A Surprising Effect of Leaving Group on the Nucleophilic Aromatic Substitution Reaction Catalyzed by 4-Chlorobenzoyl-CoA Dehalogenase

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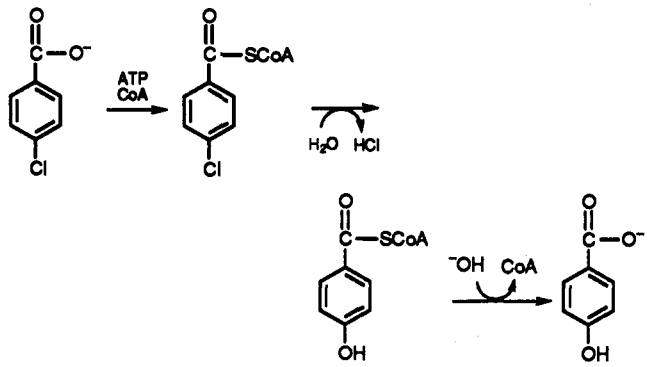
Degradation of 4-chlorobenzoate in several soil microorganisms, including *Acinetobacter* sp. 4-CB1,[1] *Pseudomonas* CBS-3,[2] and coryneform bacterium NTB-1,[3] has recently been shown to proceed by initial formation of the coenzyme A thioester of 4-chlorobenzoate, followed by dehalogenation of 4-chlorobenzoyl-CoA (see Scheme I). The dehalogenation reaction is believed to be a nucleophilic aromatic substitution in which the chloride substituent is replaced by a hydroxyl group derived from H_2O.[4,5]

Enzymic catalysis of a nucleophilic aromatic substitution reaction is intriguing for two reasons. First, nucleophilic aromatic substitution reactions are intrinsically difficult reactions and take place in nonenzymic systems only under special circumstances or very extreme conditions. Second, enzyme-catalyzed nucleophilic aromatic substitution reactions are rare. Besides 4-chlorobenzoyl-CoA dehalogenase, only some isozymes of glutathione-*S*-transferase[6] are known to catalyze a nucleophilic aromatic substitution reaction. We are interested in discovering the mechanism of this unusual enzymic dehalogenation reaction.

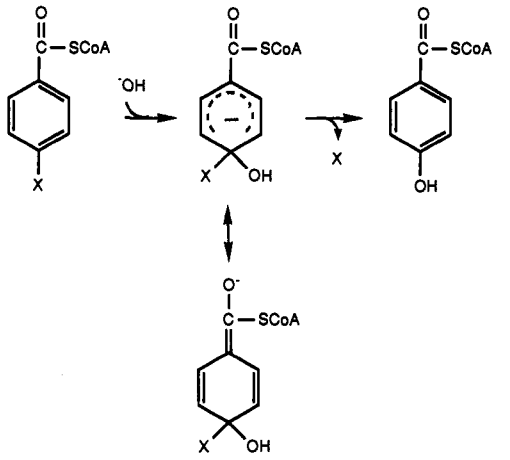
We have begun our mechanistic studies of 4-chlorobenzoyl-CoA dehalogenase with a determination of the relative leaving group abilities for bromide, chloride, and fluoride in the dehalogenation reaction. Dehalogenation of 4-bromobenzoyl-CoA is twice as fast as dehalogenation of 4-chlorobenzoyl-CoA, while dehalogenation of 4-fluorobenzoyl-CoA is over 400-fold slower. These data are in striking contrast to the relative leaving group abilities observed for the glutathione-*S*-transferase-catalyzed dehalogenation of 4-halo-3-nitro-1-(trifluoromethyl)benzenes, for which dehalogenation of the 4-fluoro compound is faster than that of the 4-chloro compound.[6] Our data also differ from those observed for nonenzymic nucleophilic aromatic substitution reactions that occur by the S_NAr mechanism, which has been proposed to be the most likely mechanism for an enzyme-catalyzed nucleophilic aromatic substitution reaction.

4-Halobenzoyl-CoA substrates for kinetic experiments were synthesized from the corresponding benzoyl chlorides. Coenzyme A was dissolved in bicarbonate buffer and stirred on ice. A stoichiometric amount of the halogenated benzoyl chloride in acetonitrile was added. The progress of the reaction was followed using the DTNB assay described by Ellman.[7] When no CoA remained, the solution was neutralized and the solvent was removed by rotary evaporation. The residue was resuspended in water and the 4-halobenzoyl-CoA purified by reverse-phase HPLC.[8] 4-Hydroxybenzoyl-CoA was synthesized as described by Merkel *et al.*[9]

Scheme I



Scheme II



The concentration of 4-hydroxybenzoyl-CoA solutions was determined by measuring the CoA released by treatment with partially purified 4-hydroxybenzoyl-CoA thioesterase. The concentration of 4-halobenzoyl-CoA substrates was determined similarly by measuring the 4-hydroxybenzoyl-CoA formed by treatment with 4-chlorobenzoyl-CoA dehalogenase.[10]

4-Chlorobenzoyl-CoA dehalogenase for use in kinetic experiments was purified from *Acinetobacter* sp. 4-CB-1.[11] Enzyme activity was assayed using a stopped-time assay. Although a continuous spectrophotometric assay is more convenient for some purposes, the stopped-time assay described here is more sensitive and also allows use of higher concentrations of substrates. (Both factors were important in obtaining good data for dehalogenation

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(8) Products are purified by chromatography on a Vydac C-18 column using a gradient of methanol in water. The solvent is removed by rotary evaporation. Purified CoA thioesters are dissolved in water and stored at −20 °C.
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(10) Aliquots of the reaction mixture were analyzed by reverse-phase HPLC at several times during the reaction. Samples were injected onto a C-18 column equilibrated with sodium acetate buffer (50 mM, pH 5.2), and compounds were eluted with a gradient of methanol to a final concentration of 80% developed over 10 min. The decrease in concentration of the 4-hydroxybenzoyl-CoA during the hydrolysis reaction is equivalent to the increase in the concentration of CoA. Therefore, the decrease in the 4-hydroxybenzoyl-CoA peak area corresponding to formation of a known amount of CoA was used to determine a calibration curve for 4-hydroxybenzoyl-CoA. This curve was used to determine the concentration of stock solutions of 4-hydroxybenzoyl-CoA. Quantitation of 4-halobenzoyl-CoA substrates was carried out similarly, except that the decrease in the 4-halobenzoyl-CoA peak area was correlated with formation of a known amount of 4-hydroxybenzoyl-CoA. Using this procedure, it was not necessary to carry the reaction to completion in order to accurately determine the concentration of 4-halobenzoyl-CoA.
(11) Purification and characterization of 4-chlorobenzoyl-CoA dehalogenase will be described in a future publication.

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Table I. Kinetic parameters for dehalogenation of various 4-X-benzoyl CoA substrates

X	K_M (μM)	k_{cat} (s^{-1})	k_{cat} (relative to X = F)
Br	34	2.31	770
Cl	34	1.30	433
F	78	0.003	1.0

of 4-fluorobenzoyl-CoA.) The assay is based upon the absorbance of the phenolate form of the product, 4-hydroxybenzoyl-CoA, that is produced upon dilution of reaction mixtures into basic solutions. The assay is carried out by mixing enzyme and substrate in potassium phosphate buffer (20 mM, pH 7.2) at 30 °C. At intervals, aliquots are taken and added to a solution of NaOH to give a final concentration of 7.5 mM NaOH. The extinction coefficient for 4-hydroxybenzoyl-CoA in 7.5 mM NaOH is 30.1 mM^{-1} cm^{-1} at 330 nm. (Measurements were taken within 20 min to eliminate any contribution from nonenzymatic dehalogenation and thioester hydrolysis to the observed signal.)

Michaelis–Menten parameters were obtained from the kinetic data using the Enzfitter program obtained from Sigma. The K_M and k_{cat} values for the various 4-halobenzoyl-CoA substrates are shown in Table I. Enzymic dehalogenation of 4-bromobenzoyl-CoA is 1.8-fold faster than dehalogenation of 4-chlorobenzoyl-CoA. Dehalogenation of 4-fluorobenzoyl-CoA is 433-fold slower than that of 4-chlorobenzoyl-CoA. If the K_Ms for the three substrates accurately reflect the respective K_Ds, then the similarity of the K_M values suggests that binding to the enzyme is affected little by the nature of the halogen substituent. In contrast, the intrinsic rate of dehalogenation, as measured by k_{cat}, is dramatically affected by the nature of the halogen leaving group.

These data provide important insight into the mechanism of the dehalogenation reaction. The most reasonable mechanism for the enzymic dehalogenation reaction is probably the S_NAr mechanism shown in Scheme II. Nucleophilic aromatic substitution reactions that take place by this mechanism generally proceed fastest when the leaving group is fluoride, even though fluoride is the poorest leaving group among the halogens. The origin of this leaving group effect lies in the relative rates of the two steps of the S_NAr reaction. The rate of the first step, attack of the nucleophile on the aromatic ring, is faster when the substrate contains the more electron-withdrawing fluoro substituent. The rate of the second step, departure of the leaving group, is slower for the fluoro compound, since fluoride is the poorest leaving group among the halogens. Since the first step is almost always rate-determining, the rate of the overall reaction is fastest when the leaving group is fluoride. For example, the rate of substitution of 4-fluoronitrobenzene with methoxide (a reasonable model for the substitution of 4-halobenzoyl-CoA with hydroxide) is 312 times faster at 50 °C than the rate of substitution of 4-chloronitrobenzene.[12] The nucleophilic aromatic substitution reaction catalyzed by glutathione-*S*-transferase shows the same order of leaving group ability as observed for nonenzymic S_NAr reactions. 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.[6]

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The nucleophilic aromatic substitution reaction catalyzed by 4-chlorobenzoyl-CoA dehalogenase shows leaving group abilities in the order *opposite* that expected on the basis of nonenzymic S_NAr reactions and the glutathione-*S*-transferase reaction. Two alternative explanations may account for these data. If the reaction proceeds via the S_NAr mechanism, then the enzyme must be so effective at catalyzing the attack of hydroxide on the aromatic ring that the first step of the S_NAr reaction is no longer rate-limiting. In this case, the rate-limiting step might be either departure of the leaving group or dissociation of either the halide or 4-hydroxybenzoyl-CoA. (4-Hydroxybenzoyl-CoA is a common product formed from all of the 4-halobenzoyl-CoA substrates. It is possible, although perhaps unlikely, that dissociation of 4-hydroxybenzoyl-CoA might be affected by the nature of the halide ion still bound to the enzyme.)

An alternative explanation for the leaving group data is that the dehalogenation reaction may proceed by some other mechanism. Other mechanisms for nucleophilic aromatic substitution that occur in nonenzymic systems include the aryne mechanism,[13] the $S_{RN}1$ mechanism[14] and the $S_{ON}2$ mechanism.[15] 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. Furthermore, the order of leaving group abilities we have observed could be consistent with some of these mechanisms. The rate-limiting step for the $S_{RN}1$ mechanism would likely be the initial reduction of the aromatic ring. This step should be slowest for the fluoro compound, based upon data indicating that the electron affinity of 4-fluoronitrobenzene is less than that of 4-chloronitrobenzene.[16] The rate-limiting step for the $S_{ON}2$ mechanism would undoubtedly be the initial oxidation of the aromatic ring. Since the ionization potential of 4-fluorobenzoic acid is higher than that of 4-bromobenzoic acid,[17] the oxidation of the fluoro compound would be expected to be slower than that of the bromo compound. A prediction of the leaving group abilities for the aryne mechanism is harder to make. Both removal of a proton from an aromatic ring and expulsion of fluoride are kinetically significant steps. If proton removal were rate-limiting, dehalogenation of the fluoro compound would be fastest. If departure of the leaving group were rate-limiting, dehalogenation of the fluoro compound would be slowest.

Our data do not permit a distinction between the various possible mechanisms for the dehalogenation reaction. We are pursuing further mechanistic studies to differentiate among these possibilities.

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