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Exploration of Possible Mechanisms for 4-Chlorobenzoyl CoA Dehalogenase: Evidence for an Aryl–Enzyme Intermediate

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Abstract: 4-Chlorobenzoyl CoA dehalogenase catalyzes the replacement of the chlorine substituent on 4-chlorobenzoyl CoA with a hydroxyl group. The S_NAr mechanism seems the most likely mechanism for this unusual and intrinsically difficult nucleophilic aromatic substitution reaction. However, the order of leaving group abilities observed for various 4-halobenzoyl CoA substrates is opposite that expected. Therefore, we have explored alternative mechanisms for the enzymic dehalogenation reaction. The aryne mechanism was ruled out by the absence of a deuterium kinetic isotope effect on the reaction. The $S_{RN}1$ and $S_{ON}2$ mechanisms were deemed unlikely because of the lack of evidence for a metal ion or organic cofactor on the enzyme. Thus, the dehalogenation reaction appears to occur via an S_NAr mechanism. Further investigations suggested that the reaction proceeds by displacement of chloride by an enzymic carboxylate, followed by hydrolysis of an aryl–enzyme intermediate. When an alternative nucleophile, hydroxylamine, was included in reaction mixtures, no product derived from direct attack of hydroxylamine upon 4-chlorobenzoyl CoA could be detected. However, inclusion of higher concentrations of hydroxylamine (100 mM) resulted in inactivation of the enzyme. These data are consistent with the formation of an aryl–enzyme intermediate that is converted to a hydroxamic acid upon attack by hydroxylamine. Enzyme activity is recovered after hydroxylamine is removed, suggesting that the enzyme is able to slowly hydrolyze the hydroxamic acid and restore the active-site carboxylate. Single-turnover ^{18}O-labeling experiments designed to confirm that the reaction occurs by direct attack of an active-site carboxylate to form an aryl–enzyme intermediate were difficult to interpret. Approximately one-half of the product contained oxygen derived from the solvent and one-half contained oxygen derived from the enzyme. Possible explanations for this phenomenon were explored, but a satisfactory explanation has not been found.

Introduction

Halogenated aromatic compounds have been introduced into the environment in enormous quantities during the past 40 years. Many of these compounds are persistent pollutants because most soil microorganisms are unable to degrade aromatic compounds bearing halogen substituents. However, a few strains, mostly isolated from toxic waste spill sites, have greatly enhanced abilities to degrade particular halogenated aromatic compounds.

Several soil microorganisms, including *Acinetobacter* sp. 4-CB1,[1] *Pseudomonas* CBS-3,[2] coryneform bacterium NTB-1,[3] and various strains of *Arthrobacter* sp.[4-6] have been reported to degrade 4-chlorobenzoate, a breakdown product of some PCB

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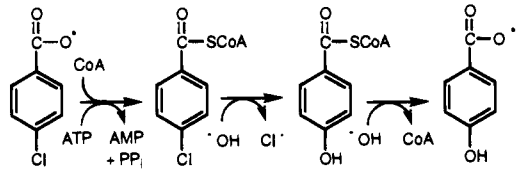
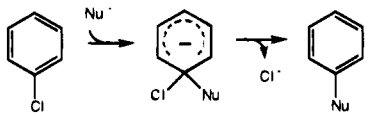
Scheme 1



Scheme 2

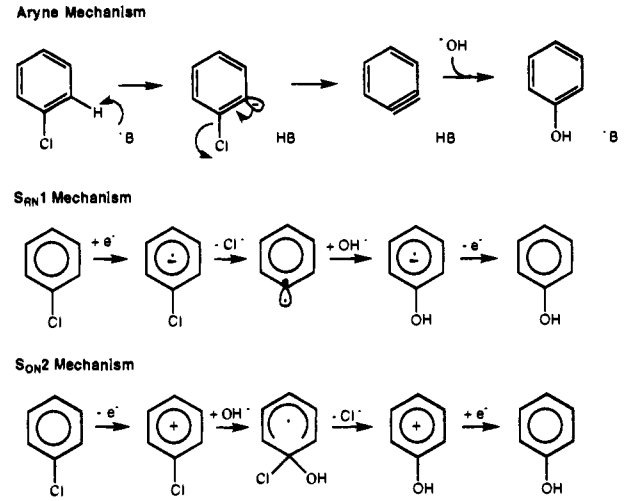


congeners and some herbicides. Our research group, working with *Arthrobacter* sp. 4-CB1 (formerly known as *Acinetobacter* sp. 4-CB1),[7] and the groups of Dunaway-Mariano[8] and Lingens,[9] working with *Pseudomonas* CBS-3, have recently shown that degradation proceeds by initial formation of the coenzyme A thioester of 4-chlorobenzoate, followed by dehalogenation of 4-chlorobenzoyl CoA (see Scheme 1).

The dehalogenation reaction catalyzed by 4-chlorobenzoyl CoA dehalogenase is a nucleophilic aromatic substitution in which the chlorine substituent is replaced by a hydroxyl group. Besides 4-chlorobenzoyl CoA dehalogenase, only some isozymes of glutathione *S*-transferase from mammalian liver are known to catalyze nucleophilic aromatic substitution reactions.[10,11] Glutathione *S*-transferase catalyzes nucleophilic aromatic substitution reactions via the S_NAr mechanism (see Scheme 2), in which the nucleophile attacks the aromatic ring to give an intermediate Meisenheimer complex which subsequently loses the leaving group to form the products.[10,11]

The S_NAr mechanism appears to be the most likely mechanism for 4-chlorobenzoyl CoA dehalogenase, as well. However, we have recently reported that the relative leaving group abilities for bromide, chloride, and fluoride in the dehalogenation reaction catalyzed by 4-chlorobenzoyl CoA dehalogenase are opposite to those expected for an S_NAr reaction.[12] S_NAr reactions generally proceed faster when the leaving group is fluoride than when the leaving group is chloride. For example, the rate of substitution of 4-fluoronitrobenzene with methoxide is 312 times faster at 50 °C than the rate of substitution of 4-chloronitrobenzene.[13] The S_NAr reactions catalyzed by glutathione *S*-transferase show a similar trend. For isozyme 4-4, V_{max} for the reaction of 4-fluoro-3-nitro-1-(trifluoromethyl)benzene is 40 times larger than V_{max} for the corresponding chloro compound. For isozyme 3-3, V_{max} for reaction of the fluoro compound is 4 times larger than V_{max} for the chloro compound.[10] The order of leaving group abilities is reversed only when the carbon–halogen bond cleavage step becomes rate-limiting. This occurs when the ring is highly activated for nucleophilic attack by several electron-withdrawing substituents, or in reactions with heavy nucleophiles such as iodide and thiocyanate.[14]

Scheme 3



The leaving group abilities for 4-chlorobenzoyl CoA dehalogenase are in striking contrast to the relative leaving group abilities observed for most non-enzymic and enzymic nucleophilic aromatic substitution reactions that occur by the S_NAr mechanism. Dehalogenation of 4-chlorobenzoyl CoA is over 400-fold *faster* than dehalogenation of 4-fluorobenzoyl CoA.[12] We initially considered two alternative explanations for these data. If the reaction proceeds via an S_NAr mechanism, then the enzyme must be such an effective catalyst that the first step of the S_NAr reaction is not rate-limiting. An alternative explanation is that the dehalogenation reaction proceeds by some other mechanism. Other mechanisms for nucleophilic aromatic substitution that occur in non-enzymic systems include the aryne mechanism,[15] the $S_{RN}1$ mechanism,[16] and the $S_{ON}2$ mechanism.[17] Scheme 3 shows these mechanisms as they might occur on the surface of an enzyme. Although none of these mechanisms is a particularly appealing candidate for an enzymic nucleophilic aromatic substitution reaction, they cannot be ruled out on the basis of the available data.

Here we describe an exploration of possible mechanisms for 4-chlorobenzoyl CoA dehalogenase. Our data suggest that the enzyme does indeed catalyze an S_NAr reaction but that the nucleophile is an active-site carboxylate rather than water. Following displacement of chloride from the substrate, the resulting ester is hydrolyzed by attack of water at the acyl carbon.

Results

Determination of the Origin of the Oxygen Incorporated into 4-Hydroxybenzoyl CoA during Multiple Turnovers. 4-Chlorobenzoyl CoA dehalogenase was incubated with 4-chlorobenzoyl CoA in phosphate buffer containing $H_2{}^{18}O$ (80% enrichment). (We were unable to do the experiments with solvent enriched 100% in ^{18}O because the enzyme loses virtually all activity upon lyophilization. Thus, the enzyme was concentrated to a minimal volume before use in these experiments.) After the dehalogenation reaction was complete, the CoA was removed from 4-hydroxybenzoyl CoA by treatment with 4-hy-

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Table 1. Incorporation of Solvent Oxygen into 4-Hydroxybenzoyl CoA under Multiple-Turnover Conditions[a]

substrate	[NaCl] (mM)	$[H_2{}^{18}O]/[H_2{}^{16}O]$	$(m/z = 95)/(m/z = 93)$[b] or $(m/z = 94)/(m/z = 92)$[c]
4-chlorobenzoyl CoA	0	3.73	3.89[b]
4-hydroxybenzoyl CoA	0	3.72	0[c]
4-hydroxybenzoyl CoA	400	3.72	0[c]

[a] [substrate]:[enzyme] or [product]:[enzyme] = 200, assuming four active sites per homotetramer. [b] Fragment ions derived from 4-methoxybenzoate. [c] Fragment ions derived from methyl 4-methoxybenzoate.

droxybenzoyl CoA thioesterase. The reaction mixture was acidified, and the 4-hydroxybenzoic acid was extracted into ether and treated with diazomethane. The mass spectrum of methyl 4-hydroxybenzoate (formed by methylation of the carboxylate group) gives a fragment ion at $m/z = 93$ due to cleavage between the aromatic ring and the carbomethoxy substituent. The mass spectrum of methyl 4-methoxybenzoate (formed by methylation of both the carboxylate and hydroxyl groups) gives a fragment ion at $m/z = 92$ due to cleavage between the aromatic ring and the carbomethoxy substituent and between the oxygen and methyl group of the methoxyl group. (The fragment ion at $m/z = 107$ due to cleavage between the aromatic ring and the carbomethoxy substituent is also potentially useful, but interference from an unidentified contaminant made these data less reliable.) Since the only oxygen atom in these fragments is in the hydroxyl group, these fragments are ideal for determining the origin of the hydroxyl group oxygen. Under multiple-turnover conditions, the enrichment of ^{18}O in 4-hydroxybenzoate formed from 4-chlorobenzoyl CoA was approximately equal to that of the solvent (see Table 1).

In order to confirm that ^{18}O was introduced into 4-hydroxybenzoyl CoA under multiple-turnover conditions during the actual dehalogenation reaction and not by enzymic or nonenzymic exchange of the hydroxyl group with the solvent, 4-hydroxybenzoyl CoA was incubated with 4-chlorobenzoyl CoA dehalogenase for 1 h and then treated with 4-hydroxybenzoyl CoA thioesterase to produce 4-hydroxybenzoate as described above. An additional experiment was carried out in the presence of 400 mM NaCl in case exchange of ^{18}O only occurs when both products are bound to the enzyme. The reaction mixtures were acidified, and the 4-hydroxybenzoate was extracted into ether, treated with diazomethane, and subjected to GC/MS analysis. Essentially no incorporation of ^{18}O into 4-hydroxybenzoate was observed under either set of conditions (see Table 1).

Analysis of Metal Content in 4-Chlorobenzoyl CoA Dehalogenase. The metal ion content of 4-chlorobenzoyl CoA dehalogenase was determined by inductively coupled plasma emission spectroscopy. Prior to analysis, the enzyme was dialyzed against a solution containing triethylammonium bicarbonate buffer and dithiothreitol in order to remove adventitious metals. The enzyme submitted for analysis retained full activity after exhaustive dialysis. The enzyme did not contain significant amounts of iron, manganese, copper, zinc, nickel, or molybdenum.

Determination of the Deuterium Kinetic Isotope Effect. The deuterium kinetic isotope effect for the dehalogenation reaction was determined by measuring k_{cat} for 4-chlorobenzoyl CoA and 4-chlorobenzoyl-d_4 CoA. The value of $^{H}k_{cat}/^{D}k_{cat}$ was 0.94 ± 0.11.

Determination of Enzyme Activity in the Presence of 4-Trifluoromethylbenzoyl CoA. 4-Chlorobenzoyl CoA dehalogenase activity was measured in the presence of various concentrations of 4-(trifluoromethyl)benzoyl CoA ranging from 0 to 250 μM. Lineweaver–Burke plots revealed that the lines for the various inhibitor concentrations intersected on the y-axis, demonstrating that 4-(trifluoromethyl)benzoyl CoA is a competitive inhibitor (see Figure 1). A replot of the slopes from the Lineweaver–Burke plot against the inhibitor concentration was used to determine that the K_i for this compound is 50 μM.

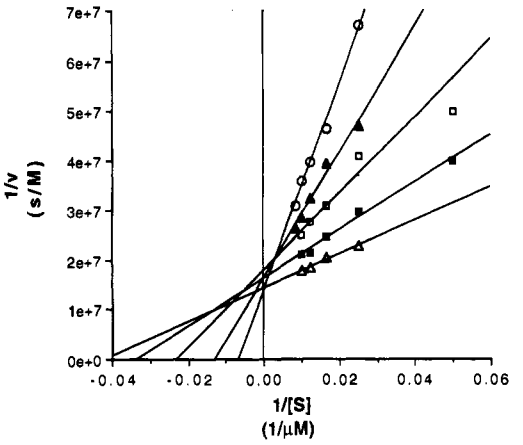

Figure 1. Inhibition of 4-chlorobenzoyl CoA dehalogenase by 4-(trifluoromethyl)benzoyl CoA. Inhibitor concentrations were 0 μM (△), 25 μM (■), 75 μM (□), 150 μM (▲), and 250 μM (○).

Titration of 4-Chlorobenzoyl CoA Dehalogenase with 4-(Trifluoromethyl)benzoyl CoA. In an effort to detect a Meisenheimer complex at the active site of 4-chlorobenzoyl CoA dehalogenase, we added 4-(trifluoromethyl)benzoyl CoA to a concentrated solution of the enzyme (0.25 mM homotetramer, 1 mM active sites, assuming four active sites per tetramer) in a quartz cuvette to give a final concentration of 6.5 mM. No peak in the visible region which could be ascribed to a Meisenheimer complex was observed.

Search for a Product Formed by Enzyme-Catalyzed Attack of Hydroxylamine on 4-Chlorobenzoyl CoA. We have carried out dehalogenation reactions in the presence of 10 mM hydroxylamine and searched for evidence of an alternative product formed by direct attack of hydroxylamine at the 4-position of 4-chlorobenzoyl CoA. HPLC was used to analyze the composition of reaction mixtures. No peaks were observed at the retention time for 4-(hydroxyamino)benzoyl CoA or for its expected oxidation product, 4-nitrosobenzoyl CoA (both of which eluted at 7.1 min).

Inactivation of 4-Chlorobenzoyl CoA Dehalogenase by Hydroxylamine. Addition of hydroxylamine to a final concentration of 100 mM to a solution of 4-chlorobenzoyl CoA dehalogenase in the presence of substrate caused an immediate loss of enzyme activity (see Figure 2). The effect of hydroxylamine was reversible. 4-Chlorobenzoyl CoA dehalogenase was treated with 200 mM hydroxylamine (in two aliquots) in the absence and presence of 4-chlorobenzoyl CoA. Residual hydroxylamine was removed by gel filtration. Removal of the hydroxylamine resulted in full recovery of enzyme activity in both cases (data not shown).

Examination of the Rate of the Non-Enzymatic Reaction of 4-Chlorobenzoyl CoA with Hydroxylamine. Attack of nucleophiles upon the thioester moiety of 4-chlorobenzoyl CoA results in cleavage of the thioester and formation of products that would not be expected to be substrates for 4-chlorobenzoyl CoA dehalogenase. In order to ensure that the inactivation of 4-chlorobenzoyl CoA dehalogenase by hydroxylamine described above was not simply due to destruction of the substrate, we examined the rate of the reaction of 4-chlorobenzoyl CoA with hydroxylamine. The disappearance of 4-chlorobenzoyl CoA (200 μM) upon treatment with an excess of hydroxylamine (10

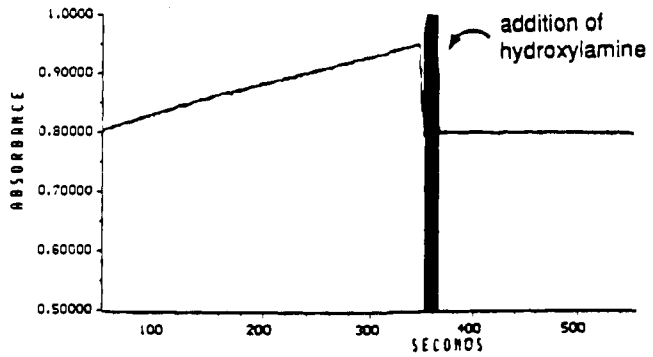

Figure 2. Inactivation of 4-chlorobenzoyl CoA dehalogenase by hydroxylamine. The initial part of the trace shows the change in absorbance at 300 nm due to the conversion of 4-chlorobenzoyl CoA to 4-hydroxybenzoyl CoA catalyzed by 4-chlorobenzoyl CoA dehalogenase. The final part of the trace shows complete cessation of activity after addition of hydroxylamine to 100 mM. (The decrease in absorbance after addition of hydroxylamine is due to dilution of sample.)

Table 2. Incorporation of Solvent Oxygen into 4-Hydroxybenzoyl CoA under Single-Turnover Conditions

expt	[enzyme]/ [4CBCoA][a]	$[H_2{}^{18}O]$/ $[H_2{}^{16}O]$	$(m/z = 94)$/ $(m/z = 92)$[b]	% O in product derived from solvent
1	3.0	2.7	0.51	46
	6.0	2.7	0.74	58
	15.0	2.7	0.64	54
2	15.0	3.9	0.62	48
	15.0	0	0	NA

[a] Assuming four active sites per homotetramer. [b] Fragment ions derived from methyl 4-methoxybenzoate.

mM) was followed by HPLC. The reaction followed pseudo-first-order kinetics (data not shown). The second-order rate constant for reaction of 4-chlorobenzoyl CoA and hydroxylamine is 0.03 M^{-1} s^{-1}. Using this second-order rate constant and the concentration of hydroxylamine (100 mM) used in the inactivation experiments (and assuming pseudo-first-order conditions) shows that it would take 3.7 min to deplete 50% of the substrate via the non-enzymatic reaction. Since the inactivation of the enzyme was essentially instantaneous, the cause was clearly not depletion of the substrate by non-enzymatic reaction with hydroxylamine.

Determination of the Origin of the Oxygen Incorporated into 4-Hydroxybenzoyl CoA during Single Turnovers. For single-turnover experiments, 4-chlorobenzoyl CoA dehalogenase was preincubated in phosphate buffer containing $H_2{}^{18}O$ for 2 or 3 h prior to addition of the substrate. In other respects, these experiments were carried out as described above for multiple-turnover experiments. The isotopic composition of the product was determined using the fragment ion at $m/z = 92$ formed from methyl 4-methoxybenzoate. (A minor contaminant interfered with the quantitation of the fragment ion at $m/z = 93$ formed from 4-methoxybenzoate when very small samples were analyzed.) The product of dehalogenation of 4-chlorobenzoyl CoA under single-turnover conditions contained a significant amount of ^{18}O (see Table 2). The isotopic content of the product does not relate directly to the amount of oxygen in the product derived from solvent, since the experiments were carried out in solvent containing less than 100% $H_2{}^{18}O$. Correction of the mass spectral data for the isotopic content of the solvent shows that approximately 52% of the oxygen in the product was derived from the bulk solvent.

Discussion

The origin of the oxygen atom that replaces the chlorine of 4-chlorobenzoate during biodegradation of 4-chlorobenzoate was addressed in 1984 by Müller et al. (working with *Pseudomonas* sp. CBS-3)[18] and Marks et al. (working with an *Arthrobacter* sp.).[19] When 4-chlorobenzoate was incubated with crude cell extracts in the presence of $H_2{}^{18}O$, ^{18}O was found to be incorporated into the hydroxyl group of *p*-hydroxybenzoate. No exchange of ^{18}O into the hydroxyl group was observed when *p*-hydroxybenzoate itself was incubated with $H_2{}^{18}O$ under the same conditions. These results must be interpreted with caution, however, since at the time the pathway for 4-chlorobenzoate degradation was not known. The pathway that has been recently elucidated in *Arthrobacter* sp. 4-CB1,[7] *Pseudomonas* sp. CBS-3,[8,9] and coryneform bacterium NTB-1[20] is shown in Scheme 1. Because the pathway was not known at the time of the original experiments, the cofactors required for the production of 4-chlorobenzoyl CoA, ATP and CoA, were not added to these reaction mixtures. Furthermore, the actual product of the dehalogenation reaction is 4-hydroxybenzoyl CoA, not *p*-hydroxybenzoate. Thus, the appropriate control for the ^{18}O-labeling experiment is the incubation of 4-hydroxybenzoyl CoA with the enzyme in $H_2{}^{18}O$ to ensure that ^{18}O does not exchange into the hydroxyl group during the incubation. We have carried out ^{18}O-labeling experiments using purified 4-chlorobenzoyl CoA dehalogenase, the correct substrate, 4-chlorobenzoyl CoA, and the correct product, 4-hydroxybenzoyl CoA. Our results confirm that the hydroxyl group of 4-hydroxybenzoyl CoA is indeed derived from water, as the isotopic content of the hydroxyl group was essentially identical to that of the water (see Table 1). The control experiment showed that no exchange of ^{18}O into the hydroxyl group of 4-hydroxybenzoyl CoA occurred during the incubation.

The results of the ^{18}O-labeling experiment and the fact that no exogenous cofactor is required by 4-chlorobenzoyl CoA dehalogenase demonstrate that this enzyme catalyzes a nucleophilic aromatic substitution reaction in which the chlorine atom is replaced by a hydroxyl group derived from water rather than from O_2. Glutathione *S*-transferase, the only other enzyme known to catalyze such a reaction, proceeds via an S_NAr mechanism. However, the order of leaving group abilities observed for 4-chlorobenzoyl CoA dehalogenase is opposite to that for glutathione *S*-transferase and, indeed, for most non-enzymatic S_NAr reactions as well. Thus, we have explored other possible mechanisms for the dehalogenase reaction.

The aryne mechanism (see Scheme 3) would proceed by elimination of HCl, followed by addition of water. This reaction requires an extremely strong base (such as KNH_2 in non-enzymic reactions) to remove the proton from the aromatic ring.[15] There is little doubt that, if the dehalogenase reaction were to proceed via the aryne mechanism, the most difficult step would be the removal of the proton ortho to the leaving group. Thus, a substantial deuterium kinetic isotope effect would be expected. The deuterium kinetic isotope effect for the reaction was 0.94 ± 0.11, thus ruling out the aryne mechanism.

The $S_{RN}1$ and $S_{ON}2$ mechanisms (see Scheme 3) both involve radical intermediates and would require the enzyme to donate or to remove, respectively, a single electron from the substrate. The ability to carry out single-electron transfer reactions could be endowed by a redox-active metal ion on the protein. Alternatively, this role could be fulfilled by an organic cofactor capable of single-electron chemistry such as a flavin or a

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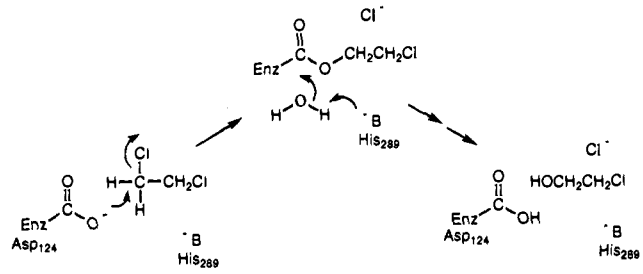
quinone. We have searched for each of these possible participants in the dehalogenation reaction. Inductively coupled plasma emission spectroscopy was used to determine the metal content of the purified enzyme. None of the redox-active metals commonly found in proteins could be detected in a sample of fully active enzyme. The possibility of organic cofactors was addressed by examining the UV/vis spectrum of purified enzyme. No long-wavelength absorbance indicative of a flavin or quinone cofactor was observed (data not shown). Thus, we have found no evidence for a single-electron donor or acceptor on the enzyme that would be required for either the $S_{RN}1$ or $S_{ON}2$ mechanism.

The lack of evidence for the three alternative mechanisms shown in Scheme 3 suggests that the most likely mechanism for the dehalogenase reaction is indeed the S_NAr mechanism. Direct observation of a Meisenheimer complex at the active site of 4-chlorobenzoyl CoA dehalogenase would support the hypothesis that the reaction occurs via an S_NAr mechanism. Armstrong and co-workers have demonstrated the presence of a Meisenheimer complex at the active site of glutathione *S*-transferase using 1,3,5-trinitrobenzene, a substrate analogue that lacks a leaving group at the position of glutathione attack. The Meisenheimer complex formed upon addition of 1,3,5-trinitrobenzene to binary complexes of glutathione *S*-transferase with glutathione had a λ_{max} of 457 nm and an extinction coefficient of 28 500 M^{-1} cm^{-1}.[11] We have attempted a similar experiment with 4-chlorobenzoyl CoA dehalogenase using 4-(trifluoromethyl)benzoyl CoA, a competitive inhibitor of 4-chlorobenzoyl CoA dehalogenase with a K_i of 50 μM. The trifluoromethyl group is strongly electron-withdrawing and should therefore maximize the stability of the addition complex at the active site. We added 6.5 mM 4-(trifluoromethyl)benzoyl CoA to 1 mM 4-chlorobenzoyl CoA dehalogenase. No increase in absorbance in the visible region was detected. This result might be attributed to formation of a Meisenheimer complex with a λ_{max} in the region of absorbance by the protein or to the formation of an undetectable amount of Meisenheimer complex. (The possibility that no Meisenheimer complex exists seems remote at this point.)

We considered further the possibility that a Meisenheimer complex at the active site of 4-chlorobenzoyl CoA dehalogenase could not be observed because the peak was obscured by the protein absorbance band centered at 280 nm. The λ_{max} of a Meisenheimer complex of 4-(trifluoromethyl)benzoyl CoA in basic solution cannot be determined directly, since the thioester moiety is more susceptible to nucleophilic attack than is the aromatic ring. Meisenheimer complexes typically absorb in the visible region, with λ_{max} values ranging from 400 to 600 nm, depending upon the substituents.[11,21] The λ_{max} of a Meisenheimer complex involving 4-(trifluoromethyl)benzoyl CoA would be expected to be blue-shifted compared to these values, since most of these values were measured for complexes bearing at least two nitro substituents. However, even if the λ_{max} is considerably blue-shifted, a peak due to a Meisenheimer complex at the active site should be readily visible.

The more likely explanation is that an insufficient amount of the Meisenheimer complex was formed to allow detection by visible spectroscopy. Under the conditions of the experiment ([active sites] = 1 mM, [I] = 6.5 mM, K_i = 50 μM), the concentration of enzyme-bound 4-(trifluoromethyl)benzoyl CoA should be 1 mM. If we assume an extinction coefficient of approximately 10 000 M^{-1} cm^{-1} and that we could detect a change in absorbance as small as 0.02, then we can calculate

Scheme 4



that less than 0.2% of the enzyme could carry the Meisenheimer complex. Thus, the putative Meisenheimer complex would have to lie >3.7 kcal in energy above the bound 4-(trifluoromethyl)-benzoyl CoA. It is certainly possible that a Meisenheimer complex is not observable for this reason.

Although the S_NAr mechanism seems the most likely mechanism for the dehalogenation reaction, preliminary stopped-flow data (not shown) are more complex than expected for a simple S_NAr reaction. Thus, we have considered the possibility that the enzyme might catalyze displacement of chloride using an enzymic nucleophile, rather than by activating water for attack on the aromatic ring. This approach is utilized by the enzymes haloalkane dehalogenase[22,23] and epoxide hydrolase.[24] The mechanism of haloalkane dehalogenase is shown in Scheme 4. Surprisingly, although the reactions catalyzed by these enzymes are considerably different (S_N2 vs S_NAR), the evidence described below suggests that 4-chlorobenzoyl CoA dehalogenase also utilizes an active-site carboxylate to displace chloride from 4-chlorobenzoyl CoA.

In order to test the possibility of displacement of chloride by an active-site carboxylate, we looked for evidence of a product formed by enzyme-catalyzed attack of hydroxylamine upon 4-chlorobenzoyl CoA. If the enzyme were able to catalyze attack of water directly upon 4-chlorobenzoyl CoA, then it is likely that it would also catalyze analogous attack of hydroxylamine. Rates of reactions involving nucleophilic attack of hydroxylamine are typically many orders of magnitude faster than those involving nucleophilic attack of water.[26] Thus, even though the concentration of hydroxylamine (10 mM) was 550-fold less than that of water, the predominant product under these conditions should be that formed from hydroxylamine, assuming that hydroxylamine and water have equal access to the active site. HPLC chromatograms showed that no 4-(hydroxyamino)-benzoyl CoA formed during turnover of 4-chlorobenzoyl CoA in the presence of 10 mM hydroxylamine. (Although arylhydroxylamines are known to undergo facile oxidation reactions, we determined that 4-(hydroxyamino)benzoyl CoA is stable under these reaction conditions for more than the 5 min required for the experiment. Furthermore, 4-nitrosobenzoyl CoA, the oxidation product of 4-(hydroxyamino)benzoyl CoA, was not found in reaction mixtures.) It is also possible that the enzyme might catalyze attack of the oxygen of hydroxylamine upon 4-chlorobenzoyl CoA to form 4-(aminooxy)benzoyl CoA. While we did not have an authentic sample of 4-(aminooxy)-benzoyl CoA to serve as a standard for HPLC, we anticipate

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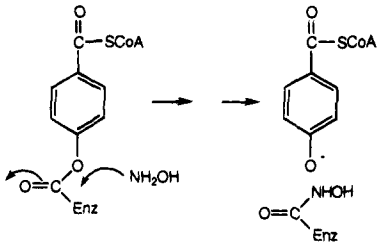
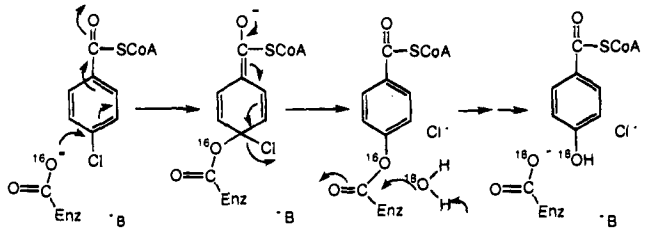
Scheme 5



Scheme 6



that the retention times of 4-(hydroxyamino)benzoyl CoA and 4-(aminooxy)benzoyl CoA would be similar. No peaks were observed within 0.9 min of the retention time for 4-(hydroxyamino)benzoyl CoA (t_R = 7.1 min). Thus, we were unable to detect formation of a product derived from displacement of chlorine by hydroxylamine. These results are consistent with a mechanism in which the substrate is initially attacked by an enzymic group rather than by water.

Further evidence for this type of mechanism is provided by the observation that addition of high concentrations of hydroxylamine to 4-chlorobenzoyl CoA dehalogenase in the presence of substrate inactivates the enzyme. Figure 2 shows the result of addition of hydroxylamine to a final concentration of 100 mM during enzymic dehalogenation of 4-chlorobenzoyl CoA. The enzyme is completely inactivated by the time the solution is mixed. These data are consistent with attack of hydroxylamine on an aryl–enzyme intermediate to yield a hydroxamic acid in place of the catalytic carboxylate (see Scheme 5). Inactivation by hydroxylamine is reversible. When hydroxylamine is removed, activity is recovered. Evidently, the enzyme is able to catalyze hydrolysis of the hydroxamic acid, probably using the same catalytic groups that are utilized to cleave the naturally-occurring aryl–enzyme intermediate. The requirement for high concentrations of hydroxylamine to achieve inactivation is presumably due to this ability, since the proportion of enzyme that is inactivated will depend on the relative rates of the attack of hydroxylamine on the aryl–enzyme intermediate (which will be proportional to the concentration of hydroxylamine) and the subsequent hydrolysis of the hydroxamic acid.

In order to further investigate the possibility of a mechanism involving an aryl–enzyme intermediate, we carried out the dehalogenation reaction in $H_2{}^{18}O$ under single-turnover conditions. As shown in Scheme 6, the oxygen in the product generated during the first turnover by this mechanism should be derived from the enzymic nucleophile, rather than from the solvent. If, however, the reaction occurs via general base-catalyzed attack of water upon the substrate, then the oxygen in the product should be derived from the solvent. Surprisingly, the results of several single-turnover experiments showed that approximately 52% of the oxygen in the product is derived from the bulk solvent. These results are clearly inconsistent with both mechanistic models. Possible reasons for the unexpectedly large amount of oxygen derived from solvent in the product are discussed below.

Similar single-turnover experiments have been carried out by Yang et al. with the 4-chlorobenzoyl CoA dehalogenase from *Pseudomonas* sp. CBS-3, who showed that about 25% of the oxygen in the product was derived from solvent.[25] These authors proposed that the extra incorporation was due to multiple turnovers catalyzed by some enzyme molecules.[25] Since the chemical step of the dehalogenase reaction is slow, the substrate will rapidly equilibrate between bound and free forms. Under these conditions, as soon as an enzyme carries out a catalytic turnover, it will reenter the pool of free enzyme. Upon binding another molecule of substrate, solvent oxygen incorporated into the active-site carboxylate during the first turnover may be transferred to the substrate during the second turnover. We tested this possibility by carrying out the single-turnover experiments using varying ratios of enzyme to substrate. If this explanation is correct, then increasing the ratio of enzyme to substrate should decrease the amount of solvent oxygen found in the product, since an enzyme molecule that has completed one turnover would have less opportunity to encounter a second molecule of substrate. This explanation clearly does not account for the excess ^{18}O observed in our experiments. Firstly, the amount of incorporation from the solvent (approximately 52%) is much higher than expected. We estimate that approximately 7% of the oxygen in the product should be derived from solvent when the ratio of active sites to substrate is 3:1 and 1.4% when the ratio of active sites to substrate is 15:1. (This calculation assumes that the active-site carboxylate rotates freely and therefore there is a 50% chance that the oxygen incorporated from the solvent during the first turnover will be transferred during the second turnover. If the active-site carboxylate is constrained and does not rotate in the active site, the corresponding estimates would be 14% and 2.8%, respectively.) Secondly, and most definitively, the amount of oxygen derived from solvent is *not* dependent upon the ratio of active sites to substrate.

The possibility that the ratio of the m/z = 94 and m/z = 92 peaks used to calculate the extent of incorporation of solvent oxygen was skewed due to contamination by a coeluting substance giving a fragment ion with m/z = 94 was ruled out by carrying out a single-turnover experiment in $H_2{}^{16}O$. The ratio of the m/z = 94 to m/z = 92 peaks was zero within the limits of detection.

A third possibility is that the active-site carboxylate undergoes rapid exchange with solvent at the active site of the enzyme in the absence of substrate. Our data suggest that such exchange cannot account for the unexpectedly high amount of incorporation of solvent oxygen into the product. In experiment 1 described in Table 2, the sample was preincubated in $H_2{}^{18}O$ for 3 h on ice prior to addition of the substrate, while in experiment 2, the preincubation time was 2 h. The 50% difference in incubation times should be reflected in different degrees of ^{18}O enrichment in the product. Thus, if preincubation for 3 h results in formation of product in which 53% of the oxygen is derived from the solvent, then preincubation for 2 h should result in formation of product in which 36% of the oxygen is derived from the solvent. The data shown in Table 2 do not support this hypothesis. Furthermore, Yang et al. observed that preincubation of the *Pseudomonas* enzyme in $H_2{}^{18}O$ for 30 min prior to addition of substrate did not change the extent of ^{18}O incorporation into product.[25]

A fourth possibility is that the enzyme catalyzes the dehalogenation by two distinct pathways: (1) displacement of chloride from 4-chlorobenzoyl CoA by an active-site carboxylate, followed by hydrolysis of the aryl–enzyme intermediate by attack of water at the carbonyl carbon and (2) displacement

of chloride from 4-chlorobenzoyl CoA by water, undoubtedly activated by a general base in the active site. Under single-turnover conditions in $H_2{}^{18}O$, reaction via the first pathway would generate product with ^{16}O in the hydroxyl group, while reaction via the second pathway would generate product with ^{18}O in the hydroxyl group. This explanation is consistent with the data from the single-turnover experiments, since the amount of incorporation of oxygen from the bulk solvent would be independent of the ratio of active sites to substrate, as it would be determined only by events occurring at the active site of the enzyme. However, this type of behavior would be unusual, if not unprecedented, for an enzyme. Furthermore, if the enzyme were able to catalyze attack of water directly upon 4-chlorobenzoyl CoA, then it is likely that it would also catalyze analogous attack of hydroxylamine. As described above, we were unable to detect formation of any product derived from direct attack of hydroxylamine on 4-chlorobenzoyl CoA. Although our results cannot definitively rule out the operation of two mechanisms when the nucleophile is water, this scenario seems unlikely.

A final possibility is that exchange of solvent oxygen into the product does in fact occur during the dehalogenation reaction, but that an irreversible step prior to the release of 4-hydroxybenzoyl CoA prohibits formation of an exchange-competent complex from the product side. Under these circumstances, 4-hydroxybenzoyl CoA could bind to the enzyme but would not undergo exchange. A reasonable candidate for such an irreversible step might be release of chloride from the enzyme. Chloride is a very poor inhibitor of the enzyme (50% inhibition is reached only at 200 mM NaCl) and thus must bind weakly. Dissociation of chloride from the enzyme into solution lacking NaCl would therefore be an effectively irreversible step. The presence of chloride might affect the orientation of the active site such that 4-hydroxybenzoyl CoA can only undergo exchange in its presence. To test this possibility, we incubated 4-hydroxybenzoyl CoA with 4-chlorobenzoyl CoA dehalogenase in $H_2{}^{18}O$ (80% enrichment) in the presence of 400 mM NaCl to ensure formation of the enzyme:4-hydroxybenzoyl CoA:chloride complex. However, exchange of solvent oxygen into 4-hydroxybenzoyl CoA was still not observed under these circumstances (see Table 1). The existence of a different irreversible step, perhaps involving an isomerization between enzyme:product complexes, may still be the explanation for the incorporation of solvent oxygen into 4-hydroxybenzoyl CoA during normal turnover. However, we currently have no data to either support or refute this possibility.

Conclusions

The data described above suggest that the reaction catalyzed by 4-chlorobenzoyl CoA dehalogenase occurs via an S_NAr reaction in which a carboxylate residue at the active site attacks the 4-position of the substrate to form a high-energy Meisenheimer complex. Subsequent loss of chloride from the intermediate results in formation of an aryl−enzyme intermediate. The product, 4-hydroxybenzoyl CoA, is generated by attack of water at the carbonyl carbon of the aryl−enzyme intermediate (see Scheme 6). Remarkably, similar mechanisms have recently been reported for haloalkane dehalogenase[22,23] and epoxide hydrolase,[24] two enzymes that catalyze nucleophilic *aliphatic* substitution reactions.

Single-turnover experiments in $H_2{}^{18}O$ were used successfully to diagnose the mechanism of epoxide hydrolase.[24] The results of our single-turnover experiments, however, were difficult to interpret. After considerable effort, we are unable to account for the 52% of the oxygen in 4-hydroxybenzoyl CoA that is derived from the solvent. Yang et al. have also proposed the aryl−enzyme intermediate mechanism for the 4-chlorobenzoyl CoA dehalogenase from *Pseudomonas* sp. CBS-3 based upon the results of single-turnover experiments in $H_2{}^{18}O$ and rapid quench experiments using radiolabeled substrate that demonstrated the existence of an intermediate containing covalently-bound radiolabel which is likely to be the aryl−enzyme intermediate.[25] Since these single-turnover experiments showed that 25% of the oxygen in the product was derived from solvent, these results are subject to the same uncertainty as are our results.

The utilization of an active-site carboxylate to displace chloride from the aromatic ring is intriguing. In model reactions such as the hydrolysis of picryl chloride, acetate is believed to act as a general base that promotes attack of water upon the substrate, rather than directly attacking the ring itself.[27] However, 4-chlorobenzoyl CoA dehalogenase may achieve more effective catalysis by using a carboxylate as a nucleophile rather than a general base. If the carboxylate can be desolvated in the active site, it would be a more powerful nucleophile than would be water, even with the assistance of a general base catalyst. The use of a carboxylate as a nucleophile is particularly clever in that regeneration of active enzyme and release of product can be accomplished by ester hydrolysis, rather than by a second S_NAr reaction.

Experimental Section

Materials. 4-Chlorobenzoyl CoA was synthesized as described previously.[7] 4-Chlorobenzoyl-d_4 CoA, 4-(trifluoromethyl)benzoyl CoA, and 4-nitrobenzoyl CoA were synthesized by an analogous procedure from 4-chlorobenzoyl-d_4 chloride (obtained from MSD Isotopes), 4-(trifluoromethyl)benzoyl chloride, and 4-nitrobenzoyl chloride, respectively. 4-Hydroxybenzoyl CoA was synthesized as described by Merkel et al.[28]

4-Nitrosobenzoyl CoA and 4-(*N*-hydroxyamino)benzoyl CoA were prepared by a minor modification of the procedure used for preparation of 4-(*N*-hydroxyamino)benzoic acid by Bauer and Rosenthal.[29] 4-Nitrobenzoyl CoA (2.8 μmol) was treated with zinc dust (18 μmol) in the presence of 0.35 M NH_4Cl. The zinc dust was removed by filtration after 10 min. Control experiments showed that the thioester moiety was not affected under these reaction conditions. Positive ion electrospray LC/MS of the reaction mixture confirmed the presence of distinct peaks with $m/z = 901$ due to 4-nitrosobenzoyl CoA ($M + H^+$) and with $m/z = 903$ due to 4-(hydroxyamino)benzoyl CoA ($M + H^+$). LC/MS was carried out on a Perkin Elmer SCIEX API III LC/MS/MS system. Chromatography for LC/MS was carried out with a gradient of acetic acid in acetonitrile on a column packed with Vydac C18 resin.

CoA thioesters were purified by preparative scale reverse-phase HPLC before use. Concentrations of CoA thioesters were determined by quantitation of the CoA released by treatment with 0.5 N NaOH or with 4-hydroxybenzoyl CoA thioesterase. (This enzyme cleaves the thioester bond of 4-chlorobenzoyl CoA, albeit much more slowly than that of 4-hydroxybenzoyl CoA.) $H_2{}^{18}O$ (98.6 atom % ^{18}O) was obtained from Isotec. All other chemicals were purchased from Aldrich.

Enzyme Purification and Assays. 4-Chlorobenzoyl CoA dehalogenase was purified from *Arthrobacter* sp. 4-CB1 as described previously.[30] Enzyme activity was measured by either a continuous assay or a stopped-time assay as described previously.[30]

Multiple-Turnover ^{18}O-Labeling Experiments. Phosphate buffer (25 μL of 100 mM, pH 7.2) and either 4-chlorobenzoyl CoA or 4-hydroxybenzoyl CoA (1.55 μmol) were combined, and the solvent was removed under reduced pressure. The residue was redissolved in

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80 μL of $H_2{}^{18}O$ (98.6% ^{18}O). Purified 4-chlorobenzoyl CoA dehalogenase (0.15−0.9 nmol of homotetramer, 0.6−3.6 nmol of active sites, assuming one active site per monomer) in 20 μL of buffer and 0.2 μL of 1 M dithiothreitol were added. Most reactions were incubated at 30 °C for 1−1.5 h. Reaction mixtures containing added NaCl were incubated at 30 °C for up to 3 h. Partially-purified 4-hydroxybenzoyl CoA thioesterase (0.002 units) was added in order to convert 4-hydroxybenzoyl CoA to 4-hydroxybenzoate. After 1.5 h, the reaction was quenched by the addition of 5 μL of 1 N HCl. The reaction mixtures were extracted five times with ether in order to collect the 4-hydroxybenzoic acid. The ether was removed and the residue redissolved in ethereal diazomethane in order to methylate the carboxylate and alcohol groups. Analysis of the content of ^{18}O was carried out by selected ion monitoring on a Hewlett Packard 5988A GC/MS using a fused silica capillary column (25 m, 0.20 mm i.d.) with a film of cross-linked 5% diphenyl- and 95% dimethylpolysiloxane.

Analysis of Metal Ion Content of 4-Chlorobenzoyl CoA Dehalogenase. The metal ion content of 4-chlorobenzoyl CoA dehalogenase was determined by inductively coupled plasma emission spectroscopy by the Colorado State University Soil, Water and Plant Testing Laboratory. Samples of enzyme were prepared for analysis by extensive dialysis against a solution of triethylammonium bicarbonate buffer (pH 7.0) containing 2 mM dithiothreitol, in order to remove adventitious metals. The buffer was treated with Chelex resin before use. Dithiothreitol and maintenance of a pH greater than 6.5 are necessary to preserve the activity of the enzyme. The enzyme was assayed after dialysis and found to be fully active. Both the enzyme sample and the final dialysate were analyzed for metal content.

Measurement of the Deuterium Kinetic Isotope Effect. Values of k_{cat} for dehalogenation of 4-chlorobenzoyl CoA and 4-chlorobenzoyl-d_4 CoA were determined using the stopped-time assay described previously.[30] Data were analyzed using the Enzfitter program from Sigma.

Determination of the K_i for 4-(Trifluoromethyl)benzoyl CoA. 4-Chlorobenzoyl CoA dehalogenase activity was measured using a range of substrate concentrations from 20 to 100 μM and of inhibitor concentrations from 0 to 250 μM. The stopped-time assay described previously was used.[30] Kinetic data were fitted using Enzfitter (obtained from Sigma) to obtain values for V_{max} and K_M at each inhibitor concentration.

Titration of 4-Chlorobenzoyl CoA Dehalogenase with 4-(Trifluoromethyl)benzoyl CoA. 4-(Trifluoromethyl)benzoyl CoA was gradually added to a solution of 4-chlorobenzoyl CoA dehalogenase (1 mM) in 20 mM MOPS buffer containing 2 mM DTT to give a final concentration of 6.5 mM. The absorbance was monitored between 320 and 600 nm in a Hewlett Packard 8452 diode array spectrophotometer.

Search for a Product Formed from Enzymatic Dehalogenation of 4-Chlorobenzoyl CoA by Hydroxylamine. 4-Chlorobenzoyl CoA dehalogenase was incubated with 4-chlorobenzoyl CoA (200 μM) and hydroxylamine (10 mM) for 5 min at 30 °C. The reaction mixture was flash-frozen to inactivate the enzyme. After being thawed, samples were injected onto a Rainin C18 column. Compounds were eluted with a gradient of acetonitrile in 50 mM sodium acetate (pH 5.2). The detector wavelength was set at 256 nm.

Inactivation of 4-Chlorobenzoyl CoA Dehalogenase by Hydroxylamine. 4-Chlorobenzoyl CoA dehalogenase (12.3 μg) was treated with 200 mM hydroxylamine (added in two aliquots) in 0.1 M potassium phosphate buffer (pH 7.2) in the absence and presence of 150 μM 4-chlorobenzoyl CoA for 30 min at 30 °C. Residual hydroxylamine was removed by gel filtration on a Superdex 200 column using a Pharmacia FPLC system. Enzyme recovered after gel filtration was assayed for activity using the continuous assay described previously.

Determination of the Second-Order Rate Constant for Reaction of 4-Chlorobenzoyl CoA with Hydroxylamine. 4-Chlorobenzoyl CoA (200 μM) was incubated with hydroxylamine (10 mM) in potassium phosphate buffer (100 mM, pH 7.2). At intervals, aliquots were injected on a Rainin C18 column and reaction components separated using a gradient of MeOH in 50 mM sodium acetate buffer (pH 5.2). The pseudo-first-order rate constant for the reaction of 4-chlorobenzoyl CoA and hydroxylamine was determined by following the disappearance of 4-chlorobenzoyl CoA over time.

Single-Turnover ^{18}O-Labeling Experiments. Purified 4-chlorobenzoyl CoA dehalogenase (15 nmol of active sites for experiment 1 and 12 nmol of active sites for experiment 2, assuming four active sites per homotetramer) in 50 mM MOPS buffer (pH 7.2) containing 150 mM KCl and 2 mM DTT was added to $H_2{}^{18}O$ (97.5% enrichment) to give a final ratio of $H_2{}^{18}O/H_2{}^{16}O$ of 2.7 (experiment 1) or 3.9 (experiment 2). (4-Chlorobenzoyl CoA dehalogenase from *Arthrobacter* sp. 4CB-1 does not survive lyophilization, so the enzyme was concentrated and added in a minimal volume to avoid excessive dilution of the $H_2{}^{18}O$. The enzyme was assayed for activity immediately prior to use.) The enzyme was preincubated on ice for 3 h (experiment 1) or 2 h (experiment 2). 4-Chlorobenzoyl CoA (15, 2.5, or 1 nmol in experiment 1 and 0.8 nmol in experiment 2) was added, and the reaction was incubated at 30 °C for 15 min. Partially-purified 4-hydroxybenzoyl CoA thioesterase (0.005 units) was added in order to convert 4-hydroxybenzoyl CoA to 4-hydroxybenzoate. After 20 min, the reaction was quenched by the addition of 1 N HCl to give a final pH of 2. 4-Hydroxybenzoate was collected, methylated, and analyzed as described above.

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