Identification of residues in the *Pseudomonas aeruginosa* elastase propeptide required for chaperone and secretion activities

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An important virulence factor of the opportunistic human pathogen *Pseudomonas aeruginosa* is elastase, a secreted thermolysin-like neutral zinc-metalloprotease (TNP). Elastase is synthesized as a larger precursor with an amino-terminal 18 kDa propeptide, and was the first TNP shown to require its propeptide as an intramolecular chaperone (IMC) for activity and secretion. This paper reports the analysis of the elastase propeptide to identify residues conserved among other TNP precursors that may be critical for its IMC function. The prosequences of TNP precursors from both Gram-negative (*Vibrio* species and *Legionella* species) and Gram-positive (*Bacillus* species) bacteria were found to show homology to the elastase propeptide. Two regions of conserved residues were observed: a hydrophilic region (ProM) found in the middle of the elastase propeptide and a more hydrophobic region (ProC) located proximal to the propeptide-processing site. To test whether such conserved motifs were important to function, single residue substitutions at eight conserved amino acids were introduced within the full-length pre-proelastase precursor by site-specific mutagenesis of *lasB*, the gene encoding elastase. Mutant *lasB* alleles were expressed from plasmids within a *lasB*-deleted *P. aeruginosa* strain, FRD740, and the effects of these propeptide alterations on elastase enzyme activity, processing, stability and accumulation inside and outside of the cell were examined. Within the ProM region, substitution at Arg74 resulted in a dramatic accumulation of proelastase in the cell, suggesting a secretion defect, and a dramatic reduction in supernatant elastolytic activity. Substitution at Asn68 adversely affected the amount of elastase in the culture supernatant, apparently as a result of the reduced stability of the mutated proelastase in the cell. Within the ProC region, mutations at Ile181 and Ala183 abolished the accumulation of a stable elastase molecule in the supernatant. Most mutations produced a phenotype consistent with a defect in protein folding and stability. Overall, the data from this preliminary study show that conserved residues within the elastase propeptide are essential for its function and begin to define the mechanisms of action of IMCs in the TNP family.

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INTRODUCTION

Most proteases secreted by eukaryotic and prokaryotic cells are synthesized as inactive proenzymes or zymogens. These precursors often have a pro-domain, generally called a propeptide, that is covalently attached to the amino and/or carboxy terminus of the mature enzyme sequence and controls the activity of the enzyme (Wandersman, 1989). The propeptide can be required for the proper folding of the protease into an active enzyme, and propeptides

exhibiting such activity are termed intramolecular chaperones (IMCs) (Inouye, 1991; McIver *et al.*, 1995; Shinde & Inouye, 1993). IMCs are generally associated with proteinases that are synthesized as pre-proenzymes with an amino-terminal signal (pre) sequence followed by a propeptide (pro) domain, and the mature enzyme located at the carboxy-terminal end of the precursor molecule. Unlike molecular chaperones, which act on multiple substrates and depend on ATP for activity, IMCs are highly specific for their respective proteases and require no energy in the form of ATP hydrolysis to accomplish their task (Inouye, 1991; Shinde & Inouye, 1993). The IMC (propeptide) is cleaved at a specific site, usually by self-processing, and is degraded once its chaperone function is complete.

Abbreviations: IMC, intramolecular chaperone; TNP, thermolysin-like neutral zinc-metalloprotease.

Subtilisin E is an alkaline serine protease secreted by *Bacillus subtilis* and was the first demonstrated IMC-containing protease. Its 77 residue propeptide is required to guide its folding into an active enzyme (Ikemura *et al.*, 1987; Zhu *et al.*, 1989). The α -lytic protease of *Lysobacter enzymogenes* is an extracellular serine proteinase that has a 166 residue propeptide that promotes folding of the enzyme into an active, secretion-competent, highly stable conformation (Fujishige *et al.*, 1992; Silen & Agard, 1989; Silen *et al.*, 1989). No obvious sequence homology exists between the propeptides of these two proteases, but both probably function by lowering the kinetic barrier between an inactive molten-globule protein and conversion to a native proteinase (Baker *et al.*, 1992; Eder *et al.*, 1993). Many eukaryotic proteases exhibit a similar requirement for the propeptide to reduce the rate of nonproductive folding, including serine proteinases (e.g. *Saccharomyces cerevisiae* carboxypeptidase Y and *Yarrowia lipolytica* alkaline extracellular protease), aspartic proteinases (e.g. *S. cerevisiae* proteinase A and *Rhizopus niveus* proteinase I) and thiol proteinases (e.g. cathepsins L and B) (reviewed by Shinde & Inouye, 2000). The diversity of proteases that depend on their propeptides for proper folding suggests a convergent evolution of this propeptide-mediated folding mechanism (Eder *et al.*, 1993).

The subtilisin family of proteinases is large, and its members are found in both prokaryotic and eukaryotic cells. The propeptide sequences of subtilisin proteases have two conserved domains, each consisting of hydrophobic residues flanked by charged amino acids (Shinde & Inouye, 1994). Random mutagenesis of the prosubtilisin propeptide identified three short hydrophobic sequences located within these conserved domains that are critical for production of active subtilisin (Kobayashi & Inouye, 1992; Lerner *et al.*, 1990). A second-site suppressor mutation of a propeptide mutation has been mapped to the mature enzyme, suggesting that functional regions within the propeptide interact with mature subtilisin during the folding process (Kobayashi & Inouye, 1992). The crystal structure of the subtilisin E-propeptide complex has been determined (Bryan *et al.*, 1995; Janknecht *et al.*, 1991), and this provided additional information on the nature of the interactions of specific residues within subtilisin and its propeptide.

Pseudomonas aeruginosa is a Gram-negative opportunistic human pathogen that secretes a large number of toxic and degradative enzymes, including several proteolytic enzymes, that play important roles in pathogenesis. The most abundant of the secreted proteases of *P. aeruginosa* is elastase. This protease has been classified as a member of family M4 (clan MA), which includes a large group of thermolysin-like, neutral zinc-metalloproteases (TNPs) that are produced by both Gram-positive and Gram-negative bacteria (Hase & Finkelstein, 1990; Kessler & Ohman, 1998; Wetmore *et al.*, 1992).

Elastase (also called LasB protease and pseudolysin) is

initially synthesized as a precursor with a pre-pro-mature domain structure consisting of a signal peptide (23 residues), a propeptide (174 residues) and a carboxy-terminal catalytic domain (301 residues) (Kessler & Ohman, 1998). The propeptide is cleaved autocatalytically within the periplasm (McIver *et al.*, 1991). There it immediately forms an inactive complex with the processed enzyme (Kessler & Safrin, 1994), and it is in this form that elastase is secreted into the extracellular environment (Kessler *et al.*, 1998). While the propeptide can be detected in the culture supernatant (Braun *et al.*, 2000; Kessler *et al.*, 1998), it is degraded shortly after secretion and only the mature moiety is stably found in the culture supernatant.

Elastase requires its propeptide for both proper folding and secretion and represents the first TNP family member for which the IMC function of its propeptide has been demonstrated (Braun *et al.*, 1996; McIver *et al.*, 1995). It has since been shown that thermolysin from *Bacillus thermoproteolyticus*, the prototype of this family of Zn-dependent metalloendopeptidases, also has a long N-terminal propeptide that is processed autocatalytically (Marie-Claire *et al.*, 1998) and plays a role in the folding of thermolysin (O'Donohue & Beaumont, 1996). TNPs are produced by a variety of bacterial species, and all appear to be synthesized as pre-proenzyme precursors with large amino-terminal propeptides, suggesting that their propeptides may function as IMCs as well. However, very little is known about the specific residues in the propeptide that are involved in IMC functions.

The propeptides of TNPs from *Bacillus* species contain conserved residues in the carboxy-terminal portion proximal to the mature processing site that are also found in the propeptide of *P. aeruginosa* elastase (Wetmore *et al.*, 1992). In the present study, we compared the *P. aeruginosa* elastase propeptide sequence to several proteins in the database in order to identify the conserved amino acids that may represent critical residues required for chaperone function. Some of these residues were then chosen as targets for site-directed mutagenesis as an initial step toward testing the prediction that sequence conservation indicates a common and critical function. Using a native system designed to study *lasB* mutations in *P. aeruginosa* (McIver *et al.*, 1995), we constructed strains with single amino acid substitutions in the propeptide of the elastase precursor. These were then tested for their effects on the accumulation of stable, extracellular elastase.

METHODS

Bacterial strains and media. *P. aeruginosa* FRD740 [Δ *lasB*9/Tn501-6] (McIver *et al.*, 1995) contains a chromosomal deletion of >95% of *lasB* that removed the ATG start codon and sequences encoding the signal peptide (pre), propeptide and the majority of the mature enzyme. This was used as an elastase-deficient strain background for expression of *lasB* alleles with mutations in the propeptide region. *Escherichia coli* BMH71-18 *mutS* [*thi supE lac-proAB mutS::Tn10* (*F'* *proA*⁺ *B*⁺ *lacI*^q ZAM15)] (Promega) was used

to propagate DNA that was subjected to site-directed mutagenesis. *E. coli* JM109 [*endA1 recA1 gyrA96 thi hsdR17* ($r_K^- m_K^+$) *relA1 supE44* $\Delta(lac-proAB)$ (*F'* *traD36 proAB lacI Δ M15*)] (Promega) was used in the manipulation of recombinant plasmids. Bacteria were cultured in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5). Media were solidified with 1.5% Bacto agar (Difco). Unless specified otherwise, antibiotics were used at the following concentrations: ampicillin, 100 $\mu\text{g ml}^{-1}$ for *E. coli*; tetracycline, 15 $\mu\text{g ml}^{-1}$ for *E. coli* or 100 $\mu\text{g ml}^{-1}$ for *P. aeruginosa*; and mercuric chloride, 18 $\mu\text{g ml}^{-1}$ for both *E. coli* and *P. aeruginosa*.

DNA manipulations. Most routine DNA manipulations were performed as described by Maniatis *et al.* (1982). DNA sequences were determined by the chain-termination technique with custom oligonucleotides. Sequence comparisons were performed using the basic local alignment search tool (BLAST) algorithm (Altschul *et al.*, 1990). Enzymes belonging to the TNP family were based upon significant homologies within the protease domain of their molecules (Hase & Finkelstein, 1990).

Construction of *lasB* alleles encoding propeptide amino acid substitutions. Oligonucleotide-directed site-specific mutagenesis was performed using the Altered Sites *in vitro* mutagenesis system (Promega) as described by the manufacturer. Single-base-pair substitutions encoding mutant LasB propeptide residues were introduced within the plasmid pKSM4 (McIver *et al.*, 1991), which contained the wild-type *lasB* gene and regulatory region on a 2.5 kb *EcoRI*–*PstI* fragment in pAlter-1 (Promega). Site-specific substitutions were verified by DNA sequence analysis before being cloned into the broad-host-range vector pLAFR3 (McIver *et al.*, 1991) to generate the pKSM66 series of plasmids (Table 1). pKSM66 clones were mobilized into the *P. aeruginosa lasB*-deficient strain FRD740 by triparental mating as previously described (Goldberg & Ohman, 1984). Plasmid pKSM3, with wild-type *lasB* in broad-host-range plasmid pLAFR3, has been previously described (McIver *et al.*, 1995).

Assay of elastolytic activity. To obtain standardized cultures of *P. aeruginosa* strains, L broth was inoculated (1:100) with an overnight culture and then grown to an OD₆₀₀ of 0.6. This was then used to inoculate (1:100) 10 ml of L broth and incubated at 37 °C with aeration for 18 h into early stationary phase, at which time extracellular elastase is stable and has reached a plateau. Elastolytic activity in 18 h culture supernatants was determined as previously described using elastin Congo red (Sigma) as substrate (Ohman *et al.*, 1980). Dilutions of samples were assayed for elastolytic activity to establish the linear range of the reactions, and activity was expressed as the increase in A₄₉₅ per min per mg total protein.

SDS-PAGE and immunoblotting. Equivalent amounts of cells in the 18 h cultures were demonstrated by turbidity (OD₆₀₀) and then centrifuged. Pellets were used for cell lysates, and supernatants were subjected to TCA precipitation to concentrate secreted proteins (McIver *et al.*, 1995). All of the respective samples were suspended in SDS sample buffer (60 mM Tris/HCl, 2% SDS, 10%, v/v, glycerol, 0.1 mg ml⁻¹ bromophenol blue, 5% 2-mercaptoethanol [pH 6.8]) and loaded onto a 12.5% polyacrylamide gel for electrophoresis (SDS-PAGE). Proteins in polyacrylamide gels were electrotransferred to nitrocellulose in a Trans-Blot apparatus (Bio-Rad) for 2 h at 160 mA at 4 °C. Immunoblotting was performed as previously described (McIver *et al.*, 1991) using rabbit anti-denatured elastase IgG (Kessler & Safran, 1988) as the primary antibody, followed by a goat anti-rabbit horseradish-peroxidase conjugate (Sigma).

Table 1. Effect of substitutions in conserved residues of the ProM and ProC regions of the elastase (LasB) propeptide on the elastolytic activities in culture supernatants of *P. aeruginosa*

Plasmid	Pre-proelastase variant	Elastolytic activity*
pKSM3	Wild-type <i>lasB</i>	100 ± 10
pKSM66.68a	Asn68Gln	23 ± 6
pKSM66.68b	Asn68Asp	20 ± 5
pKSM66.69a	Gly69Ser	47 ± 11
pKSM66.74a	Arg74Lys	31 ± 3
pKSM66.74b	Arg74Val	3 ± 3
pKSM66.81b	Gly81Glu	42 ± 4
pKSM66.84a	Val84Ala	20 ± 12
pKSM66.84b	Val84Glu	87 ± 4
pKSM66.181a	Ile181Val	79 ± 20
pKSM66.181b	Ile181Glu	2 ± 2
pKSM66.182a	Asp182Glu	103 ± 12
pKSM66.182b	Asp182Ser	112 ± 21
pKSM66.183b	Ala183Cys	3 ± 3

*Elastolytic activity in 18 h culture supernatants is shown as the percentage of that in the *lasB*⁺ control strain, FRD740(pKSM3), which typically produced 3.0 units of activity using elastin-Congo red as a substrate. The background reaction (0.4–0.5 units) produced by $\Delta lasB$ strain FRD740(pLAFR3) was subtracted. The data are means ± standard deviations based on at least two independent experiments.

RESULTS

Identification of conserved regions in the propeptide of *P. aeruginosa* elastase

The propeptide sequence of *P. aeruginosa* elastase was compared to that of other TNPs to identify conserved amino acids that might be common to IMC function. Overall, weak homologies (34% identity, 50% similarity) were observed with the propeptides of TNPs produced by other Gram-negative organisms (Fig. 1), including metalloprotease VvpE of *Vibrio vulnificus*, haemagglutinin/proteinase of *Vibrio cholerae*, zinc metalloprotease of *Vibrio anguillarum*, neutral protease/vibriolysin of *Vibrio proteolyticus* and zinc metalloproteinase of *Legionella pneumophila*. Also included were the propeptides of two TNPs from Gram-positive bacteria, *Bacillus stearothermophilus* thermolysin and *Bacillus subtilis* neutral protease A, which are known to have homology to the elastase propeptide (Wetmore *et al.*, 1992). Other IMC-containing proteases, such as subtilisin, are known to have conserved motifs in the propeptides within their subfamilies, but they had no obvious homology with the TNP propeptides.

The alignment of propeptides from eight TNPs revealed two regions with high levels of conserved residues (Fig. 1). One conserved region, called ProM (for propeptide middle), comprised residues 58 to 89 in the middle portion of the

Fig. 1. Alignment of the amino acid sequence of the *P. aeruginosa* elastase (LasB) propeptide with those of TNPs from several Gram-negative and Gram-positive bacteria. Propeptides with homology to the elastase propeptide were first identified by a search of the protein database using Basic Local Alignment Search Tool (BLAST). The amino acid sequences for the propeptide sequences were then aligned relative to the elastase propeptide according to the BLAST results. A letter indicates amino acid identity; + indicates similarity; empty spaces represent gaps in similarity introduced by the BLAST alignment program. Regions with the highest homology were designated ProM and ProC. The sequences shown are: elastase LasB of *P. aeruginosa* (P aeru); metalloprotease VvpE of *V. vulnificus* (V vuln); haemagglutinin/proteinase of *V. cholerae* (V chol); zinc metalloprotease of *V. anguillarum* (V angu); neutral protease/vibriolysin of *V. proteolyticus* (V prot); zinc metalloprotease of *L. pneumophila* (L pneu); thermolysin of *B. stearothermophilus* (B stea); and neutral protease A of *B. subtilis* (B subt). Conserved residues targeted here for mutagenesis are indicated by ^ and underlining in the *P. aeruginosa* sequence.

propeptide. Residues that were highly conserved (i.e. present in at least six out of eight sequences analysed) in ProM included Asn68, Gly69, Arg74, Gly81 and Val84. The second conserved region, called ProC (for propeptide-carboxy), comprised residues 142 to 197 and located on the carboxy end of the elastase propeptide. Residues that were most conserved included Asn144, Asn156, Ala159, Gln160, Leu161, Tyr163, Val165, Ile181, Asp182, Ala183, Leu189, Trp192 and His197. These two conserved regions were predicted to contain amino acids or motifs of functional importance to the IMC activity of proelastase.

Effects of amino acid substitutions in the propeptide on extracellular elastolytic activity

To evaluate the hypothesis that conserved amino acids in the propeptide play a role in the production of active elastase, alleles of *lasB* that encoded proteins with substitutions in conserved residues of region ProM were constructed by oligonucleotide-directed mutagenesis of the wild-type *lasB* allele in pKSM4. Following verification by sequence analysis, each altered gene was cloned into a low-copy, broad-host-range plasmid (pLAFR3) and introduced into FRD740, a *lasB*-deleted strain of *P. aeruginosa* FRD2. To determine the consequences of the mutations on extracellular elastolytic activity, supernatants from 18 h standardized cultures were obtained from the strains expressing the mutant *lasB* alleles, and the rate of hydrolysis of elastin Congo red was used to measure elastolytic activity.

The culture supernatant from FRD740(pKSM3) expressing

wild-type *lasB* from a plasmid exhibited high elastolytic activity (Table 1). FRD740(pLAFR3), the $\Delta lasB$ strain containing the cloning vector, produced low elastolytic activity, representing about 10 % of FRD740(pKSM3) levels, and this non-*LasB* value was subtracted from the other test reactions. This residual elastolytic activity in a $\Delta lasB$ background is predominantly due to the secreted *LasA* protease (Gustin *et al.*, 1996).

When the conserved, hydrophilic Asn68 residue in the ProM region was substituted with another amide residue (Gln) or an acidic residue (Asp), extracellular elastolytic activity was drastically reduced to about one-fifth of wild-type levels (Table 1). This indicated that Asn68 was a critical residue in the propeptide for production of active elastase. Likewise, when the conserved, charged Arg74 residue was substituted with another basic residue (Lys), elastolytic activity was reduced to about one-third, and introducing a hydrophobic residue (Val) reduced elastolytic activity almost completely. This suggests that a positive charge at position 74 was important for IMC function, but that there was some specificity for Arg. In contrast, when the conserved and hydrophobic Gly69 residue was substituted with hydrophilic Ser, extracellular elastolytic activity dropped only by half. Interestingly, a radical substitution at hydrophobic Gly81 with acidic Glu had a limited effect on activity. Changing the hydrophobic Val84 to another aliphatic residue (Ala) or an acidic residue (Glu) reduced extracellular elastolytic activity to about 20 or 87 %, respectively, suggesting that the size of the R group here was more important than hydrophobicity (Table 1).

Single base pair substitutions were made in the ProC region to determine the effects of alterations to a highly conserved cluster of residues: Ile181-Asp182-Ala183. These mutant alleles, cloned into pLAFR3, were then introduced into the $\Delta lasB$ mutant FRD740 and tested for elastase activity. Substitution of hydrophobic residue Ile181 with another hydrophobic residue (Val) had a minor effect, but introducing an acidic group (Glu) resulted in almost background elastolytic activity levels (Table 1). Despite its conservation among TNP propeptides, substitutions at acidic residue Asp182 with another acidic residue (Glu) or an unionized amino acid (Ser) had no adverse effect on extracellular elastolytic activity. In contrast, substitutions at the adjacent hydrophobic Ala183 with an unionized hydrophilic residue (Cys) resulted in a drastic loss of extracellular elastolytic activity (Table 1).

Effects of propeptide amino acid substitutions on elastase stability and secretion

The loss of extracellular elastolytic activity in the propeptide mutants above could be due to defects in the ability to fold the mature protease into a stable protein and/or in the ability to be secreted by the type II secretion machinery. These possibilities were addressed by immunoblot analysis in which the relative amounts of elastase antigen in cell extracts (Fig. 2a, c) and culture supernatants (Fig. 2b, d) were evaluated. Expression of each mutant allele was compared to that of the wild-type *lasB* allele (lane 2) under the same conditions. A defect in the recognition of an altered proelastase by the type II secretory apparatus would be expected to cause abnormal accumulation of periplasmic proelastase (51 kDa) and/or periplasmic elastase (33 kDa).

Fig. 2. Immunoblot showing elastase-related proteins from the $\Delta lasB$ *P. aeruginosa* strain FRD740 expressing plasmid-encoded *lasB* alleles with mutations that alter conserved residues within the propeptide domain. None of the plasmids had an adverse affect on cell growth. Samples were taken from 18 h standard cultures as described in Methods. The effects of substitutions in the ProM region on elastase in cell extracts (a) and culture supernatants (b) were determined with FRD740 harbouring the following plasmids: pLAFR3, vector (lane 1); pKSM3, wild-type *lasB* (lane 2); pKSM66.68a, Asn68Gln (lane 3); pKSM66.68b, Asn68Asp (lane 4); pKSM66.69a, Gly69Ser (lane 5); pKSM66.74a, Arg74Lys (lane 6); pKSM66.74b, Arg74Val (lane 7); pKSM66.81b, Gly81Glu (lane 8); pKSM66.84a, Val84Ala (lane 9); pKSM66.84b, Val84Glu (lane 10). The effects of substitutions in the ProC region on elastase in cell extracts (c) and culture supernatants (d) was determined with FRD740 harbouring the following plasmids: pKSM66.181a, Ile181Val (lane 11); pKSM66.181b, Ile181Glu (lane 12); pKSM66.182a, Asp182Glu (lane 13); pKSM66.182b, Asp182Ser (lane 14); pKSM66.183b, Ala183Cys (lane 15). Positions corresponding to the 51 kDa periplasmic proelastase, the 33 kDa periplasmic elastase and the extracellular 33 kDa mature elastase species are indicated. The percentage of wild-type elastolytic activity, or - if negligible, is shown.

Among the ProM mutants (Fig. 2a, b), when Arg74 was substituted with Val (lane 7), the obvious intracellular accumulation of proelastase was observed and no elastase antigen was observed in the culture supernatant. Some intracellular proelastase accumulated even with the conservative Arg74Lys substitution (lane 6), suggesting that Arg74 in the propeptide is important for both self-processing and secretion of mature elastase. The conservative Gly69Ser substitution had a minor effect on accumulation of proelastase in the periplasm and reduction of elastase antigen in the supernatant (lane 5). In addition, the Val84Glu mutation (lane 10) increased the relative amount of periplasmic proelastase but the amount of elastase in the supernatant was similar to that of the wild-type control (lane 2).

As for mutations in the ProC region (Fig. 2c, d), the conservative Ile181Val substitution generally had little effect on elastase processing or secretion (lane 11). Substitutions at Asp182 (lanes 13 and 14) also had no detrimental affect. However, the Ile181Glu (lane 12) and Ala183Cys (lane 15) substitutions resulted in a minor accumulation of periplasmic proelastase, as well as accumulation of smaller degradation products, and both blocked the formation of a stable mature elastase protein in the supernatant. Thus, the ProC residues Ile181 and Ala183 apparently play an important role, possibly in self-processing, secretion or folding of a stable elastase outside of the cell.

Another interesting phenotype observed among some of the mutant precursor proteins was an overall reduction in total elastase antigen in the culture when compared to that of wild-type *lasB* expression. For example, in the ProM region, substitution of Asn68 with Gln (lane 3) or Asp (lane 4) did not cause intracellular accumulation, but still dramatically reduced extracellular elastase antigen and enzymic activity. This presumably represents propeptide mutations that caused folding defects, suggesting that Asn68Gln- and Asn68Asp-proelastases are susceptible to nonspecific degradation by other proteases of *P. aeruginosa*. Also, the Gly81Glu substitution (lane 8) showed less intracellular proelastase and less extracellular mature elastase than wild-type (lane 2), suggesting a folding defect. The Val84Ala substitution (lane 9) resulted in reduced extracellular elastase antigen without intracellular accumulation, although a Val84Glu substitution (lane 10) was readily tolerated. In the ProC region, substitutions at Asp182 with Glu or Ser had no adverse effect on secretion of stable elastase (lanes 13 and 14). Overall, these studies support the prediction that some conserved amino acid residues, or in some cases their charge or hydrophobic character, are essential for the elastase propeptide to function efficiently as an IMC for both protein folding and secretion.

DISCUSSION

The functions that the propeptide may perform for its cognate protease as an IMC include mediating proper

folding of the protease domain, inhibiting its proteolytic activity while within the cell, delivering the protease in the proper form to the secretion machinery and facilitating its own proteolytic removal. Little is known about the propeptide-mediated functions in the family of secreted TNPs. These proteases are produced by many bacterial species and are initially synthesized as pre-proenzymes with an N-terminal propeptide. *P. aeruginosa* elastase is the first example of a TNP subfamily protein for which the IMC function, and protein-protein interactions of the propeptide with the mature enzyme, has been documented (McIver *et al.*, 1995). The *lasB*-encoded proelastase provides an excellent model system to further characterize the specific mechanisms by which the TNPs use their propeptides as an IMC in Gram-negative bacteria.

The IMC of subtilisin exhibits an interesting bias toward charged amino acids within its propeptide when compared to the mature protein; the propeptide has 36% charged residues as compared to only 12% in the mature protease (Shinde & Inouye, 1996). It has been postulated that the charged propeptide residues cover the hydrophobic surfaces of the mature protease in order to provide a more even charge distribution of the proenzyme in the cell (Shinde & Inouye, 1993). However, this mechanism is unlikely for TNP subfamily molecules because both the prodomain and the protease domain exhibit the same proportion (23–27%) of charged amino acids. The prodomain of subtilisin contains two conserved motifs essential for IMC function, which are composed of predominately hydrophobic amino acids flanked by charged residues (Shinde & Inouye, 1993). Mutations that increase the hydrophilicity of the core sequences are deleterious to subtilisin-folding reactions. In contrast, the propeptides of the TNP subfamily show no obvious homology to conserved motifs in the subtilisin propeptides, and the presence of hydrophobic amino acids flanked by charged residues is not as striking (data not shown). In addition, TNPs from Gram-negative bacteria must also cooperate with a second secretion pathway (e.g. the type II/Xcp system in *P. aeruginosa*) for export through the outer membrane (reviewed by Filloux *et al.*, 1998). Therefore, the proposed mechanisms used by subtilisin IMCs may be different from those used by the TNP IMCs to mediate chaperone function.

To begin to understand the mechanisms by which TNP propeptides act as IMCs, we first compared the propeptide sequences of several TNPs from Gram-negative and Gram-positive bacteria to identify conserved residues. Two conserved regions were observed; they were located in the middle of the propeptide and at the C-terminus and called ProM and ProC, respectively. A hydrophilicity plot of the pre-propeptide sequence of elastase (Fig. 3) showed that the ProM region was generally hydrophilic, suggesting that charge is important to its function and/or that this area may be surface-exposed. The ProC region was generally less hydrophilic than the ProM region, suggesting that it may not be surface exposed in proelastase.

Fig. 3. Overview of effects of propeptide residue-substitutions on mature elastase in the supernatant. Hydrophilicity plot of the pre-propeptide sequence of *P. aeruginosa* elastase (top panel; Kyte–Doolittle units). Aligned to this (centre) is a map showing the locations of the signal peptide and two areas of residues conserved among TNPs, called ProM and ProC. Mutated residues are underlined and an asterisk indicates a major disruption to extracellular elastase and elastolytic activity when a radical substitution was made.

We hypothesized that some of the propeptide residues conserved among TNPs were important for IMC function and if mutated would result in defects in IMC functions. To test this hypothesis, we targeted codons of several conserved residues in *lasB* (encoding pre-proelastase) for site-specific mutational analysis, and the *lasB* mutant alleles were expressed on a low-copy plasmid from the native promoter in a $\Delta lasB$ mutant, *P. aeruginosa* FRD740. Compared to wild-type *lasB* under the same conditions, the properties examined included extracellular elastolytic activity, extracellular elastase protein levels, and the accumulation of periplasmic proelastase protein. The substitutions producing major effects on the formation of stable, extracellular elastase are graphically summarized in Fig. 3. Eight conserved residues were targeted for 13 substitutions, both conservative and radical, most of which adversely affected extracellular elastolytic activity. Thus, the prediction that conserved residues in the propeptide would be critical for extracellular elastase activity from the mature domain was strongly supported by the initial analysis.

The signal peptide of cytoplasmic pre-proelastase is removed during translocation across the inner membrane into the periplasmic space, where folding occurs (Kessler & Safrin, 1988). The propeptide of elastase is secreted as a complex with the mature enzyme, suggesting that the propeptide may play an important role in trafficking elastase to the secretory machinery (Kessler *et al.*, 1998). Some of the substitutions at conserved amino acids were shown in this study to primarily affect processing and/or secretion of an active elastase into the extracellular milieu. In the ProM region, substitution of polar Arg74 with a nonpolar Val residue resulted in a drastic accumulation of proelastase within the periplasm and no detectable extracellular elastase antigen. Even substituting this Arg residue with another charged residue (Lys) had a similar effect, though less drastic. The Arg74Val-propeptide may still possess chaperone activity such that proelastase was protected from

nonspecific degradation, but the substitution prevented self-processing and caused a secretion defect, which led to the periplasmic accumulation of the unprocessed proenzyme. Since ProM is predicted to be surface-exposed, it is possible that Arg74 interacts directly with the components of the type II secretion machinery. At another ProM site, the Val84Glu substitution also resulted in increased intracellular accumulation of proelastase, indicating a secretion defect, although a substitution with Ala did not show the same effect.

With regard to the ProC region, earlier studies showed that carboxy-terminal deletions within the prodomain of a *Bacillus cereus* TNP, encompassing conserved residues corresponding to ProC of proelastase, adversely affect secretion (Wetmore *et al.*, 1992). However, these deletions removed over 40 amino acids of the propeptide and involved residues proximal to the propeptide cleavage site. Here we showed that substituting only single amino acids at Ile181 or Ala183 within the ProC region of the elastase propeptide adversely affected accumulation of stable elastase protein in the supernatant. However, a conservative substitution of Val181 for Ile, so that it retained the hydrophobic nature of the region, had no effect on the activity of the elastase prodomain. Thus, the hydrophobic character here is apparently important to function. Another study of *lasB* expression, in a *Pseudomonas putida* heterologous system, also identified a role for Ala183, in that a substitution adversely affected the inhibitor function of the propeptide on elastase activity (Braun *et al.*, 2000). Since the Ile181 and Ala183 residues are relatively close to the site of propeptide removal, they may play a role in the rate of processing and thus mature protein stability. Interestingly, our two different substitutions at the conserved Asp182 had no adverse effect on processing, secretion or protein stability.

Several substitutions in the propeptide at conserved amino acids appeared to primarily affect the folding of elastase into a conformation that can resist degradation by the other proteases in the cell and/or culture supernatant. If the propeptide is a chaperone, then this was a predictable phenotype of mutants defective in proper protein folding. In the ProM region, two different substitutions at Asn68 both permitted elastase secretion, but the levels of total elastase antigen inside and outside the cell were reduced. This suggested that Asn68 was primarily involved in the proper folding of the mature enzyme, and that the defects led to an increased degradation of proelastase within the periplasm. The strain expressing the Gly81Glu substitution contained no detectable periplasmic proelastase, suggesting an increased degradation of misfolded proelastase proteins; the extracellular elastase antigen levels and activity were less than half that of wild-type. The Val84Ala phenotype was reduced extracellular elastase antigen levels and activity, but without intracellular accumulation, suggesting a folding defect that rendered the protein more susceptible to the high level of non-elastase protease activity

in the supernatants of 18 h *P. aeruginosa* cultures. A comprehensive mutation analysis of the effects of mutations in the propeptide is in progress to identify all residues likely to be important for elastase folding and secretion. Ultimately, the crystal structure of proelastase, which has yet to be determined, will reveal how these critical residues in the propeptide interact with the mature domain to bring about proper folding and efficient secretion.

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