Replacement of tryptophan residues in haloalkane dehalogenase reduces halide binding and catalytic activity

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Haloalkane dehalogenase catalyzes the hydrolytic cleavage of carbon-halogen bonds in short-chain haloalkanes. Two tryptophan residues of the enzyme (Trp125 and Trp175) form a halide-binding site in the active-site cavity, and were proposed to play a role in catalysis. The function of these residues was studied by replacing Trp125 with phenylalanine, glutamine or arginine and Trp175 by glutamine using site-directed mutagenesis. All mutants except Trp125—Phe showed a more than 10-fold reduced $k_{cat}$ and much higher $K_m$ values with 1,2-dichloroethane and 1,2-dibromoethane than the wild-type enzyme. Fluorescence quenching experiments showed a decrease in the affinity of the mutant enzymes for halide ions. The $^3$H kinetic isotope effect observed with the wild-type enzyme in deuterium oxide was lost in the active mutants, except the Trp125—Phe enzyme. The results indicate that both tryptophans are involved in stabilizing the transition state during the nucleophilic substitution reaction that causes carbon-halogen bond cleavage.

Keywords. Haloalkane dehalogenase; 1,2-dichloroethane; tryptophan; site-directed mutagenesis; hydrolase.

Haloalkane dehalogenase (DhlA) is a bacterial enzyme that hydrolyses various environmentally important haloalkanes to the corresponding alcohols (Keuning et al., 1985; Janssen et al., 1989). The three dimensional-structure of the protein was recently solved by X-ray crystallography (Franken et al., 1991; Verschueren et al., 1993b). It is composed of two domains. The main domain is formed by an eight-stranded $\beta$ sheet surrounded by $\alpha$ helices. On top of the main domain lies a cap domain formed by five helices and loops. The active-site cavity is located between these two domains. The main domain is structurally similar to several other hydrolytic enzymes, which were classified as $\alpha/\beta$ hydrolase fold enzymes. This group includes dieneolate hydrolase from Pseudomonas sp. B13, acetylcholine esterase from Torpedo californica, carboxypeptidase I1 from wheat, and lipase from Geotrichum candidum (Ollis et al., 1992).

In haloalkane dehalogenase, Asp124, His289 and Asp260 form a catalytic triad proposed to play a central role in the dehalogenating reaction (Fig. 1). The carbon-chlorine bond of 1,2-dichloroethane, the substrate on which the dehalogenase-producing Xanthobacter autotrophicus strain was originally isolated, is attacked by the carboxylate oxygen of the nucleophilic Asp124, which yields an ester as an intermediate (Verschueren et al., 1993a; Pries et al., 1994). Hydrolysis by a water molecule activated by His289 could then lead to cleavage of the intermediate. Asp260 is hydrogen bonded to His289, which may activate the histidine by stabilizing the positive charge that develops on the imidazole ring. From sequence similarities, it is likely that a haloacid dehalogenase from Moraxella (Kawasaki et al., 1992), a 2,3,5,6-tetrachlorohydroquinone dehalogenase from Pseudomonas paucimobilis (Nagata et al., 1993), and several epoxide hydrolases (Lacourciere and Armstrong, 1993; Pries et al., 1994; Arand et al., 1994) also belong to a group of $\alpha/\beta$ hydrolase fold enzymes that use an Asp as the nucleophile. There is also a conserved histidine at the position of the haloalkane dehalogenase His289 in these enzymes.

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Fig. 1. Proposed catalytic mechanism of haloalkane dehalogenase. (A) Nucleophilic attack of Asp124 on the $\text{Ca}$ of the alkylhalide, leading to formation of the covalent alkyl-enzyme intermediate. (B) His289-catalyzed hydrolysis of the intermediate.
Table 1. Activities of wild-type haloalkane dehalogenase and tryptophan mutants.

<table>
<thead>
<tr>
<th>Haloalkane deenzymegenase</th>
<th>Activity with 1,2-dibromoethane</th>
<th>Activity with 1,2-dichloroethane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\text{max}} )</td>
<td>( K_m )</td>
</tr>
<tr>
<td>Wild type</td>
<td>4.8</td>
<td>0.007</td>
</tr>
<tr>
<td>Trp125→Gln</td>
<td>0.31</td>
<td>0.19</td>
</tr>
<tr>
<td>Trp125→Phe</td>
<td>4.4</td>
<td>0.09</td>
</tr>
<tr>
<td>Trp125→Arg</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Trp175→Gln</td>
<td>0.54</td>
<td>13</td>
</tr>
</tbody>
</table>

* No activity detectable at 10 mM substrate. Data represent detection limits at 10 mM substrate, assuming first-order kinetics.

The three-dimensional structure of dehalogenase showed that in the active site the aromatic ring nitrogen atoms of two tryptophans, Trp125 and Trp175, point towards the cavity. Further X-ray crystallographic experiments and fluorescence measurements (Verschueren et al., 1993c) showed that halide ions could be bound between the two tryptophans. It was suggested therefore that both residues could be involved in the dehalogenation reaction. In this study, we further investigate the role of the tryptophan residues by site-directed mutagenesis. Activity and fluorescence measurements of Trp125 and Trp175 mutants were used to elucidate their role in catalysis. Glutamines were chosen since the amide nitrogen of the side chain of glutamine may occupy a position equivalent to the ring nitrogen of tryptophan, while the aromatic character is lost. Arginine on position 125 was chosen since this amino acid is present at the corresponding position in the Moraxella dehalogenase mentioned above, and because arginine occupies more space than glutamine. Phenylalanine was chosen to check the importance of the indole nitrogen. The results show that the mutations have a strong effect on halide binding and catalysis.

MATERIALS AND METHODS

Materials. Restriction enzymes and T4-DNA ligase were purchased from Boehringer. Halogenated compounds were obtained from Janssen Chimica or from Merck. The synthetic oligonucleotides were (from 5' to 3') GTCAGAGCAGGAGGCAGGAGATT (W125Q haloalkane dehalogenase); GGTGGTACAGGAGCCGGCCGATT ([W125F] haloalkane dehalogenase); GGTGGTACAGGAGCCGGCCGGATT ([W125R] haloalkane dehalogenase); TTACCGCCCAGAAATACGA ([W175Q] haloalkane dehalogenase) (introduced RsaI site indicated in italics; mutated codons in bold); they were obtained from Eurogentec.

Bacterial strains and plasmids. Plasmid pPF123 is a derivative of pTTQ18 (Pharmacia) with the dhlA gene under control of the tac promoter (Schanstra et al., 1993). The pELA and pELAF expression vector are derivatives of the pET-3d vector with the dhlA gene under control of the T7 promoter (Schanstra et al., 1993). Expression of dehalogenase from pELA and pELAF derivatives was achieved in Escherichia coli BL21(DE3) (Studier et al., 1990).

DNA manipulations. Standard DNA manipulation procedures were performed according to Sambrook et al. (1989). Sequences for [W125Q] haloalkane dehalogenase and [W175Q] haloalkane dehalogenase were produced in plasmid pPF123 with the PCR mutagenesis method of Nelson and Long (1989) on a Hybaid thermal reactor. The mutated dhlA DNA fragments were recloned in plasmid pELA for expression. The mutants W125F and W125R were constructed by the Kunkel (1985) method in the same vector as used for expression. Sequencing of DNA (Sanger et al., 1977) was performed with the Pharmacia sequencing kit using [α-32P]dATP[S].

Expression and purification of dehalogenases. E. coli BL21(DE3) expressing wild-type or mutant dehalogenase was grown at 30°C in Luria-Bertani medium containing 50 μg/ml ampicillin to an A660 of about 1.0. Cultivation was continued at 17°C to avoid formation of inclusion bodies (Schanstra et al., 1993), and isopropyl-thio-β-D-galactoside was added after 1 h as an inducer for dehalogenase expression. Cells were harvested after 16 h (A660 = 2), washed, resuspended in buffer A (10 mM Tris/H2SO4, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol) and sonicated for 10 s/ml cell suspension. Cell debris and other non-soluble material were removed by centrifugation at 150000×g for 60 min.

Dehalogenases were purified as described by DEAE-cellulose chromatography using a 10 cm×2 cm column with buffer A and a 400-ml gradient from 0 to 0.3 M (NH4)2SO4 in buffer A for elution. The pooled enzyme was dialyzed against 10 mM potassium phosphate buffer, pH 6.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol. A 10 mM to 150 mM potassium phosphate gradient was used for elution. The enzyme was pooled, concentrated by ultrafiltration over an Amicon PM10 filter, and if necessary further purified on a 60 cm×4 cm Sephacryl S200 column in buffer A. The purified proteins were more than 98% pure as judged by SDS/PAGE. The purified enzymes were stored at -20°C.

Activity measurements. Dehalogenase assays were performed colorimetrically at pH 8.2 as described previously (Janssen et al., 1989). Protein concentrations were determined with Coomassie brilliant blue. Kinetic constants with 1,2-dibromoethane and 1,2-dichloroethane were determined by initial-velocity measurements using gas chromatography to determine halothanol production rates or colorimetric assays for bromide liberation.

The effect of the presence of 3H2O on the activity of wild-type dehalogenase and mutant enzymes with 1,2-dibromomethane was measured at different molar ratios of 3H2O over 1H2O in water under the same conditions as above. Substrate concentrations (10 mM) far above \( K_m \) were used to assure \( V_{\text{max}} \) conditions, except for the W175Q enzyme, which was measured at 15 mM.

Fluorescence measurements. The binding of halide to haloalkane dehalogenase was followed by steady-state fluorescence measurements at 25°C on a SLM Aminco SPF500-C spectrofluorometer as described (Verschueren et al., 1993c). Mutant dehalogenase (1 μM) was diluted in buffer A. The excitation
Table 2. Dissociation constants of halide ions with wild-type enzyme and Trp mutants.

<table>
<thead>
<tr>
<th>Haloalkane dehalogenase enzyme</th>
<th>$K_d$ of $\text{Br}^- \text{ at}$</th>
<th>$\text{Cl}^- \text{ at}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.0</td>
<td>pH 8.2</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.07 5</td>
<td>0.49 57</td>
</tr>
<tr>
<td>Trp125→Gln</td>
<td>0.5 &gt;300</td>
<td>4.0 &gt;400*</td>
</tr>
<tr>
<td>Trp125→Phe</td>
<td>65 242</td>
<td>36 150</td>
</tr>
<tr>
<td>Trp125→Arg</td>
<td>0 500*</td>
<td>b &gt;400*</td>
</tr>
<tr>
<td>Trp175→Gln</td>
<td>100 &gt;400*</td>
<td>&gt;350* &gt;400*</td>
</tr>
</tbody>
</table>

* No significant or reproducible quenching observed at concentrations up to the concentration indicated.

b Not measured due to instability.

wavelength was 290 nm, and spectra were recorded in the range of 300–500 nm.

The dissociation constants (Ward, 1985) of the mutant proteins for chloride and bromide were determined using a spreadsheet program with linear regression. Alternatively, we used non-linear-regression fitting (SigmaPlot, Jandel Scientific) of the equation $(F_o-F)/F_o = f_a*[X^-]/(X^-+K_d)$, where $F$ is the observed fluorescence at halide concentration $[X^-]$, $K_d$ is the dissociation constant, and $f_a$ is the fractional accessibility, i.e. the fraction of the total fluorescence that is quenched at $[X^-] \geq K_d$. The accuracy was $\pm 10\%$ for the wild-type enzyme, but lower with most mutants due to enzyme instability in the presence of high concentrations of halide ($\pm 30\%$).

RESULTS AND DISCUSSION

Construction and purification of mutant enzymes. [W125Q] haloalkane dehalogenase and [W175Q] haloalkane dehalogenase were constructed by PCR, and the entire sequence of the mutated genes was verified. [W125F] haloalkane dehalogenase and [W125R] haloalkane dehalogenase were constructed by the Kunkel method, with initial screening for the introduction of a RsaI restriction site, followed by sequencing. The mutant dehalogenases were expressed and purified to more than 97% homogeneity according to our standard procedures. Fluorescence spectra were recorded and were similar for the wild-type and the mutant enzymes (data not shown).

Activities and $K_m$ values. The purified mutant enzymes were examined for their kinetic characteristics. Activity measurements (Table 1) showed that compared to the wild type the $V_{max}$ values for 1,2-dibromoethane were reduced 20-fold and 11-fold for the W125Q and W175Q mutant enzymes, respectively. The Michaelis-Menten constants for 1,2-dibromoethane were increased 27-fold and 2000-fold for the W125Q and W175Q mutant enzymes, respectively. The specificity constants of the [W125Q] haloalkane dehalogenase decreased more than 500-fold both for 1,2-dibromoethane and 1,2-dichloroethane.

The activity for 1,2-dichloroethane of the [W125Q] haloalkane dehalogenase was reduced more than the activity with 1,2-dibromoethane. A 35-fold reduction in $V_{max}$ and a 18-fold increase in $K_m$ with 1,2-dichloroethane were found. The W175Q mutant was even less active with 1,2-dichloroethane. Rates did not show detectable activity with either 1,2-dibromoethane or 1,2-dichloroethane.

Halide binding by mutant enzymes. Fluorescence measurements were performed with bromide and chloride at pH 6.0 and pH 8.2. The fractional accessibility, which is determined by the number of tryptophan residues that is accessible to the quenching agent, decreased from 0.3 to around 0.2 in both Trp→Gln mutants. This is in agreement with two of the total of six tryptophans being quenched by halide ions in the wild type and one of the five tryptophans in the mutants.

The calculated dissociation constants show that the affinity of the mutant enzymes for bromide and chloride ions had decreased from 7-fold to more than 1500-fold, depending on the mutant and on the pH (Table 2). Halides were bound stronger to the W125Q mutant than to the W175Q mutant, which was correlated with the lower $K_m$ values of the W125Q mutant for 1,2-dichloroethane and 1,2-dibromoethane. Fluorescence quenching of the W125Q mutant enzyme was irreproducible at pH 6, but the $K_m$ values at pH 8 were again much higher than in the wild type. No quenching of fluorescence of the catalytically inactive [W125R] haloalkane dehalogenase was observed with bromide or chloride. Halide binding generally increased at lower pH, both with wild-type and mutant enzyme. This can be explained by easier protonation of His289 which may be required to com-

![Fig. 2. Kinetic isotope effects of deuterium oxide on wild-type haloalkane dehalogenase and Trp125 and Trp175 mutants.](image-url)
pensate the negative charge on Asp124, which is close to the halide-binding site. The properties of these mutants indicate that interaction of Trp125 and Trp175 with the halogen is essential for the cleavage of the carbon-halogen bond.

Assuming that the reaction follows the scheme

\[ E + S \xrightarrow{h} E \cdot S \xrightarrow{k_4} E \cdot R \cdot X \xrightarrow{k_1} E + R + X \]

it follows that \( K_m = k_4/k_1 \), \( k_{cat} = k_4(k_2 + k_3) \), and \( k_{cat}/K_m = k_4/K_4 \). Thus, both an increase in the substrate-binding constant \( K_m \) and a decrease in the rate of reaction of bound substrate to the covalent intermediate \( (k_4) \) can explain the increased \( K_m \) of the mutants for 1,2-dichloroethane and 1,2-dibromoethane. The decrease in \( k_{cat} \) suggests a decrease of \( k_4 \).

Effect of deuterium oxide on rate. The proposed mechanism of haloalkane dehalogenase (Fig. 1) predicts that proton transfer and breakage of N-H or O-H bonds only occurs during hydrolysis of the alkyl-enzyme intermediate and during product (proton) release \( (k_4) \), but not during formation of the covalent intermediate \( (k_2) \). Therefore, a kinetic isotope effect of \( ^2\text{H}_2\text{O} \) is expected only if one of these last steps in the reaction cycle is significantly rate limiting.

With the wild-type enzyme and 1,2-dichloroethane, 1,2-dibromoethane, or 1-chlorobutane as the substrate, a strong \(^2\text{H}\) kinetic isotope effect on \( V_{\text{max}} \) was observed (Fig. 2A). For the W125Q mutant, no \(^2\text{H}\) isotope effect was observed with 1,2-dibromoethane, indicating that the cleavage of the carbon-halogen bond has become rate limiting in this mutant. The W125F enzyme still showed an isotope effect with 1,2-dibromoethane, for which the \( V_{\text{max}} \) was hardly reduced, but no longer with 1,2-dichloroethane, which was converted much more slowly than in the wild type. For the [W175Q]-haloalkane dehalogenase, an increase in activity rather than a decrease was found, suggesting that in this mutant the rate-limiting step is different from that in the wild type. The W175Q mutant showed a large inverse \(^3\text{H}_2\text{O} \) isotope effect with 1,2-dibromoethane. As the assays could only be performed with substrate concentrations up to the \( K_m \) the results cannot be directly compared with the wild-type activities.

In conclusion, the results suggest that both tryptophans are involved in catalysis, predominantly by substrate binding and transition-state stabilization during carbon-halogen bond cleavage. This does not seem to be the rate-limiting step in the wild-type haloalkane dehalogenase during conversion of 1,2-dichloroethane and 1,2-dibromoethane. The aromaticity of the ring and the indole nitrogen may both contribute to the interaction with the halogen.

Role of tryptophans in halide binding and leaving group stabilization. The electronegative aromatic indole nitrogens of the tryptophans in the active site of haloalkane dehalogenase can be expected to provide some polarization of the N-H bond, causing a slightly positive hydrogen that can interact with the halogen. A number of other halide-binding proteins have been identified, but they do not have tryptophan residues in the halide-binding site. In human color-vision pigments, a His and a Lys residue (Wang et al., 1993), or a Glu and a Lys (Kleinschmidt and Harosi, 1992) bind a chloride ion. In carbonic anhydrase II, a Thr is involved in bromide binding (Jönsson et al., 1993), while in pig pancreatic a-amylase two Arg and an Asn bind a chloride ion (Qian et al., 1993). An Arg is also involved in chloride binding in halorhodopsin which has a Kd with chloride of 60 nM (Ames et al., 1992).

That residues other than Trp may be involved in leaving-group stabilization of hydrolytic dehalogenases is suggested by the sequence of the Moraxella haloacid dehalogenase encoded by the dehH1 gene (Kawasaki et al., 1992). The enzyme has considerable sequence similarity to dehalogenase, including the catalytic triad residues, but the residue C-terminal of the tentative nucleophilic Asp is an Arg instead of a Trp (Fig. 3). Replacement of the corresponding Trp125 with Arg in haloalkane dehalogenase yielded an enzyme with no detectable activity, indicating that other interactions influence the nature of the required halide-binding residue. This could be related to differences in positioning of the side chains in the various enzymes.

We have observed that there is no quenching of tryptophan fluorescence of the haloacid dehalogenase of X. autotrophicus (Van der Ploeg et al., 1991) by substrates or halide ions, indicating a different halide-binding site in this enzyme.
Of the other known tentative αβ fold hydrolases that use an aspartate as the nucleophile, the tetrachlorodihydroquinone dehalogenase of *P. paucimobilis* UT2 (Nagata et al., 1993), and microsomal and cytosolic epoxide hydrolases (Arand et al., 1994; Pries et al., 1994; Lacourciere and Armstrong, 1993) do have a Trp residue next to the nucleophilic Asp (Fig. 3) but the second catalytic Trp of dehalogenase is not conserved in the sequence of these enzymes. We found that a Phe at this position yields a protein with good catalytic activity towards 1,2-dibromoethane, indicating that the aromatic character of the side chain is important for the interaction with the substrate.

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**REFERENCES**


