Elastase and the LasA Protease of *Pseudomonas aeruginosa* Are Secreted with Their Propeptides*

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**Pseudomonas aeruginosa** elastase and the LasA protease are synthesized as preproenzymes with long amino-terminal propeptides. The elastase propeptide is cleaved autacatalytically in the periplasm to form a transient, inactive elastase-propeptide complex. In contrast, the processing of proLasA does not involve autoproteolysis. In this study, we analyzed short-term *P. aeruginosa* cultures under conditions that minimize proteolysis and found that an elastase-propeptide complex is secreted, and then the propeptide is degraded extracellularly, apparently by elastase itself. LasA protease, on the other hand, was found to be secreted in its unprocessed 42-kDa proenzyme form. The processing of proLasA occurred extracellularly, and it involved the transient appearance of a 28-kDa intermediate and the respective 14-kDa LasA propeptide fragment. The processing of proLasA in *P. aeruginosa* strain FRD740, which does not express elastase, also proceeded via the 28-kDa intermediate, but the rate of processing was greatly reduced. This low rate of proLasA processing was further reduced when the activity of a secreted lysine-specific protease was blocked. Purified secreted proteases of *P. aeruginosa* (i.e. elastase, the lysine-specific protease, and alkaline proteinase) converted proLasA to the active enzyme. Processing by elastase and the lysine-specific enzyme, but not by alkaline proteinase, proceeded via the 28-kDa intermediate, and both were far more effective than alkaline proteinase in converting proLasA to the mature enzyme. We conclude that LasA protease and elastase are secreted with their propeptides, which are then degraded by secreted proteases of *P. aeruginosa*. In addition to their other functions, the propeptides may play a role in targeting their respective enzymes across the outer membrane.

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*Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen, secretes several proteases into its environment that are implied in the pathogenicity of the organism. This group of enzymes includes at least four endopeptidases, elastase (also termed pseudolysin; Refs. 1–3), alkaline proteinase (1), LasA protease (staphylolysin; Refs. 3 and 4), and a lysine-specific endopeptidase that cleaves the peptide bonds following lysine residues in peptides and proteins (5). Elastase and LasA protease can degrade elastin (1, 6, 7), and LasA protease also has high staphylolytic activity that results from cleavages of the pentaglycine cross-linkages in the cell wall peptidoglycan (3, 4, 8). LasA protease action on proteins, including elastin, is limited and directed mainly to Gly3 and Gly2-Ala sequences that are uncommon in most proteins. Elastase, on the other hand, is a potent protease that cleaves proteins at multiple sites and can degrade many host proteins in addition to elastin (1, 2). Although alkaline proteinase has a broad cleavage specificity, it is not as potent as elastase and has no elastolytic activity.

Elastase is a 33-kDa zinc metalloendopeptidase that is closely related to thermolysin (2, 3). Encoded by *lasB*, elastase is synthesized as a preproenzyme (53.4 kDa) with a classical signal peptide and a covalently linked 18-kDa amino-terminal propeptide (9–11). The 2.4-kDa signal sequence is removed upon passage through the inner membrane into the periplasm, where the propeptide is rapidly cleaved off by autoproteolysis (12). This step is apparently necessary for elastase secretion because an active site mutation blocks the secretion of elastase as well as enzymatic activity and processing (13). When *lasB* is expressed in *Escherichia coli*, autocatalytic cleavage of the propeptide occurs independently of any other *P. aeruginosa* gene product(s) (12). The elastase propeptide acts as an inhibitor of the enzyme (14, 15). After autoprocessing, it remains noncovalently associated with the mature domain to form an inactive propeptide-elastase complex, and it is in this form that elastase is found within the periplasm of *P. aeruginosa* (10, 14, 15). The propeptide also functions as an intramolecular chaperone required for correct folding and secretion competence (16, 17). Elastase translocation through the outer membrane is mediated by a complex extracellular protein export apparatus (*Xcp*) of at least 12 genes that bears significant homology to the pullulanase (type II; general secretory pathway) export apparatus of *Klebsiella oxytoca* (18). Although the propeptide is required for elastase secretion, the mechanisms involved and the fate of the propeptide remain unknown.

LasA protease is a 20-kDa zinc metalloendopeptidase belonging to the β-lytic endopeptidase family of proteases (3, 4). Like the β-lytic endopeptidase of *Achromobacter lyticus* (4), LasA protease is synthesized as a preproenzyme with an unusually long (22 kDa) amino-terminal propeptide, which is larger than the mature domain (19, 20). LasA protease seems to be exported via the Xcp system, but, as compared with elastase, little is known about its maturation and secretion. When expressed in *E. coli*, proLasA does not undergo autoprocessing and shows little or no enzymatic activity (19, 20). Substitution of His120, a putative active site residue of LasA protease (3, 4), does not prevent the processing of proLasA in *P. aeruginosa*, and an enzymatically inactive 20-kDa LasA protein is found in the culture supernatant (20). Although this suggests that proLasA processing and secretion in *P. aeruginosa* may proceed by mechanisms that do not involve autoproteolysis, no specific
proLasA-processing protease has yet been identified, and the processing site is unknown.

Here we show that in P. aeruginosa, both LasA protease and elastase are secreted with their propeptides. However, whereas LasA protease is exported in its unprocessed proenzyme form, elastase seems to be secreted as a noncovalent complex with its propeptide. The propeptides of both enzymes are degraded extracellularly by the action of elastase and other secreted proteases, and the processing of the LasA propeptide proceeds via a distinct 28-kDa intermediate. The corresponding 14-kDa propeptide fragment is also detected.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions—**E. coli strains JM109(pJK107) with lasA under the inducible trc promoter (20) and JM109:DE3(pETPRO) with the lasA propeptide coding sequence fused to a 6×His tag and cloned into the NotI site of pET28b (Invitrogen) were grown in LB broth containing ampicillin (100 mg/ml) or kanamycin (30 mg/ml), respectively. Prototrophic P. aeruginosa strain FRD2 and its lasB derivative, FRD740 (16), were grown in tryptic soy broth without dextrose (Difco; Refs. 8 and 10). In some experiments, when absorbance at 660 nm of the bacterial cell suspension reached 2 OD units, the cells were harvested by centrifugation (3,000 × g for 20 min at 4 °C) and resuspended in the original volume of fresh medium, and growth was continued for different time intervals, as indicated. P. aeruginosa strain FRD740(pSM3D7) with an amber mutation at the propeptide-mature junction of lasB (16) was grown in tryptic soy broth without dextrose plus 100 mg/ml tetracycline.

**Preparation of P. aeruginosa Cell Extracts and Culture Supernatants—**Culture supernatants for assays of proteolytic activity were prepared by centrifugation (8,000 × g for 20 min) of the cell suspensions at 4 °C. To prepare samples for SDS-PAGE1 and immunoblotting analyses, P. aeruginosa cells were harvested by centrifugation (Beckman microfuge; 1 min) at room temperature, and the cells were suspended in 10% TCA. The supernatants were recentrifuged for 4 min to remove the remaining bacteria and supplemented with bovine serum albumin (100 μg), and the proteins in the supernatants were immediately precipitated with 10% TCA. After standing overnight at room temperature, the TCA precipitates were pelleted, washed with acetone, air-dried, and dissolved in SDS sample buffer (1:10 of original volume; Ref. 21). For immunoblotting under nondenaturing conditions, samples of the cell cultures were centrifuged (Beckman microfuge; 5 min) at 4 °C, and 0.5-ml aliquots of the cell-free supernatants were immediately transferred into chilled tubes containing 50 μl of TLCK (50 mM) and 1,10-orthophenanthroline (20 mM) in water. After standing on ice for additional 20 min, these samples were frozen and stored at −70 °C until use.

**Production and In Vitro Processing of proLasA—**Production of proLasA was induced in E. coli JM109(pJK107) with 1 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma; Ref. 20). Cells from 100-ml cultures were harvested by centrifugation (8,000 × g for 20 min), suspended in 5 ml of 20 mM Tris·HCl, pH 7.5, and broken by sonication. Cell debris was removed by mild centrifugation (6,000 × g for 20 min at 4 °C), and the soluble cell fraction was then obtained by ultracentrifugation (100,000 × g for 1 h at 4 °C). Processing solutions (50 μl of 20 mM Tris·HCl and 0.25 mM CaCl2, pH 7.5) contained 50 μg of protein from the E. coli extract and 10 or 50 ng of purified elastase (22), alkaline proteinase (Nagase), or the lysine-specific propeptide of P. aeruginosa. The reaction solutions were incubated at 37 °C for 1 h, and the reaction was terminated by heating in SDS sample buffer (21). The lysine-specific endopeptidase required for this experiment was purified from P. aeruginosa strain FRD2128 by DEAE-cellulose chromatography as described previously (8), except that the column was equilibrated and eluted with 0.02 M Tris·HCl, pH 8, instead of pH 7.5. The enzyme preparation thus obtained was homogenous by SDS-PAGE and silver staining.

**Purification of LasA Protease and Its Propeptide—**LasA protease was purified from the culture supernatant of P. aeruginosa strain FRD2 by DEAE-cellulose chromatography. The Cm-cellulose column (2.2 × 23 cm) was equilibrated with 0.02 M Tris·HCl and 0.5 mM CaCl2, pH 8.0, and eluted with a linear gradient of NaCl (400 ml; 0–0.6 M). The enzyme emerged from the column at ~0.4 M NaCl and, as judged by SDS-PAGE and silver staining (data not shown), was homogenous. Fractions containing staphylococytic activity (8) were concentrated by ultrafiltration (Diaflo YM-10 membrane, dialyzed against 0.05 M Tris·HCl, pH 8, and lyophilized (23). The propeptide of LasA protease was expressed in E. coli strain JM109(DE3pETPRO) and purified from the soluble fraction of these cells by chromatography on nickel-agarose (Invitrogen).

**Antibodies—**Antibodies to LasA protease were raised in rabbits (14), purified from the IgG fraction by adsorption to LasA protease immobilized on nitrocellulose blots (23), and used at dilutions of 1:500 to 1:1,000. Antibodies to the propeptide of LasA protease were raised in rabbits as described previously (20). These antibodies were isolated from the immune serum (diluted 1:100 in 30 ml of Tris-buffereled saline plus 0.05% Tween 20 containing 1% bovine serum albumin) by adsorption to proLasA from cell extracts of E. coli JM109(pJK107) immobilized on nitrocellulose blots (24, 25). The antibody solution was concentrated with sucrose (20), dialyzed into Tris-buffereled saline plus 0.05% Tween 20, and used in immunoblots at a 1:500 dilution. Previously described rabbit antibodies to the elastase propeptide (10) were purified from the IgG fraction by adsorption to nitrocellulose-immobilized protease (23). The propeptide required for this was derived from P. aeruginosa strain FRD740(pSM3D7) (16). The cells were washed with saline and broken by sonication in 0.02 M Tris·HCl and 0.5 mM CaCl2, pH 7.5, and the propeptide eluted from the cells (16) was further purified by gel filtration on TSK HW-50 (160 cm × 1 cm), and 4-(2-aminoethyl)benzenesulfonyl fluoride (1 mM; also named Pefabloc; Merck). The soluble cell fraction was prepared by ultracentrifugation (100,000 × g for 1 h at 4 °C), and a sample of this fraction containing approximately 15 μg of protease was electrophobbed to nitrocellulose. The region corresponding to the propeptide was identified by staining with Ponceau S and used after excision for immunoadsorption (23). The elastase-propeptide antibody was used at a 1:1,000 or 1:100 dilution for immunoblots of SDS or nondenaturing gels, respectively. Immunoaffinity-purified antibodies to denatured elastase (10) were used at a dilution of 1:10,000.

**Purification of LasA Protease and Its Propeptide—**Our previous studies on the processing and secretion of LasA protease relied on analyses of overnight cultures of P. aeruginosa. The results of these studies indicated that the proteolytic processing of proLasA is not autocatalytic and may involve other secreted proteases of P. aeruginosa (20). The processing protease, its cellular location, and the stage during secretion at which processing occurs remained unknown. To better understand the mechanisms underlying the maturation and secretion of the LasA protease, in the present study, we defined conditions that allow the identification of short-lived secreted protein species. Late-log P. aeruginosa cells were suspended in fresh medium, samples were removed at short intervals after medium replacement, and TCA was added to both the cell and medium fractions to prevent proteolysis. Immunoblots with antibodies to LasA protease revealed that at any of the time points examined, the cells contained only proLasA (Fig. 1A). Although the amount of cell-associated proLasA decreased during the 2-h period of the experiment, this was not accompanied by the appearance of smaller LasA-related proteins, suggesting...
that no processing of proLasA had occurred within the cells. Consistent with this, medium samples taken at early time points contained proLasA as the only LasA-related protein (Fig. 1, B and C, 0 and 10 min). Extracellular processing was evident within 20 min and proceeded in a time-dependent manner, with just a trace of proLasA remaining after 2 h (120 min). At 20 min, small amounts of mature-size (20 kDa) LasA protease and a 28-kDa processing intermediate were detected in the medium, and their levels increased with time, in parallel to the disappearance of proLasA (40–120 min). The 28-kDa processing intermediate apparently resulted from cleavage(s) within a region in the propeptide that is sensitive to proteolysis (rather than multiple random cleavages) because a 14-kDa propeptide fragment recognized by antibodies to the LasA propeptide but not by anti-LasA protease antibodies was also detected (Fig. 1C, 20–120 min). ProLasA and the 28-kDa processing intermediate were visualized with antibodies to both mature LasA protease and the LasA propeptide (compare B and C in Fig. 1), and immunoblots of cell extracts with the LasA propeptide antibody (data not shown) yielded a pattern indistinguishable from that obtained with the LasA protease antibody (Fig. 1A), detecting proLasA as the only cell-associated propeptide-related band. Collectively, these results indicate that proLasA is not processed in the cell. Instead, proLasA is exported in its unprocessed form, and the propeptide is degraded extracellularly in a stepwise manner by the action of other secreted proteases of P. aeruginosa. Secretion of proLasA appears to occur rapidly after its formation because, at any time, the level of cell-associated proLasA was far smaller than that of the secreted precursor and its extracellular processing products together (compare A and B in Fig. 1).

Proteases Involved in Extracellular Processing of proLasA—

Elastase is the prominent and most potent protease secreted by most P. aeruginosa strains. To examine whether elastase may play a role in proLasA processing and to further establish the extracellular location of this reaction, we compared proLasA secretion and processing in strains FRD2 (wild type) and FRD740 (lasBΔ). Cells were grown continuously (i.e., without medium replacement), and aliquots were removed at 30-min intervals from the late logarithmic phase into the early stationary phase (3–7 h). Cell and media samples were both analyzed for LasA-related proteins by immunoblotting, and the level of proteolytic activity in the supernatants was determined using azocasein as the substrate. The rate of growth of both strains was also compared and found to be practically the same (Fig. 2A). The cell fractions of both strains contained comparable amounts of proLasA, and no smaller LasA-related proteins were detected in extracts from either strain (Fig. 2, B and C, compare the top panels), indicating that proLasA production and secretion in both strains were similar. However, the rate of proLasA processing in the culture supernatants of strain FRD2 (wild type) and FRD740 was reduced as compared with that found for strain FRD2 (Fig. 2, B and C, compare bottom panels). During the 3–7-h period examined, virtually no proLasA was detectable in the supernatants of strain FRD2, suggesting that processing in the supernatants of this strain occurred rapidly after secretion. Culture supernatants of strain FRD740, on the other hand, contained significant amounts of unprocessed proLasA, which decreased gradually from 6 h onward, in parallel to the limited increase in proteolytic activity in the medium (see Fig. 2A). In

FIG. 1. Secretion and extracellular processing of proLasA.

Late-log cells of P. aeruginosa strain FRD2 were suspended in fresh medium and incubated for 10 min at 37 °C for reequilibration, and aliquots removed at the time intervals thereafter (top) were concentrated with TCA and analyzed by SDS-PAGE and immunoblotting with antibodies to LasA protease (A and B, for whole cell extracts and supernatants, respectively) and its propeptide (C, supernatants). Proteins in 10-fold concentrated samples corresponding to 250 μl of the original cell culture were applied on each lane, except for lane LasA, which was loaded with 40 ng of purified LasA protease. Left, molecular masses in kDa.

FIG. 2. Extracellular processing of proLasA in P. aeruginosa strains FRD2 and FRD740: correlation with proteolytic activity and role of elastase.

Cells were grown at 37 °C, and samples taken at 30-min intervals between 3 and 7 h (OD at 660 nm, 0.8–2.4) were analyzed for both extracellular proteolytic activity and proteolytic processing of proLasA. A, growth and proteolytic activity in the media of strains FRD2 and FRD740. B and C, immunoblots with antibodies to LasA protease of cell extracts (top) and supernatants (bottom) of strains FRD2 and FRD740, respectively. Proteins in 10-fold concentrated samples corresponding to 250 μl of the original bacterial cultures were applied on all lanes. Top, time (in hours) at which each sample was taken. Left, molecular masses in kDa.
and incubated at 37 °C for 1 h with 10 and 50 ng of purified elastase (E. coli JM109(pKJ107) expressing the lasA gene from a plasmid was incubated at 37 °C for 1 h with 10 and 50 ng of purified elastase (lanes 3 and 6), or the lysine-specific endopeptidase (lanes 4 and 7), and the incubation solutions were analyzed by immunoblotting with antibodies to LasA protease. Lane 1, E. coli cell fraction alone (control). Left, molecular masses in kDa.

both strains, processing proceeded via the 28-kDa intermediate. However, this processing intermediate accumulated in the growth medium of strain FRD740 but not in that of strain FRD2. Accordingly, the levels of fully processed LasA protease in the medium of strain FRD2 exceeded those in the medium of strain FRD740 (Fig. 2, B and C, bottom panels). A correlation was evident between the rate of extracellular proLasA processing and the level of proteolytic activity in the medium, which was 6–8-fold higher at any time in supernatants of strain FRD2 than in those of strain FRD740 (Fig. 2A). These results reinforced the extracellular location of proLasA processing and indicated a role for elastase in this process. However, the demonstration that proLasA can be processed in the growth medium of strain FRD740 suggested that other secreted proteases of P. aeruginosa in addition to elastase may be involved. To investigate this, we examined which of the two best candidates, alkaline proteinase and the lysine-specific endopeptidase, could process proLasA in vitro in a manner similar to that of elastase. Samples of an E. coli extract containing recombinant (inactive) proLasA were incubated with equal amounts of purified elastase, alkaline proteinase, or the lysine-specific endopeptidase, and then proLasA processing was evaluated by immunoblotting. Fig. 3 shows that each of the three endopeptidases can convert proLasA to the 20-kDa mature-size LasA protease. However, whereas both the lysine-specific endopeptidase and elastase were highly and equally effective in proLasA processing, alkaline proteinase was relatively inefficient in this regard (compare lanes 2–4 for digests with 10 ng of protease or lanes 5–7 for digests with 50 ng of protease). Furthermore, whereas processing by elastase and the lysine-specific endopeptidase proceeded via the 28-kDa intermediate, no such intermediate was observed with alkaline proteinase (lanes 3 and 6). Apparently, alkaline proteinase cleaved the LasA propeptide randomly, generating multiple fragments that escaped from the gel and thus were not detectable. Elastase and the lysine-specific protease, on the other hand, appeared to cleave first a peptide bond(s) within the same region of the propeptide and generate the 28-kDa intermediate, and then the remaining carboxyl-terminal portion of the propeptide was degraded. Complete conversion of proLasA to the 20-kDa LasA protein by any of these proteases (for alkaline proteinase, this was achieved upon extended incubation with 50 ng of enzyme) was accompanied by a comparable increase in staphylolytic activity (data not shown). From the above-mentioned results, it appears that the lysine-specific endopeptidase, in addition to elastase, may play a role in the extracellular processing of proLasA in P. aeruginosa, although alkaline proteinase may complement their action.

To substantiate the involvement of the lysine-specific endopeptidase in the physiological processing of proLasA, we examined whether TLCK, a specific inhibitor of this enzyme (5, 7), can block proLasA processing in cultures of P. aeruginosa strain FRD740 (lasBΔ). Late-log cells were suspended in fresh medium and incubated for an additional hour to allow the initial accumulation of proLasA in the medium. The suspension was then divided into two equal portions; TLCK was added to one of these, whereas the other was supplemented with the same volume of medium, serving as a control. Both suspensions were incubated for an additional 90 min, and samples of the supernatants removed at 30-min intervals were then analyzed for LasA protease-related proteins by immunoblotting. Fig. 4 shows that at 60 min, just before the addition of TLCK, a small fraction of the secreted proLasA was already processed to mature-size LasA protease, and the 28-kDa intermediate was also detectable. In the control culture that was incubated without the addition of TLCK, proLasA processing progressed with time. At 150 min, much of the proLasA was converted to the 28-kDa intermediate, and significant amounts of the intermediate were processed to mature-size LasA protease. The addition of TLCK, on the other hand, blocked the extracellular processing of proLasA almost completely. Virtually no increase in the amount of the intermediate beyond that seen at 60 min was evident, and only a slight increase in the level of mature-size LasA protease was seen, which most likely reflected the action of alkaline proteinase. Furthermore, whereas the proLasA level decreased gradually with time in the control samples, a slight accumulation of proLasA was noted in the media from TLCK-treated cultures. TLCK seemed to affect the secretion of proLasA. However, immunoblotting with antibodies to the lysine-specific endopeptidase revealed that TLCK had no effect on the secretion of this enzyme (data not shown). Activity assays with the specific substrate, Tosyl-Gly-Pro-Lys-para-nitroanilide (5), indicated that TLCK blocked the activity of the secreted lysine-specific endopeptidase by at least 95%. This inhibition most probably accounted for the observed drop in the rate of processing of secreted proLasA, supporting a role for the lysine-specific endopeptidase in the physiological processing of proLasA. We conclude that elastase and the lysine-specific protease are key players in the extracellular processing of proLasA in P. aeruginosa. In their absence, alkaline proteinase may substitute for their action, albeit not as effectively and via...
a mechanism that does not involve the formation of distinct intermediates.

Elastase Is Secreted with Its Propeptide—When P. aeruginosa is grown under most conditions, little or no proelastase is found in the cells, nor is it detectable in the supernatants (10, 13). Instead, proelastase is rapidly processed in the periplasm to form an inactive elastase-propeptide complex (10, 15), and this seems to be required for the efficient secretion of elastase (13). Previously, we speculated that the propeptide is degraded in the periplasm (10). The dependence of elastase secretion on the propeptide, which was recognized in subsequent studies (16, 17), led us to examine the possibility that elastase is exported along with the cleaved propeptide. As in the case of LasA protease, elastase secretion was followed by analyzing samples from short-term cultures of late-log phase P. aeruginosa cells suspended in fresh medium. To detect an elastase-propeptide complex, which requires non-denaturing conditions, the culture supernatants were not treated with TCA, and the proteolysis of unstable secreted proteins was minimized by the addition of protease inhibitors (see "Experimental Procedures"). SDS-PAGE and immunoblotting revealed that supernatant samples removed during the first 40 min contained elastase (Fig. 5, top panel) as well as significant amounts of the 18-kDa propeptide (Fig. 5, bottom panel). Also, a propeptide-related protein of about 15 kDa, which is likely a degradation product of the propeptide, was observed. The intensity of the propeptide band remained approximately the same between 0 and 40 min and decreased rapidly between 40 and 60 min; at 2 h, the propeptide was no longer detectable. The rapid disappearance of the propeptide at the later times correlated well with the accumulation of elastase in the medium (Fig. 5, top panel), suggesting that elastase was involved in the degradation of its own propeptide.

Immunoblotting of the culture supernatants under nondissociative conditions revealed that the samples removed during the first 20 min contained an elastase-related protein band designated E/P that migrated more slowly than elastase. By 20 min, two additional elastase-related bands with an intermediate mobility between that of E/P and elastase were evident, but no free elastase was detectable (Fig. 6, left panel). E/P was highlighted by antibodies to both elastase and its propeptide (Fig. 6, left and right panels, respectively) and migrated identically with the periplasmic elastase-propeptide complex, suggesting that it represents a noncovalent elastase-propeptide complex. Another band with mobility between that of E/P and elastase and identified in the periplasmic preparation of E/P was visualized with both antibodies and may represent an E/P complex containing partially degraded propeptide. Free elastase appeared by 40 min, and its level increased further by 60 min, but the elastase-propeptide complex and the band with intermediate mobility were detectable throughout the experiment. Collectively, the above mentioned results indicate that elastase is secreted as a noncovalent complex with its propeptide, and then the propeptide is degraded extracellularly, most probably by elastase itself.

**DISCUSSION**

Our earlier studies on elastase processing and secretion suggested that the elastase propeptide might be degraded in the periplasm (10). The dependence of elastase secretion on its propeptide, which was subsequently established by us (16) and others (17), raised the possibility that the propeptide may provide information required for passage through the outer membrane and thus may be secreted along with its enzyme. The propeptide of the α-lytic protease from Lysobacter enzymogenes, another protease that depends on its propeptide for folding and secretion, has been demonstrated in the medium of E. coli cells expressing the α-lytic protease gene (28). However, a limitation of this study was the use of a heterologous organism, E. coli, as a host. Because the genes for the E. coli type II export machinery (gsp) are barely expressed (29), the appearance of extracellular α-lytic protease and its propeptide in the medium of these cells could result from the perturbation of membrane physiology caused by overexpression of the α-lytic protease. No evidence for the secretion of amino-terminal propeptides of other proteases has yet been obtained in the natural bacterial host. In the case of P. aeruginosa, standard culture supernatants contain high levels of proteolytic activity, precluding the survival of labile protein species such as free propeptides andzymogens. To prevent the proteolytic degradation of such short-lived proteins, protein secretion was analyzed in the present study after replacing the culture broth with fresh medium to examine the onset of protease production. In addition, proteolysis was controlled by adding either TCA or protease inhibitors to the samples. Our results clearly indicate that elastase is secreted as a noncovalent complex with its propeptide, and the propeptide is degraded extracellularly, most likely by elastase itself. This is suggested by the inverse relationship between the levels of elastase and its propeptide in medium samples removed from P. aeruginosa cultures at times later than 40 min (Fig. 5). The apparent stability of the propeptide seen at the earlier time points is
misleading. In fact, it probably represents a steady-state situation in which the rate of secretion and that of propeptide degradation are still balanced. The complete disappearance of the propeptide observed once high amounts of elastase accumulate in the medium (Fig. 5) strongly supports the involvement of elastase in the degradation of its own propeptide. Furthermore, in a previous study (15), we noted that after its isolation from the periplasm, the elastase-propeptide complex becomes extremely unstable, undergoing spontaneous activation due to the loss of the propeptide, even when stored at low temperatures. Stabilization of the purified inactive complex was achieved by adding a detergent, Brij-35. The detergent probably substituted for the stabilizing interactions of the elastase-propeptide complex with envelope components, interactions that are lost upon purification as well as after secretion. Extracellular degradation of the propeptide apparently does not require prior dissociation of the complex, because native intermediates recognized by antibodies to both elastase and its propeptide were demonstrated (Fig. 6).

Secretion of the elastase-propeptide complex, documented here for the first time, is consistent with our previous findings that indicate that autoprocessing within the periplasm is essential for efficient elastase secretion (3, 10). However, an alternative secretion pathway for elastase involving the secretion of unprocessed proelastase cannot be excluded. Extracellular proelastase can be observed when autoprocessing is prevented by site-directed mutagenesis of the elastase active site, or by growing the bacteria in media depleted of Zn and Ca (30). However, the secretion of proelastase under such conditions appears to be inefficient. The export of unprocessed proelastase probably represents a secondary pathway for elastase secretion, with the primary pathway involving export of the inactive elastase-propeptide complex.

The demonstration of proLasA as the first LasA-related protein to appear in the medium and the absence of proLasA-processing intermediates in the cells provide strong evidence that LasA protease is secreted as an unprocessed proenzyme. In contrast to elastase, this seems to be the primary and perhaps the only pathway for LasA protease secretion. In agreement with our previous findings (20), the processing of proLasA does not involve autoproteolysis. It occurs in the extracellular environment and involves secreted proteases other than LasA protease itself, in particular, elastase and the lysine-specific endopeptidase. Processing seems to occur in two steps. At first, cleavage(s) by either elastase or the lysine-specific endopeptidase takes place at a site approximately 8 kDa upstream of the mature junction. This produces a 28-kDa processing intermediate (Figs. 1–4), and a 14-kDa propeptide fragment is released (Fig. 1). Based on the cleavage specificity of the proteases involved and the size of the resulting products, we presume that the lysine-specific endopeptidase may cleave the bond on the carboxyl side of Lys168 (numbered as in preproc LasA; Ref. 20), whereas elastase may cleave a peptide bond involving one of the few alamines surrounding this lysine residue. The propeptide fragment and the carboxyl-terminal portion of the LasA propeptide that remains attached to the mature domain are then degraded by multiple cleavages, most likely by the combined action of elastase and the lysine-specific protease. Alkaline proteinase can lead to LasA protease maturation (Fig. 3), although far less effectively than elastase and the post-lysine cleaving enzyme, and this may be important in strains lacking these proteases.

Despite this processing by multiple proteases, the amino terminus of mature LasA protease is the same. This suggests that the amino-terminal portion of mature LasA protease has a structure resistant to proteolysis. However, another mechanism is required to ensure the complete removal of the short propeptide sequence(s) remaining after cleavage by any of the three endopeptidases. Conceivably, this may be accomplished by an aminopeptidase, and we have recently discovered such an enzyme in the culture media of P. aeruginosa. The two proline residues at positions 2 and 3 of the mature LasA protease may serve to stop the presumed sequential degradation of the extra propeptide sequences by the aminopeptidase at the exact mature junction, because peptide bonds involving the imino group of proline are resistant to most proteases. This may be an important mechanism in the absence of propeptide-controlled autoprocessing. The finding that the first cleavage(s) within the propeptide occurs at a specific site and generates relatively stable products suggests that the LasA propeptide may contain two functionally distinct domains. Each domain may have a considerable degree of tertiary structure, and the two domains may be connected to each other by a linker in which the proteolytic processing of the LasA propeptide is initiated. Conceivably, one of these domains may have folding and targeting functions, whereas the other may function as an inhibitor of LasA protease. Based on the sequence homology with proteins of known functions, a similar domain organization has been predicted for the propeptide of a-lytic protease (31).

Elastase and LasA protease are representatives of many prokaryotic and eukaryotic secreted proteases that are synthesized with long amino-terminal propeptides. There seems to be a close relationship between folding, propeptide autoprocessing, and secretion, and this has been established for elastase (13, 16, 17) as well as other secreted bacterial proteases bearing amino-terminal propeptides such as subtilisin E from Bacillus subtilis (32) and a-lytic protease from L. enzymogenes (28). It has been suggested that only properly folded and processed forms of such proteases are exported through the extracellular environment (29). Our demonstration that elastase and LasA protease are both secreted with their propeptides, the first experimental evidence for such a mechanism, suggests that secretion of the propeptide may be a property shared by other extracellular bacterial proteases. This also favors a role for the propeptides in targeting and recognition by the export machinery of their cognate enzymes. However, autoprocessing of the propeptide may not be a universal mechanism. This mechanism may be utilized by endopeptidases with broad specificity, whereas prosenzymes of endopeptidases with restricted specificity or those of exopeptidases may depend on heterologous endopeptidases for processing as well as the ultimate degradation of their propeptides. Proenzymes of many endopeptidases with general proteolytic activity, including subtilisin E (32), a-lytic protease (28), elastase (12, 13), thermolysin, and other thermolysin-related neutral proteases (33, 34), have indeed been shown to be processed autocatalytically, whereas procarboxypeptidase Y depends on yeast vacuolar endopeptidases for processing (35).


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