Enzymes that catalyze the conversion of organohalogen compounds have been attracting a great deal of attention, partly because of their possible applications in environmental technology and the chemical industry. We have studied the mechanisms of enzymatic degradation of various organic halo acids. In the reaction of 1.2-haloacid dehalogenase and fluoroacetate dehalogenase, the carboxylate group of the catalytic aspartate residue nucleophilically attacked the a-carbon atom of the substrates to displace the halogen atom. In the reaction catalyzed by α-2-haloacid dehalogenase, a water molecule directly attacked the substrate to displace the halogen atom. In the course of studies on the metabolism of 2-chloroacrylate, we discovered two new enzymes, 2-haloacrylate reductase catalyzed the asymmetric reduction of 2-haloacrylate to produce 1,2-haloalkanoic acid in an NADPH-dependent manner. 2-Haloacrylate hydratase catalyzed the hydration of 2-haloacrylate to produce pyruvate. The enzyme is unique in that it catalyzes the non-redox reaction in an FADH2-dependent manner.

Key words: 1,2-haloacid dehalogenase; L-2-haloacid dehalogenase; fluoroacetate dehalogenase; 2-haloacrylate reductase; 2-haloacrylate hydratase

Various organohalogen compounds occur in nature. More than 3,800 organohalogen compounds are known to be produced biologically or by natural abiogenic processes such as volcano eruption and forest fire. In addition, organohalogen compounds are produced in large quantities in the chemical industry because of their usefulness as solvents, pharmaceuticals, agrochemicals, synthetic precursors, and so on. Some of these compounds are metabolized by microorganisms and disappear. A variety of enzymes that act on organohalogen compounds have been discovered. Some of them, called dehalogenases, catalyze the removal of a halogen atom from the substrates. Enzymes that catalyze the conversion of organohalogen compounds to other organohalogen compounds are also known. These are useful in environmental technology to decontaminate environments polluted with harmful organohalogen compounds. Some other enzymes are useful in the production of pharmaceuticals, agrochemicals, and their synthetic precursors, the synthesis of which involves regiospecific and/or stereospecific reactions of organohalogen compounds. Molecular engineering of these enzymes would be beneficial to increase their utility, and mechanistic analysis of these enzymes would serve as the basis for such engineering. In this review, the mechanistic analysis of various enzymes that act on organic haloacids is described.

I. 1,2-Haloacid Dehalogenase: A Representative Enzyme of the Haloacid Dehalogenase Superfamily

1,2-Haloacid dehalogenase catalyzes the hydrolytic dehalogenation of 1,2-haloalkanoic acids to produce the corresponding 1,2-hydroxyalkanoic acids. It is a representative enzyme of the haloacid dehalogenase (HAD) superfamily, which includes the P-type ATPases and other hydrolases. Structural and mechanistic analyses of this enzyme have yielded invaluable insights into the mode of action of HAD superfamily proteins.

1,2-Haloacid dehalogenase from Pseudomonas sp. YL (L-DEX YL) is one of the best studied enzymes of the HAD superfamily. Mass spectrometry has played an important role in analyzing the reaction mechanism of L-DEX YL. 18O-incorporation experiments were performed to determine the reaction mechanism and to identify the catalytic residue. When the multiple turnover enzyme reaction was carried out in H2O with L-2-chloropropionate as substrate, the lactate produced was labeled with 18O. In contrast, when the single turnover reaction was carried out in H2O using the enzyme in large excess over the substrate, the product was not labeled with 18O. Thus, an oxygen atom of the solvent water was assumed to be first incorporated into the enzyme and then transferred to the product. To identify the amino acid residue that incorporates the oxygen atom of a water molecule during catalysis, the enzyme used for the multiple turnover reaction in H2O was subjected to
proteolysis, and the molecular masses of the peptide fragments were analyzed. The peptide containing $^{18}$O was further analyzed by tandem mass spectrometry, which revealed that Asp10 was labeled with two $^{18}$O atoms. The catalytic importance of Asp10 was also confirmed by site-directed mutagenesis experiments.\(^\text{13}\) These results indicate that Asp10 acts as a nucleophile to attack the \(\alpha\)-carbon of the substrate to form an ester intermediate, which is hydrolyzed by the attack of an activated water molecule (Fig. 1). In that two $^{18}$O atoms were incorporated into Asp10, both oxygen atoms of the carboxylate group of Asp10 can attack the substrate. The formation of an ester intermediate was confirmed by paracatalytic inactivation of the enzyme by hydroxylamine, which caused modification of Asp10 into an aspartate $\beta$-hydroximate carboxyalkyl ester residue.\(^\text{17}\) The essential role of Asp10 in the catalysis is consistent with the fact that Asp10 is conserved in the proteins of the HAD superfamily.\(^\text{7}\)

Structural changes in \(1\)-DEX YL during catalysis were monitored by mass spectrometric analysis of the enzyme (Fig. 2).\(^\text{20,21}\) Upon incubation with the substrate \(1\)-2-chloropropionate, the peak corresponding to the native enzyme disappeared, and a new peak appeared at 26,255 Da (M + 73), which is considered to be an ester intermediate. These results not only verified the validity of the proposed mechanism for \(1\)-DEX YL (Fig. 1), but also demonstrated the effectiveness of mass spectrometry in the mechanistic analysis of enzyme reactions.

X-Ray crystallographic analysis of \(1\)-DEX YL provided further evidence of the formation of an ester intermediate and information on the functions of active-site amino acid residues.\(^\text{15,16,18,19}\) In the crystal structures of the S175A mutant enzyme complexed with monochloroacetate, \(1\)-2-chlorobutyrate, \(1\)-2-chloro-3-methylbutyrate, or \(1\)-2-chloropropionamide, Asp10 was esterified with the dechlorinated moiety of the substrate.\(^\text{18,19}\) The substrate moieties in all but the monochloroacetate intermediate had a D-configuration at the C2 atom. A water molecule, which was absent in the substrate-free enzyme, was present in the vicinities of the carboxyl carbon of Asp10 and the side chains of Asp180, Asn177, and Ala175 in each intermediate. This water molecule most likely hydrolyzes the ester intermediate. The side chains of Tyr12, Gln42, Leu45, Phe60, Lys151, Asn177, and Trp179 primarily compose a hydrophobic pocket around the alkyl group of the substrate moiety. This pocket probably plays an important role in stabilizing the alkyl group of the substrate moiety through hydrophobic interactions and in determining the stereospecificity of the enzyme.

The reaction mechanism of \(1\)-DEX YL was further studied by molecular modeling based on structural information on the enzyme obtained by X-ray crystallographic analysis.\(^\text{22}\) The complexes of the wild-type enzyme and the K151A and D180A mutants with \(1\)-2-chloropropionate were constructed by docking simulation. Subsequently, molecular dynamics were studied and \textit{ab initio} fragment orbital calculations on the complexes were performed. \(1\)-2-Chloropropionate was found to interact strongly with Asp10, Arg41, Lys151, and Asp180 by calculation of the interfragment interaction energy between the fragments in the \(1\)-DEX YL complexed with \(1\)-2-chloropropionate and catalytic water. Arg41 is located near the entrance to the active site and appears to play roles in regulating active site access and in stabilizing the chloride ion released in the first step of the reaction. Lys151 probably stabilizes the orientation of the substrate and maintains a balance in the charge around the active site. Asp180 probably stabilizes the rotation of Asp10, fixes catalytic water around Asp10, and prevents Lys151 from approaching Asp10. Asp180 might also activate catalytic water on its own or with Lys151, Ser175, and Asn177. The involvement of Arg41, Lys151, Ser175, Asn177, and Asp180 in the catalytic reaction of \(1\)-DEX YL was confirmed by

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**Fig. 1.** Reaction Mechanism of \(1\)-DEX YL.

**Fig. 2.** Mass Spectrometric Monitoring of Structural Changes of \(1\)-DEX YL Incubated with \(1\)-2-Chloropropionate.\(^\text{20}\)
site-directed mutagenesis experiments in which mutations of these residues caused significant decreases in activity.\textsuperscript{13}

II. DL-2-Haloacid Dehalogenase: A Dehalogenase with Unique Catalytic Properties

DL-2-Haloacid dehalogenase catalyzes the hydrolytic dehalogenation of D- and L-2-haloalkanoic acids to produce the corresponding L- and D-2-hydroxalkanoic acids respectively.\textsuperscript{6,23,24} This enzyme is unique in that it acts on the chiral carbons of both enantiomers. Racemases also act on the chiral carbons of both enantiomers of substrates, but are different from DL-2-haloacid dehalogenase in that racemases catalyze the stereochiral but not the chemical conversion of substrates. DL-2-Haloacid dehalogenase has a sequence similarity with D-2-haloacid dehalogenase, which specifically acts on D-2-haloalkanoic acids, but has no sequence similarity with L-2-haloacid dehalogenase.\textsuperscript{25}

The reaction mechanism of DL-2-haloacid dehalogenase of 	extit{Pseudomonas} sp. 113 (DL-DEX 113) was studied by site-directed mutagenesis and kinetic analysis.\textsuperscript{25} The results suggest that the enzyme has a single common active site for both the D- and the L-enantiomers. The mechanism was further studied by \textsuperscript{18}O-incorporation experiments.\textsuperscript{26} When a single turnover reaction of DL-DEX 113 was carried out using a large excess of the substrate, D- or L-2-chloropropionate, the major product was found to be \textsuperscript{18}O-labeled lactate. Mass spectrometric analysis of the enzyme after a multiple turnover reaction in H\textsubscript{2}\textsuperscript{18}O revealed that \textsuperscript{18}O was not incorporated into the enzyme during the reaction. These results indicate that the solvent water molecule attacks the α-carbon of the substrate directly to displace the halogen atom (Fig. 3). Thus enzymatic hydrolytic dehalogenation of 2-haloalkanoic acids proceeds through two different mechanisms: L-2-haloacid dehalogenase involves the formation of an ester intermediate, whereas DL-2-haloacid dehalogenase does not.

Haloalkane dehalogenase, 4-chlorobenzoyl-CoA dehalogenase, and fluorooacetate dehalogenase (described in section III below) employ a mechanism similar to that of L-2-haloacid dehalogenase, in which a nucleophilic aspartate residue attacks the carbon atom of the substrate to displace the halogen atom, leading to the formation of an ester intermediate.\textsuperscript{27–29} DL-2-Haloacid dehalogenase is unique in that a water molecule attacks the substrate directly to displace the halogen atom without the formation of an ester intermediate. Considering the sequence similarity between DL-2-haloacid dehalogenase and D-2-haloacid dehalogenase, the reaction catalyzed by D-2-haloacid dehalogenase probably proceeds through the same mechanism as that of DL-2-haloacid dehalogenase.\textsuperscript{25,26}

Analysis of solvent deuterium and chlorine kinetic isotope effects on the reactions catalyzed by DL-DEX 113 for D- and L-2-chloropropionate were also carried out.\textsuperscript{30} It was found that a step preceding dehalogenation is partly rate-limiting in the case of D-2-chloropropionate, and that the overall reaction rates are controlled by different steps in the catalysis of D- and L-2-chloropropionate.

The crystal structure of DL-2-haloacid dehalogenase of 	extit{Pseudomonas putida} strain PP3 (DehI), which shares 40% sequence identity with DL-DEX 113, was determined by Schmidberger et al.\textsuperscript{31} The crystallographic structure of the DehI homodimer was the first reported structure of DL-2-haloacid dehalogenase. The monomer was found to be highly α-helical and composed of a repeated motif. The repeats (residues 1–130 and 166–296) share only 16% sequence identity, yet can be superposed with an RMSD of 1.67 Å (116 out of 130 C\textalpha atoms). The repeats form a “pseudo-dimer” from a single protein chain, each repeat related by pseudo 2-fold symmetry. The structure as a whole may be considered to be a dimer of pseudo-dimers. Each monomer of DehI forms a single substrate-binding cavity within the pseudo-dimer interface. The site is solvent- and substrate-inaccessible except for a narrow channel, which is ~15 Å in length and extends from the substrate-binding site to the surface of the molecule.

We mutated each of charged and polar amino acid residues of DL-DEX that are conserved among DL-DEX 113, DL-2-haloacid dehalogenase of 	extit{Alcaligenes xylosoxidans} subsp. 	extit{xylosoxidans} ABIV, and D-2-haloacid dehalogenase of 	extit{Pseudomonas putida} AJ1 to probe their roles in catalysis.\textsuperscript{25} Thr65 (Thr62), Glu69 (Glu66), and Asp194 (Asp189) were found to be essential for catalytic activity (the equivalent residue of DehI is indicated in brackets). Of these residues, only Asp189 is present in the active-site cavity of DehI.\textsuperscript{31} The Asp189 site chain occurs at the edge of the cavity and is likely to be directly involved in the catalytic reaction mechanism. The positions of the D- and L-2-chloropropionate in the active site were predicted by \textit{in silico} docking. The α-carbon atom of the substrate, which is attacked by a water molecule, was placed approximately 4.7 Å from the Oδ1 atoms of both Asp189 and Asn114. A water
molecule was docked into the active site in the absence of the ligand, and the only binding site for the water was adjacent to Asp189 and Asn114, positioned appropriately for nucleophilic attack of the α-carbon atom of the docked substrate. These results suggest that Asp194 (Asp189) is responsible for water activation and is assisted by Asn117 (Asn114). More detailed insight into the mechanism of DL-2-haloacid dehalogenase would probably be obtained by crystallographic analysis of the enzyme complexed with the substrate. Crystallographic analysis of DL-2-haloacid dehalogenase of *Methylobacterium sp.* CPA1 is in progress.\(^{32}\)

### III. Fluoroacetate Dehalogenase: An Enzyme That Catalyzes the Cleavage of a Strong Carbon-Fluorine Bond

Fluoroacetate is a naturally occurring organohalogen compound produced by an actinomycete, *Streptomyces cattleya*, and some plants found in Australia, Africa, and Central America, such as *Dichapetalum cymosum*.\(^{33,34}\) It is highly toxic to mammals and is used as a rodenticide.\(^{35,36}\) It is metabolized to (2\(\rightarrow\)3)-fluorocitrate by citrate synthase and further converted to 4-hydroxy-trans-acoinitate, a competitive inhibitor of aconitase, and thereby blocks the citric acid cycle to exhibit toxicity.\(^{37}\)

Fluoroacetate dehalogenase, produced by bacteria such as *Delftia acidovorans* strain B (formerly *Moraxella* sp. strain B) and *Burkholderia* sp. FA1, catalyzes the hydrolytic defluorination of fluoroacetate to produce a fluoride ion and glycolate.\(^{38-40}\) It is unique in that it proceeds through irreversible dehalogenation with no preceding isotope-insensitive step being partly rate-determining.\(^{45}\)

Fluoroacetate dehalogenase from *Burkholderia* sp. FA1 (FAc-DEX FA1) shows high sequence similarity to FAc-DEX H1 (61% identity), and Asp105 and His272 of FAc-DEX H1 are conserved as Asp104 and His271 respectively in FAc-DEX FA1.\(^{46}\) Mutation of Asp104 and His271 of FAc-DEX FA1 resulted in a total loss of the enzyme activity, suggesting that the reaction mechanism is conserved between these enzymes.\(^{46}\)

To investigate the reaction mechanism of fluoroacetate dehalogenase further, the crystal structure of FAc-DEX FA1 was determined.\(^{46}\) This enzyme belongs to the α/β hydrolase superfamily and shares three-dimensional structural similarity with epoxide hydrolase and haloalkane dehalogenase.\(^{38}\) FAc-DEX FA1 exists as a homodimer, and each subunit consists of core and cap domains. The catalytic triad Asp104-His271-Asp128 was found in the core domain at the domain interface. The active site was composed of Phe34, Asp104, Arg105, Arg108, Asp128, His271, and Phe272 of the core domain and Tyr147, His149, Trp150, and Tyr212 of the cap domain. The main-chain NH groups of Arg105 and Phe34 in the core domain form hydrogen bonds with one of the carboxylate oxygens of Asp104, and are supposed to act as an oxygen ion hole stabilizing the oxanyion formed during the hydrolysis of the ester intermediate. In the vicinity of the N\(^{\text{1}}\) atom of Trp150 and the N\(^{\text{2}}\) atom of His149, an electron density peak corresponding to a chloride ion was found, suggesting that these are the halide ion acceptors.

The active-site channel is located at the domain interface. It is characterized by a number of charged or hydrophilic residues accommodating a hydrophilic substrate, fluoroacetate. This is in contrast with the

![Fig. 4. Reaction Mechanism of FAc-DEX H1.](image-url)
active-site channel of haloalkane dehalogenases from Sphingobium japonicum UT26, Xanthobacter autotrophicus GJ10, and Rhodococcus sp., which is mainly surrounded by hydrophobic residues. The entrance to the active-site channel of FAC-DEX FA1 is open, and a substrate can freely gain access to its binding site through the channel. Upon binding of the substrate, the conformation around the substrate entrance may change yielding a closed form preventing the free access of water molecules to the active site. The most probable candidate for the door to the entrance is Trp179, on the extended loop between α-helices 8 and 9, because this loop is mobile and is directed toward the solvent side.

Each of the active-site residues of FAC-DEX FA1 was mutated to investigate the roles of these residues in catalysis. The following mutant enzymes showed no activity toward fluoroacetate and chloroacetate: F34A, D104A, R105A, R108A, D128A, Y147A, H149A, W179A, Y212A, H271A, and F272A. In contrast, replacement of Trp150 with Ala did not significantly affect the activity toward chloroacetate, whereas that toward fluoroacetate was completely lost due to this replacement of Trp150 with Ala did not significantly affect the activity toward chloroacetate, whereas that toward fluoroacetate was completely lost due to this mutation. Trp150 was replaced also with Phe, Gln, Lys, Tyr, and Arg. None of the resulting mutants showed defluorination activity, whereas they had various degrees of dechlorination activity. The loss of defluorination activity due to the replacement of Trp150 with Phe was found not to be due to the loss of fluoroacetate binding to the active site. Hydrogen-bonding interaction between Trp150 and the fluorene atom of fluoroacetate is probably an absolute requirement for reduction of the activation energy for the cleavage of the rigid carbon-fluorine bond.

The reaction mechanism of fluoroacetate dehalogenase was further studied using quantum mechanical/molecular mechanical (QM/MM) calculations for a whole-enzyme model. To build an initial structure for the QM/MM calculations, the binding mode of fluoroacetate in the active site of fluoroacetate dehalogenase was estimated from the crystal structure of haloalkane dehalogenase complexed with 1,2-dichloroethane. The chlorine atom hydrogen-bonded to the halide ion acceptors (Trp125 and Trp175) of haloalkane dehalogenase was replaced by a fluoride atom, and the other chlorine atom was replaced by a carboxyl oxygen atom. Figure 5A shows the QM/MM-optimized structure of fluoroacetate dehalogenase complexed with fluoroacetate at pH 9.5, the optimum pH for the enzyme. A hydrogen-bonding network between the substrate and the surrounding amino acid residues (Arg105, Arg108, His149, Trp150, and Tyr212) was assumed to play an essential role in substrate binding. In addition to Trp150, which corresponds to Trp175 in haloalkane dehalogenase, His149 and Tyr212 are hydrogen-bonded to the fluorine atom of the substrate.

The QM/MM-optimized structure of the transition state for the formation of the ester intermediate is shown in Fig. 5B. The activation energy for the S2 reaction, in which Asp104 attacks the α-carbon of fluoroacetate displacing a fluoride ion, was calculated to be 2.7 kcal mol$^{-1}$ (Fig. 6). The hydrogen bonds between the fluorine atom and His149, Trp150, and Tyr212 are shortened in the transition state structure (Fig. 5B) as compared with those in the enzyme-substrate complex (Fig. 5A). The hydrogen-bonding interactions would be the main factor that reduces the activation barrier for the cleavage of the strong C–F bond. Among the three residues, Tyr212, which forms the shortest hydrogen bond with the fluorine atom, is probably the most important residue reducing the barrier.

The QM/MM optimized structure of the ester intermediate was also obtained (Fig. 5C). The results showed that the formation of the ester intermediate from the enzyme-substrate complex is highly exothermic: the relative energy of the ester intermediate is −19.3 kcal mol$^{-1}$ (Fig. 6). A water molecule, which is involved in the hydrolysis of the ester intermediate, occurs in close contact with the ε-nitrogen of His271 and the γ-carbon of Asp104.

QM/MM calculations indicated that hydrolysis of the ester intermediate is facilitated by simultaneous proton transfer from the water molecule to His271. As a result of nucleophilic attack of the water molecule on the carbonyl carbon atom of the ester intermediate, a tetrahedral intermediate is produced, in which one δ-oxygen atom of...
Asp104 is negatively charged (Fig. 6). The negative charge is stabilized by hydrogen-bonding interaction with the oxyanion hole composed of the main-chain NH groups from Phe34 and Arg105. Asp128, a residue in the catalytic triad, stabilizes the tetrahedral intermediate through electrostatic interaction with the protonated His271. Subsequent proton transfer from His271 to the α-oxygen of Asp104, the one that is not hydrogen-bonded to the oxyanion hole, occurs simultaneously with heterolytic cleavage of the bond between the α-oxygen and the γ-carbon of Asp104 (Fig. 6).

The energy diagram of the overall reaction suggests that the nucleophilic attack of the water molecule on the carbonyl carbon of the ester intermediate is the rate-determining step in the fluoroacetate dehalogenase reaction (Fig. 6).

IV. 2-Haloacrylate Reductase: Catalyst for Asymmetric Reduction of an Unsaturated Organohalogen Compound

l-2-Haloacid dehalogenase, dl-2-haloacid dehalogenase, and fluoroacetate dehalogenase, described above, catalyze the removal of a halogen atom from an sp³-hybridized carbon atom of saturated organohalogen compounds with a halogen atom bound to an sp²-hybridized carbon atom. Other dehalogenases, such as corrinoid/iron-sulfur cluster-containing reductive dehalogenases, are known to act on unsaturated organohalogen compounds, but information on the mechanism of enzymatic degradation of such compounds is limited. To get insight into the enzymatic dehalogenation of unsaturated organohalogen compounds, we searched for microorganisms that grow on 2-chloroacrylate (2-CAA) as sole source of carbon and energy. 2-CAA is a bacterial metabolite of 2-chloroallylalcohol, an intermediate or by-product in industrial herbicide synthesis. Rats excrete 2-CAA when they ingest herbicides containing a haloallyl substituent. Red algae produce various halogenated haloacrylate. By screening soil samples, we isolated three 2-CAA-utilizable bacterial strains: Burkholderia sp. WS (formerly Pseudomonas sp. WS), Pseudomonas sp. YL, and Pseudomonas sp. WL.

When 2-CAA was incubated with the cell-free extract of 2-CAA-grown Burkholderia sp. WS in the presence of NADPH, 2-chloropropionate was produced from 2-CAA, suggesting that an NADPH-dependent reductase acting on 2-CAA occurs in the 2-CAA-grown cells. NADH did not replace NADPH. 2-Chloropropionate was not produced when cell-free extract from the lactate-grown cells was used. Thus the enzyme catalyzing the reduction of 2-CAA is inducibly synthesized when cells are grown on 2-CAA.

To identify the 2-CAA-inducible proteins involved in the reduction of 2-CAA, proteins from 2-CAA-grown Burkholderia sp. WS were compared by two-dimensional polyacrylamide gel electrophoresis (PAGE) (Fig. 7). Two major 2-CAA-inducible proteins, named CAA43 and CAA67, were identified. Their genes were found to be located next to each other on the genomic DNA (GenBank accession no. AB196962). The CAA43 gene was located downstream of the CAA67 gene. The CAA43 gene contained an open reading frame of 1,002 nucleotides coding for 333 amino acid residues (Mr 35,788), and the CAA67 gene contained an open reading frame of 1,644 nucleotides coding for 547 amino acid residues (Mr 58,684). CAA43 has a nucleotide-binding motif, AXGGXXG, in the region from 153 to 159 and 38.2% amino acid
sequence identity with NADPH-dependent quinone oxidoreductase of *Escherichia coli* (NCBI accession no. P28304), which catalyzes the NADPH-dependent reduction of various quinones. Polar and positively charged residues of the quinone oxidoreductase surrounding the 2'-phosphate group of NADPH are conserved in CAA43, suggesting that it is an NADPH-dependent enzyme.

CAA67 has a nucleotide-binding motif, VXGXGXXGXXA, in the region from 11 to 22, and weak but significant sequence similarity with FAD enzymes such as l-aspartate oxidase of *E. coli* (NCBI accession no. 1CHU_A) (17.6% identity) and fumarate reductase (subunit A) of *Wolinella succinogenes* (NCBI accession no. P17412) (17.2% identity). Thus FAD probably binds to CAA67.

Amino acid sequence analyses have suggested that CAA43 is involved in the reduction of 2-CAA, because NADPH was required for the reduction of 2-CAA by an extract of the 2-CAA-grown cells, as described above. We constructed a recombinant *E. coli* strain that produces CAA43 and purified the protein. The purified CAA43 was found to catalyze the conversion of 2-CAA into 2-chloropropionate in an NADPH-dependent manner. The configuration of the 2-chloropropionate produced was (S), indicating that CAA43 catalyzes the asymmetric reduction of 2-CAA (Fig. 7). 2-Bromoacrylate also served as substrate, whereas 2-haloacrylate analogs such as acrylate and methacrylate did not. The inducible nature and the substrate specificity suggest that 2-haloacrylate is a physiological substrate of CAA43. Hence we named this novel enzyme 2-haloacrylate reductase. In *Burkholderia* sp. WS grown on 2-CAA, l-2-chloropropionate produced by 2-haloacrylate reductase is probably further metabolized to d-lactate by l-2-haloacid dehalogenase (Fig. 7).

The function of CAA67 of *Burkholderia* sp. WS is yet to be determined because of difficulty in its purification from this strain and in the construction of an expression system with recombinant *E. coli*. Hence we purified and characterized a homolog of CAA67 from another 2-CAA-utilizable bacterium, *Pseudomonas* sp. YL, as described in section V below.

2-Haloacrylate reductase is useful in the production of l-2-chloropropionate, a building block in the synthesis of aryloxyphenoxypropionate herbicides. It catalyzes the asymmetric reduction of a carbon-carbon double bond of 2-CAA, producing l-2-chloropropionate. We constructed a system for the production of l-2-chloropropionate with recombinant *E. coli* cells producing 2-haloacrylate reductase from *Burkholderia* sp. WS for the reduction of 2-CAA and glucose dehydrogenase from *Bacillus subtilis* for the regeneration of NADPH. The system provided 37.4 g/L (346 mM) of l-2-chloropropionate from 385 mM 2-CAA at more than 99.9% e.e. The conversion yield was 90%, much higher than that obtained by the conventional method employing optical resolution of a racemic mixture of 2-chloropropionate (<50%).

V. 2-Haloacrylate Hydratase: A Unique Flavoenzyme That Catalyzes a Non-Redox Reaction

As described in the preceding section, *Pseudomonas* sp. YL was isolated from soil as a 2-CAA-utilizable bacterium. It grows on 2-CAA and lactate as sole source of carbon and energy. By comparison between proteins from 2-CAA- and lactate-grown cells by two-dimensional PAGE, a major 2-CAA-inducible protein, named CAA67_YL, was identified (Fig. 8). Its inducible nature suggests its involvement in the metabolism of 2-CAA.

The caa67_YL gene coded for a protein of 547 amino acid residues with a predicted molecular weight of 59,301. A homology search revealed that CAA67_YL shares 84.6% sequence identity with CAA67 from another 2-CAA-utilizable bacterium, *Burkholderia* sp. WS (referred to as CAA67_WS in this section to avoid confusion). CAA67_YL and CAA67_WS have a nucleotide-binding motif (VXGXGXXGXXA) in the region from position 11 to position 22, and showed weak but significant sequence similarity to several flavoproteins, including l-aspartate oxidase (e.g., NCBI accession no. YP,783305), the succinate dehydrogenase flavoprotein subunit (e.g., NCBI accession no.
AAB85977), and thiol:fumarate reductase subunit A (e.g., NCBI accession no. CA04398). Sequence similarity to various flavoproteins and the occurrence of a nucleotide-binding motif suggest that CAA67YL and CAA67WS require FAD to function.

CAA67YL was purified from recombinant E. coli cells. The purified protein was found to catalyze the conversion of 2-CAA into pyruvate in the presence of a reduced form of FAD (Fig. 8). The purified protein contained an oxidized form of FAD, the molar ratio of which to the protein was 0.25. The ratio increased to 0.91 after incubation with externally added FAD. However, the protein did not show activity toward 2-CAA without reduction of FAD. The apoenzyme showed no activity, and FMNH2 did not replace FADH2. Thus the catalytic action of CAA67YL depends on a reduced form of FAD.

CAA67YL also catalyzes the conversion of 2-bromoacrylate into pyruvate, whereas the following compounds did not serve as substrates: 2-fluorocrotyl, methacrylate, acrylate, 3-chloroacrylonitrile, 2-chloro-1-propene, fumarate, phosphoenolpyruvate, D- and L-2-chloropropionate, and D- and L-lactate.

An 18O incorporation experiment using H218O indicated that an oxygen atom of a water molecule is introduced into the substrate. 2-Chloro-2-hydroxypropionate, a chemically unstable geminal halohydrin, is probably produced by the hydration of 2-CAA, and probably spontaneously decomposes into pyruvate upon the removal of HCl (Fig. 8). Since CAA67YL acts specifically on 2-CAA and 2-bromoacrylate, it was named 2-haloacrylate hydratase.

For the catalytic activity of 2-haloacrylate hydratase, FAD in the reaction mixture must be reduced. A high concentration of NADH, NADPH, or sodium dithionite can be used for the reduction of FAD. We found that NADH used for the reduction of FAD does not decrease stoichiometrically for the hydratase reaction. FADH2, once produced by a reductant such as NADH, is probably regenerated in the catalytic cycle and not consumed during the reaction.

The requirement of FADH2 for hydration of the substrate is notable because most flavoenzymes catalyze net redox reactions, such as oxidation, reduction, oxygenation, and electron transfer. CAA67YL requires FADH2 for its catalytic action, but the reaction does not involve a net change in the redox state of the coenzyme or the substrate. A possible explanation for the FADH2 requirement is that it functions as a general acid-base catalyst. Type-2 isopentenyl diphosphate isomerase was recently found to catalyze the reaction in an FMNH2-dependent manner; the coenzyme plays a general acid-base role. FADH2 in the 2-haloacrylate hydratase reaction is possibly involved in protonation of the C-3 position of 2-CAA or in activation of a water molecule that attacks the C-2 position of 2-CAA. Another possible mechanism is that FADH2 acts as a radical catalyst. One electron transfer from FADH2 to 2-CAA and protonation at the C-3 position would produce a 2-chloropropionate radical, which might subsequently be hydroxylated to produce 2-chloro-2-hydroxypropionate. Such a free-radical mechanism has been reported for other flavoenzymes, such as chorismate synthase. Further studies are in progress to elucidate the mechanism of this very unusual flavoenzyme.

CAA67WS of Burkholderia sp. WS shows high sequence similarity to CAA67YL, and probably catalyzes the same reaction as CAA67YL. As described in section IV above, Burkholderia sp. WS produces 2-haloacrylate reductase (CAA43) in addition to CAA67WS as a 2-CAA-inducible protein. 2-Haloacrylate reductase catalyzes the reduction of 2-CAA, producing D-2-chloropropionate, which is assumed to be dehalogenated, producing D-lactate by L-2-haloacid dehalogenase. Thus the bacterium has two metabolic pathways for the dissimilation of 2-CAA. Genetic analysis is required to reveal which pathway plays the principal role in the metabolism of 2-CAA in Burkholderia sp. WS. In contrast to Burkholderia sp. WS, Pseudomonas sp. YL apparently does not have 2-haloacrylate reductase, as judged by the results of two-dimensional PAGE analysis of the proteins and genomic DNA analysis. Thus 2-haloacrylate hydratase probably plays a major role in the metabolism of 2-CAA in Pseudomonas sp. YL.
2-Haloacrylate hydratase resembles cis- and trans-3-chloroacrylic acid dehalogenases\(^1,2\) in that it catalyzes the dehalogenation of the substrate by the addition of a water molecule to the carbon-carbon double bond, but it is significantly different from 3-chloroacrylic acid dehalogenases not only in its substrate specificity and primary structure but also in its cofactor requirement. No cofactor is required for 3-chloroacrylic acid dehalogenases, whereas FADH\(_2\) is an absolute requirement for the 2-haloacrylate hydratase reaction. Thus 2-haloacrylate hydratase is a new class of dehalogenases that degrades unsaturated aliphatic organohalogen compounds.

VI. Conclusions and Perspectives

The reaction mechanisms of hydrolytic dehalogenases (\(L\)-2-haloacid dehalogenase, \(D,L\)-2-haloacid dehalogenase, and fluoroacetate dehalogenase) have been studied by mass spectrometry, crystallography, site-directed mutagenesis, computational analysis, and other methods. These dehalogenases employ two distinct mechanisms. In the reactions of \(L\)-2-haloacid dehalogenase and fluoroacetate dehalogenase, the carboxylate group of Asp nucleophilically attacks the \(C\)\(_1\) carbon atom of the substrate, displacing the halogen atom and producing an ester intermediate in which the Asp residue is covalently bound to the substrate. The ester intermediate is subsequently hydrolyzed to produce 2-hydroxyxalkanoic acid and regenerate the catalytic Asp residue. Computational analyses have been performed based on the three-dimensional structures of these enzymes, and the roles of the amino acid residues involved in catalysis, such as those facilitating cleavage of the carbon-halogen bond, have been elucidated. In the reaction of \(D,L\)-2-haloacid dehalogenase, a water molecule activated by the enzyme directly attacks the substrate to displace the halogen atom. The mechanism is distinct from that of \(L\)-2-haloacid dehalogenase and fluoroacetate dehalogenase in that it does not involve the formation of a covalent intermediate. The amino acid residue responsible for activation of the water molecule that attacks the substrate was predicted by crystallographic analysis of \(D,L\)-2-haloacid dehalogenase. These mechanistic studies of dehalogenases should provide insight into rational protein engineering to modify the stability, activity, and substrate specificity of the enzymes for their use in the chemical industry and in environmental technology.

By studying the metabolism of 2-CAA in soil bacteria, two novel enzymes acting on unsaturated organohalogen compounds were discovered. 2-Haloacrylate reductase catalyzes the reduction of 2-haloacrylate in an NADPH-dependent manner. 2-Haloacrylate hydratase catalyzes the addition of a water molecule to the carbon-carbon double bond of 2-haloacrylate in an FADH\(_2\)-dependent manner. In the pathway in which 2-haloacrylate reductase is involved, 2-CAA is first converted to \(L\)-2-chloropropionate, which is subsequently dehalogenated by \(L\)-2-haloacid dehalogenase. In the pathway in which 2-haloacrylate hydratase is involved, 2-CAA is hydrated to produce 2-chloro-2-hydroxypropionic acid, a chemically unstable geminal halohydrin, which is spontaneously degraded to produce pyruvate.

The occurrence of 2-haloacrylate hydratase is remarkable because it catalyzes a non-redox reaction in an FADH\(_2\)-dependent manner. Mechanistic analysis of this enzyme should reveal a new catalytic function of flavin coenzymes.

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