Crystallographic analysis of the catalytic mechanism of haloalkane dehalogenase

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Crystal structures of haloalkane dehalogenase were determined in the presence of the substrate 1,2-dichloroethane. At pH 5 and 4°C, substrate is bound in the active site without being converted; warming to room temperature causes the substrate's carbon-chlorine bond to be broken, producing a chloride ion with concomitant alkylation of the active-site residue ASP124. At pH 6 and room temperature the alkylated enzyme is hydrolysed by a water molecule activated by the His289-Asp260 pair in the active site. These results show that catalysis by the dehalogenase proceeds by a two-step mechanism involving an ester intermediate covalently bound at ASP124.

The putative active site is located between the two domains of the protein in an internal, predominantly hydrophobic cavity. ASP124, His289 and Asp260, which are located in this cavity, have been proposed to be the catalytic residues. They form a catalytic triad, with the triad residues occurring at the same topological positions as in other members of the α/β hydrolase-fold family, despite any clear sequence homology. To this family also belong acetylcholinesterase from Torpedo californica, dienelactone hydrolase from Pseudomonas sp. B13 (ref. 7), carboxypeptidase II from wheat and lipases. All members of this family have a similar core formed by an α/β sheet of eight β-strands connected by α-helices. In addition, they all have a small pocket near the amide nitrogen of the residue following the nucleophile, which could be the oxyanion hole, indicating that they may share a similar reaction mechanism. The proposed nucleophilic residue for the dehalogenase (ASP124) is quite different from that of the other enzymes (Ser or Cys), however, and the bond to be converted is a carbon-chlorine bond while the nucleophile is a chlorine atom.

The nitrogen-fixing hydrogen bacterium Xanthobacter autotrophicus GJ10 can grow on a medium containing 1,2-dichloroethane or 2-chloroethanol as the sole carbon and energy source. The organism is being investigated for application in the clean up of environmentally harmful halogenated compounds, some of which are industrially produced in large amounts for use as cleaning agents, pesticides and solvents. The first step in the degradation of these compounds by the bacterium is catalysed by haloalkane dehalogenase, an enzyme having an M, of 36,000 and a known nucleotide and amino-acid sequence. It converts l-haloalkanes into primary alcohols and a halide ion by hydrolytic cleavage of the carbon-halogen bond, with water as a co-substrate and without any need for oxygen or cofactors, at an optimal pH of 8.2. The crystal structure of the enzyme has been determined at 2.4 Å resolution and refined to 1.9 Å (ref. 27).

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hydrolysed (carbon-halogen) is rather different from the ester
ature of the active site of dehalogenase, there are two possible
reaction mechanisms for hydrolytic dehalogenation: the first
could be a nucleophilic substitution by the carboxylate anion of
Asp$_{124}$, resulting in a covalently bound intermediate ester that
could be a nucleophilic substitution by the carboxylate anion of
or amide bond cleaved by the other enzymes. From the architec-
ture by His$_{289}$as base (Fig. 1b), the carboxylate anion of
might subsequently be hydrolysed by a water molecule, activated
by His$_{289}$as base catalysis with a water molecule activated by His$_{289}$ (ref. 11; Fig. 1b).

To discriminate between these two possibilities and to com-
pare the dehalogenase catalytic mechanism with that of the other
members of the α/β hydrolase-fold family, we analysed the cata-
lytic pathway in dehalogenase as a function of pH and tempera-
ture by high-resolution X-ray crystallography. Both putative
dehalogenase catalytic mechanisms require that the His$_{289}$ side
chain is mainly deprotonated at the pH of optimal activity. A
Crystal structure determined at pH 8.2 shows that this is indeed
the case$^{27}$. But at low pH, with the His$_{289}$ side chain protonated
and no longer acting as a base, according to the first mechanism
the covalently bound intermediate could accumulate in the active
site, whereas by the second mechanism, substrate should bind
without being degraded. We therefore soaked dehalogenase crys-
tals in mother liquor containing the substrate 1,2-dichloroethane
without being degraded. We therefore soaked dehalogenase crys-
tals for three hours in mother liquor$^{12}$ with 10 mM 1,2-
dichloroethane molecule could be fitted. The final structure, refined at
2.4 Å resolution, shows that one chlorine atom of the substrate
covalently bound in the active site. From these struc-
tures we conclude that the reaction catalysed by the
dehalogenase proceeds through a two-step catalytic mechanism
with a covalently bound intermediate.

**Substrate binding**

Substrate was bound in the active site of dehalogenase by soak-
ing crystals for three hours in mother liquor$^{37}$ with 10 mM 1,2-
dichloroethane at pH 5.0 and 4 °C. A difference Fourier showed
clear density in the cavity in which a 1,2-dichloro-
ethane molecule could be fitted. The final structure, refined at
2.4 Å resolution, shows that one chlorine atom of the substrate
(Cl−) is bound to the ring nitrogen atoms of Trp$_{125}$ and Trp$_{175}$
(Cl−N distances are 3.6 Å and 3.2 Å, respectively) (Fig. 2a). Cl− lies near the intersection of the planes of the two tryptophan
rings, thus maximizing the favourable interactions with the
slightly positively charged hydrogen atoms of the aromatic ring
nitrogens. The second chloride atom (Cl−2) is not bound very
tightly and may be flexible, interacting with the side chains of
Phe$_{128}$, Phe$_{172}$ and perhaps Phe$_{164}$. The substrate’s C$_1$ carbon
atom, which is connected to Cl−1, is near the O$_{2α}$ atom of Asp$_{124}$,
at 3.8 Å. The protein atom closest to C$_2$ is C$_2$ of Val$_{126}$, at
3.3 Å; the Asp$_{124}$ O$_{2α}$ atom is at 3.6 Å. At pH 6.2 as well as
under the present conditions, the Asp$_{124}$ side chain is hydrogen-
bonded to the N$_{ε2}$ atom of His$_{289}$ (distance is 2.6-2.7 Å). At
pH 8.2, the pH optimum of the dehalogenase, this hydrogen bond is lost and both the Asp124 and His289 side chains have moved apart (by 0.6 Å). Assuming that the substrate position at pH 8.2 is the same as at pH 5.0, the Asp124 Oδ1 atom at pH 8.2 would be somewhat closer to the substrate's C1 atom (at about 3.7 Å), and could perform a nucleophilic attack. There are no water molecules close to one of the substrate's carbon atoms, the nearest water molecule being at 5.7 Å from C1. This makes a direct attack of a water molecule on the C1 carbon atom of the substrate extremely unlikely. However, there is a water molecule (H2O485 in Fig. 2a) present close to the Cγ atom of Asp124 (distance 2.8 Å), which could hydrolyse the proposed ester intermediate at Asp124. This water molecule is near the Nε2 atom of residue His289, which also hydrogen-bonds with its Nδ1 atom to the side chain of Asp260. A schematic view of the residues important for substrate binding is given in Fig. 3a.
The alkyl-enzyme
Soaking native dehalogenase crystals for one day in 10 mM 1,2-dichloroethane at pH 5 and at room temperature caused the accumulation of a covalent intermediate in the crystal (Fig. 2b). Clear extra density extends from the O$_{\text{ax}}$ atom of the side chain of Asp$_{124}$, and the covalently bound intermediate can easily be built into this density. A spherical electron density, consistent with the density for a chloride ion in an $(|F_{\text{obs}}|_{\text{substrate}} - |F_{\text{obs}}|_{\text{native}})$ difference Fourier is located between the side chains of two tryptophan residues (residues 125 and 175). This density is not connected to the density extending from the Asp$_{124}$ side chain, indicating that the carbon-halogen bond has been cleaved, and that a chloride ion and a covalently bound intermediate have been formed. This is clear evidence for the proposed two-step mechanism, with the Asp$_{124}$ side chain acting as the first nucleophile. The chloride ion is at the same position as the CI-1 atom in the bound substrate, and interacts with the ring nitrogen atoms of the Trp residues (at 3.5 Å and 3.2 Å from the N$_{\text{ax}}$ atoms of Trp$_{125}$ and Trp$_{175}$, respectively). The two carbon atoms of the covalently bound substrate make van der Waals contacts with Phe$_{128}$, Trp$_{175}$ and His$_{289}$. In contrast to the weak density observed for the CI-2 atom in the enzyme-substrate complex, the density for CI-2 in the alkyl-enzyme complex is unambiguous (Fig. 2b). CI-2 lies in the plane of the Phe$_{172}$ side chain and may be stabilized by interactions with the hydrogen atoms at the C$_{\gamma}$ and C$_{\text{ax}}$ atoms of this residue. The Asp$_{124}$ and His$_{289}$ side chains have barely moved relative to their position in the enzyme-substrate complex. The carbonyl oxygen of the ester intermediate (Asp$_{124}$ O$_{\text{ax}}$ in the free enzyme) is hydrogen-bonded to the peptide nitrogen atoms of residues 56 and 125, whereas the ester oxygen atom (Asp$_{124}$ O$_{\text{ax}}$) is hydrogen-bonded to the His$_{289}$ N$_{\text{ax}}$ atom. Also the water molecule close to the C$_{\gamma}$ atom of Asp$_{124}$ in the complex with substrate, is still present, although its electron density has decreased (H$_2$O$_{413}$ in Fig. 2b). Assuming the temperature factor of this water to be similar to those of the surrounding atoms, its occupancy refined to about 0.4. This strongly suggests that, under our experimental conditions, this water molecule is slowly acting as the nucleophile in the second reaction step, in which the ester intermediate is hydrolysed, without being replenished. This water is completely secluded from the solvent and it is probably replenished from the active site cavity (see below). Some new electron density is already observed for an incoming water molecule near the CI-2 atom. In agreement with the partial occupancy of the hydrolytic water, we estimate the occupancy of the covalently bound intermediate to also be about 0.4 to 0.5. Figure 3b gives a schematic overview of the interactions of the alkyl group with the enzyme.

After hydrolysis of the alkyl intermediate
Soaking native dehalogenase crystals for two days at room temperature in a mother liquor with 10 mM 1,2-dichloroethane at pH 6.2 results in a complex of dehalogenase with chloride as the product (Fig. 2c). There is no longer any density corresponding to the covalently bound intermediate, or the product 2-chloroethanol. But we still observe a spherical density consistent with a chloride ion located between the side chains of Trp$_{125}$ and Trp$_{175}$ (Fig. 2c). The O$_{\text{ax}}$ atom is also hydrogen-bonded to the His$_{289}$ N$_{\text{ax}}$ atom, as we found in the native dehalogenase structure at pH 6.2 (refs 4, 27). To our surprise, although the density of the hydrolytic water molecule is clear near the C$_{\gamma}$ atom of Asp$_{124}$ in the native structure at pH 6.2 and in the enzyme-substrate complex and the alkyl-enzyme, it has almost completely disappeared in the

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**FIG. 3** Schematic view of the interactions of the active site residues of dehalogenase a, with the substrate, before the start of the reaction; b, with the alkyl intermediate and the chloride ion, during the reaction; c, with the chloride ion and the water molecules, after hydrolysis.
present structure (H₂O₃₉₉ in Fig. 2c). This indicates that this is the water molecule essential for the hydrolysis of the covalently bound intermediate, but that it is replenished after hydrolysing the alkyl-enzyme only very slowly under the conditions applied. The water molecule most likely to replenish the hydrolytic water is the one hydrogen-bonded to the Asp₁₂₄ side chain. Presumably at pH 6.2, the hydrogen bond between the Asp₁₂₄ and His₂₈₉ side chains is strong enough to limit the mobility of Asp₁₂₄ and to restrict the accessibility to the binding site of the hydrolytic water molecule. Indeed, if the experiment is done at pH 8.2, we find that the hydrolytic water molecule has been (partly) replenished. Interestingly, in contrast to the native dehalogenase structure at
pH 8.2, in which there is no hydrogen bond between the Asp₁₂₄ and His₂₈₉ side chains, a hydrogen bond exists between Asp₁₂₄ and His₂₈₉ even at pH 8.2 if a chloride ion is present in the active site. This suggests that the release of chloride from the substrate increases the pKₐ of the His₂₈₉ side chain, thereby facilitating its function as a general base to activate the hydrolytic water molecule.

Discussion

By variation of pH and temperature in soaking experiments of native haloalkane dehalogenase crystals with the substrate 1,2-dichloroethane, we succeeded in trapping different stages in the enzyme's reaction pathway. The first step of the reaction, the formation of the enzyme-substrate complex, could be trapped at low pH and low temperature. Increasing the temperature resulted in the accumulation of a covalently bound intermediate, and increasing, in addition, the pH to 6.2 allowed the release of the alcohol product but left the chloride product still bound in the active site. The reaction intermediates that we observe are reasonably stable under the experimental conditions. Their lifetimes are long enough to allow collection of diffraction data sets by standard methods over two-day periods without completely being converted. The resulting structures show the intermediates in significant occupancies.

Our experiments confirm the two-step catalytic mechanism proposed earlier, in which Asp₁₂₄ is the nucleophile in the first step of the reaction and a water molecule hydrolyses the covalent ester intermediate in the second step (see Fig. 4 for a summary of the reaction mechanism). In this respect they are consistent with the reaction mechanisms in other α/β hydrolase-fold enzymes, in which a covalent intermediate is also formed that is hydrolysed by a water molecule (for example, acetylcholinesterase and serine carboxypeptidases). But whereas in these other enzymes the same carbonyl carbon atom of the substrate molecule is attacked twice (first by the enzyme's nucleophile (Ser or Cys), and subsequently by a water molecule), in dehalogenase two different atoms are targets of nucleophilic attack; first the C₁ carbon atom of the substrate is attacked by the O₄α atom of Asp₁₂₄ to form a covalently bound ester, and next this ester is hydrolysed by a water molecule that attacks the C₄ atom of the Asp₁₂₄ residue. As a consequence, the dehalogenase reaction pathway proceeds through completely different transition states, each with its own stabilization. In the first step, the transition state is presumably a penta-coordinated C₁ carbon atom of the substrate, as occurs in SN₂ substitution reactions at sp³ carbon atoms, with the negative charge developing on the sp³ carbon atoms of the Trp₁₂₅ and Trp₁₇₅ side chains. This step is unique for the dehalogenase. The second reaction step proceeds through a tetrahedral intermediate at the C₄ atom of Asp₁₂₄. The transition states for the formation and breakdown of this tetrahedral intermediate may be stabilized by interactions with the peptide nitrogen atoms of Gly₁₈₀ and Trp₁₂₅, which are in the small oxanion pocket. The O₄α atom of Asp₁₂₄, which is negatively charged in the tetrahedral intermediate, hydrogen-bonds with these backbone NH groups in all the native and complexed dehalogenase structures analysed so far. All α/β-fold hydrolases have such a small pocket in common, near the amide nitrogen of the residue following the nucleophile. In the wheat serine carboxypeptidase, the peptide nitrogen of Gly₁₅₃ was suggested to be part of the oxanion-binding site. It is located at the C terminus of β-strand 3 (α/β hydrolase-fold numbering) at a position equivalent to that of Gly₁₈₀ in dehalogenase. In addition, in dehalogenase the O₄α atom of Asp₁₂₄ is positioned very close to the axis of helix C, at the N-terminal side of the helix. Thus, the negative charge developing on the O₄α atom may be further stabilized by the α-helix dipole and β-strand 5, the nucleophilic residue, and helix C form the so-called nucleophile elbow, which is the most conserved structure in all α/β hydrolases. Therefore it seems likely that the method of stabilization of the tetrahedral intermediate is the same for all α/β hydrolases, with conservation of the actual mechanism for the hydrolysis of the intermediate ester. In agreement with this is the observation that the interatomic interactions in the active sites are very similar. Dehalogenase extracts a proton from the hydrolytic water molecule at the N₂ of the active-site histidine, while Asp₁₂₀ interacts with the N₂ atom of His₂₈₉ through a syn-type hydrogen bond. Identical interactions are present in the active sites of dienelactone hydrolase, serine carboxypeptidase, Geotrichum candidum lipase and acetylcholinesterase, although these last two enzymes have a glutamic acid interacting with the histidine instead of an aspartic acid residue. Moreover, in the members of the α/β hydrolases with an Asp-His interaction, the plane of the Asp side chain makes an angle of ∼45° with the plane of the imidazole ring, unlike in the serine proteases, in which the carboxylate side chains are nearly coplanar with the imidazole ring.

Normally, the lifetime of reaction intermediates in wild-type enzymes is too short for crystallographic observation and millisecond Laue crystallography or site-directed mutagenesis have to be used. Here, the combination of low pH and low temperature, and the disparity between alkylation and dealkylation mechanisms in the dehalogenase, with dealkylation as the rate-limiting step under our conditions, allowed us to accumulate different reaction intermediates in the crystals and to analyse these intermediates by conventional protein crystallographic methods. We hope to clarify further details of the reaction mechanism using appropriate mutations.