A Secreted Aminopeptidase of Pseudomonas aeruginosa

IDENTIFICATION, PRIMARY STRUCTURE, AND RELATIONSHIP TO OTHER AMINOPEPIDASES*

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Using leucine-\(p\)-nitroanilide (Leu-pNA) as a substrate, we demonstrated aminopeptidase activity in the culture filtrates of several Pseudomonas aeruginosa strains. The aminopeptidase was partially purified by DEAE-cellulose chromatography and found to be heat stable. The apparent molecular mass of the enzyme was \(\approx 56\) kDa; hence, it was designated AP\textsubscript{56}. Heating (70 °C) of the partially purified aminopeptidase preparations led to the conversion of AP\textsubscript{56} to a \(\approx 28\) kDa protein (AP\textsubscript{28}) that retained enzyme activity, a reaction that depended on elastase (LasB). The pH optimum for Leu-pNA hydrolysis by AP\textsubscript{28} was 8.5. This activity was inhibited by Zn chelators but not by inhibitors of serine- or thiol-proteases, suggesting that activity was inhibited by Zn chelators but not by in-

Pseudomonas aeruginosa is an opportunistic pathogen commonly associated with infections in patients suffering from cystic fibrosis, extensive skin burns, or suppressed immunity (1). Virulence of P. aeruginosa is largely related to its ability to secrete into the environment a variety of toxic and degradative enzymes. Most notable in this regard are several proteases that
can cause extensive tissue damage, interfere with host defenses, and promote bacterial propagation and invasion during infection. Of the four endopeptidases known to be secreted by P. aeruginosa, elastase (also termed pseudolysin; encoded by lasB) is a leading virulence factor (2–4). It cleaves preferentially peptide bonds on the amino side of hydrophobic residues and can degrade numerous host proteins in addition to elastin (3). In many P. aeruginosa strains, elastase is also the major secreted protein. Alkaline proteinase (aeruginolysin) (5), another endopeptidase secreted by P. aeruginosa, can cleave a wide range of peptide bonds but its specific activity against proteins such as casein is 10-fold lower than that of elastase, and it has no elastinolysis activity (4–6). LasA protease (staphylysin) (7) has high staphylolytic activity that results from cleavages of the pentaglycine cross-linkages within the peptidoglycan of Staphylococcus aureus cells (8). LasA protease can also nick elastin at certain Gly-Gly sequences (9, 10), an activity that increases the susceptibility of elastin to other proteases and contributes to the elastinolytic potential of P. aeruginosa (9, 11, 12). The fourth endopeptidase secreted by P. aeruginosa (lysine-specific endopeptidase; protease IV) cleaves peptide bonds on the carboxyl side of lysine residues in peptides and proteins and can act on a number of host proteins including complement components, IgG, and fibrinogen (13–15). LasD, a secreted protein first described as a second staphylolytic pro-
tease of P. aeruginosa (16), is apparently not a protease but may function as a chitin-binding protein (17).

One of the roles of bacterial extracellular proteases is provi-
sion of readily available nutrients required for rapid bacterial growth (18). Small peptides and free amino acids released by the proteases from proteins in the environment can be taken up by the bacteria and utilized as a source of both carbon and nitrogen (19). The action of secreted endopeptidases, proteases that cleave internal peptide bonds, is complemented by exopepti-
dases such as amino- or carboxypeptidases that can release single amino acids from the respective ends of peptides and proteins. Bacterial aminopeptidases may be located in the cytoplasm, periplasm, on membranes, associated with the cell envelope, or secreted into the environment (for review see Ref. 20). Of the secreted aminopeptidases, those produced by Vibrio proteolyticus (formerly Aeromonas proteolytica) and Streptomy-
ces griseus (designated below as VpAP\textsuperscript{1} and SgAP, respective-
ly), have been studied thoroughly (21, 22). Both belong to the same family (M28) of Zn-dependent proteolytic enzymes with co-catalytic metal centers (23). Unlike most intracellular aminopeptidases which form oligomeric structures (20, 24), VpAP

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\(\text{VpAP}\textsuperscript{1}\) The abbreviations used are: Vp, Vibrio proteolyticus; AP, aminopeptidase; Sg, Streptomyces griseus; Ac, Aeromonas caviae; Pa, Pseudomo-
nas aeruginosa; PAGE, polyacrylamide gel electrophoresis; APY, yeast aminopeptidase Y; Vc, Vibrio cholerae; Z, benzyl-oxy-carbonyl.
and SgAP appear to act as monomers of about 30 kDa molecular mass. VpAP and SgAP are also distinguished by their heat (70 °C) stability (25–27). The substrate specificity of VpAP and SgAP is similar, both cleaving preferentially hydrophobic amino acids especially leucine occupying the N-terminal position of proteins or peptides (25, 28, 29). Although the amino acid sequences of VpAP and SgAP show relatively low homology (−29% identity) their three-dimensional structures are almost superimposable (30). Both contain two adjacent zinc atoms in their active sites that are required for activity, and the five amino acid residues involved in coordination of the two active site zinc ions (two His, two Asp, and one Glu) are identical in the two enzymes and overlap closely (30). The gene coding for VpAP (but not SgAP) has been cloned (31, 32). The deduced amino acid sequence indicated that VpAP is produced as a pre-proenzyme containing a signal peptide (21 amino acid residues), an N-terminal propeptide (81 residues), a mature domain of 299 residues, and a C-terminal propeptide (100 residues) (32). Expression studies suggested that VpAP is secreted as an active thermosensitive 43-kDa protein that is readily transformed to two thermostable forms, each of ~30 kDa in size (31). More recently, genes coding for aminopeptidases secreted by *Vibrio cholerae* (33) and *Aeromonas caviae* (34) have also been cloned, and their deduced amino acid sequences revealed that both are translated as preproenzymes and show high homology to VpAP. Unlike VpAP, however, the *A. caviae* aminopeptidase (AcAP) lacks a C-terminal propeptide domain. The N-terminal propeptides of both AcAP and VpAP were found to be required for correct folding of their respective enzymes, and they also possess inhibitory activity (35–37). The aminopeptidase of AcAP has been isolated (38) and found to share many of its properties with VpAP and SgAP.

Here we describe for the first time an extracellular aminopeptidase of *P. aeruginosa* (PaAP) and show that an active 56-kDa form of the enzyme (designated AP$_{56}$) can be converted to an active form of ~28 kDa molecular mass (AP$_{28}$) with properties similar to those of SgAP, VpAP, and AcAP. Based on the N-terminal sequences determined for AP$_{28}$ and AP$_{56}$ we identified the gene coding for PaAP. The deduced amino acid sequence of the respective translation product revealed that the active 28-kDa domain corresponds to the C-terminal portion of AP$_{56}$ and shares high homology with those of SgAP and related aminopeptidases, placing PaAP in the same family of co-catalytic Zn-dependent metallopeptidases.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—*P. aeruginosa* strains used in this study include wild type strains Habs serotype 1, FRD2, and PA01 and mutant derivatives of the latter two strains, FRD2128 (lax$_A$) (39), FRD740 (lax$_B$) (40), and PAO-E64 (lax$_A$), a temperature-sensitive lax$_A$ mutant of strain PA01 (41). Cells were grown at 37 °C with aeration for 18 h in tryptic soy broth without dextrose (Difco).

**Partial Purification of the Aminopeptidase**—The aminopeptidase was purified from 400 to 800 ml of the bacterial culture filtrate. The cells were removed by centrifugation, and proteins in the supernatant were precipitated with ammonium sulfate (Ultrapure, Schwarz-Mann; 80% saturation). The precipitate was collected by centrifugation (20,000 g; 1 h), dissolved in 0.02 M Tris-Cl, 0.5 mM CaCl$_2$, pH 8.3 (Buffer A), and dialyzed extensively against the same buffer. The dialyzed solutions (40–75 ml) were further concentrated with Aquacide II (Calbiochem), and 10–15 ml samples containing ~100 mg of protein were subjected to DAE-cellulose (Whatman DE 52) chromatography. The column (2.1 × 27 cm) was equilibrated and washed with buffer A at a flow rate of 20 ml/h. 3-ml fractions were collected. Adsorbed proteins were eluted with a linear gradient of NaCl (0–0.6 M; 540 ml) in buffer A. All procedures were conducted at 4 °C.

**Enzyme Assays**—Aminopeptidase activity was determined spectrophotometrically with Leu-$p$-nitroanilide (Sigma; 0.6 mm in 1 ml of 0.05 M Tris-Cl, pH 8.3) by continuously following the increase in absorbance at 405 nm due to the release of $p$-nitroaniline. In assays with inhibitors, the enzyme was preincubated with the inhibitor for 30 or 90 min, as indicated in the legend to Table I. One unit of activity is the amount of enzyme that causes an increase in optical density at 405 nm of 1 unit/h. Enzyme input was in the range of 0.1–3 units.

**Identification and Partial Purification of the Secreted Aminopeptidase**—As a first step toward identification of the aminopeptidase, the concentrated culture filtrate of the elastase-

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*Pseudomonas aeruginosa* Aminopeptidase: Examination of culture filtrates of *P. aeruginosa* wild type strains Habs serotype 1, FRD2, and PA01 for aminopeptidase activity revealed that all culture filtrates contained Leu-$p$-nitroanilide hydrolyzing activity. This suggested that all three strains secrete an aminopeptidase(s) into their environment. The specific activity of the putative aminopeptidase (1.5–4 units/mg protein) was comparable among these strains. Similar specific activity values (2.2–3 units/mg protein) were obtained for culture filtrates from the mutant strains FRD2128, FRD740, and PAO-E64, indicating that aminopeptidase production is independent of either elastase or Lax$_A$ protease production.

To examine whether the aminopeptidase is thermostable, the concentrated culture filtrates from strains FRD2, FRD740, and FRD2128 wild type (lax$_A$ and lax$_B$, respectively) were each incubated at 70 °C for 1–3 h, conditions that have been used in the past to demonstrate the heat stability of enzymes such as SgAP and VpAP (25, 26). Heating for up to 3 h had virtually no effect on the aminopeptidase activity, indicating that PaAP is resistant to heat.

**RESULTS**

*P. aeruginosa* Secreates a Heat Stable Aminopeptidase—Examination of culture filtrates of *P. aeruginosa* wild type strains Habs serotype 1, FRD2, and PA01 for aminopeptidase activity revealed that all culture filtrates contained Leu-$p$-nitroanilide hydrolyzing activity. This suggested that all three strains secrete an aminopeptidase(s) into their environment. The specific activity of the putative aminopeptidase (1.5–4 units/mg protein) was comparable among these strains. Similar specific activity values (2.2–3 units/mg protein) were obtained for culture filtrates from the mutant strains FRD2128, FRD740, and PAO-E64, indicating that aminopeptidase production is independent of either elastase or Lax$_A$ protease production.

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Identification and Partial Purification of the Secreted Aminopeptidase—As a first step toward identification of the aminopeptidase, the concentrated culture filtrate of the elastase-
negative *P. aeruginosa* strain FRD740 and that of strain FRD2128 that does not express LasA protease were each chromatographed on a DEAE-cellulose column, equilibrated, and washed with 0.02 M Tris·HCl, 0.5 mM CaCl$_2$, pH 8.3, (buffer A). Adsorbed proteins were eluted from the column with a linear gradient of NaCl (0–0.6 M) in the same buffer (see "Experimental Procedures"). All fractions were examined for aminopeptidase as well as elastinolytic and proteolytic activities. Fig. 1 shows that, with both strains, the aminopeptidase activity adsorbed to the column and was eluted from it as a single peak between 0.2 and 0.3 M NaCl (7–11 mmho). The active peak was followed by a peak of absorbance at 280 nm (fractions 275–325) that contained brown material but, as revealed by SDS-PAGE analysis, showed no detectable protein bands (data not shown). Elastase in the culture filtrate of strain FRD2128 adsorbed to the column and was eluted from it between 0.1 and 0.2 M NaCl (3–6 mmho), in a peak preceding that of the aminopeptidase (Fig. 1A). The recovery of the aminopeptidase activity after chromatography was 60–70%.

SDS-PAGE analysis of the aminopeptidase containing fractions from strains FRD2128 and FRD740 revealed that both contained a protein with an apparent molecular mass of ~56 kDa (designated AP$_{56}$) as the major constituent (Fig. 2, left panel, lanes 2 and 5). The aminopeptidase fraction from strain FRD2128 (but not FRD740) also contained a small amount of elastase (Fig. 2, left panel, compare lanes 2 and 5). This was also reflected in assays of elastinolytic activity showing basal levels of elastinolytic activity in the ascending part of the aminopeptidase peak (Fig. 1A). Heating (70 °C, 1 h) of the DEAE-cellulose-purified aminopeptidase fraction from strain...
FRD2128 but not FRD740 led to the disappearance of AP$_{56}$ (as well as elastase and other contaminating proteins) and a concomitant appearance of a new band migrating as a protein of about 28 kDa (AP$_{28}$; Fig. 2, compare lanes 2 and 3 with 5 and 6 in left panel). While heating had virtually no effect on the aminopeptidase activity, it reduced the elastinolytic and proteolytic activities (strain FRD2128) by at least 90%. Immunoblotting analysis revealed that antibodies to AP$_{28}$ recognized both AP$_{56}$ and AP$_{56}$. (Fig. 2, right panel). This immunological cross-reactivity and the finding that the heated enzyme fraction from strain FRD2128 (which contained AP$_{28}$ as the only component) was as active as the unheated fraction, both suggested that AP$_{56}$ and AP$_{28}$ represent two active forms of PaAP. Apparently, AP$_{56}$ corresponds to the secreted form of the enzyme, whereas AP$_{28}$ is generated from it upon heating, a process that appears to depend on elastase. Observations made with the culture filtrate from the wild type strain, FRD2, were consistent with this conclusion. By immunoblotting analysis (data not shown), the crude culture filtrate from strain FRD2 as well as the aminopeptidase-enriched fraction obtained from it after DEAE-cellulose chromatography contained AP$_{56}$ but not AP$_{28}$. Furthermore, heating of the partially purified fraction led to a complete disappearance of AP$_{56}$ and concomitant appearance of AP$_{28}$ without effect on the aminopeptidase activity.

 Conversion of AP$_{56}$ to AP$_{28}$ Depends on Elastase—To demonstrate that elastase indeed plays a role in the conversion of AP$_{56}$ to AP$_{28}$, a sample of the DEAE-cellulose-purified aminopeptidase fraction from strain FRD2128, which contained some elastase, was heated to 70 °C with or without addition of the elastase inhibitor phosphoramidon. Immunoblotting analyses of the incubation solutions revealed that phosphoramidon completely blocked the conversion of AP$_{56}$ to AP$_{28}$ (Fig. 3A, compare lanes 2 and 3), supporting a role for elastase in the conversion of AP$_{56}$ to its 28-kDa form. In another experiment, the DEAE-cellulose-purified aminopeptidase fraction from strain FRD740 (lacking elastase) was heated in the absence or presence of exogenously added elastase, and the reaction solutions were analyzed by immunoblotting. As shown in Fig. 3B, at 70 °C the addition of 0.15 μg of elastase was sufficient to fully convert AP$_{56}$ to AP$_{28}$ (lane 7). The same result was obtained when heating was performed in the presence of 3 μg of elastase (Fig. 3B, lane 6). Heating in the absence of elastase had no effect on either intensity or migration position of AP$_{56}$ (Fig. 3B, lane 5). When incubated at 37 °C with either 0.15 or 3 μg of elastase, however, conversion of AP$_{56}$ to its smaller form did not take place (Fig. 3B, lanes 4 and 3, respectively). This suggested that partial proteolysis of AP$_{56}$ by elastase might depend on a conformational change within AP$_{56}$, likely to occur at elevated temperatures. Consistent with this, when the partially purified AP$_{56}$ fraction from strain FRD740 was incubated with elastase at various temperatures ranging from 37 °C to 70 °C, we found that the lowest temperature at which the conversion of AP$_{56}$ to AP$_{28}$ took place was 55 °C (data not shown).

Enzymatic Properties—To gain some insight on the enzymatic properties of PaAP, we studied the pH dependence, sensitivity to inhibitors, and cleavage preference of AP$_{28}$. The effect of pH and those of various protease inhibitors on the activity of AP$_{28}$ were examined with Leu-$p$-nitroanilide as the substrate. Hydrolysis was evident in the pH range of 7–9.5 with maximal activity observed at pH 8.5 (data not shown). As shown in Table I, the activity of AP$_{28}$ was completely inhibited in the presence of dithiothreitol and Zn chelators such as tetraethylene pentamine and 1,10-phenanthroline. 4,7- and 1,7-phenanthroline, two non-chelating isomers of 1,10-phenanthroline, were not inhibitory. AP$_{28}$ was also inhibited by EDTA and EGTA, but as opposed to 1,10-phenanthroline inhibition was partial and required both higher inhibitor concentrations and longer exposure times. Thus, at 10 mM 50–60% inhibition was exerted by these chelators after 90 min of incubation. AP$_{28}$ was insensitive to serine-proteases inhibitors such as diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, 3,4-di-chloroisocoumarin, and tosyl-lysine chloromethylketone. N-ethylmaleimide, an inhibitor of cysteine proteinases, and the elastase specific inhibitor, phosphoramidon, had no effect on the activity of AP$_{28}$. Together, these results strongly suggest that *P. aeruginosa* aminopeptidase is a Zn-dependent metalloproteinase.

To ascertain that the activity of PaAP requires a free N-terminal amino group, a property that defines aminopeptidases, we compared the action of AP$_{28}$ on peptides with free N-terminal ends such as Leu-Ala and Ala$_4$ and their respective N-blocked derivatives, Z-Leu-Ala and N-acetyl-Ala$_4$. Cleavage was assessed by thin layer chromatography (8). The results (data not shown) indicated that hydrolysis was dependent on the presence of a free N-terminal amino group in the substrate. The N-blocked peptide derivatives, Z-Leu-Ala and N-acetyl-Ala$_4$ were both resistant to hydrolysis, whereas their respective peptides, Leu-Ala and Ala$_4$, were each readily hydrolyzed, releasing either free alanine (Ala$_4$) or a mixture of alanine and leucine (Leu-Ala). These observations further supported the nature of PaAP as an aminopeptidase.

To characterize the preference of cleavage of AP$_{28}$, the relative rates of hydrolysis of various amino acid $p$-nitroanilide derivatives were compared. As shown in Table II, Leu-$p$-nitro-
anilide was found to be the preferred substrate. It was hydrolyzed at a rate one to two orders of magnitude higher than the cleavage rates of Met-, Ala-, Pro-, Val- or Phe-

**Table I**

**Inhibitors of AP**<sub>28</sub>

| Inhibitor | Concentration | Activity |
|-----------|---------------|----------|
| none      | mM | % |
| 1,10-phenanthroline | 0.5 | 50 |
| 4,7-phenanthroline | 0.5 | 95 |
| EDTA<sup>a</sup> | 10 | 60 |
| EGTA<sup>a</sup> | 10 | 48 |
| DTT        | 0.5 | 100 |
| Phosphoramidon | 0.1 | 100 |
| TLCK       | 5   | 100 |
| DFP        | 5   | 100 |
| PMSF       | 0.4 | 100 |
| DCI        | 0.5 | 107 |
| NEM        | 5   | 95 |

<sup>a</sup> Preincubation time was 90 min.

**Table II**

**Relative rates of hydrolysis of various amino acid p-nitroanilide derivatives by AP**<sub>28</sub>

| Substrate | Relative activity |
|-----------|-----------------|
| Leu-pNA   | 100             |
| Met-pNA   | 14              |
| Ala-pNA   | 3               |
| Pro-pNA   | 1.4             |
| Val-pNA   | 1.0             |
| Phe-pNA   | 0.6             |
| Glu-pNA   | 0               |
| Gly-pNA   | 0               |

**Experimental Procedures.**

To identify the gene coding for PaAP, we determined the N-terminal sequences of the first 20 residues of AP<sub>28</sub> and AP<sub>56</sub> from *P. aeruginosa* strain FRD2 (wild type). The sequences obtained, GKPNSIAKSPVLSPLGL (AP<sub>56</sub>) and TETYNNVAETRRGNPNVVL (AP<sub>28</sub>), served as probes to search for the complete amino acid sequence of the aminopeptidase gene in the genome bank of *P. aeruginosa* PAO1 (47). The results revealed an exact match of these sequences with two sequences, SGAP and APY, from *Saccharomyces cerevisiae* (APY), and a hypothetical aminopeptidase from *Bacillus subtilis*, were retrieved with the highest scores. The sequences of VpAP, VcAP, and AcAP were also retrieved, though with lower scores. A pairwise alignment between the PaAP sequence and those of each of the other aminopeptidases revealed 52% identity with SGAP, 35–36% identity with each APY and B. subtilis aminopeptidase, and 29–32% identity with each of the remaining enzymes, VpAP, VcAP, and AcAP. Multiple alignment of the various sequences using the PILEUP program revealed that the similarity was highest within the C-terminal regions of the various enzymes, in particular those corresponding to positions 287–471 in PaAP that comprise the protease domain in each of the enzymes (Fig. 5). The five amino acid residues involved in coordination of the two zinc atoms in SGAP and VpAP (marked with an asterisk in Fig. 5) were found to be conserved in PaAP as well as all of the other aminopeptidases. The respective residues, His-296, Asp-308, Glu-314, Asp-369, and His-467 in PaAP, are therefore likely to be involved in the binding of zinc by PaAP. This suggests that PaAP contains two atoms of zinc in its active site and may belong to the same family of metalloendopeptidases as do SGAP, VpAP, and their homologs.

**DISCUSSION**

While the extracellular endopeptidases of *P. aeruginosa* and their role in pathogenicity have been studied in great detail, little is known about exopeptidases secreted by *P. aeruginosa*. A hint that *P. aeruginosa* may secrete an aminopeptidase came from a recent study by Braun et al. (49) in which the authors have shown that the N-terminal sequence of a 58-kDa protein secreted by a *P. aeruginosa* mutant lacking both elastase and Apr corresponds to an unknown protein in the *P. aeruginosa* data base that exhibits...
43% identity with the C-terminal end of SgAP. Although the authors have speculated that this protein might be an aminopeptidase, the protein has not been characterized further, and its function remains unknown. In this study, we describe for the first time an aminopeptidase secreted by \textit{P. aeruginosa}. Examination of the primary structure of PaAP reveals that the sequence APSEAQQFTE found previously (49) for the N terminus of the 58-kDa putative aminopeptidase (designated below AP58) corresponds to positions 25–34 in the deduced amino acid sequence of PaAP (Fig. 4A). This indicates that PaAP and AP58 are identical, and thus assigns a function to the previously unknown protein. The finding that the N terminus of AP56 is located somewhat downstream of the respective end of AP58 is consistent with the smaller size of AP56. Apparently, in the presence of elastase and Apr (as is the case in the culture media of \textit{P. aeruginosa} strains FRD2 and FRD2128) or even in the presence of Apr alone (strain FRD740), AP58 undergoes limited proteolysis. The identification of a putative signal peptide in PaAP suggests that it is secreted via the general secretion pathway, which requires the Xcp machinery (50). In support of this, the band corresponding to the putative aminopeptidase (AP58) is not detectable in the culture medium of an xcp mutant of \textit{P. aeruginosa} (49).

In searching for the aminopeptidase, we used Leu-pNA as the substrate because it is hydrolyzed rapidly by most of the known aminopeptidases but not by endopeptidases. The nature of the Leu-pNA hydrolyzing enzyme as an aminopeptidase was
further established by demonstrating that hydrolysis was dependent on the presence in the substrate of a free N-terminal amino group. The resistance of N-acetyl-Ala$_4$ to hydrolysis by AP$_{28}$ (the heated aminopeptidase preparation) has also indicated that the activities of elastase and alkaline proteinase, which were present in the aminopeptidase preparation before heating, were practically eliminated upon heating. The availability of this, almost homogenous, endopeptidase-free enzyme preparation has permitted an initial characterization of the enzymatic properties of PaAP to explore potential resemblance to other bacterial aminopeptidases. The results of this series of experiments showed that PaAP indeed shares several properties with known bacterial aminopeptidases such as SgAP, VpAP, and AcAP. These include preference to substrates with N-terminal leucine, pH optimum, and apparent dependence on Zn for activity. The approximate size of the active domain, 28 kDa, is also close to those found for the known bacterial aminopeptidases produced by other bacteria, a possibility strongly supported by our demonstration of the sequence homology it shares with a number of such enzymes. Most important in this regard is the fact that significant sequence similarity was evident mainly in the regions corresponding to the catalytic domain (residues 273–489 in PaAP), with the highest degree of homology observed in sequences surrounding the five amino acid residues that have been identified as the ligands of the two Zn atoms in SgAP and VpAP (Fig. 5; Refs. 30, 51). Based on the conservation of the Zn-binding residues in SgAP, VpAP, VcAP, and APY all of these aminopeptidases have been assigned to family M28 (clan MH) that comprises varied co-catalytic metallopeptidases (23, 52). Because the same potential zinc binding residues are conserved in PaAP, we presume that PaAP may also contain two atoms of zinc in its active site and thus represents a new member of family M28 of the co-catalytic metallopeptidases. In view of its remarkable sequence identity with SgAP (52%), it is conceivable that the tertiary structure of the active domain of PaAP (AP$_{28}$) may be similar to those of SgAP and VpAP. The three-dimensional structures of the latter two aminopeptidases overlap closely even though they exhibit only 29% sequence identity (30).

Of the four bacterial aminopeptidases VpAP, AcAP, VcAP, and SgAP, the latter is an exception in that its gene has not been characterized. Instead, its primary structure has been determined at the protein level (21, 53). Thus, while SgAP appears to occur in the S. griseus culture filtrates as an active 30-kDa protein (21, 26), little is known about its biosynthetic and secretory pathways. VpAP, AcAP, and VcAP are also found in their respective culture filtrates as active enzymes of about 30 kDa in size (25, 33, 38). However, these enzymes are known to be secreted as larger proenzyme molecules that are converted to their respective 30-kDa forms after secretion (31, 33, 35). Although the mature forms of these enzymes are heat-stable, extracellular processing of their precursors by accompanying endopeptidases occurs at physiological temperatures and is independent of heating. Furthermore, VpAP and VcAP contain C-terminal propeptides and these too are removed soon after secretion (31, 33). The N-propeptides of AcAP and VpAP possess inhibitory activity, and they also appear to act as intramolecular chaperones involved in enzyme folding and secretion (35–37). The C-propeptides of VpAP and VcAP show considerable homology to those of several otherwise unrelated extracellular bacterial proteases, including the endopeptidase responsible for pro-AcAP processing (54) and the hemagglutinin/protease of V. cholerae that appears to be involved in the processing of pro-VcAP (33). The function(s) of the C-terminal propeptide domains is not known but they do not seem to be required for activity (36). Our comparison of the PaAP amino acid sequence with that of the other aminopeptidases shows that PaAP does not possess a C-terminal prosequence such as those of VpAP and VcAP. A more striking difference between PaAP and the other bacterial aminopeptidases is that PaAP does not undergo extensive extracellular processing under normal growth conditions. In the wild type background as well as P. aeruginosa mutants that produce elastase or Apr, a short N-terminal sequence (14 residues in strain FRD2) is removed as AP$_{28}$ is converted to AP$_{56}$ and no further processing is evident even after prolonged incubation at 37 °C. It is conceivable that, as in the case of APY (55), the short N-terminal prosequence is inhibitory to the enzyme so that its removal leads to the activation of the putatively inactive AP$_{56}$, and we already have evidence supporting this possibility. The function of the long N-terminal sequence that is present in AP$_{56}$ but not in AP$_{28}$ is not known. It is not unlikely however that this sequence is also involved in the control of the enzyme activity. In favor of this, we found that when heated briefly in the absence of elastase the conversion of AP$_{56}$ to AP$_{28}$ is associated with an increase in the enzyme activity. A similar situation has been described recently for AcAP as well as VpAP whose proenzymes are active, but the activities of the fully processed enzymes are increased at least 10-fold due to a marked increase in their respective catalytic constants (35–37).

Since the formation of AP$_{28}$ depends on heating, its biological relevance is not obvious. While elastase is still highly active at 70 °C (56), a property that could account for its involvement in this reaction, P. aeruginosa does not grow normally at elevated temperatures. The dependence of the conversion reaction on heat does suggest, however, that a conformational change in AP$_{56}$ may be required to render it susceptible to elastase. It is conceivable that under certain growth conditions, for instance in P. aeruginosa biofilms or upon bacterial contact with host tissues or cells in the course of infection, AP$_{56}$ may interact with certain cell surface components that are absent in the culture medium, and this may elicit the conformational change(s) required for processing of AP$_{56}$ into its fully active form at physiological temperatures. Understanding the mechanisms and significance of the transition from AP$_{56}$ to AP$_{28}$ requires highly purified AP$_{56}$ and experiments toward this end are underway.

It has been speculated that the principal role of secreted aminopeptidases is liberation of free amino acids from exogenous peptides required for nutrition and cell proliferation (18, 20). Complementary specificities between endopeptidases and exopeptidases of the same cellular origin have been reported (29) and seem to be critical in this regard. The cleavage specificities of the four endopeptidases secreted by P. aeruginosa suggest that hydrolysis of proteins by each of these enzymes is likely to generate fragments with hydrophobic or apolar residues at the N-terminal position as potential substrates for PaAP. Thus, the primary role of PaAP may indeed be to complement the action of the endopeptidases in the provision of free amino acids and small peptides for nutrition, propagation, and infection. During infection, PaAP may also alter the activity of biologically active peptides such as hormones and cytokines. An example for such an action is the inactivation of interleukin-8 by aminopeptidase-N (57).

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2 I. Axelrad, M. Safrin, and E. Kessler, unpublished observations.

3 I. Axelrad, M. Safrin, and E. Kessler, manuscript in preparation.
A Secreted Aminopeptidase of *Pseudomonas aeruginosa*: IDENTIFICATION, PRIMARY STRUCTURE, AND RELATIONSHIP TO OTHER AMINOPEPTIDASES

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