Identification of Potential Active-site Residues in the *Escherichia coli* Leader Peptidase*

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Leader peptidase of *Escherichia coli* cleaves the leader sequence from the amino terminus of membrane and secreted proteins after these proteins insert across the membrane. Despite considerable research, the mechanism of catalysis of leader peptidase remains unknown. This peptidase cannot be classified using protease inhibitors to the serine, cysteine, aspartic acid, or metallo- classes of proteases (Zwijinski, C., Date, T., and Wickner, W. (1981) *J. Biol. Chem.* 256, 3593-3597). Using site-directed mutagenesis, we have attempted to place leader peptidase in one of these groups. We found that leader peptidase, lacking all of the cysteine residues, can cleave the leader peptide from procoat, the precursor to bacteriophage M13 coat protein. Replacement of each histidine residue with an alanine residue was without effect on catalysis. Among all the serine and aspartic acid residues, serine 90 and serine 185 as well as asparatic acid 99, 153, 273, and 276 are necessary to cleave procoat in a detergent extract. However, only serine 90 and aspartic acid 153 were required for processing using a highly sensitive *in vivo* assay. In addition to the residues directly affecting catalysis, asparatic acid 99 plays a role in maintaining the structure of leader peptidase. Replacement of this residue with alanine results in a very unstable leader peptidase protein. This study thus defines two critical residues, serine 90 and aspartic acid 153, that may be directly involved in catalysis and provides evidence that leader peptidase belongs to a novel class of serine proteases.

Proteins destined for secretion in both prokaryotic and eukaryotic organisms are initially synthesized as precursor molecules with an extra 15–30 amino acid residues at the amino terminus. This extension sequence, termed signal (or leader) peptide, is removed by a signal (leader) peptidase that spans the membrane with its active site facing the lumenal side of the membrane. Cleavage of preproteins by leader peptidase functions to release exported proteins from the outer surface of the *Escherichia coli* plasma membrane (Dalbey and Wickner, 1985).

In *E. coli*, there are two signal peptidases that process proteins destined to the bacterial cell surface. Lipoprotein signal peptidase (Innis *et al.*, 1984), which is an integral protein of the plasma membrane, cleaves lipoproteins exported to the outer and inner membrane. All other known preproteins are processed by leader peptidase, an inner membrane protein of 37,000 Da (Zwijinski and Wickner, 1980; Wolfe *et al.*, 1982). Its orientation across the membrane is shown in Fig. 1. Apolar domains H1 and H2 are transmembrane, the short polar P1 region is in the cytoplasm, and the third apolar domain H3 and the large carboxyl-terminal polar P2 region protrude into the periplasm (Wolfe *et al.*, 1983a; Moore and Miura, 1987). The regions of bacterial leader peptidase needed for catalysis have recently been explored using a genetic deletion approach (Bilgin *et al.*, 1989). H1 and P1 are not directly involved in catalysis, but H2 and the region immediately following it are important for this activity, suggesting that these latter regions may be close to the active site of leader peptidase.

The substrate specificity of leader peptidase has been investigated extensively (Watts *et al.*, 1983; Koshland *et al.*, 1982; Fikes *et al.*, 1990; Kuhn and Wickner, 1985; Shen *et al.*, 1991). In detergent extracts, it cleaves preproteins, ranging from bacterial exported proteins through yeast preacid phosphatase, honey bee prepromellitin, and human prehormones. Although there is no sequence similarity in the leader peptides of these preproteins, there is a common pattern of small amino acids at -1 and -3 with respect to the cleavage site and a helix-breaking residue in the region -4 to -6. Mutations that block processing indeed have been isolated to these leader peptide positions in β-lactamase (Koshland *et al.*, 1982), maltose-binding protein (Fikes *et al.*, 1990), and procoat (Kuhn and Wickner, 1985). Furthermore, we have recently found that the M13 precursor protein, termed procoat, can be efficiently cleaved only if it has the helix breaker proline at -6, a leucine, valine, threonine, glycine, or a serine at -3, and a glycine, serine, proline, or an alanine at -1 (Shen *et al.*, 1991). With few exceptions, almost any residue can be tolerated for leader peptidase processing at -1, -2, -4, and -5 of the M13 procoat.

While major progress has been made in understanding the substrate specificity, the mechanism of catalysis of leader peptidase remains elusive. Protease inhibitors such as phenylmethylsulfonyl fluoride, tosylamido-2-phenylethylchloromethylketone, EDTA, o-phenanthroline, N-ethylmaleimide, di-nitrophenol, carboxyphenanthrolone, or 2,6-pyridinedicarboxylic acid are ineffective at inhibiting leader peptidase (Zwijinski *et al.*, 1981). Using site-directed mutagenesis, we now show that leader peptidase does not require a histidine or a cysteine for catalysis. Of all the serine and aspartic acid residues, serine 90 and 185 as well as asparatic acid 99, 153, 273, and 276 are required for processing of the M13 procoat protein in a Triton X-100 extract. In contrast, only the serine 90 and aspartic acid 153 mutants are inactive using a more sensitive *in vivo* assay. These results, along with sequence similarity with other prokaryotic leader peptidases and the *Saccharomyces cerevisiae* mitochondrial leader peptidase, sug-
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EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—MC1061 (ΔlacX74, ara-D139, Δara, leu7687, gusU, gusK, her, hsm, strA) and JM103 (Δlacpro) thi, strA, supE, endA, sbcB, hsdR, traD36, proAB, lacQZ M15) were from our collection. The pING plasmid (Johnston et al., 1985) has the arabinose operon regulatory elements and the arabinose promoter, strA, supE, endA, sbcB, hsdR, traD36, proAB, lacQZ M15) were from Dr. Gary Wilcox (Ingene, Inc). Insertion of the leader peptidase gene into the PING plasmid was described in Dalbey and Wickner (1987). The techniques described in Maniatis et al. (1982) were used for the DNA manipulations. Restriction enzymes were purchased from BRL and New England Biolabs. For DNA transformations, the method of Cohen et al. (1973) was used.

In Vitro Assay—Leader peptidase activity was measured by the posttranslational conversion of procoat to coat protein and leader peptide. In this assay, procoat was synthesized in vitro according to the procedure of Zaikin et al. (1974), except for minor modifications (Yamane et al., 1987). Cultures (2 ml) of MC1061 expressing different leader peptidase mutations were grown to an optical density at 600 nm of 0.2, induced with arabinose for 2 h, followed by centrifugation for 1 min to collect the cells. After resuspending in 0.3 ml of lysis buffer containing 20% sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% Triton X-100, lysozyme (1 mg/ml), deoxyribonuclease (5 μg/ml), ribonuclease (1 μg/ml), and phenylmethylsulfonyl fluoride (5 mM) and incubating for 30 min at room temperature, cell extracts were used directly or diluted (1:10, etc.) and incubated with 35S-labeled procoat at 37 °C for 30 min. The processing of procoat was analyzed on a 23% polyacrylamide gel (Boeke et al., 1980).

In Vivo Assay—Leader peptidase activity of the mutants was measured by examining the processing of outer membrane protein A precursor (pro-OmpA) in IT41, a temperature-sensitive leader peptidase strain (Inada et al., 1989). Briefly, IT41 was grown at 32 °C in M9 medium containing 0.5% fructose and 50 μg/ml each amino acid (except methionine). After reaching the early-log phase, cultures were shifted to 42 °C for 1 h to inactivate the temperature-sensitive leader peptidase. Arabinose (0.2%) was added to the medium to induce synthesis of leader peptidase. Cells were labeled with [35S]methionine for 15 s, and unlabeled methionine was added to a final concentration of 500 μg/ml. At indicated times, samples were removed and quenched in 20% trichloroacetic acid. Samples were immunoprecipitated with antibody directed against outer membrane protein A and analyzed on a 12% SDS–polyacrylamide gel (acylamide:decarboxilic, 30:0.8) with a 5% stacking gel using a discontinuous buffer system. The gels were then fixed and subjected to fluorography (Ito et al., 1989).

RESULTS

We have used site-directed mutagenesis in an attempt to place leader peptidase in either the cysteine, serine, aspartic acid, or metallopeptidase class of proteases. All oligonucleotide-directed mutations were made as described by Zoller and Smith (1983) with some modifications (Dalbey and Wickner, 1987). The mutants were designated XNY (X, amino acid in the wild-type protein; N, the position of the amino acid; Y, the new substituted amino acid). The sequences of the mutagenic primers are shown in Table I, and the sites of the mutations are indicated in Fig. 1B. Each of the mutants was evaluated first for its catalytic activity using an in vitro assay (Table II).

As a first step, we replaced all of the cysteine residues with serines. This was achieved in two successive steps. In the first, the cysteine at position 21 was replaced with a serine. In the second step, the cysteine residues at positions 170 and 176 were replaced with serines using as a template a leader peptidase gene encoding the C21S substitution. Leader peptidase lacking all three cysteines was then assayed for enzymatic activity with an in vitro assay (Fig. 2). In this assay, radiochemically labeled [35S]procoat was used as a substrate. Leader peptidase cleaves procoat to the coat protein (Fig. 2, bottom right panel). Briefly, the in vitro assay is based on a detergent extract of MC1061 cells transformed with the appropriate plasmid. The plasmid pRD8 encodes for wild-type leader peptidase. As can be seen in Fig. 2, a 200-fold diluted extract of MC1061 pRD8 catalyzes comparable cleavage of procoat, as does undiluted extract of MC1061 without plasmid (compare no plasmid and pRD8 panels). The background activity is due to the leader peptidase coded by the chromosomal lepB gene. Cell extract containing leader peptidase C21S, C710S, and C716S, which lacks all cysteines, has full enzymatic activity. This indicates that leader peptidase is not a cysteine protease.

In a second step, we evaluated whether leader peptidase can be grouped into the classical metallo- or serine class of proteases. Since a histidine residue is essential in these groups we replaced each of the histidine residues with an alanine. Cell extracts bearing H124A, H235A, or H323A leader peptidase were analyzed by the in vivo assay (Fig. 3A). It can be seen that these substitutions caused a 1000-fold decrease in the enzymatic activity of leader peptidase. Cell extracts bearing H124A, H235A, or H323A leader peptidase were analyzed by the in vivo assay (Fig. 3A). It can be seen that these substitutions caused a 1000-fold decrease in the enzymatic activity of leader peptidase.
dase have normal enzyme activity (Fig. 2 and Table II). This shows that leader peptidase is not a standard serine protease. Nor is this result consistent with leader peptidase being a typical zinc metalloprotease since in this class of proteases histidine residues have been shown to chelate the metal ion (Dunn, 1989) and, hence, are likely to be indispensable for catalysis.

To determine whether aspartic acid and serine residues are required for catalysis, we replaced each of them with alanines. In this study, to simplify the number of oligonucleotides required for catalysis, we replaced each of them with alanines. The codons, which were changed to make the mutations, are underlined.

The synthetic oligonucleotides were prepared on an Applied Biosystem model 380B instrument by the Ohio State University Biochemical Instrument Center to change potential residues involved in catalysis. The codons, which were changed to make the mutations, are underlined.

Table I shows that three leader peptidase D280A, S281A was inactive, each of the wild-type protein. In addition, two of the six double mutant of the triple mutants, leader peptidase D153A, D158A, S161A, peptidase S90A (see also Fig. 3). Various IT41 strains containing either no plasmid, pRD8 (expressing wild-type leader peptidase), or a plasmid encoding the mutant proteins (S90A, S153A or S185A) were pulse-labeled for 15 s with [35S]methionine and chased with an excess of unlabeled methionine. At the indicated times, aliquots were removed and analyzed by immunoprecipitation to OmpA, SDS-polyacrylamide gel electrophoresis, and fluorography. While processing, this indicates that serine 185 as well as aspartic acid mutants had normal substrate processing (100-200-fold higher). It is interesting to note that while the double mutant leader peptidase D280A, S281A was inactive, each of the mutant proteins containing the single mutation was active (Table III). We believe this is due to a conformational change that arises by the replacement of two amino acids.

As a second assay, we tested the activity of selected leader peptidase mutants using a sensitive in vivo assay (Bilgin et al., 1990). It is based on the fact that overproduction of leader peptidase accelerates the processing of the precursor to outer membrane protein A (pro-OmpA) at the nonpermissive temperature in IT41 (Inada et al., 1989). This strain bears a temperature-sensitive leader peptidase encoded by the chromosomal lepB gene (Fig. 3). Various IT41 strains containing either no plasmid, pRD8 (expressing wild-type leader peptidase), or a plasmid encoding the mutant proteins (S90A, D153A or S185A) were pulse-labeled for 15 s with [35S]methionine and chased with an excess of unlabeled methionine. At the indicated times, aliquots were removed and analyzed by immunoprecipitation to OmpA, SDS-polyacrylamide gel electrophoresis, and fluorography. While processing is very rapid with a $t_{1/2} \approx 90$ s. However, extracts prepared from cells expressing either leader peptidase S90A or D153A have very low activity comparable with that of IT41 without plasmid. Slightly higher activity is observed in cells expressing leader peptidase S185A (Fig. 3), and normal rapid processing is seen with leader peptidase D273A and D276A (data not shown). Since these latter residues are not required for in vivo processing, this indicates that serine 185 as well as aspartic
acids 273 and 276 do not play a catalytic role. However, the critical residues, serine 90 and aspartic acid 153, may participate directly in catalysis.

In contrast to the serine 90 and aspartic acid 153 mutant proteins, leader peptidase D99A was not detected by immunoblot analysis (data not shown). Pulse-chase experiments revealed that leader peptidase D99A is unstable (Fig. 4), with a half-life of roughly 2 min. This is compared to a half-life of >60 min for wild-type leader peptidase (data not shown).

**DISCUSSION**

Proteases can be typically classified into four different groups: serine and cysteine proteases that form covalent enzyme complexes and aspartic acid and metalloproteases that do not form covalent intermediates (Nerurath, 1989). To date all serine, cysteine, and metallopeptidase always have a histidine residue that is required for catalysis. The histidine residue acts as the proton donor and acceptor in serine and cysteine proteases. The metal ion, which is a zinc ion in all known metalloproteases, functions to polarize the carbonyl bond of the substrate and is coordinated by two histidine residues and usually a glutamic acid (Dunn, 1989). Aspartic acid proteases almost always have a consensus sequence comprised of the motif Asp-Thr-Gly (Davies, 1990).

In this report, we have used site-directed mutagenesis to place leader peptidase into one of these four classes of proteases. Except for cysteines (which were replaced with serines), each of the histidine, aspartic acid, and serine residues was replaced with alanine. This analysis has revealed that cysteine residues are not needed for catalysis, showing that leader peptidase is not a cysteine protease. In addition, since histidine residues are not required for cleavage, it rules out leader peptidase as a classical serine protease. It is also highly unlikely that leader peptidase is a classical aspartic acid protease since it does not contain the motif Asp-Thr-Gly, which is found for all aspartic acid protease (Davies, 1990). The other group of proteases, metalloproteases, has a zinc atom that chelates two histidine residues (Dunn, 1989). Three lines of evidence indicate that leader peptidase is not a standard metalloprotease. First, it does not contain a critical histidine, a result not consistent with a histidine being required to coordinate the metal ion. Second, the enzyme is not inhibited by metal chelating agents (Zwizinski et al., 1981). Third, atomic absorption analysis shows that leader peptidase does not contain a zinc atom.

Although leader peptidase is not a standard serine protease or an aspartic acid protease, it does require several serine and aspartic acid residues for optimal activity. With an in vitro system, we found that serine 90 and serine 185, as well as aspartic acid 99, 153, 273, and 276, were essential for cleaving the M13 procoat protein and was separated from the processed coat protein by using a 23% SDS-polyacrylamide gel. In the bottom right panel, in vitro synthesized procoat (10 μl) was incubated for 30 min at 37°C with 2 μl of 1 mg/ml leader peptidase.

**TABLE II**

| Mutation | Relative enzymatic activity* |
|----------|-------------------------------|
| WT       | ++++                          |
| H124A    | ++                            |
| H235A    | ++                            |
| H323A    | ++++                          |
| C21S, C170S, C176S | ++++                        |
| S65A     | ++++                          |
| S78A     | +                             |
| S90A     | -                             |
| S161A    | ++                            |
| S185A    | +                             |
| S190A    | +                             |
| S197A    | ++                            |
| S206A    | ++                            |
| S218A    | ++++                          |
| S281A    | ++                            |
| S317A    | ++                            |
| S88A, S90A | +                         |
| S171A, S172A | ++                      |
| D25A     | ++                            |
| D99A     | ++                            |
| D112A    | ++                            |
| D129A    | +                             |
| D138A    | ++++                          |
| D158A    | ++++                          |
| D191A    | ++++                          |
| D245A    | ++++                          |
| D273A    | +                             |
| D276A    | +                             |
| D280A    | ++++                          |
| D304A, S302A | +                     |
| D45A, D46A, S44A | ++++                  |
| D138A, D145A | ++                    |
| D232A, S224A | +++                  |
| D280A, S281A | -                     |
| D304A, S302A | +                     |
| D45A, D46A, S44A | ++++                |
| D156A, D158A, S161A | -                |
| D191A, S185, S190A | -                |
| D273A, D276A, S278A | -                |

*Enzymatic activity is defined relative to the activity in a MC1061 extract (without plasmid), which is arbitrarily defined as ~. Each + corresponds to roughly 50-fold higher activity than MC1061. Activity of the leader peptidase mutants in the cell extract was determined by comparing which dilutions (1:1, 1:10, 1:200, and 1:400) were comparable with the undiluted MC1061 extract. For example, 1:200-diluted extracts from pRD8 cells have processing comparable with that of undiluted extracts without plasmid.

*Unstable protein.

**FIG. 2. In vitro leader peptidase processing of the M13 procoat protein.** Cultures expressing the wild-type or mutant leader peptidase proteins were tested for leader peptidase activity by measuring the posttranslational processing of the M13 procoat at various dilutions of the cell extract. For procoat synthesis, a transcription/translation system was used as described under “Experimental Procedures.” Briefly, cell extracts were prepared as described in the “Experimental Procedures.” Undiluted or diluted (1:10, 1:50, 1:200, and 1:400) extracts were incubated for 30 min at 37°C with in vitro synthesized [35S]procoat. [35S]Procoat was separated from the processed coat protein by using a 23% SDS-polyacrylamide gel. In the bottom right panel, in vitro synthesized procoat (10 μl) was incubated for 30 min at 37°C with 2 μl of 1 mg/ml leader peptidase.

| Mutation | Relative enzymatic activity* |
|----------|-------------------------------|
| C21S, C170S, C176S | ++++                        |
| H124A    | ++                            |
| H235A    | ++                            |
| H323A    | ++++                          |
| S90A     | ++                            |

*G. Renkes and R. Dalbey, unpublished data.
Therefore, it is unlikely that this residue is directly involved in catalysis, or that alanine results in a very unstable leader peptidase molecule. These latter serine and aspartic acid residues are not catalytic. However, recent studies strongly suggest that the serine is catalytic. We have been able to generate an acrylamide leader peptidase enzyme by replacing serine 90 with a cysteine. In contrast to the wild-type protein, this thiol leader peptidase is inactive by N-ethylmaleimide, a cysteine-specific reagent. Current effort in this laboratory is centered around isolating a catalytically active leader peptidase comprising the periplasmic domain. The long term objective is to solve the structure by x-ray crystallography to determine which residues are at the active site.

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