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Crystal structure of a family I.3 lipase from *Pseudomonas* sp. MIS38 in a closed conformation

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Abstract The crystal structure of a family I.3 lipase from *Pseudomonas* sp. MIS38 in a closed conformation was determined at 1.5 Å resolution. This structure highly resembles that of *Serratia marcescens* LipA in an open conformation, except for the structures of two lids. Lid1 is anchored by a Ca^{2+} ion (Ca1) in an open conformation, but lacks this Ca1 site and greatly changes its structure and position in a closed conformation. Lid2 forms a helical hairpin in an open conformation, but does not form it and covers the active site in a closed conformation. Based on these results, we discuss on the lid-opening mechanism.

Keywords: Family I.3 lipase; Crystal structure; Closed conformation; Lid; Ca^{2+}-binding site; *Pseudomonas* sp. MIS38

1. Introduction

Family I.3 lipases are distinguished from other lipases not only by their amino acid sequences, but also by their secretion mechanism [1]. They are secreted via the type I secretion system (T1SS) [2]. T1SS consists of three subunit proteins, which form a channel that spans the inner and outer membranes of Gram-negative bacteria. Secretion by this system occurs in a single step, directly from the cytoplasm to the extracellular milieu, bypassing the periplasm. Proteins that are secreted via the T1SS usually have a C-terminal secretion signal and several repeats of a GGxGxDxux (u: hydrophobic residue) upstream of the secretion signal, termed the repeat in toxin (RTX) motif [2]. These repeats bind Ca^{2+} ions, forming a β-roll motif [3]. The first six residues form a Ca^{2+}-binding loop and the last three form a short β-strand.

Pseudomonas sp. MIS38 produces a family I.3 lipase (PML) [4]. This lipase (PML) consists of 617 amino acid residues and two domains. The N-catalytic domain (residues 1–370) contains the active site residues, Ser^{207}, Asp^{255}, and His^{313} [4,5]. The C-domain contains several repeats of the RTX motif and a putative secretion signal near the C-terminus. PML requires Ca^{2+} for activity and folding, but does not require molecular chaperones for folding [4,6]. PML undergoes interfacial activation [4], suggesting that PML has a lid structure which is open upon contact with the micelle-forming substrate like other lipases [7,8]. The RTX motif of PML has been proposed to function as an intramolecular chaperone [2], because deletion [9] or mutation [10] of this motif generates inactive proteins, which are incompletely folded. The C-domain of PML can be used as a secretion tag for extracellular production of a heterologous protein via the T1SS [11].

The crystal structure of *Serratia marcescens* LipA (SML), which is a family I.3 lipase and shows the amino acid sequence identity of 61% to PML, has recently been determined [12]. The SML structure consists of the N-terminal lipase domain and C-terminal RTX domain. The lid of the lipase domain assumes an open conformation, where this lid is anchored by one Ca^{2+} ion (Ca1). In parallel, we determined the crystal structure of PML in a closed conformation. In this structure, not only the lid (lid1) identified in the SML structure, but also an additional lid (lid2), undergo significant changes in their positions and structures, so that they cover the active site. In addition, the Ca1 site is missing in this structure.

2. Materials and methods

2.1. Protein production, crystallization, and data collection

PML was overproduced, purified and crystallized as a secreted protein, using the methods described previously [13]. In short, PML was purified from the LB culture supernatant of *Escherichia coli* DH5 containing two plasmids, pUC-PML containing the PML gene and pYBCD20 containing the genes for the T1SS of *S. marcescens* lipase [14]. Following purification, PML crystals belonging to space group $P2_1$ were obtained by incubating the mixture of 2 μl of 10 mg/ml protein solution with 1 μl of the optimized mother liquor [0.1 M MES (pH 6.0), 0.2 M Ca-acetate, 5 mM Zn-acetate, 10% PEG20K] against 100 μl of the mother liquor using hanging drop vapor diffusion method [13]. Data collection was done at SPring8 (Harima, Japan) using beamline BL38B1 at −173 °C. Before data collection crystals were flash-cooled in a nitrogen stream at −173 °C after soaking the crystal in the mother liquor containing 20% ethylene glycol as a cryoprotectant [13]. A total of 352 images were recorded with an exposure time of 10 s per image and an oscillation angle of 1.0°. Diffraction images were indexed, integrated and scaled using the HKL-2000 program suite [15]. PML crystal has cell parameters of $a = 49.97$ Å, $b = 84.30$Å, $c = 86.85$ Å and contains one molecule per asymmetric unit.

2.2. Site-directed mutagenesis and heavy atom derivatization

Attempts to do phasing using derivatized wild-type PML crystals did not produce satisfactory results because the crystals did not specifically bind some heavy atoms or were broken upon incubation with other heavy atom solutions. Moreover, PML could not be overproduced extracellularly in selenomethionine-containing media. Therefore,

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Abbreviations: PML, family I.3 lipase from *Pseudomonas* sp. MIS38; SML, *Serratia marcescens* LipA; RTX, repeat in toxin (GGxGxDxux)

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cysteine mutagenesis was employed to introduce a heavy atom binding site to the protein. Previous report showed that mutations of serine to cysteine have high success rates for heavy atom binding [16]. Twelve serine residues (Ser^{95}, Ser^{144}, Ser^{220}, Ser^{242}, Ser^{270}, Ser^{312}, Ser^{335}, Ser^{384}, Ser^{445}, Ser^{472}, Ser^{538} and Ser^{608}) were picked randomly and were individually mutated to cysteine by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA). Mutations were confirmed by sequencing with an ABI PRISM 310 Genetic Analyzer (Perkin–Elmer). Purification and crystallization of the mutant proteins were done using the same procedures as those of the wild-type protein.

For heavy atom derivatization, crystals of PML and its mutants were soaked in the mother liquor containing 40 mM of K_2PtCl_4 for one week. After soaking, the crystals were back soaked in the mother liquor containing 20% ethylene glycol. Difference Patterson map showed that one mutant, S445C, has one Pt bound specifically (data not shown). Data collection of the heavy atom derivatives was done as described for the native crystal.

2.3. Structure solution and refinement

The structure was solved by the single isomorphous replacement with anomalous scattering (SIRAS) method using the HKL2MAP [17,18] graphical user interface, employing the Pt-derivative of S445C crystal. Automated model building was done by using ArpWarp [19]. Refinement was carried out by using REFMAC [20] of the CCP4 suite [21], and the model was completed and corrected using COOT [22]. The statistics for data collection and refinement are presented in Table 1. The atomic coordinates and structure factors are available from the Protein Data Bank (PDB) under the entry code 2Z8X. Figures were prepared using PyMol (http://www.pymol.org).

3. Results and discussion

3.1. Overall structure

The PML structure was determined at 1.5 Å resolution by the SIRAS method using a Pt-derivatized crystal of a cysteine mutant. The entire amino acid sequence of PML was observed in the electron density except the N-terminal methionine residue. PML consists of two domains, an N-domain that is rich in α-helices and a C-domain that is rich in β-strands (Fig. 1A). This structure highly resembles the SML structure [12], except for the lid structures (residues 46–74 and 146–167) (Fig. 1B). The steric configurations of the active site residues (Ser^{207}, Asp^{255} and His^{313}), that form a catalytic triad, are nearly identical to those of SML (Fig. 1B). The rms deviation of the PML and SML structures is 0.7 Å for the entire structure, excluding the lid structure, and 0.15 Å for the side chains of the active site residues. Besides the protein molecule, the density of 10 Ca^{2+} ions, 2 Zn^{2+} ions, and 769 water molecules were observed in the asymmetric unit. The Zn^{2+} ions are located at the interfaces of the protein molecule, and each is coordinated by two protein molecules, indicating that Zn^{2+} ions are required for crystal formation.

3.2. Ca^{2+}-binding sites in the lipase domain

The N-terminal lipase domain contains two Ca^{2+}-binding sites, like that of SML. However, the Ca1 site of SML is missing in the PML structure. The conserved and unique Ca^{2+}-binding sites of the lipase domain of PML are therefore termed Ca2 and Ca3, respectively. At the Ca3 site, the Ca^{2+} ion is heptacoordinated by the side chains of Lys^{278}, Asp^{283}, and Asp^{337} (both oxygen atoms), carbonyl O of Ala^{281}, and two water molecules. Because the residues forming this site is relatively well conserved in the SML structure and the concentration of the Ca^{2+} ion used for crystallization is 200 mM for PML and 10 mM for SML, this site may represent a weak Ca^{2+}-binding site. The role of the Ca2 and Ca3 sites remains to be elucidated.

Table 1
Statistics on data processing and structure determination[c]

	Native wild-type	Pt-derivative S445C
Space group	$P2_1$	$P2_1$
Unit cell	a = 49.972, b = 84.300, c = 86.849	a = 49.879, b = 84.404, c = 87.012
Wavelength (Å)	0.9	1.071784
Resolution range (Å)	50.00–1.48 (1.53–1.48)	50.0–1.80 (1.86–1.80)
Unique reflections	118,695	65,946
Redundancy	7.2 (5.8)	7.2 (6.3)
Completeness	96.9 (79,.3)	95.9 (82.5)
I/σ	38.3 (3.8)	26.9 (3.4)
R_{merge}[a] (%)	5.5 (33.3)	7.4 (29.8)
Refinement statistics		
Resolution range (Å)	37.08–1.48	
No. of reflections	107,798	
Cutoff	None	
R-value (%)	17.7	
Free *R*-value[b] (%)	19.4	
No. of protein atoms	4553	
No. of solvent atoms	769	
Rms deviations from ideal values		
Bond lengths (Å)	0.008	
Bond angles (°)	1.175	
Ramachandran plot statistics. Percentage of residues in regions:		
Most favored	91.8	
Additionally allowed	7.4	
Generously allowed	0.8	
Disallowed	0.0	

[a] $R_{merge} = \sum | I_{hkl} - (I_{hkl}) | / \sum I_{hkl}$.
[b] Free *R*-value was calculated using 5% of reflections omitted from the refinement.
[c] Numbers in parentheses are for the highest resolution shell.

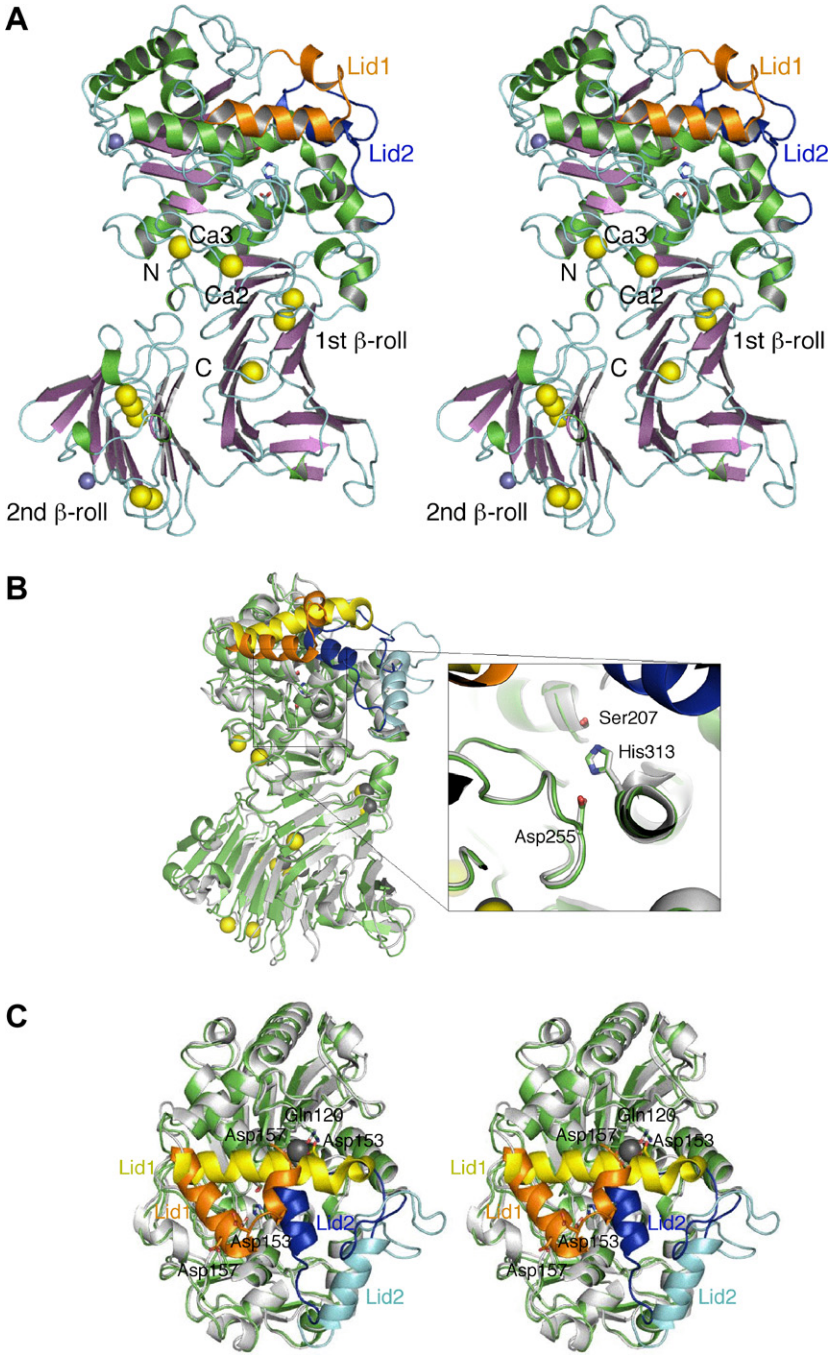

Fig. 1. (A) Stereo view of the cartoon model of PML structure, colored based on secondary structures. Ca^{2+} and Zn^{2+} ions are shown in yellow and blue spheres, respectively. Two Ca^{2+}-binding sites (Ca2 and Ca3) in the N-terminal lipase domain and the first and second β-roll motifs are indicated. Lid1 and lid2 are colored orange and blue, respectively. Three active site residues (Ser^{207}, Asp^{255}, and His^{313}) are indicated by stick models. N and C represent the N- and C-termini. (B) Superposition of the structures of PML and SML (PDB ID: 2QUB), which are colored green and gray, respectively. Lid1 and lid2 of PML are colored orange and blue, while those of SML are colored yellow and light blue, respectively. Ca^{2+} ions in the PML and SML structures are shown in yellow and dark-gray spheres, respectively. Zn^{2+} ions are shown in blue spheres. Three active site residues (Ser^{207}, Asp^{255}, and His^{313}) are indicated by stick models. (C) Stereo view of the lipase domain. The structures of the lipase domains of PML and SML are superimposed. The structures shown in Fig. 1B are viewed from the top. The Ca^{2+} ion bound to the Ca1 site is shown in dark gray sphere. The side chains of Asp^{153}, Asp^{157}, and Gln^{120}, which coordinate with this Ca^{2+} ion in an open conformation, are indicated by stick models, in addition to those of three active site residues. The side chains of Asp^{153} and Asp^{157} in a closed conformation are also indicated.

3.3. Lid structures

Comparison of the PML and SML structures indicate that the conformations of helix α6 and the region containing helix α3, which are termed lid1 and lid2, respectively, greatly vary for these structures (Fig. 1C). Lid1 (residues 146–167 in PML), which corresponds to the well known lid of lipases, assumes an open conformation in the SML structure, while it assumes a closed conformation in the PML structure. In

addition, it is sharply bent at the middle of the helix in the PML structure. Lid1 of SML is anchored by the Ca^{2+} ion bound to the Ca1 site to its position, while that of PML is not. This Ca^{2+} ion is buried inside the protein molecule and is coordinated by the side chains of Asp^{153}, Asp^{157}, and Gln^{120}, and the carbonyl oxygens of Thr^{118} and Ser^{144}. The Ca1 site is missing in the closed conformation, because Asp^{153} and Asp^{157}, which are located in helix α6, greatly change their positions in the closed conformation as compared to those in the open conformation. Previous studies showed that PML requires one Ca^{2+} ion for activity [6]. The observation that the Ca1 site is formed only in the open conformation and the conformation of the active site is not seriously changed regardless of the lid structures suggest that this catalytically essential Ca^{2+} ion binds to the Ca1 site to stabilize the open conformation. In the absence of this Ca^{2+} ion, the lid structure in the open conformation may be unstable because of the buried charged residues of lid1.

Like lid1, lid 2 (residues 46–74 in PML) assumes an open conformation, in which helix α3 forms an α-helix hairpin with α2, in the SML structure, while it assumes a closed conformation, in which helix α3 covers the active site, in the PML structure. Because none of the lipases so far reported contains this second lid, family I.3 lipases may be distinguished from other lipases by the presence of this second lid. It is noted that the hydrophobic side chains of both lid1 and lid2 are buried toward the active site in the closed conformation, while they are exposed to the solvent in the open conformation. Because PML and SML are crystallized in the absence and presence of detergent, respectively, the hydrophobic side chains exposed to the solvent in SML may be stabilized by detergent. We propose that the lids of family I.3 lipases open upon interaction with the hydrophobic surface of micellar substrate and this open conformation is stabilized by the Ca^{2+} ion bound to the Ca1 site.

3.4. β-roll motif

The C-domain of PML contains two β-roll motifs, laterally stacked together forming the so-called β-roll sandwich [12], similar to that of SML. The first β-roll motif consists of residues 373–417, containing five RTX repeats and binds three Ca^{2+} ions that are "dry", i.e. has no coordination with water molecules. The second β-roll motif consists of residues 493–568, containing eight RTX repeats and binds five Ca^{2+} ions. The last two Ca^{2+} ions that bind to this motif have coordination with water molecules, while the other three are "dry". Because each Ca^{2+} ion binds between a pair of the loops formed by the RTX motifs, the first and second β-roll motifs contain at most three and six Ca^{2+}-binding sites, respectively. All possible Ca^{2+}-binding sites are occupied by the Ca^{2+} ion in the first β-roll motifs of PML and SML. In contrast, only five and three Ca^{2+}-binding sites are occupied by the Ca^{2+} ion in the second β-roll motifs of PML and SML, respectively. In the second β-roll motif of PML, the sixth aspartic acid residue of the RTX motif, which coordinates with the Ca^{2+} ion, is fully conserved in all repeats. Nevertheless, the space between the loops of the second and fourth RTX motifs is occupied by water molecule, instead of Ca^{2+} ion. In the second β-roll motif of SML, the sixth aspartic acid residue is replaced by Ala, Asn, and Asn in the second, fourth, and sixth RTX motifs, respectively. As a result, only three Ca^{2+} ions bind to only one side of the β-roll motif.

It has previously shown that six functional RTX repeats are enough to maintain the functionality and structural integrity of PML [9]. In fact, some family I.3 lipases contain only six RTX repeats [2]. Further deletion of the repeats or mutation abolishes the repeats' ability to form β-roll motif, and greatly decreases secretion efficiency, proteolysis stability, and enzymatic activity [9,10]. These results allowed us to propose that the β-roll motif has a chaperone-like function [2,12]. Because the parallel β-sheet of one side of the first β-roll motif is extended to form a long parallel β-sheet with three β-strands (β6–β8) of the lipase domain (Fig. 1A), formation of the first β-roll motif is probably required to promote folding of the lipase domain. However, the first β-roll motif contains only five RTX repeats, which are not sufficient for formation of a β-roll motif. Therefore, formation of the β-roll sandwich may be required to stabilize the first β-roll motif. Further studies will be necessary to elucidate the precise mechanism of the chaperone-like function of the β-roll motif.

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