Fluoroacetate dehalogenase (EC 3.8.1.3) catalyzes the dehalogenation of fluoroacetate and other haloacetates. The amino acid sequence of fluoroacetate dehalogenase from *Moraxella* sp. B is similar to that of haloalkane dehalogenase (EC 3.8.1.5) from *Xanthobacter autotrophicus* GJ10 in the regions around Asp-105 and His-272, which correspond to the active site nucleophile Asp-124 and the base catalyst His-289 of the haloalkane dehalogenase, respectively (Krooshof, G. H., Kwant, E. M., Dambsky, J., Koča, J., and Janssen, D. B. (1997) *Biochemistry* 36, 9571–9580). After multiple turnovers of the enzyme-inhibitor complex has revealed that 4-hydroxy-2-ethylpyruvate (identity 14%). Moreover, Asp-124 and His-289 of the haloalkane dehalogenase and Asp-226 and His-431 act as a nucleophile causing the release of a halide ion and the formation of an ester intermediate, which is subsequently hydrolyzed by the nucleophilic attack of a water molecule on the carbonyl carbon atom. A His-272 → Asn mutant (H272N) showed no activity with either fluoroacetate or chloroacetate. However, ion-spray mass spectrometry revealed that the H272N mutant enzyme was covalently alkylated with the substrate. The reaction of the H272N mutant enzyme with [14C]chloroacetate also showed the incorporation of radioactivity into the enzyme. These results suggest that His-272 probably acts as a base catalyst for the hydrolysis of the covalent ester intermediate.

Fluoroacetate is known to be one of the most toxic compounds for mammals (1). Its toxicity is due at least partly to its conversion to (2R,3R)-erythro-2-fluorocitrate by citrate synthase (EC 4.1.3.7); the latter compound is a strong inhibitor of aconitate (EC 4.1.3.1). An x-ray crystallographic analysis of the enzyme-inhibitor complex has revealed that 4-hydroxy-transaconitate formed from (2R,3R)-erythro-2-fluorocitrate is tightly bound to the enzyme (2). Some plants found in Australia, Africa, and Central America synthesize high concentrations of fluoroacetate from fluoride and β-hydroxypropyruvate.

The dissociation energy of the C–F bond of aliphatic fluoro compounds is among the highest found in natural products (5). Various dehalogenases, e.g. L-2-haloacid dehalogenase from *Pseudomonas* sp. YL (6), haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (7), and 4-chlorobenzoyl-CoA dehalogenases from *Pseudomonas* sp. CBS-3 (8) and *Artrobacter* sp. 4-CB1 (9) have been characterized. However, none of them catalyze the hydrolytic cleavage of a C–F bond.

Fluoroacetate dehalogenases have been demonstrated in a few *Pseudomonas* strains and characterized (10–12). Kawasaki et al. have also isolated a *Moraxella* strain producing the enzyme (13) and have cloned its gene in *Echerichia coli* (14). Recently the reaction catalyzed by rat liver microsomal epoxide hydrolase (EC 3.3.2.3) has also been shown to proceed via an ester intermediate: Asp-105 was labeled with two 18O atoms. These results indicate that Asp-105 acts react with 18O, the enzyme was digested with trypsin, and the molecular masses of the peptide fragments formed were measured by ion-spray mass spectrometry. Two 18O atoms were shown to be incorporated into the octapeptide, Phe-99–Arg-106. Tandem mass spectrometric analysis of this peptide revealed that Asp-105 was labeled with two 18O atoms. After multiple turnovers of the enzyme was covalently alkylated with the substrate. The molecular masses of the peptide fragments formed were measured by ion-spray mass spectrometry. Two 18O atoms were shown to be incorporated into the octapeptide, Phe-99–Arg-106. Tandem mass spectrometric analysis of this peptide revealed that Asp-105 was labeled with two 18O atoms.
Reaction Mechanism of Fluoroacetate Dehalogenase

**EXPERIMENTAL PROCEDURES**

Materials—Sodium fluoroacetate was purchased from Wako Pure Chemical Industries (Osaka, Japan), trypsin and chloroacetic acid from Nacalai Tesque (Kyoto, Japan), DNA-Toyopearl from Tosoh (Tokyo, Japan), Gigapite from Toyogusite Chemical Industries (Tokyo, Japan), Superose 12 HR from Amersham Pharmacia Biotech (Uppsala, Sweden), H_{3}^{14}O (95–98%) from Cambridge Isotope Laboratories (Andover, MA) or Nippon Sanso (Tokyo, Japan), and [1-14C]chloroacetic acid (4.4 × 10^{7} Bq/ml, 3.3 × 10^{8} Bq/ml in toluene) from Sigma. Other chemicals were of analytical grade.

**DNA Techniques**—General procedures for DNA manipulations were carried out as described previously (20). The 1.5-kilobase SalI-KpnI fragment including the fluoroacetate dehalogenase gene was isolated from the recombinant plasmid pBREF1 (21) and then amplified by polymerase chain reaction, with HindIII sites being introduced to both ends of the polymerase chain reaction-amplified structural gene: the restriction sequences for HindIII (AAGCTT) were introduced in the region from 59 to 64 nucleotides upstream of the initiation codon ATG and in the region from 58 to 63 nucleotides downstream of the termination codon TGA (at position 943 to 948 from the initiation codon). The synthetic mutagenic primers used were as follows (underlines indicate the mutagenized nucleotides): forward primer, 5'-CTCAAGGGTAAAGGTGAAAGCTGCGGC; reverse primer, 5'-CTCAAGGGTAAAGGTGAAAGCTGCGGC. The resultant DNA was ligated into the HindIII-digested pUC119 to yield pUC1. The plasmid pUC1 contained an insert of about 1 kilobase pair. The nucleotide sequence of the fluoroacetate dehalogenase gene in pUC1 was identical to that of the original one in pBREF1 except for two bases: G substituted for A at position 244 which led to an alteration from Thr to Ala, and T substituted for C at position 390 which gave a different codon for the same amino acid, Ile. The recombinant plasmid pUC1 encoding fluorooacetate dehalogenase was mutagenized by the method of Kunkel (22). The synthetic mutagenic primers used were as follows (underlines indicate the mutagenized nucleotides): D105A, 5'-GGGAGACATGTTCC; D105G, 5'-GGGAGACATGTTCC; D105V, 5'-GGGAGACATGTTCC; H272N, 5'-GAAGAATTGCCTCTCG; H272D, 5'-GAAGAATTGCCTCTCG; H272Y, 5'-GAAGAATTGCCTCTCG. The substitutions were confirmed by DNA sequencing with a Dye Terminator sequencing kit and an Applied Biosystem 370A DNA sequencer. Mutant enzymes were produced in E. coli JM109.

**Enzyme Purification**—E. coli JM109 cells were grown aerobically at 37 °C in 1 liter of Luria Bertani (LB) medium containing 200 μg/ml ampicillin, 0.2 mM isopropyl-1-thio-β-D-galactoside, and 0.1 mg/ml bromoacetic acid. The cells were harvested, rinsed with 50 mM potassium phosphate (pH 7.5), and suspended in 20 ml of the same buffer. The cell suspension was subjected to ultrasonic oscillation at 4 °C for 25 min with a Seiko Instruments model 7500 ultrasonic disintegrator, followed by centrifugation at 12,000 rpm for 15 min. The supernatant was fractionated with ammonium sulfate, and the precipitate formed between 40% and 70% saturation of the salt was dissolved in 50 mM potassium phosphate (pH 7.5), dialyzed against the same buffer, and subsequently applied to a DEAE-Toyopearl 650M column (3 × 27 cm). The elution was carried out with a linear gradient of 50–300 mM potassium phosphate (pH 7.5). The active fractions were pooled, dialyzed against 10 mM potassium phosphate (pH 7.5), and applied to a Gigapite column (1 × 13 cm). The elution was carried out with a linear gradient of 10–300 mM potassium phosphate (pH 7.5). The active fractions were pooled and used as the purified enzyme.

**Determination of Enzyme Activity and Protein**—Fluoroacetate dehalogenase was routinely assayed by the determination of chloride formed from chloroacetic acid by the method of Iwasaki et al. (23). Glycolate formed from fluorooacetate and other haloacetates was determined by HPLC<sup>1</sup>: column, Millipore Puresil 5 μ C18, 120 Å (4.6 × 150 mm); eluent, 0.05% trifluoroacetic acid in 50 mM sodium phosphate at 215 nm. The standard assay mixture (100 μl) comprised 25 mM sodium chloroacetate (or other haloacetate), 100 mM Tris sulfate (pH 9.5), and the enzyme. The reaction was terminated by the addition of 10 μl of 1.5 mM sulfuric acid after incubation at 30 °C for 10 min. One unit of the enzyme was defined as the amount of enzyme that catalyzes the dehalogenation of 1 mM chloroacetate per min. Protein was determined with a Bio-Rad protein assay kit.

**Reaction of Fluoroacetate Dehalogenase in H_{3}^{18}O and Digestion of H_{3}^{18}O-Labeled Enzyme with Trypsin**—The wild-type fluoroacetate dehalogenase (10 nmol) was lyophilized. The dried enzyme was dissolved in 50 μl of H_{3}^{14}O containing 1 μmol of sodium fluorooacetate and 20 μmol of Tris sulfate (pH 9.5), and the mixture was incubated at 30 °C for 12 h. Fluoroacetate was omitted in a control experiment. The reaction was stopped by the addition of 100 μl of 5 mM urea solution in H_{3}^{14}O and was followed by incubation at 37 °C for 1 h. The enzyme was then digested with 5 μg of trypsin at 37 °C for 12 h. The proteolysate was loaded onto a packed capillary perfusion column (Poros II R/H, 320 μm × 10 cm LC Packings, San Francisco, CA) connected to a mass spectrometer, PE-Siex mass spectrometer (Applied Biosystems, Framingham, MA). The ion-spray ion source operated in a positive ion scan mode by selectively introducing the peptides containing Asp-105 into the mass spectrometer. The quadrupole was scanned from 300 to 2000 atomic mass units with a step size of 0.25 atomic mass unit and with a dwell time of 0.5 ms/step. The ion-spray 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 PE-Siex.

**MS Analysis of Peptides Containing Asp-105**—The proteolytic enzymes described above were applied to a C<sub>18</sub> reverse phase HPLC column (Millipore Puresil 5 μ C18, 120 Å, 4.6 × 150 mm) and eluted with 0.05% trifluoroacetic acid for 5 min, followed by a linear gradient of 0–80% acetonitrile in 0.05% trifluoroacetic acid over 60 min at a flow rate of 1.0 ml/min. The elution was monitored at 215 nm with a UV detector, and the fractions were collected manually and injected into the PE-Siex API III mass spectrometer in single-quadrupole mode under the same conditions as described above.

**Tandem MS/MS Analysis of the Peptides Containing Asp-105**—The MS/MS product ion spectra were obtained in triple-quadrupole product ion mode by selecting the peptide containing Asp-105 (m/z 984.8 or m/z 980.8) from Q1 into a collision cell (Q2) and observing the product ions in Q3. Q1 was locked on m/z 984.8 or 980.8. Q3 was scanned from 50 to just above the molecular weight of the peptides with a step size of 0.1 and with a dwell time of 1 ms/step. The ion-spray voltage was set at 5 kV, and the orifice potential was 100 V. The collision energy was 30 eV.

**Synthesis of H272N Mutant Enzyme with [1-14C]Chloroacetic Acid**—Commercially available [1-14C]chloroacetic acid is dissolved in toluene. However, we found that the wild-type enzyme is not affected by toluene when suspended in a reaction mixture at a ratio of 10% (v/v). The reaction mixture containing 10 μmol of Tris sulfate (pH 9.5), 0.75 μmol of sodium hydroxide, the wild-type or the H272N mutant enzyme (1 mg each), and 10 μl of toluene solution containing [1-14C]chloroacetic acid in a final volume of 100 μl was incubated at 30 °C for 2 h. The reaction mixture was dialyzed against 50 mM potassium phosphate (pH 7.5) for 14 h and then applied to a C<sub>18</sub> reverse phase HPLC column. The elution was carried out with 0.05% trifluoroacetic acid for 5 min, followed by a linear gradient of 0–80% acetonitrile in 0.05% trifluoroacetic acid as described above. The radioactivity of the eluate was determined with a Packard Tri-Carb scintillation spectrometer with Clear-sol-1 (Nacalai Tesque, Japan) as a scintillator.

**MS Analysis of H272N Mutant Enzyme and Its Proteolytic Peptides**—The H272N mutant enzyme (400 μg) previously lyophilized was dissolved in a mixture (40 μl) containing 0.5 mM Tris sulfate (pH 9.5) and 125 mM sodium fluorooacetate, and the solution was incubated at 30 °C for 1 h. After incubation was ended by a control experiment, a solution of 5 mM urea (100 μl) was added to the mixture, and the solution was further incubated at 37 °C for 1 h. The enzyme was then digested with 5 μg of

<sup>1</sup>The abbreviations used are: HPLC, high performance liquid chromatography; MS, mass spectrometry; PAGE, polyacylamide gel electrophoresis.
The enzyme was purified as described under "Experimental Procedures." The dehalogenase activities of pooled fractions were determined by measuring the chloride ions released from 25 mM chloroacetate.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (--)</th>
<th>Yield (%)</th>
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<td>726</td>
<td>2.2</td>
<td>1</td>
<td>100</td>
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<tr>
<td>Ammonium sulfate precipitation</td>
<td>125</td>
<td>710</td>
<td>5.7</td>
<td>2.6</td>
<td>98</td>
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<tr>
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<td>13</td>
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<td>59</td>
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<tr>
<td>Gigapre</td>
<td>14.5</td>
<td>260</td>
<td>18</td>
<td>8.2</td>
<td>36</td>
</tr>
</tbody>
</table>

The enzyme was then digested with trypsin, and the resultant peptide fragments were analyzed by the ion-spray mass spectrometer as described above.

RESULTS

Purification of Fluoroacetate Dehalogenase from Recombinant E. coli Cells—Fluoroacetate dehalogenase was purified to homogeneity (Table I). The purity of the final preparation was judged by SDS-PAGE (data not shown). The molecular weight of the enzyme was determined to be about 67,000 by Superose 12HR gel filtration chromatography in a native state, whereas it was about 33,000 by SDS-PAGE. Therefore, the enzyme appears to be composed of two identical subunits. The enzyme had a maximum activity at pH 9.5 and was stable in the pH range from 6.0 to 10.0. The highest enzyme activity in terms of initial velocities was observed at 50 °C, but 70% of the initial activity was lost upon incubation of the enzyme at this temperature for 30 min. The relative activities for several haloacetates were as follows: fluoroacetate, 480; chloroacetate, 100; bromoacetate, 60; iodoacetate, 2.6; dichloroacetate, 1.0. The following halogen compounds were inert: trichloroacetate, 2-chloropropionate, chloroethane, 2-chloroacetamide, and chloromethane. Glycolate was produced from monohaloacetates by dehalogenation, and it inhibited the enzyme at concentrations higher than 10 mM. These properties of the enzyme from Moraxella sp. B are similar to those reported for the Pseudomonas enzymes (10, 12).

Site-directed Mutagenesis of Asp-105 and His-272 of Fluoroacetate Dehalogenase—The sequence analysis of haloalkane dehalogenase from X. autotrophicus GJ10 (24) showed that it has around an 18% similarity with fluoroacetate dehalogenase from Moraxella sp. B, suggesting structural and mechanistic similarities (Fig. 2). In particular, the regions around the nucleophilic aspartate residue and the base histidine residue of the haloalkane dehalogenase are conserved as Asp-105 and His-272, respectively, in the fluoroacetate dehalogenase (Fig. 2). Asp-105 was replaced by Ala, Gly, or Val by site-directed mutagenesis and His-272 by Asn, Asp, or Tyr. All six mutant enzymes were highly expressed in E. coli (about 5–10% of the total amount of soluble cellular proteins), but none of them showed activity. Therefore, both Asp-105 and His-272 of the fluoroacetate dehalogenase play the essential roles in catalysis.

18O Labeling of the Enzyme and Isolation of the Labeled Peptide—If we assume that Asp-105 of the fluoroacetate dehalogenase acts as a nucleophile as shown in Fig. 1A, we can expect that the carboxyl group of the residue is labeled with 18O when the enzymatic defluorination of fluoroacetate is carried out in H2 18O. Therefore, we performed the reaction in H2 18O as described above. The enzyme was then digested with trypsin, and the resultant peptide fragments were separated and analyzed by ion-spray mass spectrometry. When the spectrometer was in single-quadrupole mode, the total ion current chromatogram displayed several peaks; one could clearly be assigned to be an octapeptide Phe-99–Arg-106 containing Asp-105 (data not shown). This peptide was isolated further by reverse phase HPLC and sequenced with a Shimadzu Protein Sequencer PPSQ-10 (Kyoto, Japan), giving the following: Phe-His-Leu-Gly-His-Asp-Arg, which is identical with that predicted by the nucleotide sequence. Ion-spray mass spectrometry of the octapeptide derived from the native enzyme showed a peak at m/z 980.8, which was assigned as the M + H+ ion based on the calculated mass (979.5) of the octapeptide (Fig. 3). The octapeptide isolated from the enzyme incubated with fluoroacetate in H2 18O showed two new peaks at m/z 982.8 and 984.8 corresponding to M + H+ ions formed due to the incubation (Fig. 3A). However, when fluoroacetate was omitted from the reaction mixture in H2 18O, only the peak at m/z 980.8 as observed for the native enzyme appeared (Fig. 3B). These results indicate that one or two 18O atoms are introduced into the enzyme during incubation with the substrate fluoroacetate in H2 18O.

Tandem MS/MS Analysis of the 18O-Labeled Peptide—We determined the amino acid residue labeled with 18O of the octapeptide by tandem MS/MS spectrometry. The precursor ions of m/z 984.8 and m/z 980.8 corresponding to the 18O-labeled and unlabeled octapeptide, respectively, were selected in the first quadrupole and subjected to collision-induced fragmentation in the second quadrupole. The product ions produced are shown in Fig. 4. The Y+ series ions at m/z 700.7, 587.5, 488.3, 431.4, and 294.3 derived from the 18O-labeled octapeptide were assigned as the fragment ions due to Leu-Val-Gly-His-Asp-Arg, Val-Gly-His-Asp-Arg, Gly-His-Asp-Arg, and Asp-Arg, respectively. These ions are about 4 Da higher than those derived from the unlabeled peptide at m/z 696.6, 583.5, 484.4, 427.4, and 290.0, respectively. However, the molecular ions of the fragment ions for the C-terminal Arg derived from the labeled peptide was similar to that from the unlabeled peptide. These results indicate that both of the two 18O atoms occur at Asp next to the Arg; the carboxyl group of Asp-105 is labeled with 18O atoms during the enzymatic dehalogenation in H2 18O.

Labeling of H272N Mutant Enzyme with [14C]Chloroacetate—The H272N mutant enzyme was incubated with [1-14C]chloroacetate and then deionized by C18 reverse phase HPLC. The H272N mutant enzyme was clearly labeled with chloroacetate (2,710 dpm), whereas only a little radioactivity at a background level (65 dpm) was incorporated into the wild-type enzyme. His-272 probably plays an essential role in the hydrolysis of the alkylated enzyme derived from the substrate, and the H272N mutant enzyme provided the alkylated enzyme upon incubation with the substrate.

MS Analysis of the Labeled Peptide Derived from H272N Mutant Enzyme—We examined by ion-spray mass spectrometry whether the H272N mutant enzyme is also modified with fluoroacetate. The molecular mass of the H272N mutant enzyme incubated with fluoroacetate was 33,316 Da, which is 58 Da higher than that of the unreacted mutant enzyme (33,258 Da). The molecular mass of the deprotonated form of the carboxymethylene moiety of fluoroacetic acid is 58 Da. Therefore, the mutant enzyme is probably modified by the carboxymethylene moiety of fluoroacetate.

The modified H272N mutant enzyme was digested with trypsin, and the resultant peptide fragments were analyzed by ion-spray mass spectrometry. When the spectrometer was in single-quadrupole mode, the total ion current chromatogram displayed several peaks. Peak A at m/z 980.6 and Peak B at m/z 1038.6 were assigned as the M + H+ ions derived from the octapeptide Phe-99–Arg-106 (Fig. 5); A and B are derived from the unmodified and modified enzymes, respectively. The other peaks were those derived from other regions of the enzyme.
(data not shown). The difference in mass between Peaks A and B (58 Da) agrees well with the calculated mass of the carboxymethylene moiety of fluoroacetate (Fig. 5). Although we did not determine by tandem MS/MS analysis the modified residue of the octapeptide, Asp-105 is the most probable candidate for the site of modification because it was specifically labeled with $^{18}O$ as described above. The carboxylate anion of Asp-105 probably acts as the nucleophile to attack the α-carbon of fluoroacetate with the release of fluoride anion in an $S_{N}$2-type reaction. The putative ester intermediate formed is hydrolyzed by an activated water molecule in the wild-type enzyme. However, the H272N mutant enzyme is probably unable to activate a water molecule, and the ester intermediate accumulates.

**DISCUSSION**

Fluoroacetate dehalogenase from *Moraxella* sp. B has an amino acid sequence with a limited but significant similarity to that of haloalkane dehalogenase from *X. autotrophicus* GJ10.

**FIG. 2.** Amino acid sequence alignment of fluoroacetate dehalogenase from *Moraxella* sp. B (DeFH1) and haloalkane dehalogenase from *X. autotrophicus* GJ10 (DhlA) (24). The sequence similarity was examined with the program MegAlign (DNASTAR Inc., Madison, WI). The conserved residues are shaded. Numbers on the left indicate the residue numbers in each amino acid sequence. Symbols are as follows. ●, the conserved aspartate and histidine residues; ▼, Cl-1 atom-binding residues of the haloalkane dehalogenase; ●, Cl-2 atom binding residue of the haloalkane dehalogenase.

**FIG. 3.** Ion-spray mass spectra of the peptides containing Asp-105. Mass spectrum of the octapeptide Phe-99–Arg-106 derived from the enzyme incubated in H$_2^{18}$O with (A) or without (B) fluoroacetate.

**FIG. 4.** Tandem MS/MS product ion spectra of $^{18}O$-labeled and unlabeled octapeptides Phe-99–Arg-106. The calculated value of each peptide fragment is indicated in the sequence of the peptide. A, $^{18}O$-labeled peptide (the precursor ion: m/z 492.9 (bivalent)). B, unlabeled peptide (the precursor ion: m/z 490.9 (bivalent)). The mass values of only monovalent ions are indicated.
Both enzymes are members of the same family of $\alpha/\beta$ hydrolases (25) and contain aspartate and histidine as catalytic residues conserved in all members (Fig. 2). We here propose a catalytic mechanism for fluoroacetate dehalogenase: Asp-105 acts as a nucleophile which substitutes for a halogen atom in the substrate, and the ester intermediate formed is then hydrolyzed by a water molecule activated by His-272 (Fig. 6). A similar mechanism has been proposed for the reaction catalyzed by the haloalkane dehalogenase. The substrate specificities of the two dehalogenases, however, are quite different from each other: the haloalkane dehalogenase catalyzes the hydrolysis of chlorinated and brominated $n$-alkanes, and the fluoroacetate dehalogenase catalyzes the dehalogenation of fluoroacetate and other haloacetates. Both dehalogenases probably originated from a common ancestor protein but have diverged by evolution to the present enzymes with different substrate specificities.

Phe-128 of the haloalkane dehalogenase serves as a binding site for the Cl-2 atom, which is not dechlorinated, of the substrate 1,2-dichloroethane (16). The amino acid sequence around this residue is conserved in the fluoroacetate dehalogenase, and the residue is replaced by Arg-109 in the latter enzyme (Fig. 2). Arginine is the most common binding site of various enzymes for the carboxyl group of substrates. Therefore, Arg-109 of the fluoroacetate dehalogenase probably acts as the binding site for the carboxyl group of haloacetates.

The halogen-binding sites of various enzymes have been clarified by x-ray crystallography. His-197 and Lys-200 of human color vision pigments for chloride ion (26); Thr-199 of carboxic anhydrase II for bromide ion (27); Arg-337, Arg-195, and Asn-296 of pig pancreatic a-amylase for chloride ion (28). Trp-125 and Trp-175 of the haloalkane dehalogenase serve as the binding site of the Cl-1 atom, which is dechlorinated, of 1,2-dichloroethane (16). They are conserved as Arg-106 and Trp-151 in the fluoroacetate dehalogenase, respectively (Fig. 2), suggesting that they possibly function as the binding site for the fluorine atom of fluoroacetate. Kennes et al. (29) have replaced Trp-125 of the haloalkane dehalogenase with arginine in order to mimic the putative halogen-binding site of fluoroacetate dehalogenase. However, the resulting enzyme showed no activity on fluoroacetate and other haloacetates. The halogen-binding site of the fluoroacetate dehalogenase is probably constructed by a complicated network with various polar and charged amino acid residues around Arg-106.

Recently, we have clarified the reaction mechanism of 1,2-haloacid dehalogenase from Pseudomonas sp. YL: Asp-10 acts as a nucleophile to attack the $\alpha$-carbon atom of a 1,2-haloalkanoic acid, producing an ester intermediate that is hydrolyzed by a water molecule (6). We have also shown by ion-spray mass spectrometry that the rate-limiting step of the 1,2-haloacid dehalogenase reaction is the hydrolysis of the ester intermediate. Epoxide hydrolase is a homolog of the fluoroacetate dehalogenase belonging to the same family of $\alpha/\beta$-hydrolases. The enzyme reaction also proceeds through an ester intermediate in the same manner as the fluoroacetate dehalogenase reaction. The rate-limiting step in the epoxide hydrolase reaction is also the hydrolysis of the ester intermediate (30). However, the slowest step of the reaction catalyzed by the haloalkane dehalogenase is the release of the halide ion (31, 32). The active center of the haloalkane dehalogenase is surrounded by a hydrophilic environment composed of 4 phenylalanines, 2 tryptophans, 2 leucines, a valine, and a proline (33). This provides a clear contrast to the hydrophilic environment of that of the 1,2-haloacid dehalogenase (34). Although no sequence similarity occurs between the 1,2-haloacid dehalogenase and the fluoroacetate dehalogenase (35), they are similar to each other in reaction mechanism and substrate specificity. The rate-limiting step of the fluoroacetate dehalogenase is probably the hydrolysis of the ester intermediate in the same manner as 1,2-haloacid dehalogenase. The kinetics of the ester intermediate formation by the fluoroacetate dehalogenase is being studied by means of mass spectrometric analyses.

In Australia, rumen bacteria genetically modified to express recombinant fluoroacetate dehalogenase have been applied to the detoxification of poisonous plants containing high concentrations of fluoroacetate to prepare cases in which these plants are ingested by domestic animals (36). Fluoroacetate dehalogenase can possibly be engineered through protein engineering to change its activity, making it capable of decomposing various organofluorine compounds other than fluoroacetate such as flons, which have caused environmental problems. Therefore, we are now studying more detailed characteristics of the enzyme by various methods, including x-ray crystallography of the enzyme.

REFERENCES

Fig. 5. Reverse phase HPLC elution profile of proteolytic fragments of H272N mutant enzyme incubated with fluoroacetate. The structure of the unmodified peptide (A) and a possible structure of the modified peptides (B) are shown.
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Reaction Mechanism of Fluoroacetate Dehalogenase

Reaction Mechanism of Fluoroacetate Dehalogenase from *Moraxella* sp. B

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