Identification of the histidine and aspartic acid residues essential for enzymatic activity of a family I.3 lipase by site-directed mutagenesis

Kwon Hyun-Ju, Kei Amada, Mitsuru Haruki, Masaaki Morikawa, Shigenori Kanaya*

Department of Material and Life Science, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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Abstract A lipase from *Pseudomonas* sp. MIS38 (PML) is a member of the lipase family I.3. We analyzed the roles of the five histidine residues (His^{30}, His^{274}, His^{291}, His^{313}, and His^{365}) and five acidic amino acid residues (Glu^{253}, Asp^{255}, Asp^{262}, Asp^{275}, and Asp^{290}), which are fully conserved in the amino acid sequences of family I.3 lipases, by site-directed mutagenesis. We showed that the mutation of His^{313} or Asp^{255} to Ala almost fully inactivated the enzyme, whereas the mutations of other residues to Ala did not seriously affect the enzymatic activity. Measurement of the far- and near-UV circular dichroism spectra suggests that inactivation by the mutation of His^{313} or Asp^{255} is not due to marked changes in the tertiary structure. We propose that His^{313} and Asp^{255}, together with Ser^{207}, form a catalytic triad in PML.

Key words: Lipase; Site-directed mutagenesis; Catalytic triad; Active-site; *Pseudomonas*

1. Introduction

Family I.3 lipases, which are represented by lipases from *Pseudomonas fluorescens* and *Serratia marcescens*, are distinct from other lipases not only in an amino acid sequence but also in a secretion mechanism [1]. They are secreted by a one-step pathway using an ATP-binding cassette (ABC) system [2]. They contain a typical secretion signal at the extreme C-terminal region [3]. In addition, they contain a repeat of a nine-residue GGXGXDXUX sequence motif (where X represents any amino acid residue and U represents a large hydrophobic residue) at the C-terminal regions [3–6]. The presence of this repetitive nine-residue sequence motif discriminates family I.3 lipases from other lipases. The extreme C-terminal secretion signal [7], as well as the C-terminal repetitive nine-residue sequence motif [8–10], were also identified in metalloproteases secreted by ABC transporters. X-ray crystallographic studies revealed that these metalloproteases consist of N-terminal catalytic and C-terminal β-sandwich domains [11–13]. Seven or eight repeats of a nine-residue GGXGXDXUX sequence motif form a parallel β-roll structure in the C-terminal domain, to which 7–8 Ca^{2+} ions bind.

A lipase from *Pseudomonas* sp. MIS38 (PML), which is a member of the lipase family I.3, is composed of 617 amino acid residues and acts in a monomeric form [14]. The enzyme showed both lipase and esterase activities with relatively broad substrate specificities. It was active only in the form of holo-enzyme, in which at least 12 Ca^{2+} ions bound. Because it did not show any lipase/esterase activity in the presence of other divalent cations, only the Ca^{2+} ion seems to be responsible for the formation of holo-enzyme. Site-directed mutagenesis revealed that Ser^{207} within a GXSXG motif, which is conserved in most of lipases and esterases, is required for the enzymatic activity of PML [14]. The PML sequence contains 12 repeats of a nine-residue GGXGXDXUX sequence motif at the C-terminal region. The limited proteolysis indicated that PML was digested into two fragments [14]. The N- and C-fragments contain the active-site serine residue (Ser^{207}) and entire 12 repeats of a nine-residue sequence motif, respectively. These results strongly suggest that PML consists of an N-terminal catalytic domain and C-terminal β-sandwich domain like metalloproteases. However, it remained to be determined as to whether all the active-site residues are contained in a possible N-terminal domain.

Lipases and esterase usually have a catalytic triad consisting of Ser, Asp/Glu, and His [15]. Therefore, PML must have catalytically essential histidine and acidic residues besides Ser^{207}. However, it seems difficult to predict these residues only from sequence analyses, because family I.3 lipases show poor sequence similarity to other lipases, whose active-site residues have been identified, and because only a limited number of the amino acid sequences are available for family I.3 lipases. In this report, we constructed 10 mutant proteins, in which either one of the conserved histidine and aspartic or glutamic acid residues in PML were replaced by Ala, and analyzed for their lipase and esterase activities. The results suggest that His^{313} and Asp^{255} are the components of a catalytic triad.

2. Materials and methods

2.1. Cells and plasmids

The plasmid pET-25b(+) derivative for the overproduction of PML was previously constructed [14]. In this plasmid, the expression of the gene encoding PML is under the control of the T7 promoter. *Escherichia coli* HMS174(DE3)pLysS [F^-, *recA*1, *hsdR*($r^-_{K12}m_{K12+}$) *RifR* (DE3) pLysS(Cm^r)], which was used as a host, was obtained from Novagen. All transformants were grown in L broth at 37°C in the presence of 50 μg/ml ampicillin.

2.2. Preparation of mutant proteins

Site-directed mutagenesis was performed by an oligodeoxyribonucleotide-directed dual amber method [16] using Mutant-Express Km kit (Takara Shuzo Co., Ltd.) according to the procedure recommended by the supplier. Oligonucleotides (29–30 bases), which were used as mutagenic primers, were designed to alter the codons for

*Corresponding author. Fax: (81)-6-6879 7938.
E-mail: kanaya@ap.chem.eng.osaka-u.ac.jp

Abbreviations: PML, *Pseudomonas* sp. MIS38 lipase; CD, circular dichroism

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His^{30}, His^{274}, His^{291}, His^{313}, His^{365}, Glu^{253}, Asp^{262}, Asp^{275}, and Asp^{290} to those for Ala, and the codon for Asp^{255} to those for Ala and Asn. The codon for His^{313} was changed from CAT to GCT, the codons for other His residues were equally changed from CAC to GCC, the codon for Glu^{253} was changed from GAA to GCA, the codons for Asp^{262} and Asp^{275} were equally changed from GAC to GCC, the codon for Asp^{290} was changed from GAT to GCG, and the codon for Asp^{255} was changed from GAT to GCT for Ala and AAT for Asn. All oligonucleotides were synthesized by Sawady Technology Co., Ltd. The nucleotide sequences of the genes encoding mutant proteins were confirmed using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer).

The overproducing strains were constructed by transforming *E. coli* HMS174(DE3)pLysS with the plasmid pET-25b(+) derivatives, in which the gene encoding the wild-type protein was replaced by the mutated genes. Cultivation of these *E. coli* HMS174(DE3)pLysS transformants, overproduction of the mutant proteins, and purification of the mutant proteins from cells were carried out as described previously for the wild-type protein [14]. The purities of the mutant proteins were analyzed by SDS–PAGE [17].

2.3. Enzymatic activity

The lipase activity was determined by using olive oil as a substrate [14]. The reaction was carried out in 1.5 ml of 25 mM Tris–HCl (pH 7.5) containing 10 mM $CaCl_2$ and 55 μl of olive oil at 30°C for 30 min. The reaction was terminated by the addition of 5 ml of acetone–ethanol (1:1, v/v) and the liberated fatty acid was titrated with 10 mM NaOH. The esterase activity was determined by using *p*-nitrophenyl laurate (C_{12}) as a substrate [14]. The reaction was carried out in 100 μl of 25 mM Tris–HCl buffer (pH 7.5) containing 10% acetonitrile, 10 mM $CaCl_2$, and 0.5 mM of *p*-nitrophenyl laurate at 30°C for 30 min. The reaction was terminated by the addition of SDS (final concentration of 0.2%) and the amount of the liberated *p*-nitrophenol was determined from the molar absorption coefficient value of 14 200 M^{-1} cm^{-1} at 412 nm. One unit of enzymatic activity was defined as the amount of enzyme that liberated 1 μmol of fatty acid or *p*-nitrophenol per min. The specific activity was defined as the enzymatic activity per mg of protein.

Protein concentration was determined from UV absorption on the assumption that all mutant proteins have the same absorption coefficient as that of the wild-type protein. We use an $A_{280}^{0.1\%}$ value of 1.14, which was calculated by using ε values of 1576 M^{-1} cm^{-1} for Tyr and 5225 M^{-1} cm^{-1} for Trp at 280 nm [18].

2.4. Circular dichroism (CD)

The far-UV (200–260 nm) and near-UV (250–320 nm) CD spectra were measured in 20 mM Tris–HCl (pH 7.5) containing 10 mM $CaCl_2$ at 25°C on a J-725 automatic spectropolarimeter (Japan Spectroscopic Co., Ltd.). The protein concentration and optical path length were 0.2 mg/ml and 2 mm for far-UV CD spectra, and 1.0 mg/ml and 10 mm for near-UV CD spectra, respectively. The mean residue ellipticity, $[\Theta]$, which has the units of ° cm^{-2} $dmol^{-1}$, was calculated by using an average amino acid molecular weight of 110.

3. Results and discussion

Multiple sequence alignment for the proteins that functionally and structurally resembled with one another is thought to be effective to predict the active-site residues, because these residues are fully conserved in these amino acid sequences. For family I.3 lipases, the sequence information is available for PML (accession No. AB025596), *P. fluorescens* SIK W1 lipase (accession No. AF083061), *P. fluorescens* B52 lipase (accession No. M86350), *P. fluorescens* No. 33 lipase (accession No. AB015053), *S. marcescens* Sr41 8000 lipase (accession No. D13253), and *S. marcescens* SM6 lipase (accession No. U11258). Alignment of these sequences indicated that six of 12 histidine residues and 32 of 69 acidic amino acid residues (Asp+Glu) are fully conserved (data not shown). Because so many acidic residues are conserved in the PML sequence, it would be laborious to identify a catalytically essential acidic residue by site-directed mutagenesis. However, the order of the active-site residues in the amino acid sequences of lipases and esterases had been reported to be evolutionarily conserved to be Ser, Asp/Glu, and His [1,19]. Therefore, we decided to identify the active-site histidine residue first. Once it was identified, the number of the candidates for the active-site acidic residue would be greatly reduced, because this residue should be located between Ser^{207} and the active-site histidine residue.

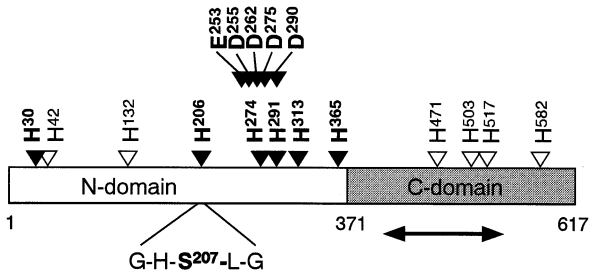

Fig. 1. Schematic representation of the amino acid sequence of PML. The positions of the conserved histidine residues, which are designated in boldface, are shown in solid inverted triangles. The positions of other histidine residues are shown in open inverted triangles. The positions of the conserved acidic amino acid residues, which are located between Ser^{207} and His^{313}, are also shown in solid inverted triangles. A possible N-terminal catalytic domain and a C-terminal β-sandwich domain, to which a number of the Ca^{2+} ions bound, are indicated. The position of a GXSXG motif, in which the active-site serine residue is designated in boldface, is also shown. The range of the amino acid sequence (Val^{405}–Asp^{543}), which is deleted in the amino acid sequences of *P. fluorescens* SIK W1 lipase and *P. fluorescens* B52 lipase, is shown by an arrow. Numbers represent the positions of the amino acid residues that start from Met^1.

Localization of the six conserved histidine residues in the PML sequence is schematically shown in Fig. 1. They are His^{30}, His^{206}, His^{274}, His^{291}, His^{313}, and His^{365}. All of them are located in a possible N-terminal domain. Of them, His^{206} is a component of a GHSLG motif, which contains the active-site serine residue (Ser^{207}). A GXSXG motif containing the active-site serine residue is conserved in most esterases and lipases [20]. This motif is usually located between β-strand and α-helix and assumes an extremely sharp turn called nucleophile elbow [19]. Because the second residue of this motif has been proposed to be required to maintain the active-site serine residue in a precise orientation [21,22], it is unlikely that His^{206} at the equivalent position is involved in a catalytic function. Therefore, we constructed five mutant proteins of PML, in which either His^{30}, His^{274}, His^{291}, His^{313} and His^{365} was replaced by Ala. His^{30} may not be a candidate for the active-site histidine residue, because it is located at the N-terminal region of Ser^{207}, but was also mutated as a reference. The resultant mutant proteins, H30A, H274A, H291A, H313A, and H365A, were overproduced in *E. coli* and purified to give a single band on SDS–PAGE (data not shown).

The lipase and esterase activities of the mutant proteins are summarized in Table 1. Of the five conserved histidine residues, His^{30}, His^{274}, His^{291}, and His^{365} are apparently not involved in the active-site, because the mutation of any one of these residues to Ala neither seriously affected the lipase activity nor the esterase activity. In contrast, the mutation of His^{313} to Ala resulted in almost complete loss of activity, indicating that this residue is essential for activity. Because

Table 1
Lipase and esterase activities of the mutant proteins of PML

Proteins	Lipase		Esterase	
	specific activity (U/mg)	relative activity (%)	specific activity (U/mg)	relative activity (%)
Wild-type	2970	100	84.4	100
H30A	2770	93	68.8	82
H274A	2200	74	79.8	94
H291A	2410	81	75.8	89
H313A	< 10	< 0.3	< 0.05	< 0.06
H365A	2770	93	71.6	85
E253A	2780	94	51.5	61
D255A	< 10	< 0.3	< 0.05	< 0.06
D255N	< 10	< 0.3	< 0.05	< 0.06
D262A	3070	103	85.2	101
D275A	2300	77	70.1	83
D290A	2610	88	67.5	80

The lipase and esterase activities were determined by using olive oil and *p*-nitrophenyl laurate as a substrate, respectively, as described under Section 2. The specific activity is defined as the amount of fatty acid or *p*-nitrophenol (μmol) liberated per min per mg of enzyme. The experiment was carried out in duplicate, and errors from the average values were within 9% and 6% of the values reported for lipase and esterase activities, respectively.

the CD spectra of the mutant protein H313A in the far- and near-UV regions were similar to those of the wild-type protein (Fig. 2), inactivation by the mutation of His^{313} is not due to marked changes in the tertiary structure. These results suggest that His^{313} is one of the active-site residues that form a catalytic triad.

Identification of His^{313} as one of the active-site residues allowed us to predict that one of the acidic residues located between Ser^{207} and His^{313} is an active-site residue. It is Glu^{253}, Asp^{255}, Asp^{262}, Asp^{275}, and Asp^{290} (Fig. 1). Therefore, we constructed the mutant proteins E253A, D255A, D262A, D275A, and D290A, in which Glu^{253}, Asp^{255}, Asp^{262}, Asp^{275}, and Asp^{290} are individually replaced by Ala, respectively. We also constructed the mutant protein D255N, in which Asp^{255} is replaced by Asn. This mutation was designed to minimize a conformational change caused by the mutation, because the spatial volumes and hydrophilicities of the side chains of Asp and Asn are similar to each other. These mutant proteins were overproduced in *E. coli*, purified to give a single band on SDS–PAGE, and examined for the lipase and esterase activities. The results are summarized in Table 1. Of the five conserved acidic residues located between Ser^{207} and His^{313}, Glu^{253}, Asp^{262}, Asp^{275}, and Asp^{290} are not essential for activity, because the mutant proteins E253A, D262A, D275A, and D290A retained more than 60% of the lipase and esterase activities of the wild-type protein. In contrast, the mutant proteins D255A and D255N showed little lipase and esterase activities. The far- and near-UV CD spectra of the mutant proteins D255A and D255N were similar to those of the wild-type protein (Fig. 2), suggesting that inactivation by the mutation of Asp^{255} is not due to marked changes in the tertiary structure. These results indicate that the carboxyl group of Asp^{255} is required for activity. According to the catalytic mechanism proposed for lipases and esterases [2], the ionized carboxyl group is required to stabilize a protonated form of the imidazole group. Therefore, it is highly likely that Asp^{255} is one of the active-site residues that form a catalytic triad.

In summary, we have probed different candidate residues for the active-site histidine and acidic residues of PML by site-directed mutagenesis. The results strongly suggest that His^{313} and Asp^{255}, together with previously identified Ser^{207} [14], form a catalytic triad in PML. Identifications of all the active-site residues that form a catalytic triad in PML support a

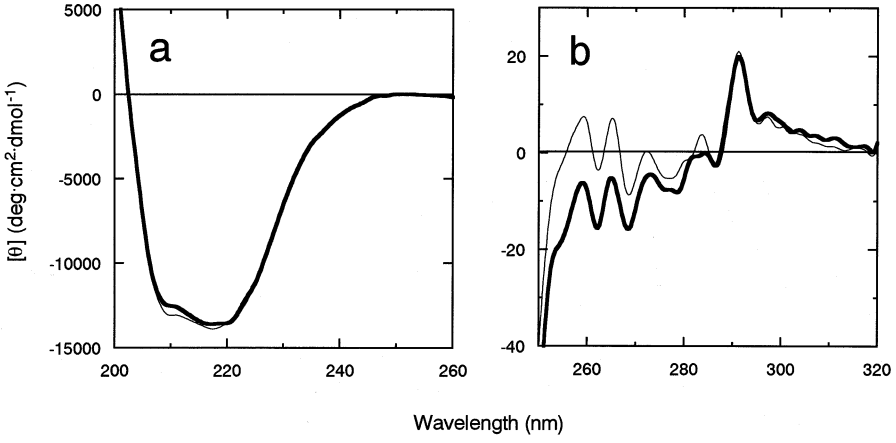

Fig. 2. CD spectra of the wild-type and mutant proteins. The far-UV (a) and near-UV (b) CD spectra of all mutant proteins were measured and those of the mutant protein H313A (thick line), which showed the largest difference from those of the wild-type protein (thin line), are shown as representatives. All spectra were measured as described under Section 2.

proposal that family I.3 lipases consist of N-terminal catalytic domain and C-terminal Ca^{2+}-binding domain [14]. These lipases showed Ca^{2+}-dependent lipase activity [14,23,24], probably because they do not assume a functional conformation in the absence of the Ca^{2+} ion. The C-terminal domains of these lipases may not be correctly folded in the absence of the Ca^{2+} ion and this incorrect folding may seriously affect the conformation of their N-terminal catalytic domains, as proposed for a metalloprotease with similar domain structure [25].

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