Crystal Structures of Reaction Intermediates of L-2-Haloacid Dehalogenase and Implications for the Reaction Mechanism*

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Crystal structures of L-2-haloacid dehalogenase from Pseudomonas sp. YL complexed with monochloroacetate, L-2-chlorobutyrate, L-2-chloro-3-methylbutyrate, or L-2-chloro-4-methylvalerate were determined at 1.83-, 2.0-, 2.2-, and 2.2-Å resolutions, respectively, using the complex crystals prepared with the S175A mutant, which are isomorphous with those of the wild-type enzyme. These structures exhibit unique structural features that correspond to those of the reaction intermediates. In each case, the nucleophile Asp-10 is esterified with the dechlorinated moiety of the substrate. The substrate moieties in all but the monochloroacetate intermediate have a D-configuration at the C2 atom. The over-structure moieties in all but the monochloroacetate intermediates correspond to those of the reaction intermediates. The substrate-free enzyme, is present in the vicinities of the substrate moiety; this residue probably serves as a binding residue for the substrate carboxyl group. The hydrophobic pocket, which is primarily composed of the Tyr-12, Gln-42, Leu-45, Phe-60, Lys-151, Asn-177, and Trp-179 side chains, exists around the alkyl group of the substrate moiety. This pocket may play an important role in stabilizing the alkyl group of the substrate moiety through hydrophobic interactions, and may also play a role in determining the stereospecificity of the enzyme. Moreover, a water molecule, which is absent in the substrate-free enzyme, is present in the vicinities of the carboxyl carbon of Asp-10 and the side chains of Asp-180, Asn-177, and Ala-175 in each intermediate. This water molecule may hydrolyze the ester intermediate and its substrate. These findings crystallographically demonstrate that the enzyme reaction proceeds through the formation of an ester intermediate with the enzyme's nucleophile Asp-10.

1-2-Haloacid dehalogenase (l-DEX)1 is a unique enzyme that catalyzes the hydrolytic dehalogenation of l-2-haloacids to produce the corresponding L-2-hydroxyacids with an inversion of the C2-configuration. Various l-DEXs exhibiting very similar sequences have been isolated from different bacterial sources. Of all the enzymes, l-DEX YL, isolated from a 2-chloroacrylate-utilizable bacterium, Pseudomonas sp. YL, is an unusual l-DEX, in that it is relatively thermostable even though it is derived from a mesophilic bacterium (1). It is a dimeric enzyme formed by two identical subunits of 232 amino acid residues. We have already reported the crystallization of l-DEX YL (2) and its crystal structure determined by a 2.5-Å resolution x-ray analysis (3). The enzyme has a core domain of α/β-structure, which differs topologically from those of the α/β hydrolase fold family proteins (4), along with a subdomain having a four-helix-bundle structure. Our ion spray mass spectrometric study showed that the dehalogenation of the l-2-haloacid catalyzed by l-DEX YL proceeds in the two-step mechanism through an ester intermediate (5). In the proposed reaction mechanism, the nucleophile Asp-10 first attacks the C2 atom of the substrate to form an ester intermediate and a halide ion, and, subsequently, a water molecule hydrolyzes the intermediate by attacking the C7 atom of Asp-10 to produce a L-2-hydroxyacid, restoring the side-chain carboxyl group of Asp-10. This mechanism has been further investigated by mass spectral analyses of the enzyme paracatalytically inactivated by hydroxylamine (6); the enzyme forms an adduct of the dehalogenated substrate moiety and hydroxylamine specifically at the Asp-10 position. We replaced each of the charged or polar amino acid residues (total 36 residues) of l-DEX YL, which are conserved in most of the l-DEXs, with another residue and found that, in addition to Asp-10, the residues of Thr-14, Arg-41, Ser-118, Lys-151, Tyr-157, Ser-175, Asn-177, and Asp-180 probably play an essential role in catalysis (7). X-ray crystallography revealed that all the residues except Arg-41 are located around the putative active site, nucleophile Asp-10, and that nine water molecules in this region form a complicated hydrogen bond network with the functionally important residues. All of the residues are thought to be involved in the catalytic process, which comprises selective recognition of the L-enantiomer of the 2-haloacid, binding of the carboxyl group of the substrate 1,2-haloacid, capture or withdrawal of the halide ion liberated from the 1,2-haloacid upon nucleophilic attack with

1 The abbreviations used are: l-DEX, L-2-haloacid dehalogenase; l-DEX YL, L-2-haloacid dehalogenase from Pseudomonas sp. YL; MCA, monochloroacetate; CPA, L-2-chloropropionate; CBT, 1,L-2-chloro-3-methylbutyrate; CMV, 1,L-2-chloro-4-methylvalerate; MCA, CBT, CMV, and CMV intermediates, reaction intermediates between the S175A mutant and the MCA, CBT, CMV, or CMV reagents, respectively; r.m.s., root mean square.

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by the side-chain carboxylate of Asp-10, formation of the ester intermediate, and activation of a water molecule for hydrolysis.

The reactions catalyzed by haloalkane dehalogenase from \textit{Xanthobacter autotrophicus} GJ10 (8–10), rat liver microsomal epoxide hydrolase (11), and 4-chlorobenzoyl-CoA dehalogenases from \textit{Pseudomonas} sp. strain CBS3 (12–14) and \textit{Arthrobacter} sp. 4-CB1 (15) also proceed through mechanisms similar to that of L-DEX YL, which involve the formation of an enzyme-substrate ester intermediate for which the acyl moiety is derived from a carbonyl group of the enzyme.

Haloalkane dehalogenase, epoxide hydrolase, and 4-chlorobenzoyl-CoA dehalogenase each represent individual families of proteins that differ from the L-DEX family. The X-ray crystallographic studies of haloalkane dehalogenase showed that the enzyme is a member of the \( \beta/\beta \) hydrolase fold family, which includes acetylcholinesterase, carboxypeptidase, and lipase (4). Members of the family have a common fold comprising an eight-stranded parallel \( \beta \)-sheet flanked by \( \alpha \)-helices, where the catalytic triad residue are located at topologically equivalent positions on particular turns and loops. In the haloalkane dehalogenase (9, 10), Asp-124 carries out a nucleophilic attack on the \( \text{C}_3 \) atom of substrates such as 1,2-dichloroethane to give an ester intermediate that is subsequently hydrolyzed by a water molecule activated by His-289. The halogen atom released from the substrate is captured by Trp-125 and Trp-175. In L-DEX YL, however, no histidine residue is present in the putative active site. His-19, which is the only histidine residue conserved of all L-DEXs, is not essential for the catalysis of L-DEX YL (16). These findings suggest that L-DEX YL utilizes a residue other than histidine to activate a water molecule for hydrolysis

Like the haloalkane dehalogenase, a conserved tryptophan residue, Trp-179, is located in the vicinity of the nucleophile Asp-10 in L-DEX YL, but it is not essential for the catalysis of the enzyme. The other three conserved tryptophan residues, Trp-40, Trp-49, and Trp-193, are located outside the active site. It is therefore assumed that in the case of L-DEX YL, the abstraction of the halogen is performed by residues other than tryptophan.

In order to elucidate the mechanism of catalysis and stereoselectivity of L-DEX YL by identifying the amino acid residues involved, it is indispensable to study tertiary structures of L-DEX YL complexed with its substrate in each step of the catalysis. We crystallized several mutants of L-DEX YL that are suitable for this purpose. Of these mutants, the S175A mutant provided good crystals, which allowed us to perform a high resolution X-ray crystallographic study. We prepared crystals of the S175A mutant complexed with various kinds of substrates to analyze their crystal structures by X-ray diffraction techniques. Four kinds of complexes gave electron density maps that unequivocally showed the formation of ester intermediates. Here we report the X-ray crystal structures of (E)-MCA, (E)-CBT, and CMV intermediates at 2.2–1.83-Å resolutions. The structures of these intermediates are essential not only for the identification of amino acid residues participating in each step of the catalysis but also for the elucidation of the mechanism of catalysis and stereoselectivity of L-DEX YL.

**MATERIALS AND METHODS**

**Preparation and Assay of Enzymes**—The wild-type and mutant enzymes of L-DEX YL were overproduced in recombinant \textit{Escherichia coli} cells and purified by ammonium sulfate fractionation followed by DEAE-Toyopearl chromatography (7, 17). The mutants Y12F and S175A of L-DEX YL were prepared as described previously (7), and the Y12A (GTA → GGC), Y12L (GTA → GAG), and Y12W (GTA → CCA) mutants were prepared by the method of Kunkel (18).

The activity of the wild-type and mutant enzymes for halide release was assayed with 25 mM MCA, CPA, CBT, CMB, and CMV as substrates. The reagents is(αS)-2-chloro-n-butyrate, L-(αS)-2-chloro-3-methyl-n-butyrate, and L-(αS)-4-chloro-n-methylpyruvate were used as the substrates for CPA, CBT, and CMV, respectively. Polyeethylene glycol 400 was added to the reaction mixture to a final concentration of 15% (v/v) to increase the solubility of these reagents. The amount of chloride ion released from the substrate was spectrophotometrically determined according to the method of Iwasaki \textit{et al.} (19). One enzyme unit of halide release activity was defined as the amount of enzyme that releases 1 pmol of halide ion/min. The amount of D-lactate produced from 25 mM L-2-chloropropionate was determined by measuring the amount of NADH produced by the reaction of D-lactate dehydrogenase with D-lactate in terms of absorbance at 340 nm.

In a typical assay, the reaction was started at 30 °C by adding 0.141 nmol of the wild-type enzyme or 13.0 nmol of the S175A mutant to a 1-mL reaction mixture of 0.1 x Tris-H\textsubscript{2}SO\textsubscript{4} solution (pH 9.5) containing 25 mM CPA and 5 mM NAD, and then 18.2 units of the D-lactate dehydrogenase (Sigma) were immediately added to the solution. The enzyme activity producing D-lactate from L-2-chloropropionate was estimated from the rate of the initial velocity to the molar absorption coefficient of 6,220 M\textsuperscript{-1} cm\textsuperscript{-1} for NADH; 1 unit was defined as the amount of enzyme needed to produce 1 pmol NADH/min. The amount of enzyme was estimated with the Bio-Rad protein assay kit. The activities of the wild-type enzyme and the S175A mutant are listed in Table I. Our ion-spray mass spectrometry study suggests that the production of D-hydroxyacid by hydrolysis after the halogen abstraction is the rate-determining step in the dehalogenation of L-2-haloacids catalyzed by L-DEX YL. These results suggest that the S175A mutant could be virtually inactive toward MCA, CBT, CMB, and CMV in the crystal at pH 5.5, although the mutant does display detectable activity toward these substrates in the solution at optimum pH.

**Crystal Preparation**—Crystals of the S175A mutant were grown by a method similar to that for the wild-type enzyme reported previously (2, 3). Rombhedral crystals were obtained by the vapor diffusion of a 15 mg/ml enzyme solution against a reservoir solution (pH 5.5) containing 50 mM potassium dihydrogenphosphate, 15% (w/v) polyethylene glycol 400 (approximate \( M_c \), 8000), and 1% (v/v) n-propanol at 4 °C. The typical crystal size was approximately 0.6 mm \times 0.5 mm \times 0.2 mm. The crystals belong to space group C2 with unit cell dimensions of \( a = 92.47 \) Å, \( b = 62.78 \) Å, \( c = 50.99 \) Å, and \( \beta = 122.7^\circ \), contain one subunit of the dimer molecule in the asymmetric unit, and are isomorphous with those of the wild type. They are of good quality suitable for high resolution X-ray analysis and diffract to better than 1.8-Å resolution.

Crystals of the S175A mutant complexed with each of the substrates MCA, CBT, CMB, and CMV were prepared by a soaking method. All of the substrates except MCA were dissolved in the reservoir solution containing 15% polyethylene glycol 400. The S175A crystals soaked in the reservoir solution containing 5 mM MCA diffracted well and gave diffraction intensities significantly different from those of the S175A crystals, which implied the formation of a complex. However, most of the S175A crystals were dissolved within 10 min in the reservoir solution with a concentration as low as 10 \( \mu \)M CPA, and cracked in reservoir solutions containing 1 mM CPA, CBT, CMB, or CMV. Therefore, a technique of cross-linking with glutaraldehyde was applied to reinforce the crystals before soaking them in the solutions containing each of the substrates except MCA. The cross-linking conditions for the crystals were investigated in solutions containing 10 mM CBT, CMB, or CMV by changing the concentration of glutaraldehyde and the soaking time. Finally, the crystals were cross-linked in 5% (v/v) glutaraldehyde for 5 h at 4 °C. However, even the cross-linked crystals cracked in the 1 mM CPA solution. CPA is best among all the substrates of the enzyme and has the lowest \( K_c \), value (7). This is the case with the S175A mutant, as shown in Table I. Binding of CPA to the enzyme may induce such large structural changes of the molecules in the crystal as to destroy the crystal packing. Efforts to prepare CPA-complexed crystals failed completely. Consequently, the complex with CPA was excluded from the present analysis.

The unit cell dimensions of all the complexed crystals are shown in Table II. The crystal of the MCA complex is isomorphous with that of the S175A mutant, although the crystallographic c axis is shortened by 0.51 Å (1.0%). However, the unit cell dimensions of the CBT-, CMB-, and CMV-complexed crystals shrunk by about 1.1–1.5 Å (1.2–1.6%) and a maximum of 0.7 Å (1.3%) in the directions of the crystallographic a

- Y.-F. Li, manuscript in preparation.
and c axes, respectively. These phenomena indicate that the molecules in these crystals must have moved closer together, compared with their corresponding positions in the wild-type and S175A mutant crystals. These rearrangements of the molecules may have been caused by a combination of the effects of cross-linking and conformational changes induced by the substrate binding.

Data Collection and Processing—Diffraction data for the S175A mutant and all of the complex crystals were collected at 20 °C with a Rigaku R-AXIS IIC imaging plate detector system using graphite or double focusing mirror-monochromated CuKα radiation, produced by a Rigaku RU-300 rotating anode x-ray generator operated at 40 kV and 100 mA. The data collection for each of the five kinds of crystals was performed with one crystal sealed in a glass capillary. The crystal-to-detector distance was set to 80.0 mm. Each frame of 2.6° crystal oscillation was collected for 10 min. Data processing was accomplished at 2.2–1.8-Å resolution with the R-AXIS IIC data processing software package. All of the frames of diffraction data were merged for every data set and scaled together. Data collection and processing are summarized in Table II.

Structure Determination and Refinement—First of all, the subunit structure of the S175A mutant was analyzed using the structure of the wild-type subunit containing Tyr-3–Ile-222, or 220 out of 232 residues, which has previously been reported at 2.5-Å resolution (3) and further refined at 2.0-Å resolution to an R-factor of 19.1% with the program X-PLO (20). The mutant structure refined with X-PLO was virtually identical to the wild-type structure, with an average r.m.s. deviation of 0.2 Å between the corresponding C atoms of the two structures. This structural similarity indicates that the S175A subunit is the wild-type subunit with Ser-175 in the mutant. The water molecule is hydrogen-bonded with the main-chain amido nitrogen and N of Asp-10 and the side chain of Ala-175, respectively. After the subunit for each complex was located in its asymmetric unit, the regions that had changed their conformations were checked for each complex using the 2Fo − Fc and 2Fo – Fc omit maps. These maps showed that the region of Asp–Ser–20 in the MCA complex, and the regions of Asp–Ser–20, Tyr–91–Asp–102, and Leu–117 Arg–135 in the other three complexes had moved away from their initial positions. Therefore, the conformations of these regions were manually modified on a computer workstaton IRIS INDIGO-Elan with the program TURBO-FRODO (21) based on the 2Fo − Fc and 2Fo − Fc omit maps. The structures were refined with the simulated annealing protocol in X-PLO. The refinement of each structure was initiated at 2.5-Å resolution using the modified coordinates and the individual temperature factors of the wild-type structure. In the initial stage of refinement, the structures of the protein regions were refined in all of the complexes. Some residues without any significant peaks corresponding to their side chains were set to Ala in the first several cycles until their peaks appeared. During the course of the refinement, water molecules were rebuilt on the basis of the 2Fo − Fc and 2Fo − Fc maps. In the final stage of refinement, a model of substrate moiety was added to the protein structure of each complex based on the 2Fo − Fc omit map. In the map, the electron density corresponding to the substrate moiety was connected to that of the Asp–10 side chain in each complex, as shown in Fig. 1. The density map clearly showed that the substrate moiety has no chlorine atom and has a ω-configuration at the asymmetric C atom. These findings indicated that in each crystal, the complex turned to an ester intermediate, where the C atom of the unchlorinated substrate is covalently bonded to the carboxyl oxygen atom of the Asp–10 side chain and has a ω-configuration in all but the MCA intermediate. Each of the ester intermediate structures was further refined to convergence. The refinement statistics are summarized in Table III.

RESULTS AND DISCUSSION

Overall Structures of Ester Intermediates—The structure of each ester intermediate contains the polypeptide chain of amino acid residues 3–222, the substrate moiety, and 66, 51, or 34 water molecules in the MCA, CBT, CMB, or CMV intermediates, respectively. The overall structure of the polypeptide chain in each intermediate is similar to that of the wild type and the S175A mutant, although there are significant structural differences in the Asp–10 Ser–20, Tyr–91–Asp–102, and Leu–117 Arg–135 regions. The conformational change in Asp–10 Ser–20 seems to be caused by an esterification with the substrate-derived moiety. The high quality of the appropriate omit-maps of all the intermediate structures allowed us to precisely locate the alkanoic acid moiety covalently bound to the carboxyl oxygen O of Asp–10 in each intermediate crystal, as shown in Fig. 1. The CBT, CMB, and CMV-derived carboxyalkyl groups have almost identical conformations. In the case of the CMB and CMV intermediates, the ω-enantiomers of CMB and CMV were used in preparing the soaking solutions. Consequently, only the corresponding pro-ω-2-hydroxyacids were observed in their electron density maps. In the case of the CBT intermediate, the racemate of 2,2-dichloro-n-butyrate was used in the soaking solution, but the electron density map of the CBT intermediate unambiguously showed the conformation of pro-ω-2-hydroxybutyrate. This suggests that the enzyme selectively reacts with the ω-enantiomer of 2-haloacid and that the C atom of the substrate has a completely inverted conformation in the ester intermediates. The structures around the active site in the MCA, CBT, CMB, and CMV intermediates are shown in Fig. 2.

In the MCA intermediate, the carboxymethyl group of the substrate is covalently bonded to O of Asp–10, and unesterified O of Asp–10 is hydrogen-bonded with N of Lys–151. In contrast, in the CBT, CMB, and CMV intermediates, O of Asp–10 is esterified with the substrate moiety in the O position in the MCA intermediate, and unesterified O is hydrogen-bonded with the hydroxyl oxygen of Thr–14, which is located opposite Lys–151. The CBT, CMB, and CMV-derived moieties displace most of the water molecules observed in the wild type, while the MCA-derived moiety coexists with several water molecules, probably because it is smaller than the substrate moiety in the other intermediates. In all of the intermediate structures, a new water molecule is commonly observed between the carboxyl group of Asp–10 and the side chain of Ala–175, replacing Ser–175 in the wild type. The water molecule is hydrogen-bonded with the main-chain amido nitrogen and N of Asn–177, O of Asp–180, and the esterified O of Asp–10 in

### Table I

| Substrate                      | CI – | NADH0  |
|-------------------------------|------|--------|
| Monochloroacetate             | 73.6 | 0.07   |
| L-2-Chloropropionate          | 100  | 0.20   |
| L-2-Chloro-n-butyrate         | 13.2 | 0.03   |
| L-2-Chloro-3-methyl-n-butyrate| 9.2  | 0.01   |
| L-2-Chloro-4-methyl-n-valerate| 18.4 | 0.01   |

- The activity was measured in terms of the amount of chloride released from substrates.
- The activity to produce ω-hydroxyacids from MCA, CBT, CMB, and CMV has not been estimated because any appropriate method for the activity assay has not been developed for these substrates.
- ∼ —, not determined.

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all of the intermediates, and also to the unesterified O\textsuperscript{d} of Asp-10 in the CBT, CMB, and CMV intermediates. These interactions are illustrated in Fig. 3.

Comparison of the Wild-type and Ester Intermediate Structures—Conformational differences between the four ester intermediates and the wild-type structures have been assessed by structural comparisons. The results for the MCA and CBT intermediates are shown in Fig. 4. The average r.m.s. deviations in the C\textalpha atom between the MCA, CBT, CMB, and CMV intermediates and the wild-type structures were 0.24, 0.44, 0.33, and 0.64 Å, respectively, which lie close to expected experimental errors. This finding indicates that the overall tertiary structure of the wild type is well preserved in the intermediates.

As is shown by the plots of r.m.s. C\textalpha deviation of intermediates from the wild type against residue number in Fig. 5, however, large and small deviations in the intermediate structures are observed mainly in the three regions of Asp-10–Ser-20, Tyr-91–Asp-102, and Leu-117–Arg-135, although in the MCA intermediate, only the Asp-10–Ser-20 region shows clear movements. All these regions tend to move toward the active site. The Asp-10–Ser-20 region containing the short β-strand β\texttwo (Phe-16–Asp-17) and the beginning of α\textone (His-19–Ala-28) exhibits large conformational changes in all the intermediates, including conspicuous rotations of the phenolate ring of Tyr-12 and the side-chain carboxyl group of Asp-10. As shown in Fig. 2, the nucleophile Asp-10 seems to have the ability to change its side-chain conformation depending on the size of the alkyl group in the substrate. In the ester intermediates, the carboxyl group of Asp-10 rotates by approximately 30° around the C\textalpha–C\textbeta bond.

### Table II
Summary of data collection statistics

|          | S175A | MCA   | CBT   | CMB   | CMV   |
|----------|-------|-------|-------|-------|-------|
| Soaking conditions |       |       |       |       |       |
| Concentration (mM) | 5     | 10    | 10    | 10    | 10    |
| Soaking time (days) | 3     | 6     | 6     | 5     | 5     |
| Cell parameters |       |       |       |       |       |
| a (Å) | 92.47 | 92.08 | 90.43 | 91.35 | 90.96 |
| b (Å) | 62.78 | 62.74 | 62.92 | 62.69 | 62.86 |
| c (Å) | 50.99 | 50.48 | 51.67 | 50.36 | 50.31 |
| β (°) | 122.7 | 122.1 | 122.8 | 121.9 | 121.7 |
| Data statistics |       |       |       |       |       |
| Resolution (Å) | 2.06  | 1.83  | 1.80  | 1.80  | 1.80  |
| No. of reflections observed | 40,092 | 54,764 | 37,221 | 41,537 | 51,737 |
| Unique | 15,301 | 17,918 | 14,454 | 18,048 | 18,081 |
| Completeness (%) | 93.9  | 95.0  | 87.1  | 92.9  | 92.4  |
| R\textmerge (%) | 5.58  | 5.78  | 8.30  | 4.88  | 6.31  |

*a R\textmerge (%) = \(100 \cdot \Sigma |I_i| - \langle I \rangle/\Sigma |I_i|\), where \(I_i\) is the intensity of individual measurements, and \(\langle I \rangle\) is the mean intensity over all measurements.

### Table III
Refinement statistics

|          | S175A | MCA   | CBT   | CMB   | CMV   |
|----------|-------|-------|-------|-------|-------|
| Resolution (Å) | 2.0  | 1.83  | 2.0   | 2.2   | 2.2   |
| R-factor (%) | 19.4 | 19.9  | 20.3  | 20.8  | 21.9  |
| Free R-factor (%) | 25.3 | 25.8  | 28.5  | 27.4  | 29.7  |
| No. of reflections (≥ 2σ) | 15,019 | 17,609 | 12,307 | 11,339 | 11,458 |
| Completeness (%) | 78.3  | 78.7  | 75.5  | 90.7  | 94.7  |
| No. of atoms | Protein | 1747  | 1751  | 1753  | 1754  | 1755  |
| Solvent | 82 | 66 | 51 | 40 | 34 |
| Mean β-factors (Å\textsuperscript{2}) | Main chain | 24.78 | 26.47 | 39.27 | 32.43 | 39.52 |
| Side chain | 28.82 | 32.43 | 44.32 | 38.27 | 45.89 |
| Water molecule | 39.26 | 37.50 | 50.82 | 46.30 | 49.89 |
| r.m.s. deviations\textsuperscript{c} | bond distances (Å) | 0.010 | 0.007 | 0.006 | 0.006 | 0.007 |
| bond angles (°) | 1.555 | 1.318 | 1.254 | 1.254 | 1.564 |

*a R-factor (%) = \(100 \cdot \Sigma |F_o| - \Sigma |F_c|/\Sigma |F_o|\), where \(F_o\) and \(F_c\) are the observed and calculated structure factor amplitudes, respectively.

*b The free R-factors were calculated for randomly selected 10% of the whole reflection data sets.

*c r.m.s., root mean square.
bond and by approximately 80° around the Cα–Cγ bond. Consequently, the orientation of the carboxyl group in the MCA intermediate is approximately opposite to those in the other three intermediates with respect to the C2 atom of the substrate moiety. The Cα atom of Tyr-12 moves by 1.05, 1.51, 0.67, and 0.93 Å in the MCA, CBT, CMB, and CMV intermediates, respectively, to get closer to the active site, and its phenolate ring rotates by approximately 30° around its Cα–Cβ bond in each intermediate. In the region of Tyr-91–Asp-102, which contains β3 (Ala-95–Pro-96) and the beginning of α5 (Val-100–Arg-109), Leu-94 shows the greatest Cα movement of 1.20, 0.51, and 0.54 Å in the CBT, CMB, and CMV intermediates, respec-

**FIG. 2.** Stereoscopic superpositions in the active site structure among the wild type (black) and the MCA (red) and CBT (blue) intermediates (A), and among the wild type (black) and the CMB (red) and CMV (blue) intermediates (B). The water molecules of Wat-501 and the new water molecule are shown by small balls labeled Wat1 and WatN, respectively. The figure was drawn using the program MOLSCRIPT (22).
tively. In the Leu-117–Arg-135 region, which contains the end of β4 (Lys-113–Ser-118) and the whole o6 (Pro-122–His-131), the Ser-118–Ala-127 region shows large changes with Cα-averaged shifts of 1.38, 0.64, and 0.90 Å in the CBT, CMB, and CMV intermediates, respectively. The structural changes described above in the Asp-10–Ser-20 region are caused by the nucleophilic attack of Asp-10 on the substrate, while those in the Tyr-91–Asp-102 and Leu-117–Arg-135 regions can be attributed to the bulkiness of the alkyl group in the CBT, CMB, and CMV substrates. The structural differences between the ester intermediates and the wild type indicate that, in the reaction process, conformational changes favorable to the formation of intermediates occur in the active site.

Some differences in intersubunit interactions in the molecule are observed between the intermediates and the wild type. The intersubunit hydrogen bond between Nε2 of Asn-42 in one subunit and Oε1 of Glu-46 in the other, which is observed in the wild-type enzyme, disappears in all the intermediates. Intersubunit hydrophobic interactions that occur between the side chains of Trp-49 and Leu-50, Leu-50 and Pro-152, Trp-49 and Leu-53, and Leu-53 and Pro-152 in the wild-type structure generally seem to be weakened in the ester intermediate structures.

**The Binding Site of the Substrate Carboxyl Group**—The dehalogenated moieties of the substrates in the four intermediates appear to have similar positions and orientations to one another. In particular, the carboxyl group of the substrate moiety in each structure occupies a very similar position, although some differences in the orientation of the alkyl group in the substrate-derived moieties are observed between the CBT, CMB, and CMV intermediates because of differences in the branching position. The relationships between the carboxylates of the substrate-derived moieties and their neighboring polypeptide residues are shown in Figs. 2 and 3. In the structures of the CBT, CMB, and CMV intermediates, the carboxyl oxygens of each substrate moiety are hydrogen-bonded with the Ser-118 hydroxyl and the main-chain amido nitrogens of Leu-11, Tyr-12, and Asn-119. The hydrogen bond between the carboxyl oxygen and the main-chain amido nitrogen of Asn-119 is not observed in the MCA intermediate. These structures clearly show that Ser-118 serves as the main residue for stabilizing the substrate carboxyl moiety in the reaction intermediate. The position of the hydroxyl oxygen atom of Ser-118 in the wild type is very close to those in the intermediates. Therefore, it is reasonable to expect that Ser-118 should be a residue essential for binding the carboxyl group of the substrate through the hydrogen bonds.

**The Hydrophobic Pocket for the Substrate Alkyl Group**—The alkyl groups derived from CBT, CMB, and CMV in the ester intermediates are situated in a unique binding site of the present enzyme. The hydrophobic side chains of Tyr-12, Leu-45, Phe-60, and Trp-179 are located in the vicinity of the binding site, as shown in Fig. 2. The four residues obviously form the hydrophobic pocket for the alkyl group, along with the Gln-44, Lys-151, and Asn-177 side chains. This hydrophobic pocket lies at the bottom of the cleft between the core-domain and subdomain, and some of the constituent residues are incorporated into the hydrophobic cluster, which contributes to the formation of the dimer molecule, as described previously (3). The seven residues that form the hydrophobic pocket are conserved in most L-DEXs thus far sequenced. We have shown by site-directed mutagenesis studies that Lys-151 and Asn-177 are essential for the enzyme activity (7). The mutagenesis studies also showed that the W179F mutant is only 10% as active as the wild-type enzyme (7), and that mutants for Tyr-12 show somewhat higher activities of 11, 11, 40, and 16% in

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**Fig. 3.** Schematic diagrams of the hydrogen bonds and hydrophobic interactions in the active site of the wild type (A) and the MCA (B) and CMV (C) intermediates. Hydrogen bonds and hydrophobic interactions are depicted by dotted lines with interatomic distance (Å). The names of the hydrogen-bonding residues are shown in boxes, those of the water molecules in oval circles, and those of the hydrophobic residues in shaded boxes. The water molecules of Wat-501, Wat-503, Wat-504, Wat-505, and the new water molecule are labeled Wat1, Wat3, Wat4, Wat5, and WatN, respectively.
Y12A, Y12L, Y12F, and Y12W, respectively. These Tyr-12 and Trp-179 mutations suggest that hydrophobic residues of an appropriate size are required for the residues in these positions to make sufficiently hydrophobic interactions with the substrate. In the intermediate structures, the distance from the C2 atom of the substrate moiety to the van der Waals surface at the bottom of the hydrophobic pocket is about 6 Å (Fig. 6). This value is equivalent to the size of the pocket, which can accommodate straight alkyl-chains of maximally five carbons, which explains the substrate specificity of L-DEX YL (1, 17). Based on the structure of the ester intermediate, the possibility of a D-substrate binding to the enzyme can easily be examined by exchanging the positions of the hydrogen atom and the alkyl group that are attached to the C2 atom of the substrate moiety. It turns out, however, that no alkyl groups could be accommodated properly in the place of hydrogen due to steric hindrance by the main-chain and side-chain atoms of Leu-11, Tyr-12 and their neighbors. Therefore, it seems reasonable to assume that the enzyme can select only L-enantiomers of 2-haloacids as its substrates using the hydrophobic pocket. The hydrophobic pocket of L-DEX YL may determine not only the substrate specificity of the enzyme but also its stereospecificity by selectively accommodating the alkyl side chain of L-2-haloacid in the inside of the pocket.

Proposed Catalytic Mechanism—L-DEX YL initiates the dehalogenation reaction by taking a L-2-haloacid into the active site. When the substrate approaches the entrance to the active site, the guanidino group of Arg-41 may serve as the recognition site for the substrate carboxyl group. Arg-41 is the only basic residue present at the entrance of the active site (Fig. 6). This residue is entirely conserved in all L-DEXs and is essential for the enzyme activity, as has been evidenced by site-directed mutagenesis studies (7).

The enzyme forms a Michaelis complex in the active site with the substrate. When the complex is formed, the water molecule Wat-504 is displaced or removed by the substrate carboxyl group. In the complex, the position of Wat-504 is occupied by the carboxyl group of the substrate, as is the case with all the intermediates. The situation of the substrate carboxyl group in the intermediates resembles that of the water molecule Wat-504 in the substrate-free form of the wild-type enzyme. The water molecule Wat-501, which is hydrogen-bonded to both O\textsubscript{d1} and O\textsubscript{d2} of Asp-10 in the substrate-free enzyme, is removed upon the formation of the ester intermediate. The O\textsubscript{d2} atom of Asp-10 probably serves as a nucleophile attacking the C2 atom of the substrate, because in the wild-type enzyme, the O\textsubscript{d1} atom of Asp-10 is stabilized by hydrogen bonding with O\textsubscript{g} of Ser-175 as well as Wat-501. Whichever oxygen atom of Asp-10 takes part in the reaction, the liberation of the nucleophilic oxygen atom from Wat-501 and Wat-504 upon the binding of the substrate is probably the crucial step in the reaction.

The dehalogenation of L-2-haloacid catalyzed by L-DEX YL probably proceeds through an S\textsubscript{N2} mechanism; the carboxyl group of Asp-10 in the substrate-free enzyme, is removed upon the formation of the ester intermediate. The O\textsubscript{d2} atom of Asp-10 probably serves as a nucleophile attacking the C2 atom of the substrate, because in the wild-type enzyme, the O\textsubscript{d1} atom of Asp-10 is stabilized by hydrogen bonding with O\textsubscript{g} of Ser-175 as well as Wat-501. Whichever oxygen atom of Asp-10 takes part in the reaction, the liberation of the nucleophilic oxygen atom from Wat-501 and Wat-504 upon the binding of the substrate is probably the crucial step in the reaction.

The dehalogenation of L-2-haloacid catalyzed by L-DEX YL probably proceeds through an S\textsubscript{N2} mechanism; the carboxyl group of Asp-10 is assumed to approach the C2 atom of the substrate from the opposite side to the halogen atom. In the transition state of the reaction, the groups other than the leaving group that are attached to the C2 atom become planar.
with the atom. The nucleophile O$_{\text{d}2}$ of Asp-10 attacks the C$_2$ atom from a direction perpendicular to the plane, while the leaving halide anion is abstracted concertedly by a residue located on the opposite side of the plane.

One of the issues to be solved is what residue serves as the acceptor of the halide ion released from the substrate. We previously proposed that Tyr-12 in the vicinity of Asp-10 could be an acceptor for the halide ion based on the structure of the substrate-free enzyme (3). However, further site-directed mutagenesis studies have shown that the Y12A and Y12L mutant enzymes have more than 10% of the activity of the wild-type enzyme described above. Moreover, the phenyl ring of Tyr-12 is not in a proper position with respect to the C$_2$ atom of the substrate moiety in the ester intermediate, although the residue is in the vicinity of Asp-10. These findings do not support our earlier proposal.

A candidate residue for the halide ion acceptor is Arg-41; Arg-41 is the only functional residue that appears to be in a suitable position in all ester intermediates (Fig. 6). Arg-41 is located in a proper position with respect to the C$_2$ atom of the substrate moiety. It turns out that the O$_{\text{d}2}$ atom of Asp-10, the C$_2$ atom of the substrate, and the N$_{\text{h}1}$ atom of Arg-41 line up approximately on a straight line. The angles between the Asp-10 oxygen O$_{\text{d}2}$ bound to the substrate C$_2$ atom and N$_{\text{h}1}$ of Arg-41 with respect to the C$_2$ atom are approximately 123°, 149°, 156°, and 150° in the MCA, CBT, CMB, and CMV intermediates, respectively. The distance between N$_{\text{h}1}$ of Arg-41 and the C$_2$ atom of the substrate is also short enough for Arg-41 to interact with the chlorine atom: 8.42, 5.45, 5.60, and 5.68 Å in the MCA, CBT, CMB, and CMV intermediates, respectively. These findings suggest that the guanidino group of Arg-41 serves as the halogen abstraction site. The relatively smaller angle and longer distance observed in the MCA intermediate is probably attributable to the lack of an alkyl side chain in MCA, which probably permits the presence of water molecules and therefore affects the position of the guanidino group of Arg-41.

The possibility that Arg-41 functions as the halogen abstraction residue was examined by means of a hypothetical model compound mimicking the transition-state structure in the SN$_2$ reaction of L-DEX YL. The model compound contained the C$_2$ atom, a hydrogen atom, a carboxyl group and an alkyl group that are attached to the C$_2$ atom on a plane, and also a chlorine atom bound to the C$_2$ atom in a direction perpendicular to the plane. The chlorine of the hypothetical compound was oriented toward the guanidino group of Arg-41. The carboxylic group of Asp-10 was shifted to a new position so that the O$_{\text{d}2}$–C$_2$ bond of
Asp-10 could be oriented toward the C\textsubscript{2} atom of the compound and perpendicularly to the plane of the compound. The other conditions in the transition-state model were fixed to those observed in the ester intermediates. Consequently, as shown in Fig. 7, C\textsuperscript{1} and O\textsuperscript{11} of Asp-10, and the chlorine and C\textsubscript{2} atom of the hypothetical compound lie linearly in the transition state. The model shown in Fig. 7 is a reasonable transition-state model of the proposed reaction mechanism for the complete reaction. A proposed reaction mechanism for the completion of the Walden inversion is illustrated in the figure. A detailed description is given in the text. There are two possible routes from the transition state to the ester intermediate. a, the side chain of Asp-10 remains in the conformation of the transition state. b, it rotates around the O\textsuperscript{11}–C\textsuperscript{12} bond in the other cases, resulting in a conformation markedly different from that observed with MCA.

Fig. 8. A proposed mechanism for the first step of the reaction. A proposed reaction mechanism for the completion of the Walden inversion is illustrated in the figure. A detailed description is given in the text. There are two possible routes from the transition state to the ester intermediate. a, the side chain of Asp-10 remains in the conformation of the transition state in the case of MCA; b, it rotates around the O\textsuperscript{11}–C\textsuperscript{12} bond in the other cases, resulting in a conformation markedly different from that observed with MCA.

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