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Further Molecular Characterization of the Cloned *Legionella pneumophila* Zinc Metalloprotease

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On the basis of DNA sequence similarities to other Zn metalloproteases, further studies of the synthesis, processing, and enzymatic structure of the cloned *Legionella* protease gene, *proA*, were initiated. Tn*phoA* fusions indicated that the entire *proA* open reading frame was transcribed and translated, including the 5′ leader sequence. The results also suggested that the entire polypeptide was exported to the periplasm before cleavage to produce the mature protease. A site-directed mutation in the putative active site, changing glutamate 378 to asparagine, abolished proteolytic activity and cytotoxicity.

The extracellular protease of *Legionella pneumophila* is a 38-kDa zinc metalloenzyme that may play a role in the pathogenesis of *Legionella* infection of protozoan and mammalian hosts. On the basis of DNA sequence homology and competitive inhibitor studies, the *L. pneumophila* protease is in the family of bacterial neutral proteases that includes thermolysin from *Bacillus thermoproteolyticus* and elastase from *Pseudomonas aeruginosa* (4). The DNA sequence of the gene encoding the *L. pneumophila* protease, designated the *proA* gene, contains a single 1,629-bp open reading frame (ORF), far larger than that necessary to encode a 38-kDa protein (4). The deduced polypeptide encoded by the ORF would be a 543-amino-acid polypeptide with a computed molecular mass of 60,775 Da. Analysis of the amino acid sequence placed the start of the mature protease at residue Glu-208 of the large ORF (9). A single export signal sequence was identified within the 16 N-terminal amino acids of the full ORF and was not in a position to mediate export of a peptide commencing with Glu-208. The computed molecular mass for a polypeptide commencing with residue 208 would be 37,826 Da, close to the observed 38-kDa value. Such disparity between ORF size and mature protein length is typical of this family of proteases.

Proteolytic activity of purified protease has been demonstrated against a broad spectrum of substrates, including collagen, gelatin, and casein (6). Protease purified from *L. pneumophila* and *Escherichia coli* containing the cloned gene has also shown cytotoxic effects against a variety of eukaryotic cells, such as HeLa cells (16), human embryonic lung fibroblasts (17), and Chinese hamster ovary (CHO) cells (12). The cytotoxicity of protease is inhibited by metal-chelating agents, suggesting that cytotoxicity and proteolysis are functions of a single zinc-containing site (1). With the *P. aeruginosa* elastase gene sequence (2) and three-dimensional protein structure (11) as a guide, we asked two questions regarding the molecular characterization of the protease. First, does the cloned *proA* gene encode a large preproenzyme? Second, does a glutamate residue form part of the protease active site and is this site also involved in cytotoxicity?

To address the questions concerning the synthesis and processing of the polypeptide from the full ORF into the mature protease, Tn*phoA* fusions (14) were made in the cloned *proA* gene. Tn*phoA* contains a *phoA* gene that lacks a promoter, a ribosome binding site, an export signal sequence, and a Met start codon. When Tn*phoA* inserts in frame into a gene that provides these elements, synthesis and export of active alkaline phosphatase occur and are detectable by a blue color resulting from cleavage of 5-bromo-3-chloro-indolyl phosphate (XP). Fusions of Tn*phoA* with the *proA* sequence could provide evidence as to whether the entire ORF was transcribed and translated. It was also not known whether the entire polypeptide was transported across the inner membrane to the periplasm or whether processing to the 38-kDa form occurred in the cytoplasm.

Tn*phoA* was introduced on a nonviable lambda phage in a PhoA$^-$ *E. coli* strain, CC118 (13), containing the cloned *proA* gene on plasmid pLEG4 (a fragment of the *proA* gene cloned on pBSII SK+ [Stratagene Cloning Systems, La Jolla, Calif.]). The plasmid pLEG4 carried a truncated form of *proA*, including all of the 5′ region but shortened just 3′ to the putative Glu-378 active site, to prevent expression of active enzyme, which appeared to be deleterious to *E. coli*. Colonies of *E. coli* with Tn*phoA* insertions were selected on Luria-Bertani agar containing 40 mg of XP per ml dissolved in *N,N*-dimethylformamide and 30 μg of kanamycin per ml (Sigma Chemical Co., St. Louis, Mo.). Both blue and white colonies were selected and placed in separate pools in order to obtain positive and negative fusions. Plasmid DNA was isolated from each pool by the alkaline lysis technique (3) and transformed into *E. coli* CC118 by electroporation (7). This step ensured that only Tn*phoA* fusions that had inserted into pLEG4 would be analyzed. As before, both blue (PhoA$^+$) and white (PhoA$^-$) colonies were selected, and the location of the Tn*phoA* insertion on pLEG4 was mapped by restriction analysis.

Results of the Tn*phoA* analysis are given in Fig. 1. Insertions within *proA* demonstrated activity only when oriented in the same transcriptional direction as the *proA* ORF and when 3′ of the promoter sequence. Insertions oriented in the direction of transcription opposite to that of the *proA* ORF were inactive. PhoA$^+$ fusions along the full length of the ORF indicated that the entire preproprotein was transcribed and translated and also suggested that the entire polypeptide was transported to the periplasm before cleavage, since the only available signal sequence was at the N terminus of the protein. Therefore, cleavage of the protease to its 38-kDa form must occur after translation. Similar findings have been obtained with *P. aeruginosa* elastase mutants that fail to undergo autoproteolytic

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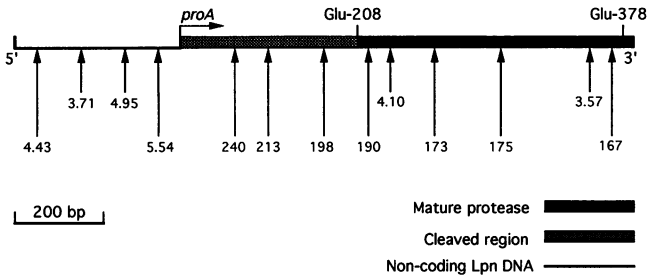

FIG. 1. Tn*phoA* insertions into the *proA* ORF. The locations of 5′-to-3′ Tn*phoA* insertions into the *proA* ORF are indicated by long arrows, and those of 3′-to-5′ insertions are indicated by short arrows. Phosphatase activity for each fusion was determined in a colorimetric assay using *p*-nitrophenyl phosphate (5). The assays were performed in triplicate for each fusion, and the mean units of phosphatase activity are shown below the arrows. For reference, the probable start site (angled arrow labeled *proA*), the Glu-208 cleavage site, and the Glu-378 putative active site are shown.

processing after synthesis and accumulate the preproenzyme in the periplasm (15).

The close similarity of the amino acid sequences of *Legionella* protease and *Pseudomonas* elastase derived by DNA sequencing led us to compare their putative active sites at the amino acid level. Previous studies performed in our laboratory demonstrated that peptide inhibitors modeled upon those that block the active site of *Pseudomonas* elastase also inhibited *Legionella* proteolytic activity (4). By crystallographic studies (2, 18) and mutational analysis (15), the center of the elastase active site has been shown to contain a glutamate residue, Glu-338 (Glu-141 in the mature enzyme), which catalyzes the peptide cleavage reaction. We therefore constructed a site-specific mutation in the *L. pneumophila* protease at a residue thought to be in the active site. Glu-378 was replaced with asparagine to test the hypothesis that the active site of the *Legionella* protease is located in that position and that the protease activity is responsible for both proteolysis and cytotoxicity.

By using the Altered Sites in vitro mutagenesis system (Promega Corp., Madison, Wis.) according to the manufacturer's instructions, a site-directed mutation was made on a fragment of the *proA* gene which extended from an internal *Eco*RI site located at nucleotide 547 through the C-terminal end of the gene; this fragment does not encode the entire ORF, and so this construct had no deleterious effect in *E. coli*. The mutation was verified by sequencing through the active-site region by the chain termination method (Sequenase version 2.0; United States Biochemical Corp., Cleveland, Ohio) (data not shown). Changing glutamate to asparagine shortened the length of the side chain from three carbons to two and changed it from a negatively charged acidic group to a neutral amide. Subsequently, the modified fragment of the *proA* gene was ligated into the wild-type *proA* clone on pSA100, replacing the wild-type fragment and producing the mutated plasmid pSA200. Both pSA100 and the ProA$^-$ mutant constructed by site-directed mutagenesis (the site-directed ProA$^-$ mutant) pSA200 were derived from pBSII SK+ and were maintained in *E. coli* DH5α (10) under selection with 100 μg of ampicillin (Sigma Chemical Co.) per ml.

Results of the analysis of the site-directed mutant are given in Table 1. To measure mutant and wild-type protease activity, *E. coli* strains carrying either pSA100, pSA200, or pBSII SK+ were grown overnight in Luria-Bertani broth with ampicillin at

TABLE 1. Proteolytic and cytotoxic activities of the cloned protease and the site-directed mutant[a]

E. coli strain	Protease activity (A_{595})	Cytotoxicity
DH5α(pBSII SK+) vector alone	0.05 ± 0.02	−
DH5α(pSA100) *proA* clone	1.70 ± 0.02	+
DH5α(pSA200) site-directed mutant	0.02 ± 0.01	−

[a] Shock products from overnight cultures of *E. coli* strains were concentrated 60-fold and then analyzed in the hide powder azure colorimetric assay. Cytotoxicity was measured by a CHO cell assay in which shock products were added to cell monolayers. After 24 h, the monolayers were examined by light microscopy for cytopathic effects, after which they were washed and stained with 1% eosin Y for a qualitative measurement of cell viability. A + sign indicates lysis of the cell monolayer as determined by uptake of stain by dead cells, and a − sign indicates complete exclusion of the stain by the cell monolayer and no detectable cytopathic effects.

37°C in a roller drum. Concentrated osmotic shock products of the periplasmic contents were prepared from the cultures, and hide powder azure (Calbiochem Corp., La Jolla, Calif.) colorimetric assays were performed in triplicate by a modification (16) of previously published methods (8). The wild-type strain containing pSA100 gave 85-fold higher activity than the mutant, whose activity did not exceed the background level of proteolytic activity of *E. coli* containing the vector alone. When concentrated shock products were added to CHO cell monolayers in an assay described by Quinn and Tompkins (16), neither the mutated protease nor the vector strains produced any cytopathic effects, as seen under light microscopy. The wild-type strain expressing protease completely lysed the monolayer, visible by uptake of the viable stain eosin Y (Sigma Chemical Co.), which cannot penetrate live cells (data not shown). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and Western blots (immunoblots) of these concentrated shock products, immunoblotted with polyclonal antisera to the cloned protease, showed no difference in the size or quantity of cross-reacting polypeptides produced by *E. coli*(pSA100) and the mutant *E. coli*(pSA200) (Fig. 2). This indicated that the lack of proteolytic activity in *E.*

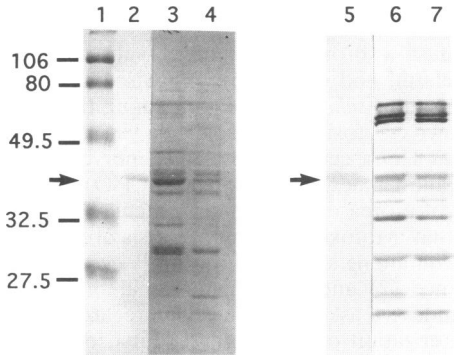

FIG. 2. SDS-PAGE gel and Western blot of wild-type, cloned, and mutant proteases. The leftmost panel shows lanes cut from a single SDS-PAGE gel showing molecular weight markers (in thousands) (lane 1), concentrated supernatant of *L. pneumophila* AA100 SG1 (lane 2), whole-cell lysate of *E. coli*(pSA100) (lane 3), and whole-cell lysate of *E. coli*(pSA200) (lane 4). Lanes 5 through 7 show the same samples as lanes 2 through 4 in a Western blot of a duplicate gel immunoblotted with polyclonal rabbit antisera raised against the cloned protease expressed in *E. coli*(pSA100). The arrows indicate the 38-kDa protease band on the SDS-PAGE gel and the cross-reacting protein on the Western blot.

coli(pSA200) was not due to a defect in protease synthesis or expression.

The results of this study show that the *Legionella* protease is similar to other bacterial zinc metalloproteases, such as *Pseudomonas* elastase, in its translation and cleavage of a long leader sequence to form the mature protein and the involvement of glutamate in the active site. The phosphatase-positive Tn*phoA* fusions made 3′ to the Glu-208 cleavage site suggest that proteolytic processing is occurring in the periplasm of *E. coli*. This conclusion cannot be applied to the situation in *L. pneumophila*, since in this organism the native protease is secreted into the medium, whereas in *E. coli* it accumulates in the periplasm. The loss of proteolysis and cytotoxicity upon substitution of asparagine for glutamate indicates that the active sites of the protease and elastase have functional homology and that both cytotoxicity and proteolysis are in the same enzymatic domain.

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