Function of the N-terminal propeptide of an aminopeptidase from *Vibrio proteolyticus*

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An aminopeptidase from *Vibrio proteolyticus* was translated as a preproprotein consisting of four domains: a signal peptide, an N-terminal propeptide, a mature region and a C-terminal propeptide. Protein expression and analysis of the activity results demonstrated that the N-terminal propeptide was essential to the formation of the active enzyme in *Escherichia coli*. Urea dissolution of inclusion bodies and dialysis indicated that the N-terminal propeptide could facilitate the correct folding of the enzyme *in vitro*. Using L-Leu-*p*-nitroanilide as the substrate,

the kinetic parameters (k_{cat} and K_m) of the pro-aminopeptidase and processed aminopeptidases were analysed. The results suggested that the N-terminal propeptide inhibited enzyme activity of the mature region. In contrast, the C-terminal propeptide did not show evidence of forming an active enzyme, of correctly folding *in vitro* or of inhibiting the active region.

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

INTRODUCTION

Aminopeptidases are exopeptidases that selectively release N-terminal amino acid residues from polypeptides and proteins. Approx. 100 bacterial aminopeptidases have been purified and biochemically studied, and nearly 40 genes encoding aminopeptidases have been cloned and characterized [1].

We have recently isolated an aminopeptidase from *Aeromonas caviae* T-64 (apAC) which exhibits debittering activity by the specific release of hydrophobic amino acid residues [2]. Gene cloning and sequencing reveal that the apAC is first translated into a prepro-aminopeptidase which includes a signal peptide, an N-terminal propeptide and a mature region [3]. We have demonstrated further that the N-terminal propeptide of the pro-apAC functions as an intramolecular chaperone and that it is also involved in determining the correct folding pattern of the enzyme *in vitro* [4]. During the course of the apAC study, another aminopeptidase was isolated from *Vibrio proteolyticus* (apVP) that displayed significant similarity to the amino acid sequence of the apAC, with 56.7% similarity [4].

V. proteolyticus, previously classified as *A. proteolytica*, is a marine bacterium producing two proteases: a neutral protease and an aminopeptidase [5]. For the apVP, the substrate specificity and kinetic properties have been characterized. This protein is thermostable and is specific for substrates possessing bulky, hydrophobic residues at the N-terminals [6]. The apVP has a molecular mass of 32 kDa and binds two zinc ions per enzyme molecule [7]. Recent crystallographic data of the apVP suggest the existence of a co-catalytic zinc unit and an active site consisting of a metal-binding site and a well-defined hydrophobic pocket [8]. Cloning and sequencing of the gene encoding the apVP indicates that it is translated as a preproprotein consisting of four domains: a signal peptide (21 amino acid residues), an N-

terminal propeptide (85 residues), a mature region (299 residues) and a C-terminal propeptide (99 residues) [9,10].

To date, little information is available on the roles of the N- and C-terminal propeptides of the pro-apVP. However, it is known that the propeptides of some protease precursors play an important role in inhibiting the active domain [11].

This paper details the function of the N-terminal propeptide of the pro-apVP. Four expression vectors with signal sequences were constructed and the resulting recombinant proteins were assayed for activity. Four expression vectors lacking signal sequences were also constructed and the resulting recombinant proteins, which all formed inclusion bodies, were dissolved in urea, refolded and assayed. In addition, the kinetic parameters (k_{cat} and K_m) of the pro-aminopeptidase and processed aminopeptidases were determined.

EXPERIMENTAL

Bacterial strains, cloning vectors and DNA manipulation

Escherichia coli JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ (lac-proAB) F' (traD36 proAB⁺ lacI^q lacZΔM15)*] and *E. coli* BL21(DE3) [*hsdS gal (ΔcIts 857 ind 1 Sam7 nin5 lacUV5-T7 gene 1)*] were used as the hosts for the recombinant plasmid and expression respectively. The plasmids pET26b and pET24a (Novagen, Madison, WI, U.S.A.) were employed for subcloning, DNA sequencing and expression. *V. proteolyticus* IFO13287 was used in a genomic DNA extraction and purification of the native apVP. Plasmid DNA was prepared using a QIAprep Spin Plasmid kit (Qiagen, Hilden, Germany). Digestion by restriction enzymes was performed in the appropriate buffer at concentrations ranging from 1 to 10 units/μg of DNA for 4–16 h at the appropriate temperatures. Completion of the reaction was con-

Abbreviations used: apAC, aminopeptidase from *Aeromonas caviae* T-64; apVP, aminopeptidase from *Vibrio proteolyticus*; PA, protease, pro-aminopeptidase-processing protease from *Aeromonas caviae* T-64.

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firmed by agarose-gel electrophoresis. A QIAEX Agarose Gel Extraction kit (Qiagen) was employed for the extraction and purification of DNA from agarose gels.

Construction of expression vectors (pVSNMC, pVSNM, pVSMC, pVSM, pVNMC, pVNM, pVMC and pVM)

In order to obtain the above gene fragments, PCRs were conducted using the genomic DNA of *V. proteolyticus* as a template. The six primers used were: primer 1, 5'-GGCTT-CCATGGAAGACAAAGTGTGGATCTCA-3' (forward for pVSNMC and pVSNM, the underlined sequence is the *NcoI* restriction-enzyme site); primer 2, 5'-AGCCACCATGGTGA-TGCCGCCGATTACACAGCAG-3' (forward for pVSMC and pVSM, *NcoI* restriction site underlined); primer 3, 5'-CACC-AAGCTTTCACTGATTGCCTGGTGTGTTGGTGTGTCAC-3' (reverse for pVSNM, pVSM, pVNM and pVM, *HindIII* restriction site underlined); primer 4, 5'-GTGAAAGCTTTCA-GAAAGTGCTGGCTTTCAGG-3' (reverse for pVSNMC, pVSMC, pVNMC and pVMC, *HindIII* restriction site underlined); primer 5, 5'-GTCAGGCTCATATGGAAGACAAAG-TGTGGATCTCA-3' (forward for pVNMC and pVNM, *NdeI* restriction site underlined) and primer 6, 5'-CCAGCTTC-CATATGCCGCCGATTACACAGCAGGCG-3' (forward for pVMC and pVM, *NdeI* restriction site underlined) based on the nucleotide sequence of the *apVP* gene [9]. The pVSNMC plasmid contained the *pelB* signal peptide and the *pro-apVP* gene; pVSNM contained *pelB* and the ΔC -*pro-apVP* gene (where ΔC indicates that the C-terminal propeptide is deleted); pVSMC contained *pelB* and the ΔN -*pro-apVP* gene (where ΔN indicates that the N-terminal propeptide is deleted); pVSM contained *pelB* and the $\Delta N\Delta C$ -*pro-apVP* gene; pVNMC contained the *pro-apVP* gene; pVNM contained the ΔC -*pro-apVP* gene; pVMC contained the ΔN -*pro-apVP* gene and pVM contained the $\Delta N\Delta C$ -*pro-apVP* gene.

DNA polymerase from *Pyrococcus kodakaraensis* KOD1 (TOYOBO, Osaka, Japan) was used at a concentration of 2.5 units/50 μ l of reaction mixture. The following PCR programme was employed: 98 °C for 3 min, then 98 °C for 15 s, 65 °C for 15 s and 72 °C for 30 s (25 cycles), and finally 72 °C for 10 min. The PCR was performed using a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT, U.S.A.). After digestion and gel purification, the fragments were ligated with the appropriately prepared plasmids: for pVSNMC, pVSNM, pVSMC and pVSM, pET26b was used with the *NcoI* and *HindIII* restriction-enzyme sites; for pVNMC, pVNM, pVMC and pVM, pET24a was used with the *NdeI* and *HindIII* restriction-enzyme sites. The resultant plasmids were sequenced by the dideoxy chain-termination procedure 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 using the GENETIX program (Software Development Co., Tokyo, Japan).

Purification of inclusion bodies and refolding

To purify the inclusion bodies of *pro-apVP*, ΔC -*pro-apVP*, ΔN -*pro-apVP* and $\Delta N\Delta C$ -*pro-apVP*, the cells 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 enzymes. To renature enzymes, 5 ml of each denatured enzyme was dialysed

at 4 °C in Tris/HCl buffer (pH 8.5) containing 1 mM $ZnCl_2$. The resulting protein solutions were tested for enzymic activity. At the same time, 30 μ l of each denatured ΔC -*pro-apVP* or $\Delta N\Delta C$ -*pro-apVP* was mixed with 2.1 ml of 0.1 % Tween-40 solution and kept at 20 °C for 5 days. At each time point, 50 μ l of each solution was taken and subjected to assay of the enzymic activity.

Purification of the *pro-apVP*, ΔC -*pro-apVP*, native *apVP* and PA protease (the pro-aminopeptidase-processing protease from *A. caviae* T-64)

The recombinant proteins were expressed and isolated as described previously [4]. The yields of the *pro-apVP* and ΔC -*pro-apVP* were approx. 5 and 10 mg/litre of culture respectively. Native *apVP* was purified from the culture filtrate of *V. proteolyticus* as described by Schalk et al. [12] and PA protease was purified from the culture filtrate of *A. caviae* T-64 as reported previously [4].

Processing of *pro-apVP* and ΔC -*pro-apVP* and determination of protein content

The processing of 52 kDa *pro-apVP* and 41 kDa ΔC -*pro-apVP* to produce 32 kDa of mature *apVP* was carried out in 20 mM Tris/HCl buffer (pH 8.5) containing 0.5 M NaCl at 30 °C for 1 h. The ratio of recombinant *pro-apVP* to PA protease was 1:1000 (w/w). To activate *pro-apVP* expressed in the culture filtrate and in the supernatant of transformant cell lysate carrying the recombinant plasmids or pET26b, 40 μ l of the solution was treated with 2 μ g of PA protease at 37 °C for 1 h. Absorptions at 280 nm were measured to determine the concentration of proteins using ϵ_{280} 71 000 M⁻¹·cm⁻¹ for *pro-apVP*, ϵ_{280} 52 400 M⁻¹·cm⁻¹ for ΔC -*pro-apVP*, ϵ_{280} 62 000 M⁻¹·cm⁻¹ for ΔN -*pro-apVP* and ϵ_{280} 43 400 M⁻¹·cm⁻¹ for $\Delta N\Delta C$ -*pro-apVP*, native *apVP*, PA protease-cleaved *pro-apVP* and PA protease-cleaved ΔC -*pro-apVP*. The absorption coefficient for each protein was calculated from the amino acid composition of Trp, Tyr, His, Met, Cys and cystine residues using the molar absorption coefficients 5070, 1190, 5, 4, 20 and 111, respectively [13].

Amino acid sequencing

In order to identify the N-terminal amino acid sequences of *pro-apVP*, ΔC -*pro-apVP*, PA protease-cleaved *pro-apVP* and PA protease-cleaved ΔC -*pro-apVP*, Edman degradation [14] was performed using a G1000A protein sequencer (Hewlett Packard, Palo Alto, CA, U.S.A.).

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

Aminopeptidase activity and K_m and k_{cat} values were determined using L-Leu-*p*-nitroanilide as a substrate as described previously [2].

Enzymes and chemicals

All restriction enzymes (from Takara Shuzo, Shiga, Japan) and DNA-ligation kits (TOYOBO) used in this study were purchased from commercial suppliers and used according to the manufacturer's instructions. All other chemicals used were of analytical-reagent grade.

RESULTS

Expression of pro-apVP, Δ N-pro-apVP, Δ C-pro-apVP and Δ N Δ C-pro-apVP

In order to investigate the role of the N- and C-terminal propeptides, four expression vectors containing the T7 *lac* promoter, *pelB* signal sequences and different gene fragments were constructed (Figure 1). The gene fragments in the plasmids were sequenced, confirming that there had been no PCR-derived errors. The data in Table 1 clearly indicate that very little aminopeptidase activity was detected in the supernatants of the cell lysates in the transformants containing the pVSMC and pVSM plasmids (both without the N-terminal propeptides). Strong enzyme activities were found in the cell lysates containing plasmids pVSNMC and pVSNM (both with N-terminal propeptides). Moreover, the enzyme activities of the supernatants for the cell lysates of pVSNMC and pVSNM increased after

Figure 1 Construction of the apVP expression vectors

pVSNMC, pVSNM, pVSMC and pVSM were incorporated into pET26b, and pVNMC, pVNM, pVMC and pVM were incorporated into pET24a. The *apVP* gene is under the control of the T7 promoter and *lac* operator. All plasmids have a gene for kanamycin resistance. *pelB* indicates the signal peptide, which directs translation products to the *E. coli* periplasmic space. *Nco*I, *Hind*III and *Nde*I indicate restriction-enzyme sites.

Table 1 Aminopeptidase activities of recombinant enzymes expressed in the cell-lysate supernatants of the transformants

The units were based on the volume of culture medium.

	Aminopeptidase activity (m-unit/ml)	
	No treatment	PA protease-cleaved
BL21(DE3)pVSNMC (pro-apVP)	230	6100
BL21(DE3)pVSNM (Δ C-pro-apVP)	360	14000
BL21(DE3)pVSMC (Δ N-pro-apVP)	1.7	1.3
BL21(DE3)pVSM (Δ N Δ C-pro-apVP)	6.7	3.8
BL21(DE3)pET26b (control)	1.9	0.090

Figure 2 SDS/PAGE analysis of the expression of the pro-apVP, and Δ C-, Δ N- and Δ N Δ C-pro-apVP in *E. coli*

Expression of each recombinant was induced by the addition of isopropyl β -D-thiogalactoside. Soluble (S) and insoluble (I) fractions of the cell lysates of *E. coli* BL21(DE3)pET26b (control), BL21(DE3)pVSNMC (pro-apVP), BL21(DE3)pVSNM (Δ C-pro-apVP), BL21(DE3)pVSMC (Δ N-pro-apVP) and BL21(DE3)pVSM (Δ N Δ C-pro-apVP) were loaded into the lanes indicated. Arrowheads indicate the positions of the expressed proteins. Loaded into each lane were 50 μ g (soluble fractions) or 20 μ g (insoluble fractions) of proteins. The molecular-mass standards (M) used were from a 10 kDa protein ladder (Gibco-BRL Life Technologies, Gaithersburg, MD, U.S.A.).

treatment of the cell lysates with a PA protease (Table 1). SDS/PAGE analysis demonstrated that all four plasmids expressed the respective recombinant proteins and that pro-apVP and Δ C-pro-apVP (both with N-terminal propeptides) were soluble, whereas Δ N-pro-apVP and Δ N Δ C-pro-apVP were not soluble (Figure 2). These data indicate that the N-terminal propeptide is essential for the expression of activity and for the solubility of the enzyme in *E. coli*, whereas the C-terminal propeptide is not.

Denaturation and renaturation of the recombinant enzymes

To facilitate an investigation of the role of the N- and C-terminal propeptides in enzyme folding *in vitro*, we constructed another four plasmids lacking signal sequences: pVNMC, pVNM, pVMC and pVM (Figure 1). The gene fragments inserted into pET24a (*Nde*I/*Hind*III) were sequenced, confirming that there were no PCR-derived errors present. As depicted in Figure 3(A), all expressed proteins formed inclusion bodies and these were purified using urea dissolution (Figure 3B). After dialysis, the activities of pro-apVP and Δ C-pro-apVP (both with the N-terminal propeptide) could be recovered, while those of Δ N-pro-apVP and Δ N Δ C-pro-apVP (both without the N-terminal propeptide) could not (Table 2). These results indicate that the N-terminal propeptide can facilitate the correct folding of the enzyme *in vitro* while the C-terminal has no such ability.

As shown in Figure 4, the activity of Δ C-pro-apVP was quickly recovered, reaching 28% of the maximum value after 24 h. After 60 h, activity reached 34% of the maximum value. In

Figure 3 SDS/PAGE analysis of the expression of the pro-apVP, and Δ C-, Δ N- and Δ N Δ C-pro-apVP in *E. coli* (without the signal peptide), and the purified inclusion bodies

(A) Expression of each recombinant was induced by the addition of isopropyl β -D-thiogalactoside. Soluble (S) and insoluble (I) fractions of the cell lysates of *E. coli* BL21(DE3)pET24a (control), BL21(DE3)pVNM (pro-apVP), BL21(DE3)pVNM (Δ C-pro-apVP), BL21(DE3)pVMC (Δ N-pro-apVP) and BL21(DE3)pVM (Δ N Δ C-pro-apVP) were loaded into the lanes indicated. Arrowheads indicate the positions of the expressed proteins. Lane M, molecular-mass standards. (B) Purified inclusion bodies of pro-apVP (lane 2), Δ C-pro-apVP (lane 3), Δ N-pro-apVP (lane 4) and Δ N Δ C-pro-apVP (lane 5) were subjected to SDS/PAGE (12.5% gel) under reducing conditions. Into each lane was loaded 1 μ g of protein. Lane 1, molecular-mass standards.

Table 2 Aminopeptidase activities of recombinant enzymes before and after dialysis

Enzyme	Aminopeptidase activity (m-unit/mg)	
	Before dialysis	After dialysis
Pro-apVP	0.21	41
Δ C-Pro-apVP	0.1	18
Δ N-Pro-apVP	0.091	0.11
Δ N Δ C-Pro-apVP	0.038	0.95

contrast, the activity of Δ N Δ C-pro-apVP recovered very slowly, reaching only 1.0% of the maximum value after 24 h, and after 60 h the activity recovered to a level of 3.7%.

Figure 4 Effect of N-terminal propeptide on activity recovery of Δ C-pro-apVP (■) and Δ N Δ C-pro-apVP (▲)

The activity was measured by mixing the denatured protein solution (1 vol.) with 0.1% Tween-40 solution (70 vol.) at 20 °C at $t = 0$. Samples were taken at the indicated times (x-axis) and activity assayed. The percentage activity values of the renatured enzymes (y-axis) were calculated using specific activities (35 unit/mg for Δ C-pro-apVP and 530 unit/mg for Δ N Δ C-pro-apVP). Determinations were performed on duplicate samples and the results are depicted as the means.

Figure 5 SDS/PAGE of pro-apVP, Δ C-pro-apVP, PA protease-cleaved pro-apVP, PA protease-cleaved Δ C-pro-apVP and native mature apVP

Purified recombinant pro-apVP (lane 2), PA protease-cleaved pro-apVP (lane 3), purified recombinant Δ C-pro-apVP (lane 4), PA protease-cleaved Δ C-pro-apVP (lane 5) and native mature apVP (lane 6) were subjected to SDS/PAGE (12.5% gel) under reducing conditions. Into each lane was loaded 1 μ g of protein. Lane 1, molecular-mass standards.

Preparation and activation of the pro-apVP and Δ C-pro-apVP

Pro-apVP and Δ C-pro-apVP were purified and subjected to SDS/PAGE (Figure 5). The results demonstrated that the enzymes were homogeneous and not contaminated by other proteins. When the N-terminal amino acid sequences of pro-apVP and Δ C-pro-apVP were analysed, they were found to be identical with the deduced gene sequence (Glu-Asp-Lys-Val-Trp), indicating that they had been correctly translated and could be used for further investigations.

Digestion with PA protease reduced the molecular mass of pro-apVP from 52 to 32 kDa and of Δ C-pro-apVP from 41 to 32 kDa (Figure 5). The resultant proteins (32 kDa) were similar to the native apVP in size. These results indicated that the N- and C-terminal propeptides were able to be cleaved by PA protease.

To identify the cleavage site, the N-terminal amino acid sequences of the PA protease-cleaved pro-apVP and Δ C-pro-

Table 3 Comparison of the kinetic parameters for the substrate L-Leu-*p*-nitroanilideData are means $\pm$ S.E.M. and were obtained from three experiments.

Enzyme	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)
Pro-apVP	12 ± 0.34	0.36 ± 0.011	30 ± 0.77
PA protease-cleaved pro-apVP	99 ± 3.3	0.15 ± 0.011	665 ± 31
Δ C-Pro-apVP	5.1 ± 0.12	0.22 ± 0.02	24 ± 0.91
PA protease-cleaved Δ C-pro-apVP	96 ± 1.7	0.15 ± 0.0058	657 ± 16
Native mature apVP	102 ± 1.3	0.14 ± 0.0043	723 ± 13

apVP were determined. The results showed that the N-terminal amino acid of the processed apVP was Met-107, identical with that of the native apVP. This indicated that the PA protease could be used to study the effects of the N- and C-terminal propeptides on the activity of the mature region.

Kinetics of the recombinant and native enzymes

Using L-Leu-*p*-nitroanilide as a substrate, the kinetic parameters of the pro-apVP, Δ C-pro-apVP, their PA protease-cleaved products and native apVP were analysed and compared. As shown in Table 3, the catalytic parameters of the PA protease-cleaved pro-apVP and PA protease-cleaved Δ C-pro-apVP are almost the same as those of the native apVP. Moreover, the processed pro-apVP, Δ C-pro-apVP and native apVP displayed an approx. 22-fold increase in the observed $k_{\text{cat}}/K_{\text{m}}$ values compared with the pro-apVP, and an approx. 27-fold increase compared with the Δ C-pro-apVP. These results suggest that the N-terminal propeptide inhibits the activity of the mature region while the C-terminal propeptide has no such apparent role.

DISCUSSION

In this paper, the function of the N-terminal propeptide of the pro-apVP was investigated. By analysing the expressed enzymes from *E. coli*, it was demonstrated that the N-terminal propeptide is essential for the formation of an active enzyme whereas the C-terminal propeptide is not. Secreted proteases are usually synthesized as inactive pro-enzymes and, following secretion, are folded into their active forms. As reported previously [15], the propeptides of cathepsins B and L (cysteine proteinases) play a role in the folding of the protein molecule. Our finding that the N-terminal propeptide of pro-apVP directly influences the activity and solubility of the enzyme suggests that the N-terminal propeptide may also play a similar role in facilitating the correct folding conformation of the enzyme *in vivo*.

The role of the N-terminal propeptide in enzyme folding was investigated further by expressing the four recombinant enzymes in *E. coli*. All of the enzymes remained within the cytoplasm of *E. coli* as inclusion bodies since they lacked signal sequences. After protein purification and refolding *in vitro*, enzyme activity was only detected in proteins containing the N-terminal propeptides, whereas enzyme activity could not be detected in the proteins without N-terminal propeptides. These observations provide further verification that the N-terminal propeptide functions to facilitate the correct folding of the polypeptide, giving rise to the observed enzyme activity *in vitro*. In contrast, the C-terminal propeptide does not appear to play a role in enzyme folding. A similar functional role of the N-terminal propeptide has been found in several bacterial and yeast

zymogens, such as pro-apAC [4], subtilisin [16] and α -lytic protease [17].

The mode of action of the propeptide in facilitating enzyme folding can be controlled in two different ways: thermodynamically or kinetically. For example, the folding of subtilisin is under thermodynamic control [16,17]. A denatured mature region of subtilisin can refold to the native state very slowly in the absence of the propeptide [16]. When catalysed by the propeptide, refolding of subtilisin is accelerated by stabilizing the folding intermediates and facilitating the folding process. Alternatively, protein folding can be under kinetic control, as is the case for α -lytic protease [17] in which a denatured mature region can form a stable folding intermediate (I state) with almost as much secondary structure (70% β -sheet) as the native state (75% β -sheet). However, the I state was not able to revert back to the native state even over an extended length of time (about 800 h) in the absence of the propeptide. This suggests that a large energy barrier blocks the conversion into the native state. The presence of the propeptide serves to accelerate the refolding process by lowering the energy barrier for the conversion. We found that the mature region of the pro-apVP (Δ NAC-pro-apVP) could recover its activity very slowly in the absence of the N-terminal propeptide whereas in the presence of N-terminal propeptide its activity recovered very rapidly. This suggested that the mature region was able to fold very slowly and that the N-terminal propeptide accelerated the folding *in vitro*. It is possible that the folding of the pro-apVP is under thermodynamic control such that the N-terminal propeptide accelerates the folding of the mature region by stabilizing the folding intermediates. Of course, this mechanism needs to be confirmed by additional supporting evidence.

By comparing the $k_{\text{cat}}/K_{\text{m}}$ values, we found that the N-terminal propeptide could inhibit the activity of the mature region of the enzyme while the C-terminal propeptide showed no such inhibitory action. The observation that the N-terminal propeptide was not able to completely inhibit the activity of the mature region is similar to that reported for pro-apAC and pro-carboxypeptidase A [4,18]. The crystal structure of pro-carboxypeptidase A shows that the N-terminal propeptide partly shields the entrance of the active centre of the enzyme, allowing partial activity to be detected [18]. In the case of pro-apVP, it is likely that the N-terminal propeptide has not completely prevented the entrance of the substrate to the active centre, resulting in the activity observed.

From our observations, it appears that the C-terminal propeptide is not essential for the formation of an active enzyme or the refolding of the mature region *in vitro*. A comparison of the nucleotide sequence shows that the C-terminal propeptide of pro-apVP shares 53% similarity with that of the serine exopeptidase of *V. alginolyticus* [19], 48% similarity with that of the protease of *V. cholera* [20] and 49% similarity with that

of the PA protease [21]. Such high levels of similarity suggest that the C-terminal propeptide may play a role in unknown species-specific systems.

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