Crystal structure and site-directed mutagenesis analyses of haloalkane dehalogenase LinB from Sphingobium sp. MI1205

Running title: Structure of LinB from Sphingobium sp. MI1205

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The enzymes LinB UT and LinB MI catalyze the hydrolytic dechlorination of β-hexachlorocyclohexane (β-HCH) and yield different products, 2,3,4,5,6-pentachlorocyclohexanol (PCHL) and 2,3,5,6-tetrachlorocyclohexane-1,4-diol (TCDL), respectively, despite their 98% identity in amino-acid sequence. To reveal the structural basis of their different enzymatic properties, we performed site-directed mutagenesis and X-ray crystallographic studies of LinB MI and its seven point mutants. The mutation analysis revealed that the seven amino-acid residues uniquely found in LinB MI were categorized into three groups based on the efficiency of the first-step (from β-HCH to PCHL) and second-step (from PCHL to TCDL) conversions. The crystal structure analyses of the wild-type LinB MI and its seven point mutants indicated how each mutated residue contributed to the first- and second-step conversions by LinB MI. The dynamics simulation analyses of the wild-type LinB MI and LinB UT revealed that the entrance of the substrate access tunnel of LinB UT was more flexible than that of LinB MI, which could lead to the different efficiencies in the dehalogenation activity between these dehalogenases.
Introduction

Hexachlorocyclohexane (HCH) is a six-chlorine-substituted cyclohexane. One of its isomers, the γ-isomer, has insecticidal properties and has been widely used as an insecticide around the world (1). Although the use of γ-HCH has been prohibited in most countries due to its toxicity and long persistence, the large-scale production, widespread use, and dumping of the other non-insecticidal isomers (α-, β-, and δ-HCHs) in the past decades still continue to create problems with HCH contamination in soil and groundwater (2). β-HCH in particular is a persistent and problematic isomer of HCH.

Several β-HCH-degrading bacteria whose β-HCH-degrading enzymes can be utilized for bioremediation have been identified (3–5). LinB MI and LinB UT are haloalkane dehalogenases isolated from Sphingobium sp. MI1205 and Sphingobium japonicum UT26, respectively, that can cleave the carbon-halogen bond in β-HCH. Haloalkane dehalogenases belong to the α/β-hydrolase family and their catalytic mechanism consists of the following steps: i) substrate binding, ii) cleavage of the carbon-halogen bond in the substrate and formation of an intermediate covalently bound to the nucleophile, iii) hydrolysis of the alkyl-enzyme intermediate, and iv) release of halide ion and alcohol (6). LinB MI and LinB UT share 98% sequence identity, with only seven different amino-acid residues (at positions 81, 112, 134, 135, 138, 247 and 253) out of 296 residues, however, these enzymes exhibit different
enzymatic properties (Fig. 1). LinB MI catalyzes the two-step dehalogenation and converts β-HCH to 2,3,4,5,6-pentachlorocyclohexanol (PCHL) and further to 2,3,5,6-tetrachlorocyclohexane-1,4-diol (TCDL) (7) in the manner of LinB2 from *Sphingomonas* sp. BHC-A (8) and LinB from *Sphingobium indicum* B90A (9), whereas LinBUT catalyzes only the first-step dehalogenation of β-HCH to PCHL (10) and cannot degrade PCHL further. Moreover, LinBMI can catalyze the first-step conversion eight times as efficiently as LinBUT (7).

In a previous site-directed mutagenesis study, the V134I, H247A and V134I/H247A mutants of LinBMI, in which one or two LinBMI-specific residue(s) were mutated to LinBUT-type residue(s), showed reduced activities in both the first- and second-step dehalogenations, with the exception that there was no reduction in the first-step dehalogenation activity of the H247A mutant (7). However, the activities of these mutants were still higher than that of LinBUT in both the first- and second-step dehalogenations, which suggested that one or more of the other five residues (T81, V112, T135, L138 and I253) uniquely found in LinBMI were also important for the high dehalogenation activity of LinBMI.

To date, the crystal structure of LinBUT has been reported (11–14), whereas the crystal structure of LinBMI has not been reported. To investigate how the seven different residues between LinBMI and LinBUT contribute to their different enzymatic properties, we performed
the site-directed mutagenesis and X-ray crystallographic studies of LinBMI and its seven point mutants where each LinBMI-specific residue is mutated to the LinBUT-type residue (T81A, V112A, V134I, T135A, L138I, H247A and I253M). Activity measurements were made for all the mutants except for V134I and H247A, which were reported previously (7).
Materials and Methods

Expression, purification, and crystallization

The expression plasmids of the wild-type LinB\textsubscript{MI} and the seven mutants (T81A, V112A, V134I, T135A, L138I, H247A and I253M) were constructed using the vector pAQNM, where the target proteins were expressed under the control of the \textit{tac} promoter and \textit{lacI}\textsuperscript{q}(7).

The wild-type LinB\textsubscript{MI} and the seven mutants were expressed and purified by the following procedures. The \textit{Escherichia coli} strain BL21(DE3) cells (Novagen) were cultured in Luria-Bertani (LB) medium containing 50 \(\mu\)g ml\(^{-1}\) ampicillin until an OD\(_{600}\) of 0.6 at 37°C. Protein expression was induced by adding IPTG (isopropyl \(\beta\)-D-thiogalactopyranoside) to a final concentration of 1 mM and the culture was continued at 25°C for 12 h. The cells were harvested by centrifugation at 4500 \(g\) at 4°C for 10 min. The harvested cells were suspended in Sol A (50 mM Tris-HCl pH 7.5, 400 mM NaCl and 5 mM imidazole) and disrupted by sonication. After centrifugation at 40,000\(g\) for 30 min at 4°C, the supernatant was loaded onto a 3-ml Ni Sepharose 6 Fast Flow column (GE Healthcare) at room temperature. After a wash step with Sol B (50 mM Tris-HCl pH 7.5, 400 mM NaCl and 50 mM imidazole), the protein was eluted with Sol C (50 mM Tris-HCl pH 7.5, 400 mM NaCl and 200 mM imidazole). The purified protein was dialyzed against 20 mM Tris-HCl pH 8.0, and then concentrated to 25 mg ml\(^{-1}\) using a Vivaspin 20 concentrator (Sartorius) at 4°C.
Initial crystallization trials of LinB\textsubscript{MI} were performed by the sitting-drop vapor-diffusion method in 96-well Intelli-Plates (Art Robbins Instruments) using sparse-matrix screening kits Crystal Screen HT, Index HT (Hampton Research) and Wizard I and II (Emerald Biosystems). Each drop was prepared by mixing equal volumes (0.7 μl) of the protein solution and a reservoir solution, and equilibrated against 70 μl of the reservoir solution at 4°C or 20°C. Further crystallization trials were carried out based on the crystallization conditions of the untagged (100 mM Tris-HCl (pH 8.8–9.0), 200 mM CaCl\textsubscript{2} and 17–19% (w/v) PEG6000) and His-tagged (100 mM Tris-HCl (pH 8.5), 200 mM MgCl\textsubscript{2} and 20% (w/v) PEG4000) LinB\textsubscript{UT} by the sitting-drop vapor diffusion method in 24-well plates (Hampton Research) (14, 15). The crystallization drops were prepared by mixing 1.0 μl protein solution and 1.0 μl reservoir solution, and were equilibrated against 0.3 ml reservoir solution.

Data collection and processing

The crystals of the wild-type LinB\textsubscript{MI} and the seven mutants were transferred to the reservoir solution containing 25% (v/v) glycerol as the cryoprotectant. The X-ray diffraction data were collected at a wavelength of 1.0000 Å in a cryogenic nitrogen gas stream at beamlines BL-5A and AR-NW12A of Photon Factory (Ibaraki, Japan). The datasets were
obtained by collecting 360 frames, with an oscillation step of 0.5°. The diffraction data were indexed, integrated, and scaled with HKL2000 (16).

Structure modeling and refinement

The crystal structure of the wild-type LinBmi was determined by the molecular replacement method using the program MOLREP (17) and the crystal structure of LinBUT (PDB code: 1CV2) (11) as the initial model. Refinements were performed using Coot (18) and Refmac5 (19). Water molecules were added using ARP/wARP (20). Then, the crystal structures of the seven mutants were solved by molecular replacement using the wild-type structure of LinBmi as the initial model. The stereochemical quality of each final model was assessed using the Ramachandran plots obtained by RAMPAGE (21).

Molecular dynamics simulation

The atomic coordinates of the crystal structures of the wild-type LinBmi (PDB code: 4H77) solved in this study and LinBUT (PDB code: 1CV2) (11) were used as the initial models. The following dynamics simulations were performed using MOE2011.10 with the default parameter settings unless stated. The missing hydrogen atoms of the wild-type LinBmi and LinBUT were generated and energy-minimized using the Merck Molecular Force Field 94x
(MMFF94x) force field with the distance-dependent dielectric electrostatics. Then, a few potassium ions for neutralization and explicit water molecules were added within a sphere of 10 Å from the protein surfaces. The resulting protein and solvent molecules in the spherical droplet were energy-minimized using MMFF94x force field with the R-field electrostatics. Tether weight was applied to all non-hydrogen atoms during the energy minimization steps. The molecular dynamics simulations were performed using the NVT ensemble and the Nosé-Poincaré-Anderson (NPA) algorithm at 303 K with a time step of 1 fs and without any bond constraint. As for the first 100-ps dynamics, the tether weight was applied to all non-hydrogen atoms and gradually reduced. After the first 100-ps dynamics, the dynamics simulations were performed for 14 ns without any positional restraint. The atomic coordinates were recorded every 1 ps after the first 100-ps dynamics and used for trajectory analysis.

**PDB accession number**

The atomic coordinates and structure factors (PDB codes: 4H77, 4H7D, 4H7E, 4H7F, 4H7H, 4H7I, 4H7J and 4H7K) have been deposited in the Protein Data Bank.
Ligand-docking simulation

The ligand-docking simulations were performed using the ASEDock, a docking program based on a shape similarity assessment between a concave portion on a protein and a ligand, in the Molecular Operating Environment (MOE) (Chemical Computing Group, Montreal, Canada). The three-dimensional structures of β-HCH and PCHL were modeled using the Molecule Builder in MOE. The initial models were energy-minimized employing the MMFF94x force field. The active site of the LinB structure was detected using the Alpha Site Finder in MOE. For each ligand, 250 conformations were generated using the default LowModeMD search parameters. The scoring function used by ASEDock was based on the protein-ligand interaction energies. The interaction energy ($U_{\text{dock}}$) of a given conformation was calculated as the sum of $U_{\text{ele}}$ (electric energy), $U_{\text{vdw}}$ (van der Waals energy) and $U_{\text{strain}}$ (difference of the minimal energies between the docked ligand and the ligand which was located nearest the docked ligand).

Enzymatic assays

For enzymatic assays, E. coli BL21 Star (DE3) cells (Invitrogen) expressing LinB and its mutants were disrupted by bacteriolysis using a CelLytic B (Sigma), and His-tagged enzymes were purified by using BD TALON Metal Affinity Resins (BD Biosciences). The purified
enzymes were incubated with 17 μM of β-HCH in 50 mM potassium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol at 30°C. The enzyme concentration in the reaction mixture was 150 μg/ml. The mixture (100 μl) was extracted with an equal volume of ethyl acetate and then analyzed by a Shimadzu GC-17A gas chromatograph with an 64Ni electron capture detector (ECD) and Rtx-1 capillary column (30 m × 0.25 μm × 0.25 μm; Restek). The column temperature was increased from 160°C to 200°C at a rate of 4°C/min for the separation of the peak of PCHL from that of TCDL, and then from 200°C to 260°C at a rate of 20°C/min. The gas flow rate was 30 ml/min. As the internal standard, 10 μM 2,4,5-trichlorophenol was used. Kinetic data were fitted to the irreversible two-step reaction structure of HCH conversion to TCDL via PCHL (Scheme 1) by using the GEPASI 3.2 software (22). The specificity constants and their standard errors for both reaction steps (k₁ and k₂) were obtained from the calculation. Evolutionary programming (23) was used to optimize the kinetic constants during the fitting of the kinetic data to Scheme 1. Values given are the mean of triplicates. Due to the low solubility (17 μM) of β-HCH in water, the k_cat and K_m values of these mutants could not be calculated.

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E + HCH \xrightarrow{k_1} E + PCHL \xrightarrow{k_2} E + TCDL
\]

Scheme 1
Results and Discussion

Site-directed mutagenesis

The wild-type LinBM used in Reference 7 and this study gave comparable data by the
same research group, as shown in Figs. 2A and 2G. We examined the dehalogenation
activities of the point mutants of LinBM, in which each of the five residues (T81, V112, T135,
L138 and I253) was mutated to the corresponding residue in LinBU (Table 1 and Figs.
2B–F). The V112A (Fig. 2C), T135A (Fig. 2D) and L138I (Fig. 2E) mutants showed reduced
activities in both the first- and second-step dehalogenations. The I253M (Fig. 2F) mutant
retained full activity in the first-step dehalogenation, but showed reduced activity in the
second-step dehalogenation as in the case of the H247A (Fig. 2I) mutant (7). On the other
hand, the T81A (Fig. 2B) mutant showed reduced activity in the first-step dehalogenation, but
retained full activity in the second-step dehalogenation. Our mutational data combined with
the previous data by Ito et al. (7) revealed that one (T81), two (H247 and I253), and four
(V112, V134, T135 and L138) of the seven different residues between LinBM and LinBU
contributed to their different efficiencies in the first step, the second step, and both the steps
of dehalogenation, respectively.

Crystallization and data collection
We obtained LinB MI crystals by combining the reported crystallization conditions for untagged and His-tagged LinB UT (14, 15). The best crystals with typical dimensions of 0.2 x 0.4 x 0.01 mm were obtained by mixing 1.0 μl of the protein solution (25 mg ml⁻¹) and 1.0 μl of the reservoir solution (100 mM Tris-HCl (pH 8.0), 20% (w/v) PEG 4000 and 200 mM CaCl₂) at 5°C. Similarly, the crystals of the seven mutants of LinB MI were obtained by mixing 1.0 μl of the protein solution (25 mg ml⁻¹) and 1.0 μl of the reservoir solution (100 mM Tris-HCl (pH 7.8–8.1), 17–20% (w/v) PEG 4000 and 200 mM CaCl₂) at 5°C. The crystal of the wild-type LinB MI belonged to the space group P₂₁2₁2 with unit cell dimensions of a = 50.4 Å, b = 72.1 Å and c = 73.5 Å, and contained one LinB MI molecule per asymmetric unit. The Matthews coefficient (24) and the solvent content were 1.96 Å³ Da⁻¹ and 37%, respectively. The crystals of the seven mutants had the same space group P₂₁2₁2 with unit cell dimensions similar to those of the crystal of the wild-type LinB MI. The diffraction data statistics for these crystals are given in Table 2.

Overall structures of the wild-type and the seven mutants of LinB MI

We have solved the crystal structures of the wild-type LinB MI at 1.60-Å resolution and of the seven mutants at 1.75–2.10-Å resolutions by molecular replacement. The LinB MI molecule existed as a monomer in the crystal, and consisted of two domains, the core domain
and the cap domain (Fig. 3A). The core domain (residues 2–132 and 214–295) had a typical
α/β-hydrolase fold as seen in other haloalkane dehalogenases (25–29). Unlike the core
domain, the cap domain varied in the number and the orientations of helices among
haloalkane dehalogenases, and the cap domain (residues 133–213) of LinB_MI was composed
of four 3_10- and six α-helices. The crystal structures of the wild-type and the seven mutants of
LinB_MI were very similar to one another, with RMSDs for C\(^\alpha\) atoms (residues 2–295) of
0.095–0.31 Å.

In LinB_MI, D108, H272 and E132 formed the catalytic triad as in LinB_UT (Fig. 3A). D108,
located on the β5 strand, acts as the nucleophile. The O\(^{δ1}\) atom of D108 formed a hydrogen
bond with the N\(^{ε}\) atom of H272, which was located on the loop between the β8 strand and η8
helix. The N\(^{δ}\) atom of H272 formed a hydrogen bond with the O\(^{ξ1}\) atom of E132, which was
located on the β6 strand.

The reservoir solution used contained 200 mM CaCl\(_2\), and the electron density of one
calcium ion was clearly observed between two adjacent LinB_MI molecules aligned in the
crystal. The calcium ion was coordinated with the O\(^{δ1}\) and O\(^{δ2}\) atoms in the side chain of
D166 in a LinB_MI molecule, the main chain O atoms of P175 and I178 of an adjacent LinB_MI
molecule and three water molecules. Thus, the calcium ion plays an important role for the
growth of this crystal by mediating the above intermolecular interaction.
The electron density of one chloride ion was observed in the active site, and the chloride ion formed hydrogen bonds with two halide-stabilizing residues, N38 and W109. These hydrogen bonds would reflect the manner of recognition of a chloride ion released from the substrate.

**Effects of different residues located near the active site on the specificity constants**

The residue at position 134 was the nearest residue to the nucleophile residue D108 among the seven different residues between LinB_Mi and LinB_UT (Fig. 3B), and is likely to bind the substrate directly. The V134I mutant of LinB_Mi retained 60% of the first-step dehalogenation activity, but showed only 11% of the second-step dehalogenation activity, when compared with the wild-type LinB_Mi (7). The superimposition of the crystal structures of the wild-type LinB_Mi and the V134I mutant revealed that the presence or absence of the C^δ_ atom at position 134 was the only difference around the active site between these two structures (Fig. 4A). To understand the effect of the C^δ_ atom at position 134, we performed co-crystallization and soaking experiments using β-HCH, but could not obtain the crystal structure of LinB_Mi complexed with β-HCH. Then, we predicted the locations and orientations of β-HCH and PCHL when bound to the wild-type LinB_Mi and the V134I mutant using the ASEDock program of MOE. The docking simulation provided reasonable binding models of β-HCH for
both the wild-type LinB\textsubscript{MI} and the V134I mutant. The \( \beta \)-HCH molecules docked in the
wild-type LinB\textsubscript{MI} and the V134I mutant were located at the same position with almost the
same orientations (data not shown). On the other hand, the docking simulation with PCHL
gave different results for the wild-type LinB\textsubscript{MI} and the V134I mutant. In the top three
solutions, the interaction energies of the PCHL molecule with the wild-type LinB\textsubscript{MI} were \(-1.6, \)
\(1.8, \) and \(3.4 \) kcal/mol, and those with the V134I mutant were \(-14.7, -1.1, \) and \(2.3 \) kcal/mol. In
the wild-type LinB\textsubscript{MI}, the manner of binding of PCHL in the top solution could explain the
occurrence of the second-step conversion from PCHL to TCDL, with the distance between
the \( O^{\delta_2} \) atom of D108 and the C4 atom of PCHL being \(3.1 \) Å (Fig. 4B). However, in the case
of the V134I mutant, the positions and orientations of the bound PCHL models in the top two
solutions (Fig. 4C, cyan and magenta) were different from those in the top solution for the
wild-type LinB\textsubscript{MI}. The C4 atoms in the two PCHL models were \(4.7 \) Å away from the \( O^{\delta_2} \)
atom of D108, and thus the second-step conversion from PCHL to TCDL was unlikely to
occur. The binding manner of the third solution (Fig. 4C, yellow) for the V134I mutant was
almost the same as that in the top solution for the wild-type LinB\textsubscript{MI}. These docking
simulation results suggested that the V134I mutant of LinB\textsubscript{MI} was not likely to bind PCHL
properly for the second-step conversion to occur because of the presence of the C\(^5\) atom at
position 134.
The residue at position 112 was located at the bottom of the substrate binding pocket (Fig. 269 3B). The V112A mutant of LinBMI retained 53% of the first-step dehalogenation activity, but showed only 23% of the second-step dehalogenation activity, when compared with the wild-type LinBMI (7). In the V112A mutant, the main chain of V134 was shifted by 0.3 Å toward the catalytic residue (D108) when compared with the corresponding region in the wild-type LinBMI, and the side chain of W109, one of the two halide-stabilizing residues, was rotated 6° relative to that in the wild-type LinBMI around the Cγ1-Cδ1 bond (Fig. 4D). Such structural differences at the two residues should be due to the absence of the Cγ2 atom rather than the Cγ1 atom in the V112A mutant of LinBMI. These structural changes within the active-site pocket should cause the reduction in the first- and second-step dehalogenation activities in the V112A mutant of LinBMI.

Effects of different residues lining the substrate access tunnel on the specificity constants

The active site of LinBMI was buried deeply inside the enzyme. Three entrances to the substrate access tunnels were found in LinBMI using the program CAVER (Fig. 5A). Two tunnel entrances (Fig. 5A, purple and cyan) were formed by the η5, α3, α5 and α6 helices, and the other tunnel entrance (Fig. 5A, pink) was formed by the two helices (η4 and α10) and
a loop between the β7 strand and the α10 helix. A tunnel entrance (Fig. 5B, purple) found in LinB_{UT}, which was formed by the α6 helix and two loops between the η4 and η5 helices and between the β7 strand and the α10 helix, was not observed in LinB_{MI} because the side chain of His247 covered the entrance. Ito et al. reported that H247 in LinB_{MI} was important for the second-step conversion of PCHL to TCDL (7). In the H247A mutant structure, the η5 helix was shifted toward the α6 helix because the H247A mutation created an extra space, which resulted in conformational changes of the side chains of F143 and P144 (Fig. 5C). Thus, the side chain of H247 would contribute to the tunnel formation suitable for substrate (PCHL) entry and product (TCDL) release.

L138 and I253 of LinB_{MI} were involved in the formation of one access tunnel (Fig. 5A, pink), while T135 was located approximately 6 Å away from the tunnel. The orientations of the side chain at position 253 were divided into two groups among the wild-type and the mutants of LinB_{MI}. In the wild-type LinB_{MI} as well as the T81A, V112A, V134I and H247A mutants, the C^β-C^γ1-C^δ1 chain of I253 faced toward the side chain of T135 (Fig. 5D). In contrast, in the T135A and L138I mutants, the C^β-C^γ1-C^δ1 chains of I253 faced toward the side chain of L138 (Figs. 5E and 5F). Thus, the T135A and L138I mutations caused the conformational changes of the side chain of I253, which resulted in the changes of the size and position of a tunnel entrance (Figs. 5D–F). In the I253M mutant (Fig. 5G), the side chain
of M253 faced toward the side chain of L138, and the side chain of L138 was rotated approximately 90° relative to that in the wild-type LinBMI along the Cβ-Cγ bond. In contrast, in LinBUT, the side chain of M253 faced toward the side chain of A135 (Fig. 6) (11). Thus, the orientation of the side chain of M253 could be influenced by the residues at position(s) 135 and/or 138. Since the residue at position 253 was located at an entrance of the access tunnel, the irregular orientation of the side chain at position 253 affected the shape of the entrance of the access tunnel in the T135A, L138I and I253M mutants (Figs. 5E–G). The irregular forms of the tunnel entrances in these mutants should lead to the reductions in the dehalogenase activities, especially the second-step dehalogenation activity.

In the wild-type LinBMI, T81 was positioned outside the active site, and the side chain of T81 formed hydrogen bonds with one water molecule and the amide nitrogen of E84. The main-chain structure of the T81A mutant was very similar to that of the wild-type LinBMI with an RMSD of 0.12 Å, and no conformational change was observed either at the active site or in the access tunnel between the wild-type LinBMI and the T81A mutant.

**Structural comparison between LinBMI and LinBUT**

LinBMI and LinBUT share 98% sequence identity. Their overall structures were very similar to each other, with an RMSD of 0.27 Å for 292 Cα atoms. The most remarkable structural
difference between LinB\textsubscript{MI} and LinB\textsubscript{UT} was observed at the N-terminal region of the cap domain (residues 134–149; Figs. 3A and 6), which plays an important role in determining the shape and size of the active site and the substrate access tunnels. A structural difference similar to that between LinB\textsubscript{MI} and LinB\textsubscript{UT} was observed between the wild-type LinB\textsubscript{MI} and the H247A mutant (Fig. 5C). The main chain of I134 in LinB\textsubscript{UT} was shifted by 0.8 Å toward the catalytic residue (D108) when compared with that in LinB\textsubscript{MI}, and the side chain of W109 in LinB\textsubscript{UT} was rotated approximately 5° relative to that in LinB\textsubscript{MI} (Fig. 6). These structural differences would be due to the size of the amino-acid residue at position 112 (Val in LinB\textsubscript{MI} vs. Ala in LinB\textsubscript{UT}), considering the structural difference between the wild-type LinB\textsubscript{MI} and the V112A mutant.

We have performed the molecular dynamics simulations for revealing the molecular mechanisms of the different efficiencies in dehalogenation between LinB\textsubscript{MI} and LinB\textsubscript{UT}. In both the cases of LinB\textsubscript{MI} and LinB\textsubscript{UT}, the C\textsuperscript{α} RMSDs against the initial coordinates increased sharply in the first one nanosecond of the simulations and the RMSDs were in the range of 1.4–1.8 Å in the last two nanoseconds (12–14 ns) (Fig. 7A), indicating that no global conformational change occurred. Figs. 7B and 7C show the superpositions of the crystal structures and the structures after the simulation of LinB\textsubscript{MI} and LinB\textsubscript{UT}, respectively. In the core domain of LinB\textsubscript{UT}, the crystal and the simulated structures were almost identical.
constrast, in the core domain of LinB<sub>MI</sub>, a conformational change was observed in a loop (residue 76-81) between β4 strand and α1 helix, which would be due to T81, the only unique residue to LinB<sub>MI</sub> in this region. The conformational change of the loop could lead to a movement of the interacting η7 helix in the cap domain toward the α4 helix and a concomitant change of the shape of the substrate binding pocket, which might cause the different efficiencies in the first-step dehalogenation activity between two enzymes. As for the cap domain, similar conformational changes were observed in both LinB<sub>MI</sub> and LinB<sub>UT</sub> in the following regions: η4-(loop)-η5, α4, α5, and α7. The conformational change at the entrance of the substrate access tunnel from η4 to η5 helices in LinB<sub>UT</sub> was larger than that in LinB<sub>MI</sub>, allowing the substrates to enter the tunnel easily (Figs. 7B and 7C). The different residues at positions 247 and 253 should cause the different conformational change of this region. Root-mean-square fluctuation (RMSF) was used as an index of structural flexibility. The RMSF analysis (Fig. 7D) clearly shows that the entrance of the substrate access tunnel from η4 to η5 helices (residues 142-146) of LinB<sub>UT</sub> is much more flexible than that of LinB<sub>MI</sub>. This high flexibility in LinB<sub>UT</sub> would lead to the large conformational change at the entrance of the substrate access tunnel as shown in Fig. 7B. In DhaA, a member of the same α/β-hydrolase family as LinB, the molecular dynamics simulation analysis revealed that the narrower substrate access tunnel in a variant than that in the wild-type enzyme shielded the...
active site from the solvent and showed a higher activity than the wild-type enzyme (30).

Similarly, the low flexibility of the tunnel entrance in LinBMI would contribute to the increase of its dehalogenation activity by inhibiting the influx of water molecules into the active site, particularly of the second-step dehalogenation activity, where the water molecules can compete with the hydroxyl group of PCHL.

Concluding Remarks

We have analyzed the dehalogenation activity of five of the seven amino-acid residues that differ between LinBMI and LinBUT. This and previous mutagenesis analyses revealed that most of the seven residues had effects on the second-step dehalogenation and none of the seven residues were critical for the degradation activity. We have determined the crystal structures of the wild-type and the seven mutants of LinBMI. The structural comparisons among the wild-type LinBMI, LinBUT and the seven mutants of LinBMI indicated that each mutant except the T81A mutant caused a small conformational change of the access tunnels or the active site that resulted in a reduction in the first- and second-step dehalogenation activities of LinBUT when compared with LinBMI. The dynamics simulations of the wild-type LinBMI and LinBUT suggested that the flexibility of the entrance of the substrate access tunnel lead to the difference of the dehalogenation activity, peculiarly the second step activity.
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References


Table 1

Specificity constants of the wild-type LinBMI and its mutants.

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<thead>
<tr>
<th>Enzyme</th>
<th>Specificity constant $k_{cat}/K_M$ (mM$^{-1}$ s$^{-1}$)</th>
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<td>HCH $\rightarrow$ PCHL</td>
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<tr>
<td>LinBMI wild-type</td>
<td>0.19 ± 0.008</td>
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<tr>
<td>LinBMI T81A</td>
<td>0.070 ± 0.003</td>
</tr>
<tr>
<td>LinBMI V112A</td>
<td>0.10 ± 0.009</td>
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<tr>
<td>LinBMI T135A</td>
<td>0.080 ± 0.005</td>
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<tr>
<td>LinBMI L138I</td>
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<tr>
<td>LinBMI I253M</td>
<td>0.21 ± 0.03</td>
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<tr>
<td>LinBMI wild-type*</td>
<td>0.205 ± 0.005</td>
</tr>
<tr>
<td>LinBMI V134I</td>
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<tr>
<td>LinBMI H247A</td>
<td>0.210 ± 0.015</td>
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<td>LinBMI V134I/H247A*</td>
<td>0.104 ± 0.003</td>
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<tr>
<td>LinBUT**</td>
<td>0.0271 ± 0.0002</td>
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</tbody>
</table>

* The same data used in Reference 7 are shown.

** LinBUT is identical to LinBMI T81A/V112A/V134I/T135A/L138I/H247A/I253M.
Table 2

Data collection and refinement statistics for the wild-type LinBMI and the seven mutants.

<table>
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<th></th>
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<td>20-1.80</td>
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<td>99.2 (99.7)</td>
<td>100.0 (100.0)</td>
<td>99.8 (99.6)</td>
<td>99.9 (99.6)</td>
<td>99.8 (99.2)</td>
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<td>Rsym (I) =</td>
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<td>0.087 (0.279)</td>
<td>0.101 (0.339)</td>
<td>0.084 (0.283)</td>
<td>0.110 (0.397)</td>
<td>0.075 (0.270)</td>
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<td>0.093 (0.367)</td>
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<tr>
<td>&lt;I&gt;/σ(I) =</td>
<td>32.0 (5.3)</td>
<td>37.7 (9.7)</td>
<td>31.1 (6.1)</td>
<td>39.0 (7.9)</td>
<td>23.7 (8.6)</td>
<td>30.5 (5.6)</td>
<td>41.7 (9.4)</td>
<td>37.3 (5.3)</td>
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Refinement statistics

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<th>Resolution range (Å)</th>
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<td>Angles (°)</td>
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<td>1.351</td>
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<td>96.9</td>
<td>96.9</td>
<td>96.9</td>
<td>96.2</td>
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<td>3.1</td>
<td>3.1</td>
<td>3.8</td>
<td>3.4</td>
<td>3.1</td>
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<td>outlier region (%)</td>
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<td>0</td>
<td>0</td>
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*Values in parentheses are for the highest-resolution shell.

\[ R_{sym} = \frac{\sum_{hkl} |I(hkl) - <I(hkl)>|}{\sum_{hkl} I(hkl)}, \text{where } <I(hkl)> \text{ is the average intensity of symmetry relation reflections} \]

\[ R_{work} = \frac{\sum_{hkl} |F_{obs} - |F_{cal}||}{\sum_{hkl} |F_{obs}|} \]

\[ R_{free} \text{ was calculated by using the 5% of reflections excluded in the refinement.} \]
Different enzymatic properties between LinB_Mi and LinB_UT. (A) The β-HCH degradation reactions catalyzed by LinB_Mi and LinB_UT. LinB_Mi converts β-HCH to PCHL and further to TCDL, while LinB_UT catalyzes only the first-step conversion of β-HCH to PCHL. The activity of LinB_Mi is approximately eight times as high as that of LinB_UT in the first-step dehalogenation of β-HCH to PCHL (7). (B) The seven different amino acid residues between LinB_Mi and LinB_UT.

Degradation of β-HCH (black circle) and appearance of its metabolites, PCHL (black triangle) and TCDL (white triangle), in reaction mixtures containing LinB_Mi wild-type (A), LinB_Mi T81A (B), LinB_Mi V112A (C), LinB_Mi T135A (D), LinB_Mi L138I (E), LinB_Mi L253M (F), LinB_Mi wild-type (G), LinB_Mi V134I (H), LinB_Mi H247A (I), LinB_Mi V134I/H247A (J) and LinB_UT wild-type (K). The same data (G–K) used in Reference 7 are also shown in this study. The activity data (A–F) of this study were obtained by the same research group as Reference 7 under the same reaction conditions except the concentration of purified enzyme (100 vs. 150 μg/ml in Reference 7 and this study, respectively).
Figure 3

Figure 4

Different amino-acid residues located around the active-site between LinB_MI and LinB_UT. (A)

The superimposition of the active sites of the wild-type (light green) and the V134I mutant

(slate) of LinB_MI. The catalytic triad residues (D108, E132 and H272), and V134/I134 are

labeled. The docking simulations of the wild-type (B) and the V134I mutant (C) with PCHL.

The chlorine, oxygen, and hydrogen atoms of the PCHL molecules are colored green, red,

and white, respectively. In the wild-type LinB_MI, the PCHL model with the lowest binding

energy is shown and its carbon atoms are colored yellow. In the V134I mutant, the carbon
atoms are colored cyan, magenta and yellow in the PCHL models with the lowest binding, the second lowest binding and the highest interaction energies, respectively. (D) The superimposition of the active sites between the wild-type (light green) and the V112A mutant (orange).

**Figure 5**

Different amino-acid residues lining the access tunnel between LinBMI and LinBUT. The three access tunnels (pink, purple and cyan) to the active site of the wild-type LinBMI (A) and LinBUT (B). The catalytic triad residues (green) and the different amino-acid residues (magenta) between the wild-type LinBMI and LinBUT are shown as stick models. The red circles represent the entrances of the access tunnels. (C) The superimposition between the wild-type (light green) and the H247A mutant (cyan) of LinBMI. The tunnel (purple) observed in the wild-type LinBMI is shown. The catalytic triad residues and the residues at positions 135, 138 and 253 are shown as sticks in the wild-type (D), the T135A mutant (E), the L138I mutant (F), and the I253M mutant (G) structures of LinBMI. The red circles show the entrances of the access tunnels of the wild-type and three mutants.

**Figure 6**
Structural comparison between the wild-type LinBMi and LinBUT. The superimposition between the wild-type LinBMi (light green) and LinBUT (cyan and dark gray). The most noteworthy difference between the wild-type LinBMi and LinBUT is colored cyan in LinBUT. The catalytic triad residues, one (W109) of two halide-stabilizing residues and six of the different residues between the wild-type LinBMi and LinBUT, are shown as stick models and labeled.

Figure 7

Molecular dynamics simulations of the wild-type LinBMi and LinBUT. (A) Time course of the Cα RMSDs from the initial structures of the wild-type LinBMi (green) and LinBUT (black) during the MD simulations. (B) The superposition between the crystal structure (gray and green) and the structure after the simulation (gray and yellow) of the wild-type LinBMi. (C) The superposition between the crystal structure (gray and black) and the structure after the simulation (gray and yellow) of the wild-type LinBUT. Green, black and yellow in (B) and (C) indicate the most different regions observed between the crystal structures and the structures after the simulations (gray and yellow). (D) The Cα RMSFs per LinBMi (green) and LinBUT (black) residues over the last 2-ns simulations.
A

B

<table>
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<tr>
<th>Residue number</th>
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<th>112</th>
<th>134</th>
<th>135</th>
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<th>247</th>
<th>253</th>
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<td>V</td>
<td>V</td>
<td>T</td>
<td>L</td>
<td>H</td>
<td>I</td>
</tr>
<tr>
<td>LinB&lt;sub&gt;UT&lt;/sub&gt;</td>
<td>A</td>
<td>A</td>
<td>I</td>
<td>A</td>
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<td>A</td>
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Figure 2