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

Amy Fujishige, Karen R. Smith, Joy L. Silen, and David A. Agard

Departments of Biochemistry and Biophysics, Pharmaceutical Chemistry, and The Howard Hughes Medical Institute, University of California at San Francisco, San Francisco, California 94143-0448

**Abstract.** $\alpha$-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, *Lyso bacter 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 $\alpha$-lytic protease are efficiently transported to the medium.

Protein 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 Bauer junctions (Filloux et al., 1990; Mackman et al., 1985; Wàndersman 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 aerugino-sa* 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*...
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 pro region 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 mitochondrial 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 pro 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 XmaI 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 pALP6 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 m33mp18 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 Eco47II/BalI fragment of pPRO1 (Silen and Agard, 1989) was ligated into pDBR2 to which the StyI sites had been removed by religation after treatment with Ncol, StyI, and Klenow fragment. pMALP4 is identical to pMALP2 (Silen et al., 1989) except that unique MluI and Xhol 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/Xhol 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 m33mp18 construct containing the pALP5 BamI-II fragment in the BamHI site of m13mpl8. The silent restriction sites and their respective mutagenic oligonucleotides are BglI 5' GATGGCCACAGATCCGCTCGAC; TGGGTCGTGCGCAACCTTTTCGGTCT-3'; HindIII, TGGGTCGTGGCGCAACCTTTTCGGTCT-3'; HindIII#2, AGCCTGGAGCTCCGTTCTGCTCGTGAC; and DraIII, TTCACCCCAACGTTGCTGCGAC. 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 pMALP2 (Fig. 1 C).

Bal31 exonuclease digests were carried out following 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* MHI cells. For NH2-terminal deletions of the pro region, Bal31 digestions were performed at the SphI site of pALP7junk. After the

The Journal of Cell Biology, Volume 118, 1992
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 NH2-terminal deletions of the pro region. The section designated junk protects the promoter and signal sequence during bi-directional Bal31 digestion starting at the SphI site. It is subsequently removed by digestion with Smal, 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.

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 MHI (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 Herpe (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-
that used with SDS gels. Immunoblots were probed with affinity-purified
ative gels, the same apparatus was used except that the low pH gel electropho-
The contents of SDS-polyacrylamide gels were transferred to nitrocellulose
Protein Quantitation and Immunoblot Analysis
A crude membrane preparation was made by the method of Mizushima and
Membrane Preparation and Fractionation
Assay for Outer Membranes
Tris, pH 8.0, using the original culture volume. EDTA-permeabilized and
Trypsin Accessibility
Precursor produced by expression of the wild-type construct (pALP5) at
Gel Electrophoresis of Proteins
protein of the pro region with glutathione transferase (Baker et al., 1992a)

Results

Localization Studies
Our initial attempts to purify the various cell-associated forms of a-lytic protease in E. coli (Fig. 2) revealed that, des-

The Journal of Cell Biology, Volume 118, 1992 36
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 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 mutant 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. Immuno- /blots 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 non-denaturing 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 Na2CO3, 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
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 (Schmitz, 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 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. 7A). 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. 7B). 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 EDTA – – + + trypsin – + – +

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 (○) or α-lytic protease content (●) as above. Major membrane bands were visible at 46 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 Na2CO3, 5 mM EDTA, pH 10.5, and loaded on sucrose gradients made up in the same buffer. KDO content (○); 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).

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 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.
to allow a-lytic protease to be secreted into the medium. In detergents, high salt, or high pH, but can be released by treatment with protein denaturants. Furthermore, the pro-region becomes tightly associated with the precursor produced by the SA195 mutation, we find that the mature 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., 1992a,b). The pro region must be present and cleaved 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.

**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.**

The Journal of Cell Biology, Volume 118, 1992 40
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 PKKRRKVY 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 Ellers 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.

We extend many thanks to Dick Shand for assistance in looking for inclusion bodies and for useful discussions. We also thank John Reidhaar-Olson, Peter Walter, and Linda Randall for critical reading of the manuscript. We are grateful to F. Szoka for the use of his scintillation counter, and to R. B. Kelly for the use of his refractometer.

Funding for this research was provided by the Howard Hughes Medical Institute.

Received for publication 9 March 1992 and in revised form 16 April 1992.

References

Fujishige et al. Requirement of Folding for Extracellular Secretion 41