Maturation of *Pseudomonas aeruginosa* Elastase

**FORMATION OF THE DISULFIDE BONDS***

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Elastase of *Pseudomonas aeruginosa* is synthesized as a preproenzyme. After propeptide-mediated folding in the periplasm, the proenzyme is autoproteolytically processed, prior to translocation of both the mature enzyme and the propeptide across the outer membrane. The formation of the two disulfide bonds present in the mature enzyme was examined by studying the expression of the wild-type enzyme and of alanine for cysteine mutant derivatives in the authentic host and in *dsb* mutants of *Escherichia coli*. It appeared that the two disulfide bonds are formed successively. First, DsbA catalyzes the formation of the disulfide bond between Cys-270 and Cys-297 within the proenzyme. This step is essential for the subsequent autoproteolytic processing to occur. The second disulfide bond between Cys-30 and Cys-57 is formed more slowly and appears to be formed after processing of the proenzyme, and its formation is catalyzed by Dof of Molecules as well. This second disulfide bond appeared to be required for the full proteolytic activity of the enzyme and contributes to its stability.

The opportunistic pathogen *Pseudomonas aeruginosa* secretes many proteins into the extracellular medium. The secreted proteins are synthesized in the cytoplasm and have to pass both membranes of the cell envelope. Four main pathways for the secretion of proteins, usually referred to as the type I, II, III, and the autotransporter pathway, have been identified in *P. aeruginosa* (1–4). The majority of the exoproteins characterized is secreted via the type II pathway, also referred to as the general secretory pathway. In addition to elastase, which is the most abundant secreted protein, lipase, alkaline phosphatase, exotoxin A, two phospholipases C, the staphylolytic protease LasA, the chitin-binding protein ChpD, and a putative aminopeptidase are secreted via this pathway (2, 5–7).

Proteins secreted via the type I or type III pathways are translocated across the two membranes of the cell envelope in a single step, without a periplasmic intermediate. In contrast, the type II pathway is a two-step mechanism. The first step is the translocation across the inner membrane, which is mediated by the Sec machinery. In the periplasm, which contains chaperones and folding catalysts, the exoproteins fold into a (near)-native conformation (8). For several proteins that are secreted via a type II mechanism (9–11), including elastase of *P. aeruginosa* (12, 13), it has been demonstrated that folding in the periplasm is essential for the subsequent translocation across the outer membrane to occur. In *P. aeruginosa*, the translocation of the periplasmic intermediates across the outer membrane is mediated by a machinery composed of at least 12 proteins, encoded by the *xcp* genes (for a review, see Ref. 2).

To investigate the biogenesis of proteins secreted via the type II pathway of *P. aeruginosa*, we have chosen elastase as a model. This metalloprotease, which is encoded by the *lasB* gene (14), is produced as a preproprotein. The pre-part is the signal peptide, which directs the translocation of the proenzyme across the inner membrane (15). The propeptide is essential for the folding of elastase in the periplasm (12, 13), and this folding allows for further processing of the proenzyme by autoproteolytic cleavage (16). The propeptide remains noncovalently associated with the mature elastase (15) and inhibits further proteolytic activity of the enzyme (17). Subsequently, the propeptide-enzyme complex is secreted and dissociates during or after translocation across the outer membrane (6, 18). Dissociation of the propeptide-enzyme complex is a well co-ordinated process, and a host-specific factor is required to induce this event (19). The propeptide is finally degraded by an extracellular protease (6, 18), probably elastase itself.

Proteins secreted via a type II pathway can acquire disulfide bonds, which are formed in the oxidizing environment of the periplasm by the presence of the Dsb system (for a review, see Ref. 20). The propeptide of elastase does not contain any cysteines, whereas the mature polyepitide contains four of them, which together form two disulfide bonds in the folded enzyme (21). Both bonds are not localized in close proximity of the active center of the protein. One disulfide bond, between Cys-30 and Cys-57, is located in the N-terminal part of the mature enzyme and connects two β-strands. The other disulfide bond, between Cys-270 and Cys-297, is located close to the C terminus and connects two α-helices. Here we demonstrate that the formation of these disulfide bonds is well ordered in time. One disulfide bond is formed in the proenzyme and is essential for subsequent autoproteolytic processing to occur. The other disulfide bond is formed only after autocatalytic

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land et al. (25) with the following modifications. The “megaparamer” was obtained by amplification of the DNA fragment with the oligonucleotides 5’ AGCGGCCGCGGCGGTTGAT and 3’ TCTGACGACGCCG- CCTGC in 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 47 °C for 1 min, and DNA synthesis at 73 °C for 2 min. The entire elastase gene with the C297A substitution was obtained using pRB1804 as template, the megaparamer, the reverse sequencing primer, and 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 47 °C for 2 min, and DNA synthesis at 73 °C for 2.5 min. The DNA fragment obtained was digested with EcoRI and PstI and cloned in the corresponding sites of pUC18 resulting in plasmid pUCP18. This plasmid was used to transform E. coli DH5α, which was made blunt with T4 DNA polymerase and cloned in the corresponding sites of pUC18 resulting in plasmid pUCP18. This plasmid was obtained by amplification of the DNA fragment with the oligonucleotides 5’ GCAGCATGGTGGCCAC and 3’ CGGTCGACAGA and transferred into E. coli DH5α. The thus 20-fold diluted plasmid was used in Western blotting analysis using a 20,000-fold dilution. For the re-
detection of disulfide bonds, DTT was added to a concentration of 20 mM. Protein patterns were analyzed by SDS-PAGE (13) on gels of 9 cm in length (Figs. 2 and 3) or on the Bio-Rad Protein II system (Figs. 1 and 4) followed, where indicated, by autoradiography. Immuno-detection by Western blot analysis was performed as described (30). To detect free thiols, proteins were incubated with N-[6-(biotinamido)]hexyl-3′(2′-pyridyldithio)propionamide (biotin-HPDP) as described (28). Briefly, proteins trichloroacetic acid-precipitated from culture supernatants were incubated for 10 min at 95 °C in the presence or absence of DTT. Subsequently 20 mM phosphate-buffered saline, 10 mM EDTA, supplemented with 0.8 mM biotin-HPDP (Pierce), was added, and the samples were incubated for 1 h at room temperature. Next the proteins were separated by SDS-PAGE and blotted onto nitrocellulose filters (Schleicher and Schuell, 0.45 µm) using a semidry electroblotting apparatus (2117 Multiblot II, LKB). Streptavidin-horseradish peroxidase was used to detect biotin-labeled proteins. The peroxidase activity was developed with a solution of 4-chloro-1-naphthol (0.5 mg/ml) in 15% methanol, 0.1% phosphate-buffered saline, and 0.01% H₂O₂.

**Enzyme Assay**—The proteolytic activity of elastase in culture supernatants of *P. aeruginosa* was determined as previously described (31). procedure described above were analyzed by SDS-PAGE (A), samples corresponding to a 180-kDa culture) or first labeled with biotin-HPDP, prior to SDS-PAGE and transfer to nitrocellulose filters (B, samples corresponding to a 90-µl culture). To detect the biotin-labeled proteins, the blot was probed with streptavidin-horseradish peroxidase. Mature elastase forms E₁ and E₂ are indicated at the right, and molecular mass markers (Mw, in kDa) are indicated at the left. On the original gel in A, the distance between the mature elastase forms E₀ and E₂ is 1.5 mm.

**RESULTS**

**Disulfide Bonds in Elastase**—Before studying the formation of disulfide bonds during the biogenesis of elastase, we first analyzed whether their presence can be demonstrated by SDS-PAGE. Frequently polypeptide chains possessing disulfide bonds have a higher electrophoretic mobility than the reduced forms of these polypeptides because of their more compact shape. Indeed when proteins from the supernatant of an overnight culture of the wild-type strain PAO25 were analyzed in the absence of DTT, elastase migrated with a higher electrophoretic mobility (Fig. 1A, lane 1) than in the presence of the reducing agent (Fig. 1A, lane 2). The absence of free cysteines in elastase was further demonstrated by incubating the proteins with biotin-HPDP, which reacts with free thiols. Biotin-HPDP reacted only with elastase after reduction of the disulfide bonds (Fig. 1B). We conclude that the presence or absence of disulfide bonds in mature elastase can indeed be demonstrated by SDS-PAGE.

To examine the formation of disulfide bonds in elastase, *E. coli* strain OE1224 was transformed with the lasB-containing plasmid pML27, and elastase maturation was studied after pulse labeling of the cells. Two forms of the proenzyme were detected, tentatively designated PE₁ and PE₂ (Fig. 2, lane 1). Reduction of the proenzymes with DTT before SDS-PAGE eliminated the PE₂ form (Fig. 2, lane 2) and resulted in a concomitant increase in the amount of the PE₁ form (Fig. 2, lane 2). This result shows that the bands represent different conformational states of the proenzyme and that the proenzyme form PE₁ contains at least one disulfide bond.

Two forms of mature elastase were detected, a faint band tentatively designated E₁ and a major band designated E₂ (Fig. 2, lane 1). When the proteins were exposed to DTT before SDS-PAGE, both the E₁ and the E₂ forms of the mature elastase were converted into another form, designated E₀, which migrated even slower in the gel than E₁ (Fig. 2, lane 2). Hence it appears that three different forms of mature elastase can be discriminated, which differ in the number of disulfide bonds. Form E₀ is the fully reduced state of the protein, whereas E₁ and E₂ contain most likely one and two disulfide bonds, respectively. Because the E₁ form could be detected after expression of elastase in vivo, it is apparently not necessary that both disulfide bonds are formed for autoproteolytic processing to occur (see also below).

**Disulfide Bond Formation in Elastase Expressed in *P. aeruginosa***—Because the experiments described above were performed in *E. coli*, the possibility that the slow formation of the second disulfide bond in mature elastase is an artifact of elastase expression in a heterologous host, rather than a gen-
Formation of Disulfide Bonds in Elastase Maturaton—To determine which of the two disulfide bonds is first formed and whether the formation of this disulfide bond is indeed essential for the processing of the proenzyme, two lasB mutants were isolated (see “Experimental Procedures”). In each of the mutants one pair of cysteines was replaced by alanines. The proteins lacking the N-terminal or the C-terminal bond were expressed from pMMB66 and pMMB8CSS, respectively. Their maturation was analyzed in P. aeruginosa using SDS-PAGE and Western blotting.

Expression of the wild-type elastase from pML27 in the lasB mutant strain PAN10 resulted in the production and secretion of mature elastase (E₂), which was detected both extracellularly (Fig. 4B, lane 2) and, as a consequence of overproduction, intracellularly (Fig. 4A, lane 2). When the gene encoding elast-
tase without the N-terminal cysteines was expressed in the same strain, the mature protein was detected in the supernatant (Fig. 4B, lane 3). As expected from the absence of a disulfide bond, the mutant protein displayed a slightly lower electrophoretic mobility than the wild-type protein. In contrast to the wild-type enzyme, the mutant enzyme did not accumulate intracellularly (Fig. 4A, lane 3). Consistent with the much lower total amount of enzyme detected, this suggests that the N-terminal disulfide bond contributes to the overall stability of the mature enzyme. Secretion of the mutant elastase was normally dependent on the Xcp machinery because the enzyme was not detected in the supernatant of the xcpR mutant strain PAN11 (Fig. 4B, lane 7) where it accumulated intracellularly (Fig. 4A, lane 7). These results demonstrate that the N-terminal disulfide bond is not required for the autoproteolytic processing of the proenzyme and for proper secretion of the mature protein. Using an antiserum directed against the propeptide, two forms of the propeptide, P$_1$ and P$_2$, were found to accumulate in the extracellular medium (Fig. 4C, lane 3). These two forms of the propeptide were previously detected in the supernatant of late-log phase cell cultures, when the extracellular proteolytic activity is still low, but disappeared during prolonged incubation when the proteolytic activity increased (6). These results suggest that, although the N-terminal disulfide bond is not essential for processing, it is required for the full proteolytic activity of elastase, which is apparently required for the extracellular degradation of the propeptide.

When the mutant elastase lacking the C-terminal disulfide bond was expressed in the lasB strain PAN10, mature elastase was neither intracellularly (Fig. 4A, lane 4) nor extracellularly (Fig. 4B, lane 4) detected. Instead, the proenzyme (PE$_0$) was found to accumulate intracellularly (Fig. 4, lane 4). Apparently the C-terminal disulfide bond is formed first, and its formation is required for the processing of the proenzyme.

**DISCUSSION**

Extracellular elastase of *P. aeruginosa* contains two disulfide bonds. In this study, we show that the two disulfide bonds are formed successively. The first disulfide bond, between Cys-270 and Cys-297, is already formed in the periplasmic proenzyme. The formation of this bond is catalyzed by DsbA and is essential for the autoproteolytic processing of the proenzyme (Fig. 4). This conclusion is in agreement with the notion that the mature form E$_0$ without disulfide bonds was never detected *in vivo*, not even in a dsbA mutant. However, in the dsbA mutant, some mature enzyme containing one disulfide bond was formed (Fig. 2, lane 4), which is probably the result of spontaneous oxidation or catalysis by other Dsb proteins. The tertiary structure of elastase is similar to that of *Bacillus cereus* thermolysin (21). However, thermolysin does not contain disulfide bonds, and such bonds are apparently not required for the autocatalytic processing of this enzyme.

The formation of the second, N-terminal disulfide bond, which is formed after cleavage of the propeptide in the processed enzyme, is almost completely inhibited in the dsbA mutant of *E. coli*, which suggests that DsbA mediates the formation of this bond. Importantly, it has been demonstrated that DsbA binds preferentially to unfolded proteins (33). Furthermore, Epps *et al.* (28) showed that the DsbA-catalyzed formation of disulfide bonds in a mutant PhoE protein is extremely rapid and precedes the formation of the (trypsin-resis-
tant) folded conformation. Our observation that DsbA mediates the formation of a disulfide bond in active and thus folded elastase is somewhat unexpected and suggests that DsbA not only mediates the formation of disulfide bonds in unfolded but also in folded polypeptides.

Recently we showed that the propeptide and the mature elastase are both secreted and that the propeptide is degraded extracellularly (6). When the proteolytic activity of extracellular elastase and other Ca<sup>2+</sup>-dependent proteases was prohibited by growth of the cells in a medium depleted of Ca<sup>2+</sup> ions, the propeptide accumulated extracellularly. Now we found that the propeptide also accumulated extracellularly, when elastase lacking the N-terminal disulfide bond was produced. The mutations did not interfere with the autoproteolytic processing and with the secretion of the mature enzyme but they prohibited the degradation of the propeptide. Consistently the proteolytic activity of the mutant enzyme in the extracellular medium was found to be severely reduced (data not shown). These results suggest that the N-terminal disulfide bond, besides its role in stabilizing the mature enzyme, is required for full proteolytic activity. In that case, the results would underscore the supposition that the propeptide is degraded by elastase itself. Alternatively the propeptide remains associated to the mutant enzyme and thereby inhibits the proteolytic activity. In that case, the formation of the N-terminal disulfide bond would be essential, the role of the propeptide associated to the mature elastase to occur. Interestingly when the wild-type elastase was co-expressed with the mutant enzyme, it was not capable of degrading the propeptide of the mutant form (data not shown). Apparently each enzyme molecule can only degrade its cognate propeptide. A possible explanation for this result is that if the propeptide remains associated with the mature enzyme, it is protected against proteolysis by the wild-type enzyme.

In the past few years, much information concerning the order of events during the biogenesis of elastase has accumulated. To include the data presented in this paper, we have adapted the model for elastase secretion presented by Kessler and Safrin (15). The new model is schematically shown in Fig. 5. First, elastase is synthesized in the cytoplasm as a prepropeptide. The pre- part is the signal peptide, which mediates translocation across the inner membrane via the Sec machinery (15). In the periplasm, the propeptide mediates the folding of the mature domain (12, 13) into its active conformation. Here we demonstrate that first a disulfide bond is formed between Cys-270 and Cys-297 and that the formation of this bond is catalyzed by DsbA. This bond is rapidly formed in the proenzyme, probably before the proenzyme is fully folded and before it is proteolytically processed. The formation of this bond is essential to allow for the subsequent autocatalytic processing of the proenzyme. After processing, which is a prerequisite for secretion (34), the propeptide remains noncovalently associated with the mature enzyme (15) and thereby inhibits the premature activation of the proteolytic activity of elastase in the periplasm (17). In addition, we showed that the second disulfide bond, between Cys-30 and Cys-57, is formed after processing in the mature enzyme and that its formation is also catalyzed by DsbA. The entire propeptide-enzyme complex is translocated across the outer membrane (6, 18). Dissociation of the propeptide-enzyme complex is a well coordinated process. The autoproteolytic processing of proelastase per se is not the trigger for the dissociation of the propeptide-enzyme complex, but interaction with a host-specific factor, possibly the Xcp secretion machinery, is required to induce this event (19). After the dissociation of the propeptide-enzyme complex, which occurs during or shortly after translocation across the outer membrane, the propeptide is degraded by an extracellular protease (6). This process requires a fully active mature elastase.

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