Chlorine Kinetic Isotope Effects on Enzymatic Dehalogenations

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ABSTRACT
Enzymatic dehalogenation reactions are important for the bioremediation of the environment because of the increasing anthropogenic pollution with halogen-containing organic compounds. Chlorine kinetic isotope effects have been measured for four hydrolytic dehalogenases. On the basis of these isotope effects, several details of the mechanisms of the enzymatic dehalogenation reactions have been revealed.

Introduction

Role of Dehalogenation Processes in the Environment. Chlorine-containing organic compounds constitute the largest group among the pollutants due to their use as herbicides, pesticides, plant growth regulators, refrigerants, and solvents.[1−3] They pose a hazard due to their carcinogenic[2] and poisonous[4] properties. Because of intentional introduction to the environment, as well as contamination due to spills, accidents, and improper disposal, they are now frequently detected also in groundwater.[5] It may soon be necessary to augment their natural biodegradation pathways, and therefore it is of high importance to learn the details of the processes that can be used for such purposes.

Many microorganisms produce a diverse range of enzymes that degrade halogenated organic compounds by the removal of the halogen substituent from the molecule. The process is called "dehalogenation", and the corresponding enzymes are called "dehalogenases". Enzymatic dehalogenation[3,6] provides a way to dispose of halogenated pollutants from the environment. We have studied four dehalogenases that catalyze hydrolysis of various halogenated compounds by means of chlorine kinetic isotope effects. These include haloalkane dehalogenase,[7] fluoroacetate dehalogenase,[8] 4-chlorobenzoyl-CoA dehalogenase,[9] and DL-haloacid dehalogenase.[10] While the mechanistic details of these reactions, vary they can be collectively described as follows:

$$E(H_2O + RCl \rightarrow H^+ + ROH + Cl^-) \qquad (1)$$

The above equation signifies that the overall reaction catalyzed by these hydrolytic dehalogenases involves one water molecule and converts a chloroorganic molecule into the corresponding alcohol, releasing chloride anions.

Kinetic Isotope Effects on Enzymatic Reactions. Two recent Accounts[11,12] in this journal provide an excellent introduction to the basics of isotope effects and their terminology. Thus, we concentrate here only on those aspects that apply to the studies presented herein. Kinetic isotope effects (KIEs) can provide information of two different types about the mechanism of a reaction. If the reaction is simple, e.g., there is only one transition state on the path from the reactants to products, then KIEs provide information about bonding changes that occur during the transition from the reactants to the transition state. This frequently allows identifying the reaction mechanism. Enzymatic reactions are complex, and therefore the analysis of their KIEs is not so straightforward. In the simplest case, an enzymatic reaction can be described by the following scheme:

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ES \xrightarrow{k_c} E + P \qquad (2)$$

where E, S, P, and ES represent enzyme, reactant, product, and Michaelis complex, respectively, and k_i are rate constants for the reactant binding to the enzyme (i = on), dissociation from the enzyme (i = off), and chemical conversion to the product (i = c). Even in this least complicated case, there are three individual rate constants that can introduce isotopic fractionation. Usually, it can be assumed that binding processes do not introduce sizable isotopic fractionation.[13] If this is true, then the kinetic isotope effect that is observed experimentally (KIE_{exp}) is related to the kinetic isotope effect on the rate constant k_c (KIE_{int}) by the equation

$$KIE_{obs} = \frac{KIE_{int} + k_c/k_{off}}{1 + k_c/k_{off}} \qquad (3)$$

KIE_{int} is called the "intrinsic kinetic isotope effect", and it can be used for the mechanistic interpretation of the chemical events in the active site of the enzyme. The ratio k_c/k_{off} is called the "commitment toward catalysis" (or the partitioning factor).[14] Varying experimental conditions can sometimes lead to the direct experimental determination of KIE_{int}.[15] It can also be modeled theoretically.[16] When the intrinsic value is known, it is possible to evaluate the commitment from the values of the intrinsic and observed KIEs. Thus, the complication introduced by the complex mechanism of the enzymatic processes can, in favorable cases, be used to advantage to produce additional information about the mechanism of the reaction studied. In the above discussion, the assumption has been made that the events in the active site after binding can be described

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by a single rate constant. This is seldom true, and further "internal commitments" need to be frequently considered (see, for example, eq 8 below).

Chlorine KIEs have been studied extensively for many chemical reactions. A comprehensive review of the leaving-group chlorine KIEs has been given by Shiner and Wilgis.[17] However, only a few reports of their application in the studies of enzymatic reactions can be found in the literature. Most frequently, chlorine KIEs were studied in the substitution reactions, with the assumption that came from early modeling studies that formally different S_N1 and S_N2 mechanisms could be distinguished on their basis. The data collected in Table 10 of ref 17 indicates that this assumption is not reflected in the experimental results. The chlorine KIEs for both mechanisms span the whole range of the experimentally determined isotope effects. This indicated that the interpretation of the mechanism based solely on the magnitude of chlorine KIE may be impaired.

Measurements of the Chlorine KIEs. Chlorine KIEs are usually smaller than 1.01, and therefore their determination requires special care. High-precision measurements were traditionally performed using isotope ratio mass spectrometers that required CH_3Cl for the analysis. Today, sufficient precision can also be obtained with other substances.[18] In our laboratory, we have developed a procedure that uses negative-ion FAB mass spectrometry of AgCl.[19,20] This is a very convenient technique, as it allows us to precipitate chloride ions directly from the reaction solution and use it with minimal further handling for isotopic analysis. Experimental KIEs can be determined on the basis of isotopic ratios R (defined as the ratio of concentrations of heavy to light isotopomer) at different fractions of reactions, f. When the isotopic composition of the product is used, then the appropriate equation is given by[21]

$$\mathrm{KIE}_{\mathrm{exp}} = \log(1 - f)/\log(1 - fR_f/R_\infty) \qquad (4)$$

where the subscript ∞ corresponds to the isotopic composition of the product after the completion of the reaction. From the mass balance, it ia s found that R_∞ is equal to R_0, the isotopic composition of the reactant before the reaction is started. If the reaction progress is restricted to a very small fraction of conversion, then eq 4 can be further simplified to

$$\mathrm{KIE}_{\mathrm{exp}} \approx R_\infty/R_f = R_0/R_f \qquad (5)$$

This relation holds to about 10−15% of the conversion. It signifies that the isotopic composition differences between the reactant before the reaction and the product are the largest and are nearly independent of the reaction progress in the initial stages of the reaction.

The chlorine KIEs discussed herein were measured by the competitive method, e.g., both isotopomers being simultaneously present in the solution, which in the case of enzymatic reactions means that the observed values reflect all the events through the first irreversible step.[14]

In this Account, we show how chlorine kinetic isotope effects allowed us to learn the details of the mechanisms of a few hydrolytic dehalogenases.

Fluoroacetate Dehalogenase

This enzyme catalyzes the hydrolytic dehalogenation of haloacetates to produce glycolate and a halide ion. The activity of this enzyme toward fluoroacetate is about 5 times higher than that toward chloroacetate, although the dissociation energy of the C−F bond of aliphatic fluorocompounds is among the highest found in bonds of natural products. Fluoroacetate dehalogenase is an example of a successful application of an enzymatic process in practice; it has been applied to the detoxification of poisonous plants containing high concentrations of fluoroacetate that are ingested by domestic animals.[4] The three-dimensional structure of this enzyme is not yet known, but studies[22] indicate that it proceeds through the nucleophilic attack of the carboxyl moiety of Asp105 on the α-carbon of the reactant, leading to the formation of an ester intermediate, which is subsequently hydrolyzed to produce glycolate:

$$\mathrm{E}_{\mathrm{H_2O}} + \mathrm{RCl} \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftharpoons}} \mathrm{E}^{\mathrm{RCl}}_{\mathrm{H_2O}} \underset{k_{\mathrm{hal}}}{\overset{k_{\mathrm{deh}}}{\rightleftharpoons}} \mathrm{E}^{\mathrm{R}}_{\mathrm{H_2O}} \cdot \mathrm{Cl}^- \overset{k_{\mathrm{hyd}}}{\rightleftharpoons} \mathrm{E}^{\mathrm{ROH}}_{\mathrm{H_+}} \cdot \mathrm{Cl}^- \rightarrow \mathrm{E}_{\mathrm{H^+}} + \mathrm{ROH} + \mathrm{Cl}^- \qquad (6)$$

with $R = CH_2COO^-$ in the present case.

The experimental chlorine KIE for the dehalogenation of chloroacetate is equal to 1.0082. Recent mass spectrometric studies[23] of the dehalogenation of fluoroacetate catalyzed by fluoroacetate dehalogenase indicate that the rate-determining step is the hydrolysis of the ester intermediate. The 5-fold difference in the overall rate for fluoro and chloro compounds can be explained in several ways. Two explanations seem to be most likely. First, there may be a shift in the rate-determining step; the dehalogenation step may become more (or even fully) rate-determining in the case of chloroacetate. One of the possible reasons for such a scenario is that the transition state for the fluoroacetate is stabilized, e.g., by a hydrogen bond to a fluorine atom, and such a stabilization is not present in the case of much larger chlorine atoms. This explanation suggests that at least a part of the active site is rather tight. Second, the rate of hydrolysis may be slower in the case of chloroacetate but still fully rate-controlling, with binding being kinetically silent. This is less probable, because this step is common for both halogenated compounds. We cannot address the question of which of these two scenarios occurs. Our results are not sensitive to these mechanistic nuances, since the experimentally observed $\mathrm{KIE}_{\mathrm{exp}}$ should be independent of the relative rates of individual steps. Consider a two-step reaction (a middle fragment of eq 6) consisting of the reversible dehalogenation step, leading to an ester intermediate and its subsequent hydrolysis:

$$\mathrm{E}^{\mathrm{RCl}}_{\mathrm{H_2O}} \underset{k_{\mathrm{hal}}}{\overset{k_{\mathrm{deh}}}{\rightleftharpoons}} \mathrm{E}^{\mathrm{R}}_{\mathrm{H_2O}} \cdot \mathrm{Cl}^- \xrightarrow{k_{\mathrm{htd}}} \qquad (7)$$

Derivation of the equation for the apparent kinetic isotope effect (KIE_{app}) for this reaction leads to an expression similar to eq 2:

$$KIE_{app} = KIE_{deh}\frac{KIE_{hyd}/KIE_{hal} + k_{hyd}/k_{hal}}{1 + k_{hyd}/k_{hal}} \qquad (8)$$

where subscripts deh, hal, and hyd correspond to elemental reactions of dehalogenation, halogenation (reverse of dehalogenation), and hydrolysis, respectively. The hydrolysis does not include the isotopic atom. In the halogenation step, the chloride ion acts as the incoming group. There are two factors influencing the magnitude of isotope effects. One is called the temperature-independent factor, and it always favors the lighter isotopomer. The second is connected with the change of bonding around the isotopic atom, and when new bonds are formed, the heavier species is favored. In the case of atoms of incoming groups, these two factors tend to cancel. Thus, it is reasonable to assume that both KIE_{hyd} and KIE_{hal} are close to unity, and therefore KIE_{app} is equal to KIE_{deh}, regardless of the commitment k_{hyd}/k_{hal}.

Theoretical calculations suggest that the experimental value of the chlorine KIE is close to the intrinsic kinetic isotope effect. To further test this possibility, we have measured chlorine KIE on the chemical dehalogenation of chloroacetate by direct nucleophilic attack of the hydroxy anion. The measured chlorine KIE is about 1.008, supporting the conclusion that the observed KIE in the case of fluoroacetate dehalogenase corresponds to the intrinsic KIE of the dehalogenation step. This implies that there are no steps preceding the dehalogenation step that contribute to the overall reaction rate.

Haloalkane Dehalogenase

Haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 catalyzes hydrolysis of the C−Cl bond of a carcinogenic pollutant, 1,2-dichloroethane. Many aspects of the mechanism of this enzyme have been thoroughly studied both experimentally[24−29] and theoretically.[30−34] It is assumed that eq 6 describes the reaction mechanism (R = CH_2Cl); Asp124 acts as a nucleophile, and released chloride ion is hydrogen-bonded to two tryptophan residues (Trp125 and Trp175). The release of the chlorine ion from the enzyme determines the overall reaction rate.[27]

There is no subsequent substitution of the second chlorine atom, while the reaction with dibromoethane proceeds with hydrolysis of both C−Br bonds, leading to glycol as the final product. It is thus impossible to measure the bromide kinetic isotope effect.[35] Chlorine kinetic isotope effects have been found to be equal to 1.0045 for 1,2-dichloroethane and 1.0066 for 1-chlorobutane, the slow substrate. The smaller value of the chlorine KIE for the natural substrate is usually explained by assuming changes in the magnitude of the commitment factor. It was postulated that the dehalogenation step is irreversible. Since the k_{cat}/K_M for 1-chlorobutane is 2 orders of magnitude smaller than that for 1,2-dichloroethane, one can expect that the commitment is very close to zero, and thus the isotope effect measured for 1-chlorobutane should be very close to the intrinsic value.

Table 1. Theoretical Chlorine Kinetic Isotope Effects on the Dehalogenation Step and Model Reactions Calculated at the B1LYP/6-31G*:PM3 Level

Trp models[a]	1-chlorobutane	1,2-dichloroethane
NH_3	1.0063	1.0065
pyrrole rings	1.0061	1.0063
indole rings	1.0065	nd[b]

[a] Part of the tryptophans included in the high level of the QM/QM calculations. [b] Not determined.

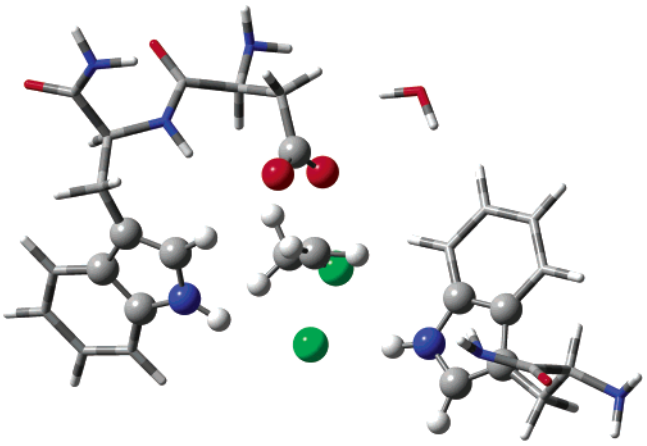


FIGURE 1. Model of the active site with the transition state for the dehalogenation step. Atoms included in the DFT layer are rendered as spheres; atoms included in the semiempirical layer are rendered as tubes.

Unlike the fluoroacetate dehalogenase, in the case of haloalkane dehalogenase the X-ray structure of the enzyme is available. It is thus possible to model the reaction in the active site of the enzyme. We have used the ONIOM method[36] as implemented in the Gaussian package.[37] The model was divided into two layers: the higher layer, which included the reactant, aspartate, truncated to formic acid and tryptophan residues truncated to ammonia, was treated at the B1LYP level,[38] while the lower layer, which included the rest of these amino acids and a water molecule, was calculated at the PM3 level.[39] Five carbon link atoms were treated as hydrogens in the higher-level calculation. This model is labeled as NH_3 in Table 1. For comparison, we have also calculated isotope effects for models in which pyrrole (Figure 1) or indole rings were used as models for tryptophans. The B1LYP method was chosen because it was optimized to yield good-quality vibrational frequencies. The isotope effects were calculated from isotopic frequencies of reactants and transition states using the ISOEFF98 program[40] from the complete equation:[21]

$$KIE_{int} = \frac{\nu^{\ddagger}_{35}}{\nu^{\ddagger}_{37}}\prod_{i}^{3n-6}\frac{u_{i35}\sinh(u_{i37}/2)}{u_{i37}\sinh(u_{i35}/2)}\prod_{i}^{3n-7}\frac{u^{\ddagger}_{i37}\sinh(u^{\ddagger}_{i35}/2)}{u^{\ddagger}_{i35}\sinh(u^{\ddagger}_{i37}/2)} \qquad (9)$$

where n is the number of atoms, $u = h\nu/kT$ (where h and k are Planck and Boltzmann constants, respectively), T is

the absolute temperature, and ν_i are the frequencies of normal modes of vibrations. Superscript "‡" indicates the properties of the transition state.

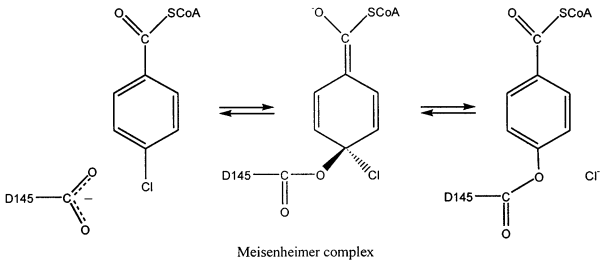
The major way by which enzymes achieve their catalytic efficiency is to stabilize the transition state by developing an active site complementary to it. Thus, the information that is sought in the rational drug design is the structure of the transition state. Our modeling allows us to learn such details of the dehalogenation step; the breaking C−Cl bond is elongated by about 0.5 Å compared to the length of this bond in the reactant, and the forming O−C bond is longer than the one in the product of the dehalogenation step by 0.64 Å. These changes can be used to estimate bond orders using the Pauling rule. In the traditional, qualitative description of transition states, this corresponds to the symmetrical, exploded (loose) transition state. The calculations confirm that the intrinsic chlorine KIE for the reaction catalyzed by haloalkane dehalogenase is close to the experimental value obtained for 1-chlorobutane. They also indicate that the carboxylic moiety of Asp124 has to move more than 1 Å toward the carbon atom of 1,2-dichloroethane to be able to act as the nucleophile. This aspartate is directly connected to tryptophan-125, which participates in the hydrogen bonding of the chlorine atom of the transition state. Thus, the optimal geometry for the hydrogen-bonding network has to be compromised to allow nucleophilic attack by the carboxyl group. As a result, the tryptophan rings are rotated out of their optimal positions during the reaction. The dehalogenation step resembles drawing a bow: the high-energy cost is compensated by the increased hydrogen-bonding to the chlorine atom. The hydrolysis triggers release of the strain and the return of the tryptophan residue to the optimal position. The reverse process is energetically unfavorable. This scenario has recently been confirmed by molecular dynamic studies of this system.[41]

One possible explanation for the difference in chlorine KIEs for 1,2-dichloroethane and 1-chlorobutane is that in both cases the intrinsic values have been observed, but they are different for these two compounds. While the observed chlorine KIEs are significantly different when the magnitude is expressed as the deviation from unity, the absolute values differ only by 0.0021. We have calculated intrinsic carbon KIEs for the L-aspartate (natural substrate) and L-cysteine sulfinate (slow substrate) equal to 1.0356 and 1.0383, respectively.[42] However, results listed in Table 1 suggest that in the case of chlorine KIEs, the difference should be an order of magnitude smaller. In theory, the reaction of 1,2-dichroethane provides an opportunity to directly measure the intrinsic chlorine KIE. This is due to the presence in the molecule of two equivalent chlorine atoms. If one of them is substituted with ^{37}Cl and the other with ^{35}Cl, then the isotopic ratio R reflects directly the intramolecular competition between isotopes and thus the intrinsic chlorine KIE. Unfortunately, neither compounds containing isotopically 100% pure ^{35}Cl nor ^{37}Cl are presently available.

The simplest explanation of the difference in chlorine KIEs for 1,2-dichloroethane and 1-chlorobutane is that there is a step preceding the dehalogenation which is slow enough to contribute to the reaction rate. Reasonable candidates for this role seems to be commitment connected with binding of the faster reactant or its conformational change, since the preferred conformation in the solution is anti, while calculations indicate that the gauche conformation is preferred in the active site. However, it is doubtful that such a conformational change has significant activation energy. Moreover, the commitment factor calculated from the isotope effects does not agree with the value that comes from the kinetic modeling of individual rate constants.[43] Thus, we favor the alternative explanation, that the dehalogenation step is reversible and the hydrolysis of the enzyme-bound intermediate is responsible for the overall irreversibility of the reaction catalyzed by haloalkane dehalogenase.

4-Chlorobenzoyl-CoA Dehalogenase

The hydrolytic dehalogenation reaction of 4-chlorobenzoyl-coenzyme A (4-CBA-CoA), catalyzed by *Pseudomonas* sp. strain 4-CBA-CoA dehalogenase,[44,45] is a step in the 4-CBA degradation pathway operational in bacteria adapted to the use of this soil pollutant as an energy source.[46] As with the other two dehalogenases described above, the 4-CBA-CoA dehalogenase employs covalent catalysis.[47,48] The mechanism differs from the one depicted by eq 7. The nucleophilic displacement of the chloride ion by a carboxylate of an active site residue (Asp145) proceeds via an S_NAr mechanism that is a stepwise process with formation of the Meisenheimer complex. This reaction is illustrated by eq 11. The only side chain positioned to interact with the chloride ion is that of Phe64. Hydrolysis of the benzoyl ester assisted by His90 generates the 4-HBA-CoA product that, along with a proton and the chloride ion, is released from the enzyme active site.



(11)

Another important difference between the mechanism of catalysis of this enzyme and the other two dehalogenases is that this reaction is reversible. Because the rates of all individual steps have been evaluated on the basis of kinetic measurement, we were able to estimate that the commitment factor for this reaction is about 0.25. This information, together with the value of the observed chlorine KIE of 1.0090, leads to the intrinsic chlorine KIE of 1.0125, matching the largest experimental value of chlorine KIE reported in the literature.[49] Interestingly, the C−Cl bond elongation occurs mostly in the first step, leading to the formation of the Meisenheimer complex, in which it reaches a value of over 2 Å, not much shorter than the bond in the subsequent transition state. Model

calculations indicate that, quite untypically, the large intrinsic KIE in this case is the result of a number of isotope-sensitive bending modes rather than related to the stretching along the reaction coordinate.

DL-Haloacid Dehalogenase

DL-2-Haloacid dehalogenase from *Pseudomonas* sp. 113 catalyzes the hydrolytic dehalogenation of both D- and L-2-haloalkanoic acids.[50] Site-directed mutagenesis studies indicate that Thr65, Glu69, and Asp194 are essential for the dehalogenation of both (*R*)-(+)-2-haloalkanoic acids and (*S*)-(−)-2-haloalkanoic acids and that both enantiomers share a common active site.

Two properties of this enzyme make it unique. First, it acts on the chiral carbons of both enantiomers, although the amino acid sequence is similar only to that of D-2-haloacid dehalogenase from *P. putida* AJ1 that specifically acts on (*R*)-(+)-2-haloalkanoic acids.[51] Of the residues, 23.5% are completely conserved between these two enzymes, while there is no sequence similarity with L-2-haloacid dehalogenases. Second, other dehalogenases studied so far have an active-site carboxylate group that attacks the substrate carbon atom bound to the halogen atom, leading to the formation of an ester intermediate, which is subsequently hydrolyzed (eq 6). The reaction catalyzed by DL-2-haloacid dehalogenase, on the other hand, proceeds without formation of an ester intermediate. ^{18}O trace studies[52] indicated that solvent directly attacks the α-carbon of 2-haloalkanoic acid to displace the halogen atom:

$$E_{H_2O} + RCl \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E^{RCl}_{H_2O} \underset{k_{hal}}{\overset{k_{deh}}{\rightleftharpoons}} E^{ROH}_{H^+} \cdot Cl^- \rightarrow E_{H^+} + ROH + Cl^- \quad (12)$$

where R = $CH(CH_3)COO^-$.

Our studies of chlorine KIEs yielded very interesting results: the observed value for the (*R*)-(+)-enantiomer (1.0090) is substantially different from the one obtained for the (*S*)-(−)-enantiomer (1.0105). Comparison of these experimental values with the results of the theoretical calculations using the AM1 Hamiltonian[53] and the SM5.4A continuum solvent model,[54,55] as implemented in the Amsol 6.5.1 program,[56] indicate that this difference can be attributed to the lack of stabilization of the departing chloride ion by a hydrogen-bond-donating group of the active side. One of the possible explanation is that (*R*)-(+)-2-chloropropionate is "correctly" oriented in the active site, as illustrated by the left-hand structure of Figure 2, allowing for contacts between the attacking water molecule, departing chloride, and carboxylic moiety of the reactant with complementary residues of the active site. In the case of (*S*)-(−)-2-chloropropionate, however, a methyl group occupies the place taken by the carboxylic moiety of (*R*)-(+)-2-chloropropionate. Rather than being attracted to the residue of the active site, the methyl group is repelled from the active site, causing the chlorine atom of the transition state to lose contact with the residue that can hydrogen-bond it, as shown in the right-hand structure of Figure 2.

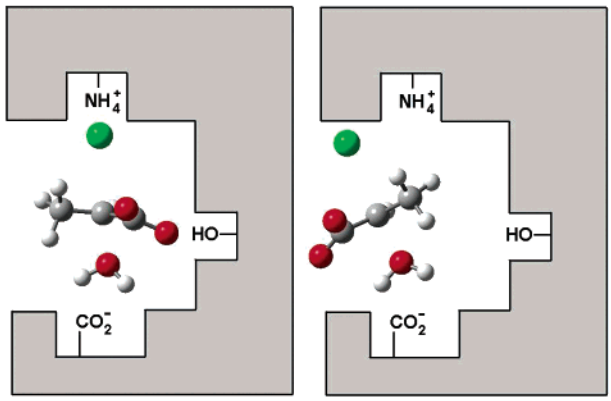


FIGURE 2. (Left) Orientation permitting hydrogen-bonding between the chlorine atom of the transition state and a residue of the active site. (Right) Perturbation of the interaction of the chlorine atom of the transition state with a residue of the active site upon change of configuration on the α-carbon.

Other explanations based on the reaction complexity cannot be ruled out, though. Regardless of the explanation of the difference in the KIEs for the two enantiomers, large values of the measured isotope effects point to the product-like transition state with a considerably elongated C−Cl bond. Model calculations indicate that this bond changes from about 1.82 Å in the reactant to about 2.47 Å in the transition state.

Summary and Future Directions

Experimental chlorine KIEs for the four hydrolytic dehalogenases range from 1.0045 to 1.0105. Also, the intrinsic KIEs measured or evaluated for these reactions lie within a very wide range of 1.0065−1.0125. This suggests that the details of the mechanisms of the reactions catalyzed by these enzymes are substantially different. There is little information in the literature on chlorine KIEs of reactions catalyzed by other enzymatic dehalogenations. Heraty and co-workers[57] reported the value of 1.0038 for the aerobic degradation of dichloromethane by a Gram-negative methylotrophic organism, MC8b. More recently, Numata and co-workers[58] reported chlorine isotopic fractionation of about 1.0056 for reductive dechlorination of trichloroethane by three strains of anaerobic bacteria. Kiefer and Copley[59] showed that the steps preceding dehalogenation are mostly rate-determining in the case of tetrachlorohydroquinone dehalogenase. Although there are no experimental data, one might thus expect that this reaction will be characterized by none, or negligible chlorine KIE. These three cases further expand the range of chlorine KIEs observed in enzymatic dehalogenations. When a sufficient number of experimental observations is collected, chlorine KIEs might turn out to be indicative of the mechanisms of action of various dehalogenases. At present, it seems that the reductive processes are characterized by smaller chlorine KIEs than the oxidative processes, although it is not a rule.

Table 2. Correlation of Energetics of C−Cl Bond and Chlorine KIEs for Enzymatic Dehalogenation

R	ΔH (kcal/mol)	Cl KIE intrinsic	Cl KIE observed
CH_2−COOH	74	1.0082	1.0082 ± 0.0005
$CH(CH_3)$-COOH	71	1.0086	1.0090 ± 0.0005[a]
		1.0101	1.0105 ± 0.0001[b]
C_6H_5	95	1.0125	1.0090 ± 0.0006
CH_2Cl	80	nd[c]	1.0038 ± 0.0003
CCl=CHCl	(90)[d]	nd	1.0057 ± 0.0007
CH_2CH_2Cl	(82)[d]	1.0066	1.0045 ± 0.0004[e]
			1.0066 ± 0.0004[f]

[a] (*R*)-(+)-2-Chloropropionate. [b] (*S*)-(−)-2-Chloropropionate. [c] Not determined. [d] Value estimated, see text. [e] 1,2-Dichloroethane. [f] 1-Chlorobutane.

On the basis of the current, insufficient data, a generalization can be made when the values of chlorine KIEs are compared with the energetics of the breaking of the C−Cl bond. Such a comparison of the gas-phase R−Cl bond dissociation enthalpy[60] with chlorine KIEs is given in Table 2. The enthalpies for 1,2-dichloroethane and trichloroethane are estimations based on the fact that the introduction of an electron-donating group (CH_3) increases the enthalpy by about 0.7 kcal/mol, while the introduction of an electron-withdrawing group lowers it by about 1.9 (CH_2Cl) or 3.4 kcal/mol (Cl), respectively. It can be seen that the observed chlorine KIE is equal to the intrinsic value for those reactants which have weaker C−Cl bonds. For those compounds for which breaking of the C−Cl bond requires more energy (over 80 kcal/mol), the mechanism becomes more complex, with the dehalogenation step not being exclusively rate-determining.

In conclusion, it is clear that even in the narrow class of hydrolytic dehalogenases, Nature developed several mechanisms. Studies involving chlorine kinetic isotope effects allow us to learn many intimate details of these mechanisms and should promote the use of enzymatic dehalogenation for detoxification, bioremediation, drug design, and other related purposes.

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