Crystal Structure of \( \text{L-2-Haloacid Dehalogenase from Pseudomonas sp. YL} \)

AN \( \alpha/\beta \) HYDROLASE STRUCTURE THAT IS DIFFERENT FROM THE \( \alpha/\beta \) HYDROLASE FOLD*  

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\( \text{L-2-Haloacid dehalogenase catalyzes the hydrolytic dehalogenation of L-2-haloalkanoic acids to yield the corresponding } \) \( \alpha/\beta \) \( \text{hydroxyalkanoic acids. The crystal structure of the homodimeric enzyme from Pseudomonas sp. YL has been determined by a multiple isomorphous replacement method and refined to 2.5 Å resolution, with a crystallographic R-factor of 19.5%.} \)  

The subunit consists of two structurally distinct domains: the core domain and the subdomain. The core domain has an \( \alpha/\beta \) structure formed by a six-stranded parallel \( \beta \)-sheet flanked by five \( \alpha \)-helices. The subdomain inserted into the core domain has a four helix bundle structure providing the greater part of the interface for dimer formation. There is an active site cavity between the domains. An experimentally identified nucleophilic residue, Asp-10, is located on a loop following the amino-terminal \( \beta \)-strand in the core domain, and other functional residues, Thr-14, Arg-41, Ser-118, Lys-151, Tyr-157, Ser-175, Asn-177, and Asp-180, detected by a site-directed mutagenesis experiment, are arranged around the nucleophile in the active site. Although the enzyme is an \( \alpha/\beta \) hydrolase, it does not belong to the \( \alpha/\beta \) hydrolase fold family, from the viewpoint of the topological feature and the position of the nucleophile.

Halogenated organic compounds are widely used as materials for various industrial products such as insecticides and solvents and cause serious environmental problems owing to their persistence and/or toxicity. Several bacterial strains have been found to take up the compounds as sole carbon and energy sources and to degrade them using enzymes called dehalogenases. Bacterial 2-haloalkanoic dehalogenases (EC 3.8.1.2) isolated from various sources and to degrade them using enzymes called dehalogenases have been found to uptake the compounds as sole carbon and energy sources and to degrade them using enzymes called dehalogenases. Several bacterial strains have been identified that can degrade these compounds.

\[ \text{RCHXCOOH} + \text{OH}^- \rightarrow \text{RCHOHCOOH} + \text{X}^- \]

**Reaction 1**

where \( R = \text{H or alkyl group and } X = \text{halogen atom, 2-Haloalkanoic acid (the substrate) and 2-hydroxyalkanoic acid (the product) are both optically active. Thus, the enzymes found so far are classified into four types on the basis of the specificity toward the configurations of chiral carbons of their substrates and their reaction products as follows. L-2-Haloide dehalogenases (L-DEXs) act on L-2-haloalkanoic acids to yield \( \alpha \)-2-hydroxyalkanoic acids (1–3, 7); D-2-haloalkanoic dehalogenases catalyze the dehalogenation of D-2-haloalkanoic acids to the corresponding L-2-hydroxyalkanoic acids (4); L-2-Haloide dehalogenases (inversion type) act on both isomers of 2-haloalkanoic acids and yield products with inversion of the \( C_2 \)-configurations (5); D-2-haloide dehalogenases (retention type) dehalogenate both L- and D-2-haloalkanoic acids to the corresponding L- and D-2-hydroxyalkanoic acids, respectively (6); L-2-Haloide dehalogenases (L-DEXs) have been isolated from several strains and most extensively studied. L-DEXs are very similar to one another in their primary structures (8), and also to haloacetae dehalogenase H-II from Moraxella sp. (9).

Recently, Pseudomonas sp. YL has been found to produce L-DEX, which is inducibly expressed by 2-chloropropionate (10). The enzyme (L-DEX YL) has been purified to homogeneity and characterized physicochemically (1). Its structural gene has been cloned and overexpressed in Escherichia coli (8, 11). L-DEX YL is a dimeric enzyme (1). Each subunit consists of 232 amino acid residues whose sequence has been deduced from the gene sequence, and its molecular weight is 26,179 (8). The enzyme shows several characteristic properties (1). It acts not only on L-2-haloalkanoic acids with short carbon chains (2–5 carbons) in aqueous solution but also on long chain substrates with up to 16 carbons in organic solvent. Thus the enzyme is useful for the production of various \( \alpha \)-2-hydroxyalkanoic acids, which are important as starting materials in the chemical industry. In addition, it is highly thermostable and is not inactivated at all by incubation at 60 °C for 30 min. Its optimal temperature is 65 °C. The catalytic mechanism of L-DEX YL has been studied by comprehensive site-directed mutagenesis (12). External incorporation (13), and chemical modification with hydroxylamine (2). These studies revealed that Asp-10, Thr-14, Arg-41, Ser-118, Lys-151, Tyr-157, Ser-175, Asn-177, and Asp-180 are important for the enzymatic activity, and that Asp-10 plays an essential role as a nucleophile in the enzymatic reaction. Consequently, the following mechanism is proposed for the hydrolytic reaction. The reaction probably starts with a nucleophilic attack by Asp-10 on the \( \alpha \)-carbon of the substrate to form an ester intermediate, followed by the hydrolysis of the product.**
intermediate by an activated water molecule, and ends with the release of the product and the simultaneous regeneration of the intact enzyme. To confirm this proposal and to obtain a more detailed description of the catalytic mechanism of the enzyme, it is indispensable to investigate at atomic level how the catalytic residues are arranged in the active site of L-DEX YL in order to interact with the substrate molecule. The crystal structure of haloalkane dehalogenase from Xanthobacter autotrophicus GJ 10 (HAL) is the only structure of dehalogenase that is available at atomic resolution (14, 15) and that leads to a proposal of the reaction mechanism based on the crystal structures of the enzyme-substrate complex under two different conditions (16). HAL is a member of the α/β-hydrolase fold family, which has a common characteristic fold comprising an eight-stranded parallel β-sheet flanked by α-helices and catalytic triad residues on the topologically and sterically equivalent loci on the specific turns and loop: the nucleophile elbow, the acidic turn, and the histidine loop (17). The catalytic triad occurs in the primary structure in the invariant order of Nu-Ac-His, where Nu is a nucleophile and Ac is an acidic amino acid residue. The nucleophile elbow has a consensus sequence of Sm-X-Nu-X-Sm-X, where Sm is a small amino acid residue and X is an arbitrary residue. Although L-DEXs catalyze a hydrolysis similar to that catalyzed by HAL, L-DEXs show low sequence similarity with HAL (8) and do not seem to have a similar catalytic triad in the order Nu-Ac-His. Therefore, it seems difficult to predict the roles of the catalytic residues of L-DEXs other than Asp-10, based on the comparison with HAL, although L-DEXs have active site nucleophiles and their reactions proceed via ester intermediates, as HAL does. In order to elucidate the mechanisms of catalysis and stereoselectivity of L-DEXs, we have determined the crystal structure of L-DEX YL at 2.5 Å resolution by multiple isomorphous replacement. This is the first report on the tertiary structure of the L-DEX family.

MATERIALS AND METHODS

Crystal Preparation—The dimeric enzyme L-DEX YL was purified (1) and crystallized (48) as described previously. Rhombohedral crystals were obtained by the vapor diffusion of a 15 mg/ml enzyme solution against a 50 mM potassium dihydrogenphosphate solution containing 150 mM (w/v) polyethylene glycol 8000 and 1% (w/v) n-propyl alcohol at 4°C. A typical crystal size was about 0.6 mm × 0.5 mm × 0.1 mm. They belong to space group C2 with unit cell dimensions a = 52.21 Å, b = 62.78 Å, c = 50.84 Å, and β = 122.4°. The crystal volume per unit mass, Vm, (18), is 2.37 Å³/Da with one subunit in the asymmetric unit, which corresponds to a solvent content of 48%. The crystals are of a good quality suitable for high resolution x-ray analysis and diffract up to at least 1.8 Å resolution. They were stored in 50 mM acetate buffer (pH 5.0) containing 15% (w/v) polyethylene glycol 8000 and 1% (w/v) n-propyl alcohol at 4°C. Preparation of heavy atom derivatives was tried under various conditions by soaking and cocrystallization methods using the same buffer solution as that used for crystal storage. Two kinds of isomorphous heavy atom derivatives were prepared by soaking crystals in 1 mM UO(NO3)2 for 6 days and 3 mM K[Au(CN)2] for 4.5 days, respectively, at 25°C. They showed diffraction patterns significantly different from that of the native crystal. Uranyl and aurate derivatives were used for high resolution solution of the structure by isomorphous replacement.

Data Collection and Processing—Diffraction data for the native and two derivative crystals were collected at 20°C on a Rigaku R-AXIS IIC imaging plate detector system using graphite-monochromatized CuKα radiation produced by a Rigaku RU-300 rotating anode generator operated at 40 kV and 100 mA. The data collection for each of the three kinds of crystals was done with one crystal sealed in a glass capillary. The crystals were mounted with the crystallographic b* axis parallel to the crystal rotation axis to record Bijvoet-mates on the same frame. The crystal-to-detector distance was set to 95.0 mm. Each frame of 3.6° crystal oscillation was collected for 10 min. Diffraction spots in a rotation range over 180° were recorded on a total of 52 frames for each data set. Data processing was accomplished at 2.5 Å resolution with the R-AXIS IIC data processing software package. Diffraction intensities of Bijvoet-pair reflections were processed for the derivatives. The diffraction data were merged and scaled to yield Rmerge values of 4.0, 6.1, and 4.6% for the native, uranyl, and aurate data sets, respectively. Data collection and processing are summarized in Table I.

Structure Determination and Refinement—The structure was solved by the isomorphous replacement method supplemented with anomalous scattering effects from both uranyl and aurate derivative crystals. All of the calculations for the isomorphous replacement were made with the PHASES program package (19). Difference Patterson maps for both derivatives calculated at 15–5.0 Å resolution showed an interpretable single heavy atom map of derivative uranyl. The major gold site was located in a 5 Å cross-difference Fourier map calculated by single isomorphous replacement with the major uranium site. The parameter refinement of heavy atom sites was carried out with a gradual increase in resolution from 5.0 to 2.5 Å. In the course of refinement, minor heavy-atom sites for both derivatives were found in difference Fourier maps calculated by double isomorphous replacement, and were included in further refinement. Finally, three uranium sites and two gold sites were identified. In the final stage of the refinement using anomalous scattering effects from both heavy atom derivatives, the refinement handedness for the enzyme molecule was selected by checking refinement statistics and difference Fourier peaks for both enantiomorphs of the heavy atom sites. The last cycle of refinement yielded multiple isomorphous replacement phases with the mean figure of merit of 0.70. Phase calculation is summarized in Table II.

A 2.5 Å resolution electron density map was calculated with multiple isomorphous replacement phases and displayed on a Silicon Graphics IRIS INDIGO-Elan workstation using the program TURBO-FRODO (20). The map showed unambiguous connectivities of electron density corresponding to the polypeptide chain and could be interpreted by reference to the published amino acid sequence (8) to build a model of L-DEX YL. The region including the residues Trp-40, Tyr-47, and Trp-49, was easily recognized on the map, forming an α-helix on the dimer interface of the molecule. Therefore, model building was initiated with this helix as the starting point. In the course of model building, a mini-map drawn on transparent sheets and a skeletonized map calculated with the program BONES in PHASES were consulted to resolve some ambiguous interpretations. Three amino-terminal residues and 10 carboxyl-terminal residues were invisible on the density map, probably due to their flexibility, and they were therefore excluded from the model. Thus the initial model for a subunit of L-DEX YL molecule was composed of 219 amino acid residues of Ile-4–Phe-222.

Refinement of the subunit model was carried out with the simulated annealing protocol of the program X-PLOR (21). Isotopic temperature factors were initially set to 15 Å² and refined in the last few cycles of refinement. Occupancies were fixed to unity for protein atoms. In the course of refinement, the model was carefully inspected with 2Fo–Fc and Fo–Fc maps and some ambiguity in the structure was resolved. Difference Fourier peaks that appeared within 3.5 Å of the protein atoms or other solvent molecules were assigned to solvent molecules. Solvent molecules approaching too close to the protein or moving far away from the protein were rejected during refinement. The initial R-factor was 42.6% for 7,848 reflections (F = 2σ(F)) in the range of 8.0–1.5 Å resolution. After eight cycles of refinement, the crystallographic R-factor was improved to 19.5% for the model comprising 2,157 protein atoms and 19 water molecules. The atomic coordinates have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

RESULTS AND DISCUSSION

Accuracy of the Model—A current model of L-DEX YL subunit comprising 219 (residues 4–222) of the total 232 residues

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**Table I**

Summary of data collection statistics

|        | Nativea | UNO3(b) | K[Au(CN)](c) |
|--------|--------|---------|--------------|
| Resolution (Å) | 2.5    | 2.5    | 2.5          |
| Observations  | 31,020 | 30,320 | 31,218       |
| Unique reflections | 8,214  | 8,087  | 8,119        |
| Bijvoet pairs   | 7,532  | 7,532  | 7,539        |
| Completeness (%)| 93.9   | 93.7   | 93.1         |
| Rmerge (%)      | 0.040  | 0.061  | 0.046        |

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(a) Data set.  
(b) Rmerge = Σ(|Fo| − |Fc|)/Σ|Fo|, where I is the intensity of individual measurements, and |l| is the mean intensity over all measurements.
and 19 water molecules was refined to an R-factor of 19.5% for data (F ≥ 2σ(F)) in the resolution range of 8.0–2.5 Å with a good agreement with the ideal stereochemistry. The root-mean-square deviations from the ideal geometry are 0.012 Å for bond angle and 1.8° for bond angles. The coordinate error estimated from a Luzzati plot (22) is 0.25 Å. The average temperature factors are 16.0 Å² for the main chain and 20.0 Å² for the side chains. No electron density is visible for the first three amino-terminal and the last 10 carboxyl-terminal residues, which may be located on the molecular surface with high flexibility. Most of the 219 residues, ca. 83%, are found in most favored regions of a Ramachandran plot (23), and the remaining are in allowed regions except only two glycines in the forbidden region. Thus, the model shows that the L-DEX YL molecule has a reasonable and well defined structure. A representative electron density map is shown in Fig. 1.

Subunit Structure—Figs. 2 and 3A show the structure and the topology of a subunit of L-DEX YL, respectively. The subunit of L-DEX YL has a unique shape resembling an elephant, with overall dimensions of about 50 Å × 40 Å × 40 Å (Fig. 2). It consists of two domains: the core domain, which looks like an elephant head, and the subdomain, which looks like an elephant head with a long trunk. The core domain comprises amino acid residues 4–15 and 97–222 (Fig. 3B), respectively. Three amino-terminal and 10 carboxyl-terminal residues, which account for about 65% of all the residues of the enzyme. The core domain has an α/β-type structure consisting of a central six-stranded parallel β-sheet (β1, β4–β8) flanked on both sides by five α-helices; three (α5, α6 and α9) are on one side and two (α7 and α8) are on the other side of the sheet. Repetitions of the typical β-α-β connectivity are observed with right-handed crossover connections, although there is a deviation from the β-α-β connectivity between β7 and β8, which are connected together through a long loop. Each β-strand consists of 3–6 amino acid residues, and each α-helix consists of approximately three turns. The order of strands in the β-sheet (β5-β4-β1-β6-β7-β8) is 3-2-1-4-5-6. Interestingly, the core domain has a switch point of the β-strand topology (24) in the middle of the central six-stranded β-sheet, where the order of the strands reverses between the third and fourth strands; the first three strands are aligned from the center to the left side, and the last three to the right side (Fig. 3A). Three 3_10-helices are formed around β5 and in front of β6. The subdomain is formed by amino acid residues 16–96 (Fig. 3B), which account for about 35% of all the residues of the enzyme. The subdomain has a distorted four-helix-bundle structure comprising four α-helices (α1-α4) packed in an approximately antiparallel manner. In the bundle structure, three of them (α1, α3, and α4) run side by side, and the longest (six-turn) α-helix (α2) crosses the others. The α-helix (α2) projects into the surface of the subunit like the trunk of an elephant, and plays an important role in dimerization of the subunits to form the L-DEX YL molecule, along with α-helix (α3) (see below). All of the α-helices are sequentially connected to each other by relatively short polypeptide chains. The subdomain is inserted between the first β-strand (β1) and the first α-helix (α5) of the core domain like an elephant head and is connected to the core domain through a two-stranded antiparallel β-sheet (β2–β3), which corresponds to an elephant neck. A 3_10-helix is formed in the loop between α4 and β3.

There is a large cavity between the two domains. In the dimer molecule of L-DEX YL, the cavity of one subunit is walled on its surface by the subdomain of the other subunit. Consequently, the cavity becomes narrower to form the active site cleft of the subunit. The carboxyl-terminal side of the parallel β-sheet in the core domain faces the bottom of the cleft, suggesting that the cleft provides an active site.

Structural Comparison with Other Proteins—The core domain of L-DEX YL has an α/β-type structure. The topology of the secondary structure elements in the core domain (Fig. 3) is quite similar to those of coenzyme-binding domains of NAD-dependent dehydrogenases, although L-DEX YL lacks an α-helix between the fifth and sixth β-strand (β7 and β8), as is observed in horse liver alcohol dehydrogenase (25) but not in most of the NAD-dependent dehydrogenases. In addition, the core domain has an insertion of the subdomain between β1 and α5. A comparison between the tertiary structures of the core domain of L-DEX YL and the NAD-binding domain of horse liver alcohol dehydrogenase shows similarities in the β-sheet region and in the helices around the sheet. It is interesting that these two proteins possessing homologous structures have different functions: hydrolysis and coenzyme-binding. Functional and evolutionary relationships between L-DEX YL and NAD-dependent dehydrogenases are unclear. The structural classification data bank (26) was searched for other proteins that have the same β-sheet topology. Among enzymes catalyzing hydrolysis, N-carbamoylsarcosine amidohydrolase from Arthrobothacter sp. (CHSase) (27) was found to represent a similar topology and spatial arrangement of secondary structure elements to those of L-DEX YL, although CHSase is a tetrameric enzyme composed of single-domain subunits having additions of an amino-terminal α-helix and a carboxyl-terminal polyproline helix. Furthermore, it was found that the functionally important residues of L-DEX YL lie on the topologically equivalent sites to those of the putative active site residues of CHSase: residues Asp-10, Thr-14, Ser-118, Lys-151, Asn-177, and Asp-180 of L-DEX YL are on the equivalently topologically sites of Asp-10, Thr-14, Ser-118, Lys-151, Asn-177, and Asp-180 of L-DEX YL are on sites topologically equivalent to those of residues Asp-51, Trp-56, Asn-94, Lys-144, Ala-172/Thr-173, and Cys-177 of the CHSase, respectively. In particular, O2 of Asp-10, N of Lys-151, and O2 of Asp-180 in the former enzyme can be approximately superimposed on O2 of Asp-51, N of Lys-144, and S of Cys-177 in the latter enzyme, respectively. However, a significant difference between the the structures of two enzymes is that the nucleophile Asp-10 of L-DEX YL is not located on the same site as the nucleophile Cys-177 of CHSase, which was tentatively identified on the basis of observations of mercury and sulfate binding (27). These findings suggest that these two enzymes might be evolutionally related, although this should be carefully confirmed by further investigations.

HAL, a monomeric enzyme that catalyzes the hydrolysis of the carbon-halogen bond of substrates such as 1,2-dichloroethane (7), is the only enzyme, other than L-DEX YL, whose crystal structure is available among dehalogenases. The tertiary struc-
The structure of HAL (14, 15) shows that it consists of two domains, domain I and domain II. Domain I has an α/β-type structure with a central, eight-stranded, mainly parallel β-sheet. A comparison between the structures of the core domain of L-DEXYL and domain I of HAL revealed a significant difference in at least three points. First, although both L-DEXYL and HAL have central domains that adopt α/β-type structures, they are not similar to each other in terms of their topological features.
L-DEX YL has a central six-stranded parallel β-sheet with strand order of 3-2-1-4-5-6, while HAL has a central eight-stranded dominantly parallel β-sheet with strand order 1-2-4-3-5-6-7-8 where strand 2 is antiparallel to the others. Second, the residues that are important for catalysis in the L-DEX YL are located in quite different topological positions from those of the catalytic triad of HAL, which is located on the specific turns or loop. Though Ser-175/Asn-177/Asp-180 of L-DEX YL are on the site topologically equivalent to Asp-124 of HAL on the "nucleophile elbow" between β7 and α3 (HAL notation), no sites of the other catalytically important residues of L-DEX YL correspond topologically to those of the other triad residues of HAL, i.e. Asp-260 (on the "acidic turn" between β9 and α11) and His-289 (on the "histidine loop" between β10 and α12) (15).

Finally, the nucleophile Asp-10 of L-DEX YL is located on the loop following the amino-terminal β-strand with dihedral (φ, ψ) angles of (100.8°, 169.0°) in the energetically favorable region of a Ramachandran plot (23), whereas the nucleophile Asp-124 of HAL sits on the turn (the nucleophile elbow) with dihedral (φ, ψ) angles of (49.0°, 135.0°) in the energetically unfavorable region. These observations suggest that these two enzymes have different structural features and that they might have evolved from different ancestors and acquired similar functions during the course of their molecular evolution.

The structure representing the features of HAL described above is called the α/β hydrolase fold (17) and is often observed in other hydrolases such as dienelactone hydrolase (28), acetyleholinesterase (29), thioesterase (30), carboxypeptidase (31, 32), lipase (33–39), and cutinase (40). Although L-DEX YL also has an α/β-type structure, it is different from the α/β hydrolase fold shown above. Thus L-DEX YL cannot be grouped into the same superfamily as the α/β hydrolase fold enzymes. The structural comparison above shows that CHSase is structurally homologous to L-DEX YL. In addition, a computer analysis of sequence homology around residues of L-DEXs (41), corresponding to Asp-10, Lys-151, and Ser-176 of L-DEX YL, shows that L-DEXs belong to a large superfamily of hydrolases with diverse substrate specificity and that the superfamily includes cytosolic epoxide hydrolases, different types of phosphatases, and numerous uncharacterized proteins from eubacteria, eu-karyotes, and Archaea. Since tertiary structures of the enzymes used in the sequence analysis are as yet unavailable except for L-DEX YL, it may presently be impossible to evaluate the result on structural viewpoints. However, the results from comparisons of both structure and sequence suggest that a new superfamily of hydrolases may be identified by further studies on tertiary structures of these enzymes including L-DEXs. Thus L-DEX YL may show a structure representative of a new superfamily of hydrolases.

Intersubunit Interaction in the L-DEX YL Dimer—The L-DEX YL molecule is a dimer consisting of two identical subunits (subunit mass, 26,179 Da). It has a compact and rotation-ellipsoidal shape with dimensions of 76 Å × 40 Å × 40 Å (Fig. 4). Two subunits, which are related by an intramolecular 2-fold
The subunits associate so as to form the intersubunit interface between the subdomain and the carboxyl-terminal half of the core domain. As a consequence, the longest \( \alpha \)-helices (\( \alpha_2 \)) running through the molecule are close to the molecular 2-fold axis. The \( \alpha \)-helices are inclined at about 30° to each other so that their amino termini are slightly farther apart than their carboxyl termini. Therefore, the molecule has a wide depression or opening around the amino termini of \( \alpha \)-helices (\( \alpha_2 \)), leading to the active site (Fig. 4). The dimer is stabilized through hydrophobic interactions and complicated hydrogen bond networks. There are five regions in each subunit contributing to the formation of the intersubunit interface: the first region (R1) of Leu-39–Arg-56 on \( \alpha \)-helix (\( \alpha_2 \)) and the second region (R2) of Asp-66–Arg-73 on \( \alpha \)-helix (\( \alpha_3 \)) lie in the subdomain, while the third region (R3) of Pro-152–Asp-153 on the loop between \( \beta \)-strand (\( \beta_5 \)) and \( \alpha \)-helix (\( \alpha_7 \)), the fourth region (R4) of Trp-179–Tyr-186 on \( \alpha \)-helix (\( \alpha_8 \)), and the fifth region (R5) of Val-200–Met-204 on the loop between \( \beta \)-strands (\( \beta7 \) and \( \beta8 \)) lie in the carboxyl-terminal half of the core domain. It appears that the regions (R1 and R2) contribute to the great part of interaction between the subunits, and thus the subdomain may be important for dimerization of the subunit. The longest \( \alpha \)-helices (\( \alpha_2 \)) from both subunits are in close contact with each other in their R1 regions around the molecular 2-fold axis. The R1-R3 interaction results from the contact between Tyr-47 from one subunit and Pro-152 from the other subunit. The R1 and R4 regions interact with each other through the helix-helix interaction. The R1-R5 and R2-R5 interactions are attributed to the interaction between helix and loop. As a consequence, a hydrophobic cluster is formed around the molecular 2-fold axis by side chains of residues Leu-39, Leu-45, Tyr-47, Trp-49, Leu-50, Leu-53, Met-54, Phe-70, Pro-152, Trp-179, Gly-183, Tyr-186, and Met-204 from each subunit. In addition, hydrogen bond networks are formed in the peripheral part of the dimer interface surrounding the hydrophobic dus.
ter. In particular, a hydrogen bond network is formed in the
depression between \(a\)-helices \(a25\) by side chains of Gln-42,
Lys-43, Glu-46, Tyr-47, Glu-202, and Glu-203 from each sub-
unit. These side chains are linearly hydrogen-bonded through
the sequential linkage across the molecular 2-fold axis. All of
the residues involved in dimer formation in \(\text{L-DEX YL}\) are
highly conserved in the \(\text{L-DEX}\) family, with the exception
of Tyr-186. The \(\text{O}^{\bullet}\) atom of Tyr-186 is also hydrogen-bonded to
the carbonyl \(\text{O}\) atom of Met-204 in the subunit, so the correspond-
ing residues of other \(\text{L-DEX}\) may be involved in the intrasu-
unit interaction rather than the intersubunit hydrophobic
interaction. These facts suggest that there are similar inter-
subunit interactions in other oligomeric \(\text{L-DEX}\) molecules from
other sources.

The Active Site—The primary structures of seven \(\text{L-2-halo}-
acid\) dehalogenases and one haloacetae dehalogenase from vari-
ous bacterial strains are similar to one another (36–70% sim-
ilarity) (8), and 36 charged and polar amino acid residues are
conserved among the enzymes. Our previous study of site-
directed mutagenesis, where all the conserved residues were
mutated in \(\text{L-DEX YL}\), revealed that replacement of each of
Asp-10, Thr-14, Arg-41, Lys-151, Tyr-157, Ser-175, Asn-177,
and Asp-180 by other amino acid residues led to a significant
decrease in the \(V_{\text{max}}\) value for \(\text{L-2-chloropropionate dehaloge}-
nation}\) (12), and that replacement of Ser-118 by alanine caused
an increase in the \(K_{\text{m}}\) value for \(\text{L-2-chloropropionate}\) (12). Re-
placement of these residues did not cause conformational de-
formation of the enzyme detectable by circular dichroism, other
spectrophotometric techniques, or gel filtration. Thus it is
likely that these residues are involved in catalysis and/or sub-
strate binding in the wild-type enzyme.

The present crystallographic study shows that all the above
residues except for Arg-41 belong to the core domain (Fig. 3B),
clustering at the bottom of the cleft between the core- and
subdomains (Fig. 5). As many as nine water molecules are
found in this region, which make a complicated hydrogen bond
network with the above residues. The cleft is wide open at its
entrance, which is consistent with the observation that \(\text{L-DEX}
\) acts on \(\text{L-2-haloalkanoic acids}\) with long alky groups such as
\(\text{L-2-bromohexadecanoic acid}\) (1). This indicates that the bot-
tom of this cleft provides the active site of the enzyme.

Most proteins with \(\alpha/\beta\)-type structures have their active sites
at topological switch points, where a cleft is formed between
two adjacent loops on opposite sides of a \(\beta\)-sheet (24). Such a
region is appropriate for substrate binding. Figs. 2 and 3A show
that the topological switch point of \(\text{L-DEX YL}\) is present be-
tween \(\beta1\) and \(\beta6\); two adjacent loops in this region are wound
clockwise and located on each side of the \(\beta\)-sheet. Five of the
above residues expected to be involved in catalysis and/or sub-
strate binding are present at this topological switch point;
Asp-10 and Thr-14 are found on the loop that follows \(\beta1\),
Ser-175 at the carboxyl end of \(\beta6\), Asn-177 on the loop between
\(\beta6\) and \(\alpha8\), and Asp-180 at the amino end of \(\alpha8\). These findings
also support the above prediction that this region is the active
site of the enzyme.

Possible Roles of the Active Site Residues of \(\text{L-DEX YL}\)—The
hydrogen-bonded triad consisting of nucleophilic amino acid,
histidine, and acidic amino acid residues plays an essential role
in the catalysis of at least four groups of hydrolases: serine
proteases such as trypsin and chymotrypsin, cysteine proteases
such as papain, subtilisins, and \(\alpha/\beta\) hydrolase fold enzymes
such as HAL (17). In the catalysis of these hydrolases, a nu-
cleophilic amino acid residue attacks a substrate to form a
covalently bonded enzyme-substrate complex such as acylated
or alkylated enzymes, and then the complex is hydrolyzed by a
water molecule activated by a histidine residue, resulting in
the yield of product and the regeneration of enzyme. Although
the tertiary structures of these four groups of hydrolases are
completely different from one another, there is a striking sim-
ilarity among these hydrolases in the spatial organization of
the key atoms of the catalytic triad (17).

\(\text{L-DEX YL}\) has a catalytic nucleophile, Asp-10 (13). The ester
intermediate is formed from Asp-10 and the substrate in the
enzyme reaction, and hydrolyzed by the solvent water. This
mechanism is quite similar to that of HAL, which belongs to the
\(\alpha/\beta\) hydrolase fold family. However, in contrast to HAL and
other hydrolases described above, \(\text{L-DEX YL}\) does not have this
kind of a catalytic triad. His-19, the only conserved histidine
residue among the \(\text{L-2-haloacid dehalogenase}\) family, is located
on \(\alpha1\) in the subdomain and is too far from Asp-10 to activate
a water molecule required for the hydrolysis of the ester inter-
mediate. The site-directed mutagenesis study also showed that
His-19 is inessential for catalysis (45). Accordingly, it is as-
sumed that \(\text{L-DEX YL}\) has a different set of residues for the
catalysis.

Amino acid residues involved in substrate binding, halide ion
abstraction, and activation of the water molecule for the hy-
drolysis of the ester intermediate are expected to be present in
the active site of \(\text{L-DEX YL}\). The candidates for halide ion
abstraction are Tyr-12, Asn-119, Lys-151, Asn-177, and Trp-
179 (Fig. 5). Of these, Asn-119 is unlikely because the mutagen-
esis study showed that the N119D enzyme had the activity
comparable with that of the wild-type enzyme (12). Trp-179 is
also unlikely because any change in the fluorescence spectrum
of the enzyme could not be detected upon addition of a substrate, suggesting that the substrate did not interact with Trp-179.\(^3\) If this is the case, the residue for halide ion abstraction in \(\text{L-DEX YL}\) is different from those in haloalkane dehalogenase, where two tryptophan residues are involved in halide ion abstraction. However, it is also possible that \(\text{L-DEX YL}\) caused only a slight change in the fluorescence spectrum of Trp-179, hardly detectable upon addition of the substrate, because the fluorescence intensity of the tryptophan residue, whose fluorescence intensities might be much higher than that of Trp-179. Asn-177 and Lys-151 are also unlikely because there is not enough space for abstraction of the halide ion between Asn-177 or Lys-151 and the nucleophile Asp-10 (the interatomic distances are about 5.0 Å between N\(^1\) of Asn-177 and O\(^2\) of Asp-10, 3.9 Å between N\(^2\) of Asn-177 and O\(^2\) of Asp-10, 5.0 Å between N\(^1\) of Lys-151 and O\(^3\) of Asp-10, and 4.8 Å between N\(^1\) of Lys-151 and O\(^2\) of Asp-10). However, if the enzyme changes its conformation upon addition of the substrate, Asn-177 and Lys-151 could be candidates for the residues interacting with the halogen atom of the substrate.

The most probable residue for halide ion abstraction is Tyr-12. In the previous site-directed mutagenesis study, it was shown that the specific activity of the mutant enzyme, Y12F, was about 40% of that of the wild-type enzyme. Assuming that an aromatic ring is required as an acceptor for a halogen atom, this result may be reasonable, because the aromatic ring can be spatially conserved in each case. The assumption could be supported by the report that negatively charged atoms have a preference for interaction with the partially positively charged edge of an aromatic ring (46, 47). Thus the aromatic ring of Tyr-12 could be an acceptor for the halide ion. In addition, there is a suitable space between Tyr-12 and Asp-10 to accommodate the substrate. Tyr-12 is completely conserved by seven L-DEXs as well as \(\text{L-DEX YL}\) will be helpful in understanding the reaction mechanism of \(\text{L-DEX}\). In addition it will be exciting to know how DEXs can express their stereoselectivity toward \(\text{C}_2\)-configurations of substrates and products. Further crystallographic studies on DEXs are in progress.

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