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

(Received for publication, June 18, 1999)

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 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-mono- and L-2-dichloropropionate and L-2-monochloropropionate 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 O₆₋–C₂ bond in a halide-stabilizing cradle made up of Arg₁₃₉, Asn₁₇₃, and Phe₁₇₅. In both structures, the Asp₈ O₂(carbonyl) oxygen atom interacts with Thr₁₁₄, Ser₁₇₁, and Asp₁₇₆, 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-mono- and L-2-dichloropropionate CH₃ group is located in a small pocket formed by side chain atoms of Lys₁₄⁷, Asn₁₇₃, 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 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 hydrolases, the haloacid dehalogenase (HAD) superfamily identified by Koonin and coworkers (5, 6). Based on three conserved sequence motifs, the L-DEXs, epoxide hydrolases, P-type ATPases, and a variety of phosphatases are recognized as members of this superfamily. Detailed information on L-DEX 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 halo alkanopropionates (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, Arg₂⁹, Ser₁₁₄, Lys₁₄⁷, Tyr₁₅₃, Ser₁₇₁, Asn₁₇₃, 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 Arg₂⁹, 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 pros...
posed to bind the carboxylate moiety of the substrate, and a halide-binding cradle formed by Asp<sup>29</sup> Tyr<sup>10</sup>, and Phe<sup>175</sup> was postulated. Conserved residues from motifs I and III, Thr<sup>15</sup>, Ser<sup>171</sup>, and Asn<sup>173</sup>, were found to interact with the nucleophile Asp<sup>8</sup> O<b>2</b> atom possibly making up an oxanion hole, and the motif III Lys<sup>147</sup> N<b>ε</b> was hydrogen-bonded to the O<sub>ε1</sub> 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 chloroalkane 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 reaction intermediates of MCAA and l-MCPA in a similar way as was done previously for haloalkane dehalogenase (14). Here we present the 1.52 Å resolution structure of the unliganded enzyme and the structures of covalent ester intermediates of the native enzyme with l-MCPA and MCAA, including a bound chloride ion. The structures experimentally confirm 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 crystallizes in two orthorhombic space groups, P<sub>1</sub>2<sub>1</sub>2<sub>1</sub> and P<sub>2</sub>1<sub>2</sub>1<sub>2</sub>. For the soaking experiment the better 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 twice in fresh synthetic mother liquid (25% (w/v) polyethylene glycol 8000, 100 mM bis-Tris, pH 7.0) in an attempt to remove the formate.

One crystal was transferred to a solution of slightly higher pH, containing 22% (w/v) polyethylene glycol 8000, 100 mM bis-Tris, pH 8 (by equilibration for 20 min at room temperature, and finally soaked for 3 h in synthetic mother liquid, pH 8.0, containing 20 mM d<sub>1</sub>-l-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 liquid 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 cryoloop (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 Laboratory Outstation at Deutsches Elektronen Synchrotron, Hamburger, beamline X11 (MCPA, pH 5 experiment) and beamline X21 (MCAA, pH 5 experiment). The ID14-3 beamline (λ = 0.9475 Å) was equipped with a MARCCD detector system, and the X11 (λ = 0.9905 Å) and X21 (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 5 experiment, the native DhlB structure was used as a starting model. Initial electron density maps clearly showed density extending from Asp<sup>5</sup> O<b>2</b> for all MCPA atoms except for the chlorine, indicating that the substrate had bound covalently and that the chlorine atom had been cleaved off. The same refinement strategy as described above was employed. Unambiguous difference electron density enabled us to build in the covalently bound substrate (Fig. 1B). A similar chloride ion was identified in the active site on the basis of residual positive difference density around a "water molecule" with a B-factor of 2.0 Å<sup>2</sup> and the chemical environment of a water molecule. A difference experiment with a 2-monoiodoacetic acid substrate gave a peak of more than double intensity in the electron density map at this position (data not shown). Topology and parameter files for the covalently modified residue were generated with XPLOR2D (30). At the end of the refinement, a final round including all data resulted in an overall crystallographic R-factor of 17.0% for data ranging from 20.0 to 1.70 Å resolution. Refinement and model statistics are given in Table I.

The DhlB structures with and without MCPA are very alike with an r.m.s. deviation of 0.2 Å for all Ca atoms of the dimer, so it can be concluded that the overall structure of the enzyme is not influenced by the binding of its substrate. The disorder observed with the pH 8 experiment in the loop that contains residues 25–27 (see "Results" and "Discussion") could be resolved in the B molecule in the MCPA experiment as two different conformations of the main chain in this region. In Molecule A, Arg<sup>29</sup>, Thr<sup>93</sup>, Lys<sup>109</sup>, Ser<sup>172</sup>, Ser<sup>173</sup>, Ser<sup>174</sup>, Met<sup>181</sup>, Thr<sup>222</sup>, and Glu<sup>225</sup> have two conformations, and in Molecule B, a second side chain position is found for Lys<sup>93</sup>, Gly<sup>94</sup>, Thr<sup>95</sup>, Ser<sup>172</sup>, Ser<sup>173</sup>, Met<sup>181</sup>, and Thr<sup>222</sup>. In both molecules, the loop formed by residues 204–207 and the C termini have been remodeled, and in molecule B, the cis-peptide bond between Ala<sup>106</sup> and Pro<sup>132</sup> has been replaced by a trans-peptide bond. Furthermore, in both molecules the helix and loop formed by residues 122–132 have shifted by 0.6 Å on average, although no structural reason is apparent.

**Refinement of the pH 5 Structure with MCPA Bound**—For the MCPA pH 5 experiment, the 1.52 Å pH 8 structure described above was used as a starting model. Initial electron density maps showed clearly shaped density extending from Asp<sup>5</sup> O<b>2</b> and for all MCPA atoms except for the chlorine, indicating that the substrate had bound covalently and that the chlorine atom had been cleaved off. The same refinement strategy as described above was employed. Unambiguous difference electron density enabled us to build in the covalently bound substrate (Fig. 1B). Also a chloride ion was identified in the active site on the basis of residual positive difference density around a "water molecule" with a B-factor of 2.0 Å<sup>2</sup> and the chemical environment of a "water molecule". A difference experiment with a 2-monoiodoacetic acid substrate gave a peak of more than double intensity in the electron density map at this position (data not shown). Topology and parameter files for the covalently modified residue were generated with XPLOR2D (30). At the end of the refinement, a final round including all data resulted in an overall crystallographic R-factor of 17.0% for data ranging from 20.0 to 1.70 Å resolution. Refinement and model statistics are given in Table I.

The DhlB structures with and without MCPA are very alike with an r.m.s. deviation of 0.2 Å for all Ca atoms of the dimer, so it can be concluded that the overall structure of the enzyme is not influenced by the binding of its substrate. The disorder observed with the pH 8 experiment in the loop that contains residues 25–27 (see "Results" and "Discussion") could be resolved in the B molecule in the MCPA experiment as two different conformations of the main chain in this region. In Molecule A, Arg<sup>29</sup>, Thr<sup>93</sup>, Lys<sup>109</sup>, Ser<sup>172</sup>, Ser<sup>173</sup>, Ser<sup>174</sup>, Met<sup>181</sup>, Thr<sup>222</sup>, and Glu<sup>225</sup> have two conformations, and in Molecule B, a second side chain position is found for Lys<sup>93</sup>, Gly<sup>94</sup>, Thr<sup>95</sup>, Glu<sup>100</sup>, Lys<sup>109</sup>, Ser<sup>172</sup>, Ser<sup>173</sup>, Ser<sup>174</sup>, Met<sup>181</sup>, and Thr<sup>222</sup>. The region from residues Ala<sup>106</sup> to Phe<sup>132</sup> is disordered as was observed in the native enzyme, pH 8 (see "Results" and "Discussion"). In the refinement of the MCPA model these characteristics were preserved.

**Refinement of the pH 5 Structure with MCAA Bound**—For the MCAA pH 5 experiment, the pH 5 DhlB structure with the covalent MCPA intermediate bound was used as a starting model. An initial electron density map clearly showed density extending from Asp<sup>5</sup> O<b>2</b> and for all MCAA atoms except for the chlorine, indicating that the substrate had bound covalently and that the chlorine atom had been cleaved off. The same refinement strategy as described above was employed. Residues with alternative conformations were preserved and their occupancies were not refined. Refinement was completed with a final round including all data, resulting in an overall crystallographic R-factor of 17.1% for data ranging from

---

*Reaction Intermediates of l-2-Haloacid Dehalogenase* 30673

---
Phe175 is not shown. Water molecules are labeled dechlorinated MCAA moiety covalently attached to Asp 8. For clarity, density is contoured at 2.25 the dimerization interface. Residues 208–213 in molecule A in the electron density. This region is part of the two-helix side chains. Moreover, in both molecules residues 208–213 data enabled the identification of alternate conformations for present in this structure as well. The high resolution of the nase displayed with final 2F - F electron density. A, 1.52 Å resolution structure with formate ion bound; B, structure with dechlorinated MCPA moiety covalently attached to Asp8; C, structure with dechlorinated MCAA moiety covalently attached to Asp8. For clarity, Phe175 is not shown. Water molecules are labeled W; the electron density is contoured at 2.25σ in A and B and at 2.0σ in C (this figure and Figs. 2, 4, and 6 were generated with BOBSCRIPT (44)). PMT, formate.

from 20.0 to 2.1 Å resolution. Refinement and model statistics are given in Table I. The enzyme structures complexed with MCPA and MCAA have an r.m.s. deviation of only 0.13 Å for all 490 Ca atoms, and consequently the results discussed below refer to both molecules unless stated otherwise.

Analysis of the Structures—The structures were analyzed using programs from the CCP4 suite (31), LSQMAN (32), VOIDOO (33), and the BIOMOL package (Protein Crystallography Group, University of Groningen). The atomic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank with the entry codes 1qq5, 1qq7, and 1qq6 for MCPA (pH 8), MCPA (pH 5), and MCAA (pH 5) experiments, respectively.

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 excursion (residues 193–219) that contributes significantly to the dimerization interface. Residues 208–213 in molecule A 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 chloride atom is observed (Fig. 1, A and C). 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 Oy 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 Oy much closer to the Oδ2 atom and at the same time increasing the distance to Thr12 Oy1 (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 oxynion intermediate might be stabilized by Thr12, Ser171, and Asn173, which together would form an oxynion hole with a tetrahedral coordination of the oxynion.

A chloride ion is present close to the substrate C2 atom, Asn115, and Arg179. 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 Arg179 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 MCPA 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 (DhLA) (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

![Figure 1](image_url)
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 side chain of Lys147. The latter interaction is facilitated by a reorientation of the lysine side chain. In the native structure, the lysine is hydrogen-bonded to Asp176 Oε2, the main chain carbonyl oxygen atom of residue 113, and Asp8 Oβ1. The geometry of the contact with the formate ion is unfavorable for hydrogen bond formation. Because of the reorientation of Lys147 in the MCPA- and MCAA-bound structures, its interaction with Asp8 Oβ1 has weakened. This is in accordance with the change of the functionality of the oxygen atom to an ether function, which is generally a weaker hydrogen bond acceptor (38). All side chains that contribute to the binding of the carboxylate moiety were shown to be catalytically critical in L-DEX YL (1).

The structure of the complex with MCPA, the only chiral substrate to be efficiently degraded by DhlB (9), shows clear electron density for the Cα atom of the substrate (Fig. 1B). The pocket in which the CH3 group is located is not particularly hydrophobic as it is lined by side chain atoms of Lys147, Asn173, Phe175, and Asp176. It is shielded from the solvent by residues from helices α2 and α10, thereby limiting its size to about 75 Å3. 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 l-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 Asp8-Ser18, Tyr89-Asp100, and Leu113-Arg131 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 L-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

---

**Table I**

Statistics of data collection and quality of the final models

|                     | Dh1B + MCPA, pH 8.0 | Dh1B + MCPA, pH 5.0 | Dh1B + MCAA, pH 5.0 |
|---------------------|---------------------|---------------------|---------------------|
| Cell dimensions (Å) | α = 57.05, b = 83.93, c = 91.23 | α = 56.75, b = 83.83, c = 90.81 | α = 56.93, b = 83.56, c = 90.06 |
| Resolution range (Å)| 30–1.52             | 99–1.70             | 99–2.10             |
| Highest resolution shell | (1.55–1.52)            | (1.73–1.70)            | (2.14–2.10)            |
| Total no. of observations | 306,585          | 202,654              | 276,852              |
| No. of unique reflections | 63,057           | 48,184               | 22,242               |
| Completeness (%) | 92.7 (81.4) | 99.0 (84.1) | 87.2 (73.1) |
| (Rmerge) (%) | 6.4 (35.1) | 3.8 (14.5) | 6.8 (22.1) |
| Quality of the final model |
| No. of atoms | 4,509 | 4,667 | 4,520 |
| Proteinb | 3,932 (155) | 4,000 (224) | 3,994 (462) |
| Substrate/formate | 6 | 10 | 8 |
| Chloride | 2 | 2 | 2 |
| Solventb | 571 (8) | 655 (9) | 516 (9) |
| Final R-factor (%) | 19.8 | 17.0 | 17.1 |
| Free R-factor (%) | (20–1.52 Å, all data) | (20–1.70 Å, all data) | (20–2.10 Å, all data) |
| r.m.s.d. from ideality for | (20–1.52 Å, test set) | (20–1.70 Å, test set) | (20–2.10 Å, test set) |
| Bond lengths (Å) | 0.006 | 0.006 | 0.007 |
| Bond angles (°) | 1.1 | 1.1 | 1.1 |
| Dihedrals (°) | 21.4 | 21.5 | 21.4 |
| Dihedral (°) | 1.6 | 1.1 | 1.0 |
| R overall (Å2) | 25.4 | 23.7 | 23.2 |
| B protein (Å2) | 22.8 | 22.5 | 21.5 |
| Estimated coordinate error (Å) | 0.14; 0.18 | 0.09; 0.17 | 0.20; 0.16 |
| Cross-validated estimated coordinate error (Å) | 0.18; 0.22 | 0.13; 0.20 | 0.27; 0.24 |

* Values in parentheses are for the highest resolution bin.

b Values in parentheses are the number of atoms in alternative conformations.

* Estimated from cross-validated σA and Luzzati plots (46), calculated from all data better than the 5.0 Å resolution.

* Estimated from cross-validated σA and Luzzati plots (46), calculated from test set data in the 5.0–1.52 Å resolution range.
residues that stabilize the Asp\textsuperscript{8} O\textsubscript{d1} 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\textsuperscript{12} and of the Asp\textsuperscript{8} O\textsubscript{d2} carbonyl oxygen atom to the Lys\textsuperscript{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{29}, 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 C\textsubscript{γ} atom of Asp\textsuperscript{8} (10). The negative charge, which develops on the O\textsubscript{d2} carbonyl oxygen atom, is stabilized by an oxyanion hole formed by side chain atoms from Thr\textsubscript{12}, Asn\textsubscript{173}, and Ser\textsubscript{171}. 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 Asp\textsubscript{8} C\textsubscript{γ} 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 Ser\textsubscript{171} O\textsubscript{γ} atom would be in the wild-type enzyme. However, the Asp\textsubscript{8} C\textsubscript{γ} 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). Lys\textsuperscript{147} and Asp\textsuperscript{176} are possible candidates to activate a water molecule that attacks the Asp\textsuperscript{8} C\textsubscript{γ}, as they are located close to the ester bond to be cleaved. The pK\textsubscript{a} 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 Lys\textsuperscript{147} in the various structures point at a flexibility of this side chain that could hamper directing a nucleophilic water molecule. Moreover, the two nearby negatively charged carboxylate moieties of the substrate and Asp\textsuperscript{176} might increase the pK\textsubscript{a} of the residue.

A comparison of the esterified nucleophile of DhlB and its immediate environment with the covalent intermediate of DhlA (14) shows that the Ne2 atom of the His\textsuperscript{289}, the residue which activates the hydrolytic water molecule, takes up a position in between Lys\textsuperscript{147} N\textsubscript{z} and Asp\textsuperscript{176} O\textsubscript{d2}, although closer to the latter (Fig. 6). Furthermore, the position of the DhlA nucleophilic water molecule is then close to Asp\textsuperscript{176} O\textsubscript{d1} and Ser\textsubscript{171} O\textsubscript{γ} 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 Asp\textsuperscript{176} and Ser\textsuperscript{171} are essential for hydrolysis of the ester. Above pH 9, the enzyme could employ the flexibility of Lys\textsuperscript{147} 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 Lys\textsuperscript{41} and Tyr\textsuperscript{68} of both molecules make hydrogen bonds to the main chain carbonyl groups of Leu\textsuperscript{216} and Ala\textsuperscript{215}, respectively, and the donor groups are only 4 Å apart. Lys\textsuperscript{147} could also play a role in the deprotonation of Asp\textsuperscript{176} after hydrolysis, although Tyr\textsuperscript{153}, the other residue hydrogen bonded to Asp\textsuperscript{176}, 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\textsuperscript{8} (Asp\textsuperscript{8}) on the phosphorus of the substrate, resulting in the formation of a phosphoenzyme intermediate (41, 42). Like in DhlB, the motif II Ser/Thr (Ser\textsuperscript{114}) and the motif III Lys (Lys\textsuperscript{147}) partly compensate the negative charge of the intermediate. Two of the three residues of the oxyanion hole in l-DEX\textsubscript{a}, Ser\textsuperscript{171}, and Asn\textsuperscript{173} 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-

---

**Fig. 5.** Reaction scheme for the proposed reaction of l-2-haloacid dehalogenase.

**Fig. 6.** Stereo view of the superimposition of the active sites of the MCPA-DhlB complex and haloalkane dehalogenase (A) and of the l-2-monochloro-n-butyrate-l-DEX YL complex and haloalkane dehalogenase (B). Only the Ca, C\textsubscript{β}, C\textsubscript{γ}, O\textsubscript{d1}, and O\textsubscript{d2} atoms of the nucleophilic aspartate residues were superimposed with r.m.s. differences for A and B of 0.19 and 0.09 Å, respectively. In B, the proposed l-DEX YL nucleophilic water molecule Wn is only 0.9 Å away from its DhlA counterpart.
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.

Acknowledgments—It is a pleasure to acknowledge Dr. D. B. Janssen and co-workers for their supply of protein and useful comments. We thank Drs. K. S. Wilson, V. S. Lamzin, and other staff of the European Molecular Biology Laboratory Outstation, Deutsches Elektronen Synchrotron, Hamburg for access to the synchrotron data collection facilities and assistance. We thank the European Union for support of the work at the European Molecular Biology Laboratory, Hamburg through the Human Capital and Mobility Programme to Large Installations Project contract no. CHGE-CT93-0040. We gratefully acknowledge Dr. the Human Capital and Mobility Programme to Large Installations and co-workers for their supply of protein and useful comments. We thank ESRF for support of the work at ESRF, Grenoble.

REFERENCES
1. Kurihara, T., Liu, J.-Q., Nardi-Dei, V., Koshikawa, H., Esaki, N., and Soda, K. (1995) J. Biochem. (Tokyo) 117, 1317–1322
2. Janssen, D. B., Scheper, A., Dijkhuizen, L., and Witholt, B. (1985) Appl. Environ. Microbiol. 49, 673–677
3. Fetzer, S. (1998) Appl. Microbiol. Biotechnol. 50, 633–657
4. Stucki, G., and Thu¨er, M. (1995) Environ. Sci. Technol. 29, 2339–2345
5. Koonin, E. V., and Tatusov, R. L. (1994) J. Mol. Biol. 244, 125–132
6. Aravind, L., Galperin, M. Y., and Koonin, E. V. (1998) Trends Biochem. Sci. 23, 127–129
7. Hooft, R. W. W., Vriend, G., Sander, C., and Abola, E. E. (1996) Nature 381, 272
8. Ridder, I. S., Rozeboom, H. J., Kalk, K. H., Janssen, D. B., and Dijkstra, B. W. (1993) Acta Crystallogr. Sect. D Biol. Crystallogr. 49, 905–921
9. van der Ploeg, J., van Hall, G., and Janssen, D. B. (1991) J. Biol. Chem. 266, 7925–7933
10. Liu, J.-Q., Kurihara, T., Miyagi, M., Esaki, N., and Soda, K. (1995) J. Biol. Chem. 270, 18309–18312
11. Liu, J.-Q., Kurihara, T., Miyagi, M., Tsunasawa, S., Nishihara, M., Esaki, N., and Soda, K. (1997) J. Biol. Chem. 272, 3363–3368
12. L. Y.-F., Hata, Y., Fujii, T., Hisano, T., Nishihara, M., Kurihara, T., and Esaki, N. (1998) J. Biol. Chem. (Tokyo) 273, 140–149
13. Verschueren, K. H. G., Seljée, F., Rozeboom, H. J., Kalk, K. H., and Dijkstra, B. W. (1993) Nature 363, 693–698
14. Ridder, I. S., Rozeboom, H. J., Kingma, J., Janssen, D. B., and Dijkstra, B. W. (1995) Methods Enzymol. 267, 307–326
15. Navaza, J. (1994) Acta Crystallogr. Sect. A Found. Crystallogr. 40, 157–163
16. Read, R. J. (1986) Acta Crystallogr. Sect. A Found. Crystallogr. 42, 140–149
17. Bhat, T. N. (1988) J. Appl. Crystallogr. 21, 279–281
18. Vellieux, F. M. D., and Dijkstra, B. W. (1997) J. Appl. Crystallogr. 30, 396–399
19. Brünger, A. T. (1992) Nature 355, 472–475
20. Rice, L. M., and Brünger, A. T. (1994) Proteins Struct. Funct. Genet. 19, 277–296
21. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grose-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
22. Engh, R. A., and Huber, R. (1991) Acta Crystallogr. Sect. A Found. Crystallogr. 47, 392–400
23. Verschueren, K. H. G., Seljée, F., Rozeboom, H. J., Kingma, J., Janssen, D. B., and Dijkstra, B. W. (1993) Acta Crystallogr. Sect. A Found. Crystallogr. 49, 905–921
24. Ridder, I. S., Rozeboom, H. J., and Dijkstra, B. W. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 1273–1290
25. Verschueren, K. H. G., Kingma, J., Rozeboom, H. J., Kalk, K. H., Janssen, D. B., and Dijkstra, B. W. (1993) Biochemistry 32, 9031–9037
26. MacArthur, M. W., and Thornton, J. M. (1996) J. Mol. Biol. 264, 1180–1195
27. Koonin, E. V., and Tatusov, R. L. (1994) J. Mol. Biol. 244, 125–132
28. Aravind, L., Galperin, M. Y., and Koonin, E. V. (1998) Trends Biochem. Sci. 23, 127–129
29. Hooft, R. W. W., Vriend, G., Sander, C., and Abola, E. E. (1996) Nature 381, 272
30. Ridder, I. S., Rozeboom, H. J., Kalk, K. H., Janssen, D. B., and Dijkstra, B. W. (1993) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 178–185
31. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
32. Kleywegt, G. J., and Jones, T. A. (1997) Methods Enzymol. 277, 208–230
33. Kleywegt, G. J., and Jones, T. A. (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
34. Verschueren, K. H. G., Kingma, J., Rozeboom, H. J., Kalk, K. H., Janssen, D. B., and Dijkstra, B. W. (1993) Biochemistry 32, 9031–9037
35. Koonin, E. V., and Tatusov, R. L. (1994) J. Mol. Biol. 244, 125–132
36. Aravind, L., Galperin, M. Y., and Koonin, E. V. (1998) Trends Biochem. Sci. 23, 127–129
37. Hooft, R. W. W., Vriend, G., Sander, C., and Abola, E. E. (1996) Nature 381, 272
38. Jeffrey, G. A., and Sanger, W. (1991) Hydrogen Bonding in Biological Structures, Springer-Verlag, New York
39. Fersht, A. R. (1985) Enzyme Structure and Mechanism, 2nd Ed., W. H. Freeman, New York
40. Ridder, I. S., and Dijkstra, B. W. (1999) Biochem. J. 339, 225–236
41. Asano, S., Tega, Y., Konishi, K., Fujisawa, M., and Takeguchi, N. (1996) J. Biol. Chem. 271, 2740–2745
42. Colet, J.-F., Gerin, I., Rider, M. H., Veiga-da-Cunha, M., and van Schaftingen, E. (1997) FEBS Lett. 408, 281–284
43. Dahms, A. S., Kanazawa, T., and Boyer, P. D. (1973) Biochemistry 12, 125–192
44. Read, R. J. (1997) Methods Enzymol. 277, 110–128