

Determination of the Chlorine Kinetic Isotope Effect on the 4-Chlorobenzoyl-CoA Dehalogenase-Catalyzed Nucleophilic Aromatic Substitution

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The chlorine kinetic isotope effect (KIE) on the dehalogenation of 4-chlorobenzoyl-CoA catalyzed by 4-chlorobenzoyl-CoA dehalogenase has been measured at room temperature and optimal pH. The measured value of $^{37}k = 1.0090 \pm 0.0006$ is larger than the KIEs recently measured for haloalkane and fluoroacetate dehalogenase. This indicates that the transition state for dissociation of chloride ion from the Meisenheimer intermediate is sensitive to the chlorine isotopic substitution. Simple modeling suggests that this sensitivity originates in the high isotopic sensitivity of the C—Cl bond bending modes. © 2002 Elsevier Science (USA)

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Kinetic isotope effects (KIEs)² can provide insight into transition state structure and, in the case of non-concerted reactions, aid in the identification of the rate-limiting transition state (1). In previous studies the Paneth laboratory measured chlorine KIEs on hydrolytic nucleophilic substitution reactions of alkyl chlorides catalyzed by the enzymes haloalkane dehalogenase (2) and fluoroacetate dehalogenase (3). Both enzymes employ an active-site carboxylate to displace the halide from the substrate (4, 5) The arylated enzyme intermediate thus formed is hydrolyzed in a second step. The chlorine KIE measured for the *Xanthobacter autotrophicus* GJ10 haloalkane dehaloge-

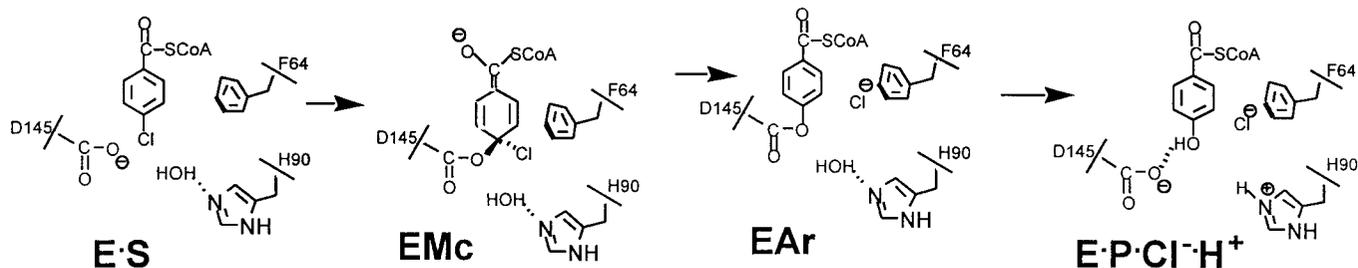
nase-catalyzed dehalogenation of 1,2-dichloroethane, a fast substrate, is 1.0045 ± 0.0045 while that measured for the slow substrate 1-chlorobutane is 1.0066 ± 0.0004 . This latter KIE is close to the calculated intrinsic isotope effect for the C—Cl bond cleavage step. The large chlorine KIE of 1.0082 ± 0.0005 measured for dehalogenation of chloroacetate by *Moraxella* sp. B fluoroacetate dehalogenase suggests a mechanism in which the dehalogenation step is solely rate limiting.

In the present study we have measured the chlorine KIE of the hydrolytic dehalogenation reaction of 4-chlorobenzoyl-coenzyme A (4-CBA-CoA) catalyzed by *Pseudomonas* sp. strain 4-CBA-CoA dehalogenase (6, 7). This enzyme participates in the 4-CBA degradation pathway operational in bacteria adapted to the use of this soil pollutant as an energy source (8). As with the two dehalogenases described above, the 4-CBA-CoA dehalogenase employs covalent catalysis (7, 9). Following substrate binding to the enzyme, nucleophilic displacement of chloride ion by attack of an active-site carboxylate residue (Asp145) on C(4) of the benzoyl ring occurs (see Scheme 1). The Meisenheimer complex formed (henceforth referred to as the EMc intermediate) expels the chloride ion to form an arylated enzyme complex (the EAr intermediate). The Cl⁻ leaving group is not stabilized by the active site and thus there is greater tendency for the EMc once formed to expel the Asp145 carboxylate. The only side chain positioned to interact with the chloride ion is that of Phe64. Hydrolysis of the benzoyl ester in EAr proceeds, assisted by His90, to generate the 4-HBA-CoA product which, along with a proton and the chloride ion, is released from the enzyme active site.

The kinetic mechanism reported (10) for the 4-CBA-CoA-catalyzed dehalogenation of 4-CBA-CoA is shown in Scheme 2. The forward and reverse rate constants

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² Abbreviations used: KIE, kinetic isotope effect; 4-CBA-CoA, 4-chlorobenzoyl-coenzyme A; DFT, density functional theory.



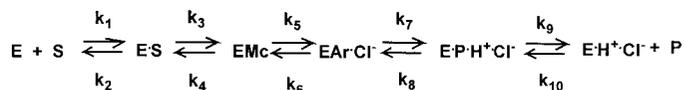
SCHEME 1

for formation of the enzyme-substrate complex ($E \cdot S$) are $k_1 = 7 \mu\text{M}^{-1} \text{s}^{-1}$, $k_2 = 30 \text{s}^{-1}$; of the enzyme-Meisenheimer complex (EMc), $k_3 = 200 \text{s}^{-1}$, $k_4 = 2000 \text{s}^{-1}$; of the arylated enzyme intermediate ($EAr \cdot Cl^-$), $k_5 = 40 \text{s}^{-1}$, $k_6 = 2 \text{s}^{-1}$; of the enzyme-product complex ($E \cdot P \cdot H^+ \cdot Cl^-$), $k_7 = 20 \text{s}^{-1}$, $k_8 = 0.25 \text{s}^{-1}$; and of enzyme plus product ($E \cdot H^+ \cdot Cl^- + P$), $k_9 = 1.5 \text{s}^{-1}$, $k_{10} = 8 \mu\text{M}^{-1} \text{s}^{-1}$. The reversal of this reaction requires H^+ (pH 7.5) and Cl^- binding ($K_d > 10 \text{mM}$) to free enzyme (E) which, under the reaction conditions, is thermodynamically unfavorable.

RESULTS AND DISCUSSION

The chlorine KIE measured for 4-CBA-CoA dehalogenase is $^{37}k = 1.0090 \pm 0.0006$ (Table I). This is the largest of the chlorine KIEs observed among the three dehalogenases studied thus far. We have modeled the dehalogenation step at the AM1 semiempirical level for all three reactions catalyzed by the dehalogenases using formate anion as the nucleophile in reaction with the appropriate chlorinated electrophile. The theoretical KIEs are listed in Table II together with the frequencies sensitive to isotopic substitution and the C—Cl bond lengths of the reactant. As can be seen, there is very good correlation between the calculated chlorine KIEs and the experimental values. The length of the C—Cl bond in the Meisenheimer complex model is the longest among the three reactant models, while in the transition-state model it is the shortest. Thus, the extent of bond breaking is not the major determinant of the magnitude of the chlorine KIE. Rather, the determinants are the strong, isotope-sensitive bending frequencies.

The chlorine KIE (^{37}k) can be expressed in terms of the microscopic rate constants shown in Scheme 2:



SCHEME 2

We have estimated (see Materials and Methods) that the equilibrium chlorine isotope effect on the dehalogenation step ($^{37}k_5/^{37}k_6$) is close to unity. By introducing the numerical values for rate constants and the equilibrium isotope effect, Eq. [1] can be rearranged:

$$^{37}k = \frac{{}^{37}k_5 + (k_5/k_4) (1 + k_3/k_2) + ({}^{37}k_5/{}^{37}k_6) [k_6/k_7 (1 + k_8/k_9)]}{1 + (k_5/k_4) (1 + k_3/k_2) + (k_6/k_7) (1 + k_8/k_9)} \quad [1]$$

$$1.0090 = {}^{37}k = \frac{{}^{37}k_5 + 0.25}{1 + 0.25} \quad [2]$$

From Eq. [2], the intrinsic chlorine KIE on the dehalogenation step can be calculated to be about $^{37}k_5 = 1.0125$. This is among the largest of the chlorine KIEs observed thus far, which are in the range 1.0056–1.0123 for the S_N2 reaction on aliphatic chloride. In fact, it matches the largest value reported in literature for the chlorine KIE (11). Moreover, it is the first chlorine KIE to be reported for an aromatic nucleophilic substitution reaction. As chlorine KIEs on aromatic nucleophilic substitution are not available, comparisons to nonenzyme-catalyzed reactions cannot be made. It should be noted that the assumption has been made that the preceding step, formation of the EMc , does not exhibit any sizable isotope effect. We have calculated the equilibrium isotope effect for this step, which can serve as an estimate of the maximum chlorine KIE, to be equal to 0.9992, justifying this assumption.

MATERIALS AND METHODS

4-CBA-CoA dehalogenase from *Pseudomonas* sp. CBS-3 was prepared as previously described (7). 4-CBA-CoA was synthesized from 4-chlorobenzoyl chloride and coenzyme A purchased from Sigma as described earlier (12). Mercuric (II) thiocyanate (Sigma), nitric acid, silver nitride (POCH, Poland), 1,3-bis[tris-(hydroxymethyl)methylamino]propane (bis-tris-propane) (CalBiochem, U.S.A.), and ferric ammonium sulfate (Technabexport, Soviet Union) were used without further purification.

TABLE I
Chlorine KIEs for Enzymatic Dehalogenation Reactions

Enzyme	Experimental	Theoretical	Isotope-sensitive frequencies (cm ⁻¹)	$r_{\text{C-Cl}}$ (Å)
Haloalkane dehalogenase ^b	1.0066 ± 0.0004	1.0074	735.5 (−3.7) ^a 331.7 (−3.7)	1.820
Fluoroacetate dehalogenase ^c	1.0082 ± 0.0005	1.0084	632.0 (−1.3) 384.8 (−3.5) 184.1 (−1.9)	1.867
4-Chlorobenzoyl-CoA dehalogenase	1.0090 ± 0.0002	1.0102	714.1 (−1.6) 417.1 (−6.6) 299.1 (−2.3)	2.036

^a Isotopic shift upon change from ³⁵Cl to ³⁷Cl. ^b Data taken from (2). ^c Data taken from (3).

Isotope effect measurements. 4-CBA-CoA [0.125 g (0.151 mmol); final concentration 15.1 mM] was dissolved in 10 ml of 50 mM chloride ion-free bis-tris-propane buffer. The pH of this solution was adjusted to 7.5 with nitric acid and thermostated at 24°C. The reaction was started by the addition of 0.5 ml of buffered 4-CBA-CoA dehalogenase (SA = 0.2 μmol per minute per milligram; 2.3 mg/ml). The progress of the reaction was monitored using a standard colorimetric assay (13) of chloride ions at 460 nm. At the desired conversion, the reaction was terminated by adjusting the solution pH to 1 with nitric acid. Chloride ions were then precipitated with silver nitrate. The precipitate was centrifuged for 3 min at 5000 rpm, washed with 2 ml of water, and centrifuged again. The washing procedure was repeated three times. The AgCl was left to dry over P₂O₅ in a vacuum desiccator in the dark. AgCl for the full conversion samples was obtained by chemical conversion. 4-Chlorobenzoyl chloride (150 mg), which was used in the synthesis of CBA-CoA, was hydrolyzed with 20 ml of 4 M NaOH at 50°C for 8 h. The progress of the reaction was monitored using ¹H NMR. The mixture was then acidified to pH 1 with nitric acid. The product, 4-chlorobenzoic acid, was extracted with 20 ml of chloroform and analyzed for chlorine content by using the colorimetric assay. Samples of 10 mg of the product were placed in combustion tubes and burned at 1050°C in the oxygen flux (30 ml/min). Gases were absorbed in a quartz tube filled with 20 ml of 6% hydrogen peroxide solution and then eluted by 70 ml of 95% ethanol. AgCl was precipitated with silver nitrate from the solution acidified to pH 1 with HNO₃ and purified by using the procedure described above.

The isotopic ratios ³⁷Cl/³⁵Cl were measured by the FAB-IRMS technique developed in our laboratory (14) and the isotope effect was calculated from the isotopic ratios of the product:

$${}^3k = k_{35}/k_{37} = \frac{1n(1-f)}{1n(1-fR_f/R_\infty)}, \quad [3]$$

TABLE II

Chlorine KIE Obtained for 4-Chlorobenzoyl-CoA Dehalogenase at 24°C

f	$R = [{}^{37}\text{Cl}/{}^{35}\text{Cl}]$	KIE
1	0.32507 ± 0.00013	—
0.286	0.32257 ± 0.00018	1.0092 ± 0.0007
0.226	0.32260 ± 0.00014	1.0087 ± 0.0006

where k_{35}/k_{37} is chlorine KIE, f is the fraction of the reaction, and R are isotopic ratios of the product after fraction of reaction f (R_f) and after full conversion (R_∞). The first equation introduces the Northrop notation of isotope effects (15), which uses a leading superscript to indicate that the symbol corresponds to an isotope effect rather than a rate constant. The leading superscripts correspond to the heavy isotope used. This notation allows using the following subscript to identify elementary reactions in the complex mechanism. Each measurement was repeated five times.

Calculations. Density functional theory (DFT) and semiempirical calculations were performed using the Gaussian98 (16) program. Chlorine KIEs were calculated using the AM1 (17) Hamiltonian using formate ion as the model for the carboxylic moiety acting as the nucleophile on chloroacetate anion and 1-chlorobutane in S_N2 reactions. An adduct of chlorobenzene and formate was used as the model of the reactant of the dehalogenation step of the 4-chlorobenzoyl-CoA dehalogenase-catalyzed reaction. In all cases transition states were identified by one imaginary frequency, corresponding to a transition from reactants to products (both having only real harmonic vibrational frequencies). Intrinsic chlorine KIEs were calculated using the ISOEFF98 program (18):

$$\frac{k_L}{k_H} = \frac{v_L^\ddagger}{v_H^\ddagger} \times \prod_i^{3n^p-6} \frac{u_{iL}^R \cdot \sinh(u_{iH}^R/2)}{u_{iH}^R \cdot \sinh(u_{iL}^R/2)} \times \prod_i^{3n^t-7} \frac{u_{iH}^\ddagger \cdot \sinh(u_{iL}^\ddagger/2)}{u_{iL}^\ddagger \cdot \sinh(u_{iH}^\ddagger/2)}, \quad [4]$$

where n is the number of atoms, $u = hv/kT$ (where h and k are Planck and Boltzmann constants, respectively, T is temperature), and v_i are the frequencies of normal modes of vibrations. Superscripts “ \ddagger ” indicate the properties of the transition state. The chlorine equilibrium isotope effect on the dehalogenation step was calculated using chlorine ion hydrogen bonded to nitrogen of an imidazole ring and Eq. [3] modified in the following ways: (a) \neq denoting the properties of the product, (b) removing the ratio $v_{35}^\ddagger/v_{37}^\ddagger$, and (c) extending multiplication to $3n - 6$ degrees of vibrational freedom.

Vibrational analysis, within the harmonic approximation, was performed for 1,2-dichloroethane, chlorophenyl, and formate using the DFT level. Becke's one-parameter exchange function (19) was used together with Lee *et al.*'s (20) exchange function and 6-31G(d) basis set (21) because B1LYP function has been developed to provide a good description of vibrations.

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