Intramolecular chaperone and inhibitor activities of a propeptide from a bacterial zinc aminopeptidase

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An aminopeptidase from *Aeromonas caviae* T-64 was translated as a preproprotein consisting of three domains; a signal peptide (19 amino acid residues), an N-terminal propeptide (101 residues) and a mature region (273 residues). We demonstrated that a proteinase, which was isolated from the culture filtrate of *A. caviae* T-64, activated the recombinant pro-aminopeptidase by removal of the majority of the propeptide. Using L-Leu-*p*-nitroanilide as a substrate, the processed aminopeptidase showed a large increase in k_{cat} when compared with the unprocessed enzyme, whereas the K_m value remained relatively unchanged. The similar K_m values for the pro-aminopeptidase and the mature aminopeptidase indicated that the N-terminal propeptide of the pro-aminopeptidase did not influence the formation of the enzyme–substrate complex, suggesting the absence of marked conformational changes in the active domain. In contrast, the marked difference in k_{cat} suggests a significant decrease in the energy of one or more of the transition states of the enzyme–substrate reaction coordinate. Moreover, we showed that the activity of the urea-denatured pro-aminopeptidase could be recovered by dialysis, whereas the activity of the urea-denatured mature aminopeptidase, which lacked the propeptide, could not. Further to this, the propeptide-deleted aminopeptidase formed an inclusion body in the cytoplasmic space in *Escherichia coli* and was not secreted at all. These results suggested that the propeptide of the pro-aminopeptidase acted as an intramolecular chaperone that was involved with the correct folding of the enzyme *in vitro* and was required for extracellular secretion in *E. coli*.

Key words: activation, folding, metalloprotease, pro-enzyme, processing.

INTRODUCTION

We have recently isolated an aminopeptidase from *Aeromonas caviae* T-64 (apAC), which exhibited debittering activity by the specific release of hydrophobic amino acid residues from bitter peptides [1]. Cloning and sequencing of this unique enzyme gene revealed that *apAC* is translated as a preproprotein consisting of three domains; a signal peptide (0.2 kDa, 19 amino acid residues), an N-terminal propeptide (10 kDa, 101 residues) and a mature region (30 kDa, 273 residues) [2].

Significant similarity with the amino acid sequence of this aminopeptidase was found in only two other enzymes. These were the aminopeptidases from *Vibrio proteolytica* [3] and *V. cholerae* [4], with 56.7 and 52.0 % identity respectively. *V. proteolytica* aminopeptidase has been studied thoroughly [5–11], and it belongs to a unique family of zinc-dependent proteolytic enzymes with co-catalytic metal centres [12,13]. The crystal structure of *V. proteolytica* aminopeptidase revealed that the active site consists of a metal-binding site and a well-defined hydrophobic pocket [13]. With the exception of one residue (Met-296 of apAC) [2,4], the amino acids involved in the binding of the two zinc ions and those associated with the hydrophobic pocket are highly conserved in these three aminopeptidases from different species. Therefore, it is conceivable that the catalytic mechanism of apAC is similar to those of the *V. proteolytica* and *V. cholerae* aminopeptidases. The pro-apAC has the same two domains as the *V. proteolytica* and *V. cholerae* aminopeptidases, as well as an additional 10-kDa domain at the C terminus [3,4]. To date, the exact role of the N-terminal propeptide domains in these pro-aminopeptidases has not been elucidated.

It is known that the propeptides in some proteolytic enzyme precursors play a significant role in the inhibition of their active domains and that during post-translational processing the propeptides are usually removed from the pro-enzymes by proteolysis. This is achieved either by autoprocessing or by the action of other proteolytic enzymes and results in the activation of the pro-enzymes [14].

It is well known that molecular chaperones function by intermolecular interactions to affect protein folding [14]. In a similar sense, some propeptides function by intramolecular interactions to affect correct protein folding. Therefore, to distinguish these types of propeptide from molecular chaperones, they are termed intramolecular chaperones [15,16]. Investigating propeptides of endo-type proteases is difficult, however, since these pro-enzymes are autocatalytically degraded into the relatively active form [14]. In the case of exo-type proteases, only two have been investigated: the propeptides of yeast pro-carboxypeptidase Y (a serine protease), which were shown to have intramolecular chaperone and inhibitor [17] activities, and the propeptide of bovine pro-carboxypeptidase A (a metalloprotease), which acted as an inhibitor [18]. However, to date there have been no studies on the aminopeptidase propeptide having both inhibition and intramolecular chaperone activities.

In the present study, we constructed a secretory expression system for the pro-apAC enzyme and demonstrated that the recombinant pro-apAC is activated by the pro-apAC protease

Abbreviations used: apAC, aminopeptidase from *Aeromonas caviae* T-64; PA protease, pro-apAC protease; IPTG, isopropyl-β-D-thiogalactopyranoside; Leu-NA, L-Leu-*p*-nitroanilide.

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The nucleotide sequence for the *apAC* gene has been deposited in the DDBJ/EMBL/GenBank databases under accession number AB015725.

(PA protease), which was isolated from the culture filtrate of *A. caviae* T-64. The k_{cat} of the activated enzyme was greatly increased, but the K_m was relatively unchanged by the proteolytic activation. Moreover, we showed that the propeptide of pro-apAC acted as an intramolecular chaperone that organized the correct folding *in vitro* and was essential for extracellular secretion from *Escherichia coli*.

EXPERIMENTAL

Bacterial strains, cloning vectors and media

E. coli JM109 (*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ(*lac-proAB*) F′ [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) and *E. coli* BL21(DE3) [*hsdS gal* (λ*c*I*ts* 857 *ind* 1 *S*am7 *nin*5 *lac*UV5-T7 gene *1*)] were used as hosts for the recombinant plasmid and expression, respectively. The plasmids pET26b and pET24a (Novagen, Madison, WI, U.S.A.) were used for subcloning, DNA sequencing and expression. The medium routinely used for *E. coli* JM109 carrying recombinant plasmids was Luria–Bertani medium [19] containing 50 μg/ml kanamycin.

DNA manipulation

Recombinant DNA techniques and methods for agarose-gel electrophoresis were followed as described by Sambrook et al. [19]. Plasmid DNA was prepared using a QIAprep Spin Plasmid kit (Qiagen, Hilden, Germany). Digestion by restriction enzymes was carried out in the appropriate buffer at concentrations of 1–10 units/μg of DNA for 4–16 h at appropriate temperatures. Completion of the reactions was confirmed by agarose-gel electrophoresis. QIAEX Agarose Gel Extraction kit (Qiagen) was used for the extraction and purification of DNA from agarose gels.

Construction of pro- and mature apAC expression vector, pASNM, pASM, pANM and pAM

To obtain the DNA encoding pro- and mature apAC, the PCR was conducted using phage DNA containing the *prepro-apAC* gene [2]. Five oligonucleotide primers: primer 1, 5′-CGCACCCATGGCCGAGCCCGTCTGGATC-3′ (forward for pASNM, the plasmid containing the *pelB* signal peptide, which directs translation products to the *E. coli* periplasmic space, and the *pro-apAC* gene; underlined sequence is the *Nco*I restriction site); primer 2, 5′-GCCCTCCATGGATCAGGGCAACATAGTCG-3′ (forward for pASM, the plasmid containing the *pelB* signal peptide and the *apAC* gene; underlining indicates the *Nco*I site); primer 3, 5′-TCGCCGCACATATGGCCGAGCCCGTCT-3′ (forward for pANM, the plasmid containing the *pro-apAC* gene; underlining is the *Nde*I site); primer 4, 5′-TGCTGCCCCATATGGATCAGGGCAACAT-3′ (forward for pAM, the plasmid containing the *apAC* gene; underlining is the *Nde*I site); and primer 5, 5′-CCCTGAATTCTCAGTTTATCTTGCCCATCTC-3′ (reverse for all; underlining is the *Eco*RI site), were prepared based on the nucleotide sequence of the *apAC* gene [2]. DNA polymerase from *Pyrococcus kodakaraensis* KOD1 (TOYOBO, Osaka, Japan) was used at 2.5 units/50 μl of reaction mixture. The PCR programme of 98 °C for 3 min, 98 °C for 15 s, 65 °C for 30 s and 72 °C for 30 s (25 times), and 72 °C for 10 min was used in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT, U.S.A.). After digestion of the PCR products with terminal restriction enzymes followed by gel purification, the amplified fragments were ligated with the appropriately prepared plasmids; for pASNM and pASM, pET26b was used using the *Nco*I and *Eco*RI sites, and for pANM and pAM, pET24a was used using the *Nde*I and *Eco*RI sites. The resulting plasmids were subjected to DNA sequencing to check the nucleotide sequences.

DNA sequencing

DNA encoding pro- and mature *apAC* were sequenced by the dideoxy chain-termination procedure [20] using the Bigdye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Foster City, CA, U.S.A.) on a 310 Genetic Analyser (Perkin-Elmer). The sequence data were analysed with the GENETIX program (Software Development Co., Tokyo, Japan).

Expression of pro- and mature apAC in *E. coli* and isolation of recombinant pro-apAC

E. coli BL21(DE3) was transformed with pASNM, pASM, pANM and pAM. Transformants were grown in Luria–Bertani medium containing 50 μg/ml kanamycin at 37 °C until the A_{600} reached 0.6. The target protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM, final concentration). Incubation was then continued at 25 °C for 16 h in the case of pASNM and pASM, and at 37 °C for 4 h in the case of pANM and pAM. After this, the cells were separated by centrifugation, suspended in 20 mM Tris/HCl buffer (pH 8.5) and sonicated using a Branson Sonifier Model 250D. The cell lysate was then centrifuged and the supernatant collected.

To isolate pro-apAC, ammonium sulphate crystals were added to the culture supernatant (4 l) to a final saturation of 70% at 4 °C. The resulting precipitate was collected by centrifugation at 10000 ***g*** for 30 min and dissolved in 40 ml of 20 mM Tris/HCl buffer (pH 8.5). The solution was then dialysed against 3 l of the same buffer at 4 °C. The dialysed solution was applied to a Q-Sepharose Fast Flow column (2.6 cm × 7.5 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated previously with 20 mM Tris/HCl buffer (pH 8.5). The column was eluted with a linear gradient of NaCl (0–0.5 M, 600 ml total volume) in the same buffer at a flow rate of 4 ml/min. Fractions were collected and assayed for aminopeptidase activity. Aminopeptidase fractions were pooled and the target protein was precipitated by the addition of ammonium sulphate to a final saturation of 70%. The precipitate was collected by centrifugation at 10000 ***g*** for 30 min and dissolved in 6 ml of 20 mM Tris/HCl buffer (pH 8.5) containing 0.5 M NaCl. The solution was applied at a flow rate of 2 ml/min to a gel-filtration column of Superdex 75pg (2.6 cm × 60 cm; Amersham) equilibrated previously with 20 mM Tris/HCl buffer (pH 8.5) containing 0.5 M NaCl. Elution with the same buffer produced active fractions that were pooled and used as purified recombinant pro-apAC. The yield from 4 l of culture filtrate was approximately 15 mg of recombinant pro-apAC.

Measurement of enzyme activities and determination of K_m and k_{cat}

Aminopeptidase activity and values for K_m and k_{cat} using L-Leu-*p*-nitroanilide (Leu-NA) as a substrate were measured as described previously [1]. The processing of 40-kDa pro-apAC to 30-kDa mature apAC was carried out in 20 mM Tris/HCl buffer (pH 8.5) containing 0.5 M NaCl at 30 °C for 1 h using 0.05% recombinant pro-apAC as a substrate. The reaction was terminated by boiling for 5 min. The progress of the conversion from pro-apAC to mature apAC was monitored by SDS/PAGE using a Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA,

U.S.A.) and Molecular Analyst™/Macintosh image-analysis software. Enzyme activity (1 unit) was defined as the activity that resulted in a 50 % conversion of 100 pmol of pro-apAC to apAC.

SDS/PAGE

Electrophoresis of proteins was performed according to the method described previously by King and Laemmli [21], using PAGEL NPU-12.5L (ATTO, Tokyo, Japan). Gels were stained with Gelcode Blue Stain Reagent (Pierce, Rockford, IL, U.S.A.).

Determination of protein content

Absorbance at 280 nm was measured to determine the concentration of proteins, using ϵ_{280} 50900 $M^{-1} \cdot cm^{-1}$ for recombinant pro-apAC, ϵ_{280} 43400 $M^{-1} \cdot cm^{-1}$ for native apAC and ϵ_{280} 44500 $M^{-1} \cdot cm^{-1}$ for PA protease-cleaved pro-apAC. The extinction coefficient for each protein was calculated from the amino acid composition of Trp, Tyr, His, Met, Cys and cystine residues by using the molar-extinction coefficients of 5070, 1190, 5, 4, 20 and 111, respectively [22]. Coomassie Protein Assay Reagent (Pierce) was used to determine the protein concentration of denatured and renatured pro- and mature apAC.

Amino acid sequencing

To identify the N-terminal amino acid sequence of recombinant pro-apAC and the PA protease-cleaved pro-apAC, Edman degradation [23] was performed using a G1000A protein sequencer (Hewlett Packard, Palo Alto, CA, U.S.A.).

Purification of native apAC and PA protease

Native apAC was purified from the culture filtrate of *A. caviae* T-64 as reported previously [1]. *A. caviae* T-64 cultivation, ammonium sulphate precipitation and DEAE-Toyopearl column chromatography were performed as reported previously [1]. After chromatography, fractions were collected and assayed for processing activity by using pro-apAC. Active fractions were pooled and dialysed against 20 mM Tris/HCl buffer (pH 8.5). A fraction of dialysed solution was applied to a Mono-Q HR5/5 column (Amersham) equilibrated with 20 mM Tris/HCl buffer (pH 8.5). The column was then eluted with a linear gradient of NaCl from 0 to 0.4 M in the same buffer, at a flow rate of 1 ml/min. The active fractions were pooled and used as purified PA protease.

Processing of recombinant pro-apAC

Recombinant pro-apAC was digested by PA protease in 20 mM Tris/HCl buffer (pH 8.5) containing 0.5 M NaCl at 37 °C for 1 h. The ratio of recombinant pro-apAC to PA protease was 1:1000 (w/w). To activate pro-apAC expressed in the culture filtrate and in the supernatant of transformant cell lysate carrying the recombinant plasmids or pET26b, 40 μl of the solution was treated with 2 μg of PA protease at 37 °C for 1 h.

Purification of inclusion bodies and refolding of pro- and mature apAC *in vitro*

To purify inclusion bodies of recombinant pro- and mature apAC, the precipitates of BL21(DE3)pANM and BL21(DE3)-pAM lysates were suspended in 20 mM Tris/HCl buffer (pH 8.5) containing 5 M urea and 10 mM 2-mercaptoethanol. After sonication and centrifugation, the precipitates were suspended in 20 mM Tris/HCl buffer (pH 8.5) containing 8 M urea, 10 mM 2-mercaptoethanol and 1 mM $ZnCl_2$. The suspensions were then sonicated and centrifuged to obtain clear supernatants that contained the denatured pro- and mature apAC. To renature pro- and mature apAC, 2 ml of denatured pro- and mature apAC solutions were dialysed against 20 mM Tris/HCl buffer (pH 8.5) containing 1 mM $ZnCl_2$ at 4 °C. The resulting solutions were obtained as renatured pro- and mature apAC.

Stability of recombinant pro- and native apAC at different temperatures

Thermostabilities of pro- and native apAC were measured as described previously [1]. To measure residual aminopeptidase activity of pro-apAC, the heat-treated pro-apAC was subjected to PA protease treatment.

Enzymes and chemicals

All restriction enzymes [from Takara Shuzo (Shiga, Japan), New England BioLabs (Beverly, MA, U.S.A.) and TOYOBO] and the DNA-ligation kit (TOYOBO) used in this study were purchased from commercial suppliers and were used according to the instructions provided by the manufacturers. All other chemicals were of a high analytical grade.

RESULTS

Expression and preparation of recombinant pro-apAC

The nucleotide sequence of the *apAC* gene codes for a polypeptide consisting of three domains: a signal peptide (19 amino acid residues), an N-terminal propeptide (101 residues) and a mature region (273 residues) [2]. To analyse the properties of the N-terminal propeptide, we constructed a pro-apAC expression vector in *E. coli*. The expression construct, pASNM, used the T7 *lac* promoter and *pelB* signal sequence fused to the *pro-apAC* gene (Figure 1). This construct enabled IPTG induction and secretion of expressed protein.

After purification from the culture filtrate, the resulting pro-apAC was homogeneous, as determined by SDS/PAGE (Figure 2, lane 2). This result showed that purified recombinant pro-apAC was not contaminated with the mature apAC. The N-terminal amino acid sequence of the pro-apAC was confirmed to

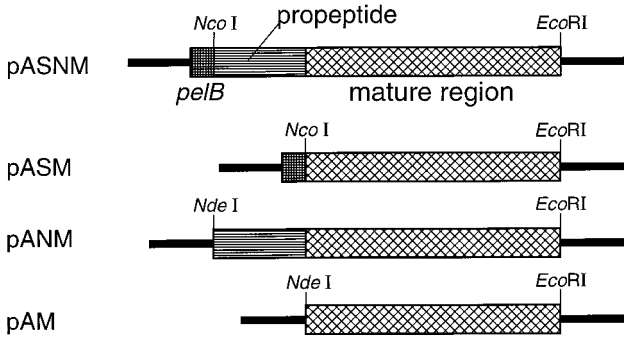

Figure 1 Construction of apAC expression vectors

pASM and pAM are incorporated into pET26b, and pANM and pAM are incorporated into pET24a. The *apAC* gene was under the control of the T7 promoter and *lac* operator. All plasmids contained a gene for kanamycin resistance. *pelB* indicates the signal peptide from *E. coli*. *Nco*I, *Eco*RI and *Nde*I restriction-enzyme sites are indicated.

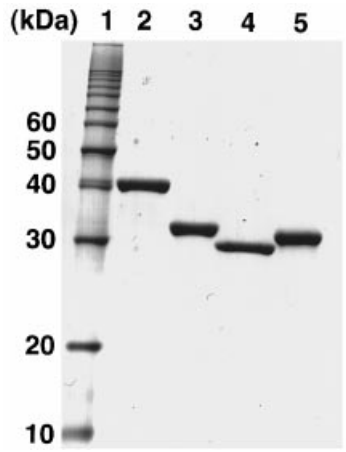

Figure 2 SDS/PAGE of recombinant pro-apAC, PA protease, native mature apAC and PA protease-cleaved pro-apAC

Purified recombinant pro-apAC (lane 2), PA protease-cleaved pro-apAC (lane 3), native mature apAC [1] (lane 4) and purified PA protease (lane 5) were subjected to SDS/PAGE (12.5% gel) analysis under reducing conditions. Into each lane was loaded 1 μg of protein. The molecular-mass standards (lane 1) used in the present study were from a 10-kDa Protein Ladder (Gibco BRL Life Technologies, Gaithersburg, MD, U.S.A.).

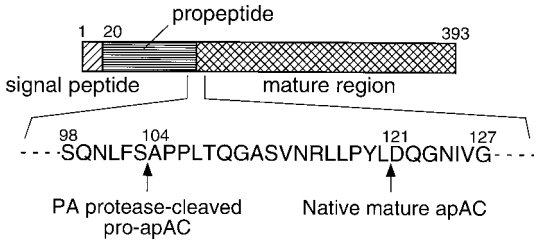

Figure 3 Cleavage positions of PA protease-cleaved pro-apAC and native mature apAC

The cleavage position of each apAC is indicated by an arrow. The first amino acid (Met) deduced from the *apAC* gene [2] was designated as position 1, and 30 amino acids between Ser-98 and Gly-127 are indicated.

Table 2 Comparison of the kinetic parameters for the substrate Leu-NA

Data are means $\pm$ S.E. and were obtained from three experiments.

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Recombinant pro-apAC	0.93 ± 0.023	0.21 ± 0.015	4.4 ± 0.21
PA protease-cleaved pro-apAC	40 ± 4.7	0.14 ± 0.03	285 ± 31
Native mature apAC	44 ± 5.3	0.14 ± 0.03	317 ± 33

be Ala-Glu-Pro-Val-Trp, which was identical to the deduced sequence from the nucleotide sequence of the gene [2]. This result showed that the signal peptide was released from expressed prepro-apAC.

Preparation of PA protease

A protease capable of converting pro-apAC to mature apAC was found in the culture filtrate of *A. caviae* T-64 at a concentration of 200 units/μl. This enzyme was termed PA protease. The protease was purified 196-fold from the culture filtrate with a recovery of 7.2%, and had a specific activity of 4750 units/μg (Table 1). As shown by SDS/PAGE (Figure 2, lane 5), the purified PA protease was homogeneous, with an estimated molecular mass of 30 kDa.

Activation of recombinant pro-apAC by PA protease

When the pro-apAC was treated with purified PA protease, a reduction of molecular mass from 40 kDa (Figure 2, lane 2) to 30 kDa (Figure 2, lane 3) was observed. The resultant protein was similar in size to the native mature apAC (Figure 2, lane 4). These results suggested that the N-terminal propeptide of pro-apAC was cleaved off by PA protease treatment.

To identify the cleavage site, the N-terminal amino acid sequence of PA protease-cleaved pro-apAC was determined. As shown in Figure 3, the N-terminal amino acid of the processed apAC was Ala-104, whereas that of the native mature apAC was Asp-121. This finding indicated that PA protease did not remove the entire propeptide, as would be expected from the sequence of the native mature apAC [1]. This result suggested the presence of yet another, undiscovered, processing enzyme that would be necessary for the cleavage of the pro-enzyme at Asp-121 to give the native mature apAC.

Kinetics of recombinant pro-apAC and PA protease-cleaved pro-apAC

The kinetic parameters of pro-apAC were compared with those of native apAC [1] using Leu-NA as a substrate at 30 °C. Although the k_{cat} of pro-apAC (0.93 s^{-1}) was less than 2.5% of that of the native enzyme (44 s^{-1}), the K_m (0.21 mM) was similar to that of the native enzyme (0.14 mM; Table 2). These results suggested that the removal of the N-terminal propeptide of pro-apAC resulted in a reduction in a kinetic barrier without markedly changing the structure of the active domain of the aminopeptidase.

Since our results showed that the PA protease-cleaved pro-apAC possessed 17 more amino acid residues at the N-terminus

Table 1 Purification of the processing enzyme from *A. caviae* T-64

Purification step	Volume (ml)	Total protein (mg)	Total units (mega-units)	Specific activity (units/μg)	Purification (-fold)	Overall yield (%)
Culture filtrate	12000	99000	2400	24	1	100.0
$(NH_4)_2SO_4$ precipitate	170	12700	1700	134	6	70.8
DEAE-Toyopearl	240	232	852	3680	152	35.5
Mono-Q	40	36.6	174	4750	196	7.2

Table 3 Aminopeptidase activities of recombinant pro- and mature apAC

	Aminopeptidase activity (m-units/ml)	
	No treatment	PA protease-cleaved
Culture filtrate		
BL21(DE3)pASNM (pro-apAC)	2.8	66
BL21(DE3)pASM (mature apAC)	0.47	1.5
BL21(DE3)pET26b (control)	< 0.00014	1.4
Supernatant of cell lysate*		
BL21(DE3)pASNM (pro-apAC)	16	74
BL21(DE3)pASM (mature apAC)	3.0	0.13
BL21(DE3)pET26b (control)	1.9	0.09

* The unit of supernatant of cell lysate was based on the volume of culture medium.

than the mature apAC, the effect of these additional residues on the enzyme's activity was investigated. Leu-NA was used as the substrate for this purpose. The k_{cat}, K_m and k_{cat}/K_m values of the PA protease-cleaved pro-apAC and native enzyme were very similar (40 and 44 s^{-1}, 0.14 and 0.14 mM, and 285 and 317 $s^{-1} \cdot mM^{-1}$, respectively, see Table 2). Since the presence of the additional 17 residues had little effect on the kinetic parameters it is highly likely that the conformation of the PA protease-cleaved pro-apAC was very close to that of the native apAC.

Expression of mature region of the aminopeptidase

To investigate the role of the N-terminal propeptide in protein folding, the propeptide-deleted apAC expression vector, pASM, was constructed (Figure 1). When *E. coli* BL21(DE3) was transformed with the plasmid of pASM [to give BL21(DE3)-pASM] and then cultured, very little aminopeptidase activity was detected in either the culture filtrate or the supernatant of the cell lysate (Table 3). In contrast, strong aminopeptidase activity was found in the culture filtrate and supernatant of the cell lysate of BL21(DE3)pASNM (2.8 and 16 m-units/ml, respectively). When BL21(DE3)pASNM was treated with PA protease, both the specific activity of the culture filtrate and that of the supernatant of the cell lysate increased. This indicated that these aminopeptidase activities were caused by expressed recombinant pro-apAC and not an aminopeptidase in *E. coli*. SDS/PAGE analysis showed that the propeptide-deleted apAC was not excreted and was not soluble after expression in *E. coli* (Figure 4). These results suggest that the N-terminal propeptide was essential for extracellular secretion from *E. coli*.

Denaturation and renaturation of pro- and mature apAC

We compared the renaturation abilities of the pro- and mature apAC proteins. In order to eliminate the effects of the signal peptide, the pro- and mature apAC expression vectors, pANM and pAM, were constructed (Figure 1). As Figure 4 indicates, both expressed proteins formed inclusion bodies. After washing the precipitates of cell lysates with 5 M urea, these precipitates were dissolved with 8 M urea to give denatured pro- and mature apAC, respectivity. The purified pro- and mature apAC were shown to be homogeneous by SDS/PAGE (Figure 4). Neither of the denatured enzymes showed any aminopeptidase activity (less than 0.05 m-units/ml). After dialysis to effect refolding of the denatured enzymes, aminopeptidase activity was observed for pro-apAC (5.6 m-units/ml), but not for mature apAC (less than 0.05 m-units/ml). The activity of the refolded pro-apAC was increased by more than seven-fold (40 m-units/ml) by PA protease treatment. These results suggest that the propeptide of pro-apAC acted as an intramolecular chaperone that organized the correct folding *in vitro*.

Thermostability of pro-apAC

To investigate whether the propeptide influenced structural stability, we compared the thermostabilities of the pro- and native apAC. As a result, the thermostability profile of pro-

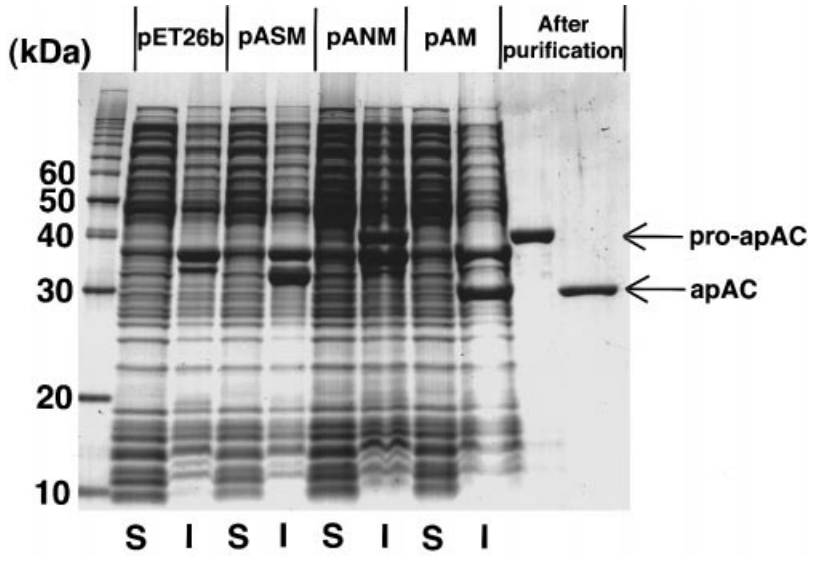

Figure 4 SDS/PAGE analysis of the expression of the N-terminal propeptide-deleted mutant, recombinant pro- and mature apAC without the signal peptide, and the purified inclusion bodies of recombinant pro- and mature apAC

Expression of each recombinant was induced by IPTG. Soluble (S) and insoluble (I) fractions were prepared as described in the Experimental section. The arrows indicate the positions of purified inclusion bodies of recombinant pro- and mature apAC. Loaded into each lane were 50 μg of proteins of the soluble or insoluble fractions and 1 μg of the purified pro- and mature apAC.

apAC (PA protease-cleaved) was found to be close to that of native apAC [1].

DISCUSSION

In the present study, the role of the propeptide in pro-apAC was examined. Firstly, we showed that the propeptide played a role in the inhibition of its active domain. We demonstrated that a newly isolated PA protease removed a propeptide of 101 amino acid residues from the 40-kDa recombinant pro-apAC to form a 30-kDa mature aminopeptidase. The molecular mass of the processed protein was similar to that of the native mature apAC [1]. As a result of removal of the pro region, the k_{cat}/K_m value increased by more than 70-fold (Table 2), indicating that the catalytic activity of the PA protease-cleaved pro-apAC was almost the same as that of the native apAC [1].

The cleavage site of PA protease on pro-apAC was found to be the Ser-103–Ala-104 bond, whereas based on the structure of the native apAC it was expected to be the Leu-120–Asp-121 bond [2]. This result suggests the presence of another, undiscovered, processing enzyme that cleaves the Leu-120–Asp-121 bond. It is known that the enzyme-maturation process frequently proceeds through stepwise proteolytic cleavages until the entire propeptide is removed [14]. In the case of pro-apAC, cleavage at Ser-103–Ala-104 is likely to allow further maturation by removal of the remaining sections of the propeptide by another processing enzyme.

The propeptide fragment consisting of 101 amino acid residues, which was removed from pro-apAC by PA protease, could not be detected by SDS/PAGE (results not shown). This fact indicates that the removed propeptide region was degraded into smaller oligopeptides by the PA protease and/or the activated apAC. It is possible that the hydrolysis of the propeptide is to ensure that the conversion process is irreversible and that the propeptide does not act as a competitive inhibitor of the active enzyme. In the case of subtilisin, digestion of the propeptide is rationalized as a means of recycling the amino acids for subsequent use by the bacterium [24].

For the substrate Leu-NA, the K_m value of the pro-apAC (0.21 mM) was similar to that of the processed apAC (0.14 mM). In contrast, the k_{cat} value of pro-apAC (0.93 s^{-1}) was less than 2.5 % of that of the PA protease-cleaved pro-apAC (40 s^{-1}). Similar K_m values indicate that the N-terminal propeptide of the pro-apAC does not influence the formation of the enzyme–substrate complex, suggesting the absence of marked conformational changes in the active domain. In contrast, the marked decrease in k_{cat} suggests a significant decrease of an activation-energy barrier in the reaction coordinate of the enzyme–substrate complex.

The synthesis of a weak active form of an aminopeptidase followed by its conversion to a more active form by the release of a propeptide has not been reported before. On the other hand, synthesis of an inactive precursor form has been observed in several other types of proteolytic enzyme, such as pro-cathepsin B (an endo-type cysteine protease) [25–27], pro-cathepsin D (an endo-type aspartic protease) [28] and pro-carboxypeptidase A (an exo-type metalloprotease) [18]. Kinetic studies of pro-carboxypeptidase A showed that its k_{cat} values were 20 times lower than those of the mature form, whereas the K_m values were similar [18]. In the cases of subtilisin (an endo-type serine protease) [29,30] and α-lytic protease (an endo-type serine protease) [31], the activities of the mature proteases were inhibited following the addition of the propeptide as a separate polypeptide. Moreover, X-ray analysis of pro-cathepsin B [32,33], pepsinogen (an endo-type aspartic protease) [34,35], pro-carboxypeptidase A [36,37] and the propeptide–subtilisin complex [38] showed that the propeptide physically closed the active site of the catalytic pocket. Similarly to the conclusions derived from the kinetic studies mentioned previously, these proteases possess the correct conformation at their active site; however, the active site is blocked sterically by the propeptide. Thus although the affinity for the active site may or may not remain the same for a given substrate, the turnover rate would be expected to decrease due to steric hindrance. As mentioned, the K_m value of pro-apAC was close to that of active apAC, and therefore the presence of the propeptide is unlikely to have affected the conformation of the active site; however, like the other proteases already mentioned, it seems probable that the propeptide of the recombinant pro-apAC acts by sterically blocking the active site. The results of crystallization and X-ray analyses of the recombinant pro-apAC, which are now in progress, will contribute to our understanding of the inhibitory mechanisms and activation pathways of this unique bacterial leucyl aminopeptidase from *A. caviae* T-64.

The propeptide of pro-apAC acted as an intramolecular chaperone that organized the correct folding *in vitro* and was required for extracellular secretion in *E. coli*. The urea-denatured pro-apAC recovered aminopeptidase activity by dialysis of the denaturant, whereas the mature apAC lacking the propeptide was not activated. This indicates that the propeptide may support the folding of apAC as an intramolecular chaperone. The propeptide-deleted apAC formed an inclusion body in the cytoplasmic space in *E. coli* (Figure 4) and was not secreted at all. These results suggest that the aggregation of propeptide-deleted apAC *in vivo* may be due to its failure to fold correctly in the cytoplasmic space.

Propeptides playing the role of an intramolecular chaperone have also been observed in several other types of protease, such as subtilisin [39–41], α-lytic protease [42], aqualysin (an endo-type serine protease) [43] and carboxypeptidase Y [17]. The propeptides in these proteases, however, have no significant sequence similarity with the known molecular chaperones and, in addition, the propeptide of apAC has no significant sequence similarity with the propeptides of other proteases. Therefore, it is possible that the propeptide of apAC may function in a different manner from the propeptides of these proteases.

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