Structure-Function of Haloacid Dehalogenase: Tertiary Structure Elucidated by X-Ray Crystallographic Study

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The structure of the dimeric L-2-haloacid dehalogenase from *Pseudomonas* sp. YL was determined at 2.5 Å resolution by X-ray crystallographic studies. The structure of the enzyme subunit consists of two domains: a core-domain and a sub-domain. The core-domain has an α/β structure where a six-stranded parallel β-sheet is flanked with five α-helices. The sub-domain has four α-helices, and is inserted between the first β-strand and α-helix of the core-domain. Several residues which were suggested to be involved in the catalysis by mutagenesis studies, including the nucleophilic residue Asp10, are found to be clustered at the bottom of the cleft encompassed by the two domains. This implies that the site around Asp10 in the cleft may be the reasonable active site. The hydrophilic environment around the active site is convenient for the enzyme to bind haloacids, substrates possessing hydrophilic carboxyl groups.

Keywords: Dehalogenation/ Hydrolase/ X-ray structure / Catalytic mechanism

Halogenated organic compounds are widely used as materials for various industrial products such as insecticides and solvents, and cause serious environmental problems owing to their persistence and/or toxicity. Several kinds of bacteria have been found to be able to take up some of such compounds as sole carbon sources and to degrade them using enzymes called dehalogenases. 2-Haloacid dehalogenases (E.C. 3.8.1.2) isolated from several bacteria catalyze the hydrolytic degradation of 2-haloacids to produce the corresponding 2-hydroxy acids as follows:

$$RCHXCOOH + OH^- \rightarrow RCHOHCOOH + X^-$$

(R = H, alkyl ; X = halogen)

The enzymes are classified into four types on the basis of the C_2 configurations of their substrates and reaction products.

L-2-Haloacid dehalogenase from *Pseudomonas* sp. YL is a dimeric enzyme composed of two identical subunits each of which comprises 232 amino acid residues corresponding to the molecular weight of 26,179. It acts on L-2-haloalkanoic acids to yield D-2-hydroxyalkanoic acids. The catalytic mechanism of the enzyme has been studied by comprehensive site-directed mutagenesis (1) and ^{18}O incorporation (2). From the results of these studies, it has been proposed that Asp10 is the active site nucleophile attacking the C_2 atom of substrates.

In order to elucidate the catalytic and stereoselective reaction mechanisms of 2-haloacid dehalogenases, we have undertaken crystallographic studies of the enzymes. Recently, we succeeded in the structure determination of dimeric L-2-haloacid dehalogenase from *Pseudomonas* sp. YL at 2.5 Å resolution by double isomorphous replacement in X-ray crystallog-

MOLECULAR BIOLOGY AND INFORMATION —Biopolymer Structure—

Scope of research

Our research aims are to elucidate structure-function relationships of biological macromolecules, mainly proteins, by using physicochemical methods such as spectroscopic and X-ray diffraction methods. The following 1attempts have been mainly made in our laboratory for that purpose. (1) Peptide secondary or supersecondary structures in aqueous or hydrophobic environments are studied to get a principle of protein architecture, employing various spectroscopic methods. (2) X-ray diffraction studies on protein structures in crystal and in solution are carried out by crystallographic and/or small-angle X-ray scattering techniques to elucidate structure-function relationships of proteins.

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raphy.

Crystals of the enzyme were grown at 4℃ by vapor diffusion, supplemented by repetitive seeding, against a 50 mM potassium dihydrogenphosphate solution (pH 4.5) containing 15% (w/v) polyethylene glycol 8000 and 1% (v/v) *n*-propanol. A typical crystal size was about 0.6 mm × 0.5 mm × 0.1 mm. The crystals belong to space group *C*2 with unit cell dimensions of a = 92.21 Å, b = 62.78 Å, c = 50.84 Å and β = 122.4°, and contain two dehalogenase dimers in the unit cell. Assuming one subunit per asymmetric unit, the specific volume Vm results in 2.37 Å^3/Da, which corresponds to a solvent content of 48.2%.

Uranyl and aurate derivative crystals were prepared at 25℃ by soaking the native crystals in the solutions of 1 mM $UO_2(NO_3)_2$ for 6 days and 3 mM $K[Au(CN)_2]$ for 4.5 days, respectively. They were isomorphous with native crystals. Diffraction data for the native and two derivative crystals were collected up to 2.5 Å resolution at 20℃ on a Rigaku R-AXIS IIC imaging plate detector system using a graphite-monochromated Cu$K\alpha$ radiation produced by a fine-focused rotating anode X-ray generator Rigaku RU-300 operated at 40 kV-100 mA.

Difference Patterson maps of the derivatives, calculated at 5.0 Å resolution, showed an interpretable single major heavy-atom site for each derivative. The minor heavy-atom sites were located by difference Fourier maps. The refinement of heavy-atom parameters was carried out at 2.5 Å resolution using program package PHASES, and finally gave three U and two Au sites. In the final cycle of refinement, the anomalous scattering effects from the metals were considered for phase calculation. The mean figure of merit was 0.70 for the phase angles.

A 2.5 Å resolution electron density map calculated with multiple isomorphous replacement phases showed an obvious connectivity of electron density for the polypeptide chain. It was used for building a model of the enzyme structure on a graphics workstation using the program TURBO-FRODO. The N-terminal three residues and C-terminal ten residues were both invisible in the density map, and then excluded from the model. Consequently, the initial model for the subunit molecule comprised amino acid residues of 4 to 222.

The model consisting of 2,157 atoms in the protein and 19 water oxygens was refined with the simulated annealing protocol of the program X-PLOR. The crystallographic R-factor for the model was converged to 19.5% for 7,848 reflections ($F \geqq 2\sigma(F)$) within the resolution range of 8.0–2.5 Å. The r.m.s. deviations from ideal bond lengths and bond angles are 0.012 Å and 1.8°, respectively.

The subunit consists of two domains: a core-domain and a sub-domain. The core-domain has an α/β type structure where a central six-stranded parallel β-sheet is flanked by three α-helices on one side and two on the other. The domain is formed by amino acid residues 4 to 19 and 74 to 222. The sub-domain consists of four α-helices packed like a four-helix-bundle. The sub-domain is formed by amino acid residues 20 to 73, and is inserted between the first β-strand and the first α-helix in the core-domain. There is a cleft formed between the two domains which are linked together by two-stranded antiparallel β-sheet.

The dimeric molecule has a compact ellipsoidal shape with dimensions of 76 Å× 40 Å× 40 Å (Figure 1). The structure of the dimer is stabilized mainly by interactions among a cluster of hydrophobic residues from both subunits. Besides, polar residues, lining up across the molecular two-fold axis, form a linear hydrogen-bond network in the vicinity of the hydrophobic cluster. Almost all of these residues involved in the intersubunit interaction are highly conserved among L-2-haloacid dehalogenases.

The site-directed mutagenesis study has shown that mutation of the residue Asp10, Thr14, Arg41, Ser118, Lys151, Tyr157, Ser175, Asn177 or Asp180 in the subunit affects the activity of the enzyme. All the residues except for Arg41 belong to the core-domain, being located on the wall of the cleft between the two domains. The site occupied by these residues is positioned at the topological switch-point in the order of strands on the central β-sheet, and has enough room to accomodate a substrate. This suggests that the site can be the reasonable active site of the enzyme subunit. The active site cleft is widely open at its entrance, which is consistent with the observation that the enzyme can act on 2-haloacids including long alkyl groups. The environment around the catalytic site seems to be so hydrophilic that the enzyme can bind haloacids possessing the hydrophilic carboxyl group.

This project has been carried out in collaboration with the division of Molecular Biofunction - Molecular Microbial Science-, Institute for Chemical Research.

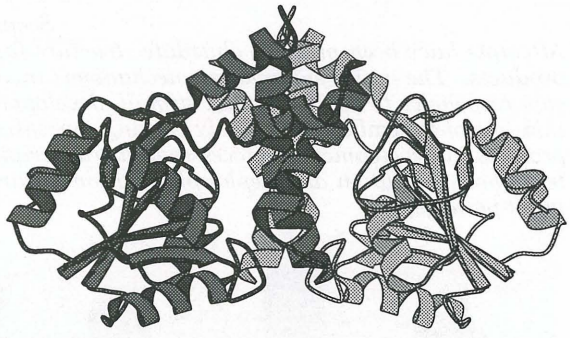

Figure 1. A dimeric molecule of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL.

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