4-CBA-CoA before it begins to decline. The kinetics of formation and disappearance of the radiolabeled enzyme suggest that it represents an intermediate along the dehalogenase reaction pathway. We proceeded to the ^{18}O-labeling experiments described below to test our hypothesis of the structure of the putative covalent enzyme intermediate.

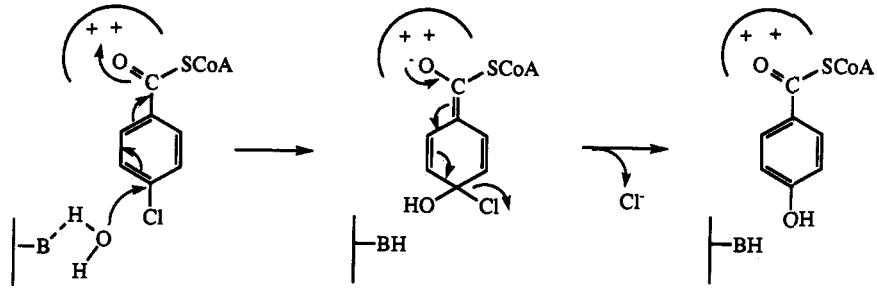
^{18}O-Labeling Studies of Oxygen Atom Transfer in the Dehalogenase Reaction. The observation of a covalent enzyme intermediate suggested that the nucleophilic aromatic substitution (S_NAr) pathway proposed earlier for the dehalogenase reaction (Liang et al., 1993) needed modification (Scheme 2). Incorporation of nucleophilic catalysis in the basic S_NAr mechanism of Scheme 2 would account for such an intermediate. Accordingly, in Scheme 3, the dehalogenase reaction is shown to proceed through a covalent enzyme adduct as a result of the addition of an active site group to C(4) of the 4-CBA-CoA and the subsequent elimination of Cl$^-$ from the resulting σ-complex. The covalent intermediate would then undergo attack at C(4) by activated water to form a second σ-complex followed by elimination of the active site group to form product. Alternatively, if the enzyme active site nucleophile is an Asp or Glu side chain, the nucleophilic catalysis pathway of Scheme 4 avails itself. In this case, the active site carboxylate residue (Asp or Glu) is used to form an aryl enzyme intermediate and this intermediate is hydrolyzed to product by attack of an activated water molecule at the aryl carbonyl carbon rather than at the ring C(4) (Scheme 4). This pathway can, in principle, be tested using oxygen-isotope labeling techniques to probe oxygen transfer from the enzyme to C(4) of the substrate.

The oxygen transfer experiment carried out to test the mechanism of Scheme 4 consisted of reacting 480 μM dehalogenase (preequilibrated in 98% $H_2{}^{18}O$ to exchange $H_2{}^{16}O$ from the active site)[2] with 114 μM 4-CBA-CoA in 98% $H_2{}^{18}O$. 4-HBA-CoA thioesterase was then added to the reaction mixture for the purpose of converting the 4-HBA-CoA produced to 4-HBA for product analysis. The 4-HBA was methylated with diazomethane to form the (comparatively volatile) methyl 4-methoxybenzoate adduct which, in turn, was analyzed by GC-MS. The mass spectrum of the resulting methyl 4-methoxybenzoate contained parent ion (M*) peaks at 166, 168, and 170 (m/e), corresponding to the all-^{16}O parent ion, the parent ion labeled with one ^{18}O at C(4) or at the methyl ester substituent (introduced by thioesterase-catalyzed hydrolysis of the 4-HBA-CoA product in $H_2{}^{18}O$),[3] and the parent ion labeled with ^{18}O at both the C(4) and methyl ester positions. The parent ion fragment corresponding to M* – $COOCH_3$ was apparent at (m/e) 107 (no^{18}O) and 109 (one ^{18}O). The peaks at 107 and 109 were used in the calculation of the $^{18}O/^{16}O$ ratio at C(4) in methyl 4-methoxybenzoate since the corresponding fragments do not contain the ^{18}O introduced by the 4-HBA-CoA thioesterase-catalyzed reaction. On the basis of the relative intensities of the 107 and 109 (m/e) peaks (Table 1), we calculate that 73% of the C(4)-OCH_3 of the methyl 4-methoxybenzoate adduct contained ^{16}O while 27% contained ^{18}O. In the second trial (in which the enzyme was not first preincubated in $H_2{}^{18}O$), we observed values of 75% and 25%, respectively (see Table 1). Hence, three-quarters of 4-CBA-CoA was converted to 4-HBA-CoA by transfer of ^{16}O originating from the enzyme and one-quarter by transfer of ^{18}O originating from the solvent.

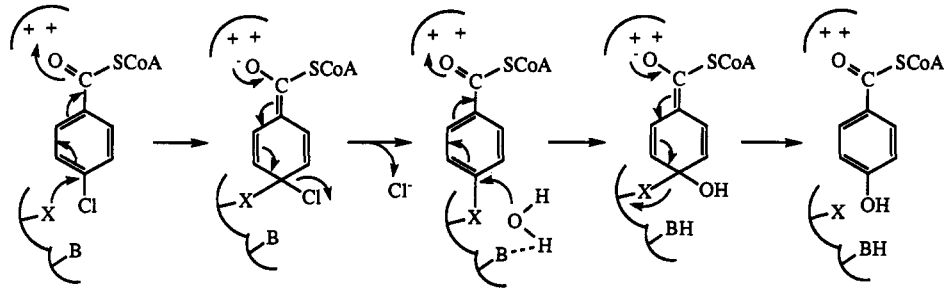
[2] Elemental analysis has shown that the 4-CBA-CoA dehalogenase does not contain a tightly bound metal ion cofactor which could otherwise serve as the source of slow exchanging H_2O (Liang, 1994).

[3] The thioesterase hydrolysis of 4-HBA-CoA was carried out in $H_2{}^{18}O$ so that the $H_2{}^{18}O$ could be recycled. Dilution of the dehalogenase reaction solution with $H_2{}^{16}O$ prior to reaction with the thioesterase would have precluded our ability to reisolate the $H_2{}^{18}O$.

Scheme 2: Dehalogenation of 4-CBA-CoA *via* an Aromatic Nucleophilic Substitution Reaction



Scheme 3: Dehalogenation of 4-CBA-CoA using Nucleophilic Catalysis



Scheme 4: Dehalogenation of 4-CBA-CoA using an Active Site Carboxylate as the Nucleophile

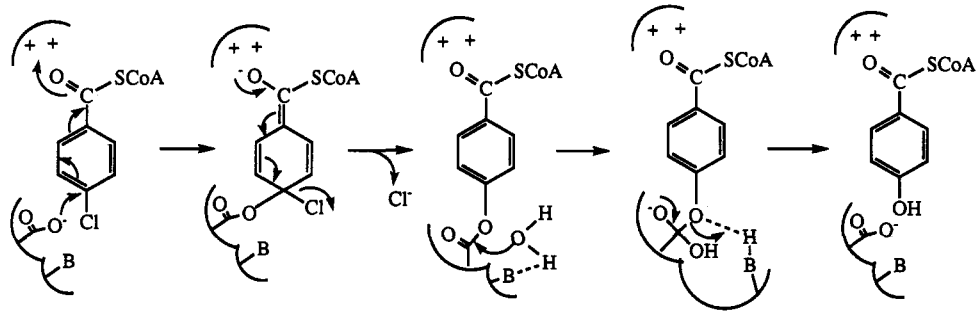


Table 1: Relative Intensities of the Peaks Observed in the Mass Spectrum of the Methyl 4-Methoxybenzoate Generated from the 4-HBA-CoA Produced from 4-CBA-CoA and Dehalogenase in $H_2{}^{18}O$ (See Methods for Details)

	M* (*m/e*)			M* – $COOCH_3$ (*m/e*)	
	166	168	170	107	109
single turnover, trial 1	0.87	2.72	1.06	1.00	0.36
single turnover, trial 2	1.23	6.11	2.31	1.00	0.34
multiple turnover[a]	1.20	13.35	36.87	1.00	8.12

[a] No peak was observed at *m/e* 172.

Our accuracy in analyzing the $^{16}O/^{18}O$ incorporation into product was checked by repeating the same reaction/analysis sequence with 2.6 μM dehalogenase and 2.7 mM 4-CBA-CoA in 93% $H_2{}^{18}O$. Under these conditions, the enzyme would turnover approximately 1000 times and, thus, the 4-HBA-CoA product is expected to contain the same ^{18}O enrichment at C(4) as the starting $H_2{}^{18}O$ solvent (viz. 93% ^{18}O, 7% ^{16}O). As indicated in Table 1, the relative intensities of the 107 and 109 (*m/e*) peaks observed in the mass spectrum of the methyl 4-methoxybenzoate derivative reflect 89% ^{18}O and 11% ^{16}O incorporation at C(4) (96% of the theoretical value). Hence, our observation of less than 100% ^{16}O transfer from the enzyme (i.e., 73–75%) suggests ^{18}O incorporation into the enzyme carboxylate from solvent and, hence, into the product. It is unlikely that $H_2{}^{18}O$ exchanged with some of the ^{16}O of the active site carboxylate prior to turnover since approximately the same amount of ^{18}O was observed in the product formed by enzyme which had been preincubated in $H_2{}^{18}O$ (trial 1, Table 1) as was observed in that formed by enzyme which had not been preincubated (trial 2, Table 1). Alternatively, the ^{18}O incorporated into product may have derived from the enzyme catalyzing a second turnover. With a turnover rate of 6.5 s^{-1}, the binding of 4-CBA-CoA will be at equilibrium, and thus, each enzyme molecule that catalyzes a reaction becomes labeled with ^{18}O, can reequilibrate with the remaining substrate, and in its second reaction insert ^{18}O into the product. For 114 μM 4-CBA-CoA and 480 μM enzyme active sites, roughly 10% of the product is predicted to be ^{18}O labeled.[4] If the concentration of active enzyme in the reaction is off by a factor of 2, then the 25% ^{18}O incorporation observed can be accounted for.[5]

DISCUSSION

The results from the rapid-quench studies suggest the formation of a covalent enzyme intermediate in the 4-CBA-CoA dehalogenase reaction. The results from the ^{18}O-labeling experiments indicate that this intermediate is an aryl enzyme, formed by attack of an active site carboxylate (Asp or Glu) onto C(4) of the benzoate ring followed by elimination of Cl^-. The catalytic advantage of this reaction pathway (illustrated in Scheme 4) derives from the partitioning of two energy-demanding tasks into separate transition states. Specifically,

[4] We thank a reviewer for pointing this out.

[5] At a concentration of 480 μM active sites, the enzyme solution is turbid, suggesting that the enzyme may have undergone some aggregation and consequential loss of activity.

nucleophilic addition to the aromatic ring, a seemingly difficult step, utilizes a protein side chain carboxylate as the attacking nucleophile. This "bimolecular" step is followed by a second "bimolecular" step in which the comparatively poor nucleophile, H_2O, cleaves the enzyme–aryl linkage by attack at the carbonyl carbon. The accumulation of the aryl enzyme intermediate during turnover (evidenced by Figure 1, bottom) suggests that the steps which lead to its formation are faster than those involved in its hydrolysis. The transition states associated with aryl enzyme formation and hydrolysis are probably close in energy, however, as evidenced by the large substituent effect observed for fluorine in the leaving group position (i.e., the k_{cat} for 4-fluorobenzoyl-CoA is 1×10^{-5} that of 4-CBA-CoA; Liang et al., 1993).

It was pointed out earlier (Babbitt et al., 1992) that the hydrolysis reaction catalyzed by the 4-CBA-CoA dehalogenase is similar in nature to the hydration reaction catalyzed by 2-enoyl-CoA hydratase and that these two enzymes appear to share common ancestry. On the other hand, the use of covalent catalysis to form an aryl enzyme intermediate distinguishes the 4-CBA-CoA dehalogenase from the 2-enoyl hydratase and links it to the microsomal epoxide hydrolase and bacterial 2-haloalkane dehalogenase (Lacourciere & Armstrong, 1993; Verschueren et al., 1993). Structurally, these latter two enzymes are related to one another, both globally and through a sequence motif containing the active site Asp (Lacourciere & Armstrong, 1993, 1994). The 4-CBA dehalogenase does not share significant sequence identity with either of these two enzymes nor does it share their common catalytic motif. Thus, the 4-CBA dehalogenase appears to be connected to these two hydrolases solely by the common use of an active site carboxylate to mediate hydrolytic substitution at a carbon center: a possible example of convergent evolution.

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