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Analysis of Prepro-α-Lytic Protease Expression in *Escherichia coli* Reveals that the Pro Region Is Required for Activity

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The α-lytic protease of *Lysobacter enzymogenes* was successfully expressed in *Escherichia coli* by fusing the promoter and signal sequence of the *E. coli phoA* gene to the proenzyme portion of the α-lytic protease gene. Following induction, active enzyme was found both within cells and in the extracellular medium, where it slowly accumulated to high levels. Use of a similar gene fusion to express the protease domain alone produced inactive enzyme, indicating that the large amino-terminal pro region is necessary for activity. The implications for protein folding are discussed. Furthermore, inactivation of the protease by mutation of the catalytic serine residue resulted in the production of a higher-molecular-weight form of the α-lytic protease, suggesting that the enzyme is self-processing in *E. coli*.

α-Lytic protease is one of a battery of extracellular enzymes secreted by the gram-negative bacterium *Lysobacter enzymogenes* to lyse and degrade soil microorganisms. By virtue of its well-studied Asp-His-Ser catalytic triad mechanism (1, 2, 5), its degree of structural homology to mammalian serine proteases (6), and its amenity to nuclear magnetic resonance studies (1, 2, 30), α-lytic protease is an ideal candidate for site-specific mutagenesis studies of substrate specificity and structure-function relationships. We recently reported the cloning and sequence analysis of the α-lytic protease gene from *L. enzymogenes* (33). The nucleotide sequence contained a large open reading frame 5′ of the coding sequence of the mature enzyme, and we proposed that the additional 199 amino acids made up a 33-amino-acid signal sequence (pre) and a 166-amino-acid pro region. Recent studies with secreted proteases from both gram-positive and gram-negative bacteria, including several *Bacillus* species (17, 32, 34, 36–38, 41), *Neisseria gonorrhoeae* (28), *Streptomyces griseus* (13), and *Serratia marcescens* (40), have shown that all of these bacterial proteases are synthesized as precursors, although the pro region varies in its amino- or carboxyl-terminal location. Recently, the 77-amino-acid amino-terminal pro region of *Bacillus subtilis* subtilisin E has been shown to be necessary for the production of active protease, suggesting a critical role for the pro region in folding (15). In this report we provide evidence that the pro region of α-lytic protease has a similar function.

We subcloned regions of the α-lytic protease gene behind the promoter and signal sequence of the *Escherichia coli phoA* gene, allowing production of α-lytic protease in *E. coli* under conditions of phosphate depletion (16). In addition to confirming the requirement for the pro region, this approach has provided evidence that expression of active enzyme is temperature sensitive in *E. coli* and that α-lytic protease has the ability to proteolytically process itself. The implications of these results for protein folding are discussed.

MATERIALS AND METHODS

Enzymes. All restriction enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.), with the exception of *Sma*I, which was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). T4 polynucleotide kinase was purchased from Pharmacia Molecular Biologicals (Piscataway, N.J.). Calf intestinal phosphatase was purchased from Boehringer Mannheim and column purified (9). The Klenow fragment of DNA polymerase I was purchased from Amersham Corp. (Arlington Heights, Ill.). All enzymes were used as recommended by the manufacturers.

Strains, media, and expression. DG98, an F′-carrying strain used for M13 phage growth, was described previously (33). MH1 (*araD139 ΔlacX74 galU galK hsr rpsL*) was used as the host for expression of α-lytic protease (11). For M13 phage growth, YT medium was used (21). LB medium containing 50 μg of ampicillin per ml was used to maintain plasmids derived from pBR329.

For *phoA*-directed expression of α-lytic protease we used MOPS (morpholinepropanesulfonic acid) medium (24) supplemented with 0.4% glucose, 0.15% vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.), and KH_2PO_4 (pH 7.4) or yeast extract to provide a controlled level of phosphate. Typically, after growth overnight from a single colony in 5 ml of MOPS with a high level of phosphate (50 mM KH_2PO_4 [pH 7.4], 50 μg of ampicillin per ml), cells were pelleted, washed three times, and suspended in phosphate-free MOPS medium. The cells were then diluted 1 to 50 into MOPS supplemented with 0.15% yeast extract (0.15% yeast extract was determined to provide 0.63 mM phosphate [total]), which allowed the cells to reach a high density (A_{600}, 3) before the phosphate was depleted. Expression was monitored either by alkaline phosphatase induction or by the production of α-lytic protease (see description of enzyme assays below). Typically, maximal production of α-lytic protease was achieved after 5 days of induction at 24°C. For plate screening, colonies were transferred to plates with low levels of phosphate (0.1 mM) for induction at room temperature and then to milk plates (LB agar with 0.5% skim milk powder) (38), where a region of clearing indicated protease activity.

Plasmid constructions. To be consistent with the published literature on α-lytic protease, we numbered the mature amino acid sequence 1 to 198 and assigned negative numbers to the prepro sequence. The putative signal sequence, therefore, was numbered −199 to −166, and the pro region was numbered −165 to −1 (33).

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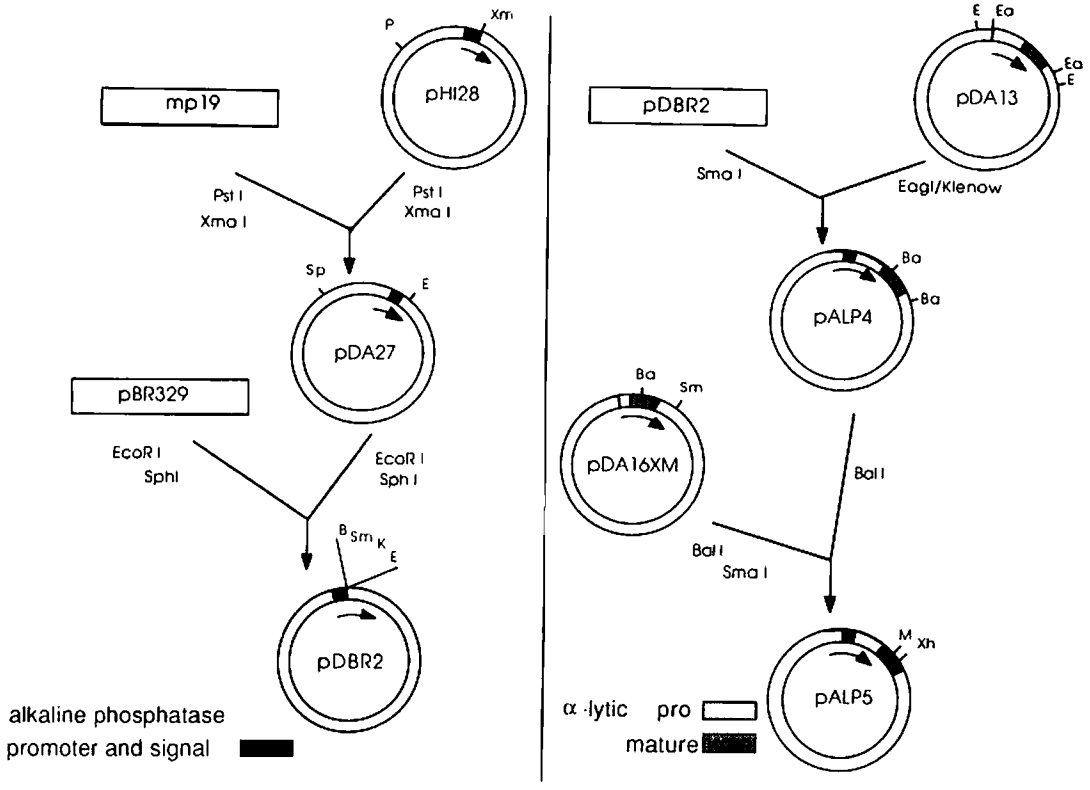

FIG. 1. Construction of expression vectors pDBR2, pALP4, and pALP5. For the construction of pDBR2, a fragment containing the *phoA* promoter and signal sequence from pHI28 was cloned into M13mp19 to increase the number of restriction sites after the signal sequence-coding region. The expanded fragment was then moved into pBR329 to create pDBR2. For the construction of pALP4, pDBR2 was ligated to a fragment containing the α-lytic coding sequence at what is likely to be the beginning of the pro region. The correct orientation was established by an activity assay. For the construction of pALP5, the sequence corresponding to the carboxy-terminal region of the mature enzyme was replaced by the corresponding fragment from pDA16XM, a mutagenized derivative that contained *Mlu*I and *Xho*I restriction sites flanking the coding sequence for binding site pocket residues. See text for details. Restriction enzyme site abbreviations: Ba, *Bal*I; B, *Bam*HI; Ea, *Eag*I; E, *Eco*RI; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; Sm, *Sma*I; Sp, *Sph*I; Xm, *Xma*I; Xh, *Xho*I.

M13mp18 and M13mp19 replicative forms (25) were purchased from Boehringer Mannheim. pHI28, which contains the *phoA* promoter and signal sequence followed by a polylinker region, was the generous gift of H. Inouye and S. Michaelis. When necessary, cohesive ends were filled in with the Klenow fragment in nick-translation buffer. All restriction fragments were isolated in 0.5 to 2.0% low-gelling-temperature agarose gels, and ligations were performed in diluted agarose (35). Transformation was accomplished by the $MgCl_2$ and $CaCl_2$ method (22). Plasmids and replicative forms were isolated by alkaline lysis (4). Restriction enzyme digest analyses were resolved on 0.7 to 2.0% agarose gels.

pDA27, an M13mp19 derivative that contains the *phoA* promoter and signal sequence, was constructed by inserting the *Pst*I-*Xma*I fragment from pHI28 into the *Pst*I-*Xma*I sites of M13mp19. pDBR2, our basic expression vector, was made by inserting the *Eco*RI-*Sph*I fragment of pDA27 into the corresponding sites of pBR329 (Fig. 1).

pALP4, the construct that placed the α-lytic proenzyme under the control of the *phoA* promoter and behind the *phoA* signal sequence, was made by inserting a 1.3-kilobase-pair filled in *Eag*I fragment from pDA13 (M13mp18 containing the α-lytic gene on a 1.741-base-pair *Eco*RI fragment [33]) into *Sma*I-cut pDBR2 (Fig. 1 and 2). Insertion in the correct orientation was ascertained first by screening for enzymatic activity and then by restriction enzyme analysis.

Oligonucleotide mutagenesis. Purified oligonucleotides were provided by the Biological Resource Center (University of California, San Francisco), with the exception of the *Xho*I mutation oligomer, which was provided by the Howard Hughes Medical Institute (University of California, San Francisco). Site-specific mutagenesis was performed by the double primer method of Norris et al. (26) with minor modifications. In all cases, the M13 sequencing primer (17-mer; Pharmacia Molecular Biologicals) was used as the second (nonmutagenic) primer. After extension with the Klenow fragment in the presence of ligase, the hybrid molecules were either digested with restriction enzymes and the appropriate fragment was gel purified and recloned into a new M13 vector or, alternatively, were directly transformed into DG98. Screening of transformants was done either by gel analysis of replicative forms (for the creation of restriction enzyme sites) or by hybridization to nitrocellulose lifts of plaques with ^{32}P-labeled oligonucleotide ([γ-^{32}P]ATP [>3,000 Ci/mmol]; Amersham). The presence of mutations was confirmed by the chain-termination sequencing method of Sanger et al. (31).

pDA16, a *Nar*I fragment in M13mp19 that contains the mature coding sequence (in the positive orientation relative to β-galactosidase transcription), was mutated by site-specific mutagenesis to contain two unique restriction sites: *Mlu*I (base 1248 of the *Eco*RI fragment), using GCGGCCC ATGCACGCGTTGCCCT, and *Xho*I (base 1375), using TCG

AACAGGCTCGAGCGCTGCGAGG, to make pDA16XM. (Underlined bases indicate changes made to the sequence.) The *Ball-Sma*I fragment from the pDA16XM replicative form was substituted for the corresponding *Ball* fragment in pALP4 to make pALP5 (Fig. 1), the expression construct for structure-function mutagenesis experiments.

pMALP2, the construct that allows expression of the protease domain (amino acids 1 to 198) alone and in which the protease is directly fused to the *phoA* signal sequence, was made by oligonucleotide-directed deletion. The mature coding region was placed 3′ of the *phoA* promoter and signal sequence by inserting a *Kpn*I–filled-in *Hin*dIII fragment from pDA15 (the *Nar*I fragment of α-lytic protease in M13mp19 in the minus orientation) into the *Kpn*I–filled-in *Eco*RI site of pDBR2 polylinker to make pMALP1. To produce single-stranded DNA for mutagenesis, the *Sph*I fragment of this construct was cloned into the M13mp18 *Sph*I site and checked by restriction analysis for the proper orientation. To eliminate the intervening 11 amino acids contributed by the M13 polylinker and the 18 amino acids from the α-lytic protease pro region, we used an oligonucleotide-directed deletion using the 30-mer 5′-CCTGTCACAAAGCC/GCCA ACATCGTCGGC-3′ (the slash indicates the fusion site; see the section on oligonucleotide mutagenesis above). The *Sph*I fragment from the mutated plasmid (which included the *phoA* signal sequence-mature α-lytic protease fusion) was transferred into *Sph*I-cut pMALP1, to make pMALP2 (Fig. 2).

The serine 195 to alanine mutation (pALP5SA195) was accomplished by site-specific mutagenesis of pDA16XM single-stranded DNA by using the 25-mer CGAACCGCCAG CATCGCCGCGGCCC. (For the purposes of describing mutations introduced into the mature α-lytic protease, we adopted the chymotrypsin numbering scheme. For example, the Asp-His-Ser catalytic triad in α-lytic protease [amino acids 63, 36, and 143] would be referenced by their homologous positions in chymotrypsin [amino acids 102, 57, and 195]. This allows a more direct comparison with the extensive literature on protease structure and function.) pALP5 DNA was cut with *Mlu*I and *Xho*I and the mutated fragment from the pDA16XM replicative form was substituted.

Protein analysis. Sodium dodecyl sulfate–15% polyacrylamide gels were run as described by Laemmli (19). Western blots (8) were probed with affinity-purified rabbit anti-α-lytic protease antibody followed by ^{125}I protein A (>300 μCi/μg; ICN Pharmaceuticals, Inc., Irvine, Calif.). Antibody immunoserum was prepared by Babco (Berkeley, Calif.) and was purified by using Affigel-10 (Bio-Rad Laboratories, Richmond, Calif.) that was derivatized (23) with native α-lytic protease. Gel samples were prepared by pelleting cells at 12,000 × *g* in a microfuge (Beckman Instruments, Inc., Fullerton, Calif.) and boiling them in sample buffer, or for the culture supernatant, dialyzed against water and then combined with sample buffer and boiled.

Protein purification and enzyme assays. Native α-lytic protease was purified from culture filtrates of *L. enzymogenes* 495 (14, 39) and migrated as a single band when it was electrophoresed on a native polyacrylamide gel at low pH (12). The enzyme was assayed by using succinyl–Ala–Pro–Ala–*p*-nitroanilide (Peninsula Chemicals) as the substrate by monitoring *p*-nitroaniline production at 410 nm (extinction coefficient, ϵ = 8,860 M^{-1} cm^{-1}). Alkaline phosphatase production was monitored at 420 nm as described by Brickman and Beckwith (7) by using *p*-nitrophenyl phosphate as the substrate.

RESULTS

Expression of active α-lytic protease. We previously reported the cloning of the entire α-lytic protease gene, including regulatory elements, contained on a single *Eco*RI fragment in M13mp18 (pDA13 [33]). We were unable to detect expression of α-lytic protease in *E. coli* strains containing this gene either by an enzymatic assay or by immunological screening. The lack of activity was attributed to the poor agreement of the promoter sequence at positions −10 and −35 and ribosome-binding site sequences relative to the *E. coli* consensus sequences (33), although it was also possible that the promoter required *L. enzymogenes*-specific expression proteins that were not present in the phylogenetically distant *E. coli*. For our α-lytic protease structure-function studies, it was necessary to develop a suitable *E. coli* expression system. As α-lytic protease is likely to require an oxidative environment for the proper formation of its three disulfide bonds, we substituted an *E. coli* signal sequence (in addition to the promoter and ribosome-binding site region) for the corresponding *L. enzymogenes* sequences to ensure transport to the periplasm. Studies with subtilisin E (15), among other bacterial proteases, have indicated that the long amino-terminal pro region is required for expression of active enzyme. As the nucleotide sequence of α-lytic protease suggested a preproenzyme structure, we fused the *phoA* promoter and signal sequence to the α-lytic pro region at the point we judged, by determination of the amino acid composition, to be the end of the α-lytic signal sequence (filled in *Eag*I site, base 342; Fig. 2).

The resulting construct, pALP4, conferred phosphate starvation-inducible protease activity when it was transformed into MH1. Surprisingly, we found that this activity was temperature sensitive and was not detectable at temperatures above 30°C. After induction in liquid culture at 24°C, the activity was detectable in both the cells and the culture medium; with increasing time the majority was found in the medium (activity corresponding to 6 mg/liter after 5 days). This distribution was confirmed by Western blot (immunoblot) analysis (Fig. 3), in which antigenically cross-reacting bands from both the cell pellet and the supernatant appeared at the same molecular weight as the purified *L. enzymogenes* enzyme. No higher-molecular-weight form was detected, indicating that processing of the preproenzyme already occurred. Purification of the enzyme from liquid culture medium established that the protease expressed in *E. coli* had identical kinetic parameters to the enzyme produced by *L. enzymogenes*. The values of enzymatic rate (k_{cat}) and binding (K_m) constants were 79 s^{-1} and 5.7 mM for the *L. enzymogenes* enzyme and 73 s^{-1} and 5.6 mM, respectively, for the enzyme produced in *E. coli*. Amino-terminal sequence analysis of the purified *E. coli* product revealed that cleavage from the pro region occurs at the same site as that in the *L. enzymogenes* enzyme (data not shown).

When foreign proteins expressed in *E. coli* are detected in the medium, there is always a question of whether the transport is specific, as in the case of immunoglobulin A protease of *N. gonorrhoeae* (28), or is caused by leakiness of the outer membrane, which is usually indicated by the presence of periplasmic enzymes in the culture medium. Unfortunately, in our current system, expression is achieved when growth ceases (20) and protease production is measured over days, not hours. The length of the expression time makes the question of selective transport from the periplasm more difficult to address, as one must compensate for cell death and lysis, in addition to the effects of the

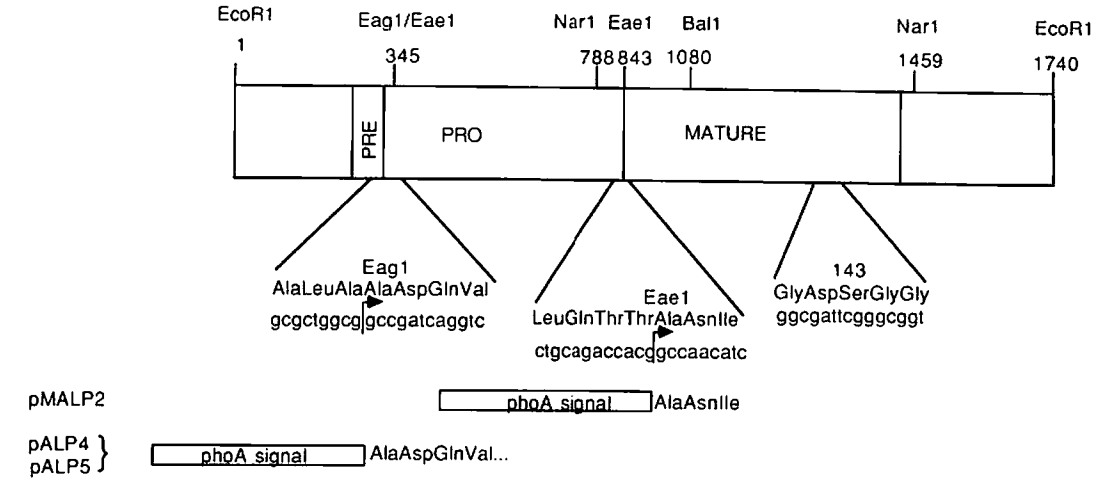

FIG. 2. Fusion sites of the *phoA* signal sequence to α-lytic protease in pMALP2, pALP4, and pALP5 and the site of the catalytic serine Ser-195 (amino acid 143) which was substituted with alanine to make an inactive protease. For amino acid numbering convention, see Materials and Methods.

expressed protein. However, when parallel cultures of MH1(pALP5) and MH1(pBR329) were compared after only 12 h of induction, the pALP5 culture had significant levels of β-lactamase, alkaline phosphatase, and cyclic phosphodiesterase (all periplasmic enzymes) in the medium, while the pBR329 culture showed no such leakage (data not shown). This apparent loss of integrity of the outer membrane suggests that transport is nonspecific.

To facilitate the generation of binding pocket mutations we designed a derivative of pALP4, pALP5, creating two unique restriction sites: *Mlu*I (base 1248 of the *Eco*RI fragment) and *Xho*I (base 1375). These sites flanked the coding sequences for amino acids 136 to 177 and did not change the amino acid sequence. Cell extracts of MH1 cells expressing protein from pALP5 gave identical results to those found with pALP4 (Fig. 3), as judged by Western blot analysis and enzymatic assay. This construct was used in all expression studies unless stated otherwise.

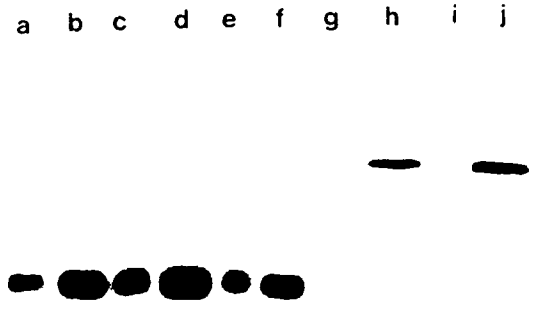

FIG. 3. Western blot (immunoblot) of cell extracts of induced α-lytic protease constructs. Cultures of MH1 containing the various constructs were grown at 24 to 25°C unless indicated otherwise and were induced for 3 days. All samples are the equivalent of 50 μl of culture, except for pALP5 grown at 37°C, which is equivalent to 100 μl. Lane a, Purified α-lytic protease from *L. enzymogenes* (100 ng); lanes b and c, pALP4 cell pellet, supernatant; lanes d and e, pALP5 cell pellet, supernatant; lanes f and g, pMALP2 cell pellet, supernatant; lanes h and i, pALP5 grown at 37°C, cell pellet, supernatant; lane j, pALP5SA195 cell pellet.

The role of the precursor in α-lytic expression in *E. coli*. To assess the role of the pro region in the expression of functional α-lytic protease, we examined the activity of the protease domain when it was expressed alone. Using an oligonucleotide-directed deletion, we made a plasmid containing a direct fusion of the final codon of the *phoA* signal sequence (Ala) to the first amino acid of mature α-lytic protease (Ala-1) (pMALP2; Fig. 2). When MH1 cells carrying this construct were grown under conditions of phosphate starvation, we were unable to detect activity in either the supernatant or cell pellets. Western blot analysis revealed an immunologically cross-reacting species that migrated identically to purified *L. enzymogenes* α-lytic protease on sodium dodecyl sulfate-polyacrylamide gels (Fig. 3). Like the active enzyme, the inactive protease domain appeared to accumulate with time, reaching levels (approximately 3 mg/liter) much greater than those required to assay even fractionally active enzyme (data not shown). However, unlike the active construct, the protease domain remained cell associated; surprisingly, little protease was detected in the medium, even though, as with cells containing pALP5, the presence of periplasmic enzymes in the medium indicated that the outer membrane was leaky. The observation that the immunogen migrated at the same position as the native enzyme indicated that the *phoA* signal sequence was cleaved and suggested localization in the periplasm. However, the exact site of cleavage was not determined. The lack of activity from this construct indicated that production of active α-lytic protease in *E. coli* requires the pro region.

An interesting feature of the production of active protease was its temperature sensitivity. Expression at 37°C resulted in no detectable activity and revealed no immunologically cross-reacting protein at the expected size of the mature form (20 kilodaltons). However, a cross-reacting protein of about 38 kilodaltons, which was the expected size of the proenzyme, was produced (the presence or absence of the signal sequence was not established; Fig. 3). As was the case with pMALP2 expression, most of the protein appeared to

remain cell associated and presumably accumulated in the periplasm. The absence of the processed form suggested that the enzymatic activity normally associated with α-lytic protease might also be responsible for the processing of the proenzyme and the release of the mature form.

To test the role of the protease in the processing of the proenzyme directly, we made a conservative mutation that changed the codon for the catalytic serine 195 (amino acid 143; for amino acid numbering convention, see Materials and Methods) to an alanine, rendering the enzyme inactive. Expression of the protein from this plasmid at 24°C gave a result similar to that seen for expression at higher temperatures: production of an inactive, higher-molecular-weight form of the predicted proenzyme size (38 kilodaltons; Fig. 3). This result indicates that the catalytic activity of the protease is necessary, if not sufficient, for normal processing, explaining why proper processing occurs in a foreign host. It is interesting that the α-lytic protease shows a strong preference for cleaving peptides on the C-terminal side of small hydrophobic amino acid residues, with Ala being the best substrate (3). That cleavage actually occurs before the Ala in the sequence Thr-Thr-Ala-Ile suggests that the three-dimensional structure at the cleavage site favors access of the enzyme to Thr over Ala.

DISCUSSION

In this report we described the successful expression of α-lytic protease in *E. coli*, using the *phoA* promoter and signal sequence. Production of active protease, which accumulates in the extracellular medium, is dependent on the presence of the large amino-terminal pro region that is part of the *L. enzymogenes* gene structure. Expression of the protease domain alone results in an inactive, but properly sized and apparently stable, protein. The fact that a mutation in the active site of α-lytic protease results in the accumulation of precursor indicates that α-lytic protease is responsible for its own processing. In all these respects, α-lytic protease mimics the behavior of subtilisins, as deduced from experiments with *B. subtilis* (15) and *B. amyloliquefaciens* (29) subtilisins (amino-terminal prepro regions of 106 and 107 amino acids, respectively). An interesting point of speculation is whether the relatively large size of the α-lytic protease precursor (199 amino acids) is indicative of some additional function in the native organism, such as transport across the second membrane of *L. enzymogenes*. In this regard, it should be pointed out that when the protease domain alone is expressed, it remains cell associated, presumably in the periplasmic space, whereas expression of the preproenzyme leads to mature α-lytic protease accumulation in the medium. The general leakiness of the outer membrane seen in our expression of the preproenzyme argues against the use of a specific transport mechanism in *E. coli*.

The phenomenon of temperature sensitivity in the production of active α-lytic protease in *E. coli* is particularly interesting when one considers the stability of the mature product. In both *L. enzymogenes* and *E. coli* cultures, α-lytic protease is sufficiently stable to accumulate in the culture medium for several days, showing an unusual resistance to autolysis. In the case of the native enzyme, greater than half the catalytic activity is maintained at 66°C (18). Apparently, in the formation of the active stable product there is a step that is highly sensitive to changes in temperature.

Our current thinking is that the remarkable temperature stability and protease resistance of the active enzyme is generated at the expense of ease of folding. Both these aspects of stability can be explained by X-ray crystallographic analysis of the active enzyme. The enzyme structure shows an exceptional degree of structural rigidity (10), suggesting that there is a high energy barrier between the folded and unfolded states. A high energy barrier could account for the inactive state of the protease domain when it is expressed alone and for the apparent inability of the enzyme, once denatured, to refold (J. Richards, personal communication). By analogy with the concept of transition state stabilization in enzyme reactions, we hypothesize that the pro region would preferentially bind and stabilize the protease domain's "transition state" for folding, essentially acting as a template on which the mature enzyme finds its active conformation. Experiments to test this hypothesis are under way.

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