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The role of the N-terminal propeptide of the pro-aminopeptidase processing protease: refolding, processing, and enzyme inhibition

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Abstract

Pro-aminopeptidase processing protease (PA protease) is an extracellular zinc metalloprotease produced by *Aeromonas caviae* T-64 and it is classified as M04.016 according to the MEROPS database. The precursor of PA protease consists of four regions; a signal peptide, an N-terminal propeptide, a C-terminal propeptide, and the mature PA protease. The in vitro refolding of the intermediate pro-PA protease containing the C-terminal propeptide (MC) was investigated in the presence and absence of the N-terminal propeptide. The results indicate that the noncovalently linked N-terminal propeptide is able to assist in the refolding of MC. In the absence of the N-terminal propeptide, MC is trapped into a folding competent state that is converted into the active form by the addition of the N-terminal propeptide. Moreover, the N-terminal propeptide was found to form a complex with the folded MC and inhibit further processing of MC into the mature PA protease. Inhibitory activity of the purified N-terminal propeptide toward mature PA protease was also observed, and the mode of this inhibition was determined to be a mixed, noncompetitive inhibition with an associated allosteric effect.

Keywords: N-terminal propeptide; C-terminal propeptide; Intramolecular chaperone; Noncompetitive inhibition; Refolding; Processing; Metalloprotease; *Aeromonas caviae*

Extracellular proteases are generally synthesized as inactive precursors consisting of a signal peptide, an N-terminal propeptide, and/or a C-terminal propeptide, along with a mature region displaying catalytic activity. The role of the signal peptide is to assist in the secretion of the enzyme across the cytoplasmic membrane. The C-terminal propeptides of some proteases are also proposed to assist in the secretion of the enzyme [1–3] and are also thought to be involved in substrate binding [4]. Typically, the N-terminal propeptides of proteases act as intramolecular chaperones and/or inhibitors of the cognate mature enzymes, and this is the case for subtilisin [5], α-lytic protease [6], carboxypeptidase Y [7], cathepsins L [8], thermolysin [9], and yeast protease Y [10].

PA protease is an extracellular zinc metalloprotease produced by *Aeromonas caviae* T-64, which has an ability to activate the pro-aminopeptidases from both *A. caviae* T-64 [11] and *Vibrio proteolyticus* [12] via the processing of their proforms. Some extracellular proteases from *Aeromonas* spp. are proposed to be associated with the virulence of the bacteria [13,14]. Although it is not clear whether or not PA protease plays a part in such virulence, a Blast 2 search reveals that the PA protease precursor has a high level of identity with the precursors of the *A. hydrophila* elastase (85.8%) [14], the *V. vulnificus* protease (53.3%) [4], and the *Pseudomonas aeruginosa* elastase (52.5%) [15], all of which have been implicated in pathogenesis. The precursor of PA protease (SNMC) comprises a 19 amino acid putative signal peptide (S), a 165 amino acid N-terminal propeptide (N), a 33,000-Da mature region possessing protease activity (M), and an 11,000-Da C-terminal propeptide (C) [16]. Active mature PA protease was found in the culture filtrate of *A. caviae* T-64, suggesting that both

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the N- and C-terminal propeptides are processed in vivo [11]. Evidence obtained from the expression of the PA protease gene in *Escherichia coli* suggests that the presence of the N-terminal propeptide is required to obtain an active PA protease, whereas the C-terminal propeptide was not necessary for the display of enzyme activity [16]. It has been reported that the in vitro refolding and processing of the proform of PA protease (NMC) occurs through a stepwise, autoprocessing mechanism [17]. Initially, the N-terminal propeptide is autocatalytically removed after the completion of the refolding step, and the C-terminal propeptide is autoprocessed after the degradation of the N-terminal propeptide. It is the degradation of the N-terminal propeptide that appears to be the rate-limiting step in the maturation of PA protease. However, the role that the N-terminal propeptide plays in the folding process and in the inhibition of the PA protease is not clear. Moreover, the mechanism of the slow C-terminal propeptide autoprocessing remains to be elucidated. Therefore, we decided to investigate the N-terminal propeptide-assisted refolding and processing of the intermediate pro-PA protease containing the C-terminal propeptide (MC), and the inhibition of the mature PA protease by the N-terminal propeptide. The results described herein demonstrate that the N-terminal propeptide is able to facilitate the refolding of MC, and that it inhibits mature PA protease in a mixed, noncompetitive manner. In addition, the N-terminal propeptide was found to inhibit the C-terminal propeptide autoprocessing of folded MC (MC_f) by forming an MC_f/N complex.

Materials and methods

Materials. The *Z*-Phe–Tyr–Leu substrate was purchased from the Peptide Institute, Osaka, Japan. The native mature PA protease from *A. caviae* T-64 was purified as described previously [11]. The restriction enzymes used in this study were purchased from Takara Shuzo, Shiga, Japan, and were used according to manufacturer's instructions.

Cloning and expression of MC and His-tagged MC. Two recombinant plasmids, pPMC and pPHMC, were constructed for the expression of MC and a His-tagged MC, respectively. Based on the nucleotide sequence of the PA protease gene [16], the coding regions of pPMC and pPHMC were engineered by PCR to add either an *Nco*I (for pPMC) or an *Nde*I (for pPHMC) restriction enzyme site upstream of the MC encoding region and *Hin*dIII sites downstream of the termination codons. The respective PCR products were subsequently cloned into the *Nco*I–*Hin*dIII or the *Nde*I–*Hin*dIII restriction enzyme sites of the pET28a plasmid (Novagen), and *E. coli* BL21(DE3) were then transformed with the pPMC and pPHMC plasmids, respectively. The resulting transformants, BL21(DE3)pPMC and BL21(DE3) pPHMC, were grown separately in LB medium containing 50 μg/ml kanamycin at 37 °C until the OD_{600} reached levels of 0.6. The target proteins were then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM final concentration) and the incubations were continued at 30 °C for a further 4 h. After expression, both MC and the His-tagged MC formed inclusion bodies. The inclusion bodies of MC and His-tagged MC were isolated and purified, employing a previously described method [17].

Preparation and purification of the N-terminal propeptide. The inclusion bodies of NMC were subjected to in vitro refolding and processing as described previously [17]. When 80–90% of NMC had converted to MC, the processed N-terminal propeptide was purified as follows: the refolding and processing solution was first dialyzed against buffer A (20 mM Mes, 1 mM EDTA, pH 6.0) at 4 °C for 4 h, followed by centrifugation at 20,000*g* for 10 min. The resulting supernatant was applied to an S-Sepharose Fast Flow column (Pharmacia Biotech, Uppsala, Sweden, 5×50 mm) equilibrated with buffer A. After washing the column with buffer A, the column was eluted with a linear concentration gradient of NaCl from 0 to 0.4 M in buffer A, at a flow rate of 0.5 ml/min. The N-terminal propeptide fraction was concentrated using a Micron YM-3 centrifugal filter (Amicon, Beverly, USA). The concentrated solution (2 ml) was dialyzed against 3×5 liters of 20 mM Tris–HCl buffer (pH 7.0) at 4 °C to remove the EDTA present, and the solution was stored at −20 °C.

The N-terminal propeptide assisted refolding of MC. The inclusion bodies of MC and the His-tagged MC were solubilized in 20 mM CAPS buffer (pH 11) containing 8 M urea and 10 mM dithiothreitol. After incubation at 30 °C for 1 h, the solutions were centrifuged at 20,000*g* for 10 min. For each of the supernatants, 1 volume of solution (20 μM of protein) was diluted with 9 volumes of 20 mM CAPS (pH 11) containing different concentrations of the N-terminal propeptide, and the diluted solutions were maintained at 25 °C for 2 h. Then, each solution was dialyzed against 20 mM Tris–HCl buffer (pH 7) at 4 °C for 48 h. To degrade the N-terminal propeptide that inhibited PA protease activity, the refolded samples were treated with subtilisin (Boehringer–Mannheim GmbH, Mannheim, Germany) at a final concentration of 0.1 μM and incubated at 30 °C for 15 min. The reactions were terminated by the addition of 5 mM PSMF (Sigma). Under these conditions, the N-terminal propeptide was fully degraded and proteolytic activity of the refolded PA protease exhibited. Thereafter, the subtilisin-treated samples were subjected to a proteolytic activity assay using casein as the substrate. The recovered activity of the refolded PA protease was compared with the activity obtained using the same amount of native mature PA protease, which was defined as having an activity level of 100%.

Separation of the MC_f/N complex. The inclusion bodies of the His-tagged MC were denatured in 8 M urea, and then refolded in the presence of the N-terminal propeptide with an [N]:[MC] ratio of 2:1, as described above. Thereafter, 40 μl of a 50% slurry of Ni–NTA resin (Qiagen, Hilden, Germany) equilibrated with buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) was added to 200 μl of the refolding solution. After gently mixing at 4 °C for 30 min, the resin was recovered by centrifugation at 15,000*g* for 10 s, and it was washed twice with 200 μl of a wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). Finally, the bound proteins were eluted with 3×40 μl of an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0).

SDS–PAGE. The method described by King and Laemmli [18] was employed for SDS–PAGE analysis. To prevent autoprocessing and self-degradation of the pro- and mature PA proteases during sample preparation, the proteins were precipitated with trichloroacetic acid at a final concentration of 20%, then washed with acetone before subjecting to SDS–PAGE.

Determinations of protein concentration. Protein concentrations were determined spectrophotometrically at 280 nm using an ε_{280} of 62,660 $M^{-1}\,cm^{-1}$ for MC and the His-tagged MC, an ε_{280} of 51,730 $M^{-1}\,cm^{-1}$ for the mature PA protease, and an ε_{280} of 25,320 $M^{-1}\,cm^{-1}$ for the N-terminal propeptide; values calculated from their respective amino acid compositions.

Enzyme activity assay. The proteolytic activity of PA protease was assayed using casein as the substrate as described previously [20]. To determine the inhibition parameters, the hydrolytic reaction was performed at 30 °C in 200 μl of 20 mM Tris–HCl (pH 7.0) containing 1 nM enzyme and 0.2 mM *Z*-Phe–Tyr–Leu as the substrate. After 30 min, the reaction was stopped by the addition of 200 μl of 0.2 M acetic acid

containing 0.2 mM EDTA. The Tyr–Leu released was monitored by capillary electrophoresis [19] on a P/ACE System 2000 (Beckman Instruments, Fullerton, USA). One unit of hydrolytic activity is defined as the amount of enzyme required to produce 1 nM of product (Tyr–Leu) per minute under the conditions described above.

Inhibition measurements. The hydrolytic activity of PA protease toward the substrate *Z*-Phe–Tyr–Leu was assayed as described above. The concentration of PA protease was fixed at 1 nM, and the N-terminal propeptide concentrations were 40, 80, and 120 nM. The reactions were initiated by the addition of the substrate dissolved in methanol, to give final concentrations of 0.1, 0.2, 0.3, and 0.4 mM. A Lineweaver–Burk plot was used to determine the type of inhibition present. Kinetic parameters were calculated using the computer program GraFit (Erithacus Software, Staines, UK) and reaction rates were calculated from the slope of the product versus time plot (within the linear range) and were fitted to the following equations:

$$v = \frac{V_{\max}[S]}{(K_m + [S])(1 + [I]/K_i)}, \quad (1)$$

$$v = \frac{V_{\max}[S]}{(K_m + [S])(1 + [I]^2/K_i^2)}, \quad (2)$$

where [S] and [I] are the concentrations of the substrate and inhibitor, respectively, V_{max} is the maximal velocity, K_m is the Michaelis constant and K_i is the dissociation constant for the inhibition of the protease by its N-terminal propeptide. Eq. (1) describes normal, noncompetitive inhibition and Eq. (2) describes noncompetitive inhibition with an associated allosteric effect produced by the inhibitor.

Results

The role of the N-terminal propeptide in the refolding of MC

The presence of the N-terminal propeptide region was reported as an essential requirement to obtaining active PA protease when the PA protease gene was expressed in *E. coli* [16], and recently it was reported that the denatured pro-PA protease (NMC) is able to refold and autoprocess in vitro [17]. In this report, the refolding of the unfolded MC (MC_u) assisted by the noncovalently linked N-terminal propeptide (N) was investigated in vitro. When different concentrations of the N-terminal propeptide were included in the refolding solution, the recovered activity increased as the [N]:[MC] ratio was increased (Fig. 1A, curve 1). In contrast, in the absence of the N-terminal propeptide, no apparent protease activity (<0.1% relative to mature PA protease activity) was detected in the refolding solution (Fig. 1A, curve 1), and most of the MC molecules remained in the soluble fraction after folding (data not shown). When an equivalent molar amount of the N-terminal propeptide was added to the soluble MC solution, and the mixture was kept at 4 °C for 5 days, 16% of the proteolytic activity was recovered from the mixture, whereas less than 0.2% of the recovered activity was observed for the control MC solution without the N-terminal propeptide present. These results indicate that the noncovalently linked N-terminal propeptide is able to assist in the refolding of MC_u, therefore, the N-terminal propeptide is proposed to act as an intramolecular chaperone in the folding of PA protease. In addition, in the absence of the N-terminal propeptide, MC might be trapped into a folding competent state that is able to be converted into the active form when the N-terminal propeptide is added.

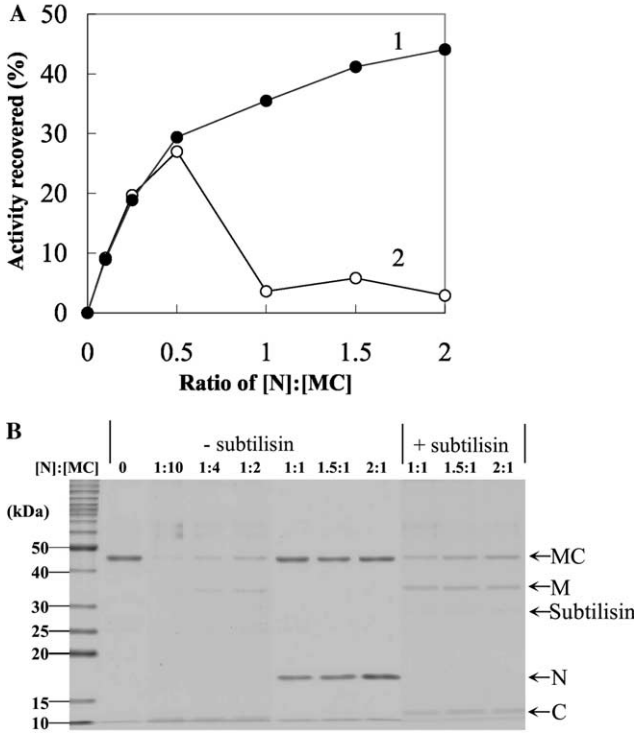

Fig. 1. The effect of the N-terminal propeptide on the refolding and processing of MC. The N-terminal propeptide-assisted refolding of unfolded MC was performed as described in Materials and methods. Before and after subtilisin treatment, the refolding solutions with different molar ratios of the N-terminal propeptide to MC ([N]:[MC]) were subjected to a proteolytic activity assay and SDS–PAGE analysis. (A) Proteolytic activity assay. The percentage of the recovered activity was calculated using native mature PA protease as a control (100%). Curve 1, before subtilisin treatment; curve 2, after subtilisin treatment. (B) SDS–PAGE analysis. The arrows indicate the positions of MC, mature PA protease (M), subtilisin, the N-terminal propeptide (N), and the processed C-terminal propeptide (C), respectively.

The role of the N-terminal propeptide in the processing of folded MC (MC_f)

Before the subtilisin treatment, the detected proteolytic activity decreased when the [N]:[MC] ratio exceeded 1 (Fig. 1A, curve 2), meanwhile, the N-terminal propeptide remained intact and the C-terminal propeptide was not processed (Fig. 1B). After subtilisin treatment, the N-terminal propeptide was degraded, accompanied by C-terminal propeptide processing (Fig. 1B) and proteolytic activity was exhibited (Fig. 1A, curve 1). These findings suggest that the N-terminal

propeptide inhibits the C-terminal processing of folded MC (MC_f). It was speculated that, after assisting the MC_u refolding to MC_f, the N-terminal propeptide was still bound to MC_f. To confirm whether or not this speculation was correct, Ni–NTA resin was employed to isolate the proposed MC_f/N complex after the N-terminal propeptide-assisted refolding of the His-tagged MC. It was found that the eluate (E) contained both the His-tagged MC and the N-terminal propeptide with an [N]:[His-tagged MC] ratio of 1:2.5 (Fig. 2). After subtilisin treatment, about 40% of the activity was recovered in the eluate, suggesting that 40% of the bound His-tagged MC existed in the correctly folded form (MC_f). Because the Ni–NTA resin has a high affinity for both folded and unfolded His-tagged MC, and based on the knowledge that the N-terminal propeptide itself could not bind to Ni–NTA resin under the same conditions, the N-terminal propeptide that was found in the eluate was expected to be associated with the MC_f that was bound to Ni–NTA resin to form a 1:1 MC_f/N complex.

In the presence of the N-terminal propeptide, the autoprocessing of the C-terminal propeptide of PA protease was a slow process, whereby 24 h and 30 min were required for the completion of the C-terminal propeptide autoprocessing at 30 and 50 °C, respectively [17]. In this study, we examined the autoprocessing of the C-terminal propeptide in the absence of the N-terminal propeptide. As illustrated in Fig. 3, the refolding solution was first treated with subtilisin at 30 °C for 10 min to fully degrade the N-terminal propeptide. After the subtilisin was inactivated by PMSF, all of the MC present was autoprocessed to the mature enzyme by

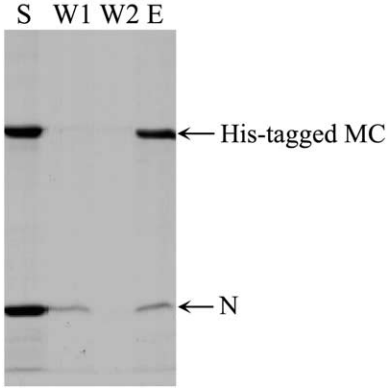

Fig. 2. The separation of the MC/N complex from the Ni–NTA resin. The denatured His-tagged MC was subjected to refolding in the presence of the N-terminal propeptide with an [N]:[MC] ratio of 2:1 as described in Materials and methods. Thereafter, the refolding solution (S) was mixed with Ni–NTA resin, and the Ni–NTA resin was recovered by centrifugation. Then, the resin was washed two times (W1, W2) with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). Finally, the bound proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) (E).

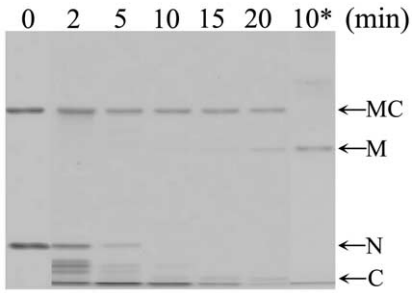

Fig. 3. The subtilisin-assisted degradation of the N-terminal propeptide and the processing of the C-terminal propeptide. The refolding solution with an [N]:[MC] ratio of 2:1 was mixed with 0.05 μM of subtilisin and incubated at 30 °C. At the times indicated, samples were taken and the reaction stopped by the addition of 5 mM PSMF, followed by SDS–PAGE. The lane denoted by an asterisk indicates the sample was first treated with subtilisin at 30 C for 10 min, and then, after the inactivation of subtilisin by PMSF, further incubated at 50 °C for 2 min.

incubation at 50 C after only 2 min, effectively demonstrating that the rate of C-terminal propeptide autoprocessing of MC_f in the absence of the N-terminal propeptide was much faster than that observed in the presence of the N-terminal propeptide.

The role of the N-terminal propeptide in the inhibition of mature PA protease

In the absence of the N-terminal propeptide, the K_m and k_{cat} values of PA protease for the substrate *Z*-Phe–Tyr–Leu were determined to be 0.26 mM and 45.9 s^{-1}, respectively. From the Lineweaver–Burk plot (Fig. 4), it can be seen that the V_{max} values decreased as the concentration of the N-terminal propeptide increased, indicating that the N-terminal propeptide noncompetitively inhibits the mature PA protease. Fitting the reaction rates to the normal, noncompetitive inhibition model (Eq. (1)) was not successful; as shown in Fig. 4A, with a relatively low concentration of the N-terminal propeptide (40 nM), a weaker inhibitory activity toward PA protease was observed, while stronger levels of inhibition of the enzyme were observed when higher concentrations of the propeptide (80 and 120 nM) were employed. In contrast, the observed reaction rate values fitted well with those calculated using Eq. (2) (Fig. 4B), indicating that there were some allosteric effects present in the inhibition of the enzyme. The K_i value was calculated to be 69.0 nM.

Discussion

The roles that the N-terminal propeptide of PA protease plays in refolding, processing, and enzyme inhibition are summarized in Fig. 5.

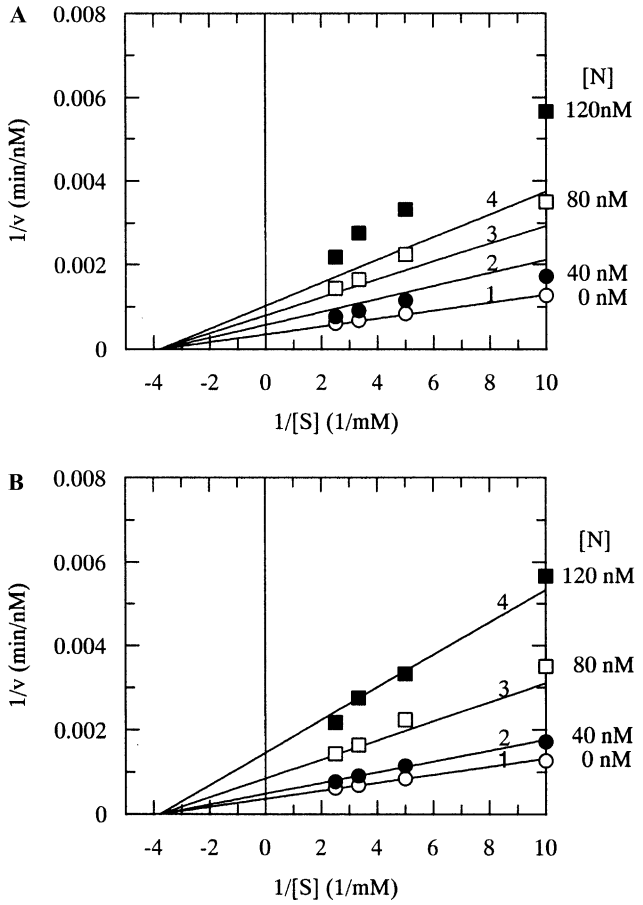

Fig. 4. Lineweaver–Burk plots of the inhibition of PA protease by its N-terminal propeptide. Using different concentrations of *Z*-Phe–Tyr–Leu (0.1, 0.2, 0.3, and 0.4 mM) as the substrate, the hydrolytic reaction rates of the PA protease in the absence and presence of different concentrations of the N-terminal propeptides were measured with a constant enzyme concentration of 1 nM. The concentrations of the N-terminal propeptides used are depicted near the respective data points. Experimental data were fitted to (A) a normal, noncompetitive model (Eq. (1)) and (B) a noncompetitive model with an allosteric effect induced by the inhibitor (Eq. (2)). Curves 1–4 depict the standard curves calculated from Eqs. (1) or (2), where the inhibitor concentrations are 0 nM (curve 1), 40 nM (curve 2), 80 nM (curve 3), and 120 nM (curve 4), respectively.

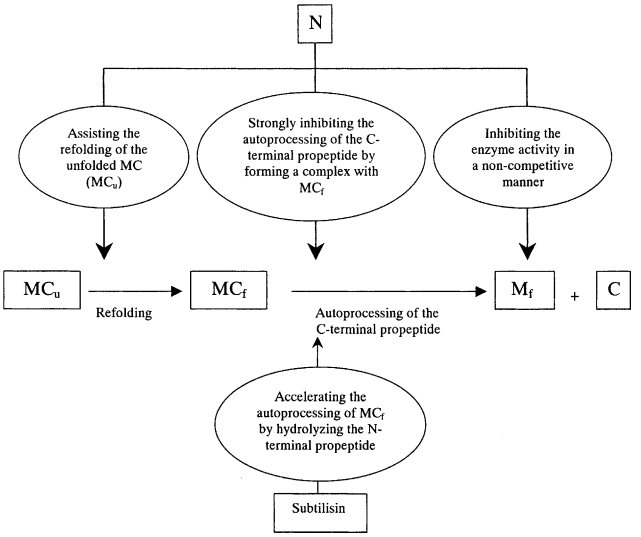

Fig. 5. Schematic representation of the roles of the N-terminal propeptide of PA protease in refolding, processing and enzyme inhibition. The N-terminal propeptide (N) assists the unfolded MC (MC_u) to refold to its correctly folded form (MC_f). However, the autoprocessing of the C-terminal propeptide (C) of refolded MC (MC_f) is strongly inhibited by the N-terminal propeptide. The N-terminal propeptide can be either autocatalytically degraded gradually or hydrolyzed by another protease (subtilisin). Upon the disappearance of the N-terminal propeptide, MC_f converts into the mature PA protease (M_f) by autoprocessing of the C-terminal propeptide. In addition, the N-terminal propeptide inhibits the activity of mature PA protease in a mixed, noncompetitive manner with an associated allosteric effect.

Two mechanisms for the propeptide-assisted folding of proteases have previously been described. In one of the mechanisms described, the N-terminal propeptides of subtilisin [20] and α-lytic protease [6] are proposed to lower the energy of a rate-limiting transition state at a late stage of folding and facilitate the polypeptide to undergo on-pathway folding. For the other mechanism, the N-terminal propeptide is proposed to prevent off-pathway misfolding or aggregation, and this is the situation described for carboxypeptidase Y [21]. Considering the evidence that, in the absence of the N-terminal propeptide, MC is trapped into a folding competent state that was able to be converted into an active form by the addition of the N-terminal propeptide, the function of the N-terminal propeptide in the folding of PA protease appears to be similar to that of subtilisin and the α-lytic protease. Further studies, such as the characterization of the proposed folding competent state of MC, are required to clarify the detailed mechanisms defining the role of the N-terminal propeptide in the folding of PA protease.

To date, the majority of the inhibitions of mature proteases by their N-terminal propeptides have been found to be of the competitive type [22–26]. The exception to this generalization is found for thermolysin, which is inhibited by its propeptide in a noncompetitive manner [9]. Likewise, the inhibition of mature PA protease by its N-terminal propeptide also involves a noncompetitive mechanism. Additionally, there is a strong likelihood that the thermolysin-like *Pseudomonas aeruginosa* elastase is also inhibited by its propeptide in a noncompetitive manner [27]. The similar propeptide inhibition types displayed amongst these types of enzymes tend to suggest there is a common propeptide inhibition mechanism for the thermolysin-like metalloproteases. As a noncompetitive inhibitor, the N-terminal propeptide binds to the mature enzyme at a region distinct from the active site, leading to a conformational change in PA protease. Thus the catalytic activity of the enzyme toward the substrate alters, probably induced by

changes in the local structure of the active site effected by an allosteric mechanism.

It is worth noting that the inhibition of mature PA protease (M_f) by the N-terminal propeptide was apparent when a high N-terminal propeptide to enzyme ($[N]:[M_f]$) ratio was employed, while a single molar equivalent of the N-terminal propeptide was enough to strongly inhibit processing of the C-terminal propeptide from the folded MC (MC_f). This apparent difference could be attributed to the different affinities of the N-terminal propeptide toward unfolded MC (MC_u) and the mature PA protease, M_f. In functioning as a molecular chaperone, it is probable that the N-terminal propeptide displays a higher affinity toward MC_u, recognizing the exposed hydrophobic residues and/or the unstructured backbone of MC_u. After assisting MC_u to refold to MC_f, the N-terminal propeptide is still bound to MC_f, forming an MC_f/N complex. Comparatively, the N-terminal propeptide has lower affinity for M_f where the hydrophobic residues and/or the unstructured backbone are buried. Therefore, a higher concentration of the N-terminal propeptide is required to enhance the efficiency of binding. On the other hand, because only one molar equivalent of the N-terminal propeptide was processed from NMC in vivo, and the processed N-terminal propeptide was susceptible to proteolysis, one can imagine that the molar ratio of the free N-terminal propeptide to mature PA protease does not exceed a value of 1:1 in vivo. At such a low $[N]:[M_f]$ ratio, the inhibition of mature PA protease by the processed N-terminal propeptide is comparatively weak. Consequently, it seems that, rather than inhibiting the activity of M_f, the main role of the N-terminal propeptide is to inhibit the C-terminal propeptide processing of MC_f to prevent premature proteolytic activity in vivo.

The slow N-terminal propeptide-mediated autoprocessing of the C-terminal propeptide seems to be a distinguishing feature of PA protease. In other proteases such as aqualysin I [28], the *Vibrio* metalloproteases [4,29–31] and the *A. hydrophila* elastase [14], of which their precursors also contain N- and C-terminal propeptides, the maturation of the precursor is accomplished by cleavage of the N-terminal propeptide and subsequent removal of the C-terminal propeptide, in a similar manner to PA protease [17]. However, no evidence has been ascribed to the roles of the N-terminal propeptides of these proteases in the autoprocessing of the C-terminal propeptide. In addition, the physiological relevance of the slow N-terminal propeptide-mediated C-terminal propeptide autoprocessing of PA protease may be related to the function of the C-terminal propeptide. It has been demonstrated that the C-terminal propeptide of the *V. vulnificus* protease (VVP) is involved in the binding of VVP to the target substance during the infection of the bacteria [4]. In addition, the C-terminal propeptides of aqualysin I [3], the extracellular proteases of *Neisseria gonorrhoeae* [1], *Serratia marcescens* [2], and *Xanthomonas campestris* [32] are able to facilitate the secretion of the enzymes. Our previous reports suggest that the C-terminal propeptide of PA protease is not necessary for the display of enzyme activity [16], and it is processed as an intact polypeptide [17], however, at present its function remains unclear. The C-terminal propeptide of PA protease has 61.1% amino acid identity with that of the C-terminal propeptide of the *X. campestris* protease whereas no such similarity was found in the mature region, and it has 45.5% identity with that of the VVP C-terminal propeptide [16]. Assuming the C-terminal propeptide of PA protease plays a role in the enzyme secretion and/or the substrate binding in vivo, a rapid separation of the C-terminal propeptide from the mature region is clearly undesirable with respect to ensuring the secretion of the enzyme and/or the infection of the bacteria.

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