Chapter 2: Structure and mechanism of bacterial dehalogenases: Different ways to cleave a carbon-halogen bond

René M. de Jong & Bauke W. Dijkstra

Abstract

Dehalogenases make use of fundamentally different strategies to cleave carbon-halogen bonds. The structurally characterized haloalkane dehalogenases, haloacid dehalogenases, and 4-chlorobenzoate-CoA dehalogenases use substitution mechanisms that proceed via a covalent aspartyl intermediate. Recent X-ray crystallographic analysis of a haloalcohol dehalogenase and a trans-3-chloroacrylic acid dehalogenase provide detailed insight into a different intramolecular substitution mechanism and a hydratase-like mechanism, respectively. The available information on the various dehalogenases supports different views on the possible evolutionary origins of their activities.

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Introduction

From the beginning of the past century, halogenated hydrocarbons have been extensively applied in industry and agriculture. Decades after the start of their widespread use, evidence started to accumulate that some of these xenobiotic halogenated compounds are highly toxic. Notorious examples are dioxins and polychlorinated biphenyls (PCBs), but also halogenated solvents like for instance 1,2-dichloroethane have been classified as probably carcinogenic to humans (Safe, 1994; Salovsky et al., 2002). Because of these noxious properties, many of these organohalogenes have now been banned from use and have been replaced by environmentally less harmful compounds.

Halogenated compounds are, however, not only of anthropogenic origin. Over 1500 organohalogenes are known that are produced naturally (Odberg, 2002; Balschmitter, 2003). They range from volatile compounds such as methylchloride to antibiotics like vancomycin and chloramphenicol. Insight into the enzymes that incorporate halogens into organic compounds is rather limited (van Pée and Unversucht, 2003). This strongly contrasts with the large amount of detailed information on enzymes that degrade halogenated hydrocarbons, releasing the halogens as halide ions.

In this review we focus on bacterial dehalogenases. These enzymes make use of a variety of distinctly different catalytic mechanisms to cleave carbon-halogen bonds. X-ray structures of haloalkane dehalogenases, haloacid dehalogenases, and 4-chlorobenzoyl-CoA dehalogenase demonstrated the power of substitution mechanisms that proceed via a covalent aspartyl intermediate (Verschueren et al., 1993; Benning et al., 1996; Hisano et al., 1996; Ridder et al., 1999). Recent structural characterizations of a haloalcohol dehalogenase and a trans-3-chloroacrylic acid dehalogenase reveal the details of two other elegant catalytic strategies, which exploit catalytic mechanisms from homologous enzymes in a different chemical context (de Jong et al., 2003; de Jong et al., submitted). The available information on the various dehalogenases supports different views on the possible origins of their individual activities towards presumed anthropogenic halogenated substrates.
Figure 1. The bacterial degradation routes of four different halogenated compounds, in which the five families of dehalogenases discussed in this chapter facilitate the enzymatic cleavage of the carbon-halogen bonds.

**Dehalogenase families**

**Haloalkane dehalogenases**

Haloalkane dehalogenases cleave the carbon-halogen bond in halogenated aliphatic hydrocarbons (Figure 1A). The haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 was the first dehalogenase of which the crystal structure was determined (Franken et al., 1991), but later also other structures have become available (Newman et al., 1999; Oakley et al., 2002). The structure consists of two domains, a main domain and a cap domain (Figure 2A). The main domain consists of a mostly parallel eight-stranded \( \beta \)-sheet (only the second \( \beta \)-strand is antiparallel), connected by \( \alpha \)-helices on both sides of the \( \beta \)-sheet. The cap domain is \( \alpha \)-helical. This fold is the hallmark of the superfamily of \( \alpha/\beta \)-hydrolases, to which, besides the haloalkane dehalogenases, also lipases, esterases, carboxypeptidases, and acetylcholinesterases belong (Ollis et al., 1992; Heikinheimo et al., 1999; Nardini & Dijkstra, 1999). These latter enzymes catalyze the hydrolysis of ester and amide bonds via a two-step nucleophilic acyl substitution mechanism similar to that of serine proteases (see *e.g.* Dodson and Wlodawer, 1998;
Jaeger et al., 1999). In the first step, the serine or cysteine of a Ser/Cys-His-Asp/Glu catalytic triad functions as a nucleophile that attacks the $sp^2$-hybridized carbonyl carbon atom of the scissile ester/amide bond, resulting in a covalently bound ester intermediate. Subsequently, a water molecule activated by the His/Asp or His/Glu pair hydrolyzes this ester intermediate in the second step.

Haloalkane dehalogenases use a very similar two-step catalytic mechanism, except that the nucleophile is an aspartate instead of a serine/cysteine residue, which, in an $S_N2$ type substitution mechanism, attacks the halogen-bearing $sp^3$-hybridized carbon atom. This leads also to a covalently bound ester intermediate that can be easily hydrolyzed by attack of a His-activated water molecule on the $Cγ$ atom of the aspartate (Verschueren et al., 1993). Thus, the substitution of the serine/cysteine of an $α/β$-hydrolase by an aspartate confers on the enzyme an essential prerequisite to hydrolyze carbon-halogen bonds in haloalkanes. In addition, haloalkane dehalogenases contain a halide-binding site to facilitate the dehalogenation reaction.

**Figure 2.** A) Structure of haloalkane dehalogenase from *X. autotrophicus* (Franken et al., 1991), showing the nucleophilic aspartate and a bound chloride ion. B) Dimeric structure of haloacid dehalogenase with a covalently bound intermediate (Ridder et al., 1999). C) Schematic of the catalytic mechanisms of haloalkane and haloacid dehalogenases.
During recent years, computational studies have unravelled the details of the catalytic mechanism. In the enzyme, the nucleophilic aspartate is nearly entirely positioned in a “near attack conformation”, with the attacking oxygen of the aspartate in line with the C-Cl bond of the substrate, at a distance favorable for attack of the halogen-bearing carbon atom. The correct positioning accounts, however, only for 25% to the lowering of the transition state free energy (Shurki et al., 2002; Hur et al., 2003). The remaining 75% probably originates from contributions of the halide-binding residues to transition state stabilization (Boháč et al., 2002) and from the half-hydrophilic/half-hydrophobic nature of the active site, which facilitates both the activation of the nucleophile and the departure of the leaving group. Computational quantification of these contributions is presently beyond reach (Hur et al., 2003).

Haloacid dehalogenases

Haloacid dehalogenases catalyze the hydrolysis of α-halogenated carboxylic acids, such as 2-chloroacetate, which is an intermediate in the degradation of 1,2-dichloroethane (Figure 1A). They are members of the haloacid dehalogenase (HAD) superfamily (Koonin and Tatusov, 1994; Ridder et al., 1999), to which also magnesium-dependent phosphatases and P-type ATPases belong. The haloacid dehalogenases are dimers with two- or three-domains per subunit: a core domain with a Rossmann-fold-like six-stranded parallel β-sheet flanked by five α-helices, a sub-domain consisting of a four-helix bundle, and, in some enzymes, a dimerization domain of two anti-parallel α-helices (Figure 2B) (Hisano et al., 1996; Ridder et al., 1997). This fold is completely different from the α/β-hydrolase fold of the haloalkane dehalogenases. Like the latter enzymes, the haloacid dehalogenase utilizes an aspartate-based catalytic mechanism that proceeds via a covalent intermediate (Figure 2C), but there is no histidine to activate a nucleophilic water molecule. Also, the halide-binding site is very different (Ridder et al., 1999). How the water molecule is activated is not known, but it has been suggested that another aspartate in the active site fulfills this function (Ridder et al., 1999). Intriguingly, at least one other unrelated haloacid dehalogenase family exists (Hill et al., 1999; Marchesi & Weightman, 2003), which was shown to bypass the classic covalent ester intermediate (Nardi-Dei et al., 1999). This suggests that enzymes with different haloacid dehalogenating strategies have evolved from different enzyme precursors, but unfortunately, crystallographic data that could provide structural evidence on the mode of action of these type II haloacid dehalogenases is still unavailable.
The 4-chlorobenzoyl-Coenzyme A (CoA) dehalogenase from Pseudomonas sp. strain CBS-3 is a homotrimer, of which each subunit folds into two domains. The N-terminal domain contains a 10-stranded $\beta$-sheet, forming two nearly perpendicular layers, which are flanked by $\alpha$-helices (Figure 3A). The C-terminal domain is composed of three amphiphilic $\alpha$-helices, and is primarily involved in trimerization. Thorough characterization of the enzyme has revealed the details of the mechanism by which a halogen is displaced from the aromatic ring of 4-chlorobenzoate, a degradation product of PCBs (Figure 1D) (Benning et al., 1996; Luo et al., 2001; Dong et al., 2002). The mechanism also proceeds via a covalent aspartyl intermediate. First, the substrate is ligated to CoA by 4-chlorobenzoate CoA ligase. The CoA-ligated product is then bound by the 4-chlorobenzyl-CoA dehalogenase, with the enolate anion of the thioester link of the CoA-ligated substrate stabilized by two backbone NH groups. This induces a partially positive charge on the halogen-bearing carbon atom, which makes it susceptible to nucleophilic attack by the aspartate (Figure 3B) (Luo et al., 2001). The $S_{N}Ar$-type substitution results in a covalent Meisenheimer intermediate, which was recently confirmed by Raman spectroscopic measurements (Dong et al., 2002). Chloride is expelled upon restoration of the aromatic ring system, producing a second arylated enzyme intermediate that is subsequently hydrolyzed by attack on the $C_\gamma$ atom of the nucleophilic aspartate by a histidine-activated water molecule, yielding 4-hydroxybenzoyl-CoA (Zhang et al., 2001; Lau et al., 2002).

The stabilization of the acyl-CoA enolate anion intermediate is shared with other hydratases, isomerases and thioesterases of the crotonase (or enoyl-CoA hydratase) superfamily (Holden et al., 2001) but the nucleophilic aspartate is only present in the dehalogenase. Like in haloalkane and haloacid dehalogenases, the aspartate confers to the enzyme its unique capability to hydrolyze the bond between a halogen and an aromatic carbon atom.
The enzymes discussed above illustrate the power of aspartate-mediated substitution mechanisms to hydrolyze organohalogens. However, several other dehalogenases exist that use completely different catalytic strategies. For instance, halorespiring anaerobic bacteria contain reductive dehalogenases, but unfortunately their structures are not yet known (Wohlfarth and Diekert, 1997; Furukawa, 2003). In contrast, X-ray structures of a haloalcohol dehalogenase and the trans-3-chloroacrylic acid dehalogenase CaaD have recently provided detailed insight into two other fundamentally different dehalogenation strategies.

**Haloalcohol dehalogenases**

Several bacteria contain enzymes that are able to displace a halogen from a vicinal haloalcohol substrate. One such enzyme is the haloalcohol dehalogenase HheC from *Agrobacterium radiobacter* AD1, which plays a role in the degradation of 1,3-dichloro-2-propanol (Figure 1B). HheC is a homotetrameric enzyme (Figure 4A): the monomer is homologous to that of members of the widespread short-chain dehydrogenase/reductase (SDR) family. SDR-enzymes are redox enzymes that catalyze alcohol-ketone conversions.
of a wide variety of alcohols, steroids and sugars (Filling et al., 2002; Oppermann et al., 2003). They have the well-known dinucleotide-binding Rossmann fold (Rossmann et al., 1974) and a Ser-Tyr-Lys/Arg catalytic triad. HheC shares the fold and catalytic triad of the SDR-family, but lacks the characteristic dinucleotide-binding Gly-X-X-Gly-X-Gly motif (Figure 4D) (van Hylckama Vlieg et al., 2001; de Jong et al., 2003). Instead, several larger residues replace the smaller ones of the motif, thus filling up the space of the cofactor-binding site. In this way, a spacious halide-binding site is created (Figure 4B). The enzyme uses an intramolecular \( S_N^2 \)-type substitution mechanism, in which the secondary hydroxyl group of the haloalcohol substrate is deprotonated by the tyrosine of the conserved Ser-Tyr-Arg catalytic triad acting as a base (Figure 4C). The hydroxyl oxygen concomitantly substitutes the vicinal halogen to yield the corresponding epoxide product, a proton and a chloride ion. Thus, while the aspartate-dependent dehalogenases discussed above catalyze a two-step hydrolysis of the carbon-halogen bond, the presence of a hydroxyl function vicinal to the halogen allows the one-step, intramolecular substitution of the halogen by the hydroxyl.

3-Chloroacrylic acid dehalogenases

Like 4-chlorobenzoyl-CoA dehalogenases, the chloroacrylic acid dehalogenases displace a halogen from an sp\(^2\)-hybridized carbon atom. The cis- and trans-3-chloroacrylic acid dehalogenases from \textit{Pseudomonas pavonaceae} 170 (Poelarends et al., 2001) play a role in the degradation of 1,3-dichloropropene, a compound applied in agriculture to kill plant-infecting nematodes (Figure 1C). After conversion of trans-1,3-dichloropropene by a haloalkane dehalogenase, oxidation of the trans-1-chloro-3-hydroxypropene product yields \textit{trans}-3-chloroacrylate. The \textit{trans}-3-chloroacrylic acid dehalogenase (CaaD) converts this product into malonate semialdehyde, a chloride ion and a proton. Subsequent decarboxylation of malonate semialdehyde yields acetaldehyde, which can be used as an alternative growth substrate.

The X-ray structures of the native and an inactivated form of \textit{trans}-3-chloroacrylic acid dehalogenase (CaaD) have recently been solved (de Jong et al., submitted). The enzyme is a trimer of \( \alpha \beta \) heterodimers (Figures 5A, 5B). Its fold is similar to that of 4-oxalocrotonate tautomerase (4-OT), a member of the tautomerase superfamily, which contains isomerases and tautomerases (Whitman, 2003). However, whereas in the isomerases and tautomerases the catalytic Pro-1 is in a hydrophobic environment, in CaaD the corresponding Pro-1\( ^\beta \) is in a hydrophilic active site near a buried glutamate. This
has a major effect on the pKₐ of the proline residue: in 4-OT the proline has a strongly reduced pKₐ (Czerwinski et al., 2001), whereas in CaaD the pKₐ is normal. This has consequences for the catalytic role of the proline: in the isomerases and tautomerases it functions as a general base, but in CaaD it functions as a general acid.

Figure 4. A) Tetrameric structure of haloalcohol dehalogenase (de Jong et al., 2003) with a bound chloride ion and a bound epoxide product. B) Detailed view of the halide binding site. Trp249* comes from another subunit. Dashed lines indicate hydrogen bonds. C) Schematic of the catalytic mechanism of haloalcohol dehalogenase. D) Sequence comparison of the haloalcohol dehalogenases HheC, HheA, and HheB with 4 SDR-family members indicated by their PDB entry number. 1FMC is an NAD-dependent enzyme, while 1YBV, 1AE1, and 2AE2 are NADP-dependent. An aspartate or glutamate in one of the boxed positions is indicative of an NAD-dependent enzyme, whereas NADP-dependent enzymes have 1 or 2 arginines or lysines in different positions. NAD-dependent SDR subfamilies are defined among others by the position of the aspartate (position 1, 2 or 3 in the box).
Figure 5. A) Heterohexameric structure of trans-3-chloroacrylic acid dehalogenase with a covalently bound malonyl adduct to Pro-1β (de Jong et al., in press). B) Structure of the trans-3-chloroacrylic acid dehalogenase monomer with a covalently bound malonyl adduct to Pro-1β. C) Stereo view of the active site showing the bound adduct. The red and pink main chains indicate chains from the α and β subunits, respectively. D) Schematic of the catalytic mechanism of trans-3-chloroacrylic acid dehalogenase.
The role of the Pro-1β in CaaD became clear from the crystal structure of CaaD inactivated by the suicide substrate 3-bromopropynoate (Figure 5C) (de Jong et al., submitted). This compound inactivates the enzyme by forming a covalent malonyl adduct with the proline residue. This modification is the result of the hydration of the carbon-carbon triple bond of the inhibitor via a Michael addition, catalyzed by the active site glutamate, which activates a water molecule, and by the proline, which donates a proton. The reactivity of the triple bond is increased by electrostatic interactions of its conjugated carboxylate group with two arginine residues. In the case of 3-bromopropynoate the product rearranges to a reactive acyl bromide, which forms a covalent bond with the deprotonated proline. In contrast, the substrate trans-3-chloroacrylate is converted to an instable halohydrin that decomposes to form malonate semialdehyde, a chloride ion and a proton (Figure 5D).

Thus, the conjugated system of the carbon-carbon double bond and the carboxylate group enables the dehalogenase to hydrate the substrate via a conjugate addition reaction, followed by spontaneous dehalogenation of the product. The active site glutamate and proline thus form the basis of a previously unidentified hydratase activity in the tautomerase superfamily.

**On the evolution of dehalogenating activities**

The structural characterization of several bacterial dehalogenases has provided detailed insight into their evolutionary relationship to other enzyme families. Some dehalogenases may have evolved from superfamily members with distinctly different activities, analogous to the creation of low-level crotonase activity in 4-chlorobenzoyl-CoA dehalogenase by a double glutamate mutation (Xiang et al., 1999). Alternatively, dehalogenation activity may emerge alongside an original enzyme activity. This is illustrated by the bacterial muconate lactonizing enzymes, some of which can also dehalogenate chlorinated muconates. A few amino acid substitutions can account for this dehalogenating activity (Kajander et al., 2003). Although these case studies nicely illustrate that minimal changes can drastically alter enzyme activities, they do not provide compelling evidence on the origins of the various isolated dehalogenases.

The recent adaptation of a precursor enzyme in response to large concentrations of a xenobiotic halogenated substrate may seem a reasonable mechanism for the emergence of these activities, but seems difficult to reconcile with the worldwide spread of some of the enzymes. Conserved gene clusters containing haloalkane-utilizing catabolic
pathways have been isolated from bacterial strains from three different continents (Poelarends et al., 2000). Although horizontal gene transfer and/or integrase-dependent gene acquisition can help in the local spread in bacteria (Poelarends et al., 2000), a global diffusion by this mechanism seems unlikely. However, the presence of haloalkane dehalogenases in various parasitic Mycobacterium strains, which colonize both animal tissues and the free environment, could suggest a possible role of parasitic microorganisms in a worldwide distribution mechanism (Jesenská et al., 2000).

The global distribution of these enzymes could also support a pre-industrial origin. Many different organohalogens occur naturally, suggesting that corresponding dehalogenating activities exist in nature. Even in the case of highly toxic dioxins an anaerobic bacterium has been isolated that can grow on them (Bunge et al., 2003). Dioxins were long believed to be exclusively produced by the recent industrial activity of mankind (Alcock and Jones, 1996), but they have entered the environment also by natural processes (Hoekstra et al., 1999) and by the pre-industrial domestic burning of peat (Meharg and Killham, 2003).

Although all dehalogenases that have been structurally characterized up to now are members of existing enzyme superfamilies, their sequences are highly divergent from superfamily members without dehalogenase activity. However, a structure-based sequence alignment of haloalcohol dehalogenases and SDR enzymes demonstrates a significant sequence identity of up to 30 to 35 % (de Jong et al., 2003). Two families of haloalcohol dehalogenases can be discerned that are related to different coenzyme-based SDR subfamilies, indicating that the two dehalogenase families independently originated from two different NAD-binding precursors, rather than NAD(P)H-binding precursors (Figure 4D). It is conceivable, that the haloalcohol could have bound in the active site of such an SDR enzyme, whereupon the catalytic tyrosine fortuitously facilitated the intramolecular substitution of the halogen by deprotonating the intramolecular hydroxyl group. The dehalogenase activity could have been improved as a result of evolutionary optimization of this rudimentary promiscuous activity. Catalytic promiscuity has been discovered in various other enzyme superfamilies that use similar structural features in a different chemical context (O'Brien and Herschlag, 1999). Strikingly, the closest relatives of 3-chloroacrylic acid dehalogenase, the tautomerase 4-OT and its homologue YwhB, have a promiscuous dehalogenating activity towards trans-3-chloroacrylic acid (Whitman, 2003). It would be interesting to see whether some existing members of the SDR family display a low haloalcohol dehalogenation activity.
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