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Biochemistry

Secretion and autoproteolytic maturation of subtilisin

(protein transport/proteolytic processing/mutagenesis)

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ABSTRACT **The sequence of the cloned *Bacillus amyloliquefaciens* subtilisin gene suggested that this secreted serine protease is produced as a larger precursor, designated preprosubtilisin [Wells, J. A., Ferrari, E., Henner, D. J., Estell, D. A. & Chen, E. Y. (1983) *Nucleic Acids Res.* 11, 7911–7925]. Biochemical evidence presented here shows that a subtilisin precursor is produced in *Bacillus subtilis* hosts. The precursor is first localized in the cell membrane, reaching a steady-state level of ≈1000 sites per cell. Mutations in the subtilisin gene that alter a catalytically critical residue (i.e., aspartate +32 → asparagine), or delete the carboxyl-terminal portion of the enzyme that contains catalytically critical residues, block the maturation of this precursor. This block occurs when these mutant genes are expressed in *B. subtilis* hosts where the chromosomal subtilisin gene has been deleted. When the mutant *B. amyloliquefaciens* subtilisins are expressed in *B. subtilis* hosts that contain an intact chromosomal subtilisin gene, the mutant precursors are processed to a mature form and released to the medium. Such processing, in *trans*, of the precursor is also demonstrated *in vitro* by addition of active subtilisin. Thus, the release of subtilisin from the cell membrane is dependent on an autoproteolytic process that appears to be novel among secreted proteins.**

Subtilisins are a class of serine endoproteases secreted into the external medium by a wide variety of *Bacillus* species (1). The enzymes from *Bacillus amyloliquefaciens, Bacillus licheniformis*, and *Bacillus amylosaccharitiucus* have been sequenced (2), and the genes coding for *B. amyloliquefaciens* and *Bacillus subtilis* have been cloned (3–6). Kinetic (1, 7, 8) and crystallographic studies (9, 10) have helped to characterize the structural and catalytic properties of these enzymes. In contrast, comparatively little is known of the biogenesis of subtilisin aside from the observation that its expression is linked to the onset of sporulation (11, 12).

The DNA sequences of the two cloned subtilisins (3–6) reveal an intervening propeptide sequence between a putative signal sequence and the mature enzyme sequence. By analogy to eukaryotic enteric proteases (for review, see ref. 13), it might be expected that subtilisin would be secreted as the zymogen, prosubtilisin. However, only mature subtilisin is detected extracellularly (3). No biochemical evidence has yet been presented to show the existence of precursor forms of subtilisin. It is shown here, by analysis of subcellular fractions of *Bacillus* strains expressing various mutations in the subtilisin gene, that a full-length precursor of subtilisin (i.e., preprosubtilisin) exists in association with the cell membrane. Furthermore, the conversion of this primary gene product into the mature enzyme is mediated by active subtilisin and therefore this processing is most likely autocatalytic.

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MATERIALS AND METHODS

T4 lysozyme was provided by Ron Wetzel (Genentech). T4 DNA kinase was from Bethesda Research Laboratories. *Bam*HI, *Eco*RI, and T4 ligase were from New England Biolabs. DNA polymerase large fragment (Klenow fragment) was obtained from Boehringer Mannheim. Enzymes were used as recommended by their respective suppliers. Eugenio Ferrari and Dennis Henner kindly provided the following *B. subtilis* strains used in these studies (6, 14): BG2036 (Apr^-, Npr^-), BG2019 (Apr^-, Npr^+), BG2044 (Apr^+, Npr^-), and I-168 (Apr^+, Npr^+).

Site-Directed Mutagenesis. Oligonucleotides were synthesized by the addition of monomers or presynthesized trimers using phosphotriester chemistry (15), except that mesitylene nitrotriazole was used as a condensing agent. Oligonucleotides were purified by polyacrylamide gel electrophoresis.

All mutants prepared are summarized in Fig. 1. Mutagenesis of aspartate +32 to asparagine +32 was directed by a phosphorylated oligonucleotide having the sequence 5'- CGGTTATC-AAC-AGCGGTAT -3'. The *B. amyloliquefaciens* subtilisin gene contained on a 1.5-kilobase *Eco*RI/*Bam*HI fragment (3) was cloned into M13 mp11, and single-stranded phage DNA was prepared (16). This template was double-primed with the 5'-phosphorylated M13 sequencing primer and the mutagenesis primer as described (17). Mutant phage were identified by hybridization with the ^{32}P-labeled mutagenic primer using a tetramethyl ammonium chloride washing procedure (18). All mutations were confirmed by M13 dideoxy sequencing (19). The mutagenized 1.5-kilobase *Eco*RI/*Bam*HI fragment was cloned back into the *E. coli–B. subtilis* shuttle plasmid, pBS42 (20). To ensure that no second-site mutation(s) had occurred, the region of DNA that was sequenced was replaced with wild-type sequence containing aspartate +32. This reconstruction restored the wild-type protease phenotype.

A primer having the sequence 5'- AAGGCACTTCCGGGAGCTCAACCCGGGTAAATACCCT -3' directed a 7-base-pair deletion and a frameshift starting at codon 163 giving rise to the plasmid pΔ166 (unpublished observations). Mutagenesis was carried out as described above. A primer having the sequence 5'- GAGAGGCAAAAAGCTTTTTGCTTTAGC -3' directed an in-frame deletion of codons −102 to −98 in the subtilisin signal sequence. Mutagenesis was carried out as described (21, 22). Plasmids were transformed into *E. coli* strain MM294 as described by Mandel and Higa (23). Plasmid DNA prepared from *E. coli* (24) was used to transform *B. subtilis* I-168 and mutants of this strain (25). Transformants were selected for chloramphenicol resistance in LB medium containing chloramphenicol at 12.5 μg/ml or LB plates containing chloramphenicol at 5 μg/ml.

In vitro transcription–translation was carried out using a kit obtained from Amersham primed with CsCl-purified plasmid DNA isolated from *E. coli* (26).

Cell Fractionation and Immunoblot Analysis. Stationary-phase cultures (1.25 ml) were treated with 1 mM phenylmethylsulfonyl fluoride to inactivate subtilisin activity. Sam-

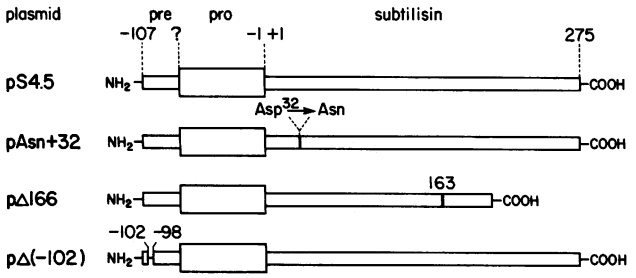

FIG. 1. Summary of mutations prepared to evaluate subtilisin processing. pS4.5 is a wild-type *B. amyloliquefaciens* subtilisin gene cloned into a *B. subtilis–Escherichia coli* shuttle plasmid (3). pAsn+32 is a point mutant in mature subtilisin sequence in which the catalytically critical aspartate +32 is mutated to asparagine +32. pΔ166 introduces a frameshift at position 163 of mature subtilisin, causing premature chain termination 21 codons downstream. pΔ−102 introduces an in-frame deletion of five amino acids from codon −102 to −98 in the signal sequence. All mutations were produced by site-directed mutagenesis. Pre, pro, and mature sequence domains are indicated in blocks. The pro–mature boundary is known (1–3). However, the boundary between pre- and prosequences is unknown and only arbitrarily assigned.

ples were centrifuged at 11,000 × *g* for 10 min and a medium fraction of 0.6 ml was mixed with 0.6 ml of 20% trichloroacetic acid. The suspension was incubated at 4°C for 30 min and the precipitated protein was recovered by centrifugation for 10 min at 19,000 rpm in a Sorvall SS34 rotor (37,000 × *g*). After washing with 0.6 ml of acetone followed by centrifugation, the pellet was dried. The sample was disassociated in $NaDodSO_4$ sample buffer [4% (wt/vol) glycerol/2% $NaDodSO_4$/10 mM Na phosphate, pH 6.8] at 95°C for 3 min.

The cell fractionation procedure was modified from Kaback (27). The cell pellet was washed with 100 μl of 10 mM Tris·HCl (pH 7.4), treated with phenylmethylsulfonyl fluoride, and centrifuged. The cells were resuspended in 100 μl of 30 mM Tris·HCl (pH 8.0) and treated with T4 lysozyme (20 μg/ml) at 37°C for 20 min. After centrifugation at 40,000 × *g* for 20 min, the supernatant (i.e., cytosol fraction) was saved. The pellet (i.e., crude membrane fraction) was resuspended in 100 μl of 50 mM Na phosphate (pH 6.6) and treated with 5 mM EDTA. The crude membrane fraction was treated with DNase (10 μg/ml)/10 mM $MgCl_2$, and incubated at 37°C for 10 min. The membranes were recovered by centrifugation at 40,000 × *g* for 20 min, washed once in 100 μl of 50 mM sodium phosphate buffer, and disassociated as described above.

Certain cell pellets were treated with exogenous subtilisin *in vitro* prior to analysis. Cells from 12.5 ml of culture were resuspended in 1.0 ml of osmotically supported Tris buffer [30 mM Tris·HCl, 25% (wt/vol) sucrose, pH 8.0] and treated with *B. licheniformis* subtilisin (1 μg/ml) (Sigma). After overnight incubation at room temperature with stirring, the cells and medium were separated by centrifugation and fractionated as described above.

The samples were electrophoresed on 12.5% polyacrylamide gels (0.75 mm × 15 cm × 15 cm) as described by Laemmli (28, 29), except that 10% (wt/vol) glycerol was added to the separating gel to enable simultaneous casting and polymerization with the stacking gel. For greater resolution, particular samples were electrophoresed in 10% polyacrylamide gels (0.4 mm × 50 cm). Proteins were transferred to nitrocellulose, treated with 10% acetic acid, neutralized, and probed with antibodies to subtilisin (30).

Preparation of Antibodies and Radioimmunoassay. Subtilisin was purified (8) and digested with CNBr at 100 mg/ml (5 mg of protein per ml) in 88% formic acid. After incubation at room temperature for 2 hr, the reaction mixture was lyophilized and resuspended in 0.1 M Tris·HCl (pH 8.0) containing 0.05% Triton X-100. This was mixed with an equal volume of complete Freund's adjuvant and distributed intracutaneously over several sites on each rabbit (1 mg per animal). Booster injections were given in the ear vein (200 μg per animal) on days 21 and 42. Blood was collected weekly after each injection.

Subtilisin antigens in the membrane, media, and cytosol fractions were quantitated by radioimmunoassay. The membrane fractions were treated with 5 M urea prior to analysis in order to solubilize the antigen. The urea extract was diluted 1:20 with assay buffer (10 mM Na phosphate, pH 7.4/0.9% NaCl/0.5% bovine serum albumin/1 mM phenylmethylsulfonyl fluoride) just before analysis. Affinity-purified anti-subtilisin immunoglobulin fraction (31) was coated at 10 μg/ml onto the 96-well microtiter plate for 2 hr at room temperature. After washing with assay buffer, samples and standards were incubated in the wells as before. After washing, 100,000 cpm of ^{125}I-labeled antibody (31) was introduced and the plate was incubated again for 2 hr. After washing, the plate was assayed.

RESULTS

Identification of a Subtilisin Precursor. The wild-type *B. amyloliquefaciens* subtilisin gene was introduced on the plasmid pS4.5 (3) into *B. subtilis* strain BG2036. In this strain, both the endogenous alkaline protease (*apr*) (i.e., subtilisin) and neutral protease (*npr*) genes have been deleted (14). The immunoblot analysis in Fig. 2*A* shows that at both early and late stages of cell growth, only the 27-kDa mature form of subtilisin (P27) was detected in media fractions. In contrast, analysis of membrane fractions shows the presence of a 42-kDa precursor (P42). Initially, the appearance of P42 in the membrane was coincident with the appearance of P27 in the medium. Subsequently, P42 was seen to disappear as P27 accumulated. The accumulation of P42 measured by RIA (Fig. 2*B*) peaked at ≈6.5 hr of cell growth, which was just prior to the expected onset of sporulation. As the cells sporulated, measured by the drop in absorbance of 550 nm, P27 continued to accumulate in the medium, while the level of P42 in the membrane decreased.

To examine the maturation process in the absence of subtilisin's proteolytic activity, aspartate +32, a member of the catalytic triad, was converted into asparagine (pAsn+32; see Fig. 1). No mature subtilisin (P27) was observed in the medium (Fig. 3). Initially, the precursor of asparagine +32, P42, appeared in the membrane with normal synthesis kinetics (data not shown). However, unlike the wild-type precursor, it continued to accumulate, reaching a plateau late in stationary phase. The asparagine +32 precursor migrated roughly 1-kDa smaller in size than the aspartate +32 precursor. This is presumably due to charge and not a size difference, as we have observed a similar difference between amide and acid substitutions in subtilisin at positions +166 and +222 (unpublished results). To further evaluate whether enzymatically active subtilisin was important in processing, pΔ166 was prepared (see Fig. 1). In pΔ166, the region of the gene coding for the carboxyl terminus of the protein was deleted, thereby removing the catalytic serine +221, which is required for subtilisin activity. Subcellular fractions of stationary-phase cells expressing pΔ166 (Fig. 3) showed only a 31-kDa membrane-bound antigen (P31). The difference in electrophoretic migration observed for P31 and P42 was consistent with the length of the pΔ166 deletion.

Evidence that P42 is Preprosubtilisin. To determine whether the membrane-bound precursor P42 contained the signal sequence, a deletion of five residues, −102 through −98, was constructed in the subtilisin gene (pΔ−102; Fig. 1). The immunoblot analysis (Fig. 4) revealed an alteration in the electrophoretic mobility of the translated precursor for pΔ−102 consistent with a five-amino acid deletion. Further-

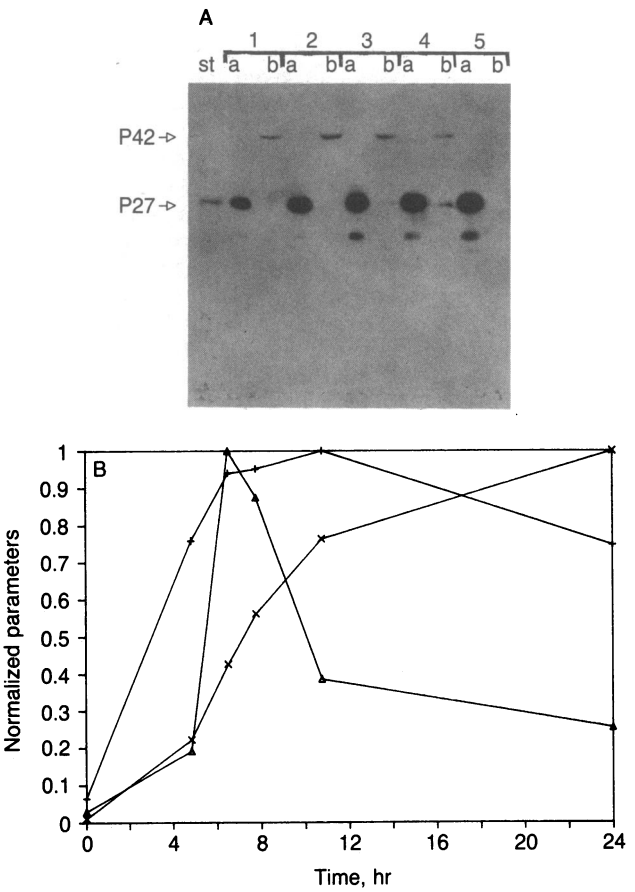

FIG. 2. Biosynthesis of subtilisin and its precursor during cell growth of a *B. subtilis* culture (BG2036) containing the *B. amyloliquefaciens* subtilisin gene on a plasmid, pS4.5. (*A*) Immunoblot analysis of media (lanes a) and membrane (lanes b) fractions sampled as a function of time of culture growth. Numbers 1–5 refer to time points of 4.8, 6.5, 7.8, 10.8, and 36 hr, respectively. st denotes 0.1 μg of a standard mature *B. amyloliquefaciens* subtilisin (P27). The band appearing in the medium below P27 is a degradation product of P27. Cells were harvested and fractionated as described. A sample containing 0.3-ml equivalents of culture for medium samples or 0.2-ml equivalents of culture for membrane samples was loaded onto the gels shown. The P27 in lane 4b is a result of contamination from lane 5a and did not show up in repeat experiments (not shown). (*B*) Time course of cell growth (+), secretion of mature subtilisin (P27) into the medium (×), and accumulation of subtilisin precursor (P42) into cell-membrane fractions (△). Cell growth was measured by absorbance at 550 nm; subtilisin was quantified by radioimmunoassay. Values were normalized to their maxima [cell growth was A_{550} = 4; mature subtilisin (P27) was 36 μg/ml; precursor (P42) was 0.06 μg/ml].

more, as shown in Fig. 5, P42 was made in an *E. coli*-derived coupled transcription–translation assay when programmed with the intact subtilisin gene *pS4.5*. The P42 band can be identified by comparison with the translation products generated with the control plasmid pBS42 (18), which lacks the subtilisin gene. Fig. 5 also shows an immunoblot of membranes from cells containing the pS4.5 plasmid, demonstrating that the membrane precursor made *in vivo* is the same size as the *in vitro*-generated translation product.

Evidence that P42 Can Be Matured *in Vivo* or *in Vitro* by Exogenous Subtilisin. In contrast to the wild-type subtilisin, the maturation of the asparagine +32 mutant was blocked when produced in a host that lacked both subtilisin (*apr*) and neutral protease (*npr*) genes (see Fig. 3). To test whether enzymatically active subtilisin or neutral protease was necessary for processing, pAsn+32 was expressed in *B. subtilis* hosts that contained in their chromosome either an intact

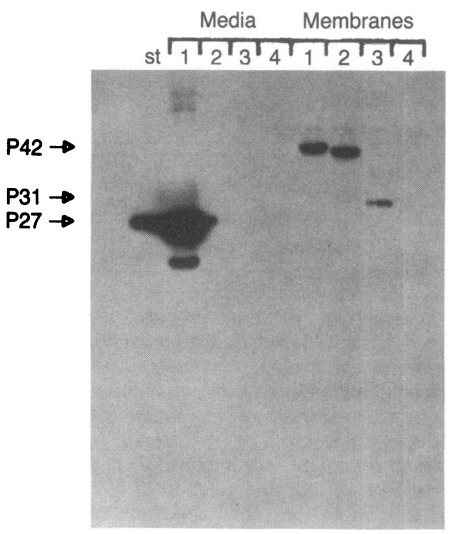

FIG. 3. Subcellular localization of wild-type and mutant subtilisins, asparagine +32 and pΔ166. Plasmids containing the indicated genes were grown in *B. subtilis* BG2036 for 20 hr. Cells were fractionated as described, and fractions were loaded onto gels as described in Fig. 2*A*. st denotes a gel lane containing 0.1 μg of subtilisin. The media and membrane fractions in lanes 1–4 were derived from pS4.5, pAsn+32, pΔ166, and the vector control pBS42, respectively.

subtilisin gene (BG2044), an intact neutral protease gene (BG2019), or both (I168). Processing of the membrane-bound precursor to the mature asparagine +32 enzyme was only observed in Apr$^+$ hosts (Fig. 6*A*). Maturation of asparagine +32 subtilisin was independent of an intact *npr* gene as shown by the continued presence of P42 in the Apr$^-$ Npr$^+$ strain, BG2019. Antibodies elicited by the *B. amyloliquefaciens* subtilisin do not cross-react as well with the *B. subtilis* subtilisin. Thus, a P27 band was not seen with the vector control, pBS42, in the Apr$^+$ hosts.

The P42 precursor of asparagine +32 subtilisin derived from the Apr$^-$ Npr$^-$ host, BG2036, could also be matured *in vitro* by addition of subtilisin (Fig. 6*B*). After overnight incubation at room temperature with *B. licheniformis* subtilisin (1 μg/ml), the majority of the P42 material was released from the membrane and converted to P27. The *B. licheniformis* enzyme was used because *B. amyloliquefaciens* subtilisin antibodies do not cross-react with it. In contrast, cells incubated in the absence of protease showed neither accumulation of P27 nor loss of P42. Cells containing only the vector plasmid pBS42 did not show any detectable P27 in the presence of added *B. licheniformis* subtilisin. These studies suggest that P42 and P27 have a precursor–product relationship.

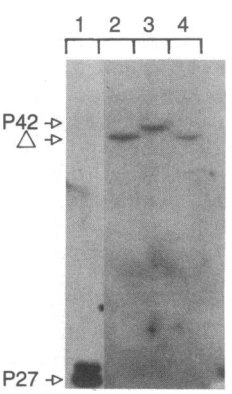

FIG. 4. High resolution (50 cm) immunoblot analysis of membrane-bound precursors from wild type (pS4.5) and a signal peptide deletion mutant (pΔ−102). Lane 1 contains 0.1 μg of mature subtilisin; lanes 2 and 4 contain membrane fractions from a pΔ−102 culture of *B. subtilis* BG2036; lane 3 contains membranes from a wild-type subtilisin (pS4.5) culture of *B. subtilis* BG2036.

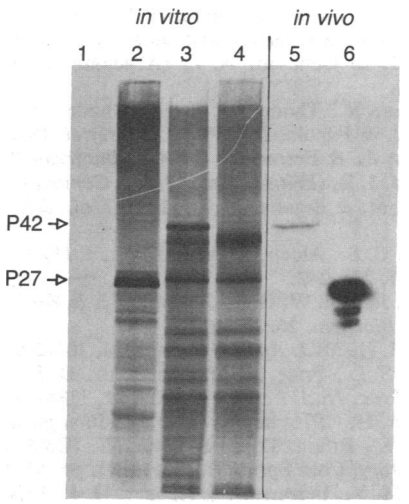

FIG. 5. *In vitro* transcription–translation and parallel immunoblot analysis of subtilisin precursors. Autoradiography of *in vitro* transcription–translation performed in the absence of DNA (lane 1), in the presence of control DNA from Amersham (lane 2), in the presence of the wild-type subtilisin plasmid pS4.5 (lane 3), or in the presence of the vector minus the subtilisin gene *pBS42* (lane 4). Lanes 5 and 6 contain membrane and media fractions, respectively, from pS4.5 cultures. Proteins from the gel were transferred to nitrocellulose; lanes 5 and 6 were subjected to immunoblot analysis. The blot was reassembled and subjected to autoradiography.

DISCUSSION

These data strongly suggest that the maturation and release of subtilisin from the membrane involves an autocatalytic process. First, the membrane-associated precursor (Fig. 2*A*) was shown to be full length through the construction of deletions in the signal and mature sequences (Figs. 3 and 4) and by *in vitro* transcription–translation of the intact gene (Fig. 5). Second, the proteolytic activity of subtilisin facilitates its own release from the cell membrane. When catalytically inactive subtilisins (i.e., pΔ166 and pAsn+32) were expressed in alkaline protease-deficient hosts, the enzymes were not released to the medium but accumulated as preproenzymes in the cell membrane (Figs. 2*A* and 6). This processing block was alleviated *in vivo* by including an intact subtilisin gene in the *B. subtilis* host (Fig. 6*A*) or *in vitro* by addition of active subtilisin to cells (Fig. 6*B*). The processing of the *B. amyloliquefaciens* enzyme in *trans* could be carried out using the *B. amyloliquefaciens* (Fig. 2*A*), *B. subtilis* (Fig. 6*A*), or the *B. licheniformis* subtilisin (Fig. 6*B*). The data have not shown the existence of processing intermediates between preprosubtilisin and subtilisin; however, such intermediates may be unstable and go undetected. Although these studies indicate that an autocatalytic step(s) can be involved in processing, these studies have not proved that the maturation of subtilisin is exclusively dependent on autocatalysis.

The maximal level of the membrane-associated precursor, P42, from pAsn+32 was <0.2% the production of mature wild-type P27. In addition, no P42 was found in the cytosol nor was P27 found in medium when expressed in an Apr$^-$ host. This suggests that when processing of P42 is blocked, further translation of P42 may be inhibited. Consistent with this possibility is that when the processing block for pAsn+32 was partially alleviated, by expression of it in an Apr$^+$ host such as I-168, 3–5 times more P27 was produced than when P42 was produced from pAsn+32 in an Apr$^-$ host, BG2036 (Fig. 6*A*). The production of subtilisin from the host, *B. subtilis* I-168, is <1% of the level of wild-type *B. amyloliquefaciens* subtilisin in I-168 produced from the high copy plasmid, pS4.5 (3, 4). Thus, it is not too surprising that the

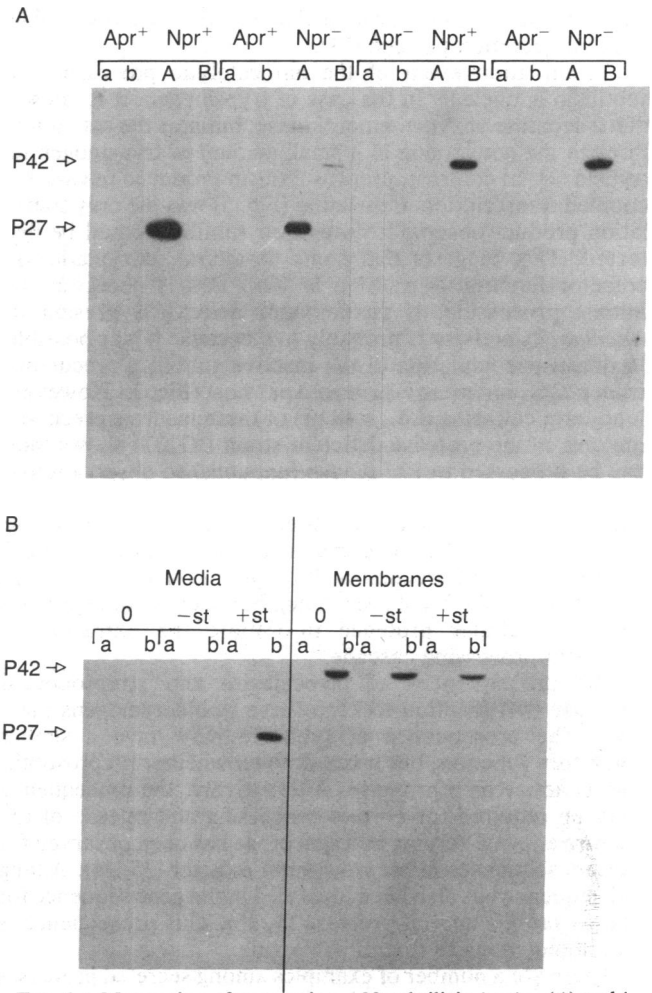

FIG. 6. Maturation of asparagine +32 subtilisin *in vivo* (*A*) and *in vitro* (*B*). (*A*) Immunoblot analysis of medium fractions (lanes a and A) and membrane fractions (lanes b and B) from *B. subtilis* cultures containing either the pAsn+32 plasmid (lanes A and B) or the vector pBS42 minus the subtilisin gene (lanes a and b). These plasmids were expressed in the following *B. subtilisin* strains: *B. subtilis* I-168 (Apr$^+$ Npr$^+$), *B. subtilis* BG2044 (Apr$^+$ Npr$^-$), *B. subtilis* BG2019 (Apr$^-$ Npr$^+$), and *B. subtilis* BG2036 (Apr$^-$ Npr$^-$). Cultures were grown and fractionated as described in Fig. 2. (*B*) Immunoblot analysis of *B. subtilis* BG2036 cells containing pAsn+32 (lanes b) or vector pBS42 (lanes a) plasmids. Cultures were grown to stationary phase (16 hr) and cells were pelleted and washed. Medium and membrane fractions are shown for samples taken immediately after culturing (labeled 0) or after overnight incubation in the presence (+st) or absence (−st) of *B. licheniformis* subtilisin (1 μg/ml).

level of P27 produced from pAsn+32 in *B. subtilis* I-168 was only ≈1% the level of P27 produced from pS4.5. It is also possible that P42 could be extensively degraded in the membrane. Although this possibility cannot be excluded, it appears less likely given the fact that proteolysis of P42 derived from Asn+32 by exogenous subtilisin *in vivo* (Fig. 6*A*) or *in vitro* (Fig. 6*B*) generates a stable mature P27 in the medium.

The production of the P42 precursor of subtilisin reaches a steady-state concentration in the membrane. This suggests that P42 may interact specifically with the membrane. From the maximal accumulation of P42 of subtilisin in the membrane (0.06 μg/ml; Fig. 2*B*) at a cell density of ≈10^9 cells per ml (at A_{550} = 4), one can calculate that there are ≈1000 molecules of P42 per cell at steady state. If P42 resides in specific secretion sites, then 1000 must be a minimum estimate of the total number of such sites. It has been estimated from the amount of membrane-associated MalE-

β-galactosidase fusion protein that *E. coli* contains 20,000 sites for secretion per cell (32).

The initiation event of the autocatalytic processing of subtilisin is unclear. In the case of trypsinogen, it is known that a separate enzyme, enterokinase, initiates the activation through the conversion of a small amount of trypsinogen to trypsin (3). In contrast, preprosubtilisin produced *in vitro* by coupled transcription–translation (Fig. 5) was the only translation product observed; no mature subtilisin could be detected. This suggests that some structural, enzymatic, or cofactor function is missing *in vitro* that is necessary to initiate processing. If an activator activity is present in *Bacillus*, its activity is probably low because it was possible to detect accumulation of the inactive subtilisin precursors from pΔ166 and pAsn+32 in an Apr$^-$ host (Fig. 3). However, long-term culturing (i.e., >48 hr) of these inactive precursor mutants in the protease-deficient strain BG2036 shows they can be processed to P27 *in vivo* (unpublished observations). Under these conditions, where the cells have completely sporulated and lysed, the internal subtilisin-like serine protease (33) would become available to process these membrane-bound precursors. It is possible, in fact, that early sporulation and lysis of a small number of cells could provide this intracellular protease to initiate the extracellular subtilisin processing cascade.

The prosequences of trypsinogen and streptococcal protease (34) function to keep these stable zymogens inactive. The prosequence of subtilisin may have a similar inhibitory function, but it is unknown whether preprosubtilisin is active as a protease. Alternatively, the prosequence may be required for correct processing and release of the mature enzyme serving as a spacer, as has been observed for certain sequences in prepro (yeast) α factor (35, 36). A long prosequence has also been identified in the gene sequence for the *B. subtilis* neutral protease (5, 30). This prosequence is not homologous to that of subtilisin.

There are a number of examples among secreted proteases in which an autoproteolytic event is important in the maturation of the enzyme. For example, streptococcal protease (33) and trypsinogen (12) can be activated in an autocatalytic fashion. Although genetically unrelated to trypsin, subtilisin evolved a similar active-site structure (9, 10). The data presented here further suggest that subtilisin, like trypsin, evolved an autocatalytic maturation strategy. It would appear that subtilisin is a unique example among secreted proteins (for review, see refs. 37–40) in that its release from the cell membrane can occur by autoproteolysis instead of proteolysis by signal peptidase.

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