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 nematicide 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 $k_e$ for bromo-compounds. However, the rate-determining step in the conversion ($k_{cat}$) 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 $K_m$ and similar $k_{cat}$ values for these compounds. The study provides a basis for the analysis of rate-determining steps in the hydrolysis of various environmentally important substrates.

Haloalkane dehalogenase is one of the few enzymes involved in degradation of xenobiotic compounds of which the x-ray structure is known (4). The reaction mechanism for 1,2-dichloroethane has been solved using x-ray crystallography and site-directed mutagenesis studies (5–7). The reaction is initiated by binding of the substrate into the Michaelis complex (E-RX), followed by nucleophilic attack of Asp-124 on the carbon atom to which the halogen is bound, leading to formation of an alkyl-enzyme intermediate (E-RX). This covalent intermediate is subsequently hydrolyzed by activated water, with His-289 acting as a general base catalyst, leading to enzyme and bound products (E-ROH-X). The final step is release of the products. We recently found that halide release follows a complex pathway and could limit the $k_{cat}$ (8). This reaction mechanism is summarized in reaction scheme shown in Scheme I.

Besides the hydrophilic catalytic residues, the active site of the dehalogenase is mainly lined with hydrophobic residues: 4 phenylalanines, 2 tryptophans, 2 leucines, a valine, and a proline (4; Fig. 1). This hydrophobic environment and its relatively small size (37 Å$^3$) predict a low affinity for polar and large molecules. The $k_{cat}$ values for 1,2-dichloroethane and 1,2-dibromoethane are similar, although the affinity for bromide ions is much higher than for chloride, and the carbon-bromine bond is less stable than the carbon-chlorine bond. Thus, the steady-state kinetics do not reflect the kinetics of carbon-halogen bond cleavage.

To obtain further insight into the kinetics and specificity of the dehalogenase, we studied the steady-state kinetics of conversion of a range of halogenated compounds, and the pre-steady-state kinetics of 1,2-dichloroethane and 1,2-dibromoethane conversion using stopped-flow fluorescence and rapid-quench-flow techniques. This paper presents a complete description of the rates of the separate steps during 1,2-dibromoethane and 1,2-dichloroethane conversion by haloalkane dehalogenase. The results show that in the dehalogenase reaction cleavage 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 release of the halide ion out of the active site cavity.

**EXPERIMENTAL PROCEDURES**

Materials—Halogenated compounds were obtained from Janssen Chimica (Beerse, Belgium) or from Merck (Darmstadt, Germany). $\text{H}_2\text{O}$ (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 f(1)+ origin for the production of single-stranded DNA (10) 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 (1). Protein concentrations were determined with Coomassie Brilliant Blue using bovine serum albumin as a standard. The concentration of purified enzyme was determined using $\varepsilon_{280} = 4.87 \times 10^4$ $\text{M}^{-1}\text{cm}^{-1}$ calculated with the program DNASAR (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...
Specificity and Kinetics of Haloalkane Dehalogenase

**RESULTS**

Specificity of Haloalkane Dehalogenase—The substrate range of the dehalogenase was tested for a variety of chlorinated and brominated compounds (Table I). For all pairs of substrates in this table, the enzyme had a significantly higher $K_m$ for chloro-compounds than for the brominated analogs. This correlated with the lower affinity of the enzyme for chloride compared with bromide, as determined with steady-state fluorescence (Table II). The $K_{cat}$ values for most brominated and chlorinated nonbranched substrates with chain lengths up to 4-carbon atoms were comparable, however, indicating a similar rate-limiting step for such compounds.

Long chain haloalkanes were poor substrates with a high $K_m$ and a low $k_{cat}$. There was no solvated kinetic isotope effect of $^{2}$H$_2$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-dibromoethane was probably also caused by a different rate-determining step since $^{2}$H$_2$O did not affect the rate of 1,2-dichloropropane conversion, whereas the rate of 1,2-dibromoethane conversion was reduced by 50% in $^{2}$H$_2$O (Fig. 2B).

Polar compounds such as haloalkylated alcohols and epoxides were poorer substrates than apolar compounds. The $K_m$ of these compounds was increased compared to their apolar counterparts. 2-Chloroethanol, the product of the conversion of 1,2-dichloroethane by the dehalogenase, was never observed in the x-ray structure (5). 2-Chloroethanol and chloroacetamide 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 $\mu$M). Thus in
Scheme I, $k_{-2}$ and $k_{-3}$ are of no kinetic relevance.

Pre-steady-state Analysis of 1,2-Dibromoethane Conversion—
The best known substrate of haloalkane dehalogenase is 1,2-dibromoethane with a $k_{cat}/K_m = 3.0 \times 10^3$ 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 ± 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

\[\begin{align*}
\text{Table I} \\
\begin{array}{cccc}
\text{Chlorinated} & K_m & k_{cat} & k_{cat}/K_m \\
\text{mM} & \text{s}^{-1} & \text{M}^{-1} \text{s}^{-1} \\
\text{CH}_2\text{X-CH}_2\text{X} & 0.53 & 3.3 & 6.2 \times 10^3 \\
\text{CH}_2\text{C(OC)}\text{H}_2\text{X-CH}_2\text{X} & 2.2 & 1.5 & 6.8 \times 10^2 \\
\text{CH}_2\text{C}\text{X-CH}_2\text{X} & 1.4 & 0.088 & 63 \\
\text{CH}_2\text{C}\text{X-CH-CH}_3 & 13 & 0.15 & 12 \\
\text{CN-CH}_2\text{X} & 6.3 & 1.5 & 2.4 \times 10^2 \\
\text{CH}_2\text{C(OC)}\text{H}_2\text{X} & 48 & 1.8 & 38 \\
\text{CH}_2\text{OH-CH}_2\text{X} & >400 & — & 0.70^b \\
\text{H}_2\text{N-CO-CH}_2\text{X} & >100 & — & 0.70^b \\
\end{array}
\end{align*}\]

\[\begin{align*}
\text{Brominated} & K_m & k_{cat} & k_{cat}/K_m \\
\text{mM} & \text{s}^{-1} & \text{M}^{-1} \text{s}^{-1} \\
\text{CH}_2\text{X-CH}_2\text{X} & 0.01 & 3.0 & 3.0 \times 10^3 \\
\text{CH}_2\text{C(OC)}\text{H}_2\text{X-CH}_2\text{X} & 0.06 & 0.94 & 1.6 \times 10^3 \\
\text{CH}_2\text{C}\text{X-CH}_2\text{X} & 0.3 & 0.64 & 2.1 \times 10^3 \\
\text{CH}_2\text{C}\text{X-CH-CH}_3 & 1.3 & 2.1 & 1.6 \times 10^3 \\
\text{CN-CH}_2\text{X} & 0.49 & 2.7 & 5.5 \times 10^3 \\
\text{CH}_2\text{C(OC)}\text{H}_2\text{X} & 2.2 & 2.6 & 1.2 \times 10^3 \\
\text{CH}_2\text{OH-CH}_2\text{X} & 4.9 & 3.9 & 1.6 \times 10^3 \\
\text{H}_2\text{N-CO-CH}_2\text{X} & 20 & 1.0 & 5.0 \times 10^3 \\
\end{align*}\]

\[a —, not detectable.\]
\[b \text{ Measured as the first order rate-constant at concentrations of 400 mM CH}_2\text{OH-CH}_2\text{Cl, 100 mM Cl-CH}_2\text{Cl, and 100 mM H}_2\text{N-CO-CH}_2\text{Cl.}\]

\[\text{Table II}
\begin{align*}
\text{Fluoride} & K_d\text{(mM)} & 75 & 10 & 5.0^a \\
\text{Chloride} & & 0.31 & 0.34 & 0.49^b \\
\text{Bromide} & & — & & \\
\text{Iodide} & & — & & \\
\end{align*}\]

\[a —, not detectable.\]
\[b \text{ Taken from Ref. 17 (25 °C).}\]
\[c \text{ The fraction of fluorescence quenched at saturating halide concentrations.}\]

\[\text{A} \]

\[\begin{align*}
\text{B} \end{align*}\]

\[\text{Fig. 2. Solvent } ^2\text{H}_2\text{O kinetic isotope effects on haloalkane dehalogenase activity.} \]

The observed rate ($V_{obs}$) as a fraction of the rate observed in $^1\text{H}_2\text{O} (V_H)$ was determined at different $^2\text{H}_2\text{O}/^1\text{H}_2\text{O}$ ratios. A, the $V_{obs}/V_H$ for 1-chlorobutane (5 mM, ●), 1-chloroethane (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 Km 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 kcat 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 k₂ and k₃ 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 k₄ 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 (k₂) and hydrolysis of this intermediate (k₃) 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).

Binding of substrate and halides quenches the intrinsic protein fluorescence of the dehalogenase because the halide ion or the halogen moiety of the substrate binds between two Trp residues. Under single turnover conditions, the kinetics of 1,2-dibromoethane conversion by haloalkane dehalogenase were sufficiently slow to be followed by stopped-flow fluorescence. When 1,2-dibromoethane was mixed with excess enzyme, the fluorescence initially rapidly decreased, reached a minimum, and then slowly increased again (Fig. 4). The initial decrease in fluorescence was related to the rate of substrate import, since a 2-fold higher concentration of substrate increased mainly the rate and amplitude of the initial fluorescence decrease (Fig. 4).

Combination of rapid-quench-flow and stopped-flow fluorescence experiments allowed extraction of rates for all the steps in a four-step reaction scheme by numerical simulation (Table III, Scheme II, fits in Fig. 3 and 4). Stopped-flow fluorescence experiments provided mainly information about the rate of substrate binding, while the rapid-quench-flow experiments in combination with the steady-state kcat and Km provided information about the rest of the reaction. Carbon-halogen bond cleavage 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 k₄ = 4 × 1.5 s⁻¹. This was close to the experimental kcat 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 kcat were identical, while the corresponding Km values were in the same order of magnitude (Table III).

Pre-steady-state experiments in the presence of ²H₂O—A 50% reduction of the kcat was found using ²H₂O as the solvent, which was correlated with the effect of ²H₂O 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 ²H₂O 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 burst, 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 kcat in ²H₂O (Table III). The effect of ²H₂O on the pre-steady-state burst must be an effect on the rate of hydrolysis of the alkyl-enzyme intermediate (k₃) since the rate of carbon-halogen bond cleavage (k₄) was not affected in a single turnover experiment with 1,2-dibromoethane in ²H₂O (Fig. 3B, lower panel). The single turnover experiment also showed that the k₃ was affected by using ²H₂O as the solvent (Fig. 3B). Furthermore, a stopped-flow fluorescence single turnover experiment showed that ²H₂O had no effect on the first part of the transient (Fig. 4, inset). The rate of the initial decrease of the fluorescence...
cence was identical in $^{2}$H$_{2}$O and $^{1}$H$_{2}$O, indicating that the first rate of halide release was the slowest step (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 $k_{cat} = k_4 k_5 k_6 (k_2 k_3 + k_2 k_4 + k_3 k_4)$, $K_m = k_2 k_5 (k^{-1} + k_2) k_2 k_3 + k_2 k_4 + k_3 k_4)$, and $k_{cat}/K_m = k_1 k_6 (k^{-1} + k_2)$.

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 (PO$_4^{3-}$, “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-chlorobenzoyl-CoA by 4-chlorobenzoyl-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-dihaloalcohols, the rate-determining step probably shifts from a step after hydrolysis of the alkyl-enzyme intermediate (as in 1,2-dibromoethane) to a
Specificity and Kinetics of Haloalkane Dehalogenase

Table III
Haloalkane dehalogenase kinetic constants

| Substrate                  | $k_1$  | $k_{-1}$ | $k_2$  | $k_3$  | $k_4$  | $k_{\text{cat}}$ | $k_{\text{cat}}$ | $K_m$  | $K_m$  | $K_{cat}/K_m$ |
|----------------------------|--------|----------|--------|--------|--------|-----------------|-----------------|--------|--------|--------------|
| 1,2-Dibromoethane          | 0.75 ± 0.1 | >20 | >130 | 10 ± 2 | 4 ± 1.5 | 2.8 | 3.0 | 4.3 | 10 | 0.30 |
| 1,2-Dibromoethane (H₂O)   | 0.75 ± 0.1 | >20 | >130 | 4 ± 1 | 3 ± 0.5 | 1.7 | 1.5 | 2.6 | 4.3 | 0.35 |
| 1,2-Dichloroethane         | 9 ± 1 × 10⁻³ | 20 ± 5 | 50 ± 10 | 14 ± 3 | 8 ± 2 | 4.6 | 3.3 | 0.72 × 10³ | 0.53 × 10³ | 6.2 × 10⁻³ |

*a* Calculated from the rate-constants given.

*b* Michaelis-Menten parameters determined in the steady-state assays.

step before formation of this intermediate with 1,2-dichloropropane, as suggested by the effect of H₂O on the conversion rate of these substrates. This seems more generally applicable, since the absence of a solvent kinetic isotope effect on 1-chlorohexane conversion also indicates that halide release is not rate-limiting.

Two steps of the dehalogenase reaction are sensitive to the use of D₂O as the solvent, i.e., hydrolysis of the alkyl-enzyme intermediate and halide release. Activation of a water molecule by proton transfer to the general base His-289 is necessary for cleavage of the alkyl-enzyme (5, 7) and explains the effect of D₂O on the rate of hydrolysis of the alkyl-enzyme, while we have recently proposed a more global effect of D₂O on the rate of halide release by decrease of the overall flexibility of a part of the protein (8).

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 $k_chloride$ 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 $k_2$. 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 $k_2$ and high $K_2$.

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.

Acknowledgments—We thank Ivo Ridder for help on Fig. 1, and Anja Ridder and Evert Bokma for experimental help.

REFERENCES
1. Keuning, S., Janssen, D. B., and Witholt, B. (1985) J. Bacteriol. 163, 635–639
2. Pries, F., van den Wijngaard, A. J., Bos, R., Pentenga, M., and Janssen, D. B. (1994) J. Biol. Chem. 269, 17480–17484
3. van den Wijngaard, A. J., van der Kamp, K. W. H. J., van der Ploeg, J., Pries, F., Kazemier, B., and Janssen, D. B. (1992) Appl. Environ. Microbiol. 58, 976–983
4. Verschueren, K. H. G., Franken, S. M., Rozeboom, H. J., Kalk, K. H., and Dijkstra, B. W. (1993) J. Mol. Biol. 232, 856–872
5. Verschueren, K. H. G., Sélée, F., Rozeboom, H. J., Kalk, K. H., and Dijkstra, B. W. (1993) Nature 363, 693–698
6. Pries, F., Kingma, J., Pentenga, M., van Pouderoyen, G., J. eronimus-Stratingh, C. M., Bruins, A. P., and Janssen, D. B. (1994) Biochemistry 33, 1242–1247
7. Pries, F., Kingma, J., Krooshof, G. H., J. eronimus-Stratingh, C. M., Bruins, A. P., and Janssen, D. B. (1995) J. Biol. Chem. 270, 10405–10411
8. Schanstra, J. P., and Janssen, D. B. (1996) Biochemistry, in press
9. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–89
10. Schanstra, J. P., Rink, R., Pries, F., and Janssen, D. B. (1993) Protein Expression Purif. 4, 479–489
11. Leatherbarrow, R. J. (1987) A Non-linear Regression Data Analysis Program for the IBM PC, Elsevier Science Publishers BV, Amsterdam
12. Johnson, K. A. (1992) Enzymes 20, 1–61
13. Mendes, P. (1993) Comput. Appl. Biosci. 9, 563–571
14. Frieden, C. (1994) Methods Enzymol. 240, 311–322
15. Fersht, A. R. (1985) Enzyme Structure and Mechanism, 2nd Ed., pp. 121–154, W. H. Freeman, New York
16. Kennes, C., Pries, F., Krooshof, G. H., Bokma, E., Kingma, J., and Janssen, D. B. (1995) Eur. J. Biochem. 228, 403–407
17. Verschueren, K. H. G., Kingma, J., Rozeboom, H. J., Kalk, K. H., and Janssen, D. B., and Dijkstra, B. W. (1993) Biocatalysis 32, 9031–9037
18. Huang, C. Y. (1979) Methods Enzymol. 63, 54–64
19. Bloch, W., and Gorby, M. S. (1980) Biochemistry 19, 5008–5018
20. Hull, W. E., Halford, S. E., Gutfreund, H., and Sykes, B. D. (1976) Biochemistry 15, 1547–1561
21. Liu, R.-Q., Liang, P.-H., Scholten, J., and Dunaway-Mariano, D. (1995) J. Am. Chem. Soc. 117, 5003–5004
22. Studki, G., and Thüler, M. (1995) Environ. Sci. Technol. 29, 2339–2345
23. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950