Rapid Communication *J. Biochem.* **124**, 20-22 (1998)

X-Ray Structure of a Reaction Intermediate of L-2-Haloacid Dehalogenase with L-2-Chloropropionamide[1]

Yong-Fu Li, Yasuo Hata,[2] Tomomi Fujii, Tatsuo Kurihara, and Nobuyoshi Esaki[2]

Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto 611-0011

Received for publication, April 8, 1998

The crystal structure of a complex prepared by soaking a single crystal of the S175A mutant of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL in a solution containing a poor substrate, L-2-chloropropionamide, has been determined by X-ray analysis at 2.15 Å resolution with a crystallographic *R* factor of 19.8%. The present analysis has revealed the structure of the reaction intermediate trapped in the crystal. In the intermediate, the substrate moiety lacking chlorine is covalently bound to the carboxyl group of D10, and adopts the pro-D-configuration at the C_2 atom. The amide group of the substrate is hydrogen-bonded with the hydroxy group of S118. The methyl group bound to the C_2 atom exists in a hydrophobic pocket which is important for recognition of the alkyl group of the substrate. The guanidino group of R41 has reasonable orientation for halogen-abstraction.

Key words: L-2-chloropropionamide, crystal structure, haloacid dehalogenase, reaction intermediate.

L-2-Haloacid dehalogenase [EC 3.8.1.2] from *Pseudomonas* sp. YL (L-DEX YL) catalyzes the hydrolytic dehalogenation of an L-2-haloacid to produce the corresponding D-2-hydroxyacid. We have identified essential residues for this enzyme catalysis by site-directed mutagenesis and mass spectrometry. In the enzyme, the carboxyl group of D10 acts as the nucleophile that attacks the C_2 atom of the substrate to form an ester intermediate, which is subsequently hydrolyzed by a water molecule (*1*). In addition to D10, residues T14, R41, S118, K151, Y157, S175, N177, and D180 play essential roles in the catalysis (*2*). We have proposed that S118 is the binding site for the carboxylate group of the substrate (*2*). The tertiary structure of the enzyme we determined has revealed that all of these residues constitute the active center around nucleophile D10 (*3*).

In order to clarify the reaction mechanism of L-DEX YL at the atomic level, we have crystallized several mutants of the enzyme which are effective for trapping complexes formed at each step of the reaction process. At present, crystals of the S175A mutant, which are isomorphous with those of the wild type and thus useful for this purpose, have been obtained under the same conditions as those for the wild type. Mass spectrometric studies have suggested that S175A has the ability of ester intermediate formation but lacks the ability to hydrolyze the ester. Its crystal structure has been found to be virtually identical to that of the wild type, with an average C^{α} r.m.s. deviation of 0.20 Å (Li, Y.-F. *et al.*, unpublished result). We soaked the crystals in various kinds of L-2-chloropropionate (CPA) solutions in order to prepare crystals of a complex, but the attempts were unsuccessful. In each of the CPA solutions, the crystals cracked immediately and were dissolved even after cross-linking with glutaraldehyde. CPA is the best substrate for the enzyme, while L-2-chloropropionamide (CPN) is an extremely poor substrate, the k_{cat}/K_m value being 4.9×10^{-5} $s^{-1}\cdot mM^{-1}$, which is about 2,600,000 times lower than that for CPA; the k_{cat} and K_m values for CPN were 0.0088 s^{-1} and 180 mM, respectively, under the standard assay conditions, while they were 47 s^{-1} and 0.37 mM for CPA, respectively (*4*). As a consequence, we have succeeded in trapping a reaction intermediate by means of CPN and the S175A mutant as follows.

A S175A crystal of $0.30\times0.28\times0.20$ mm was cross-linked with 5% glutaraldehyde for 5 h, and then soaked in 10 mM DL-2-chloropropionamide for 10 days at 4°C. The crystallographic data were: space group *C*2, cell dimensions of $a=91.00$ Å, $b=62.78$ Å, $c=52.01$ Å, and $\beta=122.5°$, and one subunit per asymmetric unit. X-Ray diffraction data of the soaked crystal were collected at 20°C with a Rigaku R-AXIS IIC, using Cu$K\alpha$ radiation produced with a double focusing mirror system and a Rigaku RU-300 rotating anode X-ray generator operated at 40 kV and 100 mA. The crystal-to-detector distance was 80.0 mm. The crystal was mounted with *b*-axis parallel to the rotation axis of the goniometer. One frame of the oscillation image

[1] This work was supported in part by Grants-in-Aid for Scientific Research, 09660088 (to Y.H.), 08760080 (to T.K.), 08680683 (to N.E.), and 09460049 (to N.E.), from the Ministry of Education, Science, Sports and Culture of Japan, and a Research Grant from the Japan Society for the Promotion of Science (Research for the Future) to N.E. Y.H. is a member of the TARA (Tsukuba Advanced Research Alliance) Project of Tsukuba University, Japan.

[2] To whom correspondence should be addressed. Tel: +81-774-38-3251, Fax: +81-774-38-3014, E-mail: hata@scl.kyoto-u.ac.jp; or Tel: +81-774-38-3240, Fax: +81-774-38-3248, E-mail: esaki@scl.kyoto-u.ac.jp

Abbreviations: CPA, L-2-chloropropionate; CPN, L-2-chloropropionamide; rmsd, root mean square deviation; L-DEX, L-2-haloacid dehalogenase; L-DEX YL, L-2-haloacid dehalogenase from *Pseudomonas* sp. YL.

was recorded on an imaging plate with an oscillation range of 2.6° and an exposure time of 10 min. A total of 70 frames covered a rotation range of over 180°. Data processing was performed up to 1.8 Å resolution using the R-AXIS IIC software package to obtain 16,631 unique reflections from 36,643 observations with an R_{merge} value of 5.7% and a completeness of 91.1%.

The previously reported subunit structure of the wild-type L-DEX YL at 2.5 Å resolution (*3*) was used as the starting model to locate the present protein subunit at an appropriate position with the rigid-body refinement program of X-PLOR (*5*). Further refinement of the model was carried out with the simulated annealing protocol of X-PLOR, followed by positional and individual temperature factor refinements. The regions exhibiting conformational changes were checked on the basis of the $2F_o - F_c$ and $F_o - F_c$ difference electron density maps, and rebuilt manually with the TURBO-FRODO program (*6*). The omit map clearly showed that the substrate moiety is connected with the carboxyl oxygen of D10 (Fig. 1). In the last stage of the refinement, a model of the dechlorinated substrate moiety was built into the corresponding density. The nitrogen atom of the substrate-derived amide was assigned to the peak with lower electron density than that for the oxygen atom. The model of the substrate moiety was included for further refinement with water molecules picked up on the basis of the $2F_o - F_c$ and $F_o - F_c$ maps. The refinement for 11,630 reflections at 8.0-2.15 Å resolution was converged to crystallographic R and R_{free} factors of 19.8 and 25.6%, respectively. The r.m.s. deviations of bonds, angles, dihedral angles and improper angles from their ideal values were 0.006 Å, 1.24°, 24.43°, and 0.6°, respectively.

The structure of the CPN intermediate consists of a polypeptide chain of amino acid residues 3-222, a dechlorinated substrate moiety, and 38 water molecules; the N-terminal 2 residues and C-terminal 10 residues of the polypeptide chain are missing probably because of disorder, as in the case of the wild type. The mean B-factors for the main chain, side chain, entire molecule, and solvents are 33.8, 36.6, 35.2, and 48.0 Å^2, respectively. The overall structure of the polypeptide chain in the intermediate is similar to those of the wild type and the S175A mutant. Significant structural differences between the intermediate and the others lie in the regions of D10-S20, Y91-D102, and L117-R135, which are spatially close to one another. These conformational changes are probably caused by the formation of the ester intermediate. The C_2 atom of the substrate moiety is dechlorinated, covalently bonded with the $O^{\delta 2}$ atom of nuleophile D10, and adopts the pro-D-configuration. A mixture of D- and L-2-chloropropionamide was used as the reagent for preparation of the CPN intermediate. However, the enzyme is expected to selectively react with L-2-chloropropionamide through the same mechanism as that for L-2-haloacid with inversion of the C_2 configuration. The amide group of the substrate moiety is mainly stabilized through hydrogen-bonding with the hydroxy group of S118 (Fig. 2). In the native enzyme, the site of the amido group is occupied by a water molecule hydrogen-bonded with S118. The situation of the amido group in the CPN intermediate is very similar to that of the water molecule in the substrate-free enzyme. In the formation of a Michaelis complex, the water molecule is probably replaced by a substrate entering the active center. It has also been found on structure analysis of L-DEX from *Xanthobator autotropicus* GJ10 that the carboxyl group of a substrate analog, formate, is hydrogen-bonded with the side-chain hydroxyl of the conserved serine residue corresponding to S118 of L-DEX YL (*7*). Therefore S118 is the main residue for recognition of the carboxyl or amido group of a substrate. The methyl group derived from CPN is situated in the hydrophobic pocket comprising the side chains of Y12, L45, F60, and W179, along with those of Q44, K151, and N177. The hydrophobic pocket seems to play an important role in the recognition of the alkyl group of a substrate with the L-configuration.

In the CPN intermediate, the water molecules located in the active center of the native enzyme are replaced by the CPN moiety, while a new water molecule, Wat500, is located between the carboxyl group of D10 and the side chain of A175. This water molecule is hydrogen-bonded with the two O^{δ} atoms of D10, the main chain and side-chain nitrogen atoms of N177, and $O^{\delta 1}$ of D180. We have found in mass spectrometric studies that N177 and D180 as well as S175 probably participate in the second step of enzyme catalysis: the activation of a water molecule for hydrolysis of the ester intermediate (Li, Y.-F. *et al.*, unpublished result). If this is the case, Wat500 is probably the water molecule that is utilized for hydrolysis.

Another new density peak, which is observed for neither the wild type nor the S175A mutant, has been found at a position close to the guanidino nitrogen of R41 (3.29 Å), and to the side-chain amido nitrogen of N119 (3.56 Å). From the surrounding conditions, it has been suggested that the density peak is probably attributable to the halogen anion

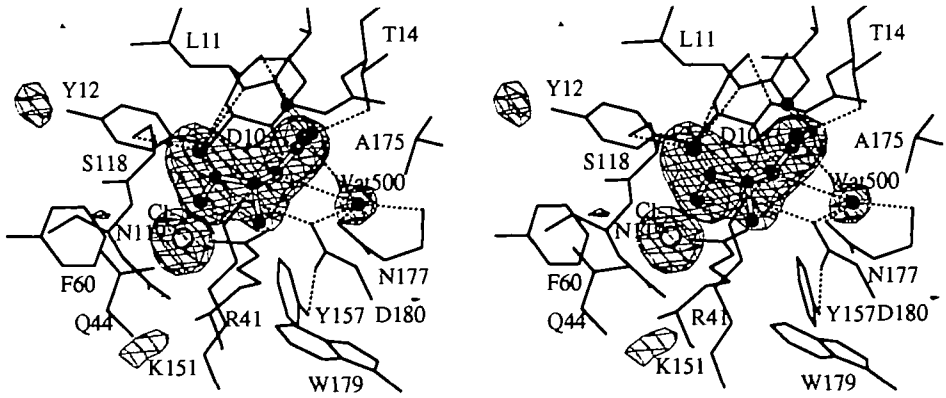

Fig. 1. Stereoview of the $F_o - F_c$ omit-map of the active site in the CPN intermediate of the S175A mutant. A difference Fourier map was obtained using the coordinates of a model which has the side chain of D10, the new water molecule and the probable chloride anion omitted. The map contoured at the 2.5σ level shows the esterified side chain of D10 as well as the new water molecule, labeled Wat500, and the chloride anion, labeled Cl^-. Hydrogen-bonds (2.4-3.5 Å) are shown as dashed lines. The figure was generated with the BOBSCRIPT program (*9*).

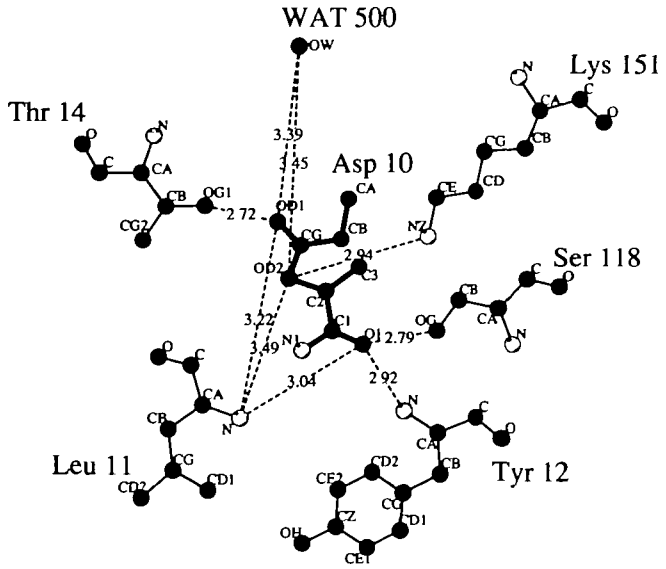

Fig. 2. **Schematic diagram of hydrogen-bonding interactions around the substrate-derived moiety in the active site of the CPN intermediate.** The C_2 atom of the CPN moiety is covalently bonded to the $O^{\delta 1}$ atom of D10. Hydrogen bonds are depicted as dashed lines with the bond lengths. The figure was produced with the LIGPLOT program (*10*).

released from the substrate, although it has not been so easy to prove this crystallographically because of the relatively high value of the average temperature factor of the protein atoms and the low occupancy of the peak. The guanidino group of R41 probably acts as a halogen abstraction residue; the side chain of R41 points to the C_2 atom of the substrate moiety, and no other basic residues which can serve as the halogen abstraction site exist around the C_2 atom. Arginine has been shown to be the binding site for chloride of a chloride pump (*8*). Moreover, the density peak appears in the vicinity of the amido nitrogen atom of the substrate moiety (3.72 Å) in the CPN intermediate, but does not appear in an ester intermediate formed between an alkanoic acid and the S175A mutant (Li, Y.-F. *et al.*, unpublished result). This demonstrates that the positively charged amido nitrogen, instead of the negatively charged carboxyl oxygen, is required for the appearance of the new peak that results from a negatively charged species. Thus, we have assigned the new peak to a chloride anion which interacts electrostatically with the positively charged nitrogens of the guanidino group of R41, the side-chain amido of N119, and the amido of the substrate moiety. Detailed studies for identification of this peak are now in progress.

We have shown by mass spectrometry that the catalysis by L-DEX YL proceeds through an ester intermediate (*1*). The present crystallographic study strongly suggests that when D10 attacks the C_2 atom of a substrate, the halogen atom must be simultaneously abstracted from the substrate by the guanidino group of R41 on the opposite side to the D10 side chain.

REFERENCES

1. Liu, J.-Q., Kurihara, T., Miyagi, M., Esaki, N., and Soda, K. (1995) Reaction mechanism of L-2-haloacid dehalogenase of *Pseudomonas* sp. YL. *J. Biol. Chem.* **270**, 18309-18312
2. Kurihara, T., Liu, J.-Q., Nardi-Dei, V., Koshikawa, H., Esaki, N., and Soda, K. (1995) Comprehensive site-directed mutagenesis of L-2-haloacid dehalogenase to probe catalytic amino acid residues. *J. Biochem.* **117**, 1317-1322
3. Hisano, T., Hata, Y., Fujii, T., Liu, J.-Q., Kurihara, T., Esaki, N., and Soda, K. (1996) Crystal structure of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL. *J. Biol. Chem.* **271**, 20322-20330
4. Iwasaki, I., Utsumi, S., Hagino, K., and Ozawa, T. (1956) A new spectrophotometric method for the determination of small amounts of chloride using the mercuric thiocyanate method. *Bull. Chem. Soc. Jpn.* **29**, 860-864
5. Brünger, A.T. (1992) *X-PLOR Manual (V.3.1)*, Yale University Press, New Haven
6. Jones, T.A. (1985) Interactive computer graphics: FRODO. *Methods Enzymol.* **115**, 157-171
7. Ridder, I.S., Rozeboom, H.J., Janssen, D.B., and Dijkstra, B.W. (1997) Three-dimensional structure of L-2-haloacid dehalogenase from *Xanthobacter autotrophicus* GJ10 complexed with the substrate-analogue formate. *J. Biol. Chem.* **272**, 33015-33022
8. Rüdinger, M., Haupts, U., Gerwert, K., and Oesterhelt, D. (1995) Chemical reconstitution of a chloride pump inactivated by a single point mutation. *EMBO J.* **14**, 1599-1606
9. Esnouf, R.M. (1997) An extensively modified version of MolScript that includes greatly enhanced coloring capabilities. *J. Mol. Graph. Model* **15**, 132-134
10. Wallace, A.C., Laskowski, R.A., and Thornton, J.M. (1995) LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng.* **8**, 127-134