Role of Repetitive Nine-Residue Sequence Motifs in Secretion, Enzymatic Activity, and Protein Conformation of a Family I.3 Lipase

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A family I.3 lipase from Pseudomonas sp. MIS38 (PML) contains 12 repeats of a nine-residue sequence motif in the C-terminal region. To elucidate the role of these repetitive sequences, mutant proteins PMLS, PML4, PML1, and PML0, in which 7, 8, 11, and all 12 of the repetitive sequences are deleted, and PMLA19, in which 19 C-terminal residues are truncated, were constructed. Escherichia coli DH5 cells carrying the Serratia marcescens Lip system permitted the secretion of the wild-type and all of the mutant proteins except for PMLA19, although they were partially accumulated in the cells in an insoluble form as well. Both the secretion level and cellular content of the proteins decreased in the order PML > PMLS > PML4 > PML1 > PML0, indicating that repetitive sequences are not required for secretion of PML but are important for its stability in the cells. All the mutant proteins were purified in a refolded form and their biochemical properties were characterized. CD spectra, the Ca
+ contents, and susceptibility to chymotryptic digestion strongly suggested that the five repetitive sequences remaining in PML5 are sufficient to form a β-roll structure, whereas the four in PML4 are not. PML5 and PMLA19 showed both lipase and esterase activities, whereas PML4, PML1, and PML0 were inactive. These results suggest that the enzymatic activity of PML is not seriously affected by a deletion or truncation at the C-terminal region as long as a succession of repetitive sequences can build a β-roll structure.

[Key words: lipase, mutation, protein conformation, secretion, Pseudomonas sp., Serratia marcescens Lip]

Family I.3 lipases, which are represented by lipases from Pseudomonas fluorescens (LipAyr) and Serratia marcescens (LipASM), are distinguished from other lipases not only by their amino acid sequences but also by their secretion mechanism (1, 2). They are secreted from gram-negative bacteria through both inner and outer membranes by a three-component ATP-binding cassette (ABC) transporter system (3–5). A hydrophobic five-residue sequence located between the 19th and 15th residues from the C-terminus of LipAyr, has been identified as a secretion signal (6).

Family I.3 lipases (7, 8), as well as most other proteins secreted by ABC transporters, such as Escherichia coli hemolysin (HlyA) (9), Erwinia chrysanthemi protease B (PrtB) (10), and Pseudomonas aeruginosa alkaline protease (AprApa) (11), contain several repeats of a nine-residue GGXGXDXUX sequence motif (X, any amino acid; U, large hydrophobic amino acid) in the upstream region of a C-terminal secretion signal (Fig. 1). According to the crystal structures of AprApa (12, 13) and S. marcescens metalloprotease (PrtASM) (14, 15), these repetitive sequences form a parallel β-roll structure (two repeats per one roll), to which 5–6 Ca
+ ions bind. The first six residues of this sequence motif form a loop and the last three form a β-strand. Each Ca
+ ion binds between a pair of these loops. The role(s) of these repetitive sequences remains to be fully understood. From analyses of the secretion of mutant proteins of HlyA (16) and PrtB (17), which lack most of the repetitive sequences, and their enzymatic activities in the culture supernatant and cell extracts, it has been suggested that the repetitive sequences are not required for secretion but are needed for activity. However, it remains to be determined whether deletion of these repetitive sequences seriously affects protein conformation and/or secretion efficiency. It has also yet to be elucidated whether all of the repetitive sequences are required for activity.

A lipase from Pseudomonas sp. MIS38 (PML), a member of family I.3, is composed of 617 amino acid residues (18). It shows amino acid sequence identities of 60.8% to LipASM and 57.4% to LipAyr, and contains 12 repeats of a nine-residue sequence motif in the C-terminal region (Fig. 1). Recombinant PML is overproduced in E. coli in inclusion bodies, solubilized in a buffer containing 8 M urea, and refolded in the presence of the Ca
+ ion (18). The enzyme is active only in a holo-form, in which ~12 Ca
+ ions bind. Site-directed mutagenesis studies have revealed that Ser207, Asp235,
and His\textsuperscript{311} form a catalytic triad (19). Limited proteolysis of recombinant PML produces an N-terminal fragment (1–370) and a C-terminal fragment (371–617), which respectively contain all of the catalytic residues and repetitive sequences. These findings suggest that PML consists of an N-terminal catalytic domain and a C-terminal \( \beta \)-roll domain, like AprA\textsubscript{A} (12) and PrtA\textsubscript{II} (14).

In the work reported here, we constructed four mutant proteins of PML, in which 7, 8, 11, and all 12 of the repetitive sequences are deleted, as well as a mutant protein with GGAATTCATATGGGT-CXGTATGACTA-3') and primer 2 (5'-TC PML gene was previously shown to be a secretion signal (6), is shown as a black box. Numbers indicate the positions of the amino acid residues starting from the initial methionine residue. PML, \emph{Pseudomonas} sp. Mis38 lipase (2DDB AB025596); LipA\textsubscript{A}, \emph{P. fluorescens} lipase (GenBank AF083061); AprA\textsubscript{A}, \emph{P. aeruginosa} alkaline protease (PIR S26699); PrtB, \emph{E. chrysanthemi} protease B (GenBank J04736); HlyA, \emph{E. coli} hemolysin (PIR P09883).

\textbf{Materials and Methods}

**Cells and plasmids** The \emph{Escherichia coli} strains used were DH5\textsuperscript{F}, hisdR17 (\( {\tau}_{C}, m_{C}^{+} \)), recA1, endA1deoR, thi-1, supE44, gyrA96, relA1) and HMS74(DE3)pLysS [F, recA1, hisD (\( {\tau}_{C}, m_{C}^{+} \)), Rif\textsuperscript{R} (DE3) pLysS(Cm\textsuperscript{R})]. Plasmid pYBCD20 harboring the \textit{lipBCD} gene from \emph{Serratia marcescens} SM8000 (20) and the plasmid pET-25b\textsuperscript{b} (original, (15) and the 3'-mutagenic primer, and the other with the 5'-mutagenic primer, and the other with the 5'-mutagenic primer and primer 2. These two PCR products were mixed and the secondary PCR product was amplified using only primers 1 and 2. The 5'-mutagenic primers for amplification of the genes encoding PML5, PML4, PML1, and PML0 were 5'-CCGT GGTGCGTACAAACACACCTCTGGTACCGGGCCTG-3', 5'-AG TACTTTTCTCATCGCGGCCAGCGCAAGAG-3', 5'-AGATCTTCTAATCAACACGGCCTG-3', and 5'-AGATCTTTTCTCATCGGTTTACGTCAACG-3'. These 3'-mutagenic primers were complementary to these primers. The PCR products were digested with EcoRI and HindIII and ligated into the EcoRI-HindIII sites of pUC18.

Plasmids pET-PML5, pET-PML4, pET-PML1, pET-PML0, and pET-PML\textsubscript{19} were amplified by PCR using primer 1 and primer 2, and the 3'-mutagenic primer, and the other with the 5'-mutagenic primer and primer 2. These two PCR products were mixed and the secondary PCR product was amplified using only primers 1 and 2. The 5'-mutagenic primers for amplification of the genes encoding PML5, PML4, PML1, and PML0 were 5'-CCGT GGTGCGTACAAACACACCTCTGGTACCGGGCCTG-3', 5'-AG TACTTTTCTCATCGCGGCCAGCGCAAGAG-3', 5'-AGATCTTCTAATCAACACGGCCTG-3', and 5'-AGATCTTTTCTCATCGGTTTACGTCAACG-3'. These 3'-mutagenic primers were complementary to these primers. The PCR products were digested with EcoRI and HindIII ligated into the EcoRI-HindIII sites of pUC18.

Plasmids pET-PML5, pET-PML4, pET-PML1, pET-PML0, and pET-PML\textsubscript{19} were overproduced by PML5, PML4, PML1, PML0, and PML19, respectively, were constructed by digesting the plasmid pUC18 derivatives with \( NdeI \) and HindIII, followed by ligation of the resultant \( NdeI \)-HindIII fragments into the HindIII sites of pET-25b\textsuperscript{b}.

PCR was performed in 30 cycles using a thermal cycler (GeneAmp PCR System 2400; Perkin-Elmer, Tokyo) and Vent polymerase (New England Biolabs, Beverly, MA, USA). The nucleotide sequences of the genes encoding mutant proteins were confirmed with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer).

**Secretion** \emph{E. coli} DH5\textsuperscript{F} cells were first transformed with plasmid pYRC120 and then with a plasmid derivative. The resultant recombinant cells carrying the two different plasmids were grown in LB medium (21) containing 50 \( \mu \)g/ml ampicillin and 30 \( \mu \)g/ml chloramphenicol at 30°C for 24 h with vigorous shaking. The \emph{S. marcescens} \textit{lipBCD} gene and the gene encoding PML or mutant PML were constitutively expressed in these recombinant cells. The culture was then centrifuged at 15,000 \( \times \)g for 10 min at 4°C to separate the cells and the supernatant. The cells were washed once with \( 50 \) mM Tris-\( \text{HCl} \) (pH 7.5) and dissolved in 0.1 M Tris-\( \text{HCl} \) (pH 6.8) containing 2% SDS, 5% (v/v) \( \beta \)-mercaptoethanol, 10% DTT, and His\textsuperscript{311} form a catalytic triad (19). Limited proteolysis of recombinant PML produces an N-terminal fragment (1–370) and a C-terminal fragment (371–617), which respectively contain all of the catalytic residues and repetitive sequences. These findings suggest that PML consists of an N-terminal catalytic domain and a C-terminal \( \beta \)-roll domain, like AprA\textsubscript{A} (12) and PrtA\textsubscript{II} (14).

In the work reported here, we constructed four mutant proteins of PML, in which 7, 8, 11, and all 12 of the repetitive sequences are deleted, as well as a mutant protein with a C-terminal 19-residue truncation, and analyzed their secretion by using recombinant \emph{E. coli} cells carrying the \emph{S. marcescens} ABC transporter system (Lip system). We also purified these mutant proteins and analyzed their biochemical properties. Based on the results, we discuss possible roles of the repetitive sequences of PML.
(v/v) glycerol, and 0.02% bromophenol blue (SDS sample buffer) to yield a whole cell extract. To estimate the amounts of the proteins accumulated intracellularly and secreted into the external medium, the culture supernatant and whole cell extract were analyzed by SDS-PAGE (22) on a 12% polyacrylamide gel, followed by staining with Coomassie brilliant blue (CBB). Because the concentrations of the proteins in the culture supernatant were too low to be detected by CBB staining, these proteins were precipitated by 70% (v/v) acetone and dissolved in the SDS sample buffer at a concentration sufficient for detection by CBB staining.

**Overproduction, purification, and refolding E. coli HMSN174(DE3)pLysS cells were transformed with pET-25b(+) derivatives and grown in L-broth containing 50 μg/ml ampicillin at 37°C. Overproduction of the mutant proteins of PML in inclusion bodies, solubilization and purification in the presence of 8 M urea, and refolding in the presence of the Ca²⁺ ion were done as previously described for recombinant PML (18).**

**Gel filtration** Gel-filtration chromatography was carried out using a column (ø2.2 × 95 cm) of Sephacryl S-300 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) equilibrated with 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. To estimate the molecular weights of the proteins, bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa) were used as standards.

**Protease digestion** The protein was digested with chymotrypsin at 25°C for 10 min in 10 mM Tris-HCl (pH 8.0) containing 2.5 mM diithiothreitol (DTT) at substrate:enzyme ratios of 50:1-500:1 (by weight). Degradation of the protein was analyzed by 12% SDS-PAGE, followed by CBB staining.

**Enzymatic activity** The lipase and esterase activities were determined in 25 mM Tris-HCl (pH 7.5) at 30°C using olive oil and p-nitrophenyl laurate (C₁₈) respectively as the substrate, as described previously (18). 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. The protein concentration was determined from UV absorption using an A₂₈₀ value of 1.1 for PML, and 1.2 for PML5, PML4, PML1, PML0, and PML19, which were calculated using 1576 M⁻¹ cm⁻¹ for Tyr and 5225 M⁻¹ cm⁻¹ for Trp at 280 nm (23). *E. coli* DH5 transformants with plasmid pYBCD20 and a pUC18 derivative were also examined for lipase production by *in situ* plate assay. The transforms were grown on LTB-agar medium (L broth supplemented with 1% (v/v) glycerol, and 0.02% bromophenol blue (SDS sample buffer) at a concentration of 10 mM CaCl₂ at 20°C on an automatic spectrophotometer (model J-725; Japan Spectroscopic, Tokyo). The protein concentration and optical path length were 0.1 mg/ml and 2 mm for far-UV CD spectra, and 1.0 mg/ml and 10 mm for near-UV CD spectra. The mean residue ellipticity, [θ] (deg cm² dmol⁻¹), was calculated using an average amino acid molecular weight of 110.

**Circular dichroism** Circular dichroism (CD) spectra, and 1.0 mg/ml and 10 mm for near-UV CD spectra were measured in 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl, at 20°C on an automatic spectropolarimeter (model J-725; Japan Spectroscopic, Tokyo). The protein concentration and optical path length were 0.1 mg/ml and 2 mm for far-UV CD spectra, and 1.0 mg/ml and 10 mm for near-UV CD spectra. The mean residue ellipticity, [θ] (deg cm² dmol⁻¹), was calculated using an average amino acid molecular weight of 110.

**Western blot (immunoblot) analysis** Western blotting was done as described previously (24). Proteins were subjected to SDS-PAGE on a 12% polyacrylamide gel and electrophoretically transferred to a PVDF membrane (Bio-Rad, Tokyo). The blots were blocked by soaking in 5% skim milk (Difco Laboratories, Detroit, MI, USA) in 25 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and incubated with antibas (diluted 1:100), which previously produced against *S. marcescens* lipase (LipA₄₈₁) (7). Signals were detected with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G.

**RESULTS**

**Mutant design** PML is similar in size to LipA₄₈₁ but much larger than LipA₄₈. Comparison of the amino acid sequence of PML with that of LipA₄₈ reveals that the PML sequence has a 138-residue insertion in the C-terminal region where a putative nine-residue sequence motif is repeated (Fig. 1). If this insertion is excluded, PML shows an amino acid sequence identity of 74.4% to LipA₄₈. Since this insertion contains seven repetitive sequences, LipA₄₈ contains only five such sequences in total at positions 373–405. Thus, PML and LipA₄₈ differ considerably in the number of their repetitive sequences. This raises the question whether the repetitive sequences can be reduced from 12 to 5, or less than 5, without seriously affecting the secretion efficiency and/or the enzymatic activity of PML. Therefore, we constructed the mutant proteins PML5, PML4, PML1, and PML0 (Fig. 2) and examined their secretion and enzymatic activities. PML5 lacks the 138-residue insertion (residues 406–543) and therefore contains five repetitive sequences like LipA₄₈. PML4 lacks the first repetitive sequence (residues 373–381) in addition to the 138-residue insertion, and thus contains four repetitive sequences. PML1 and PML0 lack the initial 11 (residues 373–546) and all 12 (residues 373–557) of the repetitive sequences, respectively. In addition, we constructed the mutant protein PML19, in which 19 C-terminal residues are truncated, to examine whether it is not secreted into the medium but is enzymatically active. PML19 does not contain a secretion signal which, according to a recent report by Omori et al. (6), is located at positions 19–15 from the C-terminus of PML.

**PML secretion system** To examine whether *E. coli* DH5 cells carrying the *S. marcescens* Lip system (20) can be used to secrete PML, these cells were transformed with the plasmid pUC-PML. The resultant recombinant cells grew rather poorly and the OD₆₀₀ value of the culture reached only 1.3 even in the stationary phase. Protein analysis in the culture supernatant and whole cell extracts by SDS-PAGE indicated that a relatively large portion of the PML was secreted into the external medium, while the rest accumulated intracellularly (Fig. 3). Sonication lysis of the cells followed by centrifugation and SDS-PAGE revealed that PML accumulated intracellularly in an insoluble form (data not shown). From the intensities of the bands visualized by CBB staining, the amounts of PML secreted into the external medium and accumulated insolubly in the cells were estimated to be roughly 50 and 30 mg/l culture, respectively. In contrast, when *E. coli* DH5 cells not carrying the Lip system were transformed with pUC-PML, PML was not secreted into the external medium but accumulated intracellularly in an insoluble form. The secretion of PML is thus dependent on the Lip system.

PML was purified from the culture supernatant by applying an aliquot of it onto a gel-filtration column. PML was eluted from the column as a single peak. It gave a single band on SDS-PAGE and had the same far-UV and near-UV CD spectra as the refolded protein (data not shown). The molecular weights of PML estimated by gel-filtration chromatography and SDS-PAGE were 68,000 and 66,000, respectively, suggesting that, like the refolded protein, it ex-
FIG. 2. Schematic representations of the primary structures of PML and its mutant proteins. Numbers indicate the positions of the amino acid residues located at the C-terminus of each sequence block, except for that for the initial methionine residue. Each arrow represents the nine-residue GGGXGXDXU sequence motif. The amounts of proteins secreted into the external medium (external) and accumulated within the cells (internal), which were estimated from the intensities of bands visualized by Coomassie brilliant blue staining or immunoblotting analysis following SDS-PAGE, are also shown.

FIG. 3. SDS–PAGE analysis of proteins secreted into the external medium and accumulated in cells. Recombinant E. coli DH5 cells were cultivated and the culture supernatant and whole cell extract were prepared as described in Materials and Methods. The culture supernatant (A) and whole cell extract (B, C) (100 μl culture equivalent each) were applied to 12% SDS-PAGE. The gels were stained with Coomassie brilliant blue (A, B) or analyzed by immunoblotting with antisera against LipAα (C). M, A low molecular weight marker kit (Amersham Pharmacia Biotech); lane 1, purified PML (1 μg); lanes 2–7, E. coli DH5 carrying plasmids pYBCD20 and pUC-PML (lane 2), pUC-PML5 (lane 3), pUC-PML4 (lane 4), pUC-PML1 (lane 5), pUC-PML0 (lane 6), pUC-PMLΔ19 (lane 7), or pUC18 (lane 8).
of the proteins, which are summarized in Fig. 2, decreased in the order PML > PML5 > PML4 > PML1 > PML0. The secretion level and the cellular content of PML0 were 50- and 600-fold lower than those of PML. In contrast, PMLA19 was not secreted into the external medium but it did accumulate in the cells (Fig. 3).— probably because this mutant does not contain a secretion signal. Its absence from the culture supernatant was confirmed by immunoblotting analysis (data not shown). The amount of PMLA19 that accumulated in the cells was 100-fold lower than that of PML.

Purification of mutant proteins The mutant proteins were overproduced in E. coli HMS174(DE3)pLysS cells transformed with pET-25b(+) derivatives in inclusion bodies, solubilized and purified in the presence of 8 M urea, and refolded in the presence of the Ca²⁺ ion. The molecular weights of PML5, PML4, PML1, and PML0 were comparable to that of PML (roughly 50 mg/l culture), whereas that of PMLA19 was 2–3 times lower (Fig. 4). All these mutant proteins were purified to give a single band on SDS-PAGE with a yield of approximately 70% (data not shown). The production levels of PML5, PML4, PML1, and PML0 were comparable to that of PML (approximately 50 mg/l culture), whereas that of PMLA19 was 2–3 times lower (Fig. 4). All these mutant proteins were purified to give a single band on SDS-PAGE with a yield of approximately 70% (data not shown). The production levels of PML5, PML4, PML1, and PML0 were comparable to that of PML (approximately 50 mg/l culture), whereas that of PMLA19 was 2–3 times lower (Fig. 4).

Enzyme activities The lipase and esterase activities of the refolded proteins, which were respectively determined at 30°C using olive oil and p-nitrophenyl laurate as the substrates, are summarized in Table 1. PML5 showed both lipase and esterase activities. The specific lipase and esterase activities of PML5 were roughly comparable to those of PML. In contrast, PML4, PML1, and PML0 showed neither lipase nor esterase activity. PMLA19 also showed both lipase and esterase activities, but the specific activities were much lower than those of PML.

The enzymatic activities of these mutant proteins were further confirmed by an in situ plate assay using an LTB-agar plate. If E. coli transformants producing lipase or esterase grew on the plate, a halo would be formed around each colony due to the hydrolysis of tributyrin emulsified in the LTB-agar medium by the enzyme. When recombinant E. coli DH5 cells carrying the plasmid pYBCD20 and a pUC18 derivative were grown on the plate, those secreting PML and PML5 gave a clear halo, whereas those secreting PML4, PML1, and PML0 gave no halo at all. Recombinant cells producing PMLA19 gave a weak halo, despite the fact that this mutant protein accumulated only intracellularly, which was probably due to leakage from the cells.

CD spectra The near-UV CD spectra of the wild-type and all the mutant proteins were similar to one another (Fig. 5B), suggesting that the three-dimensional environments of the aromatic residues in the protein were not seriously affected by the mutations. In contrast, the far-UV CD spectra of the mutant proteins differed from that of PML (Fig. 5A), suggesting that the protein secondary structures were altered by the mutations. The spectrum of PML exhibited a broad trough with two minimum [θ] values of -12,600 at 209 nm and -12,800 at 216 nm, whereas that of PML5 had a trough with a minimum [θ] value of -13,000 at 208 nm followed by a shoulder with a [θ] value of 11,000 at 220 nm. The spectrum of PML4 was similar to that of PML5 in the depth of the trough at 208 nm, but the shoulder at around 220 nm was higher than that of PML5. The spectra of PML1 and PML0 were basically identical to that of PML4 and the spectrum of PMLA19 was very similar to that of PML5.

Ca²⁺ content The number of the Ca²⁺ ions which remained bound to the protein after extensive dialysis was determined to be 11 ± 0.5 for PML, 5.0 ± 0.2 for PML5, 1.8 ± 0.2 for PML4, 1.6 ± 0.2 for PML1, and 1.4 ± 0.2 for PML0, and 6.5 ± 0.2 for PMLA19 by atomic absorption spectrometry on a Jarrel-Ash A-8500 Mark II spectrometer. These values represent an average of the values obtained from two independent experiments.

Stability PML, PML5, and PMLA19 (0.01 mg/ml) were incubated in 20 mM Tris-HCl (pH 7.5) containing 10 mM CaCl₂ at 50°C, and the remaining activities at 30°C were de-

![FIG 4. SDS-PAGE analysis of overproduced proteins. PML and its mutant proteins were overproduced in E. coli HMS174(DE3)pLysS cells transformed with plasmid pET-25b(+) derivatives as described in Materials and Methods. The whole cell extract (50 μl culture equivalent) was applied to 12% SDS-PAGE. The gel was stained with Coomassie brilliant blue. M. A low molecular weight marker kit (Amer- sham Pharmacia Biotech); lane 1, purified PML (1 μg); lanes 2–7, E. coli HMS174(DE3)pLysS carrying plasmid pET-PML (lane 2), pET-PML5 (lane 3), pET-PML4 (lane 4), pET-PML1 (lane 5), pET-PML0 (lane 6), pET-PMLA19 (lane 7), or pET-25b(+) (lane 8).]
FIG. 5. CD spectra of wild-type and mutant proteins. The spectra of the wild-type and all the mutant proteins were measured in 10 mM Tris-HCl (pH 7.5) containing 10 mM CaCl₂ at 20°C as described in Materials and Methods. (A) The far-UV CD spectra of PML (thick line), PMLS (thin line), and PML4 (broken line) are shown. The far-UV CD spectra of PML₁, PML₀, and PML∆₁₉ are not shown because the spectra of PML₁ and PML₀ were nearly identical to that of PML4 and the spectrum of PML∆₁₉ was almost the same as that of PML₅. (B) The near-UV CD spectrum of PML₁ (thin line), which exhibited the largest difference from that of PML (thick line), is shown as a representative spectrum. The mean residue ellipticity, [θ], is given in deg cm² dmol⁻¹.

FIG. 6. Susceptibility of proteins to chymotryptic digestion. Proteins were digested with chymotrypsin at 25°C for 10 min in 10 mM Tris-HCl (pH 8.0) containing 2.5 mM DTT at a substrate:enzyme ratio of 50:1 for PML (lane 2) and PMLS (lane 4), and 500:1 for PML4 (lane 6). Samples were analyzed by 12% SDS-PAGE, followed by CBB staining. M, A low molecular weight marker kit (Amersham Pharmacia Biotech); lane 1, undigested PML; lane 3, undigested PMLS; lane 5, undigested PML4.

terminated at appropriate intervals using p-nitrophenyl laurate as a substrate. PML, PMLS, and PML∆₁₉ lost half of their activities upon incubation for 20, 15, and 3 min, respectively, indicating that PML₅ and PML∆₁₉ are slightly and markedly less stable against heat inactivation than PML.

Susceptibility to chymotryptic digestion

Upon digestion with chymotrypsin at 25°C for 10 min at a substrate:enzyme ratio of 50:1, roughly 80% and 50% respectively of the PML and PMLS molecules remained intact (Fig. 6). In contrast, most of the PML₄ molecules failed to stay intact, even at a substrate:enzyme ratio of 500:1 (Fig. 6). Most of the PML₁, PML₀, and PML∆₁₉ molecules also did not remain intact upon chymotryptic digestion at a substrate:enzyme ratio of 500:1 (data not shown). These results indicate that the susceptibilities of PML₄, PML₁, PML₀, and PML∆₁₉ to chymotryptic digestion are much higher than that of PML₅, while PML₅ itself is slightly more susceptible to chymotryptic digestion than PML.

DISCUSSION

Validity of using refolded proteins for characterization

We used refolded proteins for biochemical characterization in this study because we were not able to purify all the mutant proteins secreted into the external medium. We could purify PML₅ from the culture supernatant in the same manner as PML by applying an aliquot of it onto a gel-filtration column. However, PML₄, PML₁, and PML₀ could not be purified from the supernatant by the same procedure because their concentrations were too low. Precipitation of the proteins with ammonium sulfate, acetone, or ethanol was not effective in concentrating them because they were not solubilized in their native form once they were precipitated. Ultrafiltration was also ineffective because the culture supernatant contained various substances at relatively high concentrations. Purification by ion-exchange column chromatography was tried, but the proteins were not retained in either an anion-exchange or a cation-exchange column under any of the conditions examined. The specific activity of the refolded protein of PML is lower than but comparable to that of the secreted one, suggesting that PML is refolded into the native enzyme but with a yield of less than 100%. In addition, insofar as could be ascertained from the far- and near-UV CD spectra data, the refolded protein was structurally identical to the secreted one. These findings strongly suggest that the biochemical properties of the refolded proteins reflect those of the native ones.

Role of repetitive sequences in protein conformation

When the far-UV CD spectra of PML, PMLS, and PML₄ are compared, it can be seen that the depth of the trough at 210–230 nm decreases in line with a reduction in the number of repetitive sequences in the protein (Fig. 5A). Because a typical spectrum for a β-structure gives a broad trough in this region with a minimum [θ] value at 218 nm (25), and the last three residues of each repetitive sequence form a β-strand in a β-roll structure, the decrease in the depth of the trough at 210–230 nm may result from a lower β-roll content in the protein. However, PML₁ and PML₀ showed the
same spectrum as PML4, despite the absence of most or all of the repetitive sequences. These results suggest that in PML4, which contains four repetitive sequences, a β-roll structure is not formed, or is only poorly formed. The determinations of Ca^{2+} ions bound to these proteins and their susceptibility to chymotryptic digestion support this hypothesis. When the Ca^{2+} contents of PML, PML5, and PML0 are compared, the Ca^{2+} content is observed to decrease in proportion to the reduction in the number of repetitive sequences. However, PML4 has a similar Ca^{2+} content to PML0. Likewise, when the susceptibility of each of the mutant proteins to chymotryptic digestion is compared with that of PML, the susceptibility of PML5 is seen to be rather similar to that of PML, whereas PML4, PML1, and PML0 have comparable susceptibilities, which are much higher than that of PML. It has been reported that digestion by protease proceeds mainly via the unfolded state of proteins (26). Because the equilibrium between the folded and unfolded states of the protein must be shifted so that the fraction of the unfolded state increases, when a part of the protein molecule is unfolded, the dramatic increase in the susceptibility of PML4 to chymotryptic digestion as compared to PML5 may result from an inability of PML4 to form a β-roll structure.

The near-UV CD spectra of PML5, PML4, PML1, and PML0 were similar to that of PML (Fig. 5B). Because the near-UV CD spectrum reveals the three-dimensional environments of aromatic residues and most of the aromatic residues (six out of eight tryptophan and 16 out of 20 tyrosine residues) are located in the putative N-terminal catalytic domain of PML, these spectra may reflect the conformation of its N-terminal catalytic domain. These results suggest that removal of the repetitive sequences alters the β-roll structure of PML without seriously affecting the conformation of its N-terminal catalytic domain.

Role of repetitive sequences in enzymatic activity

The lipase and esterase activities of PML5 were comparable to those of PML, indicating that the number of repetitive sequences in PML can be reduced to five without seriously affecting the enzymatic activity. In contrast, PML4 showed neither lipase nor esterase activity, indicating that removal of an additional single repetitive sequence inactivates the protein. As mentioned above, a β-roll structure seems to be fully formed in PML5, but poorly formed in PML4. Therefore, formation of a β-roll structure may be required to make the conformation of a putative N-terminal catalytic domain functional. This conformational change may be too subtle to be detected by the near-UV CD spectra. A similar role has been proposed for the β-roll structure of AprA₁₉ (27). This protease lost enzymatic activity by the mutation of Asp (to Ala), which is located within a repetitive sequence and provides a ligand for Ca^{2+} binding. It has been suggested that denaturation or imperfect folding of the C-terminal domain affects folding of the N-terminal catalytic domain. Likewise, HlyA lost hemolytic activity upon deletion of 11 of 13 repetitive sequences (16), probably because the conformation of a catalytic domain is slightly altered in the absence of a β-roll structure.

It is noted that the stability of PML5 against heat inactivation was slightly lower than, but comparable to, that of PML, suggesting that the number of repetitive sequences in PML can be reduced to five without seriously affecting the protein stability as well.

Role(s) of repetitive sequences in secretion

The fact that PML0 was secreted into the external medium indicates that the repetitive sequences are not required for secretion. However, we found that the repetitive sequences are important for the stability of the protein in the cells, because both the amount of the mutant protein secreted into the external medium and that accumulated in the cells decreased as the number of repetitive sequences in the mutant protein was reduced. If the repetitive sequences were not required for the stability of the protein in the cells, but were required to increase the secretion efficiency, the amount of the protein accumulated in the cells would increase as the number of repetitive sequences was reduced. Mutant proteins lacking most of the repetitive sequences may be highly unstable and rapidly degraded by proteases in the cells before they are recognized by the ABC transporters. It seems unlikely that the genes encoding these mutant proteins are poorly expressed, because all the genes encoding the mutant and wild-type proteins were expressed with comparable efficiencies when the pET system was used. It has been suggested that proteins secreted by ABC transporters are synthesized in the cells 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 the binding of Ca^{2+} ions to the C-terminal domain (12, 28). If this is the case, the repetitive sequences may be required to increase the stability of the imperfectly folded structure.

Based on analyses of the ability of a C-terminal fragment of HlyA and PrtB to promote the secretion of passenger proteins (29–31), it has also been suggested that repetitive sequences are required to separate the C-terminal secretion signal from the passenger domain and thereby facilitate recognition of the signal by ABC transporters. The repetitive sequences may play such a role in PML as well.

The repetitive sequences of PML are not consecutive but are interrupted by a large peptide insertion (Fig. 1). Similarities in the enzymatic activity, stability, and secretion level between PML5 and PML suggest that this inserted sequence is not functionally important. However, it remains to be determined whether the PML repetitive sequences constitute a single large β-roll structure or two independent β-roll structures.

Roles of the 19 C-terminal residues

Truncation of 19 C-terminal residues creates a mutant protein that lacks the ability to be secreted via the Lip system. In addition, it greatly affects the enzymatic activity, susceptibility to chymotryptic digestion, and stability of PML. Because the depth of the trough in the far-UV CD spectrum of PML1₉ at 210–230 nm was less than that of PML (Fig. 5A) and only 6–7 Ca^{2+} ions bound to it, a β-roll structure is probably not fully formed in PML1₉. Thus, the 19 C-terminal residues are required not only for the secretion of PML but also to make the conformation of the protein fully functional.
REFERENCES