Haloalkane dehalogenase converts halogenated alkanes to their corresponding alcohols. The active site is buried inside the protein and lined with hydrophobic residues. The reaction proceeds via a covalent substrate-enzyme complex. This paper describes a steady-state and pre-steady-state kinetic analysis of the conversion of a number of substrates of the dehalogenase. The kinetic mechanism for the “natural” substrate 1,2-dichloroethane and for the brominated analog and nematocide 1,2-dibromoethane are given. In general, brominated substrates had a lower $K_m$ but a similar $k_{cat}$ than the chlorinated analogs. The rate of C-Br bond cleavage was higher than the rate of C-Cl bond cleavage, which is in agreement with the leaving group abilities of these halogens. The lower $K_m$ for brominated compounds therefore originates both from the higher rate of C-Br bond cleavage and from a lower $K_s$ for bromo-compounds. However, the rate-determining step in the conversion (Scheme I) of 1,2-dibromoethane and 1,2-dichloroethane was found to be release of the charged halide ion out of the active site cavity, explaining the different rates of 1,2-dichloroethane and 1,2-dibromoethane conversion by haloalkan dehalogenase. The results showed that in the dehalogenase reaction deactivation of C-Br bonds is faster than C-Cl bonds, in agreement with the leaving group abilities of these halogens. The rate-limiting step for 1,2-dichloroethane and 1,2-dibromoethane conversion, however, is not carbon-halogen bond cleavage but halide release from the active site cavity.

EXPERIMENTAL PROCEDURES

Materials—Halogenated compounds were obtained from Janssen Chimica (Beerse, Belgium) or from Merck Darmstadt, Germany. [2H2O] (99.8% v/v) was purchased from Merck or from Isotec Inc. (Miamisburg, OH).

Bacterial Strains and Plasmids—pGELAF+, an expression vector based on pET-3d (9) with the dehalogenase gene (dhlA) under the control of the T7-promoter and an additional f1-origin for the production of single-stranded DNA was used to overexpress the dehalogenase in Escherichia coli strain BL21(DE3) (9).

Protein Expression and Purification—The enzyme was expressed and purified as described earlier (10). The buffers used during purification were TEMAG (10 mM Tris-sulfate, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 3 mM sodium azide, and 10% (v/v) glycerol) and PEGAG (10 mM sodium phosphate, pH 6.0, 1 mM EDTA, 1 mM 2-mercaptoethanol, 3 mM sodium azide, and 10% (v/v) glycerol). The enzyme was concentrated with an Amicon ultrafiltration cell using a PM10 filter.

Dehalogenase Assays and Protein Analysis—Dehalogenase assays were performed using calorimetric detection of halide release as described (11). Protein concentrations were determined with Coomassie Brilliant Blue using bovine serum albumin as a standard. The concentration of purified enzyme was determined using $\varepsilon_{280} = 4.87 \times 10^4$ M$^{-1}$ cm$^{-1}$ calculated with the program DNASTAR (DNASTAR Inc., Madison, WI). The purified enzyme was analyzed with SDS-polyacrylamide gel electrophoresis, which showed that the purity of the preparations was greater than 99% (data not shown). Solvent kinetic isotope...
effects were determined with substrate dissolved in D$_2$O. Assays were performed as described above using increased concentrations of D$_2$O.

For the determination of the $K_m$, alcohol or halide production was measured in 4.5 ml incubations containing various concentrations of the substrate in 50 mM Tris-sulfate buffer, pH 8.2, and a suitable amount of enzyme. Samples were incubated at 30 °C. The amount of enzyme was adjusted such that the amount of substrate converted in the incubation period was less than 10%. The amount of alcohol produced was determined by gas chromatography, and halide production was determined colorimetrically as described (1).

The reversibility of the dehalogenase reaction was tested by incubating 6 U of enzyme with 100 mM 2-chloroethanol, 100 mM 1-butanol, or 50 mM 2-bromoethanol and 100 mM bromide or chloride in 4.5 ml of Tris-sulfate buffer, pH 8.2. At different points in time, 1-ml samples were taken and transferred to an ice-cold mixture of 3.5 ml of water and 1.5 ml of dithiothreitol. Gas chromatography was used to study the kinetics of substrate conversion. All experiments were performed on an Applied Photophysics, model SX177MV, fitted with a xenon arc lamp with excitation at 290 nm. Fluorescence emission from Trp residues was observed through a 320-nm cut-off filter supplied with the instrument. All reactions were performed at 30 °C and the reported concentrations are those in the reaction chamber. Each trace shown is the average of 4–7 individual experiments (depending on the signal).

Rapid-quench Experiments—Rapid-quench-flow experiments were performed at 30 °C on a rapid-quench instrument (ROF-63) from KinTek Instruments (12). Typically, the experiments were carried out by loading the enzyme in one sample loop (50 μl) and substrate in the second loop (50 μl). The reaction was started by rapidly mixing the two reactants and quenched with 120 μl of 0.4 M H$_2$SO$_4$ (final concentration) after time intervals ranging from 2 ms to 1 s. The quenched mixture was directly injected into 3.5 ml of ice-cold diethylether containing 0.05 M 1-bromohexane as the internal standard. After extraction, the diethylether phase was analyzed with gas chromatography.

Stopped-flow Fluorescence Experiments—Stopped-flow fluorescence was used to study the kinetics of substrate conversion. All experiments were performed on an Applied Photophysics, model SX177MV, fitted with a xenon arc lamp with excitation at 290 nm. Fluorescence emission from Trp residues was observed through a 320-nm cut-off filter supplied with the instrument. All reactions were performed at 30 °C and the reported concentrations are those in the reaction chamber. Each trace shown is the average of 4–7 individual experiments (depending on the signal).

Long chain haloalkanes were poor substrates with a high $K_m$ and a low $k_{cat}$. There was no solvent kinetic isotope effect of 2H$_2$O on 1-chlorohexane conversion, while such an effect was observed for the conversion of 1-chlorobutane and 1,2-dichloroethane (Fig. 2A), which suggests a different rate-determining step for 1-chlorohexane. The large difference in $k_{cat}$ between the branched substrates 1,2-dichloropropane and 1,2-dibromopropane is probably also caused by a different rate-determining step since 1,2-dichloroethane by the dehalogenase, was never observed in the x-ray structure (5). 2-Chloroethanol and chloracetamide did not allow extraction of the steady-state affinity and rate constants of the enzyme, since the $K_m$ values were above solubility of these compounds in water.

The enzyme exhibited no activity toward the fluorinated compounds that we have tested (1-fluoropentane and 1-fluorohexane). Binding of fluorides to the enzyme was measured by fluorescence quenching, and by competition experiments with bromide using steady-state fluorescence (17). No binding of fluoride and no inhibition of 1,2-dichloroethane activity by fluoride was found.

The reaction of the dehalogenase can be regarded as irreversible, since we could not detect any product for the reverse reaction and no inhibition of the dehalogenase activity by alcohol (1-butanol) was found. When enzyme was incubated with 100 mM 2-chloroethanol, 100 mM 1-butanol, or 50 mM 2-bromoethanol and 100 mM of halide, no formation of halogenated products could be detected (detection limit of 70 μM). Thus in
Pre-steady-state Analysis of 1,2-Dibromoethane Conversion—
The best known substrate of haloalkane dehalogenase is 1,2-dibromoethane with a $k_{cat}/K_m = 3.0 \times 10^5$ M$^{-1}$ s$^{-1}$ (Table I).

The concentration of both substrate and product (2-bromoethanol) can be detected by gas chromatography in the micromolar range. Because of the high affinity of the enzyme for this substrate and the availability of sensitive assays for substrate and product, we selected 1,2-dibromoethane as the model compound to study pre-steady-state kinetics.

Upon mixing dehalogenase with excess of 1,2-dibromoethane, a clear burst of 2-bromoethanol was observed in rapid-quench experiments (Fig. 3A). The steady-state production rate ($3 \pm 0.2$ s$^{-1}$) of 2-bromoethanol between 0.2 s and 1 s was identical to the steady-state $k_{cat}$ of the enzyme (3.0 s$^{-1}$, Table I). A burst in substrate decrease was not observed due to a background of excess substrate. The occurrence of a pre-steady-state product burst at excess substrate indicates that all the steps before the main rate-limiting step in the steady state are fast (12). Thus, all steps before and including hydrolysis of the alkyl-enzyme intermediate were not rate-limiting. The slowest step therefore must be product release.

Release of the alcohol from the enzyme active site was probably not rate-limiting for the following reasons. First, the alcohol produced was never observed in the crystal structure (5). Second, the $K_m$ for the alcohol was about 1000-fold higher than $K_m$ for the substrate.

Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
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</thead>
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<tr>
<td>CH$_2$X-CH$_2$X</td>
<td>0.53</td>
<td>3.3</td>
<td>$6.2 \times 10^3$</td>
</tr>
<tr>
<td>CH$_3$-O-CH$_2$X</td>
<td>48</td>
<td>1.8</td>
<td>38</td>
</tr>
<tr>
<td>CH$_3$-C-O-CH$_2$X</td>
<td>&gt;400</td>
<td>—</td>
<td>0.70$^b$</td>
</tr>
<tr>
<td>CH$_3$-C-H$_2$X</td>
<td>&gt;100</td>
<td>—</td>
<td>0.70$^b$</td>
</tr>
<tr>
<td>H$_2$N-CO-CH$_2$X</td>
<td>&gt;100</td>
<td>—</td>
<td>0.046$^b$</td>
</tr>
</tbody>
</table>

$^a$ — not detectable.

$^b$ Measured as the first order rate-constant at concentrations of 400 mM CH$_2$OH-CH$_2$Cl, 100 mM Cl-CH$_2$Cl, and 100 mM H$_2$N-CO-CH$_2$Cl.

Fig. 1. Stereo picture of substrate bound into the hydrophobic active site cavity of haloalkane dehalogenase (see Ref. 5; created with MOLSCRIPT, Ref. 23).

Fig. 2. Solvent $^2$H$_2$O kinetic isotope effects on haloalkane dehalogenase activity. The observed rate ($V_{obs}$) as a fraction of the rate observed in $^1$H$_2$O ($V_H$) was determined at different $^2$H$_2$O/$^1$H$_2$O ratios. A, the $V_{obs}/V_H$ for 1-chlorobutane (5 mM, ●), 1-chlorohexane (3 mM, ○), and 1,2-dichloroethane (5 mM, ■) conversion. B, the $V_{obs}/V_H$ for 1,2-dibromo propane (4 mM, ●) and 1,2-dichloropropane (25 mM, ○) conversion.
the $K_m$ for the corresponding substrate (Table I), indicating that as soon as this polar uncharged product was formed, it diffused out of the hydrophobic active site cavity. The step in the mechanism (Scheme I) that is left to limit the $k_{cat}$ is halide release. We have shown recently with stopped-flow fluorescence studies of halide binding, that release of the charged halide ion to the solvent is limited by a slow enzyme isomerization that occurs at a rate of 9 s$^{-1}$ and 14 s$^{-1}$ for bromide and chloride release, respectively (8).

These observations allow simplification of Scheme I to Scheme II, where $k_{-2}$ and $k_{-3}$ are omitted since step 2 and 3 are regarded as irreversible, and the release of the alcohol is included in step 3. Under initial rate conditions, the contribution of $k_{-4}$ is also negligible (Scheme II).

A pre-steady-state burst experiment with substrate in excess over enzyme provides little information about the rates of the separate steps buried within the burst. For the dehalogenase reaction, the rates of formation of the alkyl-enzyme intermediate ($k_2$) and hydrolysis of this intermediate ($k_6$) are not distinguishable in such an experiment if alcohol production is followed. A single turnover experiment with enzyme in excess over substrate provides more information in this case. Fig. 3B shows an experiment where enzyme (445 μM) was in excess over 1,2-dibromoethane (250 μM). Within 150 ms, all substrate was consumed, while at around 400 ms, all 2-bromoethanol was produced. Substrate decrease and product increase curves crossed at 40 μM, indicating that the rate of formation of the alkyl-enzyme intermediate was much faster than its hydrolysis, and that alkyl-enzyme accumulated (Fig. 3B, upper panel).

The effect of the solvent on the pre-steady-state burst was found to occur at a rate of at least 130 s$^{-1}$. The slow step under steady-state conditions is release of the halide ion with a rate of $k_4 = 4 \pm 1.5$ s$^{-1}$. This was close to the experimental $k_{cat}$ and in the same order of magnitude as the rate of the slow step in the bromide release sequence as determined by stopped-flow fluorescence analysis of halide release (9 s$^{-1}$; Ref. 8). The calculated and experimental steady-state $k_{cat}$ were identical, while the corresponding $K_m$ values were in the same order of magnitude (Table III).

Pre-steady-state experiments in the presence of $^3$H$_2$O—A 50% reduction of the $k_{cat}$ was found using $^3$H$_2$O as the solvent, which was correlated with the effect of $^3$H$_2$O on the rate of halide release (8). Pre-steady-state experiments were performed to identify which other steps in the reaction mechanism were also affected by the use of $^3$H$_2$O as the solvent. A clear effect of the solvent on the pre-steady-state burst was found when enzyme was mixed with excess 1,2-dibromoethane. Both the amplitude and the rate of the burst decreased (Fig. 3A). The steady-state turnover, determined from the last part of the curve (0.2-1 s), reduced to 1.4 ± 0.3 s$^{-1}$, a value similar to the $k_{cat}$ in $^3$H$_2$O (Table III). The effect of $^3$H$_2$O on the pre-steady-state burst must be an effect on the rate of hydrolysis of the alkyl-enzyme intermediate ($k_6$) since the rate of carbon-halogen bond cleavage ($k_5$) was not affected in a single turnover experiment with 1,2-dibromoethane in $^3$H$_2$O (Fig. 3B, lower panel). The single turnover experiment also showed that the $k_{cat}$ was affected by using $^3$H$_2$O as the solvent (Fig. 3B). Furthermore, a stopped-flow fluorescence single turnover experiment showed that $^3$H$_2$O had no effect on the first part of the transient (Fig. 4, inset). The rate of the initial decrease of the fluorescence...
specificity was identical in $^1$H$_2$O and $^3$H$_2$O, indicating that the first part of the reaction was not sensitive to the use of $^3$H$_2$O as the solvent. The minimal fluorescence shifted in time in $^3$H$_2$O and also the fluorescence increase in the second part of the transient was slower than in $^1$H$_2$O. The shift in the time point of minimal fluorescence is caused by a decrease in rate of $k_3$ and $k_4$ (Table III), allowing more accumulation of the alkyl-enzyme intermediate in $^3$H$_2$O than in $^1$H$_2$O (Fig. 3B).

As with experiments in $^1$H$_2$O, rate constants could be extracted from numerical simulation of Scheme II (Table III). Compared to $^1$H$_2$O, both the rates of hydrolysis of the alkyl-enzyme intermediate and halide release were slower in $^3$H$_2$O.

Pre-steady-state Analysis of 1,2-Dichloroethane Conversion—

Similar pre-steady-state experiments as with 1,2-dibromoethane were performed for the "natural" substrate 1,2-dichloroethane and are displayed in Fig. 5 and 6. Experiments with excess substrate over enzyme showed a smaller alcohol burst than with 1,2-dibromoethane (Fig. 5A), indicating that the rates of either $k_2$ and/or $k_3$ (Scheme II) were close to the steady-state conversion rate. A single turnover experiment with enzyme in excess over substrate (Fig. 5B), where the decrease in substrate and the increase of product crossed almost in the middle of the plot (450 $\mu$M substrate and product), did not yield unique solutions for $k_2$ and $k_3$. Both rates of $k_2$ and $k_3$ could be varied between $10^2$ s$^{-1}$ and $50$ s$^{-1}$ when the second order rate constant for association of enzyme and substrate was varied between 0.01 and 0.04 $\mu$M$^{-1}$s$^{-1}$. Rates for binding of substrate could be extracted from stopped-flow fluorescence experiments with 1,2-dichloroethane (Fig. 6). The rate and amplitude of the initial decrease in fluorescence is related to the substrate concentration, as with 1,2-dibromoethane conversion. These stopped-flow fluorescence experiments showed that binding of substrate occurred at a rate of $(9 \pm 1) \times 10^3$ $\mu$M$^{-1}$s$^{-1}$, yielding in combination with the rapid-quench experiments a $k_2$ of $50 \pm 10$ s$^{-1}$ and a $k_3$ of $14 \pm 3$ s$^{-1}$ (Table III). As with 1,2-dibromoethane, the rate of halide release was the slowest step ($k_4 = 8 \pm 2$ s$^{-1}$) in the reaction and close to the rate of the slow step in chloride release (14 s$^{-1}$) as determined earlier by transient kinetic analysis of chloride binding and release (8). The $k_{cat}$ and $K_m$ calculated from the pre-steady-state kinetic analysis were close to the experimentally determined steady-state $k_{cat}$ and $K_m$ (Table III).

**DISCUSSION**

Haloalkane dehalogenase is one of the few enzymes involved in degradation of xenobiotic compounds of which both the x-ray structure and reaction mechanism are known. The "natural" substrate is 1,2-dichloroethane, a compound that did not occur in the biosphere until it was synthesized by man. Thus, it is not surprising that the catalytic performance of haloalkane dehalogenase is rather poor. This paper describes the kinetics of the conversion of a range of halogenated aliphatics by the dehalogenase as studied by a combination of steady-state, rapid-quench-flow, and stopped-flow fluorescence experiments.

The steady-state $k_{cat}$, $K_m$, and $k_{cat}/K_m$ for a four-step kinetic model (Scheme II) were derived using the determinant method described by Huang (18), which gives $k_{cat} = k_2k_3k_4/(k_2k_5 + k_2k_4 + k_3k_4)$, $K_m = k_2k_3(k_1/k + k_2)/k_2k_3 + k_2k_4 + k_3k_4$, and $k_{cat}/K_m = k_1k_2(k_1/k + k_2)$.

The steady-state $k_{cat}$ and $K_m$ calculated from the separate rate constants that were determined by pre-steady-state measurements (Table III) were close to the experimentally determined steady-state values, both for 1,2-dibromoethane and 1,2-dichloroethane conversion. Scheme II, together with derived rate constants, thus gives a good description of the kinetic mechanism and steady-state kinetics of the dehalogenase reaction. The slowest step for both 1,2-dichloroethane and 1,2-dibromoethane conversion was the release of the halide ion, as was also suggested on bases of transient kinetic analysis of halide release of the dehalogenase. Export of the charged halide ion was limited by a slow enzyme isomerization preceding the actual halide release (8). Both the experiments described here and our previous study gave a 1.5-2-fold higher rate of chloride release than bromide release, which was caused by a higher rate of the enzyme isomerization in the presence of chloride. Both binding and release of neutral substrates and products were fast. A slow release of a charged product (PO₄, "sticky acid") from the noncovalent complex also occurs in alkaline phosphatase (19), although in this study no evidence was found that release was preceded by an enzyme isomerization, as was suggested by others (20).

The actual cleavage of the carbon-halogen bond was not found to be rate-limiting for both 1,2-dichloroethane and 1,2-dibromoethane conversion. The rate of cleavage of the C-Br bond is, however, faster than the rate of cleavage of the C-Cl bond, which is in agreement with bromine being a better leaving group in bimolecular nucleophilic substitutions than chlorine.

This order for rates of C-X cleavage was recently also found by stopped-flow analysis of the dehalogenation of 4-chlorobenzylo-CoA by 4-chlorobenzylo-CoA dehalogenase (21). The affinity of the dehalogenase for halide ions, as measured with steady-state fluorescence, showed the same order: I, Br > Cl > F.

The difference between the rates of cleavage of a C-Cl and C-Br bond and the difference of the second-order association constants for enzyme and substrate are the main determinants in the difference between the Michaelis constant ($K_m$) of the enzyme for chlorinated and brominated compounds. The lower rate of C-Cl cleavage is especially unfavorable in cases where other properties of the substrate lower the binding affinity of the enzyme, such as in long, branched, or polar substrates. In some cases, as with the branched 1,2-dihalopropane, the rate-determining step probably shifts from a step after hydrolysis of the alkyl-enzyme intermediate (as in 1,2-dibromopropane) to a
The cleavage of the alkyl-enzyme (5, 7) and explains the effect of proton transfer to the general base His-289 is necessary for intermediate and halide release. Activation of a water molecule \(2H_2O\) on the rate of hydrolysis of the alkyl-enzyme, while we have recently proposed a more global effect of \(2H_2O\) on the rate of halide release by decrease of the overall flexibility of a part of the protein (8).

**TABLE III**

Haloalkane dehalogenase kinetic constants

<table>
<thead>
<tr>
<th>Compounds</th>
<th>(k_1)</th>
<th>(k_{-1})</th>
<th>(k_2)</th>
<th>(k_3)</th>
<th>(k_4)</th>
<th>(k_{cat}) calc</th>
<th>(k_{cat}) exp</th>
<th>(K_m) calc</th>
<th>(K_m) exp</th>
<th>(k_{cat}/K_m) exp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-Dibromoethane</td>
<td>0.75 ± 0.1</td>
<td>&gt;20</td>
<td>&gt;130</td>
<td>10 ± 2</td>
<td>4 ± 1.5</td>
<td>2.8</td>
<td>3.0</td>
<td>4.3</td>
<td>10</td>
<td>0.30</td>
</tr>
<tr>
<td>1,2-Dibromoethane (2H_2O)</td>
<td>0.75 ± 0.1</td>
<td>&gt;20</td>
<td>&gt;130</td>
<td>4 ± 1</td>
<td>3 ± 0.5</td>
<td>1.7</td>
<td>1.5</td>
<td>2.6</td>
<td>4.3</td>
<td>0.35</td>
</tr>
<tr>
<td>1,2-Dichloroethane</td>
<td>9 ± 1 \times 10^{-3}</td>
<td>20 ± 5</td>
<td>50 ± 10</td>
<td>14 ± 3</td>
<td>8 ± 2</td>
<td>4.6</td>
<td>3.3</td>
<td>0.72 \times 10^{3}</td>
<td>0.53 \times 10^{3}</td>
<td>6.2 \times 10^{-3}</td>
</tr>
</tbody>
</table>

\(^a\) Calculated from the rate-constants given.

\(^b\) Michaelis-Menten parameters determined in the steady-state assays.

Most brominated polar substrates had a high \(K_m\) and a high \(k_{cat}\). Upon examination of the equations for these steady-state parameters, the substantial increase in \(K_m\) is most likely caused by an increase in \(k_{cat}/K_m\). Initial binding of the polar substrate into the Michaelis complex in the apolar active site cavity is unfavorable, but the rate of C-Br bond cleavage is still high, thereby maintaining a high \(k_{cat}\) at saturating substrate concentrations.

It is possible with the knowledge of the x-ray structure, mechanism, and kinetics of the dehalogenase to indicate rate-determining step(s) for the substrates listed in Table I. Chlorinated long chain alkanes and branched alkanes, for example, will have their rate-determining step at C-Cl cleavage. The \(K_m\) for such compounds is not extremely high indicating that binding will occur, but proper positioning of the halogen moiety between the two tryptophans and the position of the C-Cl bond toward the nucleophilic Asp-124 could be less optimal than with 1,2-dichloroethane. The increase in \(K_m\) would then mainly be caused by the low \(k_{cat}\). Brominated long alkanes and branched alkanes will probably still have their rate-determining steps at the end of the reaction sequence. The better leaving group characteristics and the fact that bromine is easier to polarize may allow a less precise fit for brominated compounds to be stabilized in the transition state.

The activity of the dehalogenase toward dibromomethane and dichloromethane is completely different. The high \(k_{cat}\) for dibromomethane suggests that the rate-determining step is still at the end of the reaction sequence, whereas the reasonably high \(K_m\) originates from the increase in \(k_{cat}\) most likely caused by the less tight binding of this small substrate in the active site cavity. Cleavage of the C-Cl bond is definitely rate-limiting for conversion of dichloromethane. The extremely high \(K_m\) is most likely the combination of a very low \(k_{cat}\) and high \(K_m\).

An example of polar halogenated alkanes has already been discussed above.
This kinetic model and detailed knowledge of the reaction mechanism by x-ray crystallography and site-directed mutagenesis studies, provide a good starting point for protein engineering studies aimed at improving the catalytic performance of the enzyme. The Xanthobacter strain expressing the wild type haloalkane dehalogenase has now successfully been used for the removal of 1,2-dichloroethane from contaminated groundwater at full scale (22). Expanding the substrate range of the enzyme would be an important step toward developing suitable biocatalysts for the removal of halogenated aliphatic compounds that are recalcitrant so far.

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REFERENCES