DL-2-Haloacid Dehalogenase from *Pseudomonas* sp. 113 Is a New Class of Dehalogenase Catalyzing Hydrolytic Dehalogenation Not Involving Enzyme-Substrate Ester Intermediate*

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Various enzymes catalyzing hydrolytic dehalogenation of organohalogen compounds have been isolated and characterized (1–3). These enzymes include 2-haloacid dehalogenases (EC 3.8.1.2), haloacetate dehalogenases (EC 3.8.1.3), haloalkane dehalogenases (EC 3.8.1.5), and 4-chlorobenzoyl-CoA dehalogenases (EC 3.8.1.6). 2-Haloacid dehalogenases are further classified into three groups based on their substrate specificities (4). L-2-Haloacid dehalogenase (L-DEX) specifically acts on L-2-haloalkanoic acids, and the corresponding d-2-hydroxyalkanoic acids are produced. D-2-Haloacid dehalogenase (D-DEX) catalyzes the conversion of d-2-hydroxyalkanoic acids into L-2-hydroxyalkanoic acids. DL-2-Haloacid dehalogenase (DL-DEX) dehalogenates both D- and L-2-haloalkanoic acids, and the corresponding L- and d-2-hydroxyalkanoic acids are produced. DL-DEX is similar to racemases and epimerases in that it acts indiscriminately on the chiral center of both D- and L-enantiomers. However, this enzyme is unique in that it catalyzes a chemical conversion on the chiral centers of both enantiomers.

Thus far, the reaction mechanisms of L-DEX from *Pseudomonas* sp. YL (L-DEX YL) (5–7), haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (8–10), and 4-chlorobenzoyl-CoA dehalogenases from *Pseudomonas* sp. strain CBS3 (11, 12) and *Arthrobacter* sp. 4-CB1 (13) have been analyzed. Their reactions proceed as shown in Fig. 1A. Each of these dehalogenases has an acidic amino acid residue whose carboxylate group attacks the carbon atom of the substrate to which the halogen atom is bound. Asp105 of L-DEXYL, Asp124 of haloalkane dehalogenase from *X. autotrophicus* GJ10, and Asp145 of 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp. strain CBS3 were identified to play this essential role for respective enzymes. The ester intermediates produced in the course of these reactions are subsequently hydrolyzed releasing the products and restoring the carboxylate groups of the enzymes. These were confirmed by chemical modification, site-directed mutagenesis, mass spectrometry, and x-ray crystallographic analysis (5–13).

DL-DEXs have been purified from *Pseudomonas* sp. 113 (DL-DEX 113) (14), *Pseudomonas putida* PP3 (15), and *Rhizobium* sp. (16). However, none of the reaction mechanisms of these DL-DEXs have been studied, and it is unknown whether the reaction mechanism of DL-DEX is similar to that of other haloalkoholases (dehalogenases that catalyze the hydrolytic dehalogenation). We previously determined the primary structure of DL-DEX 113 (Fig. 2), and found that it is similar to that of d-DEX from *Pseudomonas putida* AJ1 (17). We also showed that DL-DEX 113 has a single and common catalytic site for both D- and L-enantiomers based on a site-directed mutagenesis experiment and kinetic analysis (17). In the present study, we analyzed the reaction mechanism of DL-DEX 113 by means of 18O incorporation experiments, and found that the reaction does not involve the formation of an enzyme-substrate ester intermediate. A water molecule is probably activated by a catalytic base of the enzyme, directly attacking the α-carbon of D- and L-2-haloalkanoic acids to displace the halogen atom (Fig. 2).

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† The abbreviations used are: DL-DEX, DL-2-haloacid dehalogenase; L-DEX, L-2-haloacid dehalogenase; D-DEX, D-2-haloacid dehalogenase; L-2-haloalkanoic acids, and the corresponding d-2-hydroxyalkanoic acids are produced. D-2-Haloacid dehalogenase (D-DEX) catalyzes the conversion of d-2-hydroxyalkanoic acids into L-2-hydroxyalkanoic acids. DL-2-Haloacid dehalogenase (DL-DEX) dehalogenates both D- and L-2-haloalkanoic acids, and the corresponding L- and d-2-hydroxyalkanoic acids are produced. DL-DEX is similar to racemases and epimerases in that it acts indiscriminately on the chiral center of both D- and L-enantiomers. However, this enzyme is unique in that it catalyzes a chemical conversion on the chiral centers of both enantiomers.

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DL-DEX 113, DL-DEX from *Pseudomonas* sp. 113; L-DEX YL, L-DEX from *Pseudomonas* sp. YL; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.
FIG. 1. Reaction mechanisms of dehalogenases catalyzing the hydrolytic dehalogenation. A, reaction mechanisms of: a, t-DEX; b, haloalkane dehalogenase; and c, 4-chlorobenzoyl-CoA dehalogenase. B, reaction mechanism which does not involve an ester intermediate.

FIG. 2. Amino acid sequence of t-DEX 113.  

1B). This is the first example of an enzymatic dehalogenation that proceeds through the mechanism shown in Fig. 1B.

EXPERIMENTAL PROCEDURES

Materials—H$_{18}$O (95–98%) was obtained from Cambridge Isotope Laboratories (Andover, MA) and Nippon Sanso (Tokyo, Japan). t- and l-2-chloropropionate were purchased from Sigma. Lysyl endopeptidase of Akromobacter lyticus M497-1 and trypsin (TPCK treated) were from Wako Industry Co., Ltd. (Osaka, Japan) and Sigma, respectively. Other chemicals were of analytical grade.

Purification of t-DEX 113—Recombinant Escherichia coli JM109 cells harboring p4b (1) encoding t-DEX 113 (17) were cultivated at 37°C for 14–18 h in a Luria-Bertani medium (1% polypeptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) containing 150 μg/ml ampicillin. The cells were collected by centrifugation, suspended in a 50 mM potassium phosphate buffer (pH 7.0) containing 150 μg/ml ampicillin and 0.2 mM isopropyl-1-thio-β-D-galactoside. The cells were collected by centrifugation, suspended in a 50 mM potassium phosphate buffer (pH 7.0), and disrupted by ultrasonic oscillation at 4°C for 20 min with a Seiko Instruments ultrasonic disintegrator model 7500. The cell debris was removed by centrifugation. The supernatant solution was brought to 40% saturation with ammonium sulfate, and the precipitate was removed by centrifugation. The supernatant solution was brought to 40% saturation with ammonium sulfate, and the precipitate was removed by centrifugation. The supernatant was applied to a Butyl chromatography (YMC Co., Kyoto, Japan) connected to the mass spectrometer and then eluted with a PE-Sciex API mass spectrometer equipped with an ionspray ion source. The quadrupole was scanned from 300 to 2000 atomic mass units with a step size of 0.25 atomic mass units and a 0.5-ms dwell time per step. Ionspray voltage was set at 5 kV, and the orifice potential was 80 V. The molecular mass of each peptide was calculated with MacSpec software supplied by Sciex.

Digestion of t-DEX 113 with Trypsin—Lyophilized 10 nmol of t-DEX 113, 1.2 μmol of t- or l-2-chloropropionate (neutralized with NaOH), and 1.25 μmol of Tris-H$_2$SO$_4$ (pH 9.5) were mixed in 50 μl of H$_{18}$O and incubated at 30°C for 24 h. The enzyme was inactivated by incubating the reaction mixture for 10 min at 80°C and then denatured by the addition of 100 μl of 3 M urea in 100 mM Tris-H$_2$SO$_4$ (pH 7.5). Subsequently, the volume was adjusted to 500 μl with 120 mM Tris-H$_2$SO$_4$ (pH 7.5) in order to reduce the urea concentration to 1 M and the pH to approximately 8.0. t-DEX 113 in this solution was digested with 5 μg of TPCK-treated trypsin at 37°C for 12 h.

To digest t-DEX 113 in H$_{18}$O, the protein was denatured by the addition of 100 μl of 3 M urea in 100 mM Tris-H$_2$SO$_4$ (pH 7.0) prepared with H$_{18}$O. Thereafter, 150 μl of 100 mM Tris-H$_2$SO$_4$ (pH 7.5) containing 5 μg of trypsin prepared with H$_{18}$O was added to this solution and incubated at 37°C for 12 h.

Digestion of t-DEX 113 with Lysyl Endopeptidase—Lyophilized 10 nmol of t-DEX 113, 1.2 μmol of t- or l-2-chloropropionate (neutralized with NaOH), and 2.5 μmol of Tris-H$_2$SO$_4$ (pH 9.5) were mixed in 50 μl of H$_{18}$O and incubated at 30°C for 24 h. The enzyme was inactivated by incubating the reaction mixture at 80°C for 10 min, denatured with 8 M urea, and subsequently digested with 825 pmol of lysyl endopeptidase at 37°C for 12 h.

Liquid Chromatography/Mass Spectrometry Analysis of the Proteolytic Digests—The proteolytic digests of the enzyme were loaded onto a YMC-PackC4-AP column (100 × 1.0-mm inner diameter) (YMC Co., Kyoto, Japan) connected to the mass spectrometer and then eluted with a linear gradient of 0–80% acetonitrile in 0.05% trifluoroacetic acid over 80 min at a flow rate of 10 μl/min. A total ion current chromatogram was recorded in the single-quadrupole mode with a PE-Sciex API III mass spectrometer equipped with an ionspray ion source. The quadrupole was scanned from 300 to 2000 atomic mass units with a step size of 0.25 atomic mass units and a 0.5-ms dwell time per step. Ionspray voltage was set at 5 kV, and the orifice potential was 80 V. The molecular mass of each peptide was calculated with MacSpec software supplied by Sciex.

Site-directed Mutagenesis of t-DEX 113—Plasmid p4b (1) was mutagenized by the method of Kunel et al. (18). The mutant enzymes and synthetic mutagenic primers were as follows (the underlines indicate the mutagenized nucleotides): D181A, 5'-TCACGGATTTC-3'; D181R, 5'-TCACGGATGCGCCTGAG-3'; D181R, 5'-TCACGGATGCGCCTGAG-3'; D181R, 5'-TCACGGATGCGCCTGAG-3'. Mutant enzymes were produced by E. coli BMH 71-18 mutS.

Treatment of t-DEX 113 and t-DEXs with Hydroxylamine—t-DEX...
113, L-DEX YL (19), and L-DEX from Pseudomonas putida no. 109 (20) (final 0.45 mg/ml) were mixed with 1 M hydroxylamine in 1 M Tris-

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ular masses of all peptides were virtually indistinguishable

255–272, and 35–43, respectively (Table I, Fig. 2). The molec-

77–105, 273–283, 44–66, 163–180 (dimer), 197–210, 120–134, 9–21, 184–196, 108–119, 239–249, 152–162, 22–34, 211–228, 108–119, 239–249, 152–162, 22–34, 211–228, 152–162, 22–34, 211–228, 22–34, 211–228, 9–21, 184–196, 108–119, 239–249, 152–162, 22–34, 211–228, 77–105, 273–283, 44–66, 163–180 (dimer), 197–210, 120–134, 255–272, and 35–43, respectively (Table I, Fig. 2). The molecular masses of all peptides were virtually indistinguishable

18O—

L-2-chloropropionate, and

D-2-chloropropionate, and

An oxygen atom of

A

B

FIG. 3. Ionspray mass spectra of lactate produced with DL-DEX 113 (A and B) and L-DEX YL (C) in H$_2$O. L-2-Chloropropionate was used as a substrate in A and C, and D-2-chloropropionate was used in B. The spectra were obtained between 85 and 95 atomic mass units. Step size was 0.1 atomic mass unit, and dwell time was 10 ms/step. Ionspray voltage was set at ~3.5 kV, and the orifice potential was ~50 V.

RESULTS

Single Turnover Reaction of DL-DEX 113 in H$_2$O—We conducted the single turnover reaction of DL-DEX 113 in H$_2$O with D- or L-2-chloropropionate as a substrate. A peak of lactate was found at m/z 85, only 10% of the peak from the predicted one (Table II, Fig. 2). The molecular masses of proteolytic fragments of DL-DEX 113 after multiple turnover reaction in H$_2$O with A, L-2-chloropropionate, and B, D-2-chloropropionate as a substrate.

Peptide Proteolytically Formed from DL-DEX 113—

Peptide

Molecular masses of proteolytic fragments of DL-DEX 113 incubated with substrates in H$_2$O

| Peak | Fragment | Predicted (average mass) | Measured |
|------|----------|--------------------------|----------|
| 1    | 144–151  | 933.99                   | 933.5    |
| 2    | 143–151  | 1090.18                  | 1089.5   |
| 3    | 67–76    | 1138.22                  | 1137.5   |
| 4    | 2–8      | 851.04                   | 850.5    |
| 5    | 286–298  | 1368.40                  | 1367.8   |
| 6    | 301–307  | 816.83                   | 816.5    |
| 7    | 9–21     | 1465.57                  | 1465.0   |
| 8    | 184–196  | 1486.59                  | 1486.0   |
| 9    | 108–119  | 1263.39                  | 1262.8   |
| 10   | 239–249  | 1093.19                  | 1092.8   |
| 11   | 152–162  | 1201.41                  | 1200.8   |
| 12   | 22–34    | 1545.69                  | 1545.9   |
| 13   | 211–228  | 2023.29                  | 2022.7   |
| 14   | 77–105   | 2919.26                  | 2919.1   |
| 15   | 273–283  | 1232.51                  | 1231.8   |
| 16   | 44–66    | 2674.10                  | 2674.4   |
| 17   | 163–180  | 4165.72                  | 4166.2   |
| 18   | 197–210  | 1664.95                  | 1665.5   |
| 19   | 120–134  | 1830.10                  | 1830.5   |
| 20   | 255–272  | 2043.42                  | 2043.2   |
| 21   | 35–43    | 1045.28                  | 1046.0   |

* DL-DEX 113 was incubated with L-2-chloropropionate (l-CPA) or D-2-chloropropionate (d-CPA) in H$_2$O, and digested with trypsin.

from the predicted ones whether the reaction was conducted with D- or L-2-chloropropionate.

Since peptides containing amino acid residues 1, 106–107, 135–142, 181–183, 228–238, 250–254, 284–285, and 299–300 were not found in the trypsin-digested sample, we also analyzed l-lysyl endopeptidase-digested enzyme by the same method. Peptides 120–142, 232–238, and 299–300 were identified, and their molecular masses were virtually indistinguishable from the predicted ones (Table II, Fig. 2). The molecular masses of the peptides containing amino acid residues 1, 106–107, 181–183, and 228–231 could not be measured.

Liquid Chromatography/Mass Spectrometry Analysis of the Proteolytic Fragments Formed in H$_2$O—An oxygen atom of...
the catalytic carboxylic group of haloalkane dehalogenase from *X. autotrophicus* GJ10 is rapidly replaced by an oxygen atom of the solvent water even in the absence of the substrate (9). If this is the case for DL-DEX 113, $^{18}$O once incorporated into the acidic amino acid residue during the dehalogenation should have been replaced by the $^{16}$O of the solvent water during the treatment with trypsin or lysyl endopeptidase, which raised the possibility that the increase in the molecular mass of the peptides might not be detectable in the above experiments.

To examine this possibility, we carried out the denaturation and trypsin digestion of the enzyme in $H_2^{18}$O, and digested with lysyl endopeptidase.

![Table II](image)

| Peak | Fragment | Predicted (average mass) | Measured | $^{18}$O | $^{16}$O |
|------|----------|--------------------------|----------|--------|--------|
| 1    | 144–151  | 933.99                   | 937.8    | 937.8  |
| 2    | 67–76    | 1138.22                  | 1142.0   | 1141.8 |
| 3    | 2–8     | 851.04                   | 855.0    | 855.0  |
| 4    | 9–21    | 1485.57                  | 1493.2   | 1490.0 |
| 5    | 301–307 | 816.93                   | 816.6    | 816.8  |
| 6    | 184–196 | 1486.59                  | 1490.2   | 1490.0 |
| 7    | 108–119 | 1263.39                  | 1267.0   | 1267.2 |
| 8    | 239–249 | 1083.19                  | 1097.0   | 1097.0 |
| 9    | 152–162 | 1201.41                  | 1205.2   | 1205.0 |
| 10   | 22–34   | 1545.69                  | 1549.2   | 1549.2 |
| 11   | 211–228 | 2023.29                  | 2027.3   | 2026.9 |
| 12   | 77–105  | 2919.26                  | 2923.5   | 2923.2 |
| 13   | 35–43   | 1045.28                  | 1049.2   | 1048.8 |
| 14   | 273–285 | 1232.51                  | 1236.0   | 1236.2 |
| 15   | 44–66   | 2674.10                  | 2678.4   | 2677.8 |
| 16   | 163–180 (dimer) | 4165.72 | 4173.6   | 4173.6 |
| 17   | 197–210 | 1664.95                  | 1669.0   | 1668.8 |
| 18   | 120–134 | 1803.10                  | 1835.9   | 1834.4 |
| 19   | 255–272 | 2043.42                  | 2047.3   | 2047.3 |

$^a$ DL-DEX 113 was incubated with L-2-chloropropionate (L-CPA) or D-2-chloropropionate (D-CPA) in $H_2^{18}$O, and digested with lysyl endopeptidase.

![Table III](image)

| Peak | Fragment | Predicted (average mass) | Measured | $^{18}$O | $^{16}$O |
|------|----------|--------------------------|----------|--------|--------|
| 299–306 | 1063.25 | 1062.8                  | 1062.8   | 1062.8 |

$^a$ DL-DEX 113 was incubated with L-2-chloropropionate (L-CPA) or D-2-chloropropionate (D-CPA) in $H_2^{18}$O, and digested with lysyl endopeptidase.

![Table IV](image)

| Peak | Fragment | Predicted (average mass) | Measured | $^{18}$O | $^{16}$O |
|------|----------|--------------------------|----------|--------|--------|
| 1    | 144–151  | 933.99                   | 937.8    | 937.8  |
| 2    | 67–76    | 1138.22                  | 1142.0   | 1141.8 |
| 3    | 2–8     | 851.04                   | 855.0    | 855.0  |
| 4    | 9–21    | 1485.57                  | 1493.2   | 1490.0 |
| 5    | 301–307 | 816.93                   | 816.6    | 816.8  |
| 6    | 184–196 | 1486.59                  | 1490.2   | 1490.0 |
| 7    | 108–119 | 1263.39                  | 1267.0   | 1267.2 |
| 8    | 239–249 | 1083.19                  | 1097.0   | 1097.0 |
| 9    | 152–162 | 1201.41                  | 1205.2   | 1205.0 |
| 10   | 22–34   | 1545.69                  | 1549.2   | 1549.2 |
| 11   | 211–228 | 2023.29                  | 2027.3   | 2026.9 |
| 12   | 77–105  | 2919.26                  | 2923.5   | 2923.2 |
| 13   | 35–43   | 1045.28                  | 1049.2   | 1048.8 |
| 14   | 273–285 | 1232.51                  | 1236.0   | 1236.2 |
| 15   | 44–66   | 2674.10                  | 2678.4   | 2677.8 |
| 16   | 163–180 (dimer) | 4165.72 | 4173.6   | 4173.6 |
| 17   | 197–210 | 1664.95                  | 1669.0   | 1668.8 |
| 18   | 120–134 | 1803.10                  | 1835.9   | 1834.4 |
| 19   | 255–272 | 2043.42                  | 2047.3   | 2047.3 |

$^a$ DL-DEX 113 was incubated with L-2-chloropropionate (L-CPA) or D-2-chloropropionate (D-CPA) in $H_2^{18}$O, and digested with lysyl endopeptidase.

![Table V](image)

| Enzymes | Activity (units/mg) |
|---------|---------------------|
| L-Cysteine | 1.07               |
| L-Cystine | 1.01               |
| D-Cysteine | 1.24               |
| D-Cystine | 1.26               |
| Wild-type | 1.10               |
| D181A | 1.24               |
| D181R | 0.98               |
| D181E | 1.06               |

$^a$ DL-DEX 113 was incubated with L-2-chloropropionate (L-CPA) or D-2-chloropropionate (D-CPA) in $H_2^{18}$O, and digested with lysyl endopeptidase.

![Table VI](image)

| Additions | Activity (units/mg) |
|-----------|---------------------|
| Hydroxylamine | 0.06               |
| Substrate | 0.00               |

$^a$ DL-DEX 113 was incubated with L-2-chloropropionate (L-CPA) or D-2-chloropropionate (D-CPA) in $H_2^{18}$O, and digested with lysyl endopeptidase.

Effect of the Replacement of Asp181 on Enzyme Activity—The molecular masses of peptides 1 (M), 106–107 (LK), 143 (R), 181–183 (DIR), and 229–231 (IRK) could not be determined in the above experiments. Therefore, we could not exclude the possibility that $^{18}$O was incorporated into Asp or Glu in these peptides. However, among these peptides, only peptide 181–183 contains an acidic residue, Asp181. We replaced Asp181 with Ala, Arg, and Glu by site-directed mutagenesis to clarify whether Asp181 is involved in the catalytic reaction shown in Fig. 1A as Asp10 of L-DEX YL is. The activities of these mutant enzymes were similar to that of the wild-type enzyme (Table V), indicating that Asp181 is not essential for the catalysis.

Effect of Hydroxylamine on Enzyme Activity—We previously found that hydroxylamine performs a nucleophilic attack on the active site aspartate residue (Asp181) of l-DEX YL (6). This inactivation was observed only in the presence of the substrate, and an active intermediate formed from Asp10 and the substrate was thought to be a target of hydroxylamine. This was confirmed by mass spectrometric analysis of the inactivated enzyme, which showed that the modified Asp10 residue contained both hydroxylamine- and substrate-derived moieties. The inactivation of 4-chlorobenzylo-Ca dehalogenase by hydroxylamine was also reported (13). In contrast, no inactivation of DL-DEX 113 was observed (Table VI).
The reaction mechanism of DL-DEX 113 was studied by $^{18}$O incorporation experiments and site-directed mutagenesis. Single turnover reactions carried out in $\text{H}_2\text{O}^{18}$ indicated that an oxygen atom of the solvent water is directly incorporated into the product (Fig. 3, A and B). We also found that an oxygen atom of the solvent water is not incorporated into the side chain carboxylate groups of the acidic amino acid residues of the enzyme in the dehalogenation reaction (except for Asp$^{181}$, whose molecular mass could not be measured) (Tables I-IV). A site-directed mutagenesis experiment showed that Asp$^{181}$ is not essential in the catalysis (Table V). These results are consistent with the general base mechanism shown in Fig. 1B, but not with the mechanism shown in Fig. 1A. This applies to the dehalogenations of both enantiomers of 2-haloalkanoic acids because the results obtained for both enantiomers were virtually the same. We previously reported that DL-DEX 113 has a single and common catalytic site for both L- and D-enantiomers based on a site-directed mutagenesis experiment and kinetic analysis (17). This conclusion is supported by our present data showing that the enzymatic dehalogenations of both enantiomers proceed through the same mechanism as shown in Fig. 1B.

The reaction mechanisms of three kinds of halidohydrolases have been analyzed: haloalkane dehalogenase from *X. autotrophicus* GJ10 (8–10), 4-chlorobenzoyl-CoA dehalogenases from *Pseudomonas* sp. strain CBS3 (11, 12), and *Arthrobacter* sp. strain CBS3 (11, 12), and *Pseudomonas* sp. strain CBS3 (11, 12), and *Pseudomonas* phicus X. autotrophicus have been analyzed: haloalkane dehalogenase from *P. putida* 20981 (14). Recently, we found that Glu 69 and Asp194 are essential for the reaction mechanism in the dehalogenation reaction (except for Asp181, which an essential ester intermediate is produced from the substrate. Further studies including crystallographic analysis of the enzyme are now being carried out to identify the active site residues and to clarify their roles in the catalysis.

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