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Catalysis-Linked Inactivation of Fluoroacetate Dehalogenase by Ammonia: A Novel Approach to Probe the Active-Site Environment[1]

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Fluoroacetate dehalogenase from *Moraxella* sp. B (FAc-DEX) catalyzes the hydrolytic dehalogenation of fluoroacetate and other haloacetates. Asp[105] of the enzyme acts as a nucleophile to attack the α-carbon of haloacetate to form an ester intermediate, which is subsequently hydrolyzed by a water molecule activated by His[272] [Liu, J.Q., Kurihara, T., Ichiyama, S., Miyagi, M., Tsunasawa, S., Kawasaki, H., Soda, K., and Esaki, N. (1998) *J. Biol. Chem.* 273, 30897–30902]. In this study, we found that FAc-DEX is inactivated concomitantly with defluorination of fluoroacetate by incubation with ammonia. Mass spectrometric analyses revealed that the inactivation of FAc-DEX is caused by nucleophilic attack of ammonia on the ester intermediate to convert the catalytic residue, Asp[105], into an asparagine residue. The results indicate that ammonia reaches the active site of FAc-DEX without losing its nucleophilicity. Analysis of the three-dimensional structure of the enzyme by homology modeling showed that the active site of the enzyme is mainly composed of hydrophobic and basic residues, which are considered to be essential for an ammonia molecule to retain its nucleophilicity. In a normal enzyme reaction, the hydrophobic environment is supposed to prevent hydration of the highly electronegative fluorine atom of the substrate and contribute to fluorine recognition by the enzyme. Basic residues probably play a role in counterbalancing the electronegativity of the substrate. These results demonstrate that catalysis-linked inactivation is useful for characterizing the active-site environment as well as for identifying the catalytic residue.

Key words: ammonia, catalysis-linked inactivation, fluoroacetate dehalogenase, homology modeling, mass spectrometry.

Fluoroacetate dehalogenase from *Moraxella* sp. B (FAc-DEX; EC 3.8.1.3) catalyzes the dehalogenation of fluoroacetate and other haloacetates to produce glycolate (*1*). To our knowledge, FAc-DEX is the only enzyme that catalyzes the hydrolytic defluorination of aliphatic compounds. Carbon-fluorine bonds in aliphatic compounds are highly stable, and no other dehalogenases catalyze the cleavage of carbon-fluorine bonds. L-2-Haloacid dehalogenase from *Pseudomonas* sp. YL (L-DEX YL; EC 3.8.1.2) catalyzes the hydrolytic dehalogenation of various L-2-haloalkanoates and haloacetates, except for fluorinated compounds, to produce the corresponding D-2-hydroxyalkanoates and glycolate, respectively (*2*).

We have studied the reaction mechanisms of FAc-DEX and L-DEX YL, and showed that the reactions proceed in a similar manner (*3, 4*), although little amino acid sequence similarity is found between the two enzymes (*5*). Asp[105] of FAc-DEX and Asp[10] of L-DEX YL attack the substrate in the first step, causing the release of a halide ion and the formation of an ester intermediate, which is hydrolyzed in the second step by an activated water molecule (Scheme 1). In FAc-DEX, His[272] has been shown to function as a base to activate a water molecule in the second step of the reaction. Hydrolytic dehalogenation catalyzed by all the dehalogenases characterized so far, except for DL-2-haloacid dehalogenase from *Pseudomonas* sp. 113, proceeds in a similar manner through an ester intermediate (*6*).

Although the mechanistic pathway of FAc-DEX can be outlined as above, an important problem remains to be solved: why only FAc-DEX catalyzes the hydrolytic cleavage of an aliphatic carbon-fluorine bond. In the present study, we characterized FAc-DEX in comparison with L-DEX YL by catalysis-linked enzyme inactivation to address this problem. Catalysis-linked enzyme inactivation involves

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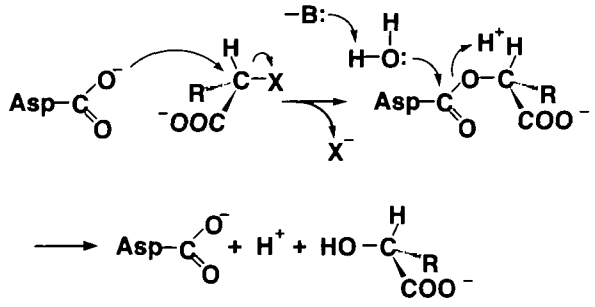
Abbreviations: BpoA2, bromoperoxidase A2 from *Streptomyces aureofaciens*; DhlA, haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10; FAc-DEX, fluoroacetate dehalogenase from *Moraxella* sp. B; HPLC, high performance liquid chromatography; L-DEX YL, L-2-haloacid dehalogenase from *Pseudomonas* sp. YL; MS, mass spectrometry.



Scheme 1. **Mechanism of the FAc-DEX and L-DEX YL reactions.**

a chemical reaction between an enzyme-substrate complex and an extrinsic reagent that is not a constituent of the normal enzyme-substrate reaction, causing irreversible covalent modification of a catalytic residue (*7, 8*). FAc-DEX and L-DEX YL are expected to be inactivated when a nucleophilic reagent added to the reaction mixture attacks the ester intermediate, taking the place of a water molecule in the second step of the reaction. Here, we used ammonia as a nucleophilic reagent, expecting that the experiment would yield information on the hydrophobicity and/or basicity of the active-site environment, because ammonia easily loses its nucleophilicity if it is present in a hydrophilic and acidic environment. As a result, we found that the reagent caused the inactivation of FAc-DEX and L-DEX YL in different manners. FAc-DEX was completely inactivated by catalysis-linked inactivation with ammonia, whereas L-DEX YL was not. Mass spectrometric analyses revealed that the inactivation occurred through modification of the nucleophile Asp^{105} of FAc-DEX. The difference in the reactivity of the enzymes with ammonia might be related to the difference in hydrophobicity and basicity of the active sites by three-dimensional structure analyses. The results demonstrate the validity of catalysis-linked inactivation in characterizing the active-site environment as well as in identifying the catalytic residue.

EXPERIMENTAL PROCEDURES

Materials—Sodium fluoroacetate and protamine sulfate were purchased from Wako Pure Chemical Industries (Osaka), chloroacetic acid, L-2-chloropropionic acid, and ammonium sulfate from Nacalai Tesque (Kyoto), and Butyl- and DEAE-Toyopearl from Tosoh (Tokyo); chromotropic acid was obtained from Merck (Darmstadt, Germany), and *N*-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Enzyme Purification—All operations were performed at 4°C. The recombinant *Escherichia coli* JM109 cells producing the enzyme (*4*) were grown aerobically at 37°C for 14 h in LB medium containing 200 μg/ml ampicillin and 0.2 mM isopropyl-1-thio-β-D-galactoside. The cells harvested from a 4-l culture were disrupted by sonication. DNA and its binding proteins were removed from the supernatant with 0.2% protamine sulfate. The supernatant was fractionated with ammonium sulfate. The 40–70% saturation fraction was dissolved in 50 mM potassium phosphate (pH 7.5), and then applied to a Butyl-Toyopearl 650 column (3 × 25 cm). The column was washed with 500 ml of buffer supplemented with 30% (w/v) ammonium sulfate, and the enzyme was eluted with a linear gradient of 30–0% ammonium sulfate in buffer with a total volume of 1 liter. The active fractions, dialyzed against 10 mM potassium phosphate (pH 7.5), were applied to a DEAE-Toyopearl 650M column (1.7 × 5 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 10–50 mM potassium phosphate (pH 7.5). The active fractions were pooled and used as the purified enzyme preparation.

Determination of the Enzyme Activity and Protein Concentration—FAc-DEX and L-DEX YL were routinely assayed by measuring the chloride ions produced from chloroacetate by the method of Iwasaki *et al.* (*9*). The standard assay mixture (100 μl) contained 25 mM chloroacetate, 100 mM Tris-sulfate buffer (pH 9.5), and the enzyme. The reaction was terminated by the addition of 10 μl of 1.5 M 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 μmol of substrate per min. FAc-DEX was also assayed by measuring glycolate ions produced from fluoroacetate by the method of Dagley and Rodgers (*10*). Protein concentration was determined with a Bio-Rad protein assay kit (Hercules, CA).

Inactivation of FAc-DEX and L-DEX YL—(A) The enzyme (FAc-DEX, 650 nM; L-DEX YL, 3.2 μM) was incubated at 30°C for 1 h in 100 μl of a solution containing 150 mM Tris-sulfate (pH 9.0), 100 mM substrate (fluoroacetate for FAc-DEX; L-2-chloropropionate for L-DEX YL), and 1.25 M ammonium sulfate. After incubation, the reaction mixture was dialyzed against about 3×10^8 volumes of 50 mM potassium phosphate (pH 7.5), and the residual enzyme activities were measured by the method described above. (B) For mass spectrometric analysis, 60 μM FAc-DEX was incubated at 30°C in 500 μl of a solution of 150 mM Tris-sulfate (pH 9.0), 100 mM sodium fluoroacetate, and 1 M ammonium sulfate. FAc-DEX was inactivated less efficiently in B than in A, because the high concentration of the enzyme caused a rapid decrease in the concentration of fluoroacetate and lower concentration of ammonium sulfate was added in B. Thus, after 1 h, 50 μl of 1 M fluoroacetate was added, and the mixture was incubated for another 1 h to decrease the residual activity of the enzyme to about 50% of the original activity. After the reaction, the mixture was dialyzed against 8×10^6 volumes of 50 mM potassium phosphate (pH 7.5).

Digestion of FAc-DEX with Trypsin—The enzyme inactivated and dialyzed as described above was lyophilized, dissolved in 100 μl of 8 M urea in 200 mM Tris-sulfate buffer (pH 7.2), and incubated at 37°C for 1 h. The enzyme was digested by the addition of 7.5 μg trypsin dissolved in 400 μl of 120 mM Tris sulfate buffer (pH 7.5) at 37°C for 12 h.

LC/MS Analysis of Peptides—The proteolysates (20 μl) described above were mixed with 1 μl of 20% (v/v) 2-mercaptoethanol, incubated for 1 h, and loaded onto a C_{18} reverse-phase HPLC column (Michrom BioResources 5 μ 100 Å, 1.0 × 150 mm) connected to a mass spectrometer, PE-Sciex API 300. Elution was carried out with 2% acetonitrile containing 0.1% formic acid for 5 min, followed by a linear gradient of 2–62% acetonitrile containing 0.1% formic acid over 60 min at a flow rate of 40 μl/min. The total ion current chromatogram was recorded in the single-quadrupole mode. The quadrupole was scanned from 300 to 2,000

atomic mass units with a step size of 0.2 atomic mass unit and a dwell time of 0.5 ms/step. The ion-spray voltage was set at 5 kV, and the orifice potential was 30 V.

LC/MS/MS Analysis of the Peptides—The proteolysates were applied to the HPLC column connected to the mass spectrometer and eluted in the same manner as described above. Daughter ion spectra were obtained in the triple-quadrupole daughter ion scan mode by introducing peptides containing Asp^{105} ($[M + 2H]^{2+}$ at m/z 491.0 or 490.4) from Q1 into a collision cell (Q2) and observing the daughter ions in Q3. Q3 was scanned from 30 to 1,000 with a step size of 0.2 and a dwell time of 0.5 ms/step. The ion-spray voltage was set at 4.8 kV, and the orifice potential was 30 V.

Molecular Modeling of FAc-DEX—The structure of FAc-DEX was predicted by homology modeling on the basis of the structures of haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (DhlA) (*11, 12*) and bromoperoxidase A2 from *Streptomyces aureofaciens* (BpoA2) (*13*) using the program MODELLER, version 4 (*14*). The PHD secondary structure prediction (*15*) was used to make structure-based sequence alignments. Five models were generated for FAc-DEX using complete optimization cycles, conjugate gradients, and simulated annealing. The quality of these structures was examined by PROCHECK (*16*) and the Protein Health and 3D profile (*17*) modules of Quanta 4.0 (Molecular Simulations, Burlington, MA). All other estimates of structural parameters were obtained with the software packages Quanta 4.0 and Insight II (Molecular Simulations, Burlington, MA). Calculations were performed on a Silicon Graphics Indigo 2 workstation.

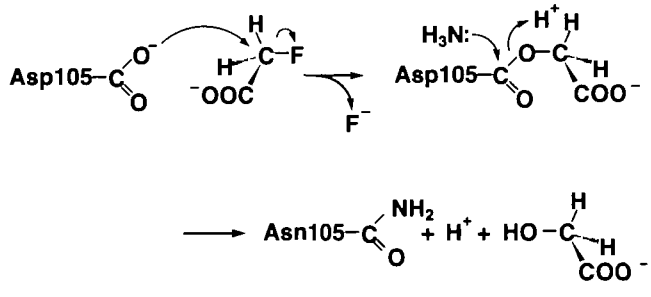
Site-Directed Mutagenesis—Replacement of amino acid residues was carried out by the method of Kunkel *et al.* (*18*). The synthetic mutagenic primers used were as follows (underlines indicate the mutagenized nucleotides): R106G, 5′-GCCGCCATCATGTCC; R106K, 5′-GGTACGCCCGCCTTTATCATGTCCGACGAG; R106S, 5′-GCCGCTATCATGTCC; R106W, 5′-CCCGCCCCAATCATGTCCGACG. The substitutions were confirmed with an Applied Biosystem 373A DNA sequencer (Foster City, CA). Mutant enzymes were produced in *Escherichia coli* JM109.

RESULTS

Catalysis-Linked Inactivation of FAc-DEX by Ammonia—We found that FAc-DEX was nearly completely inactivated after 1-h incubation with fluoroacetate and ammonia (Fig. 1A, Samples 1, 4). In 1 min, the residual activity of the enzyme decreased to about 50% of the original activity. Extensive dialysis of the inactivated enzyme did not result in reactivation, indicating a covalent modification of the enzyme. Since omission of either fluoroacetate or ammonia caused virtually no alteration in the enzyme activity (Fig. 1A, Samples 2, 3, 4), an ammonia molecule probably attacks the ester intermediate of the enzyme reaction (Scheme 2).

We next examined the effect of ammonia on the activity of L-DEX YL. In contrast to the case of FAc-DEX, no significant inactivation occurred when L-DEX YL was incubated with ammonia in the presence of its substrate, L-2-chloropropionate (Fig. 1B, Samples 1, 4). Thus, ammonia does not act as a catalysis-linked inactivator for L-DEX YL. This is not due to low activity of L-DEX YL in the presence of ammonia: each L-DEX YL molecule catalyzed the hydrolysis of 23,000 molecules of L-2-chloropropionate during the incubation, whereas each FAc-DEX molecule catalyzed the hydrolysis of only 840 molecules of fluoroacetate, indicating that the ester intermediate is produced more often in the L-DEX YL reaction than in the FAc-DEX reaction. Because hydroxylamine, which is more bulky than ammonia, was shown in our previous study (*19*) to cause catalysis-linked inactivation of L-DEX YL, it is unlikely that ammonia fails to reach the active site of L-DEX YL. Ammonia apparently gains access to the active site of L-DEX YL, but it probably does not have nucleophilicity.

Mass Spectrometric Analysis of FAc-DEX Modified by Ammonia in the Presence of Fluoroacetate—For mass spec-



Scheme 2. **Catalysis-linked inactivation of FAc-DEX by ammonia.**

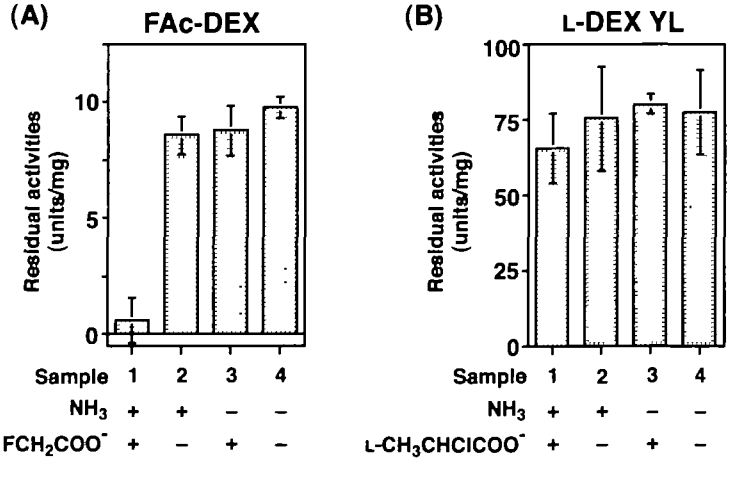


Fig. 1. **Effect of ammonia on FAc-DEX (A) and L-DEX YL (B).** The enzymes were incubated in the presence (+) or absence (–) of ammonia and respective substrates. Residual activities were measured after extensive dialysis against 50 mM potassium phosphate (pH 7.5) [n = 3 except for Sample 1 in A (n = 2)].

trometric analysis, FAc-DEX was incubated with ammonia and fluoroacetate until the residual activity of the enzyme decreased to about 50% of the original activity as described in "EXPERIMENTAL PROCEDURES." The enzyme was then digested with trypsin, and the resultant peptides were analyzed by LC/MS. When the spectrometer was in the single-quadrupole mode, the total ion current chromatogram displayed several peaks (Fig. 2A). Peak 2, which eluted at 13.76 min, provided an ion-spray mass spectrum showing two peaks at m/z 980.8 and 491.0, which were assigned to $[M + H]^+$ and $[M + 2H]^{2+}$ ions, respectively (Fig. 2B). Thus, the molecular mass of the peptide eluted as Peak 2 is 979.9 Da (the average of the molecular masses determined based on the m/z values of the two peaks), indicating that the peptide is the octapeptide Phe^{99}–Arg^{106}, whose predicted molecular mass is 979.5 Da. Peak 1, which eluted at 12.84 min, provided a mass spectrum showing two peaks at m/z 979.6 and 490.4 (Fig. 2C), corresponding to $[M + H]^+$ and $[M + 2H]^{2+}$ ions, respectively, and the determined molecular mass of the peptide is 978.7 Da. The molecular mass is 1.2-Da smaller than that of the octapeptide Phe^{99}–Arg^{106}, suggesting that a residue in the peptide is modified by incubation with ammonia.

The amino acid residue modified by ammonia was determined by tandem LC/MS/MS analysis. The parent ions of $[M + 2H]^{2+}$ at m/z 490.4 (Fig. 2C) and 491.0 (Fig. 2B), corresponding to the modified and unmodified octapeptides, respectively, were selected in the first quadrupole and subjected to collision-induced fragmentation in the second quadrupole. The daughter ions are shown in Fig. 3. The Y series monovalent ions at m/z 695.4, 582.2, 483.2, 426.0, and 289.0 (Fig. 3B) derived from the parent divalent ion at m/z 490.4 were assigned to derivatives of Leu-Val-Gly-His-Asp-Arg, Val-Gly-His-Asp-Arg, Gly-His-Asp-Arg, His-Asp-Arg, and Asp-Arg, respectively. These values are about 1-Da lower than those derived from the unmodified peptide at m/z 696.4, 583.2, 484.4, 427.4, and 290.0, respectively (Fig. 3A). However, the molecular mass of the fragment ion for the C-terminal Arg derived from the modified peptide (m/z 175.2) was virtually identical to that from the unmodified one (m/z 175.0). These results indicate that Asp^{105} adjacent to the C-terminal Arg is specifically modified so that its molecular mass is 1-Da lower than the original value: the side-chain carboxyl group of Asp^{105} is most probably converted to an amido group (Scheme 2).

Since the amounts of the two peptides appearing as Peak 1 and Peak 2 in Fig. 2A are in the ratio of about 1:1, the 50% loss of enzyme activity in the sample for mass spectrometric analyses is considered to be caused by the modification of Asp^{105} by ammonia.

Confirmation of Asn Formation by Protein Sequencing—The modified peptide (Peak 1 in Fig. 2A) was isolated by HPLC on a C_{18} reverse-phase HPLC column (Millipore Puresil 5 μ C_{18} 120 Å, 4.6 × 150 mm) and sequenced with a Shimadzu PPSQ-10 protein sequencer (Kyoto). The following sequence was obtained (boldface indicates the amino acid at residue number 105): Phe-His-Leu-Val-Gly-His-**Asn**-Arg, which is identical to that predicted from the wild-type nucleotide sequence with the exception of Asn. This result indicates that Asn was produced from Asp^{105} by catalysis-linked modification by ammonia.

Homology Modeling of FAc-DEX—The results obtained in this study indicate that an ammonia molecule nucleophilically attacked the active-site Asp^{105} of FAc-DEX to convert the residue into Asn (Scheme 2). To elucidate the reason why an ammonia molecule has nucleophilicity at the active site of FAc-DEX, we built a three-dimensional structure of FAc-DEX by homology modeling using DhlA and BpoA2 as reference structures. All of these enzymes are considered to belong to the α/β hydrolase family: DhlA and BpoA2 were shown to belong to this family based on

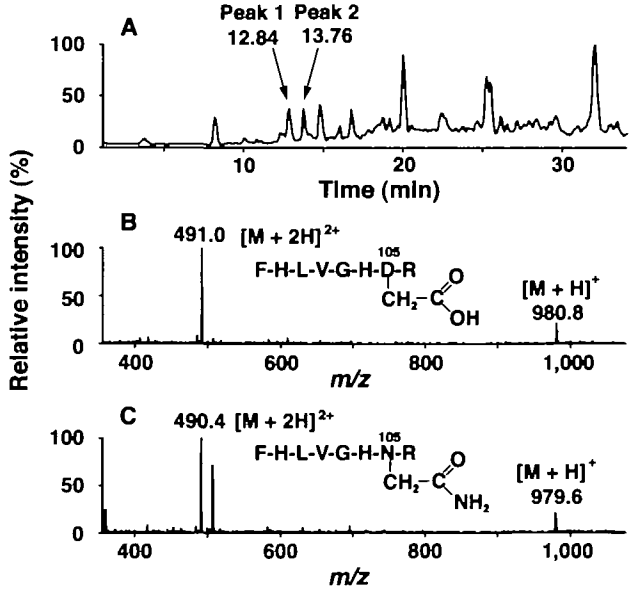


Fig. 2. **LC/MS analyses of the proteolytic peptides of FAc-DEX inactivated by ammonia in the presence of fluoroacetate.** A, total ion current chromatogram of proteolytic peptides; B and C, ion-spray mass spectra of the octapeptides Phe^{99}–Arg^{106} from Peaks 2 and 1 in A, respectively. The probable structures of these peptides are also shown.

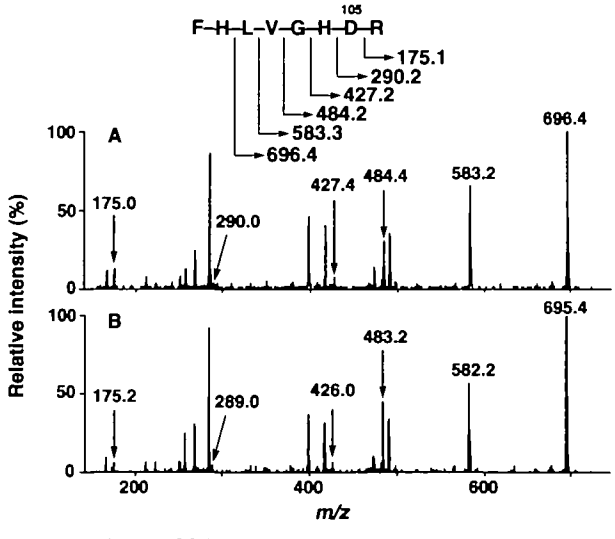


Fig. 3. **Tandem LC/MS/MS daughter ion spectra of the octapeptides Phe^{99}–Arg^{106} from unmodified (A) and modified (B) FAc-DEX.** The calculated m/z values of fragment ions produced from the unmodified peptide are indicated. The MS/MS spectra were obtained with the precursor ions of $[M + 2H]^{2+}$ at m/z 491.0 and 490.4, respectively. The mass values of only monovalent ions are indicated.

their crystal structures, and FAc-DEX has sequence similarity to these enzymes as well as a consensus sequence for this family (G-X-Nu-X-G: Nu = catalytic nucleophile) (*20*). The sequence alignment shown in Fig. 4A used for homology modeling is appropriate in that the catalytic nucleophiles (Asp^{106} of FAc-DEX, Asp^{124} of DhlA, and Ser^{99} of BpoA2) and the basic residues that activate a water molecule for hydrolysis (His^{272} of FAc-DEX, His^{289} of DhlA, and His^{258} of BpoA2) align well with one another (*13*). Figure 4B shows the constructed three-dimensional model structure of the active site of FAc-DEX. We found that replacing Arg^{106} (which is located in a position corresponding to the Trp^{125} of DhlA that functions as a halide ion acceptor) with Gly, Lys, Ser, or Trp by site-directed mutagenesis results in a total loss of the dehalogenation activity of FAc-DEX, indicating that Arg^{106} plays an essential role in the catalytic reaction. This supports the appropriateness of the model structure. The active site of FAc-DEX in the vicinity of the catalytic nucleophile, Asp^{105}, was found to be composed mainly of hydrophobic (Phe^{35}, Tyr^{148}, Trp^{151}, Tyr^{213}, and Phe^{273}) and basic (His^{104}, Arg^{106}, Arg^{109}, and His^{272}) amino acid residues (Fig. 4B). The hydrophobic and basic environment is thought to be suitable for an ammonia molecule to be present in a non-protonated form to retain its nucleophilicity: because of the lack of acidic residues, an ammonia molecule is unlikely to be protonated in the active site. Some of the basic residues may also play a role in increasing the nucleophilicity of the ammonia molecule by withdrawing a proton from it.

A

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                                  β    β         β          α
FAc-DEX  M--DFP----------------GFKNSTVTVDGVDIAYTVSGEGPP--VLM-LHGFPQNRAMWARVAPQLAEH-H  53
DhlA     MINAIRTPDQRFSNLDQYPFSPNYLDDLPGYPGLRAHYLDEGNSDAEDVFLCLHGEPTWSYLYRKMIPVFAESGA  75
BpoA2    M--P-----------------FITVGQENSTSIDLYYEDHGTGQP--VVL-IHGFPLSGHSWERQSAALLDAGY  52

         β                    α          β*        β         β
FAc-DEX  TVVCADLRGYGDSDKPKCLPDRSNYSFRAFAHDQLCVMRHLGFERFHLVGHDRGGRTGHRMALDHPEAVLSLTVM 128
DhlA     RVIAPDFFGFGKSDKPV---DEEDYTFEFHRNFLLALIERLDLRNITLVVQDWGGFLGLTLPMADPSRFKRLIIM 147
BpoA2    RVITYDRRGFGQSSQPT-----TGYDYDTFAADLNTVLETLDLQDAVLVGFSMGTGEVARYVSSYGTARIAKVAF 122

              β          α          β         α
FAc-DEX  D-IV---PTYA-MFMNTNRLVAASYWHW-YFLQQPEPFPEHMIGQDPDF---FYETCLFGWGATKVSDFDQQMLN 194
DhlA     NACLMTDPVTQPAFSAFVTQPADGFTAWKYDLVTPSDLR-------------LDQFMKRWAPT----LTEAEAS 204
BpoA2    ---L---ASLEPFLLKTDDNPDGA---------APQEFFDGIVAAVKADRYAFYTGFFNDFYNLDENLGTRISEE 182

         .....    β          β    β          α
FAc-DEX  AYRESWRNPAMIHGSCSDYRAAAT------IDLEHDSADIQR-KVECPTLVFYGSKGQMGQLFDIPAEWAKRC-- 260
DhlA     AYAAPF-PDTSYQAGVRKFPKMVAQRDQACIDISTEAISFWQNDWNGQTFMAIGMKDK---LLGPDVMYPMKALI 275
BpoA2    AVRNSWNTAAS--GGF--FAAAAAPTT--W--YTDFRADIPR--IDVPALILHGT-GDRTLPIENTARVFHKALP 246

            β     *    α
FAc-DEX  NN--TTNASLPGGHFFVDQFPAETSEILLKFLARNG* 294
DhlA     NGCPEPLEIADAGHF-VQEFGEQVAREALKHFAETE* 310
BpoA2    SA--EYVEVEGAPHGLLWTHAEEVNTALLAFLAK*   278
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B

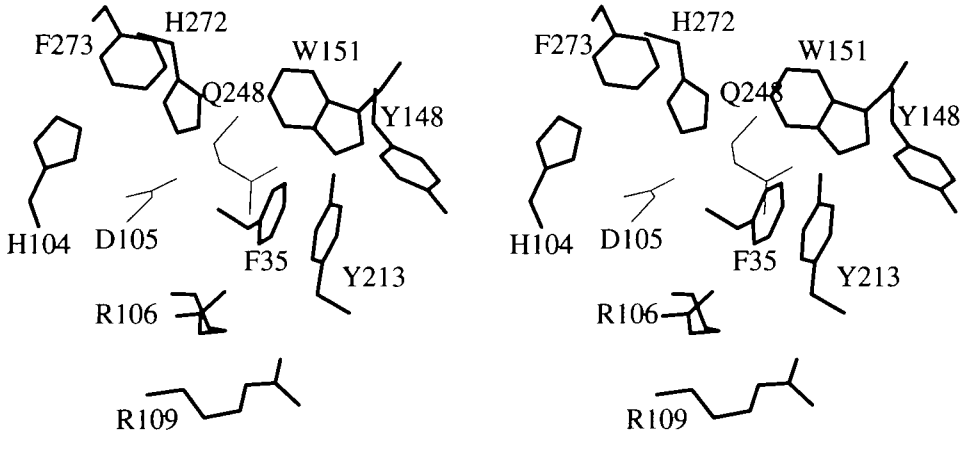

Fig. 4. **Sequence alignment and molecular modeling of FAc-DEX.** A, sequence alignment of FAc-DEX with DhlA (GenBank accession M26950) and BpoA2 (M84990). Predicted secondary structures of FAc-DEX are also shown. The catalytic nucleophiles and the catalytic basic residues are indicated by asterisks. B, stereoview of the active site model of FAc-DEX prepared using program MOLSCRIPT (22). Hydrophobic and basic residues are drawn with thick lines; other types of amino acid residues are drawn with thin lines.

DISCUSSION

To our knowledge FAc-DEX is the only enzyme that catalyzes the degradation of an aliphatic fluorinated compound by cleaving the carbon-fluorine bond, whose dissociation energy is among the highest found in natural compounds. To elucidate the structural basis for the unique activity of FAc-DEX, we characterized the enzyme by catalysis-linked inactivation and homology modeling of the three-dimensional structure.

We found that FAc-DEX is inactivated by ammonia concomitantly with enzymatic defluorination of the substrate. An ammonia molecule was shown to attack the active-site residue, Asp^{105}, nucleophilically to convert it into an asparagine residue. Since the modification did not occur in the absence of the substrate, an ammonia molecule probably attacks the carbonyl carbon atom of the ester intermediate (Scheme 2). We observed similar catalysis-linked inactivation for L-DEX YL when hydroxylamine was used as a nucleophilic reagent (*19*). Hydroxylamine nucleophilically attacks the carbonyl carbon atom of the ester intermediate formed transiently during the course of the L-DEX YL reaction.

Ammonia is a much weaker nucleophile than hydroxylamine and is easily protonated in aqueous solution to lose its nucleophilicity. Indeed, L-DEX YL is not efficiently inactivated by ammonia. This presents a striking contrast to the case for FAc-DEX, which is completely inactivated by ammonia. Thus, the active site of FAc-DEX is considered to have unique characteristics that prevent the protonation of an ammonia molecule. We constructed a three-dimensional model of FAc-DEX and found that its active site is composed mainly of hydrophobic (Phe^{35}, Tyr^{148}, Trp^{151}, Tyr^{213}, and Phe^{273}) and basic (His^{104}, Arg^{106}, Arg^{109}, and His^{272}) residues. In contrast, the active site of L-DEX YL, whose three-dimensional structure was determined by X-ray crystallography, is highly hydrophilic (*21*). Thus, it is conceivable that an ammonia molecule is protonated in the active site of L-DEX YL but not in the active site of FAc-DEX.

Since fluorine is the most electronegative element, the fluorine atom in fluoroacetate tends to be hydrated. Hydrophobic residues in the active site of FAc-DEX are probably important in keeping the fluorine atom unhydrated, which is supposed to be essential for the halogen-accepting residue in the enzyme, most likely Arg^{106}, to recognize the halogen atom of the substrate: an interaction between the fluorine atom and Arg^{106} is believed to be essential for the polarization of the carbon-fluorine bond, which increases the electrophilicity of the α-carbon atom to facilitate the nucleophilic attack of Asp^{105} on the α-carbon atom of the substrate. Basic amino acid residues in the active site of FAc-DEX are thought to play a role in counterbalancing the negative charge of the substrate.

We previously showed that hydroxylamine is useful for identifying the active-site nucleophile of L-DEX YL. Here, we found that ammonia can be used to identify the catalytic residue of FAc-DEX. FAc-DEX was inactivated much more efficiently by ammonia than by hydroxylamine (data not shown), probably due to the size of the molecule: hydroxylamine is more bulky than ammonia and does not easily access a position suitable for nucleophilic attack on the carbonyl carbon atom of the ester intermediate in the active site of FAc-DEX. Thus, ammonia could provide an alternative to hydroxylamine for identifying a catalytic residue by catalysis-linked modification when hydroxylamine does not work as an efficient inactivator. When ammonia is used as a catalysis-linked inactivator, an active-site carboxylate group is converted into an amido group: Asp into Asn, or Glu into Gln. Thus, it is possible to identify the converted residues with a conventional protein sequencer.

The present study demonstrates that the catalysis-linked inactivation of enzymes provides a useful tool for probing the active-site environment as well as for identifying a catalytic residue. The modification of Asp^{105} by ammonia confirmed that this residue acts as a catalytic nucleophile in the FAc-DEX reaction. Characterization of FAc-DEX by X-ray crystallography and genetic engineering is in progress to examine whether the hydrophobic and basic environment in the active site is essential for the degradation of fluorinated compounds.

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