JOURNAL OF BACTERIOLOGY, Nov. 1988, p. 5241–5247
0021-9193/88/115241-07$02.00/0

Vol. 170, No. 11

Synthesis, Processing, and Transport of *Pseudomonas aeruginosa* Elastase

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Received 8 April 1988/Accepted 2 August 1988

Three cell-associated elastase precursors with approximate molecular weights of 60,000 (P), 56,000 (Pro I), and 36,000 (Pro II) were identified in *Pseudomonas aeruginosa* cells by pulse-labeling with [^{35}S]methionine and immunoprecipitation. In the absence of inhibitors, cells of a wild-type strain as well as those of the secretion-defective mutant PAKS 18 accumulated Pro II as the only elastase-related radioactive protein. EDTA but not EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] inhibited the formation of Pro II, and this inhibition was accompanied by the accumulation of Pro I. P accumulated in cells labeled in the presence of ethanol (with or without EDTA), dinitrophenol plus EDTA, or carbonyl cyanide *m*-chlorophenyl hydrazone plus EDTA. Pro I and Pro II were localized to the periplasm, and as evident from pulse-chase experiments, Pro I was converted to the mature extracellular enzyme with Pro II as an intermediate of the reaction. P was located to the membrane fraction. Pro I but not Pro II was immunoprecipitated by antibodies specific to a protein of about 20,000 molecular weight (P20), which, as we showed before (Kessler and Safrin, J. Bacteriol. 170:1215–1219, 1988), forms a complex with an inactive periplasmic elastase precursor of about 36,000 molecular weight. Our results suggest that the elastase is made by the cells as a preproenzyme (P), containing a signal sequence of about 4,000 molecular weight and a "pro" sequence of about 20,000 molecular weight. Processing and export of the preproenzyme involve the formation of two periplasmic proenzyme species: proelastase I (56 kilodaltons [kDa]) and proelastase II (36 kDa). The former is short-lived, whereas proelastase II accumulates temporarily in the periplasm, most likely as a complex with the 20-kDa propeptide released from proelastase I upon conversion to proelastase II. The final step in elastase secretion seems to require both the proteolytic removal of a small peptide from proelastase II and dissociation of the latter from P20.

Unlike most gram-negative bacteria, *Pseudomonas aeruginosa* secretes several proteins into the medium. Many of these proteins, including exotoxins A and S, the proteases elastase and alkaline proteinase, and phospholipase C, are toxic to humans and animals and are thought to enhance the virulence of the organism (5, 13, 25, 29, 42). Although the properties of most *Pseudomonas* exoenzymes and the role that each of them may play in the pathogenesis of *Pseudomonas* infections have been studied in detail, relatively little is known about their synthesis and secretion.

DNA sequencing of the structural genes of exotoxin A (10) and phospholipase C (34) indicated that like most exported proteins of other bacteria or higher organisms (for reviews, see references 2 and 35), both the exotoxin and the phospholipase are synthesized by the cells as larger precursors, each containing a typical amino-terminal leader sequence which is removed during the secretion process. Lory et al. (27) demonstrated that processing and secretion of the exotoxin precursor are both inhibited in the presence of ethanol, leading to accumulation of the precursor in the outer membrane. No exotoxin was found in the periplasm. These authors proposed that the exotoxin precursor is secreted cotranslationally and directly to the outer membrane via zones of adhesion between the inner and outer membranes. According to their model, the exotoxin precursor is proteolytically processed to the mature toxin upon release into the medium. Secretion of phospholipase C and of the elastase seems to follow a different route, since phospholipase activity (32) and an inactive elastase precursor (19, 22) were demonstrated in the periplasmic fraction of *P. aeruginosa* cells. The inactive periplasmic elastase precursor is activated by controlled proteolysis (19, 22), yet this precursor was reported to have the same molecular weight and the same N-terminal amino acid (alanine) as the extracellular elastase (7, 8, 19, 24). Fecycz and Campbell (7) suggested that activation did not result from proteolytic processing of the precursor itself, but rather involved dissociation of the elastase from a non-covalently bound elastase inhibitor.

We have recently described the purification of the inactive periplasmic elastase precursor and demonstrated that it is slightly larger than the elastase (22); the apparent molecular weight of the precursor, P36, was ca. 36,000, whereas that of the enzyme was ca. 35,500; activation involved proteolytic processing of the precursor itself. We also found that this periplasmic precursor was complexed with another protein of about 20,000 molecular weight, P20, whose identity and function were unclear. A membrane-associated larger elastase precursor of about 47,000 molecular weight was recently identified by Goldberg and Ohman (9).

In the present investigation we studied the biosynthesis and export of *Pseudomonas* elastase by pulse-labeling with [^{35}S]methionine. Our results suggest that the elastase is synthesized by the cells as a preproenzyme of about 60,000 molecular weight, whose processing and export involve two periplasmic intermediate proelastases with approximate molecular weights of 56,000 (proelastase I) and 36,000 (proelastase II). Proelastase II is probably identical with the

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previously described P36 (22), and P20 represents the extra "pro" sequence removed from proelastase I upon conversion to proelastase II.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* Habs serotype I, an elastase-producing wild-type strain isolated from a human corneal ulcer (20), and the elastase-deficient mutant PAKS 18 (43) (a gift of B. Wretlind, Danderyd Hospital, Sweden) were used in this study. Cells were grown at 37°C with shaking in the defined medium of Jensen et al. (18), modified to contain 8.3 mM glucose.

Radioactive labeling of proteins. After growth to the end of the logarithmic phase (A_{660}, 1.8 to 2), the cells were centrifuged (8,000 × *g*, 20 min, 4°C), suspended to a fifth of the initial volume in fresh medium, and incubated with or without added EDTA (0.15 or 0.3 mM as specified) for 9 min. [^{35}S]Methionine (1,030 to 1,400 Ci/mmol; Amersham) was then added to 100 μCi/ml, and unless otherwise specified, the cell suspensions were further incubated for 3 min. In some experiments, dinitrophenol (DNP), carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), or ethanol was added to the concentrations given in the text 3 min before the radioactive amino acid was added. To terminate methionine incorporation, the cell suspensions were briefly centrifuged (30 s) in a Beckman Microfuge, and the cell pellets were suspended rapidly in 10% trichloroacetic acid (TCA; 0.5 ml). In pulse-chase experiments, incorporation of radioactive methionine was blocked by adding nonradioactive methionine to a final concentration of 100 μg/ml. In addition, the supernatants obtained after the initial pelletting of the cells were recentrifuged for 4 min to remove remaining bacteria, and after 100 μg of bovine serum albumin (BSA) was added, the medium proteins were precipitated with TCA.

Immunoprecipitation and electrophoretic analysis. TCA precipitates were washed with acetone, air dried, and dissolved by heating to 100°C in 1% sodium dodecyl sulfate (SDS)–0.05 M Tris hydrochloride (pH 7.5). Insoluble material was removed by centrifugation, and the radioactive solutions were diluted 10-fold with immunoprecipitation buffer (0.05 M Tris hydrochloride [pH 7.5], 0.15 M NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.1% SDS, and 0.5 mg of BSA per ml) from which SDS was omitted. To prevent nonspecific binding of proteins, the diluted radioactive solutions were treated with preimmune serum (20 μl) and Formalin-fixed *Staphylococcus aureus* cells (BioMakor, Rehovot, Israel) as described by Tai et al. (39). Portions of the solutions obtained after removal of the staphylococci were incubated (2 h, 25°C) with 2.5 μg of immunoaffinity-purified antibodies to denatured elastase or, as in the experiment of Fig. 7, with 10 μg of DEAE-cellulose-purified antibodies against P20, followed by the addition of 10 or 50 μl, respectively, of the *Staphylococcus aureus* cell suspension and further incubation for 1 h at 4°C. The cells were washed four to five times with immunoprecipitation buffer, and bound proteins were dissociated by boiling in 2% SDS sample buffer (23). The immunoprecipitated proteins were then reduced with 2-mercaptoethanol (4%) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide gels (23), followed by fluorography (1). The molecular weights of the immunoprecipitated radioactive proteins were determined by using calibrated 7 to 15% polyacrylamide gradient gels.

Preparation of antibodies. Antibodies against denatured elastase were raised in rabbits as described previously (22), and the immunoglobulin G (IgG) fraction was isolated from the immune sera by DEAE-cellulose chromatography (17). The antibodies were further purified by immunoaffinity chromatography on a column of Sepharose coupled to denatured elastase as follows: 5 mg of elastase was precipitated with 10% TCA, dissolved in 0.1 N NaOH, and after neutralization with 1 M Tris hydrochloride (pH 7.5), the solution was dialyzed against 0.1 M $NaHCO_3$–0.5 M NaCl, pH 8.0, and the denatured enzyme was coupled to CNBr-activated Sepharose-4B (33). The substituted resin (7 ml, 0.7 mg of denatured elastase per ml) was suspended in 0.1% SDS for 48 h, washed thoroughly with phosphate-buffered saline (PBS) containing 20 mM EDTA and 0.4 mM phenylmethylsulfonyl fluoride, and packed into a column. The DEAE-cellulose-purified IgG fraction in the same buffer was applied to the column, and after removal of unbound proteins by washing with equilibration buffer, bound antibodies were released from the column by elution with 8 M urea in the same buffer. The purified antibodies were dialyzed against PBS and stored at −80°C.

Pure P20 for immunization was obtained by SDS-PAGE of the partially purified elastase precursor fraction described previously (22). After rapid staining of the gels with Coomassie blue (16), gel regions containing the protein were removed, washed with PBS, and stored at −80°C. Gel slices, each containing 70 to 100 μg of the antigen, were homogenized in PBS, mixed with an equal volume of complete Freund adjuvant, and injected subcutaneously into rabbits at 2-week intervals. Blood was collected after the third and fourth injections, and the IgG fraction was purified from the pooled sera by DEAE-cellulose chromatography (17).

Immunoblotting. Electrophoretic transfer of proteins from SDS gels to nitrocellulose paper was performed with 25 mM ethanolamine–38 mM glycine–20% methanol–0.01% SDS, pH 9.4 (38), and the papers were blocked with 5% BSA. The papers were then incubated (2 h, 37°C) with antiserum to denatured elastase or to denatured P20 (diluted 1:200 and 1:25, respectively), followed by incubation (4 h, 25°C) with ^{125}I-labeled protein A (a gift of N. Savion of our institute; ~ 250,000 cpm/ml). Immunologically reactive proteins were visualized by autoradiography.

Cell fractionation. Cells were converted to spheroplasts essentially as described by Cheng et al. (3). Suspensions (1 ml) of radioactively labeled cells were washed once with 1 ml of medium. The cells were suspended in 1 ml of 0.2 M $MgCl_2$–0.5 mg lysozyme per ml–0.01 M Tris hydrochloride (pH 8.4) and incubated at 4°C for 20 min. After centrifugation (1.5 min; Beckman Microfuge), the cells were suspended in 1 ml of 0.01 M $MgCl_2$–0.01 M Tris hydrochloride (pH 8.4) and incubated at 4°C for 20 min. The resulting spheroplasts were centrifuged, suspended in 1 ml of 0.02 M Tris hydrochloride (pH 7.5) containing 20 mM EDTA, 10 mM benzamidine, 0.4 mM phenylmethylsulfonyl fluoride, and 1 μg of leupeptin per ml, and broken by sonication. The membrane fraction was pelletted by ultracentrifugation (100,000 × *g*, 60 min) and resuspended in 1 ml of the above protease inhibitor-containing Tris buffer. During the procedure, samples were removed from the supernatants and the membrane fractions for assays of glucose-6-phosphate dehydrogenase activity (28), and the remaining portion of each fraction was prepared for immunoprecipitation by adding 250 μg of BSA and precipitation of total proteins with 10% TCA.

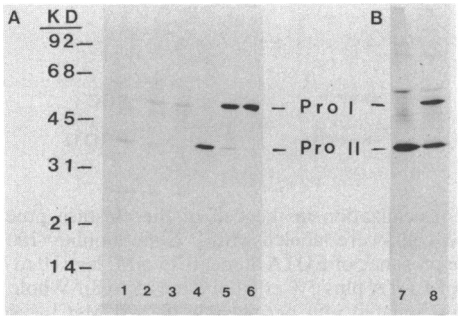

FIG. 1. Effect of EDTA on accumulation of elastase precursors by *P. aeruginosa* wild-type strain Habs serotype I (A) and by the elastase-deficient mutant PAKS 18 (B). Cells were pulse-labeled with [^{35}S]methionine (100 μCi/ml) for 3 min without additions (lanes 1, 4, and 7) or in the presence of 0.15 mM (lanes 2, 5, and 8) or 0.3 mM (lanes 3 and 6) EDTA. The cells were processed and immunoprecipitated with antibodies against denatured elastase as described in the text with the exception of the samples in lanes 1 to 3, which were immunoprecipitated after adding to each sample 5 μg of unlabeled denatured elastase. M_r values are indicated to the left (in thousands).

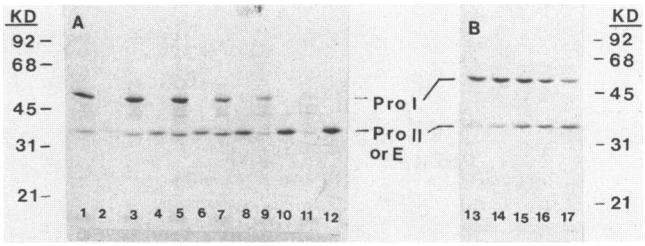

FIG. 2. Pulse-chase analysis of Pro I and Pro II. (A) Cells of *P. aeruginosa* Habs serotype I (wild type) were pulsed with [^{35}S]methionine (100 μCi/ml) in the presence of 0.15 mM EDTA. After 3 min, nonradioactive methionine was added to 100 μg/ml, and samples were removed at 0 (lanes 1 and 2), 1.25 (lanes 3 and 4), 2.5 (lanes 5 and 6), 5 (lanes 7 and 8), 10 (lanes 9 and 10), and 20 (lanes 11 and 12) min postchase. Cells were separated from the medium, and portions corresponding to a bacterial OD at 660 nm of 0.14 were immunoprecipitated with antielastase antibodies as described in the text. Odd-numbered lanes, cells; even-numbered lanes, medium. (B) Cells of *P. aeruginosa* PAKS 18 were pulsed with [^{35}S]methionine (100 μCi/ml) in the presence of 0.3 mM EDTA. After 1.5 min, unlabeled methionine was added to 100 μg/ml, and samples were removed and processed for immunoprecipitation at 0, 2.5, 5, 7.5, and 10 min (lanes 13 to 17, respectively) postchase. Only the fluorograms obtained from the cell fractions are shown, and the amount of radioactivity applied in each lane corresponds to a bacterial OD at 660 nm of about 0.3.

RESULTS

Accumulation and processing of elastase precursors in the presence of EDTA. *P. aeruginosa* wild-type cells were pulse-labeled with [^{35}S]methionine for 3 min in the absence (control) or presence of EDTA at concentrations of the chelator that do not lyse the cells. The labeled cells were solubilized in SDS and subjected to immunoprecipitation with antibodies against denatured elastase. Figure 1 shows that the immunoprecipitate derived from control cells labeled in the absence of EDTA contained a single radioactive protein with an apparent molecular weight of 36,000 (Pro II, lane 4). Formation of this protein was inhibited in the presence of EDTA, leading to accumulation in the cells of a larger radioactive protein with an apparent molecular weight of 56,000 (Pro I, lanes 5 and 6). Immunoprecipitation of Pro I and Pro II was specific, since it was inhibited in the presence of excess unlabeled elastase (Fig. 1, lanes 1 to 3). Also, no radioactive proteins were precipitated when the antielastase antibodies were substituted by preimmune serum (data not shown). Essentially the same results were obtained with the elastase-deficient mutant PAKS 18; however, when labeled in the absence of EDTA, the mutant cells accumulated higher amounts of Pro II than the wild-type cells (compare lanes 4 and 7 in Fig. 1), and the inhibition by EDTA of Pro II formation was less pronounced in the mutant cells than in those of the wild-type strain (compare lanes 5 and 8 in Fig. 1). Immunoprecipitates obtained from wild-type cells labeled in the presence of EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] contained only Pro II, i.e., were indistinguishable from those of the control cells (data not shown). Together, the above results suggest that Pro I and Pro II represent specific elastase precursors and that Pro II might be an intermediate in the processing of Pro I to the mature enzyme. Also suggested from these results is that conversion of Pro I to Pro II is dependent on divalent cations other than Ca^{2+}.

To demonstrate that Pro I and Pro II were indeed specific elastase precursors, we performed a pulse-chase experiment in which *P. aeruginosa* wild-type cells were labeled with [^{35}S]methionine for 3 min in the presence of 0.15 mM EDTA and chased after excess unlabeled methionine was added to the labeling suspension. Samples were removed at the times specified in Fig. 2A. Each sample was separated into a cell pellet and a supernatant, which were then separately immunoprecipitated with antibodies against denatured elastase. Figure 2A shows that at the end of the pulse, most of the precipitable radioactivity was intracellular and associated mainly with Pro I; only a trace of radioactive elastase was detectable at that time in the medium (lanes 1 and 2). The amount of radioactive Pro I decreased as a function of chase time, concomitantly with the appearance of increasing amounts of radioactive elastase in the medium. At 10 min, most of the precipitable radioactivity was found in the extracellular elastase (lanes 9 and 10), and at 20 min, practically all of it was associated with the extracellular enzyme (lanes 11 and 12). The disappearance of Pro I and the appearance of radioactive elastase in the medium were accompanied by a transient increase in the level of the cell-associated Pro II that was maximal between 2.5 and 5 min (lanes 5 and 7). These results clearly indicate that Pro I is an elastase precursor and suggest that Pro II is an intermediate in the processing of Pro I to the elastase. To substantiate the latter conclusion, we performed a similar pulse-chase experiment in which cells of the mutant strain PAKS 18, which accumulate Pro II (see Fig. 1B), were labeled. Figure 2B shows that in the mutant cells the time-dependent decrease in the amount of Pro I was accompanied by a proportional increase in the level of radioactive Pro II, indicating that Pro II indeed serves as an intermediate in the proteolytic conversion of Pro I to the mature elastase.

Accumulation of elastase precursors in the presence of CCCP, DNP, or ethanol. When labeled in the presence of either CCCP or DNP alone, *P. aeruginosa* wild-type cells accumulated Pro II as the only immunoprecipitable radioactive protein, i.e., neither CCCP nor DNP alone altered normal processing of elastase precursors (data not shown). When each of the uncouplers was added in combination with EDTA, a new radioactive protein was immunoprecipitated by antielastase antibodies, migrating in the gel slightly more

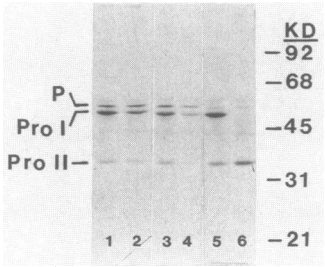

FIG. 3. Effect of CCCP and DNP on accumulation of elastase precursors. *P. aeruginosa* cells were labeled with [^{35}S]methionine (100 μCi/ml) for 3 min without additions (lane 6), in the presence of 0.15 mM EDTA plus CCCP (5 and 20 μM [lanes 1 and 2, respectively]), EDTA plus DNP (5 and 10 mM [lanes 3 and 4, respectively]), or EDTA alone (lane 5). Cells were immunoprecipitated with antielastase antibodies as described in the text.

slowly than Pro I and having an apparent molecular weight of 60,000 (band P in Fig. 3). A protein of the same electrophoretic mobility also accumulated when the cells were labeled in the presence of 5 or 7.5% ethanol (Fig. 4, lanes 2, 3, 5, and 6) but not in the presence of 2.5% ethanol (lanes 1 and 4 in Fig. 4). In contrast to CCCP and DNP, the effect of ethanol was independent of the presence of EDTA (compare lanes 2 and 5 or 3 and 6 in Fig. 4). CCCP, DNP, and ethanol are known to inhibit translocation through the cytoplasmic membrane and processing of the signal peptide of secreted proteins in *E. coli* (4, 6) and other bacteria (14, 27, 40). Accordingly, we conclude that P represents the elastase precursor containing the signal peptide-preproelastase; Pro I and Pro II apparently represent two proelastase species, the former being most likely the immediate product of the signal peptidase reaction.

Localization of the elastase precursors. To determine the cellular location of the elastase precursors, *P. aeruginosa* cells were labeled with [^{35}S]methionine in the presence of EDTA alone or EDTA plus ethanol. The cells were then separated into the periplasmic (0.2 M $MgCl_2$ extract and 0.01 M $MgCl_2$ shock fluid), cytoplasmic, and membrane fractions, and each fraction was examined for the presence of elastase precursors by immunoprecipitation with antielastase antibodies. Figure 5 shows that Pro I and Pro II were practically confined to the $MgCl_2$ supernatants (lanes 1, 2, 5, and 6), indicating that both proelastases reside in the periplasm. During the incubation of the cells in the $MgCl_2$

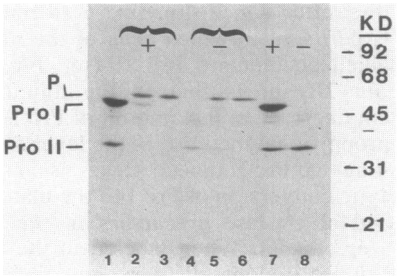

FIG. 4. Effect of ethanol on the accumulation of elastase precursors. *P. aeruginosa* cells were labeled with [^{35}S]methionine (100 μCi/ml) for 3 min in the absence of ethanol (lanes 7 and 8) or in the presence of 2.5% (lanes 1 and 4), 5% (lanes 2 and 5), or 7.5% ethanol (lanes 3 and 6); + and − indicate the presence and absence of 0.15 mM EDTA, respectively. Cell proteins were immunoprecipitated with antielastase antibodies as described in the text.

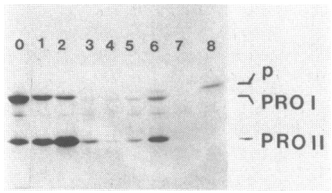

FIG. 5. Localization in the cell of the elastase precursors. *P. aeruginosa* cells were labeled with [^{35}S]methionine (100 μCi/ml; 3 min) in the presence of EDTA alone (0.15 mM; lanes 0 to 4) or in the presence of EDTA plus 5% ethanol (lanes 5 to 8). Whole cells (lane 0) were fractionated into periplasmic (0.2 M $MgCl_2$-lysozyme extract [lanes 1 and 5] and 0.01 M $MgCl_2$ shock fluid [lanes 2 and 6]), cytoplasmic (lanes 3 and 7), and membrane (lanes 4 and 8) fractions, and the elastase precursors were immunoprecipitated from each fraction as described in the text. The amounts of radioactivity applied to the gel correspond to a bacterial OD at 660 nm of 0.36 (lane 0), 0.6 (lanes 1 and 2), or 1.6 (lanes 3 to 8). Portions of each fraction were also assayed for glucose-6-phosphate dehydrogenase activity. At least 80% of the total cellular activity of this enzyme was found in the cytoplasmic fraction, indicating that release of the periplasmic fluids did not involve significant leakage of cytoplasmic proteins.

solutions, however, a considerable proportion of proelastase I was converted to proelastase II (compare lanes 1 and 2 with lane 0). Thus, even though the interactions of the individual precursors within the periplasm might not necessarily be the same, such differences cannot be inferred from the relative distribution of the two proelastases between the two $MgCl_2$ eluents. P, the elastase precursor accumulating in the presence of ethanol, was associated with the membrane fraction (lane 8), whereas the soluble cytoplasmic contents were essentially free of radioactive precursor proteins (lanes 3 and 7).

P20 is the major portion of the pro sequence of proelastase I. We recently reported the purification from *P. aeruginosa* cells of an elastase precursor of about 36,000 molecular weight (P36) and showed that it was complexed with an immunologically nonrelated protein of about 20,000 molecular weight (P20) (22). Since conversion of proelastase I to proelastase II involves the removal from proelastase I of an extra amino acid sequence of about 20,000 molecular weight, the possibility arose that P20 represented the cleavage product released from proelastase I upon conversion to proelastase II. To test this possibility, we prepared antibodies against P20 and examined their ability to immunoprecipitate the radioactive elastase precursors. The specificity of the antibodies was established by immunoblotting. Figure 6 shows that antibodies against P20 bound exclusively to this protein and did not recognize the elastase precursor P36. Correspondingly, antibodies to the elastase reacted with P36 but not with P20. The radioactive proteins from *P. aeruginosa* cells labeled with [^{35}S]methionine in the presence of EDTA and containing proelastase I and proelastase II were then subjected to immunoprecipitation with antibodies against P20, and the results were compared with those obtained after immunoprecipitation with antielastase antibodies. Figure 7 shows that the immunoprecipitate obtained with antibodies to P20 contained only proelastase I (lane 2), whereas both proelastase I and proelastase II were present in the immunoprecipitate obtained with antielastase antibodies (lane 1). These results identified P20 as the main cleavage product released upon processing of proelastase I and suggest that it accounts for most if not all of the pro sequence of proelastase I.

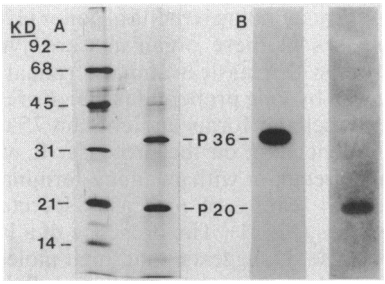

FIG. 6. Specificity of antibodies against P20 and against the elastase. (A) SDS-PAGE of molecular weight standards (left) and of the immunoaffinity-purified elastase precursor fraction (right; ~0.3 μg of protein; see reference 22 for details), silver stained (31). (B) Immunoblot of the proelastase fraction shown in A (~2.4 μg of protein per lane). Electrotransferred proteins were visualized by incubating the nitrocellulose sheets with either antielastase antibodies (left) or antibodies against P20 (right), followed by incubation with ^{125}I-protein A and autoradiography as described in the text. P36, Inactive elastase precursor; P20, 20-kDa protein that copurified with P36 (22).

DISCUSSION

An inactive elastase precursor with the same molecular weight as the mature elastase has been demonstrated in the periplasmic space of both wild-type and mutant strains of *P. aeruginosa* (7, 8, 19, 24). Recently, we found that this precursor is in fact slightly larger than the extracellular enzyme (22) and proposed that it represents a proenzyme form of the elastase, free of the signal peptide. We assumed that the elastase is made by the cells as a larger precursor, preproelastase, which was rapidly processed and therefore not detected. To demonstrate a larger elastase precursor(s), we studied the biosynthesis of the enzyme in the presence of processing inhibitors. Newly synthesized proteins were radioactively labeled at the end of the logarithmic phase of growth, as this is the time of maximal elastase production (19, 21). Further processing of the cells involved treatment with TCA and SDS, leading to protein denaturation. Denatured elastase was poorly recognized by antibodies against the native enzyme. Consequently, immunoprecipitation of elastase-related proteins was performed with antibodies raised by immunization with the denatured enzyme.

Altogether, we identified in the cells three distinct elastase-related radioactive proteins with apparent molecular weights of about 60,000 (P), 56,000 (Pro I), and 36,000 (Pro II). The last is most likely identical with the elastase precursors identified previously in the periplasmic fraction of *P. aeruginosa* cells (7, 8, 19, 22, 24). Its molecular weight is close to that of the enzyme, it resides in the periplasmic space, and it was the only radioactive elastase-related protein accumulating in cells labeled in the absence of inhibitors. The results of the pulse-chase experiments and the observation that accumulation of Pro I is associated with a marked reduction in the level of Pro II identify Pro I as an earlier precursor of the elastase and suggest that Pro II serves as an intermediate in the proteolytic conversion of Pro I to the mature enzyme. The possibility that Pro II is a nonphysiological cleavage product of Pro I formed during processing of the cells in the absence of inhibitors is unlikely because (i) Pro II was the only radioactive elastase-related protein detected even when the cells were rapidly treated with TCA before further processing, (ii) relatively large amounts of Pro II and no Pro I were found in PAKS 18 cells (these mutant cells normally accumulate about 90% of the elastase they produce as an inactive precursor having approximately the same molecular weight as the mature enzyme [7]), and (iii) the decrease in the amount of radioactivity of Pro I during the chase of PAKS 18 cells was accompanied by a proportional increase in the amount of radioactive Pro II. The following observations favor P as the initial translation product of the elastase structural gene, containing the signal peptide. (i) Processing of P was inhibited in the presence of DNP, CCCP, and ethanol, agents known to inhibit the removal of the signal peptide and translocation through the cytoplasmic membrane of precursor proteins of several bacteria (4, 6, 14, 27, 40). (ii) P accumulating in the presence of ethanol was membrane associated. (iii) P was heavier than Pro I, the larger of the two periplasmic elastase precursors, by about 4,000 daltons. This molecular weight value is in the order of those reported for many leader sequences, including those of *P. aeruginosa* phospholipase C (34) and the two extracellular proteases of *Streptomyces griseus* (12), each containing 38 amino acid residues.

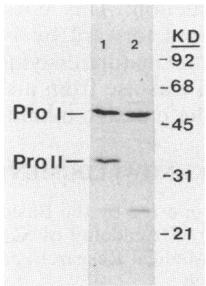

FIG. 7. Identification of P20 as the major pro sequence of proelastase I. *P. aeruginosa* cells were labeled with [^{35}S]methionine (100 μCi/ml) for 3 min in the presence of 0.15 mM EDTA. The cells were processed and immunoprecipitated with antibodies against elastase (lane 1) or with antibodies against P20 (lane 2) as described in the text.

With the exception of the 47,000-molecular-weight elastase precursor reported by Goldberg and Ohman (9), no high-molecular-weight elastase precursors such as P and Pro I have been observed so far, most likely because these species have short half-lives. Indeed, the 47,000-M_r elastase precursor was found to accumulate in *P. aeruginosa* cells defective in processing and secretion of the elastase due to a mutation in their *lasA* gene (9, 36), and in the present study, demonstration of the high-molecular-weight precursors was made possible by use of processing inhibitors. The reason for the marked difference in size between the 47,000-molecular-weight precursor observed by Goldberg and Ohman (9) and proelastase I, for which we calculated a molecular weight of 56,000, is not clear. It may reflect differences in the procedures used to estimate molecular weights; however, the possibility that the 47,000-M_r protein is a degradation product of a larger precursor cannot be excluded.

Inhibition of processing of P by DNP and CCCP was dependent on the presence of EDTA. We attribute this dependence to the hydrophobic character of both uncouplers. *P. aeruginosa* cells do not normally take up hydrophobic compounds (26, 30). However, uptake of such substances is observed when the cells are treated with agents that permeabilize the outer membrane, such as EDTA. This divalent cation chelator is thought to act by removing Mg^{2+} ions bound to lipopolysaccharide molecules and required for stabilization of the outer membrane (11). Expression of the

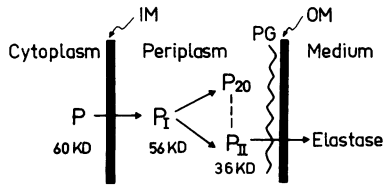

FIG. 8. Diagram illustrating the sequence of events involved in elastase secretion. P, Preproelastase; P_I and P_{II}, proelastase I and proelastase II, respectively; IM, inner membrane; OM, outer membrane; PG, peptidoglycan. The dashed line designates the possibility that, within the periplasm, P20 and proelastase II are noncovalently associated. KD, kilodaltons.

inhibitory activity of DNP and CCCP in the presence of the chelator might therefore result from the permeabilizing effect of EDTA, allowing the inhibitors to reach their target sites in the inner membrane. Processing of Pro I was inhibited by EDTA but not by EGTA, i.e., this reaction is dependent on metal ions other than Ca^{2+}. The mechanism by which EDTA inhibits the proteolytic processing of Pro I is not clear. It is plausible that conversion of Pro I to Pro II depends on a specific metalloprotease which is inhibited in the presence of the chelator. An alternative explanation for this effect is that under physiological conditions, the processing reaction takes place at or in association with the outer membrane and thus depends on a fully functional membrane. If this is indeed the case, the inhibition could be an outcome of the well-documented disorganizing effect of EDTA on the outer membrane of *P. aeruginosa* cells (11).

Based on the present observations, we propose the following sequence of events for the biosynthesis and export of the elastase (Fig. 8). The enzyme is synthesized by the cells as a much larger precursor, preproelastase, having a molecular weight of about 60,000. Translocation of this precursor through the cytoplasmic membrane and processing of the signal peptide occur cotranslationally, or possibly shortly afterwards, because in the absence of suitable inhibitors, the preproenzyme is practically undetectable even when the cells are pulsed for periods as short as 15 s (Kessler and Safrin, unpublished observation). Removal of the signal sequence from preproelastase yields another short-lived elastase precursor, proelastase I, having a molecular weight of about 56,000. This proelastase is found in the periplasm, possibly in association with a component(s) of the outer membrane, and is rapidly processed to a smaller proenzyme form, proelastase II (molecular weight of about 36,000). Proelastase II accumulates temporarily in the periplasm until the final removal from it of a small extra peptide and secretion of the mature enzyme into the medium. Conversion of proelastase I to proelastase II involves an en bloc proteolytic removal from proelastase I of a polypeptide (propeptide) of about 20,000 molecular weight which remains noncovalently associated with proelastase II. This is evident from our finding that proelastase I but not proelastase II is recognized by antibodies specific to P20, a protein of approximately 20,000 molecular weight which we have shown recently is complexed with P36, the 36,000-molecular-weight periplasmic elastase precursor (22). Thus, the last step in elastase secretion is complex, involving not only the proteolytic processing of proelastase II but also its dissociation from P20.

High-molecular-weight precursors have been demonstrated for the extracellular proteases of several gram-positive bacteria, including *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Streptomyces griseus*. As indicated from studies of the corresponding structural genes (12, 37, 41), the secreted proteases of these organisms are synthesized as large precursors with classical amino-terminal signal (pre) peptides followed by long propeptides which are not found in the mature extracellular forms and contain 75 to 194 amino acid residues, depending on the protease. It was therefore proposed that a precursor with an amino-terminal propeptide may be a general feature of proteases secreted by gram-positive organisms (12, 41). The presence of a large propeptide in proelastase I suggests that high-molecular-weight proenzymes may be common to the extracellular proteases of both gram-negative and gram-positive bacteria. In favor of this possibility is the recent demonstration of a large precursor for an extracellular serine protease of *Serratia marcescens* (44). It is not known whether in all instances secretion and maturation of the extracellular bacterial proteases involve the formation of a smaller intermediate proenzyme, as is the case for *Pseudomonas* elastase. The localization in the molecule of the extra sequences of the elastase is also not known. It is reasonable to assume that these sequences, like the propeptides of the extracellular proteases of *Bacillus* spp. (37, 41) and those of *Streptomyces griseus* (12), are N-terminal. The possibility that the entire pro sequence of proelastase I or at least the short additional sequence of proelastase II is carboxy terminal cannot, however, be excluded. A short carboxy-terminal pro sequence which is removed after secretion was recently demonstrated for the extracellular aerolysin of *Aeromonas hydrophila*, and the removal of this extra peptide from aerolysin was shown to be associated with a significant enhancement of the hemolytic activity of this protein (15). Also, the proenzyme of the extracellular serine protease of *Serratia marcescens* was shown to contain a long C-terminal sequence which is missing in the mature form of the enzyme (44).

The functions of the propeptides are not known. The long propeptide may play a role in secretion by binding the proenzyme to the cell membrane (41), the peptidoglycan matrix (12), or, in a gram-negative organism, to the outer membrane. It may also function in keeping the cell-associated enzyme inactive, preventing proteolysis of cell envelope proteins. Consistent with the latter hypothesis are the observations that P20, the polypeptide cleaved from proelastase I, remains noncovalently associated with proelastase II and that the proelastase secreted by *lasA* mutants of *P. aeruginosa* possesses proteolytic but not elastolytic activity (9). It is plausible that the small propeptide of proelastase II confers on the proenzyme a conformation having basal proteolytic activity and that the association of P20 with this partially active proenzyme serves as a protective mechanism to prevent self-destruction. This possibility is compatible with the hypothesis put forward by Fecycz and Campbell (7), that secretion of the mature enzyme involves separation of the cell-associated elastase from a specific inhibitor. P20 may well represent the putative inhibitor.

ACKNOWLEDGMENTS

This research was supported by the Basic Research Foundation, administered by the Israel Academy of Sciences and Humanities, the Recanati Fund for Medical Research, and the Benno and Alice Gitter Fund for Research.

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