Correct Folding of α-Lytic Protease Is Required for Its Extracellular Secretion from *Escherichia coli*

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Abstract. α-Lytic protease is a bacterial serine protease of the trypsin family that is synthesized as a 39-kD preproenzyme (Silen, J. L., C. N. McGrath, K. R. Smith, and D. A. Agard. 1988. *Gene (Amst.)*. 69: 237–244). The 198–amino acid mature protease is secreted into the culture medium by the native host, *Lysobacter enzymogenes* (Whitaker, D. R. 1970. *Methods Enzymol.* 19:599–613). Expression experiments in *Escherichia coli* revealed that the 166–amino acid pro region is transiently required either in cis (Silen, J. L., D. Frank, A. Fujishige, R. Bone, and D. A. Agard. 1989. *J. Bacteriol.* 171:1320–1325) or in trans (Silen, J. L., and D. A. Agard. 1989. *Nature (Lond.)*. 341: 462–464) for the proper folding and extracellular accumulation of the enzyme. The maturation process is temperature sensitive in *E. coli*; unprocessed precursor accumulates in the cells at temperatures above 30°C (Silen, J. L., D. Frank, A. Fujishige, R. Bone, and D. A. Agard. 1989. *J. Bacteriol.* 171:1320–1325).

Here we show that full-length precursor produced at nonpermissive temperatures is tightly associated with the *E. coli* outer membrane. The active site mutant Ser 195→Ala (SA195), which is incapable of self-processing, also accumulates as a precursor in the outer membrane, even when expressed at permissive temperatures. When the protease domain is expressed in the absence of the pro region, the misfolded, inactive protease also cofractionates with the outer membrane. However, when the folding requirement for either wild-type or mutant protease domains is provided by expressing the pro region in trans, both are efficiently secreted into the extracellular medium. Attempts to separate folding and secretion functions by extensive deletion mutagenesis within the pro region were unsuccessful. Taken together, these results suggest that only properly folded and processed forms of α-lytic protease are efficiently transported to the medium.

P ROTEIN targeting has been a topic of intense study in recent years (Pugsley, 1989). In gram-negative bacteria, there are four noncytoplasmic destinations: the inner membrane, the periplasm, the outer membrane, and the extracellular medium. While targeting to the inner membrane or the periplasmic space is fairly well understood, general mechanisms and cues for targeting proteins to the outer membrane or the extracellular medium have not yet been identified (for review see Model and Russel, 1990). It is unclear to what extent transport to the extracellular medium and to the outer membrane share mechanistic features with transport to the inner membrane and periplasm. In certain cases, extracellular proteins seem to translocate without a signal sequence, and it has been suggested that they do so directly via the Bayer junctions (Filloux et al., 1990; Mackman et al., 1985; Wandersman and Delepelaire, 1990). In other cases, proteins translocate first to the periplasm using the signal sequence–dependent pathway and are subsequently translocated to the outer membrane (Sen and Nikaido, 1990) or external medium with the help of accessory proteins. The recent demonstration that 14 genes of the *pul* operon are required for the extracellular secretion of the *Klebsiella pneumoniae* enzyme pullulanase (Pugsley et al., 1990) highlights the potential complexity of extracellular transport systems. However, the finding that two of the *xcp* genes required for protein secretion from *Pseudomonas aeruginosa* show significant homology to *pul* genes (Filloux et al., 1990) suggests that at least some aspects of extracellular transport from gram-negative bacteria may share common mechanisms.

Many extracellularly secreted proteases bear large amino- or carboxy-terminal pro regions. Where examined, secretion of these molecules to the extracellular medium appears to be dependent on the presence of a signal sequence as well as the presence of the pro region. The *Serratia marcescens* serine protease is synthesized as a 112-kD preproenzyme, whose amino-terminal signal sequence and 52-kD carboxy-terminal pro region are cleaved during export through the inner and outer membranes, respectively (Miyazaki et al., 1989). Similarly, aqualysin I is produced by *Thermophilus*

aquaticus with an amino-terminal signal sequence and pro region in addition to a large carboxy-terminal pro region (Terada et al., 1990). Both of these proteases can be expressed in *Escherichia coli*, and become processed and secreted under proper conditions. For the IgA protease of *Neisseria gonorrhoeae*, it has been proposed that the pro region forms a pore in the outer membrane through which the protease is translocated. After translocation, the mature enzyme is released from the pro region by proteolysis (Pohlner et al., 1987).

α-Lytic protease is an extracellular serine protease from the gram-negative soil bacterium *Lysobacter enzymogenes* (Whitaker, 1970). This protein has been extensively studied as a model system for investigating serine protease mechanism (Hunkapiller et al., 1973; Bachovchin et al., 1988; Bone et al., 1987) and for understanding the structural basis for enzyme specificity (Bone et al., 1989a,b, 1991). α-Lytic protease is synthesized as a preproenzyme (Silen et al., 1988); expression experiments in *E. coli* have revealed that the 166-amino acid pro region plays an obligatory role in the folding of the 198-amino acid protease domain (Silen et al., 1989). For this reason, the mechanism of folding of α-lytic protease has been examined. Constructs lacking the pro region (Δ-pro-α-lytic protease) give rise to inactive α-lytic protease (Silen et al., 1989). Remarkably, it is possible to complement the folding defect of this Δ-pro molecule by in vivo coexpression of the pro region in trans (Silen and Agard, 1989). Similarly, in vitro experiments demonstrate that it is possible to refold denatured α-lytic protease only in the presence of the pro region (Baker et al., 1992a,b). As in the in vivo system, the pro region can effect folding in either the presence or the absence of a covalent attachment to the protease region. Moreover, the in vitro folding is temperature sensitive; folding is greatly retarded at temperatures above 30°C, (Fujishige, A., D. Baker, and D. A. Agard, manuscript in preparation).

When wild-type α-lytic protease is expressed in *E. coli*, the mature (proteolytically processed and active) protease first appears in the periplasm and then accumulates in the medium at temperatures below 30°C. When the same construct is expressed at temperatures above 30°C, where it is incompetent to fold, an inactive precursor accumulates in the cells. Inactivation of the protease by mutation of the catalytic serine residue results in a similar accumulation of cell-associated precursor even at permissive temperatures, indicating that α-lytic protease is self-processing in *E. coli*. The misfolded Δ-pro-α-lytic protease is also found to be cell associated, independent of expression temperature (Silen et al., 1989).

In this paper we provide evidence that the Δ-pro-α-lytic protease, mutant inactive precursor, and the wild-type precursor synthesized at the restrictive temperature are all tightly associated with the *E. coli* outer membrane. In addition, deletion analysis of the pro region reveals that the folding and the secretion functions are not easily separable and that only proteolytically active molecules are efficiently secreted across the outer membrane. A folded but inactive protease produced by mutation of the protease active site in complementation with a wild-type pro region is efficiently transported to the medium, indicating that enzyme activity per se is not required for secretion. We therefore propose that, unlike translocation through the cytoplasmic or the mi-

tochondrial membrane, which requires that the nascent protein be in an unfolded state (Randall and Hardy, 1986; Eilers and Schatz, 1986), efficient translocation through the outer membrane appears to require that the protein be correctly folded.

Materials and Methods

Plasmid Constructions

pALP5, pALP5-SA195, and pMMALP2 are pBR329 derivatives that have been described previously (Silen et al., 1989). pALP5 contains a copy of the α-lytic proenzyme under the control of the phoA promoter and fused to the phoA signal sequence. pALP5-SA195 is the same vector with the active site serine 195 (chymotrypsin numbering, James et al., 1978) mutated to alanine to inactivate the enzyme. pMALP2 contains the protease region fused directly to the phoA signal sequence, producing the inactive protease referred to as Δ-pro-α-lytic protease.
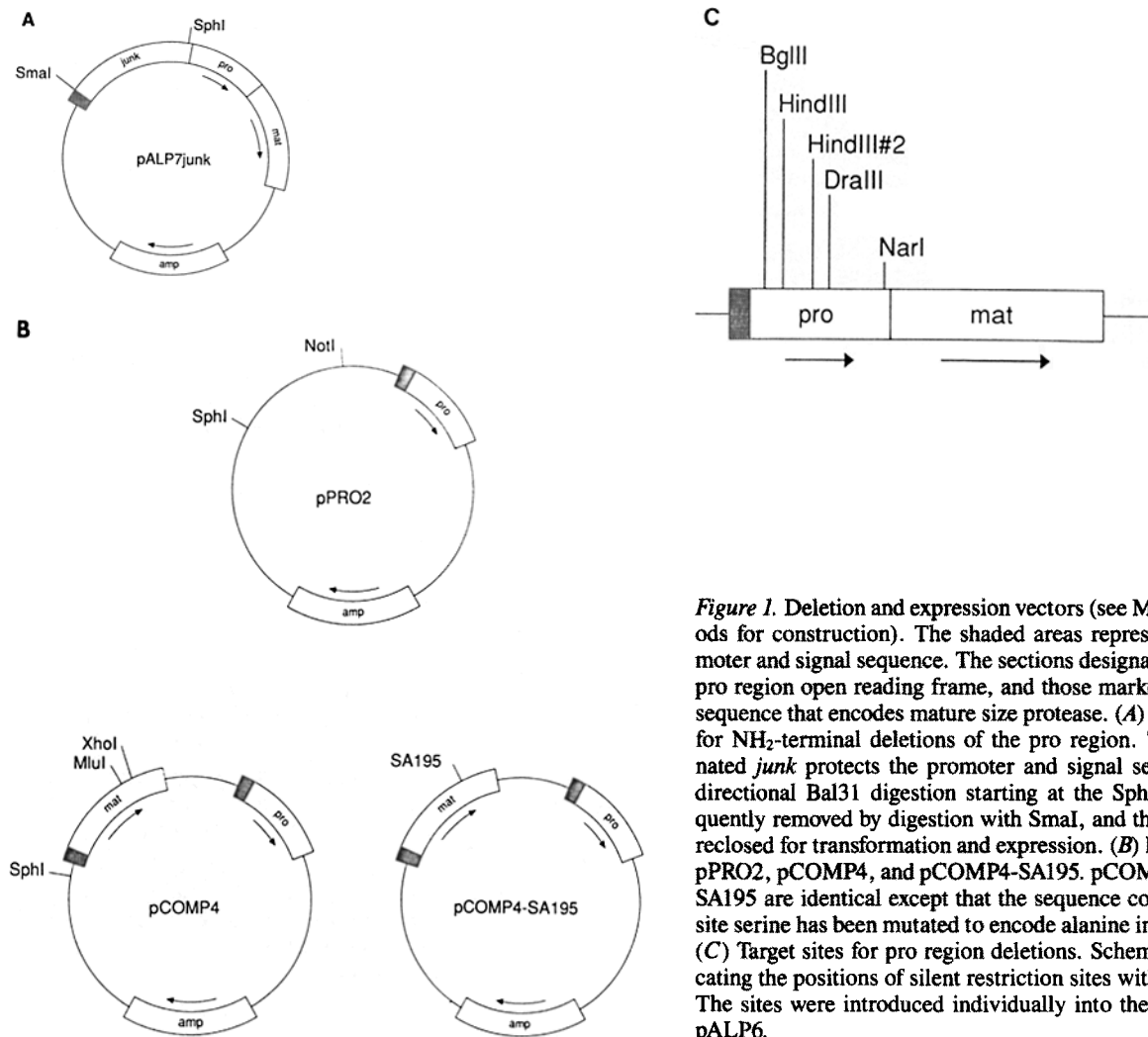
pALP6 was created to introduce unique restriction sites in pALP5 in order to conveniently subclone and express deletion mutants of the pro region (see below). It was constructed by ligating the filled-in EagI/XbaI restriction fragment of pALP5 containing the α-lytic protease gene into the SmaI site of the phoA expression vector pDBR2 (Silen et al., 1988) that had previously been digested with NotI and NarI, filled in with Klenow (New England Biolabs, Beverly, MA), and religated. pALP7junk was generated to allow deletion mutagenesis at the NH2 terminus of the pro region without subcloning. The "junk" portion protects the promoter and is removed after Bal31 deletion and before ligations (see below). It was produced in two cloning steps. First the fill-in EagI/XbaI restriction fragment of pALP5 was cloned into the filled-in XmaI site of pDBR2 (NotI/NarI dropout) creating the out-of-frame construct pALP7. Then the 686-bp filled-in BglII/AccI fragment of ml3mpl8 was cloned into the pALP7 filled-in XmaI site in the minus orientation (Fig. 1 A).

For the construction of the pro region vector pPRO2, the Eco47III/BalI fragment of pPRO1 (Silen and Agard, 1989) was ligated into pDBR2 from which the StyI sites had been removed by religation after treatment with NcoI, StyI, and Klenow fragment. pMALP4 is identical to pMALP2 (Silen et al., 1989) except that unique MluI and XhoI sites have been introduced by replacing the BalI/NcoI fragment with the analogous fragment from pALP5 (Silen et al., 1989). For the construction of the complementation vector pCOMP4, the coding region for the protease was excised from pMALP4 by cutting first with NcoI, filling in with Klenow fragment, and then cutting with SphI. This fragment was ligated into pPRO2 that had been treated with NotI, Klenow, and SphI. For the construction of pCOMP4-SA195, the MluI/XhoI fragment of pALP5-SA195 (Silen et al., 1989) was ligated into the analogous sites of pCOMP4 (Fig. 1 B).

Deletion Mutagenesis of the Pro Region

Silent restriction sites within the coding sequence for the pro region were made by oligonucleotide-directed mutagenesis as described by Kunkel (1985) using an ml3mpl8 construct containing the pALP5 BamHI fragment in the BamHI site of ml3mpl8. The silent restriction sites and their respective mutagenic oligonucleotides are Bgl I, 5'-GATGCCCAGATCTCG-CTGCATTGCG-3'; HindIII, TGGGTGCGCGCAAGCTTTTCGGTCT-3'; HindIII#2, AGCTTGAAGCTTCCGTCTTCGTTGC; and DraIII, TTC-CACCCCACCCAGTGTGCTCGAC. The oligonucleotides were synthesized at Operon Technologies, Inc. (Alameda, CA). For internal sites in the pro region, the mutated BamHI fragments were ligated individually into pALP6 (Fig. 1 C).

Bal31 exonuclease digestions were carried out as follows. 10 μg plasmid was digested to completion with the endonuclease for the targeted site in a 50-μl reaction. The reaction was adjusted to 90 μl containing 12.5 mM MgCl2, 12.5 mM CaCl2, 50 mM NaCl, 20 mM Tris (pH 8), and 1 mM EDTA; divided into three samples, each with a different level (0.01 U, 0.1 U, and 1.0 U) of Bal31 Exonuclease (Boehringer Mannheim Biochemicals, Indianapolis, IN). Each sample was incubated at 30°C and 7.5-μl aliquots were withdrawn after 1, 2, 5, and 10 min. Reactions were stopped by addition of 10 μl 0.1 M EDTA/0.1 M EGTA. Time points were phenol/chloroform extracted, ethanol precipitated, and treated with Klenow. To determine the extent of Bal31 digestion, a portion of each sample was analyzed by gel electrophoresis; appropriate samples were ligated and transformed into *E. coli* MH1 cells. For NH2-terminal deletions of the pro region, Bal31 digestions were performed at the SphI site of pALP7junk. After the

A

SphI

SmaI

pALP7junk

amp

B

NotI

SphI

pPRO2

amp

XhoI
MluI

SphI

mat

pCOMP4

amp

SA195

mat   pro

pCOMP4-SA195

amp

C

BglII

HindIII

HindIII#2

DraIII

NarI

pro   mat

Figure 1. Deletion and expression vectors (see Materials and Methods for construction). The shaded areas represent the PhoA promoter and signal sequence. The sections designated *pro* refer to the pro region open reading frame, and those marked *mat* refer to the sequence that encodes mature size protease. (*A*) pALP7junk vector for NH$_2$-terminal deletions of the pro region. The section designated *junk* protects the promoter and signal sequence during bidirectional Bal31 digestion starting at the SphI site. It is subsequently removed by digestion with SmaI, and the vector is cleanly reclosed for transformation and expression. (*B*) Expression vectors pPRO2, pCOMP4, and pCOMP4-SA195. pCOMP4 and pCOMP4-SA195 are identical except that the sequence coding for the active site serine has been mutated to encode alanine in pCOMP4-SA195. (*C*) Target sites for pro region deletions. Schematic diagram indicating the positions of silent restriction sites within the pro region. The sites were introduced individually into the expression vector pALP6.

extraction and precipitation, the DNA was digested with a second endonuclease, SmaI, treated with Klenow, ligated, and transformed into *E. coli* MH1 cells (see Fig. 1 *B*).

The deletion mutants were prescreened for expression by colony immunoblot. MH1 cells transformed with mutant-bearing plasmids were plated on LB plates. After 12 h at 37°C, a nitrocellulose circle (Schleicher & Schuell Inc., Keene, NH; 0.45-μm pore) was placed on top of the growing colonies. The colonies were allowed to adsorb to the filter for 1 min before carefully removing the nitrocellulose filter. The filter was then placed colony side up on an inducing plate (1.5% agar containing the modified MOPS medium described below). The colonies were allowed to grow and express protein for 12–36 h at 25°C. At that point, the cells were washed off the filters with blot buffer and subjected to immunogold and silver staining with AuroProbe BLplus and IntenSE BL reagents (Janssen Life Science Products, Piscataway, NJ) using affinity-purified rabbit anti-α-lytic protease antibody (Silen et al., 1989) as the primary antibody. Colonies producing protease were then subjected to sequencing, activity analysis, and gel electrophoresis. Activity assays were performed as described (Silen et al., 1989) after treatment with trypsin to remove bound pro region (see below).

Double-stranded Sequencing

DNA was extracted from 5 ml overnight culture by the alkaline extraction method of Birnboim and Doly (1979). The DNA was further purified by polyethylene glycol precipitation as described by Hattori and Sakaki (1986). Double-stranded sequencing was performed on the entire DNA preparation according to the methods outlined in Tabor and Richardson (1987).

Strains, Media, and Expression

E. coli MH1 (*araD139 ΔlacX74 galU galK hsr rpsL*; Hall et al., 1984) was

used as the host for expression of all forms of α-lytic protease described above. Luria-Bertoni medium containing 100 μg/ml carbenecillin was used to maintain expression of plasmids. For phoA-directed expression, single colonies were picked into Luria-Bertoni medium containing 100 μg/ml carbenecillin and allowed to grow overnight at 37°C. The cells were then pelleted, washed three times in MOPS medium (Neidhardt et al., 1974), and diluted 1:50 into MOPS medium supplemented with 0.2% glucose, 0.15% vitamin free Casamino acids (Difco Laboratories Inc., Detroit, MI), and 0.05% Yeast Extract (Difco Laboratories Inc.). This modified MOPS medium provides a low phosphate environment that allows the cells to reach high density before induction by phosphate depletion. The cells were grown at either 37°C or 22°C as indicated, and harvested after 36 h.

Initial Localization and Solubility Studies

Whole cells were examined at a magnification of 1,000 using wet mount phase-contrast microscopy. Although all cells carrying α-lytic–bearing plasmids were irregular in form (elongated, some incomplete septation), those that produce cell-associated forms did not differ from their secreting counterparts. Large refractile bodies or inclusions were not observed.

Cells and media were separated by centrifugation. The presence of α-lytic protease in either cells or supernatants was determined by immunoblots of SDS–polyacrylamide gels (see below). Cells were resuspended in Laemmli sample buffer (Laemmli, 1970) and boiled for 5 min to effect lysis. Supernatants were dialyzed and concentrated where necessary, and boiled in sample buffer for 1 min.

The periplasm was extracted by osmotic shock using the method of Neu and Heppel (1965). Alternatively, complete cell lysis was effected by treatment in 8% sucrose, 45 mM EDTA, 5% Triton X-100, 50 mM Tris, pH 8.0, with 10 mg/ml lysozyme. The lysed cells were further extracted with 2.5% β-octyl glucoside or 0.2% deoxycholate in 5 mM potassium phos-

phate, pH 7. In separate studies, whole or lysed cells were treated with 1% Triton X-100 in the absence or presence of 5 mM EDTA (differential extraction of inner and outer membranes). In other analyses, the lysed cells were extracted with buffers from pH 4 to pH 10.5. After each extraction method mentioned, the sample was centrifuged. The supernatants were dialyzed where necessary, and both the supernatant and the pellets were analyzed by SDS-PAGE followed by immunoblotting (see below).

Membrane Preparation and Fractionation

A crude membrane preparation was made by the method of Mizushima and Yamada (1975). The membranes were resuspended in either 1% EDTA, pH 7; 1% EDTA, pH 7, with 200 mM NaCl; 1% EDTA, pH 7, with 1 M NaCl; or 1% EDTA with 200 mM Na_2CO_3, pH 10.5. Sucrose gradients were poured in 5% steps, 35–55% (wt/wt) sucrose. For each sample, the sucrose gradient contained 5 mM EDTA and the same salt and pH as the sample resuspension buffer. 100–400 μl of treated membranes was separated by isopycnic sucrose density gradient centrifugation at 125,000 g for 4 h using an SW60 rotor (Beckman Instrs., Inc., Fullerton, CA). 200-μl fractions were collected from the top of each gradient. The bottom of each tube was washed with Laemmli sample buffer and is referred to as the gradient pellet. Sucrose concentration of the fractions was assessed by refractive index. Similar studies were performed with 1% Triton X-100 or 1 M urea in the resuspension and sucrose buffers.

For flotation experiments, the crude membranes were mixed with sucrose and the appropriate buffer to produce 55% sucrose samples. These were placed on the bottom of the SW60 tube and the step gradients were carefully overlaid on top. Each flotation experiment was performed with an analogous density gradient experiment. The pairs were centrifuged at 125,000 g for 5 h, at which time the membrane bands appeared to be in the same location within the experimental error and variations due to applied sample density and volume.

Assay for Outer Membranes

50 μl of each fraction was diluted 10-fold to 500 μl with distilled water and the membranes were recovered by centrifugation at 35,000 g for 45 min. The pellets were assayed for the lipopolysaccharide marker, 2-keto-3-deoxyoctonate (KDO),[1] by the microassay of Karkhanis et al. (1978).

Trypsin Accessibility

Precursor produced by expression of the wild-type construct (pALP5) at 37°C was tested for trypsin sensitivity in whole cells versus EDTA-permeabilized cells. For EDTA-permeabilization, cells were washed twice with ice cold 10 mM Tris, pH 8.0. Next, they were resuspended in cold 5 mM EDTA, 10 mM Tris, pH 8, and placed on ice for 15 min. The treated cells were microfuged and carefully resuspended in 0.5 mM EDTA, 10 mM Tris, pH 8.0, using the original culture volume. EDTA-permeabilized and whole cells (in culture medium) were treated with 10 μg/ml trypsin on ice for 15 min. The cells were recovered by centrifugation and immediately resuspended in preheated Laemmli sample buffer that had been adjusted to pH 4.5 in order to stop the reaction. The samples were boiled for 5 min and microfuged for 1 min before analysis by immunoblots of SDS gels.

Gel Electrophoresis of Proteins

SDS-PAGE was carried out on a minigel apparatus (Hoefer Sci. Instrs., San Francisco, CA). Samples were combined with Laemmli sample buffer, boiled for 1 min, and microfuged for 1 min. The supernatant was run on a discontinuous (4–12%) SDS-polyacrylamide gel. Native PAGE was carried out at low pH on discontinuous, nondenaturing gels prepared and run as described by Hames (1981). pCOMP4-SA195 samples were trypsinized briefly to remove bound pro region (see below).

Protein Quantitation and Immunoblot Analysis

The contents of SDS-polyacrylamide gels were transferred to nitrocellulose using a polyblot apparatus (American BioNetics, Emeryville, CA). For native gels, the same apparatus was used except that the low pH gel electrophoresis running buffer was used for electrotransfer with reverse polarity from that used with SDS gels. Immunoblots were probed with affinity-purified rabbit anti-α-lytic protease antibody or an antibody directed against a fusion

protein of the pro region with glutathione transferase (Baker et al., 1992a) followed by ^{125}I-protein A (>300 μCi/μg; ICN Pharmaceuticals, Inc., Irvine, CA) or alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad Laboratories, Richmond, CA). For relative quantitation of α-lytic protease in the sucrose gradients, the lanes of ^{125}I-protein A probed immunoblots were counted dry on a Gamma 8000 scintillation counter (Beckman Instrs., Inc.).

Results

Localization Studies

Our initial attempts to purify the various cell-associated forms of α-lytic protease in *E. coli* (Fig. 2) revealed that, despite the presence of a signal sequence, the molecules were not present in periplasmic extracts. Instead, they were found in the pellet rather than the supernatant of total cell lysates. Although many extraction methods were tried, only those that disrupt strong protein–protein interactions (8 M urea, 6 M guanidine, SDS) were effective in releasing α-lytic protease from the cell pellet. Nonionic detergents such as β-octyl glucoside and Triton X-100, which are effective in extracting integral membrane proteins of the inner membrane but not usually those of the outer membrane, did not extract the cell-associated forms of α-lytic protease. Deoxycholate solubilized only a small fraction of cell-associated precursor. Variation of pH (5–10.5) was similarly ineffective in solubilizing the cell-associated forms. Inclusion bodies were not detectable by phase-contrast microscopy (data not shown). These results led us to believe that the molecules might be associated with the outer membrane.

Examination of a partially purified crude membrane fraction (as defined by Mizushima and Yamada, 1975) confirmed that the wild-type precursor to α-lytic protease (produced at 37°C) was present almost exclusively in the membrane fraction. Notably, the wild-type precursor accumulates in *E. coli* only at temperatures above the natural growth temperature of the native host, *Lysobacter enzymogenes*. To determine whether the precursor location was solely due to growth temperature, we examined the localization of an active site mutant, pALP5-SA195, which had previously been shown to accumulate as a cell-associated, high molecular weight precursor even at temperatures that permit processing and secretion of the wild-type protease (Silen et al., 1989). The mutant precursor was also found exclusively in the membrane fraction. Since both of these molecules retain a covalently attached pro region, we considered the possibility that the pro region mediated all interactions with the membrane. Therefore, we examined the location of the wild-type protease expressed with the phoA signal sequence but without the pro region (Δ-pro-α-lytic protease, pMALP2). This molecule is also inactive and cell associated, but appears by SDS-PAGE to have its signal sequence removed (Silen et al., 1989). Despite the absence of the large pro region, this molecule was also found in the crude membrane fraction.
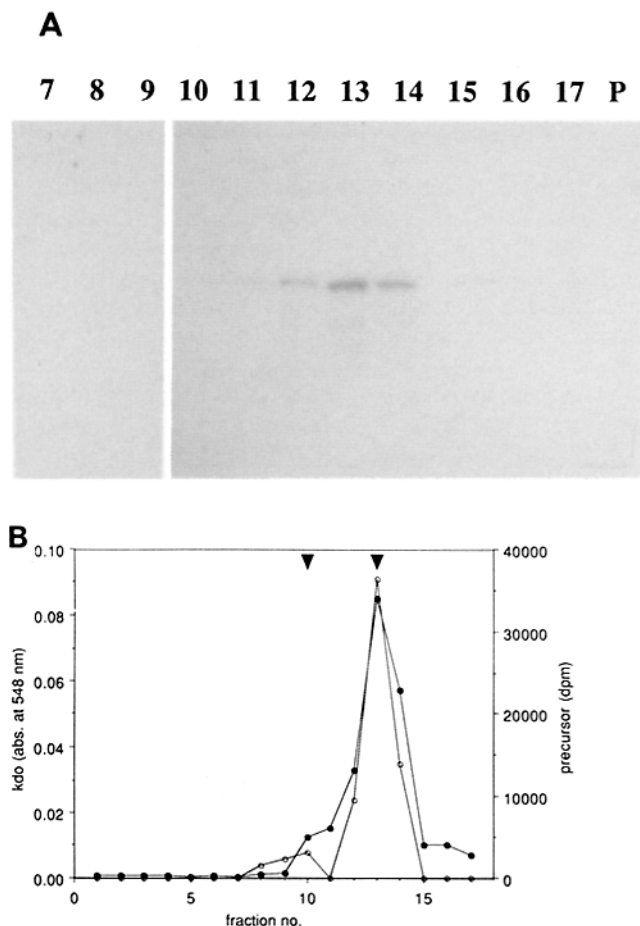
Separation of the crude membrane fractions into inner and outer membranes on sucrose density gradients revealed that the precursor produced at 37°C, the active site mutant produced at 22°C, and the Δ-pro-α-lytic protease produced at 22°C cofractionated with the outer membrane. Two major membrane bands were visible in the gradients: the lighter band, corresponding to inner membranes were observed at 45 to 48% sucrose, while the denser band consistently

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1. *Abbreviation used in this paper:* KDO, 2-keto-3-deoxyoctanate.

	pre	pro	α-lytic protease	Expression Temperature	Activity	Extracellular Secretion
pALP5				22°C	+	+
pCOMP4				22°C	+	+
pMALP2				22°C	−	−
pALP5				37°C	−	−
pALP5-SA195			ser195 → ala *	22°C	−	−
pCOMP4-SA195			ser195 → ala *	22°C	−	+

Figure 2. Characteristics of expression constructs. Cells and media were subjected to α-lytic protease assays as well as immunoblot analyses. The wild-type construct (*pALP5*) expressed at permissive temperatures allows proper folding of the protease and cleavage of the pro region. α-Lytic protease activity is required for the cleavage. Both the pro region and the mature protease are secreted into the medium. Physical linkage of the pro region is not required for proper folding and secretion, as shown by the complementation construct, pCOMP4. Deletion of the pro region (*pMALP2*) results in an inactive, cell-associated molecule. Uncleaved precursors accumulate in the cell upon expression of wild type at nonpermissive temperatures or by mutation of the active site serine (*pALP5-SA195*) with growth at permissive temperatures. Mutation of the active site serine in the complementation construct (*pCOMP4-SA195*) allows secretion of the mature, folded, but inactive protease region.

Figure 3. Location of the proenzyme produced at 37°C. Prepro-α-lytic protease was expressed at 37°C from plasmid pALP5 in the *E. coli* host cell, MH1. The inner and outer membranes were separated by isopycnic sucrose density gradient centrifugation. Fractions were collected from the top of the gradient. Major membrane bands were visible at 46 (*arrowhead*) and 53% (*arrowhead*) sucrose. (*A*) Autoradiograms of the fractions probed for α-lytic protease. The proteins of each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and probed first with anti-α-lytic protease antibody and then with [125I]protein A. All of the precursor

migrated between 52 and 54% sucrose. In one case, a ghost band was observed at 39% sucrose. KDO assays verified that the densest membrane band contained outer membrane lipopolysaccharide, whereas the lighter bands did not. Immunoblots revealed that each of the cell-associated forms was present only in those fractions that contained outer membrane material (Figs. 3–5). The same result was obtained when the crude membrane fraction was loaded on the bottom of the gradient (data not shown) and allowed to float upwards, indicating that the cell-associated forms were not aggregates sedimenting to the position of the outer membrane.

In addition, we attempted to dislodge the Δ-pro-α-lytic protease from the membrane by mild treatments that are generally nondenaturing to proteins but that could disrupt weak associations with the membrane or membrane components. We therefore included 1% Triton X-100 or 1 M urea in the resuspension of the crude membranes as well as in the sucrose gradient itself. Neither treatment altered the comigration of Δ-pro-α-lytic protease with the outer membrane (data not shown).

Significantly, neither the precursors nor the Δ-pro-α-lytic protease could be dislodged from the outer membrane by treatment with either high pH (200 mM Na_2CO_3, pH 10.5, Fig. 6), or by salt (200 mM or 1 M NaCl, data not shown). These procedures are known to be effective in the extraction of peripheral membrane proteins. We conclude that the various cell-associated forms, which are strongly cationic, are not associated with the outer membrane solely by ionic interactions with negatively charged outer membrane components, and that their association with the outer membrane is strong. Treatment with Triton X-100 in conjunction with

containing fractions are shown; no signal was observed elsewhere. The emptied gradient tube was washed in Laemmli sample buffer to solubilize any proteins that might have pelleted at the bottom of the tube; this fraction is marked *P*. (*B*) The outer membrane content of each fraction as determined by KDO assay (○) and relative quantification of the α-lytic precursor content as determined by gamma count of individual lanes of the immunoblot shown above (●).
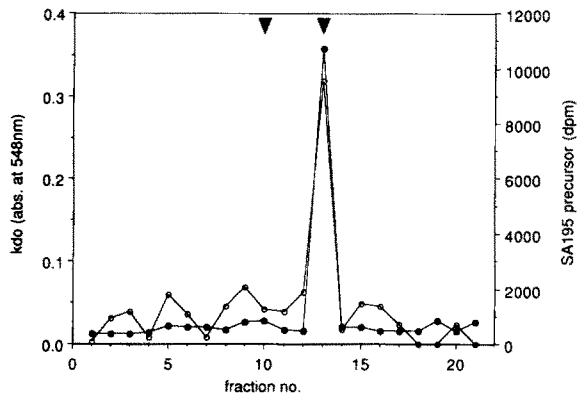
Figure 4. Location of the active site mutant precursor produced at 22°C. The active site mutant produced from vector pALP5-SA195 gives rise to a precursor that cofractionates with *E. coli* outer membranes. Crude membranes were fractionated on sucrose density gradients containing 5 mM EDTA, pH 7.0. Fractions were taken from the top of each gradient and assayed for KDO content (O) or α-lytic proenzyme content (●) as above. Major membrane bands were visible at 46 and 52% sucrose, as indicated by arrowheads. Similar results were obtained using high salt or high pH (data not shown).

EDTA appeared to deplete the outer membrane of all three cellular forms of α-lytic protease, but did not effect complete extraction (data not shown). This result is consistent with the behavior of outer membrane proteins OmpA, OmpC, and OmpF, which are partially extracted (~50%) by Triton-EDTA (Schnaitman, 1974; Hindennach and Henning, 1975) and outer membrane-localized fusions of LamB-LacZ, which are extracted to varying degrees by this treatment (Hall et al., 1982).

The accumulation of the mutant form of α-lytic protease precursor (pALP5-SA195) in the outer membrane of *E. coli* suggests that the association is not a simple artifact of growth temperature, but is likely to reflect a physical property of the
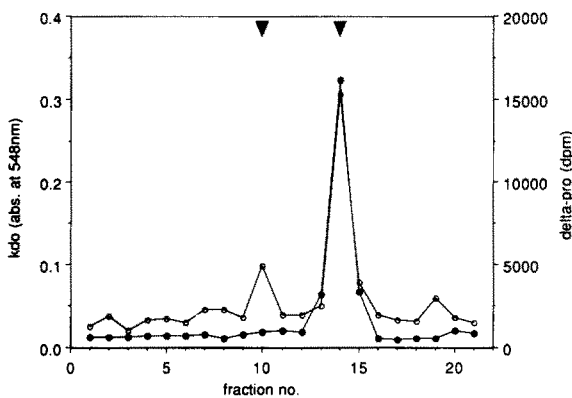
Figure 5. Location of Δ-pro-α-lytic protease. pMALP2 has a signal sequence fused directly to the protease region, and lacks the pro region altogether. The protein produced from this vector is also found in the outer membrane of *E. coli*. Membranes were fractioned in sucrose density gradients containing 5 mM EDTA, pH 7.0. Fractions were taken from the top of each gradient and assayed for KDO content (O) or α-lytic protease content (●) as above. Major membrane bands were visible at 48 and 52% sucrose, as indicated by arrowheads.
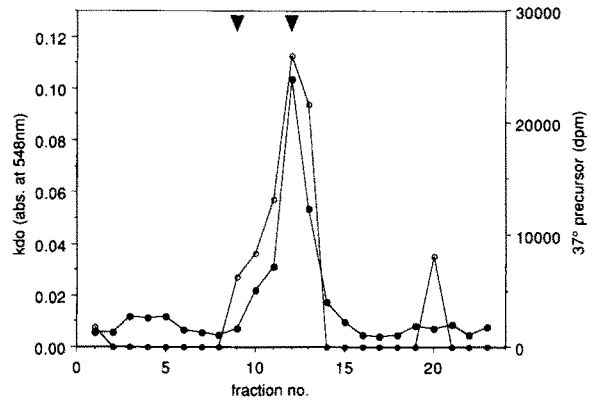

Figure 6. Fractionation at high pH. Prepro-α-lytic protease was expressed at 37°C and prepared as above. Crude membranes were resuspended in 200 mM Na$_2$CO$_3$, 5 mM EDTA, pH 10.5, and loaded on sucrose gradients made up in the same buffer. KDO content (O); α-lytic proenzyme content (●). Major membrane bands were visible at 46 and 54% sucrose, as indicated by arrowheads. Similar results were obtained for the active site mutant (palp5-SA195) and Δ-pro-α-lytic protease (pMALP2) expressed at 22°C (data not shown).

precursor. Furthermore, strong association with the outer membrane can occur even in the absence of the pro region.

The precursor produced at 37°C is degraded by endogenous proteases but was only mildly accessible to trypsin that was added to intact cells (Fig. 7 *A*). By contrast, noticeable loss of the high molecular weight precursor was observed after 15 min of trypsinization when the outer membrane of the cells was first permeabilized by treatment with EDTA (Fig. 7 *B*). A fragment of intermediate size between precursor and mature forms of α-lytic protease appeared and persisted. This suggests that a region of the precursor is protected from
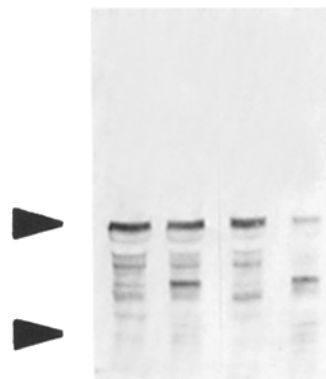
Figure 7. Accessibility to trypsin. Sensitivity of the wild-type precursor to trypsin was tested for whole cells and EDTA-permeabilized cells as indicated. The original state of degradation by endogenous proteases is indicated in the first and third lanes (whole cells and EDTA-permeabilized cells, respectively), and their sensitivity to exogenously added trypsin is shown in the second lane (whole cells) and fourth lane (EDTA permeabilized cells). The positions of the precursor and the mature protease are indicated by the arrowheads.

proteolysis. Such protection could occur from folding, interaction with the membrane, or tight interaction with another protein.

Correlation of Secretion and Folding

The pro region has apparent roles in both folding and secretion (Silen et al., 1989; Baker et al., 1992a,b). We sought to distinguish these two functions by deletion analysis. Six areas throughout the pro region were targeted for deletion mutagenesis in an attempt to find a minimal unit responsible for either folding or secretion or to disable one but not both functions. Silent restriction sites were introduced within the pro region; no changes in α-lytic protease expression levels due to the resulting codon changes were observed. Deletions at the restriction sites did, however, affect accumulation of mature protease in the medium. More than 1,000 colonies per deletion site were screened for activity by plate assay. More than 30 colonies per site were further characterized by sequencing, SDS-PAGE immunoblots, and solution activity assays. These analyses showed that deletions of more than five amino acids at any site abolished protease activity in both the cells and the medium. Furthermore, deletions within the pro region that produced inactive protease invariably disturbed secretion to the medium and vice versa, despite the fact that the protease-coding segment itself was not mutated. The characteristics of a representative sample of the deletion mutants are shown in Fig. 8. One explanation of these data is that the folding and secretion functions of the pro region may not be separable. Another interpretation is that secretion requires not only an intact pro region, but protease activity as well.
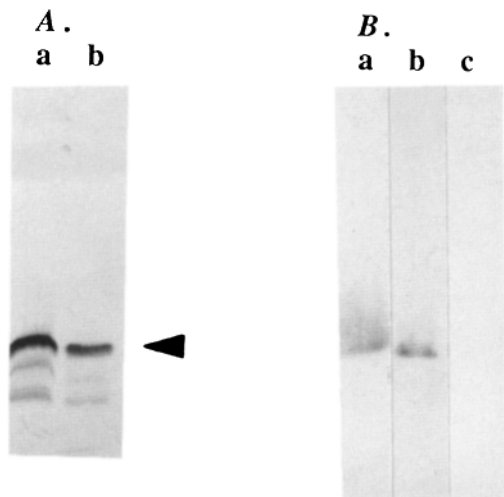
Accordingly, we investigated whether enzymatic activity was required for export across the outer membrane, or whether proper folding alone was sufficient. To produce an inactive but correctly folded protease, we constructed the complementation vector pCOMP4-SA195, which supplies a wild-type copy of the pro region in trans to the protease region containing the active site mutation. Previous results had shown that the pro region can supply the necessary folding information to the protease region in trans (Silen and Agard, 1989). Such a construct should allow proper folding of the protease region to occur while producing inactive protease, and precludes the necessity for the auto-proteolytic processing between the pro and protease regions. In contrast to both the unprocessed form of this mutant and to the misfolded Δ-pro-α-lytic protease, the folded but inactive mutant protease partitions between the medium and in the cells (Fig. 9 A) in much the same manner as does the wild-type gene. It is important to note that this fractionation of protease between cells and medium is seen in all constructs expressing functional α-lytic protease (either the intact protein or via complementation) in E. coli. Over a period of days, α-lytic protease continues to accumulate in the medium, and the cell-associated fraction remains constant or decreases. In these cases, the cell-associated form is mature sized, shows α-lytic protease activity, and is presumed to be a soluble periplasmic species.

The inactive mutant protease secreted by the complementation system comigrates with wild-type α-lytic protease on a nondenaturing native gel (Fig. 9 B), verifying that the two have substantially similar tertiary structure. Because the pro region is known to bind tightly to the protease region until
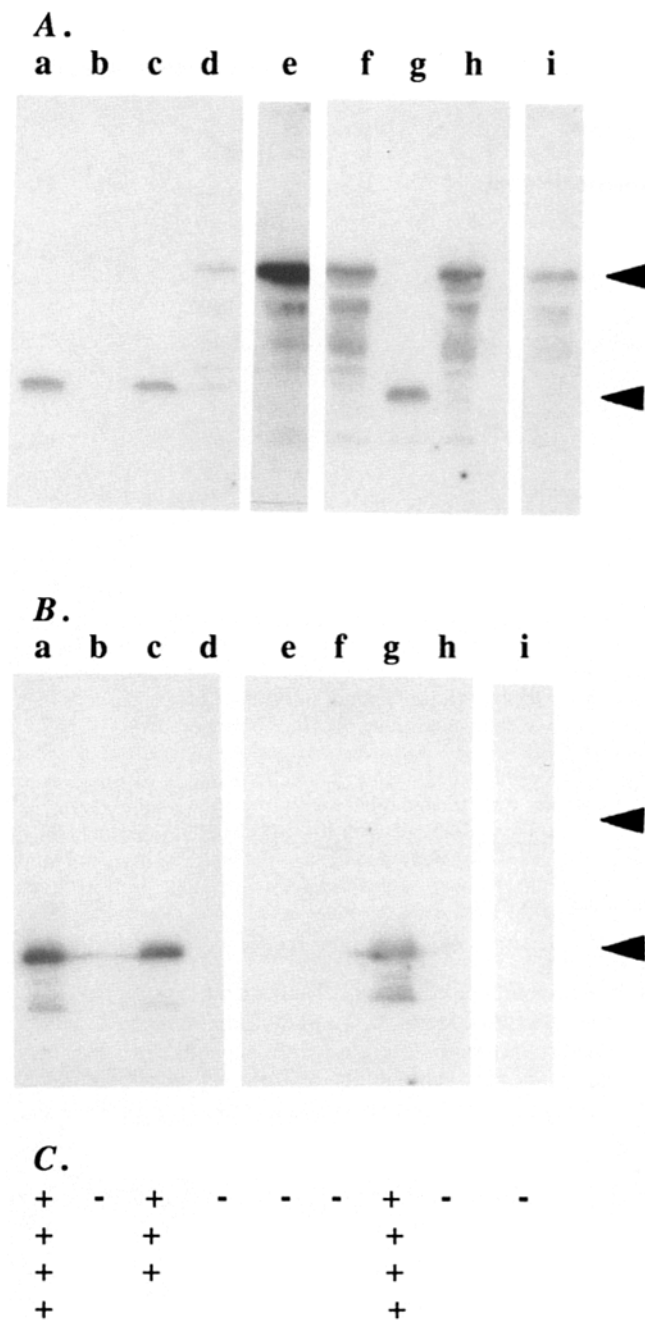
Figure 8. Correlation of activity and secretion. Representative immunoblots of cell extracts and dialyzed media from pro region deletion mutants. The arrowheads indicate the positions of the precursor and the mature protease. (A) Cells: (lane a) pALP6 (wild type); (lane b) uninduced pALP6; (lane c) NH$_2$-terminal deletion of three amino acids; (lane d) NH$_2$-terminal deletion of six amino acids; (lane e) 3–amino acid deletion at the BglI site; (lane f) 13–amino acid deletion at the HindIII site; (lane g) 1–amino acid deletion at the DraI site; (lane h) 10–amino acid deletion at the DraI site; (lane i) 4–amino acid deletion at the NarI. (B) Media, samples as above. (C) Activity observed in the media.

it is proteolyzed (Baker et al., 1992a), the fractionated samples containing inactive mutant protease (pCOMP4-SA195) were trypsinized briefly before electrophoresis. The fact that the mutant protease region is trypsin resistant provides additional evidence that it is compactly folded.
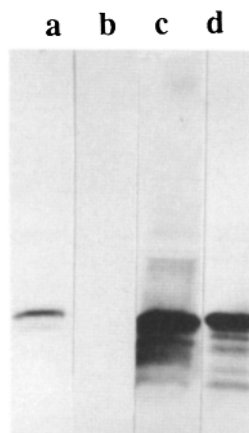
Figure 10. Location of the pro region. Immunoblots of pPRO2 and pALP5, using a primary antibody directed against a pro region fusion protein (see Materials and Methods). The pro region content of the cells and the media were compared under conditions where the pro region is produced alone (pPRO2) versus in the wild-type construct where it is produced with mature protease (pALP5). Pro region content of pPRO2-bearing cells (lane *a*) and pPRO2 medium (lane *b*), pALP5-bearing cells (lane *c*), and medium (lane *d*).

Figure 9. Complementation of the folding defect in the active site mutant. (*A*) pCOMP4-SA195 was expressed at 22°C in *E. coli* MH1 cells (see Materials and Methods for conditions). The media and cells were separated by centrifugation and equivalent amounts were loaded on a discontinuous SDS gel. The arrowhead indicates the migration position of mature α-lytic protease. The immunoblot shows that the mutant is of mature size and is found in the cells (lane *a*) as well as the media (lane *b*). (*B*) The supernatants of pALP5 and pCOMP4-SA195 were each dialyzed against distilled water, lyophilized, and brought up in a small volume of distilled water. The samples were treated briefly with trypsin to remove bound pro region and then combined with low pH sample buffer and loaded on a nondenaturing, nonreducing, low pH gel. The immunoblot of the gel is shown above. (Lane *a*) Wild-type α-lytic protease; (lane *b*) pCOMP4-SA195 treated with trypsin; (lane *c*) trypsin.

Immunoblots of cell extracts and media containing wild-type α-lytic protease have revealed that cleavage of the pro region occurs before secretion into the medium. Furthermore, when the same blots are probed with antibodies directed against the pro region, it can be seen that the pro region itself is released into the medium. Nevertheless, when expressed alone in the pPRO2 construct, the pro region remains cell associated (Fig. 10). Attempts at further fractionation have been hampered by the apparent instability of the pro region when expressed alone.

Discussion

In the present study we have sought to understand factors that interfere with the extracellular secretion of α-lytic protease. It has been established that the pro region plays a crucial role in the folding of the protease region both in vivo (Silen et al., 1989; Silen and Agard, 1989) and in vitro (Baker et al., 1992*a,b*). The pro region must be present and cleaved to allow α-lytic protease to be secreted into the medium. In all other cases, such as the Δ-pro-α-lytic protease or the precursor produced by the SA195 mutation, we find that the α-lytic protease molecule becomes tightly associated with the outer membrane in a manner that cannot be released by detergents, high salt, or high pH, but can be released by treatment with protein denaturants. Furthermore, the precursor is considerably more sensitive to proteolysis by added trypsin in EDTA-permeabilized cells than in whole cells. The possibility existed that these proteins had aggregated and associated with the outer membrane either in vivo or during cell lysis. The appearance of a protected band upon trypsinization argues against the possibility that the precursor exists as an aggregate. We have also tried to address the problem of aggregates sedimenting to the level of the outer membrane by using a combination of flotation and sedimentation density gradient experiments, by varying the extraction and gradient conditions, and by testing the solubility properties of all cell-associated species in Triton X-100 with EDTA. The results support a bona fide association of the precursors and Δ-pro-α-lytic protease with the outer membrane, rather than an artifactual comigration of aggregates with the outer membrane. Beyond this, the extraction properties cited above are suggestive of a molecule arrested in its interaction with protein docking or translocation machinery, or tightly bound to an outer membrane protein in a nontranslocation-competent fashion.

What is the role of the pro region in the secretion process? One possibility had been that the pro region carries the "targeting signal" that mediates association with the outer membrane. This does not appear to be the case with α-lytic protease. The fact that Δ-pro-α-lytic protease is also tightly associated with the outer membrane indicates that, although the pro region is required to complete transport across the outer membrane, it is not required for association with the membrane. Another possibility, as suggested for the IgA protease (Pohlner et al., 1987), is that the pro region actually forms the channel through which the protease domain passes. For wild-type α-lytic protease, the pro region is cleaved from the mature region in the periplasmic space and accumulates in the medium. When expressed in trans to the protease region, it also appears in the medium. When expressed alone, however, the pro region accumulates in the cells but not in the medium. This data must be interpreted with some caution, however, as it is possible that the pro region is unstable in the absence of the α-lytic protease region. Nevertheless, for the wild-type construct, there is an apparent interdependence for secretion of the pro region and the protease region. Furthermore, we have demonstrated a strong correlation between secretion and folding of the protease region in mutants with disrupted pro regions. Finally, the correctly folded but inactive version of α-lytic protease (pCOMP4-SA195) is efficiently transported. Taken together with the in vitro folding studies (Baker et al., 1992*a,b*), these

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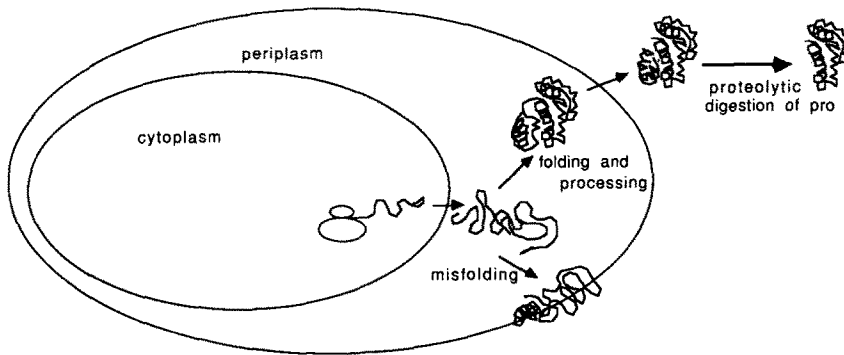
Figure 11. Maturation of α-lytic protease. The preproenzyme is transported across the inner membrane to the periplasmic space, where the signal (pre) sequence is removed. Upon proper folding, the precursor is cleaved between the pro region and the protease region. α-Lytic protease activity is required for the cleavage. The two regions have a high affinity for one another, and therefore remain as a complex while they are conveyed across the outer membrane to the media. Over a period of time, the pro region is further degraded, leaving the mature protease. If the protein does not fold correctly or cleavage between the pro region and the protease region does not occur, the misfolded/precursor form becomes tightly associated with other membrane.

data suggest that the primary function of the pro region is to effect proper folding of the protease region, and that only properly folded molecules are efficiently transported across the outer membrane. It is not yet possible in our system to distinguish between selective transport of folded proteins (comparable to eukaryotic nuclear import; Dingwall and Laskey, 1986) and selective retention of misfolded proteins (comparable to BiP-mediated retention of misfolded proteins in the endoplasmic reticulum; Gething et al., 1986).

α-Lytic protease is one of several proenzymes that have been shown to possess pro region–dependent export characteristics when expressed in *E. coli* (Miyazaki et al., 1989; Silen et al., 1989; Terada et al., 1990). The conditions for export appear to be protease specific and to correlate with the conditions for maturation to the active form, which vary widely for the different proteases. For example, aqualysin I requires temperatures of 65°C for final processing and release from the outer membrane (Terada et al., 1990), while α-lytic protease is efficiently processed and secreted only at temperatures below 30°C (Silen et al., 1989). Other evidence for the involvement of folding in targeting to or across the outer membrane comes from studies of the Omp F porin. Sen and Nikaido (1990) have successfully trimerized Omp F after incorporation of folded monomers into cell envelope preparations in the presence of small amounts of Triton X-100. Their work suggests that the outer membrane can accept prefolded molecules. In contrast, the inner membrane appears to require linear insertion of proteins.

In the best-characterized cases, it appears that targeting to various eukaryotic and prokaryotic cellular locations is mediated by primary structural cues (e.g., signal sequences [Blobel and Dobberstein, 1975] or specific targeting signals such as KDEL for ER retention [Munro and Pelham, 1987], or PKKKRKV for nuclear import of SV40 large T antigen [Kalderon et al., 1984]). Many attempts have been made to discover a primary sequence responsible for targeting proteins into or across the outer membrane of gram-negative bacteria (Nikaido and Vaara, 1987). Because no such signal or independently transporting domain has yet been discovered, it has been suggested that insertion into or transport across the outer membrane may be encoded in a tertiary structural cue (Sen and Nikaido, 1990; Model and Russel, 1990; Dornmair et al., 1990).

We propose a model (Fig. 11) for transport of proteins across the outer membrane of *E. coli*, wherein tertiary and quaternary structure play a significant role. However, our data suggest that while completion of translocation or release from the outer membrane requires that the protein be properly folded (a tertiary cue), association with the outer membrane and perhaps initiation of transport does not require a tertiary signal (misfolded proteins associate very strongly). In this model, the role of pro regions can be understood in that they are necessary for assisting in the folding of the protein to be secreted, and not directly involved in the transport process per se. Furthermore, this model proposes that outer membrane translocation involves mechanisms very different from those elucidated for transport across the inner membrane of *E. coli*, mitochondrial or chloroplast membranes, or the ER membrane, where a targeting sequence and the absence of tertiary structure are the major requirements (for review see Eilers and Schatz, 1988). Further studies are required to determine whether the folded state is being recognized for transport or the incorrectly folded state is being recognized for retention. We hope to utilize in vitro folding reactions in the presence and absence of outer membrane components to answer this question.

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