Ca^{2+}-induced folding of a family I.3 lipase with repetitive Ca^{2+} binding motifs at the C-terminus

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Abstract In order to understand a role of the Ca^{2+} ion on the structure and function of a Ca^{2+}-dependent family I.3 lipase from *Pseudomonas* sp. MIS38, apo-PML, holo-PML, holo-PML*, and the N-terminal domain alone (N-fragment) were prepared and biochemically characterized. Apo-PML and holo-PML represent refolded proteins in the absence and presence of the Ca^{2+} ion, respectively. Holo-PML* represents a holo-PML dialyzed against 20 mM Tris–HCl (pH 7.5). The results suggest that the C-terminal domain of PML is almost fully unfolded in the apo-form and its folding is induced by Ca^{2+} binding. The folding of this C-terminal domain may be required to make a conformation of the N-terminal catalytic domain functional.
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Key words: Lipase; Protein folding; Ca^{2+} binding; Circular dichroism; *Pseudomonas*

1. Introduction

Lipase from *Pseudomonas* sp. MIS38 (PML) is a member of the family I.3 lipase and is composed of 617 amino acid residues [1]. Family I.3 lipases, which are represented by lipases from *Pseudomonas fluorescens* [2,3] and *Serratia marcescens* [4,5], are distinguished from other lipases not only in amino acid sequence but also in their ability to be secreted by a three-component ATP-binding cassette (ABC) transporter system [6,7]. The genes encoding three components of the ABC transporters, which are involved in the extracellular secretion of lipases from *P. fluorescens* [8] and *S. marcescens* [9], have been identified.

In addition to family I.3 lipases, a variety of proteins are secreted from Gram-negative bacteria through both inner and outer membranes by ABC transporters [10,11]. They include hemolysin from *Escherichia coli*, leukotoxin from *Pasteurella haemolytica*, cyclolysin from *Bordetella pertussis*, a heme-binding protein (HasA) from *S. marcescens*, and metalloproteases from *Erwinia chrysanthemi*, *S. marcescens*, and *Pseudomonas aeruginosa*. These proteins do not contain a typical N-terminal signal sequence, but contain an uncleavable C-terminal secretion signal within the last 50–60 residues from the C-terminus [12–14].

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Abbreviations: PML, *Pseudomonas* sp. MIS38 lipase; ANS, 1-anilino-8-naphthalenesulfonic acid

Most of the proteins secreted by ABC transporters, including family I.3 lipases, contain multiple repeats of a nine-residue GGXGXDXUX sequence motif (X, any amino acid; U, large hydrophobic amino acid), which are located just upstream of a C-terminal secretion signal. It has been reported that a repeat of this sequence motif is not required for secretion of low-molecular-weight proteins, but is required for efficient secretion of high-molecular-weight proteins [13,15]. According to the crystal structures of metalloproteases from *P. aeruginosa* [16] and *S. marcescens* [17], these proteins consist of the N-terminal catalytic and C-terminal β-roll domains. Seven or eight repeats of a nine-residue sequence motif form a parallel β-roll structure in the C-terminal domain, to which five Ca^{2+} ions bind. The first six residues of this motif form a loop and each Ca^{2+} ion binds between a pair of these loops. However, it remains to be determined whether a β-roll structure is formed prior to the binding of Ca^{2+} ions or it requires Ca^{2+} ions for correct folding.

PML contains 12 repeats of a nine-residue sequence motif at the C-terminal region. It was overproduced in *E. coli* as inclusion bodies, solubilized in buffer containing 8 M urea, and refolded in the presence of the Ca^{2+} ion [1]. The enzyme was active only in the holo-form, in which $\sim$12 Ca^{2+} ions were bound. Site-directed mutagenesis studies revealed that all the catalytic residues are located at the N-terminal region [18]. These results suggest that PML consists of the N-terminal catalytic domain and the C-terminal β-roll domain like metalloproteases from *S. marcescens* and *P. aeruginosa*. In this report, we show that the conformation of apo-PML, which is refolded in the absence of the Ca^{2+} ion, is different from that of holo-PML, which is refolded in the presence of the Ca^{2+} ion. Biochemical characterizations of these two proteins, as well as those of the N-terminal domain alone, suggest that the C-terminal domain of PML is unfolded in the absence of Ca^{2+} and that its folding is induced by Ca^{2+} binding.

2. Materials and methods

2.1. Preparation of apo-PML, holo-PML, and holo-PML*

Recombinant PML was overproduced in *E. coli* as inclusion bodies, and solubilized and purified in the presence of 8 M urea, as described previously [1]. The purified protein was refolded by dialyzing against 20 mM Tris–HCl (pH 7.5) in the presence and absence of 5 mM $CaCl_2$ to produce holo-PML and apo-PML, respectively. Holo-PML* was prepared by dialyzing holo-PML against 20 mM Tris–HCl (pH 7.5).

2.2. Preparation of the N-fragment

The plasmid for the overproduction of the peptide from Met^1 to Ile^{372} of PML (N-fragment) was constructed by introducing a termi-

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nation codon into the 373th codon of the PML gene in the plasmid pET-25b(+) derivative for the overproduction of PML, by site-directed mutagenesis [19]. The oligonucleotide used as a mutagenic primer was designed to alter the 373th codon of the PML gene from GGC for Gly^{373} to TGA. The resultant plasmid was used to transform *E. coli* HMS174(DE3)pLysS (Novagen) to construct an overproducing strain. The nucleotide sequence of the mutated gene was confirmed using ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Norwalk, CT, USA).

Overproduction of the N-fragment as inclusion bodies, solubilization and purification of the recombinant protein in the presence of 8 M urea, and refolding were carried out as described previously for the intact protein [1]. The purity of the refolded protein was analyzed by SDS–PAGE [20] on a 12% polyacrylamide gel, followed by staining with Coomassie brilliant blue.

2.3. Biochemical characterizations

The molecular masses of the proteins were estimated by using a column (1.6×60 cm) of Superdex 200 (Pharmacia Biotech) equilibrated with 25 mM Tris–HCl (pH 7.5) containing 150 mM NaCl. Bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa) were used as standard proteins.

For native PAGE, samples were dissolved in 50 mM Tris–HCl (pH 6.8) containing 10% (v/v) glycerol and 0.5% (w/v) bromophenol blue, and applied to a polyacrylamide gel, in which a 4.75% gel (stacking gel) is laid on a 12% gel (running gel). Stacking and running gels were prepared in 0.125 M Tris–HCl (pH 6.8) and 0.375 M Tris–HCl (pH 8.8), respectively. Electrophoresis was carried out using 0.25 M Tris–glycine (pH 7.9) at 30 mA for 2 h, so that proteins migrated from cathode to anode. Proteins were stained with Coomassie brilliant blue.

The intrinsic fluorescence spectra of the proteins were measured with a Hitachi-2000 fluorescence spectrophotometer using a 1-cm cuvette at 25°C. The protein concentration was 10 μg/ml. The buffer was 20 mM Tris–HCl (pH 7.5) for apo-PML and holo-PML*, the same buffer containing 5 mM $CaCl_2$ for holo-PML, and the same buffer containing 4 M guanidine hydrochloride (GdnHCl) for PML in a denatured form. The excitation wavelength was 280 nm, and the emission was monitored from 300 to 400 nm.

Binding of 1-anilino-8-naphthalenesulfonic acid (ANS) (Sigma, St. Louis, MO, USA) to the protein was also analyzed by measuring the fluorescence of ANS at 25°C. The protein and ANS were dissolved in the buffer mentioned above. The concentration of the protein was 10 μg/ml (0.15 μM), and that of the ANS was 50 μM. The excitation wavelength was 376 nm, and the emission was monitored from 400 to 600 nm. The spectrum obtained in the absence of the protein was used as a blank.

The far-UV (200–260 nm) circular dichroism (CD) spectra were measured in 10 mM Tris–HCl (pH 7.5) in the presence or absence of 5 mM $CaCl_2$ at 20°C on a J-725 automatic spectropolarimeter (Japan Spectroscopic, Japan), unless otherwise stated. The protein concentration and optical path length were 0.07 mg/ml and 2 mm. The mean residue ellipticity, $[\theta]$, which has the unit deg cm^2/dmol, was calculated by using an average amino acid molecular weight of 110.

2.4. Enzymatic activity

The lipase and esterase activities were determined in 25 mM Tris–HCl (pH 7.5) at 30°C by using olive oil and *p*-nitrophenyl laurate (C_{12}) as a substrate, respectively, as described previously [1]. 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 concentrations were determined from UV absorption using an $A_{280}^{0.1\%}$ value of 1.14 for apo-PML or holo-PML and 1.45 for the N-fragment, which were calculated by using 1576 M^{-1} cm^{-1} for Tyr and 5225 M^{-1} cm^{-1} for Trp at 280 nm [21].

3. Results

3.1. Ca^{2+} content

The number of Ca^{2+} ions bound to holo-PML* was determined to be 11.3 ± 0.5 (average of the values obtained from two independent experiments) by atomic absorption spectrometry (Jarrel-Ash A-8500 Mark II), which was comparable to that (11.8) previously determined [1]. In contrast, the number of Ca^{2+} ions bound to the N-fragment, which was refolded in the presence of 10 mM $CaCl_2$ and then dialyzed against 20 mM Tris–HCl (pH 7.5), was determined to be <0.3. These results indicate that holo-PML* contains ~11 Ca^{2+} ions, whereas the N-fragment does not contain any.

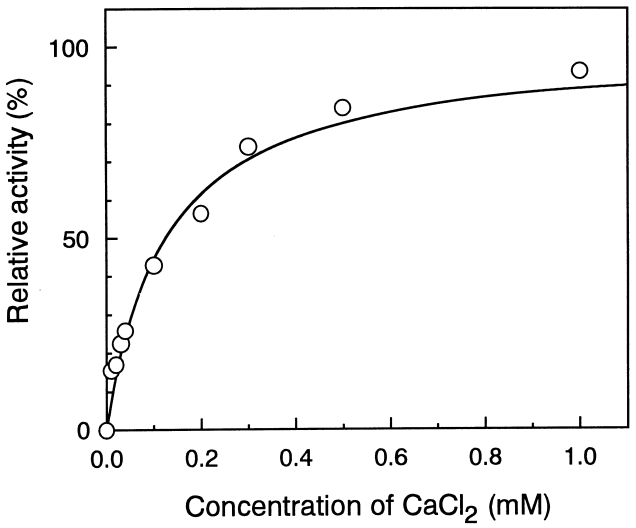

Fig. 1. Ca^{2+} dependence of the PML activity. The enzymatic activity of holo-PML* relative to that determined in the presence of 10 mM $CaCl_2$ is shown as a function of the $CaCl_2$ concentration. The PML activity was determined at 30°C for 10 min by using *p*-nitrophenyl laurate as a substrate [1]. The curve represents the theoretical data, from which the number of Ca^{2+} ions required for activity and its dissociation constant are determined to be 1 and 0.13 mM, respectively, by Scatchard analysis [22].

3.2. Enzymatic activity

Apo-PML and holo-PML* exhibited little enzymatic activity in the absence of $CaCl_2$ but fully exhibited both lipase and esterase activities in the presence of 10 mM $CaCl_2$. The specific activities of these proteins, which were similar to each other, were 2800 ± 300 U/mg for the hydrolysis of olive oil and 90 ± 10 U/mg for the hydrolysis of *p*-nitrophenyl laurate (C_{12}). These values were comparable to those of holo-PML.

Dependence of the enzymatic activity of holo-PML* on the $CaCl_2$ concentrations, which was analyzed by using *p*-nitrophenyl laurate (C_{12}) as a substrate, is shown in Fig. 1. From these data, the number and the dissociation constant of the Ca^{2+} ion(s) required for activity were determined to be 1.06 ± 0.03 and 0.13 ± 0.01 mM (S.E.M., $n=7$), respectively, by Scatchard analyses [22]. These results suggest that holo-PML* requires a single Ca^{2+} ion for activity and its dissociation constant is 0.13 mM. Interestingly, the Ca^{2+} dependence of the enzymatic activity of apo-PML was similar to that of holo-PML* (data not shown). Apo-PML is probably converted to holo-PML* if the Ca^{2+} concentration is much lower than 0.13 mM.

3.3. Molecular size

The molecular weights of holo-PML and holo-PML* were estimated to be 68 000 on gel filtration column chromatography, whereas that of apo-PML was estimated to be 90 000 (Fig. 2). The molecular weights of these proteins were equally estimated to be 66 000 on SDS–PAGE, suggesting that these

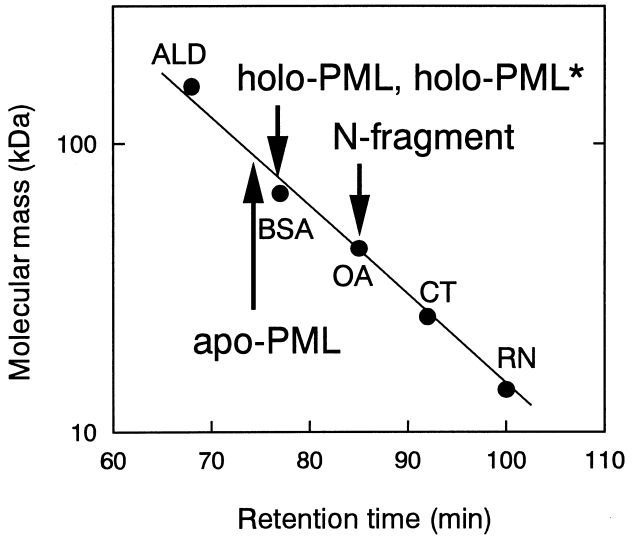

Fig. 2. Estimation of molecular weight. Apo-PML, holo-PML, holo-PML*, and the N-fragment were independently applied to a column of Superdex 200. Markers (Pharmacia Biotech): ALD, aldolase (158 kDa); BSA, bovine serum albumin (67 kDa); OA, ovalbumin (43 kDa); CT, chymotrypsinogen A (25 kDa); RN, ribonuclease A (14 kDa). The retention times of these proteins are indicated by arrows.

proteins exist in a monomeric form but apo-PML assumes a more extended conformation than does holo-PML or holo-PML*. A consistent result was obtained by native PAGE. Because the isoelectric point of holo-PML* must be higher than that of apo-PML due to the binding of the Ca^{2+} ions, and because the protein migrated in the gel from cathode to anode, holo-PML* should migrate more slowly than does apo-PML, if the conformational sizes of these proteins are similar to each other. However, as shown in Fig. 3, apo-PML migrated more slowly in the gel than did holo-PML*.

3.4. Intrinsic fluorescence spectra

The intrinsic fluorescence spectra of holo-PML, holo-PML*, and apo-PML, which were excited at 280 nm, gave the maximum intensities at 330 nm, 330 nm, and 337 nm, respectively (Fig. 4). These spectra were red-shifted in the presence of 4 M GdnHCl, so that they equally gave the maximum intensity at 346 nm, probably because the aromatic residues are fully exposed to the solvent. The wavelength maximum for intrinsic fluorescence of apo-PML is red-shifted as compared to that of holo-PML or holo-PML*, suggesting that apo-PML assumes a partially folded structure or a structure in which one part of the molecule is fully folded whereas the other part of the molecule is fully unfolded.

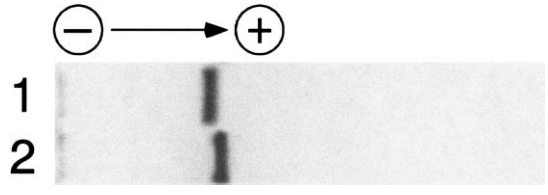

Fig. 3. Native PAGE of apo-PML and holo-PML*. Native PAGE was carried out using a 12% polyacrylamide gel as described in Section 2, followed by staining with Coomassie brilliant blue. Lanes: 1: apo-PML (1 μg); 2: holo-PML* (1 μg).

3.5. ANS fluorescence spectra

ANS binds more effectively to a protein with a partially folded structure than to a protein with a fully folded or unfolded structure [23]. However, the ANS fluorescence spectra of apo-PML, holo-PML*, and holo-PML were nearly identical to one another and gave the highest intensity at 480 nm (data not shown). These results suggest that a part of the holo-PML or holo-PML* structure is almost fully unfolded in the apo-PML structure.

3.6. CD spectra

In order to obtain more information on the conformations of apo-PML and holo-PML*, the far-UV CD spectra of apo-PML and holo-PML* were measured in the absence of $CaCl_2$. These spectra were considerably different from each other as shown in Fig. 5, suggesting that the content of the secondary structures of holo-PML* is higher than that of apo-PML. The difference spectrum, which represents the spectrum of holo-PML* relative to that of apo-PML, showed a broad trough with a minimum $[\theta]$ value at 219 nm (Fig. 5). This spectrum is similar to a typical one for β-structure [24], suggesting that the difference in the CD spectra of apo-PML and holo-PML* reflects the difference in their β-structure contents. The CD spectrum of holo-PML, which was measured in the presence of 5 mM $CaCl_2$, was identical to that of holo-PML*, suggesting that the conformation of holo-PML* is not seriously changed upon binding of the catalytically essential Ca^{2+} ion.

Holo-PML* gave the same spectrum as apo-PML in the presence of 10 mM EDTA, indicating that holo-PML* is converted to apo-PML upon EDTA treatment. Likewise, apo-PML gave the same spectrum as holo-PML or holo-PML* in the presence of 5 mM $CaCl_2$. This latter spectral change was not caused by other metal ions, such as Zn^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+}, suggesting that binding

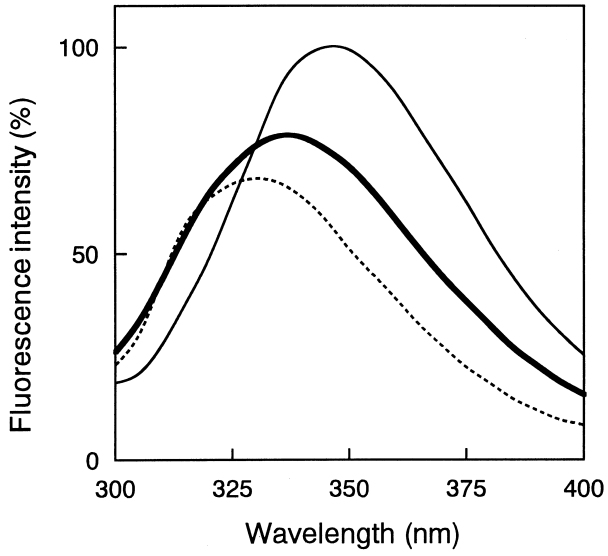

Fig. 4. Intrinsic fluorescence spectra of apo-PML and holo-PML*. The spectra of apo-PML (thick line), holo-PML* (broken line), and GdnHCl-denatured PML (thin line) were measured at 25°C, as described in Section 2. The protein concentrations were 10 μg/ml. The fluorescence intensities relative to that of GdnHCl-denatured PML at 346 nm are shown. The spectrum of holo-PML was measured as well, but is not shown because it was identical to that of holo-PML*.

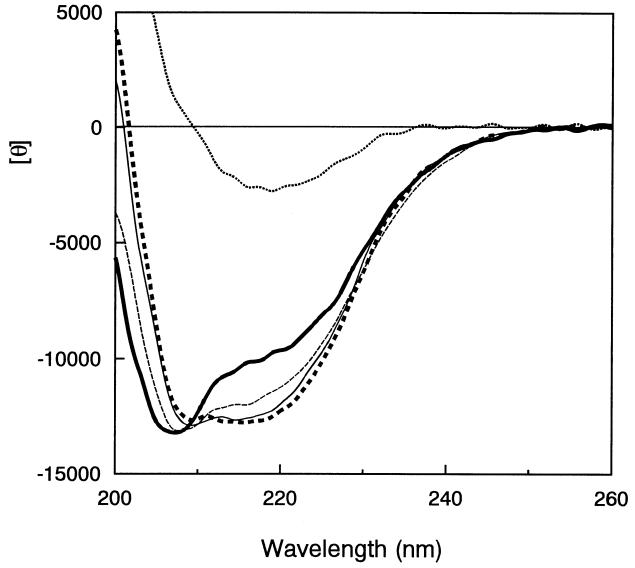

Fig. 5. Far-UV CD spectra. The far-UV CD spectrum of apo-PML measured in 10 mM Tris–HCl (pH 7.5) (thick line), and those measured in 10 mM Tris–HCl (pH 7.5) containing 5 μM (thin broken line) and 30 μM (thin line) $CaCl_2$ are shown. The spectrum of holo-PML* (thick broken line) measured in 10 mM Tris–HCl (pH 7.5) is also shown. All these spectra were measured at 20°C. The spectra of holo-PML and the N-fragment were measured as well, but are not shown because the former was identical to that of holo-PML* and the latter was almost identical to that of apo-PML. The protein concentration was 0.07 mg/ml (~1 μM). The difference spectrum between the spectra of apo-PML and holo-PML*, which was calculated by subtracting the CD values of apo-PML from those of holo-PML*, is represented by a dotted line.

of the Ca^{2+} ions to apo-PML specifically induces a conformational change to produce holo-PML or holo-PML*.

The CD spectrum of apo-PML (1 μM) measured in the presence of 30 μM $CaCl_2$ was nearly identical to that of holo-PML*, whereas that measured in the presence of 5 μM $CaCl_2$ was different from those of apo-PML and holo-PML* (Fig. 5), suggesting that apo-PML is almost fully converted to holo-PML* in the presence of 30 μM $CaCl_2$ but is partially converted to it in the presence of 5 μM $CaCl_2$. Apo-PML gave various different CD spectra at 0–30 μM $CaCl_2$. However, superposition of these spectra did not give a clear isodichroic point (data not shown), suggesting that apo-PML is not converted to holo-PML* in a two-state mechanism but converted to it through several intermediate forms which differ in Ca^{2+} content.

3.7. Properties of the N-fragment

The N-fragment (1–372) was prepared to examine whether the N-terminal catalytic domain of PML alone exhibits the enzymatic activity. Good agreement in the molecular weights estimated from gel filtration column chromatography (Fig. 3) and calculated from the amino acid sequence suggests that the N-fragment exists in a monomeric form. The far-UV CD spectrum of this protein was nearly identical to that of apo-PML (Fig. 5). This spectrum was basically unchanged in the absence or presence of Ca^{2+}. The N-fragment exhibited little lipase and esterase activity even in the presence of 10 mM $CaCl_2$.

4. Discussion

*4.1. Conformations of apo-PML and holo-PML**

In this article, we showed that apo-PML assumes an extended conformation as compared to that of holo-PML*. Intrinsic and ANS fluorescence spectra of this protein suggest that one part of this protein molecule is folded whereas the other part of it is unfolded. Because it has been proposed that PML consists of the N-terminal catalytic domain and the C-terminal β-roll domain [1], and because the N-fragment of PML (1–372) assumes a similar conformation as that of apo-PML as judged by CD spectra, apo-PML may represent an intermediate form, in which the N-domain is almost fully folded whereas the C-domain is almost fully unfolded. We also showed that folding of the C-terminal domain of PML is induced by Ca^{2+} binding. This folding is probably initiated by the formation of a β-roll structure, which is built of a succession of a nine-residue GGXGXDXUX sequence motif, because this sequence motif has been shown to bind Ca^{2+} ions internally in the β-roll structure [16,17]. It would therefore be informative to examine whether the C-terminal domain alone assumes a β-roll structure in a Ca^{2+}-dependent manner. However, attempts to overproduce the C-fragment of PML (373–617) in *E. coli* have so far been unsuccessful, probably because it is unstable in the *E. coli* cells.

4.2. Role of the C-domain

The observation that the N-fragment of PML did not exhibit enzymatic activity, despite the possession of all the catalytic residues, suggests that the C-terminal domain indirectly affects the conformation of the N-terminal catalytic domain of PML. Therefore, the conformation of the N-fragment may represent an inactive one. Because the N-domain of apo-PML assumes a similar conformation as that of the N-fragment, folding of the C-terminal domain of PML might be required to make the conformation of the N-terminal catalytic domain functional. However, the active conformation of the N-terminal catalytic domain may not be considerably different from the inactive one, because the major peptides generated from the N-fragment upon limited chymotryptic digestion were also generated from holo-PML, although holo-PML* was much more resistant to chymotryptic digestion than the N-fragment (H.-J. Kwon, personal communication). A similar role has been proposed for the C-terminal β-roll domain of metalloprotease from *P. aeruginosa* [25]. This protease lost the enzymatic activity by the mutation of Asp (to Ala), which is located within a nine-residue sequence motif and provides ligand for Ca^{2+} binding. It has been suggested that denaturation or imperfect folding of the C-terminal domain due to the lack of some Ca^{2+} ions affects folding of the N-terminal catalytic domain. Requirement of a repeat of a nine-residue sequence motif for cytolytic activity has also been reported for *E. coli* hemolysin [26].

Another role, which is related to secretion and the subsequent extracellular folding process, has been proposed for the β-roll structure [16,27]. According to this proposal, the proteins secreted by ABC transporters are synthesized in the cells in a form with an imperfectly folded structure, in which the C-terminal domain is unfolded, secreted across the inner and outer membranes in this form, and finally refolded into a functional conformation in an extracellular medium upon

binding of the Ca^{2+} ions to the C-terminal domain. Our results support this proposal, because the concentration of the free Ca^{2+} ions in the *E. coli* cells (<1 μM) is too low to fully convert apo-PML to holo-PML*, whereas that in the extracellular medium (>0.1 mM) is sufficiently high to fully convert it. The genes encoding the ABC transporter required for the extracellular secretion of PML have not been identified. However, we recently found that PML was effectively secreted into the extracellular medium from the *E. coli* cells carrying the *lipBCD* genes from *S. marcescens* (H. Kwon, K. Omori and S. Kanaya, unpublished). These *E. coli* cells were previously shown to effectively secrete *S. marcescens* lipase into the extracellular medium [9]. Therefore, there seems no doubt that PML is secreted by ABC transporters.

4.3. Requirement of Ca^{2+} for activity

In this article, we showed that PML requires Ca^{2+} not only for the formation of a functional structure but also for enzymatic activity. Enzymatically active holo-PML is stable only in the presence of the Ca^{2+} ion and is converted to inactive holo-PML* in the absence of the Ca^{2+} ion. The catalytically essential Ca^{2+} ion may bind to or in the vicinity of the active site of PML, like the Zn^{2+} ion of metalloproteases from *S. marcescens* [17] and *P. aeruginosa* [16]. However, it remains to be determined whether the catalytically essential Ca^{2+} ion binds to the N-terminal catalytic domain of PML, because it rather weakly binds to PML with a dissociation constant of 0.13 mM and can be dissociated from the protein upon dialysis. Determination of the crystal structure of PML complexed with Ca^{2+} will be required to identify this Ca^{2+} binding site.

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