The Role of Spontaneous Cap Domain Mutations in Haloalkane Dehalogenase Specificity and Evolution*

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The first step in the utilization of the xenobiotic chlorinated hydrocarbon 1,2-dichloroethane by Xanthobacter autotrophicus is catalyzed by haloalkane dehalogenase (DhlA). The enzyme hydrolyses 1-haloalkanes to the corresponding alcohols. This allows the organism to grow also on short-chain (C_2-C_4) 1-chloro-n-alkanes. We have expressed DhlA in a strain of Pseudomonas that grows on long-chain alcohols and have selected 12 independent mutants that utilize 1-chlorobutane. Six different mutant enzymes with improved $K_m$ or $V_{max}$ values with 1-chlorobutane were obtained. The sequences of the mutated dhlA genes showed that several mutants had the same 11-amino acid deletion, two mutants carried a different point mutation, and three mutants had different tandem repeats. All mutations occurred in a region encoding the N-terminal part of the cap domain of DhlA, and it is concluded that this part of the protein is involved in the evolution of activity toward xenobiotic substrates.

Some bacterial strains of the species Xanthobacter autotrophicus (1, 2) and Ancylobacter aquaticus (3) are capable of growing on the synthetic chlorinated hydrocarbon 1,2-dichloroethane. Degradation proceeds via 2-chloroethanol, chloroacet-aldehyde, and chloroacetic acid to glycolate (1). Haloalkane dehalogenase (DhlA), a 310-amino acid cytoplasmic protein, catalyzes the first hydrolytic reaction step. The gene has been cloned and sequenced (4), and identical dehalogenases were detected in different strains that grow on 1,2-dichloroethane (3).

The three-dimensional structure of DhlA was recently solved by x-ray crystallography (5, 6). DhlA is composed of a globular main domain with an $\alpha/\beta$-hydrolase fold structure and a separate cap domain (5–7). The active site cavity is located between the two domains. X-ray crystallographic studies indicated that the enzyme catalyzes cleavage of halogenated compounds by nucleophilic displacement of the halogen by Asp260 (8). It was shown that during incubations of DhlA with substrate in H_2O/H_218O incorporation of $^{18}$O occurred both in Asp260 and product (9). This indicates that the covalent alkyl-enzyme intermediate, formed by nucleophilic displacement of the halogen by Asp260, is hydrolyzed by nucleophilic attack of a water molecule on the carbonyl carbon atom. His289 probably activates this water molecule by substrate binding, with assistance of Asp260 (8). Two tryptophan residues, Trp129 and Trp179, are involved in substrate and halide binding (8, 10).

The $\alpha/\beta$-hydrolase fold domain of DhlA is a structurally conserved fold shared by several hydrolytic proteins (7). The overall topology of DhlA consists of an eight-stranded $\beta$-sheet, with connecting $\alpha$-helices. The nucleophilic residues, either Ser, Cys, or Asp, are positioned on a sharp bend between the fifth $\beta$-strand and the following $\alpha$-helix. The positions of the other two active site residues, His289 and Asp260, are conserved as well. The proteins differ by the presence, position, and sequences of cap domains, and therefore this part of the proteins was suggested to influence substrate specificity (7).

Haloalkane dehalogenase is active with several 1-chloro-alkanes and $\alpha,\omega$-dichloro-alkanes, but there is hardly any activity with 1-chlorohexane or chloroalkanes of longer chain length (1). The enzyme has some activity with long-chain bromoalkanes (C_5-C_12), suggesting that the active site can accommodate longer substrates and that differences in binding affinities or reaction rates are responsible for the lower activity with long-chain chloroalkanes.

Since 1,2-dichloroethane is not known to occur or to be produced naturally, it is likely that an enzyme capable of hydrolyzing this compound evolved to its present form after industrial 1,2-dichloroethane production and emission started in 1922 (11, 12). No sequences that are similar to DhlA and from which the present enzyme could be derived are known, however. We decided to investigate how DhlA can evolve to convert new substrates by selecting spontaneous mutants that degrade 1-chlorohexane, an approach termed experimental enzyme evolution (13–15). The sequences and activity of the mutant enzymes that were obtained indicate that short direct repeats in the N-terminal part of the cap domain play an important role in the evolution of haloalkane dehalogenase specificity.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—Pseudomonas GJ31 is a chlorobenzene utilizing organism described earlier (4, 16). Escherichia coli JM101 was used as host strain for isolation of single strand DNA and sequencing. Plasmid pJ20 is a broad host range plasmid (4) derived from pLAFR1 (17). It contains the dhlA gene with its own promoter and constitutively produces DhlA in several Gram-negative bacteria (4).

**Selection of Mutants**—Selection of spontaneous mutant enzymes was achieved with the recombinant bacterial strain Pseudomonas GJ31(pJ20). Pseudomonas GJ31 is capable of growing on long-chain alcohols. The plasmid was introduced by triparental mating (17) and allowed the organism to grow on 1-chlorobutane by expression of the dhlA gene up to levels of 25% of the total cellular protein (4). Mutants of Pseudomonas GJ31(pJ20) that could utilize 1-chlorobutane as a growth substrate were selected (see Fig. 1) at 22°C by cultivation in 1-liter serum flasks containing 400 nI of synthetic medium (4). The medium contained 2 mM 1-chlorobutane, which did support growth of the recombinant, and 2 mM 1-chlorobutane, which is hardly hydrolyzed by the wild type enzyme and did not support growth. After 4 weeks of incubation, some cultures showed higher turbidities than a control containing no 1-chlorobutane. After three serial transfers in medium containing 2 mM 1-chlorobutane as the sole carbon source, mutants were purified on nutrient broth agar plates containing tetracycline (12 $\mu$g/ml).

**Sequencing**—The dhlA gene of mutant V152–153 was determined by cloning Ala-HindIII and Sau3A DNA fragments in phage M13mp18.
and M13mp19 (18), followed by dyeoxy sequencing (19). The other mutant dhIA genes were amplified by the polymerase chain reaction (20), followed by dyeoxy sequencing of the double-stranded polymerase chain reaction DNA. The primers used for sequencing are as follows (listed from 3' to 5' with the starting nucleotide number (4) given and c and n indicating coding and non-coding strand, respectively): 989 ATGCAGGCACGGCACG (c), 1503 ACCGCACTTCCTGCACG (c), 1590 CTGACCAGGTCGACCC (c), 1528 TCTGCTGCGGCAAAGTG (n), 1628 GAAAGGCCGACGATATC (c), 1322 TGCAACAGGAGTCCGGC (n).

**Purification of Haloalkane Dehalogenase**—Mutants of *Pseudomonas GJ31(pPJ20)* were grown at 30 °C in a mineral medium (1) containing 4 mM 1-chloroethane as the sole carbon source. After 4 days, cells were harvested by centrifugation, washed in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 1 mM β-mercaptoethanol, and sonicated after reassembly in this buffer. Mutant haloalkane dehalogenases were purified as described before (1).

**Determination of Dehalogenase Activities**—The activities of the mutant enzymes were tested with a variety of substrates by spectrophotometric measurement of the rates of halide production (1) using purified enzymes (wild type and mutants P168S, Δ164-174, and V152-153) or crude extracts (mutants D170H, V172-174, and V145-154). The concentration of 1-chlorohexane in the assays was 3 mM, the other substrates were used at 5 mM. The dehalogenase content of cell-free extracts was determined by Coomassie Brilliant Blue staining of the protein bands of SDS-polyacrylamide gels, and subsequent peak integration of scans of the gels was taken with a desktop scanner. Accuracy was better than 10%.

For the determination of *K*, *V*, and *k*/*K*, alcohol production was measured in 4.5-ml incubations containing 0.014 mM substrate in 50 mM Tris-HCl buffer, pH 8.2, and 10 milliunits of 1,2-dibromoethane dehalogenase or 100 milliunits of 1-chlorohexane or 1,2-dichloroethane dehalogenase. Samples were incubated for 15 min at 30 °C, and the amount of alcohol produced was determined on a Chrompack 438s gas chromatograph with a CPWax 52 CB column, using an ECD detector for 2-bromoethanol and a FID detector for 2-chloroethanol and 1-hexanol. The carrier gas was nitrogen (60 KPa), and the temperature program was 3-min isothermal at 45 °C followed by an increase to 200 °C at 10 °C/min. *K*, *V*, and *V* max values were calculated from the rates of alcohol production by nonlinear regression analysis using the Michaelis-Menten equation and the Enzfitter program of Leatherbarrow (21).

**RESULTS**

**Isolation of 1-Chlorohexane-utilizing Mutants**—The dehalogenase-expressing strain *Pseudomonas GJ331(pPJ20)* was found to be capable of growth on 1-chlorobutane, but not on 1-chlorohexane. The specific activity of purified haloalkane dehalogenase with 1-chlorohexane was 2.5 units/mg for this mutant, which apparently was not sufficient for growth, although the species was able to utilize 1-chloro-n-alkanes. To isolate mutants with improved dehalogenase activities, *Pseudomonas GJ31(pPJ20)* were grown at 30 °C in a mineral medium (1) containing 1-chlorohexane as the sole carbon source. The other two duplication mutant enzymes had the largest activities than the wild type for all chlorinated compounds except for 1-chloro-n-alkanes (Fig. 3). Activities of *in Vivo* Mutants—All mutants produced a dehalogenase with increased activity toward 1-chlorohexane and other C<sub>2</sub>-C<sub>6</sub> chloroalkanes. Summarizing, the *Δ164-174* deletion enzyme clearly had a higher activity, whereas mutants V172-174 and V145-154 produced dehalogenases that migrated somewhat slower than the wild type.

No plasmids could be isolated from the Δ170H and V145-154 mutants suggesting that the plasmid had integrated into the chromosome of *Pseudomonas GJ31*. These mutants still produced haloalkane dehalogenase, but the Δ170H mutant expressed the enzyme at a lower level than the wild type and the other mutants (Fig. 3).

**Sequences of Mutated Dehalogenases**—From sequencing the dhIA genes of the 1-chlorohexane utilizing mutants, six different mutants were identified. Only two mutants carried a single base substitution, leading to the mutations Asp<sup>168</sup>→ His, and Pro<sup>168</sup>→ Ser. The other mutants had much larger changes, being deletions or insertions. A large in frame deletion of 33 bp, causing an 11-amino acid deletion of Phe<sup>164</sup>-Ala<sup>165</sup>, was detected in six independently selected mutants. The duplications that were found were in frame direct repeats, varying in length from 6 to 30 bp. One mutant that carried an 8-bp duplication leading to a tandem repeat of Met<sup>152</sup>-Thr<sup>153</sup> was found twice. The other duplications were detected only once, and caused tandem repeats of Phe<sup>172</sup>-Ala<sup>174</sup> and Ile<sup>145</sup>-Asp<sup>146</sup>, respectively. The 9-bp duplication in mutant V172-174 encompassed the last 9 bp of the 33-bp deletion in mutant Δ164-174 (see also Fig. 2).

The deletions and duplications caused slight changes in electro

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**Fig. 1. Selection of mutants of haloalkane dehalogenase.** Plasmid pPJ20 is a broad host range vector, expressing the dhIA gene product. It was introduced in *Pseudomonas GJ31* by conjugation (4). Strains were cultivated as described under "Experimental Procedures."
is deleted in the Δ164-174 mutant enzymes. The other repeats detected in the mutants are located in the N-terminal part that encodes the N-terminal part of the cap domain (Fig. 2). In the other mutants have failed so far, probably due to disturbance of the ends of the deleted sequence with the amino acid sequence. Residues forming the active site cavity are observed with bromoalkanes, as illustrated by the high activity of 1-chlorohexane conversion is achieved. Probably, from the active site Asp174, the loss of 1-amino acid residue, which is located in a cis proline turn between helices 4 and 5, is replaced. This allows the formation of the cis proline turn. In the A164-174 mutant enzyme, Phe164 and Phe174 are lost. The other mutations thus have indirect effects.

The mutations were all located in a segment of the dhLA gene that encodes the N-terminal part of the cap domain (Fig. 2). In the mutant enzymes D170H, P168S, Δ164-174, and V172-174, the changes affect the structurally important salt bridge Asp170-Lys261 (Fig. 4). Although the Asp170 O61 is 15.6 Å away from the active site Asp188 O61, the loss of the Asp170-Lys261 salt bridge appears to change the active site cavity in such a way that improved 1-chlorohexane conversion is achieved. Probably, the active site cavity residues Phe164 and Phe174 are more mobile or have a slightly different position in the mutants (Fig. 4). In the P168S enzyme, the proline which is located in a cis proline turn between helices 4 and 5, is substituted. This allows rotation around the Cα-N bond of residue 168 and a change of the cis bond in a trans bond (22), which could lead to disturbance of the Asp170-Lys261 salt bridge that is in its proximity. In the V172-174 mutant enzyme, Phe172 is preceded by an Ala instead of Gly171, which has unusual ϕ-ψ-angles for Ala in the structure of wild type DhIA and forms the N-cap of helix 5, where Ala is less favored (23, 24). This will affect the cavity geometry via Phe164 or via the salt bridge by increasing the size of the cis proline turn. In the Δ164-174 mutant enzyme, Phe164 and Phe172, which point to the cavity, are deleted, as is the salt bridge residue Asp170. In the other two mutant enzymes, V152-153 and V145-154, cavity size may be influenced, but this is less clear since the amino acids surrounding the duplication are not in contact with the active site.

**DISCUSSION**

**Structure-Activity Relationships of Mutant Enzymes**—The mutants of haloalkane dehalogenase described here show that the cap domain of DhIA acts as an activity-modifying domain that can accommodate spontaneous mutations which can be selected in vivo and lead to alterations of substrate specificity. Since all mutants showed improved kinetics for 1-chlorohexane, the substrate binding site must have been altered in these enzymes such that improved binding and/or conversion is achieved. It is known that two tryptophans in binding of the halide moiety of the substrate (10), but other residues are probably responsible for determining the substrate specificity. Candidates for residues that interact with the R-group of the substrate are Phe158, Phe164, Phe172, Phe222, Pro252, Val256, Leu261, and Leu265 (5, 6). All mutations that influence substrate range were located in the part of the cap domain that is N-terminal of Trp237. This part is mainly stabilized by a surface located salt bridge between Asp170 and Lys261 of the main domain (Fig. 4) and hydrophobic interactions of 4 buried residues (Phe164, Phe164, Phe172, and Gly171). Since buried resi-
Note the differences in mobility between mutants V152-153; V145-154; and 3, three independent mutants A164-174; and from duplications. The different lanes indicate crude extracts from mutant proteins carrying the deletion of the cap domain could have undergone 1,2-dibromoethane activation (25) and because of the low number of hydrogen bonds and significant changes that allow improved binding or conversion of the position and role of the residues influenced by the mu-
tain A~p'~~-Lys~~' salt bridge, as indicated by examination of the template strand, but if strand slippage occurred during lagging strand synthesis (Fig. 5A), excision and mismatch repair occurred only in the template strand, but if strand slippage occurred during lagging strand synthesis (Fig. 5B), excision and mismatch repair took place in both strands, which seems less likely.

The occurrence of three different mutations that are direct tandem repeats of varying length, rather than insertions of random sequence, suggests a specific mechanism for their origin. Formation of direct repeats has been found to occur at sites flanked by short repeats that create DNA structures that are easily misaligned, recombinated and extended (30, 35, 36). The tandem repeats in the mutated dhIA genes are not flanked by repeated sequences, however, indicating that specific sequences are not required for their generation. The results suggest that an aspecific stuttering type of process can lead to the formation of short repeats, irrespective of existing repetitions.

Evolution of Haloalkane Dehalogenase—1,2-Dichloroethane is a synthetic compound. Although traces of it may be formed by haloperoxidases (37), it is unlikely that sufficient selective pressure to generate a dehalogenase for this chlorinated hydrocarbon existed until its industrial production and emission started in 1922. DhIA could have adapted to 1,2-dichloroethane by similar modifications of the cap domain as found here in the spontaneous mutants. Our results present experimental evidence that the cap domain, which is an excursion on the \(\alpha/\beta\)-hydrolase fold structure (7), is involved in the evolutionary modification of specificity, and that generation of repeats is an important mutational event during this process. The DNA coding for the N-terminal part of the cap domain of the wild type enzyme already contains two in frame direct repeats: a 15-bp perfect direct repeat encoding the sequence Val-Thr-Gln-Pro-

The table below shows the kinetic constants of dehalogenase mutants:

<table>
<thead>
<tr>
<th>Strain</th>
<th>1,2-Dibromoethane</th>
<th>1-Chloroethane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_v)</td>
<td>(k_m/K_v)</td>
</tr>
<tr>
<td>Wild type</td>
<td>6.0</td>
<td>0.0072</td>
</tr>
<tr>
<td>D170H</td>
<td>4.4</td>
<td>0.087</td>
</tr>
<tr>
<td>P168S</td>
<td>8.1</td>
<td>0.031</td>
</tr>
<tr>
<td>(\Delta)164-174</td>
<td>4.06</td>
<td>0.092</td>
</tr>
<tr>
<td>(\Delta)172-174</td>
<td>4.5</td>
<td>0.18</td>
</tr>
<tr>
<td>(\Delta)152-153</td>
<td>4.5</td>
<td>0.18</td>
</tr>
<tr>
<td>(\Delta)145-154</td>
<td>6.4</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Dyes serve as structural anchors and strongly resist translocation (25) and because of the low number of hydrogen bonds and salt bridges, structural changes will be easily accommodated by the N-terminal part of the cap domain. If the mutations influence the Asp~Phe-Lys~Phe~repeat, then this part of the cap domain could have undergone significant changes that allow improved binding or conversion of long chain chloroalkanes.

The mutations all influence the binding and/or reaction rate of various substrates (Tables II and III). The higher \(V_{max}\) for 1-chlorohexane shows that the reaction of bound substrate is faster in the mutants. Since this effect is observed with chlorinated but not with brominated substrates, formation of the covalent alkyl-enzyme intermediate must be the rate-limiting step in the wild type that is accelerated in the mutants. This may be caused by a better average positioning of the Cys of the bound substrate for nucleophilic attack by Asp~Phe~and stabilization of the reaction intermediate. The lower \(K_v\) could indicate that substrate binding is improved in the mutants, but it can also be explained by a higher rate of formation of the covalent intermediate. How 1-chlorohexane is bound is unknown, but a larger or more flexible hydrophobic cavity that increases the binding energy of the larger substrate is likely. The wild type enzyme also must have considerable flexibility to allow substrate binding, since the active site cavity (5, 6) is too small for binding 1-bromohexane, which is a substrate for wild type haloalkane dehalogenase. Furthermore, the cavity is buried which requires some cap domain residues to move for substrate entrance. Large substrate-induced structural changes are observed in the mobile surface loops of lipase of Rhizomucor miehei (26), human pancreatic lipase (27), and lipoprotein lipase (28, 29), which are also \(\alpha/\beta\)-hydrolase fold excursions (7).
FIG. 4. Part of the structure of haloalkane dehalogenase with the mutations indicated. Stereoepitaxial depiction of the active site of wild type haloalkane dehalogenase. Asp124 acts as the nucleophile (9), His289 and Asp280 are probably involved in the general-base catalyzed hydrolysis of the covalent intermediate, and Trp175 and Thr171 play a role in substrate binding and halide release (10). The two spheres represent water molecules in the active site cavity that occupy the substrate binding site (5). Side chains of the residues Asp124, Trp175, Phe182, and His289 are shown in italics.

FIG. 5. Proposed models for the formation of the A164-174 deletion during replication. Replication of the plasmid and transcription of dhlA are in the same direction (4, 34). The two direct repeats which are present in wild type dhlA are indicated by lines. The broken line indicates the 9-bp imperfect repeat that can be involved in strand slippage. A loop of DNA is excised and a gap (bold) is mismatch repaired to C. The result is a deletion of 33 bases. In A, leading strand synthesis during replication is indicated by the arrow. Mismatch repair occurs only in the template strand. In B, the arrow indicates lagging strand synthesis, and repair must occur in both strands. The slipped DNA is shown in italics.

process similar to the selection of the mutations that arose during cultivation on 1-chlorohexane, with a few additional fine tuning modifications leading to the present wild type DhlA. Thus, we hypothesize that the direct repeats in the DNA sequence encoding the N-terminal part of the cap domain are of recent evolutionary origin and were selected during the adaptation of an older dehalogenating enzyme to industrially produced 1,2-dichloroethane.

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REFERENCES