Crystal Structures of Intermediates in the Dehalogenation of Haloalkanoates by L-2-Haloacid Dehalogenase*

Ivo S. Ridder, Henriëtte J. Rozeboom, Kor H. Kalk, and Bauke W. Dijkstra‡

From the Laboratory of Biophysical Chemistry and BIOSON Research Institute, Department of Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

The L-2-haloacid dehalogenase from the 1,2-dichloroethane-degrading bacterium Xanthobacter autotrophicus GJ10 catalyzes the hydrolytic dehalogenation of small L-2-haloalkanoates to their corresponding d-2-hydroxyalkanoates, with inversion of the configuration at the C2 atom. The structure of the apoenzyme at pH 8 was refined at 1.5 Å resolution. By lowering the pH, the catalytic activity of the enzyme was considerably reduced, allowing the crystal structure determination of the complexes with L-2-monochloropropionate and monochloroacetate at 1.7 and 2.1 Å resolution, respectively. Both complexes showed unambiguous electron density extending from the nucleophile Asp8 to the C2 atom of the dechlorinated substrates, corresponding to a covalent enzyme-ester reaction intermediate. The halide ion that is cleaved off is found in line with the Asp8 O6δ-C2 bond in a halide-stabilizing cradle made up of Arg116, Asn115, and Phe175. In both structures, the Asp8 Oδ2 carbonyl oxygen atom interacts with Thr12, Ser171, and Asn179, which possibly constitute the oxyanion hole in the hydrolysis of the ester bond. The carboxyl moiety of the substrate is held in position by interactions with Ser114, Lys147, and main chain NH groups. The L-2-monochloropropionate CH3 group is located in a small pocket formed by side chain atoms of Lys147, Asn179, Phe175, and Asp176. The size and position of the pocket explain the stereospecificity and the limited substrate specificity of the enzyme. These crystallographic results demonstrate that the reaction of the enzyme proceeds via the formation of a covalent enzyme-ester intermediate at the nucleophile Asp8.

L-2-Haloacid dehalogenase (L-DEX) catalyzes the hydrolytic dehalogenation of L-2-haloalkanoates to the corresponding d-2-hydroxyalkanoates with inversion of the configuration at the C2 atom. Several homologous L-DEXs have been found in various Pseudomonas species and in Xanthobacter autotrophicus GJ10, a bacterium that is able to degrade the xenobiotic compound 1,2-dichloroethane (1, 2). This halogenated hydrocarbon is industrially produced in large quantities and is applied as a solvent and as an intermediate in the production of plastics (3). Because microorganisms that contain dehalogenases can be used in a biotechnological approach to detoxify halogenated aliphatics (4), such enzymes are a fascinating target for research. In addition, the stereospecificity of L-DEXs could make them useful for the biosynthesis of chiral 2-hydroxyalkanoic acids. Furthermore, L-2-haloacid dehalogenase is the prototypical member of a large superfamily of hydrolyases, the haloglicid dehalogenase (HAD) superfamily identified by Koonin and coworkers (5, 6). Based on three conserved sequence motifs, the L-DEXs, epoxide hydrolyases, P-type ATPases, and a variety of phosphatases are recognized as members of this superfamily. Detailed information on L-DEXs is of interest as the enzyme is the only member of the HAD superfamily that has been structurally characterized so far.

The x-ray structures of two L-2-haloacid dehalogenases have been reported, L-DEX YL from Pseudomonas sp. YL (Protein Data Bank code 1JUD (7)) and DhlB from X. autotrophicus GJ10 (Protein Data Bank code 1AQ6 (8)). The enzymes share a sequence identity of 40%, and their structures are closely related. Both enzymes have a mixed α/β core domain in a Rossmann fold with a four-helix bundle subdomain insertion. DhlB is somewhat larger, and the 21 extra residues form a two-helix excursion from the α/β core domain on the same side as the four-helix bundle. Together these helical domains provide a tight dimer interface and limit the substrate specificity of the X. autotrophicus enzyme to short substrates such as haloacetates and halopropionates (8, 9).

Comprehensive biochemical data have been obtained for the Pseudomonas enzyme (1, 10, 11). Asp8 was identified as the nucleophile in the first step of the enzymatic reaction, the formation of a covalent enzyme-ester intermediate. Furthermore, these studies revealed eight more charged and polar amino acids (Thr12, Arg19, Ser114, Lys147, Tyr153, Ser171, Asn179, and Asp176) that are involved in substrate binding and catalysis. Most of the catalytically critical residues are conserved in the HAD superfamily and they stand out from the main domain. The only exception is Arg19, which is provided by the four-helix bundle domain. The x-ray structures enabled a detailed discussion of the role of these residues, and in particular useful information could be extracted from a model of a bound L-2-monochloropropionate (MCPA) substrate, which was based on the position of a formate ion in the active site of DhlB (8). The conserved serine residue in motif II, Ser114, was proposed to determine the position of the formate ion in the active site of DhlB.

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† To whom correspondence should be addressed: Laboratory of Biophysical Chemistry, Dept. of Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands. Tel.: 31-503634378; Fax: 31-503634800; E-mail: b.w.dijkstra@chem.rug.nl.

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The atomic coordinates and structure factors (codes 1qq5, 1qq7, and 1qq6) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The abbreviations used are: L-DEX, L-2-haloacid dehalogenase; L-DEX YL, L-2-haloacid dehalogenase from Pseudomonas sp. YL; DhlB, L-2-haloacid dehalogenase from X. autotrophicus GJ10; MCPA, 2-monochloropropionate; bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; MCAA, monochloroacetate; r.m.s., root mean square; DhlA, haloalkane dehalogenase from X. autotrophicus GJ10.
posed to bind the carboxylate moiety of the substrate, and a halide-binding cradle formed by Arg²⁹, Tyr¹⁰, and Phe⁷⁵ was postulated. Conserved residues from motifs I and III, Thr¹², Ser¹⁷¹, and Asn¹⁷³, were found to interact with the nucleophile Asp⁸ O₂⁻ atom possibly making up an oxyanion hole, and the motif III Lys¹⁴⁷ Nɛ was hydrogen-bonded to the Oδ₁ atom of the nucleophile.

These proposals were recently corroborated by crystal structures of an inactive S171A l-Dex YL mutant enzyme covalently bound to several chloroalkanoate substrates (12, 13). However, structural evidence for the location of the halide ion is still lacking. To gain more insight into the abstraction of the halide ion and the reaction of the enzyme with MCPA, one of its best substrates (9), we extended our crystallographic studies on DhlB. One soaking experiment with MCPCA was done at pH 8, at which the enzyme is active, to obtain information about the enzyme structure during or after processing of the substrate. Two other experiments were performed at low pH to trap the enzyme structure during or after processing of the substrate.

Refinement of the pH 5 Structure—For the MCPA pH 5 experiment, the 1.52 Å resolution structure described above was used as a starting model. Initial σ₂-weighted 2Fobs − Fcal, Fobs − F, electron density maps were calculated and inspected with O (27) to check the agreement of the model with the data. PROCHECK (28) and WHATCHECK (29) were used to assess the stereochemical quality. Whenever necessary the model was manually adjusted in O. When the refinement gave no further decrease in Rfree nor any improvement in stereochemistry, it was considered completed. A final round of refinement including all data resulted in an overall crystallographic R-factor of 19.8% for data ranging from 20.0 to 1.52 Å resolution. Refinement and model statistics are given in Table I, and the quality of the electron density can be judged from Fig. 1A.

The asymmetric unit contains two protein molecules of residues 1–245 forming the DhlB dimer, 571 water molecules, and two formate ions. The substrate binding model proposed earlier (8) and contribute to the detailed understanding of the reaction mechanism of the enzyme.

Materials and Methods

Crystal Preparation—Crystals of l-2-haloacid dehalogenase were grown in the presence of sodium formate by macroseeding as described previously (15). The enzyme crystalizes in two orthorhombic space groups: P2₁2₁2₁ and C2₁. For the soaking experiment the diffracting primitive crystal form was chosen (a = 56.75 Å, b = 83.83 Å, c = 90.81 Å, 2 mol/asymmetric unit). As formate binds in the active site of the enzyme, crystals were washed two times in fresh synthetic mother liquor (25% (w/v) polyethylene glycol 8000, 100 mM bis-Tris, pH 8.0, 4 °C) in an attempt to remove the formate.

One crystal was transferred to a solution of slightly higher pH, containing 25% (w/v) polyethylene glycol 8000, 100 mM sodium phosphate, pH 7.5, equilibrated for 20 min at room temperature, and finally soaked for 3 h in synthetic mother liquor, pH 8.0, containing 20 mM d,l-2-monochloropropionic acid. A second crystal was transferred to a more acidic solution of 25% (w/v) polyethylene glycol 8000, 100 mM citrate, pH 5.0, at 4 °C, equilibrated for 20 min, and soaked for 45 min at 4 °C in synthetic mother liquor containing 20 mM l-MCPA. A third, analogous experiment was done at pH 5.0 using 10 mM monochloroacetic acid (MCAA) instead of MCPCA.

Diffraction Data Collection—Diffraction data were collected at cryotemperatures (100 K) from single crystals at experimental station ID14-3, European Synchrotron Radiation Facility (ESRF), Grenoble (MCPA, pH 5 experiment) and at the European Molecular Biology Outstation at Deutsches Elektronen Synchrotron, Homburg, beamline X11 (MCPA, pH 5 experiment) and beamline X31 (MCAA, pH 5 experiment). The ID14-3 beamline (λ = 0.9475 Å) was equipped with a MAR CCD detector system, and the X11 (λ = 0.9095 Å) and X31 (λ = 1.00 Å) beamlines with a 30-cm and a 18-cm MAR image plate detector, respectively. Data were integrated and merged with DENVZ/SCALEPACK (16). Data processing statistics are given in Table I.

Refinement of the pH 8 Structure—For the MCPA pH 8 experiment, the native DhlB structure (Protein Data Bank entry 1AQ6 (8)) without any atom. This was done to allow for the large variability of the angle observed in atomic resolution structures (25, 26). After the adjustment, no deviating bond angles were flagged anymore. However, the O symmetry of the nucleophile Asp⁸ became 163°, which is far off the normal value for a trans-peptide bond.

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Reaction Intermediates of L-2-Haloacid Dehalogenase

**RESULTS AND DISCUSSION**

**Native Structure at pH 8 and 1.52 Å Resolution**—The structure of dimeric L-2-haloacid dehalogenase at pH 8 is very similar to the previously reported 1.95 Å resolution structure (8), with an r.m.s. difference of 0.47 Å for all 490 Ca atoms. This difference can be attributed to a few regions in the protein where the molecule has been modeled differently (see “Materials and Methods”). The disorder observed before in two loops and the C terminus (residues 25–27, 204–206, and 243–245) is present in this structure as well. The high resolution of the data enabled the identification of alternate conformations for 17 side chains. Moreover, in both molecules residues 208–213 were modeled in two different ways to account for ambiguities in the electron density. This region is part of the two-helix cradle of positively charged and polar amino acids most likely interact with the same residues in molecule B (Fig. 2). It is surprising to see that equally good dimerization contacts can be made by two different conformations.

The A and B molecules of the dimer can be superimposed with an r.m.s. difference of 0.29 Å for 245 Ca atoms. The major differences are found around the rebuilt residues (Pro107, the loop from residues 204–207, and the C terminus). None of these differences are considered functionally relevant, and therefore all results discussed below pertain to both molecules unless stated otherwise. The active site of the enzyme at pH 8 contains a formate ion that originates from the crystallization solution. This ion was observed in the 1.95 Å resolution structure as well, and its position was used to construct a model for the binding of an L-MCPA substrate (8). To replace the formate ion by MCPA, the crystal was washed several times. Apparently, the washing procedure was not sufficiently adequate to remove the ion.

**Active Site Structure of the Enzyme-Ester Intermediates**—In the experiments with MCPA and MCAA at pH 5, a substrate is covalently bound in the active site of DhlB. In these structures, it has replaced the formate ion and the water molecules that were present in the native structure. Very clearly, continuous electron density extends from the Asp8 Oδ1 atom to the C2 atom of the substrates, whereas no density for a covalently attached chlorine atom is observed (Fig. 1, A and B). This indicates that a covalent bond has formed between the nucleophilic Asp8 residue and the substrate and that the covalent Cl–C bond in the substrate has been cleaved. The Asp8 Oδ2 atom has a carbonyl functionality in the enzyme-ester intermediate structure. It is hydrogen-bonded to the hydroxyl group of the Thr12 side chain and the side chain amide group of Asn173 (Fig. 3A). Furthermore, in the MCAA covalent intermediate Ser171 Oγ is located within a 3.3-Å distance, but it is not in the plane of the carbonyl oxygen atom lone electron pairs. In the MCAA bound structure, however, the derivatized Asp8 side chain is rotated ~30° about the Cβ–Cγ bond, bringing Ser171 Oγ much closer to the Oδ2 atom and at the same time increasing the distance to Thr12 Oγ1 (Fig. 3B). This suggests a rotational freedom in the enzyme-ester intermediate, which might be used to optimize the interactions of the Oδ2 atom with the enzyme in the hydrolysis step of the reaction, when a negative charge develops on the Asp8 Oδ2 atom. The negative charge of this oxyanion intermediate might be stabilized by Thr12, Ser171, and Asn173, which together would form an oxyanion hole with a tetrahedral coordination of the oxyanion.

A chloride ion is present close to the substrate C2 atom, Asn115, and Arg239. It is found in line with the Asp8 Oδ1–C2 bond, at a distance of 3.6 Å from the C2 atom of the substrate, and the ion is close to both N7 atoms of Arg239 and to the side chain amide group of Asn115. Both amino acid residues were shown to be catalytically essential in the l-DEX YL enzyme (1), but the chloride ion was not found in the covalent intermediate structures of this enzyme (12). Furthermore, the chloride is located in the plane of the aromatic ring of Phe175 at a closest distance of 4.7 and 4.2 Å in the MCPA and MCAA experiments, respectively. The difference is because of the interaction of the phenyl ring with the methyl group of the MCAA substrate, which is absent in MCAA. Aromatic ring systems are known to be partially positively charged in the plane of the ring (34) and binding interactions of tryptophan and tyrosine rings with halide ions have been observed before in haloalkane dehalogenase (DhIA) (35, 36). In 4-chlorobenzoyl-CoA dehalogenase the active site is surrounded by aromatic residues as well (37). Other aromatic residues near the DhlB active site, Tyr10 and Phe58, have a less favorable ring orientation. The stabilizing cradle of positively charged and polar amino acids most likely...
The structure of the complex with MCPA, the only chiral substrate to be efficiently degraded by DhlB (5), shows clear electron density for the C\(_6\) atom of the substrate (Fig. 1B). The pocket in which the CH\(_3\) group is located is not particularly hydrophobic as it is lined by side chain atoms of Lys\(_{147}\), Asn\(_{173}\), Phe\(_{175}\), and Asp\(_{176}\). It is shielded from the solvent by residues from helices a2 and a10, thereby limiting its size to about 75 Å\(^3\). This explains why the substrate specificity of DhlB is restricted to small haloalkanoates (9). The position of the small pocket also resolves the preference of the enzyme for \(\alpha\)-substrates; as for any substrate with the methyl group and the hydrogen atom interchanged at the C2 position, steric clashes with main chain atoms of residues 10 and 11 would occur (Fig. 4A).

The overall structures of native DhlB and the reaction intermediates are very alike (see "Materials and Methods"), demonstrating that the native enzyme is in an active conformation. All DhlB structures represent a form of the enzyme in which the active site is very compact and shielded from the solvent. This is in contrast with L-DEX YL, in which a significant movement of the Asp\(_8\)-Ser\(_{19}\) and Tyr\(_{89}\)-Asp\(_{100}\) regions toward the active site was observed in the structures of the covalent substrate complexes of L-DEX YL compared with the wild-type enzyme, which is more open (12). The compact form of DhlB could be caused by the extra two-helix subdomain that is not present in L-DEX YL and that in part closes off the entrance to the active site (8). Li et al. (12, 13) have succeeded in trapping a covalent intermediate using L-2-monochloro-n-butyrate and the S171A mutant of L-DEX YL, which has an impaired oxyanion hole. Comparison of the DhlB and \(\alpha\)-DEX YL covalent intermediate structures shows that the alkyl tail of the butyrate is close to the position of the chloride ion in DhlB (Fig. 4, A and B). In view of the 40% amino acid sequence identity between the two enzymes, one would expect that the catalytic residues and the

functions to bind the halogen moiety of the substrate and to counterbalance the negative charge that develops on the halide during cleavage of the C–Cl bond.

The carboxylate moiety of the substrate is bound in the same position as the formate ion in the native DhlB structure. It is held in position by electrostatic interactions of its O1 atom with the main chain NH groups of residues 9 and 10 and with the side chain of Ser\(_{115}\) (Fig. 3). The other carboxylate oxygen atom functions to bind the halogen moiety of the substrate and to restrict the size of the active site to about 75 Å\(^3\). The position of the small pocket also resolves the preference of the enzyme for \(\alpha\)-substrates; as for any substrate with the methyl group and the hydrogen atom interchanged at the C2 position, steric clashes with main chain atoms of residues 10 and 11 would occur (Fig. 4A).

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residues that stabilize the Asp\textsuperscript{8} O\textsubscript{d2} oxyanion and the halide ion would be absolutely conserved. In DhlB, Asn\textsuperscript{173} is part of the oxyanion hole and Phe\textsuperscript{175} is involved in halide binding, and it is hard to imagine that the same residues in L-DEX YL would contribute only to the binding of the alkyl tail of the substrate as Li et al. (12) suggest. Another difference is found with the MCAA binding experiments. All DhlB structures and all but the MCAA-bound L-DEX YL structures are very similar with respect to the orientation of the Asp\textsuperscript{8} side chain. The aberrant side chain conformation of Asp\textsuperscript{8} in the MCAA-bound L-DEX YL structure is stabilized by hydrogen bonds of the Asp\textsuperscript{8} O\textsubscript{d1} ether oxygen atom to Thr\textsubscript{12} and of the Asp\textsuperscript{8} O\textsubscript{d2} carbonyl oxygen atom to the Lys\textsubscript{147} ε-amino group (Fig. 4C). This might be because of the L-DEX YL S171A mutation, which caused the loss of the attractive interaction between Asp\textsuperscript{8} O\textsubscript{d2} and Ser\textsuperscript{171} O\textsubscript{γ}, present in the wild-type enzyme. Further research is required to establish whether the binding modes of the covalently bound complexes of L-DEX YL with MCAA and n-butyrate are of functional relevance.

**Implications for the Reaction Mechanism**—The dehalogenation reaction catalyzed by DhlB starts with the import of the negatively charged substrate via the cleft between the main domain and the four-helix bundle subdomain. The substrate can be either "pulled in" by an overall electrostatic dipole, which is directed along the dimer axis that is parallel to the import route (8), or via a guidance mechanism in which Arg\textsuperscript{39} plays a major role (12). The substrate is bound through specific binding interactions of its functional groups with conserved active site residues. The structures presented here demonstrate that the reaction proceeds through a nucleophilic attack of the Asp\textsuperscript{8} O\textsubscript{d1} atom on the C\textsubscript{2} of the substrate, resulting in the formation of a covalent enzyme-ester intermediate (Fig. 5). The salt bridge to the positively charged Lys\textsuperscript{147} side chain reduces the pK\textsubscript{a} of the aspartate, thereby increasing its nucleophilicity. The formation of the O\textsubscript{d1}–C\textsubscript{2} bond is accompanied by the cleavage of the C\textsubscript{2}–Cl bond. These three atoms are found in line, with the chloride ion located in a halide-stabilizing cradle formed by the side chains of Arg\textsuperscript{39}, Asn\textsuperscript{115}, and Phe\textsuperscript{175}. As a result, the configuration of the other three substituents of the C\textsubscript{2} atom is inverted, which is in agreement with the observed inversion of configuration at the chiral center of the substrate (9).

In the next step of the reaction, the ester bond is hydrolyzed by a nucleophilic attack of a water molecule (or hydroxyl ion) on
the Cγ atom of Asp8 (10). The negative charge, which develops on the Oδ2 carbonyl oxygen atom, is stabilized by an oxyanion hole formed by side chain atoms from Thr12, Asn173, and Ser171. The nature of the hydrolytic water molecule and its activation is still subject to speculation, as there is no such molecule present within 6 Å from the Asp8 Cγ atom in the DhlB covalent intermediates. A movement of a part of the enzyme would be required to allow a water molecule to enter the active site. In the S171AL-DEX YL mutant one water molecule (Wn) was found near the ester bond (12), but it occupies a position near where the Ser171 Oγ atom would be in the wild-type enzyme. However, the Asp8 Cγ atom is accessible from this side only, as main chain atoms from residues 9–12 prohibit the approach of a water molecule from the other side (Fig. 4A). Lys147 and Asp176 are possible candidates to activate a water molecule that attacks the Asp8 Cγ, as they are located close to the ester bond to be cleaved. The pKₐ of a lysine side chain (−10) (39) is around the relatively high pH optimum of 9.5 for DhlB (9), but the different orientations of Lys147, the residue which activates the hydrolytic water molecule, takes up a position in between Lys147 Nζ and Asp176 Oδ2, although closer to the latter (Fig. 6). Furthermore, the position of the DhlA nucleophilic water molecule is then close to Asp176 Oδ1 and Ser171 Oγ and very near Wn in the l-2-monochloro-n-butyrate-l-DEX YL complex. This supports the suggestion from Li et al. (12) that Asp176 and Ser171 are essential for hydrolysis of the ester. Above pH 9, the enzyme could employ the flexibility of Lys147 to vacate the space needed for the water, driven by a pH effect. We have not been able to obtain stable crystals at pH 9 or higher, which is indicative of structural changes around this pH. Also the dimer interface might be susceptible to change around the pH optimum, as Lys41 and Tyr68 of both molecules make hydrogen bonds to the main chain carbonyl groups of Leu216 and Ala215, respectively, and the donor groups are only 4 Å apart. Lys147 could also play a role in the deprotonation of Asp176 after hydrolysis, although Tyr153, the other residue hydrogen bonded to Asp176, is another candidate for this function.

Many of the residues in the active site are conserved among the members of the HAD superfamily (5, 6). The model, which was constructed for the structure and mechanism of the phosphatase and P-type ATPase members of the superfamily (40), is fully corroborated by the DhlB enzyme-ester intermediate structures. These enzymes cleave covalent bonds of phosphorylated substrates by nucleophilic attack of the motif I Asp (Asp8) on the phosphorus of the substrate, resulting in the formation of a phosphoenzyme intermediate (41, 42). Like in DhlB, the motif II Ser/Thr (Ser114) and the motif III Lys (Lys147) partly compensate the negative charge of the intermediate. Two of the three residues of the oxyanion hole in l-DEXs, Ser171 and Asn173, are not found in the phosphatase and P-type ATPase members of the superfamily. This is not surprising as the proposed hydrolytic mechanism does not include the formation of an oxyanion intermediate, but instead the phosphoenzyme intermediate is hydrolyzed by attack on the phos-
phorus atom (43). The negative charge that develops on the phosphoryl group was proposed to be counterbalanced by a magnesium ion (40). Although many details of the l-DEX mechanism are now well understood, it is evident that further research is required to resolve the hydrolysis step of the dehalogenation mechanism of l-2-haloacid dehalogenase. In particular, a three-dimensional structure of the native enzyme at the pH optimum of 9.5 could provide useful information about the position of the nucleophilic water molecule, although so far we have not been able to obtain crystals that are stable at this pH.

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