Chlorine Kinetic Isotope Effect on the Fluoroacetate Dehalogenase Reaction

Andrzej Lewandowicz,[†] Daria Sicinska,[†] Juliusz Rudzinski,[†] Susumu Ichiyama,[‡] Tatsuo Kurihara,[‡] Nobuyoshi Esaki,[‡] and Piotr Paneth*,[†]

Institute of Applied Radiation Chemistry
Technical University of Lodz, Zeromskiego 116
90-924 Lodz, Poland
Laboratory of Microbial Biochemistry
Institute for Chemical Research, Kyoto University
Uji, Kyoto 611-0011, Japan

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Dehalogenases have attracted much attention recently,[1] because of their potential use in the synthesis of various pharmaceuticals and agrochemicals and in bioremediation of halocompound-polluted environment. In fact, fluoroacetate dehalogenase has been applied to the detoxification of poisonous plants containing high concentrations of fluoroacetate ingested by domestic animals.[2] This enzyme catalyzes the hydrolytic dehalogenation of haloacetates to produce glycolate and a halide ion, with the highest activity toward fluoroacetate. This is unusual because the dissociation energy of the C−F bond of aliphatic fluorocompounds is among the highest found in bonds of natural products. Nevertheless, the activity of this enzyme toward fluoroacetate is about 5 times higher than toward chloroacetate.

Although the three-dimensional structure of this enzyme has not been solved yet, it is known that the amino acid sequence is similar to that of haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10, especially in the regions around Asp105 and His272, which correspond to the active site nucleophile Asp124 and the base catalyst, His289, of the haloalkane dehalogenase, respectively. Studies of the mechanism of fluoroacetate dehalogenase indicate that it proceeds through the nucleophilic attack of the carboxyl moiety of Asp105 on the α-carbon of the substrate, leading to the formation of an ester intermediate, which is subsequently hydrolyzed to glycolate. Enzyme activity exhibits a broad *plateau* in the pH range 7.5−10 as illustrated in Figure 1.

We have recently reported[3] chlorine kinetic isotope effects (KIEs) on the haloalkane dehalogenase reaction. From the difference in chlorine KIEs for "natural" (1,2-dichloroethane) and "slow" (1-chlorobutane) substrates, we were able to learn new details about the catalytic steps.

We have taken advantage of the fact that when a KIE on an enzymatic reaction is measured by the competitive method it provides information about catalytic events through the first irreversible step. This frequently allows learning details of the steps not otherwise amenable to direct analysis. We have reported earlier chlorine KIEs for haloalkane dehalogenase, which proceeds through a similar mechanism. We have found values of 1.0045 for dichloroethane and 1.0066 for chlorobutane. The substantially larger value of the chlorine KIE for the slow substrate, chlorobutane, indicates that in the case of dichloroethane an isotopically insensitive step is partly rate-determining. From the analysis of the kinetic complexity, we have suggested that this is the result

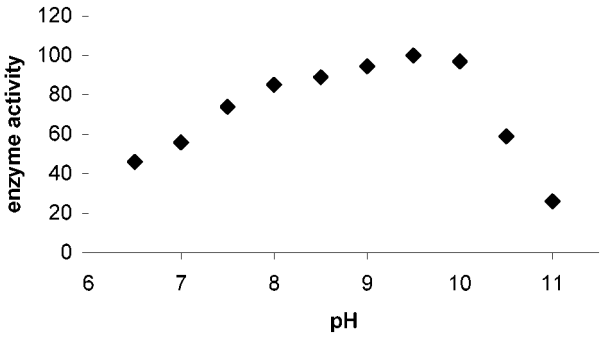

Figure 1. Fluoroacetate dehalogenase activity as a function of pH.

Table 1. Chlorine KIEs on Enzyme-Catalyzed and Chemical Dehalogenation of Chloroacetate at 34 °C

f[a]	enzymatic	chemical
0.04	1.0086	
0.06		1.0084
0.06		1.0080
0.12		1.0077
0.12		1.0073
0.12		1.0082
0.13		1.0076
0.29	1.0085	
0.30	1.0082	
0.44	1.0076	
average	1.0082 ± 0.0005	1.0079 ± 0.0004

[a] Fraction of reaction.

of the partial reversibility of the dehalogenation step. We have now applied the same probe to the mechanism of the reaction catalyzed by fluoroacetate dehalogenase. Herein, we report results of studies of the chlorine KIE on the fluoroacetate dehalogenase reaction using chloroacetate as the substrate.

We have found (Table 1) the chlorine KIE for this reaction to be 1.0082 ± 0.0005, much larger than either of the isotope effects measured for haloalkane dehalogenase.

Because the crystal structure of the fluoroacetate dehalogenase is not available, we were unable to model the elementary S_N2 step for this reaction with inclusion of the amino acids of the active site, as we did in the case of haloalkane dehalogenase. We have shown previously that density functional theory calculations with gas-phase models lead to the overestimation of chlorine KIEs.[3] We have, therefore, performed simple calculations in which formate anion (used as the model of the carboxylic moiety of Asp105) acts as the nucleophile on the α-carbon of chloroacetate ion. We have used the AM1 semiempirical Hamiltonian,[4] following our previous finding that it yields chlorine KIEs in good agreement with experimental values (Paneth, P.; Westaway, K.; Matsson, O., in preparation) The calculated value of the chlorine KIE was found to be 1.0084 in excellent agreement with the measured value. In addition, we have measured chlorine KIE on chemical dehalogenation of chloroacetate (Table 1) which can serve as a relevant chemical model of the S_N2 chlorine displacement on chloroacetate. At pH = 11 the reaction proceeds by direct nucleophilic attack by hydroxide. The measured chlorine KIE is 1.0079 ± 0.0004, again in very good agreement with the value found for the enzymatic reaction. Both isotope effects on enzymatic and chemical reactions reported here are the first examples of chlorine KIEs on haloacids. It is thus worth noting that these are somewhat larger than the corresponding values observed for the S_N2 reactions of chloroalkanes, indicating that the transition states in the present cases are more product-like.

[†] Technical University of Lodz.
[‡] Kyoto University.

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The large chlorine KIE suggests that in the case of fluoroacetate dehalogenase reaction the experimentally observed value corresponds to the intrinsic value for the dehalogenation step. This is further supported by the agreement of the experimental values of the chlorine KIE with the one predicted theoretically for the S_N2 step and with the experimental chlorine KIE on the chemical dehalogenation at high pH. Alternatively, one might expect that the equilibrium chlorine isotope effect might be of considerable size. However, extensive experimental studies (Esaki et al., unpublished) exclude reversibility of this reaction. Furthermore, the preliminary value of the k_{18}/k_{19} KIE for the reaction with flouroacetate is equal to 1.0142 (Matsson et al., unpublished). Comparison of this value to the theoretical maximum expected for the C−F bond breaking[5] indicates that the commitment for catalysis must be smaller than one. Since the reaction of chloroacetate is 4.8 times slower,[1] the commitment for this reaction will decrease considerably. All of the above arguments indicate that, unlike the case of the haloalkane dehalogenase, the mechanism of the fluoroacetate dehalogenase proceeds through the irreversible dehalogenation with no preceding isotope-insensitive step being partly rate-determining. It will be interesting to model this step once the active site structure becomes available.

Materials. Fluoroacetate dehalogenase from *Moraxella* sp. B was prepared as described earlier.[6] Chloroacetic acid (Sigma, Germany) was used as the substrate. Mercuric (II) thiocyanate (Sigma), nitric acid, silver nitride (POCh, Poland), ferric ammonium sulfate (Technabexport, Soviet Union), and 1,3-bis[tris-(hydroxymethyl)methylamino]propane (BTP, bis-tris-propane) (CalBiochem, U.S.A.) were used without further purification.

Isotope Effect Measurements. In the case of low conversion samples, 0.0889 g (0.941 mmol) of chloroacetic acid (final concentration 18.63 mM) was dissolved in 50 mL of 50 mM chloride-ion-free bis-tris-propane buffer, the pH was adjusted to 7.7 with nitric acid, and the mixture was thermostated at 34 °C. The reaction was started by the addition of 0.5 mL of fluoroacetate dehalogenase (final concentration 203.7 units/L) prepared from lyophilized 10.29 units of the enzyme. Reaction progress was monitored by using a fixed-point colorimetric assay[7] of chloride at 460 nm. At the desired conversion, the reaction was stopped by acidification to pH = 11 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, and then AgCl was left to dry over P_2O_5 in a vacuum desiccator in the dark.

AgCl for full conversion was obtained by hydrolysis of 0.1 g of chloroacetic acid in 30 mL of 0.1 mM NaOH at 50 °C for 5 h, followed by acidification to pH = 1 with nitric acid, precipitation with $AgNO_3$, centrifugation, and washing as described above for low-conversion samples.

Isotopic ratios of ^{37}Cl to ^{35}Cl (R) were measured by the FAB-IRMS technique developed in our laboratory,[8,9] and the isotope effect was calculated from isotopic ratios of the product after a small fraction of the reaction, f, and after full conversion, from the equation:[10]

$$\frac{k_{35}}{k_{37}} = \frac{\ln(1-f)}{\ln(1-fR_f/R_\infty)} \qquad (1)$$

where k_{35}/k_{37} is chlorine kinetic isotope effect, f is the fraction of reaction, and R_i are isotopic ratios of the product after the fraction of reaction f (R_f), and after full conversion (R_∞). Each isotope ratio was calculated from five individual measurements. Resulting chlorine KIEs are given in Table 1.

Calculations. Semiempirical AM1 calculations were performed using the Gaussian98[11] program. The transition state was identified by one imaginary frequency, corresponding to transition from reactants to products. Intrinsic chlorine KIE was calculated using the ISOEFF98 program[12] from the complete equation.[10]

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