Specificity of 4-Chlorobenzoyl Coenzyme A Dehalogenase Catalyzed Dehalogenation of Halogenated Aromatics†

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ABSTRACT: Steady-state and transient kinetic techniques were used to evaluate the efficiency of 4-chlorobenzoyl coenzyme A (4-CBA-CoA) turnover catalyzed by 4-CBA-CoA dehalogenase from *Pseudomonas* sp. CBS-3. The k_{cat} for a single turnover on the enzyme was found to be 2 s^{-1}, while that for multiple turnovers was found to be 0.6 s^{-1}. Catalysis rather than product release was judged to be rate limiting. Comparison of the rates of turnover of 4-bromobenzoyl-CoA (1.4 s^{-1}), 4-iodobenzoyl-CoA (1.1 s^{-1}), and 4-fluorobenzoyl-CoA (8×10^{-6} s^{-1}) indicated that cleavage of the carbon–halogen bond occurs in the rate-limiting transition state of the reaction. Structure–activity measurements made with 4-CBA-CoA analogs bearing electron-donating or -withdrawing substituents at C(2) or C(3) suggested the importance of steric/solvation effects on the enzymatic reaction and failed to provide insight into the nature of the reaction intermediate. The inhibition constants measured for benzoyl-CoA (72 μM), CoA (140 μM), and 4-chlorobenzoate (21 mM) compared to the K_m measured for 4-CBA-CoA (4 μM) suggest the dominant role played by the CoA moiety in substrate anchoring.

Because of their widespread use as industrial and agricultural agents, halogenated hydrocarbons constitute a particularly formidable class of environmental pollutants. In recent years much attention has focused on the bioremediation of these compounds [for recent reviews, see Abramowicz (1990) and Commandeur and Parsons (1990)]. Our own studies have concentrated on the bacteria-mediated degradation of halogenated aromatics, 4-chlorobenzoate in particular. The chemical strategy that we have observed for the biodegradation of 4-chlorobenzoate (4-CBA)[1] in *Pseudomonas* sp. CBS-3 involves the coupling of three catabolic pathways (Scholten et al., 1991). The first pathway, unique to this particular pseudomonad,[2] converts the 4-CBA to 4-hydroxybenzoate (4-HBA) in a three-step process (see Scheme I). The 4-HBA thus formed is oxidized to protocatechuate and then to carboxymuconate via the *ortho*-cleavage pathway (Ornston, 1990). β-Ketoadipate is ultimately formed and processed through the β-ketoadipate pathway to succinyl-CoA and acetyl-CoA.

As illustrated in Scheme I, the *Pseudomonas* sp. CBS-3 dehalogenase pathway consists of three enzymes: a 4-CBA:CoA ligase, a 4-CBA-CoA dehalogenase, and a 4-HBA-CoA thioesterase (Chang et al., 1992; Löffler et al., 1992). Of these three enzymes, the one of particular novelty is the 4-CBA-CoA dehalogenase, which catalyzes an aromatic substitution reaction involving the replacement of the chloride substituent with the hydroxyl group from a molecule of water. The ligase and thioesterase are auxiliary to the dehydrogenase, functioning in the formation and then removal of the CoA thioester ring functionality.

In principle, one can write several possible chemical pathways for the 4-CBA-CoA dehalogenase reaction, including an elimination–addition mechanism (in which a benzyne intermediate is formed by ring deprotonation and Cl^- departure) (Miller, 1968), an $S_{RN}1$ radical chain reaction (in which a radical anion intermediate is formed) (Bunnett, 1978), and a nucleophilic substitution reaction (Miller, 1968). However, in the absence of evidence for a strong base to deprotonate the aromatic ring or a cofactor to serve as a radical initiator in the dehalogenase active site, it is not unreasonable to dismiss the first two pathways in favor of the nucleophilic substitution pathway. This pathway is in fact supported by the finding that the 4-CBA-CoA dehalogenase shares ancestry with the 2-enoyl-CoA hydratase (crotonase) of the fatty acid β-oxidation pathway, suggesting that, in parallel to the hydratase reaction, the dehalogenase catalyzes nucleophilic addition of a water molecule on an sp^2-hybridized carbon in conjugation with the CoA thioester substituent (Babbitt et al., 1992).

The generally accepted mechanism for aromatic nucleophilic substitution on activated arenes in polar hydroxylic solvents involves rate-limiting ipso addition giving a Meisenheimer (σ) complex (Miller, 1968; Bernasconi, 1980). Armstrong and co-workers have reported this mechanism for the glutathione *S*-transferase catalyzed substitution of glutathione for halogen on highly activated halogenated arenes (Chen et al., 1988). Evidence for this mechanism derives from structure–activity studies which show direct correlation between the electron-withdrawing capacity of the substrate ring substituents and k_{cat} (ρ = 1.2) (Chen et al., 1988) and from the observation of the 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate anion formed from glutathione and 1,3,5-trinitrobenzene in the enzyme active site (Graminski et al., 1989). The turnover rate reported for 1-chloro-2,4-dinitrobenzene is 5 s^{-1} with isozyme 4-4 and 20 s^{-1} with isozyme 3-3 (Graminski et al., 1989).

The σ value of the CoA thioester substituent on the dehalogenase substrate 4-CBA-CoA is less than that of a para nitro substituent[3] and hardly seems adequate to resonance-stabilize the σ complex resulting from "HO^-" addition to C(4)

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[1] Abbreviations: CBA, chlorobenzoate; HBA, hydroxybenzoate; 4-CBA-CoA, 4-chlorobenzoyl coenzyme A; 4-HBA–CoA, 4-hydroxybenzoyl coenzyme A; CoA, coenzyme A; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography.

[2] This pathway has also been demonstrated to operate in the 4-chlorobenzoate-degrading bacterium *Acinetobacter* sp. strain 4-CB1 (Copley & Crooks, 1992).

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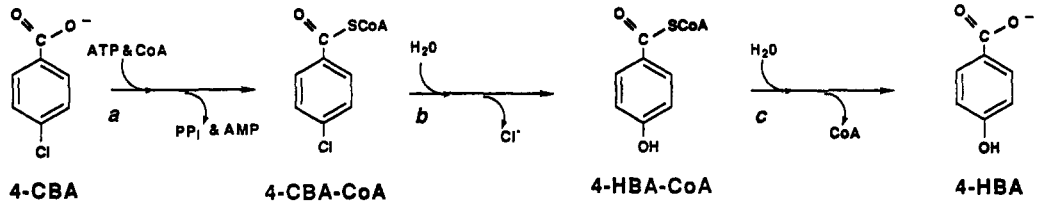
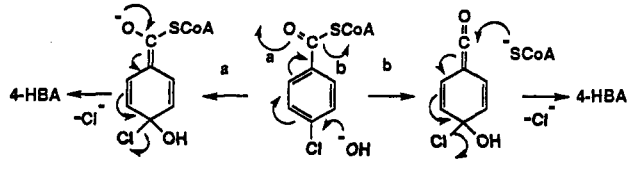
Scheme I: Reaction Steps of the Dehalogenation of 4-CBA in *Pseudomonas* sp. CBS-3 Catalyzed by (a) 4-CBA:CoA Ligase, (b) 4-CBA-CoA Dehalogenase, and (c) 4-HBA-CoA Thioesterase



Scheme II: 4-CBA-CoA Dehalogenase Catalyzed Nucleophilic Aromatic Substitution on 4-CBA-CoA Shown as Occurring via a Meisenheimer Intermediate (Pathway a) or a Ketene Intermediate (Pathway b)



(Scheme II). On the other hand, additional stabilization may be made available through the interaction of the C=O of the CoA thioester substituent with electron-withdrawing groups (H-bonding or ion pairing) in the enzyme active site (Scheme II). Alternatively, the CoA substituent might play a quite different role in this reaction. As depicted in Scheme II, nucleophilic attack on the 4-CBA-CoA ring could result in displacement of $CoAS^-$ and formation of a ketene intermediate (Scheme II). Recapture of the ketene with $CoAS^-$ would then lead to expulsion of the Cl^-. An analogy to this reaction pathway is found in the dehydrohalogenation of acyl halides to ketenes (Darling & Kidwell, 1968) and in the ketene formation from thiol ester α-carbanions [as recently reviewed by Douglas (1986)].

The present study was undertaken to examine the efficiency of the 4-CBA-CoA dehalogenase in binding and in catalyzing the dehalogenation of structural analogs of 4-CBA-CoA for the purpose of probing the chemical pathway of the reaction. Herein we report our findings.

MATERIALS AND METHODS

General. [^{14}C]4-CBA was purchased from California Bionuclear Corp. CoA and buffers were purchased from Sigma Chemical Co., while all other chemicals were obtained from Aldrich Chemical Co.

Purification of 4-CBA-CoA Dehalogenase. This enzyme was purified from *Escherichia coli* K38 cells transformed with the dehalogenase-encoding plasmid *SmaI–SalI*–pT7.5 as described in Chang et al. (1992) except that a phenyl-Sepharose CL-4B column chromatographic step was inserted between the DEAE-cellulose and Sephadex G-200 column chromatographic steps. The dehalogenase-containing fractions from the DEAE-cellulose column were combined and loaded onto a 4- × 10-cm phenyl-Sepharose column equilibrated with 10% ammonium sulfate in 50 mM K^+-Hepes (pH 7.5)/1 mM DTT. The column was eluted first with 240 mL of a linear gradient of 10 → 0% ammonium sulfate in 50 mM K^+-Hepes (pH 7.5)/1 mM DTT and then with 500 mL of buffer. Following the Sephadex G-200 column chromatography, the dehalogenase was obtained in 10% overall yield with a specific activity of 2 μmol of 4-HBA-CoA [min(mg of protein)] at pH 7.5 and 25 °C.

Synthesis of Dehalogenase Substrates and Products. 4-Fluoro-, 4-chloro-, 4-bromo-, 4-iodo-, 2,4-dichloro-, 3,4-dichloro-, 3-(chloromethyl)-, 2-chloro-, and 3-chlorobenzoyl-CoA were prepared by adaptation of the procedure described by Mieyl et al. (1974). Accordingly, 50 mg (64 μmol) of CoA lithium salt was dissolved in 1 mL of deionized water, and the pH of the resulting solution was adjusted to 8 with 1 M LiOH. A 5-fold molar excess of the benzoyl chloride derivative was added at 25 °C to the rapidly stirring solution under N_2. The pH of the reaction solution was maintained between 7.5 and 8.0 by the addition of 0.2 M LiOH. After the reaction solution was stirred for 30 min, the pH was adjusted to 4 with 1 M HCl. Following an additional 30 min of stirring, the solution was centrifuged to remove the precipitated benzoic acid derivative, and the supernatant was chromatographed on a 2- × 110-cm Sephadex G-25 column with deionized water (0.2 mL/min). The column fractions were assayed using the HPLC method of Merkel et al. (1989). The product-containing fractions were pooled and evaporated to dryness on a rotary evaporator (average yield ≈ 60%). 2-HBA-CoA was prepared by this same basic procedure except that the reaction was carried out with CoA dissolved in 1 mL of 0.5 M sodium bicarbonate buffer (pH 8) and the 2-hydroxybenzoyl chloride was prepared from 2-HBA and oxalyl chloride.

4-Chloro-2-nitro-, 4-chloro-3-nitro-, 4-chloro-3-methyl-, 3-chloro-4-hydroxy-, 3-hydroxy-, and 4-hydroxybenzoyl-CoA were prepared using the basic procedure reported by Merkel et al. (1989). To a stirred solution of the benzoic acid derivative (440 μmol) in dry THF (20 mL) was added ethyl chloroformate (44 μL, 460 μmol) and triethylamine (60 μL, 430 μmol) at 25 °C under N_2. A white precipitate which formed over a period of 1 h was removed by filtration. The resulting clear solution was then added dropwise (over a 45-min period) to a N_2-purged solution of 32 mg of CoA lithium salt (41 μmol) in 10 mL of deionized water. The pH of the solution was maintained between 7.5 and 8.0 by the addition of 0.2 M LiOH. After being stirred for 1 h under N_2, the solution was adjusted to pH 4 with 1 M HCl. Unconsumed acid was removed by three extractions with ethyl acetate (10 mL each). The product was purified as described above (20% average yield).

2-Chloro-4-hydroxy-, 4-hydroxy-2-nitro-, and 4-hydroxy-3-nitrobenzoyl-CoA were prepared from 2,4-dichloro-, 4-chloro-2-nitro-, and 4-chloro-3-nitrobenzoyl-CoA (1 mM) by treatment with 4-CBA-CoA dehalogenase in 50 mM K^+-Hepes (pH 7.5, 25 °C) and purified by Sephadex-G25 column chromatography.

The spectral properties of all of the above compounds are reported in the supplementary material.

Initial Velocity Studies. The substrate activity of each analog was first tested by using HPLC techniques to monitor the disappearance of reactant and the appearance of product.

[3] We predict that the σ value of the thioester CoA substituent would be close to that of an ester or a ketone, ≈0.8–0.9, which is less than that of a nitro substituent (σ = 1.27) (Miller, 1968). The hydrolytic dehalogenation of 4-chloronitrobenzene requires harsh conditions (Bunnett & Zahler, 1951).

The expected product was independently prepared as described above and used as an HPLC standard. The HPLC set up included an ultrasphere 4.6-mm × 25-cm ODS-C_{18} reversed-phase column monitored at 260 nm and eluted with an acetonitrile (solvent A)/ammonium acetate (solvent B) gradient.

The kinetic parameters K_m and V_{max} for (fast reacting) dehalogenase substrates were determined from the initial velocity data measured using 1-mL reaction solutions buffered at 25 °C with 50 mM K^+-Hepes (pH 7.5) and containing 4-CBA-CoA (3-30 μM), 4-bromobenzoyl-CoA (6–40 μM), 4-iodobenzoyl-CoA (4-40 μM), 2,4-dichlorobenzoyl-CoA (9-180 μM), 3,4-dichlorobenzoyl-CoA (25-310 μM), 4-chloro-2-nitrobenzoyl-CoA (12-120 μM), or 4-chloro-3-nitrobenzoyl-CoA (11-110 μM). The reactions were monitored spectrophotometrically. The difference between the molar extinction coefficients of the substrate and the corresponding 4-hydroxybenzoyl-CoA product is 8200 M^{-1} cm^{-1} for 4-CBA-CoA and 6500 M^{-1} cm^{-1} for 4-bromobenzoyl-CoA at 300 nm, 2400 M^{-1} cm^{-1} for 4-iodobenzoyl-CoA at 330 nm, 12 510 M^{-1} cm^{-1} for 2,4-dichlorobenzoyl-CoA at 322 nm, 15 880 M^{-1} cm^{-1} for 3,4-dichlorobenzoyl-CoA at 334 nm, and 4540 M^{-1} cm^{-1} for 4-chloro-2-nitrobenzoyl-CoA and 8580 M^{-1} cm^{-1} for 4-chloro-3-nitrobenzoyl-CoA at 310 nm. The initial velocity data were analyzed using eq 1 and the Fortran program of Cleland (1979).

$$V_o = V_{max}[A]/([A] + K_m) \quad (1)$$

In eq 1, V_o = initial velocity, V_{max} = maximal velocity, [A] = substrate concentration, and K_m = Michaelis constant. The k_{cat} values were determined from the ratio of the V_{max} values and the enzyme concentrations used in the reactions. The concentrations of enzyme stock solutions were determined using the Bradford method (Bradford, 1976) and a subunit molecular mass of 30 kD (Chang et al., 1992).

The k_{cat} for 4-fluorobenzoyl-CoA dehalogenation was determined from the initial velocity of the reaction of 500 μM 4-fluorobenzoyl-CoA with 160 μM dehalogenase in 50 mM K^+-Hepes (pH 7.5)/1 mM DTT. The reaction was monitored at varying conversion by HPLC.

The substrate activities of 2-chlorobenzoyl- and 3-chlorobenzoyl-CoA were examined by testing product (2-hydroxy- and 3-hydroxybenzoyl-CoA, respectively) formation in 50-μL reaction solutions containing 500 μM reactant and 60 μM enzyme, over a 12-h period, using HPLC techniques (see above). The substrate activity of 4-chloro-3-methylbenzoyl-CoA was measured by incubating 100-300 μM reactant with 10-20 μM enzyme and assaying the reaction mixture for the product (4-hydroxy-3-methylbenzoyl-CoA) over a 2.5-h period, using HPLC techniques.

The K_i values for benzoyl-CoA (80 and 160 μM), 4-fluorobenzoyl-CoA (60 and 120 μM), 4-chloro-3-methylbenzoyl-CoA (100 μM), 4-CBA (10 mM), and CoA (400 μM) were determined from the initial velocity of the dehalogenase-catalyzed reaction of 4-CBA-CoA (5-40 μM) measured in the presence and absence of the respective inhibitors. The initial velocity data were analyzed using eq 2 and the Fortran program of Cleland (1979).

$$V_o = \frac{V_{max}[A]}{[K_m(1 + [I]/K_i) + [A]]} \quad (2)$$

In eq 2, [I] = inhibitor concentration and K_i = the inhibition constant.

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. Forty-three microliters of enzyme in 50 mM K^+-Hepes/1 mM DTT (pH 7.5) was mixed with 43 μL of [^{14}C]4-CBA-CoA (sp act. = 4.6 mCi/mmol) in 50 mM K^+-Hepes/1 mM DTT. The reactions were quenched after a specified period of time with 134 μL of 0.12 N HCl. Next, 100 μL of CCl_4 was added, and the resulting solution was vortexed vigorously to precipitate the protein. The resulting mixture was then centrifuged, and the supernatant was siphoned from the protein pellet, mixed with unlabeled 4-CBA-CoA and 4-HBA-CoA, and separated on an HPLC 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 $[P]_t = [P]_{max}[1 - \exp(-kt)]$.

Incubation of 3-(Chloromethyl)benzoyl-CoA with 4-CBA-CoA Dehalogenase. Ten micromolar dehalogenase in 50 mM K^+-Hepes (pH 7.5) was incubated at 25 °C with 30 μM 3-(chloromethyl)benzoyl-CoA for 4 h. One-hundred-microliter aliquots of this mixture were taken during this period and added to a dehalogenase activity assay system consisting of 100 μM 4-CBA-CoA in 50 mM K^+-Hepes/1 mM DTT (pH 7.5) in a 1-mL volume. The specific activity of the dehalogenase incubated with 3-(chloromethyl)benzoyl-CoA was compared to that of dehalogenase incubated in buffer alone. Neither enzyme sample showed a significant decrease in specific activity over the 4-h incubation period. The substrate activity of 3-(chloromethyl)benzoyl-CoA (500 μM) was tested by incubation with 4-CBA-CoA dehalogenase (140 μM) in 50 mM K^+-Hepes (pH 7.5) at 25 °C. Over a 24-h period aliquots were removed from the reaction mixture and analyzed for 4-hydroxy-3-methylbenzoyl-CoA by HPLC.

Table I: Kinetic Constants of 4-CBA-CoA Dehalogenase Determined at 25 °C in 50 mM K^+-Hepes/1 mM DTT (pH 7.5)[a]

substrate or inhibitor	k_{cat} (s^{-1})	K_m or K_i (μM)
4-CBA-CoA	0.60 ± 0.01	3.7 ± 0.3
4-bromobenzoyl-CoA	1.35 ± 0.03	4.2 ± 0.3
4-iodobenzoyl-CoA	1.07 ± 0.01	6.5 ± 0.3
4-fluorobenzoyl-CoA	8×10^{-6} [b] 1×10^{-5} [c]	40 ± 5[d]
2,4-dichlorobenzoyl-CoA	0.511 ± 0.005	10.4 ± 0.4
3,4-dichlorobenzoyl-CoA	0.052 ± 0.001	42 ± 3
4-chloro-2-nitrobenzoyl-CoA	0.063 ± 0.002	30 ± 2
4-chloro-3-nitrobenzoyl-CoA	0.0256 ± 0.002	5.5 ± 0.2
4-chloro-3-methylbenzoyl-CoA	5×10^{-4}	75; 76 ± 7[d]
benzoyl-CoA		72 ± 8[d]
CoA		140 ± 10[d]
4-CBA		21000 ± 2000[d]

[a] See Materials and Methods for details. [b] Defluorination. [c] Thioester hydrolysis. [d] K_i.

RESULTS AND DISCUSSION

Kinetics of 4-CBA-CoA Dehalogenation. Initial velocity and transient kinetic techniques were used to examine the efficiency of 4-CBA-CoA dehalogenase catalysis of 4-CBA-CoA dechlorination. The K_m and k_{cat} values determined from the initial velocity experiments are listed in Table I, and the reaction time courses obtained from the transient kinetic experiments are shown in Figure 1. Figure 1A shows the time course for a single turnover in the dehalogenase active site, while Figure 1B shows the time course for approximately three turnovers, and Figure 1C, 12 turnovers. The rate data of Figure 1A were fit to a first-order rate equation to obtain an apparent rate constant of $k = 2.4 \pm 0.1$ s^{-1}. The same turnover rate was observed by using approximately twice the concen-

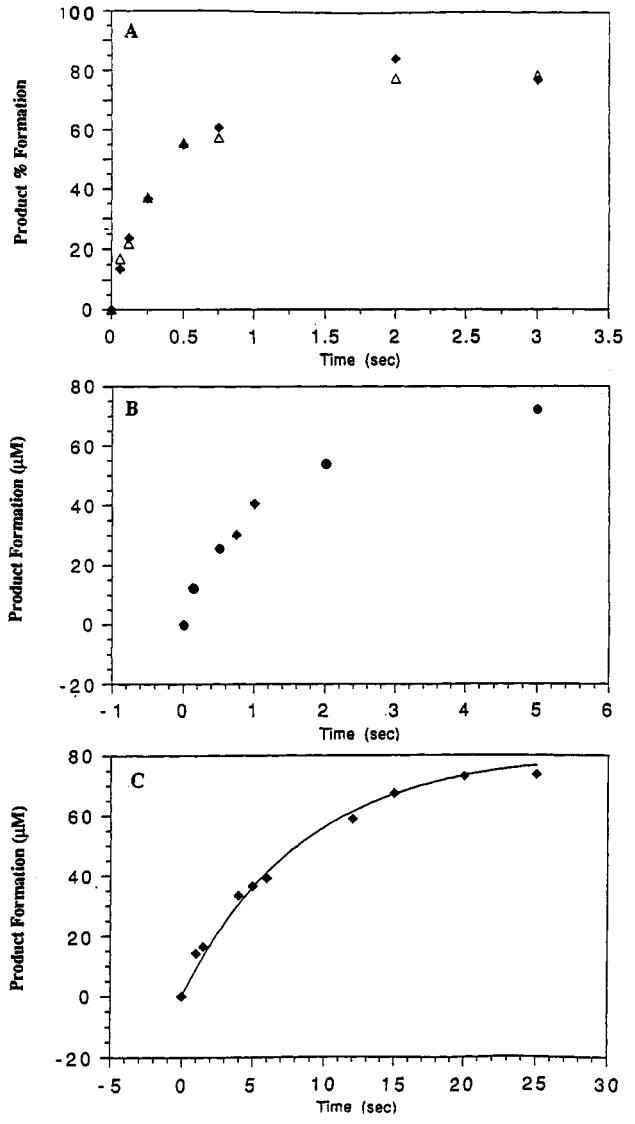


FIGURE 1: Time courses for the reaction of (A) 68 μM [^{14}C]4-CBA-CoA with 146 (◆) or 86 μM enzyme (△), (B) 123 μM [^{14}C]4-CBA-CoA and 43 μM enzyme, or (C) 153 μM [^{14}C]4-CBA-CoA with 13 μM enzyme in 50 mM K$^+$-Hepes (pH 7.5), 25 °C. The time courses were fit to a first-order kinetic equation by computer (see Materials and Methods for details).

tration of dehalogenase, indicating that catalysis, rather than substrate binding, is rate limiting under the reaction conditions used in this experiment.

A comparison of the apparent first-order rate constant measured for a single turnover on the enzyme (k_{cat} = 2.4 s^{-1}) with that measured by initial velocity techniques for multiple turnovers (k_{cat} = 0.6 s^{-1}; Table I) indicates that catalysis, rather than product release, may be partially rate limiting in the steady-state turnover rate. This conclusion is also suggested by the data presented in panels B and C of Figure 1 which fail to show a clear "burst" of product formation associated with the first turnover. The $t_{1/2}$ for a single turnover calculated from the data of Figure 1A is 0.30 s, while that averaged over three turnovers (Figure 1B) is 0.35 s, and that averaged over 12 turnovers (Figure 1C) is 0.50 s. The $t_{1/2}$ averaged over 12 turnovers approaches that measured under steady-state conditions, $t_{1/2}$ = 1 s, and suggests that, at higher levels of substrate conversion, product inhibition may be reducing the turnover rate.[4]

The chemical steps of the 4-CBA-CoA dehalogenase reaction thus appear to be slow, possibly reflecting the difficulty associated with catalysis of the nucleophilic substitution reaction. Crotonase, which catalyzes the analogous Micheal addition of H_2O to 2-enoyl-CoA, turns over at a rate of 1000 s^{-1} (Bahnson and Anderson, 1991).

Substrate Anchoring and Regiospecificity of Dehalogenation. In order to evaluate the contributions made by the CoA moiety, the 4-chlorobenzoyl moiety, and the 4-chloro substituent of 4-CBA-CoA to the enzyme binding affinity, the inhibition constants of CoA, 4-CBA, and benzoyl-CoA were measured. All three compounds displayed competitive inhibition vs 4-CBA-CoA. Because catalysis is rate limiting the K_m measured for 4-CBA-CoA defines the dissociation constant for the enzyme–substrate complex. Comparison of this K_m (4 μM) to the benzoyl-CoA K_i (70 μM) suggests that the 4-chloro substituent makes a small contribution to substrate binding to the active site surface (perhaps through van der Waals interactions). Comparison of the K_i for benzoyl-CoA (70 μM) to that of CoA (K_i = 140 μM) indicates that the CoA moiety of 4-CBA-CoA plays a dominant role in substrate anchoring. This is further evidenced by the large K_i (21 mM) observed for 4-CBA alone, although unfavorable electrostatic interactions arising from the charged carboxylate might interfere with and, therefore, mask tighter binding interactions. The fact that the CoA substituent of 4-CBA-CoA generates most of the observed binding energy provides at least a partial explanation of why it, as opposed to the carboxylate precursor, is required for the dehalogenation reaction.

Next, the regiospecificity of the dehalogenase reaction was examined by testing the substrate activities of 2-CBA-CoA and 3-CBA-CoA. Failure of the enzyme to turn over either regioisomer indicates a stringent requirement for nucleophilic addition para to the CoA thioester ring substituent. Nucleophilic attack at C(3) could not be assisted by resonance stabilization via the CoA thioester, but in principle, attack at C(2) could be. The similar kinetic constants observed for 4-CBA-CoA (k_{cat} = 0.6 s^{-1}) and 2,4-dichlorobenzoyl-CoA (k_{cat} = 0.5 s^{-1}) (Table I) indicate that the presence of the chloro substituent at C(2) does not interfere with productive substrate binding. Thus, the failure of 2-CBA-CoA to undergo turnover suggests that the nucleophile cannot access C(2) and/or that the enzyme plays an essential role in activating/steering the nucleophile for attack at C(4) [but not at C(2)]. On the basis of the contributions made by the CoA and chlorobenzoyl moieties to substrate binding, we can conclude that these regions are anchored at fixed sites in the enzyme active site. The attacking nucleophile and its activating/binding group(s) must therefore be located close to C(4) of the bound substrate.

Substituent Effects and Leaving Group Mobilities in the 4-CBA-CoA Dehalogenase Reaction. The characteristic leaving group mobility pattern observed for aromatic nucleophilic substitution with anionic nucleophiles in protic solvents is F ≫ Cl > Br > I (Miller, 1968). This order is ascribed to the effects of differing electronegativity, combined with the unimportance of bond breaking in an addition–elimination pathway involving a σ intermediate in which formation of the first transition state is rate limiting (Miller, 1968). With heavy nucleophiles the mobility of fluorine relative to the other halogens is reversed (i.e., Cl ≫ F) as a consequence of the second (product forming) transition state being rate limiting (Miller, 1968). For the glutathione *S*-transferase catalyzed dehalogenation of 4-halo-3-nitro-1-

[4] The K_d for the dehalogenase–4-HBA-CoA complex has been measured by spectrophotometry-based titration experiments to be ~1 μM (P.-H. Liang and D. Dunaway-Mariano, unpublished data).

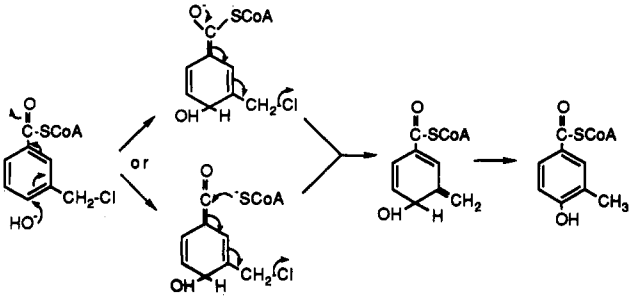
(trifluoromethyl)benzene with glutathione, the F/Cl mobility ratio is 40, consistent with rate-limiting σ complex formation (Chen et al., 1988). From Table I the leaving group mobility in the dehalogenation of 4-halobenzoyl-CoA substrates catalyzed by the 4-CBA-CoA dehalogenase can be seen as Br, I > Cl ≫ F. Noting that chemistry, not product release, controls the rate of the dehalogenase reaction, the very small F/Cl mobility ratio (1×10^{-5}) indicates that carbon–halogen bond cleavage occurs in the rate-limiting transition state.[5] As illustrated in Scheme II, this product-forming transition state could, in principle, follow the formation of either a σ or a ketene intermediate. Irrespective of which intermediate is involved, the magnitude of the F/Cl mobility ratio would suggest that the inductive effect of the F substituent does not significantly offset its high bond-dissociation energy.[6]

The effect of C(2) and C(3) chloro, nitro, and methyl ring substituents on the rate of substrate turnover is reflected by the kinetic constants listed in Table I. In all cases (except C(2) chloro) the substituent placed on the 4-CBA ring significantly slowed catalysis. The C(3) methyl substituent displayed the greatest inhibitory effect on catalysis. It is clear from these substituent effects that the 4-CBA-CoA dehalogenase catalyzed reaction is governed by different geometric/electronic/solvation constraints than might be expected for the corresponding solvolytic reaction. Thus, a Hammett structure–activity correlation is not valid for this particular enzymatic reaction. On the other hand, the high level of substrate specificity exhibited by this enzyme holds important implications concerning its evolution and its use as a prototype for the design of aromatic dehalogenases.

Alternate Reaction Pathways. As described earlier, the generally accepted mechanism for nucleophilic aromatic substitution involves the formation of a resonance-stabilized carbanion intermediate. Adaptation of this mechanism to the dehalogenase reaction leads to two hypothetical pathways, one involving formation of a thioester enol(ate) intermediate as a result of C=O bond cleavage and the other a ketene–thiol(ate) intermediate complex as a result of C—S bond cleavage (Scheme II). Product formation from either intermediate involves redirection of electrons into the ring with departure of Cl^-. Recognizing that the driving force for the elimination of Cl^- from either intermediate of Scheme II is aromatization and that product formation is irreversible, we thought, *a priori*, that the enzyme might catalyze the dehalogenation of 3-(chloromethyl)benzoyl-CoA. The trienol formed from H_2O addition and Cl^- elimination from this analog could possibly be captured by an active site nucleophile or, alternatively, undergo tautomerization to 4-hydroxy-3-methylbenzoyl-CoA (Scheme III). Accordingly, 3-(chloromethyl)benzoyl-CoA was prepared and tested as a 4-CBA-CoA dehalogenase substrate and as an enzyme inactivator. No inactivation was observed, nor was product formed.

The failure of the dehalogenase to catalyze the dehalogenation of 3-(chloromethyl)benzoyl-CoA has several possible interpretations. First, the "electron-rich intermediate" may invariably partition back to the (aromatic) substrate rather than forward to the trienol structure (Scheme III). Alternatively, it is possible that the combination of the absence of a chloro substituent at C(4) with the presence of a (deactivating) chloromethyl group at C(3) results in an insurmountable energy barrier to H_2O nucleophilic addition (see Table I). Finally, contrary to Scheme III, the reaction may not pass through an electron-rich intermediate. Regardless of why the 3-(chloromethyl)benzoyl-CoA is inert, our attempt to evidence a σ or ketene intermediate by demonstrating an alternate elimination mode was unsuccessful.

Scheme III: Possible Chemical Pathways following 4-CBA-CoA Dehalogenase Catalyzed Nucleophilic Addition of Water to 3-(Chloromethyl)benzoyl-CoA



The final issue we wish to address in this paper is the rationale behind the proposal of the ketene pathway shown in Scheme II. In principle, this mechanism could be applied to any enzyme-catalyzed conjugate addition to an unsaturated CoA thioester, provided that the ketene is an accessible intermediate. At the present we lack an adequate model to evaluate the energy of such an intermediate. Nevertheless, experimentally, we have observed that the 4-CBA-CoA dehalogenase catalyzes hydrolysis of the thioester substituent ($k_{cat} = 1 \times 10^{-5}$ s^{-1}) of 4-fluorobenzoyl-CoA at a rate which is competitive with that of ring defluorination ($k_{cat} = 0.8 \times 10^{-5}$ s^{-1}) (Table I). Aside from trivial explanations for the CoA thioester hydrolysis which must be considered (e.g., contaminating thioesterase and slow indigenous thioesterase activity), one intriguing possibility is leakage of the C(4) fluoroketene intermediate from the dehalogenase active site and its capture by solvent water. Further studies may provide further insight into the mechanism of catalysis by this novel enzyme.

NOTE IN ADDED PROOF

Recent stopped-flow experiments have revealed formation of an intermediate during 4-CBA-CoA turnover at an approximate rate of 3.5 s^{-1}, conversion to enzyme-bound product at an approximate rate of 1.5 s^{-1}, and product release at a rate of ~200 s^{-1}.

SUPPLEMENTARY MATERIAL AVAILABLE

A listing of spectral data for dehalogenase substrates and products synthesized in this study (5 pages). Ordering information is given on any current masthead page.

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[5] Because the 4-fluoro substituent is capable of H-bond formation (with an active site residue), it is conceivable that nonproductive binding of the 4-fluorobenzoyl-CoA could take place in the dehalogenase active site. However, given the dominant role played by the CoA substituent in substrate anchoring and the similarity in the K_i values for 4-fluorobenzoyl-CoA and benzoyl-CoA (Table I), the fluoro substituent is most likely exerting its inhibitory effect on catalysis strictly by increasing the energy barrier to carbon–halogen bond cleavage.

[6] The relative rate of S_N1 solvolysis of *tert*-butyl fluoride vs *tert*-butyl chloride is $\sim 1 \times 10^{-5}$. In contrast, for aromatic nucleophilic substitution reactions in which carbon–halogen bond cleavage is rate limiting, the F/Cl mobility ratio is several orders of magnitude larger (i.e., $\geq 1 \times 10^{-2}$; Miller, 1968).

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