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

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The L-2-haloacid dehalogenase from the 1,2-dichloro-ethane-degrading bacterium Xanthobacter autotrophicus GJ10 catalyzes the hydrolytic dehalogenation of small L-2-haloalkanoates to their corresponding n-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 Asp6 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 Asp6 O6a-C2 bond in a halide-stabilizing cradle made up of Arg39, Asn123, and Phe175. In both complexes, the Asp6 O6a carbonyl oxygen atom interacts with Thr12, Ser171, and Asn173, 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, 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 Asp6.

L-2-Haloacid dehalogenase (L-DEX) catalyzes the hydrolytic dehalogenation of L-2-haloalkanoates to the corresponding n-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 considered 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). Asp6 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, Arg39, 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 Arg39, 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 pro-

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‡ 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-monochloroacetate; bis-Tris, bis(2-hydroxyethyl)iminotrihydroxymethylmethane; MCAA, monochloroacetate; r.m.s., root mean square; DhlA, haloalkane dehalogenase from X. autotrophicus GJ10.

2 The residue numbering of the l-DEX YL enzyme is slightly different from that of DhlB. For clarity, homologous residues are numbered according to the DhlB sequence.
posed to bind the carboxylate moiety of the substrate, and a halide-binding cradle formed by Arg239, Tyr10, and Phe775 was postulated. Conserved residues from motifs I and III, Thr132, Ser171, and Asn174, were found to interact with the nucleophile Asp8 O2 atom possibly making up an oxyanion hole, and the motif III Lys147 Nε was hydrogen-bonded to the O6i atom of the nucleophile.

These proposals were recently corroborated by crystal structures of an inactive S171A Δ-DEX YL mutant enzyme covalently bound to several chloroalkanoic acid 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. Two other experiments were performed at low pH to trap the reaction intermediates of MCAA and L-MCPA in a similar way. 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: P212121 (molecular replacement) and P21 (MCPA, pH 8 experiment). For the soaking experiment the better diffraction 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 liquor (25% (w/v) polyethylene glycol 8000, 100 mM bis-Tris, pH 8.0) 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 bis-Tris, pH 7.8, 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-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 MCPA.

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 8 experiment) and at the European Molecular Biology Laboratory Outstation at Deutches Elektronen Synchrotron, Hamburg, beamline X11 (MCPA, pH 5 experiment) and beamline X21 (MCAA, pH 5 experiment). The ID14-3 beamline (λ = 0.947 Å) was equipped with a MarCCD detector system, and the X11 (λ = 0.9095 Å) and X21 (λ = 1.00 Å) beamlines with a 30-cm and a 18-cm MAR image plate area detector, respectively. Data were integrated and merged with DENZO/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 restraint on the main chain ω dihedral angles was relaxed using the restrained Cα positional refinement with the AQP6 structure. The set of reflections set apart to calculate Rfree values (21) in the refinement of the 1.95 Å native structure was extended to cover 5% of the additional data as well. After an initial round of rigid body refinement, the model was subjected to one refinement cycle of torsion-angle dynamics (22) and subsequent cycles of positional and B-factor refinement for all atoms and refinement of the occupancy of residues with two conformations, all performed with CNS 0.5 (23). The phase of N-Cα-C bond angles that deviated from their ideal value (24). Hence, the CNS topology and parameter files were adjusted such that the restraint on the main chain ω dihedral angle was relaxed to 20%. The final model with the native structure values for N-Cα-C bond angles was then refined using the program AMoRe (17). The refinement statistics for the model are given in Table I.

Refinement of the pH 5 Structure with MCPA Bound—For the MCPA pH 5 experiment, the 1.52 Å resolution structure was refined using the program AMoRe (17). The refinement statistics for the model are given in Table I.

Refinement of the pH 5 Structure with MCAA Bound—For the MCAA pH 5 experiment, the 1.52 Å resolution structure was refined using the program AMoRe (17). The refinement statistics for the model are given in Table I.
Reaction Intermediates of l-2-Haloacid Dehalogenase

**Fig. 1.** Stereo view of the active site of l-2-haloacid dehalogenase displayed with final $2F_o - F_e$ electron density. A, 1.52 Å resolution structure with formate ion bound; B, structure with dechlorinated MCPA moiety covalently attached to Asp 8; C, structure with dechlorinated MCAA moiety covalently attached to Asp 8. For clarity, density is contoured at 2.25 $\sigma$ in the dimerization interface. Residues 208–213 in molecule A 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 (Pro, 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 Asp 8 Oδ1 atom to the C2 atom of the substrates, whereas no density for a covalently attached chlorine atom is observed (Fig. 1, B and C). This indicates that a covalent bond has formed between the nucleophilic Asp 8 residue and the substrate and that the covalent Cl–C bond in the substrate has been cleaved. The Asp 8 Oδ2 atom has a carbonyl functionality in the enzyme-ester intermediate structure. It is hydrogen-bonded to the hydroxyl group of the Thr 12 side chain and the side chain amide group of Asn 173 (Fig. 3A). Furthermore, in the MCAA covalent intermediate Ser 171 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 Asp 8 side chain is rotated $–30^\circ$ about the Cβ–Cγ bond, bringing Ser 171 Oγ much closer to the Oδ2 atom and at the same time increasing the distance to Thr 12 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 Asp 8 Oδ2 atom. The negative charge of this oxyanion intermediate might be stabilized by Thr 12, Ser 171, and Asn 173, 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, Asn 115, and Arg 199. It is found in line with the Asp 8 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 Arg 199 and to the side chain amide group of Asn 115. 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 Phe 175 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 (DHAl) (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, Tyr 10 and Phe 58, have a less favorable ring orientation. The stabilizing cradle of positively charged and polar amino acids most likely

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. deviation 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 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.

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 (Pro, 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.

The chloride ion is present close to the substrate C2 atom, Asn 115, and Arg 199. It is found in line with the Asp 8 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 Arg 199 and to the side chain amide group of Asn 115. 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 Phe 175 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 (DHAl) (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, Tyr 10 and Phe 58, have a less favorable ring orientation. The stabilizing cradle of positively charged and polar amino acids most likely
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 carbamoyloxy 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 NH groups of residues 9 and 10 and with the main chain atoms of residues 10 and 11, 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 structure of the complex with MCPA, the only chiral substrate to be efficiently degraded by DhlB (9), shows clear electron density for the C3 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 a2 and a10, thereby limiting its size to about 75 Å3. This explains why the substrate specificity of DhlB is restricted to small haloalkanoates (9).

TABLE I

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<th>DhIB + MCAA, pH 5.0</th>
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a Values in parentheses are for the highest resolution bin.

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

c Estimated from cross-validated σA and Luzzati plots (46), calculated from test set data in the 5.0–1.52 Å resolution range.

d Estimated from cross-validated σA and Luzzati plots (46), calculated from test set data in the 5.0–1.52 Å resolution range.

Fig. 2. Two conformations of residues 208–213 of molecules A and B at the dimerization interface.
residues that stabilize the Asp$^8$ O$_{d1}$ oxyanion and the halide ion would be absolutely conserved. In DhlB, Asn$^{173}$ is part of the oxyanion hole and Phe$^{175}$ 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$^8$ side chain. The aberrant side chain conformation of Asp$^8$ in the MCAA-bound l-DEX YL structure is stabilized by hydrogen bonds of the Asp$^8$ O$_{d1}$ ether oxygen atom to Thr$^{12}$ and of the Asp$^8$ O$_{d2}$ carboxyl oxygen atom to the Lys$^{147}$ 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$^8$ O$_{d2}$ and Ser$^{171}$ O$_{Y}$ 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$^{39}$ 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$^8$ O$_{d1}$ atom on the C$_2$ of the substrate, resulting in the formation of a covalent enzyme-ester intermediate (Fig. 5). The salt bridge to the positively charged Lys$^{147}$ side chain reduces the pK$_a$ of the aspartate, thereby increasing its nucleophilicity. The formation of the O$_{d1}$–C$_2$ bond is accompanied by the cleavage of the C$_2$–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$^{29}$, Asn$^{115}$, and Phe$^{175}$. As a result, the configuration of the other three substituents of the C$_2$ 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 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 Asp176 might increase the pKₐ 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 Nε atom of the Histidine, 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 Lys147 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 Tyr531, 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 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 phospho-
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 hydrolisis 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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