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INVESTIGATIONS INTO THE CATALYTIC MECHANISM AND THE STRUCTURE/FUNCTION OF THE *ESCHERICHIA COLI* LEADER PEPTIDASE

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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The Ohio State University

1995

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To My Mother and Father
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LIST OF ABBREVIATIONS

A alanine
Amp ampicillin
APS ammonium persulfate
Bis bisacrylamide
BSA bovine serum albumin
C cysteine
CAPS (3-[cyclohexyl(amoio)]-1-propanesulfonic acid)
DNA deoxyribonucleic acid
dATP 2'-deoxyadenosine 5'-triphosphate
dCTP 2'-deoxycytosine 5'-triphosphate
dGTP 2'-deoxyguanosine 5'-triphosphate
dTTP 2'-deoxycytidine 5'-triphosphate
ddATP 2',3'-dideoxyadenosine 5'-triphosphate
ddCTP 2',3'-dideoxycytosine 5'-triphosphate
ddGTP 2',3'-dideoxyguanosine 5'-triphosphate
ddTTP 2',3'-dideoxythymidine 5'-triphosphate
DEAE diethyl(amoioethyl) cellulose
DTT dithiothreitol
EDTA ethylenediamine tetraacetic acid disodium
H histidine
Hepes (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid)
IPTG isopropyl β-D-thiogalactopyranoside
K lysine
kb kilobase pairs
KD 1000 Daltons
LB luria broth
Lep leader peptidase
MBP maltose binding protein
MWCO molecular weight cut-off
N asparagine
OmpA outer membrane protein A
PEG polyethylene glycol
pfu plaque forming units
PMSF phenylmethylsulfonyl flouride
R arginine
rATP ribose adenosine 5’-triphosphate
RF replicative form
RPM revolutions per minute
RT room temperature
S serine
SB sample buffer
SDS sodium dodecylsulfate
SDS-PAGE sodium dodecylsulfate-polyacrylamide gel electrophoresis
TBE tris-borate EDTA
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TE</td>
<td>tris/EDTA buffer</td>
</tr>
<tr>
<td>Tet</td>
<td>tetracycline</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroactic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>[tris(hydroxymethyl)aminomethane]</td>
</tr>
<tr>
<td>TX-100</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>TX-114</td>
<td>Triton X-114</td>
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<tr>
<td>UTP</td>
<td>uridine 5'-triphosphate</td>
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Chapter I

Introduction

1.1 Protein Secretion

Both prokaryotic and eukaryotic cells are divided into many compartments. Each compartment consists of a membrane and an aqueous space enclosed by a membrane containing a set of proteins which allow each compartment to have its specific function. Eukaryotic cells have up to twenty different compartments, whereas E. coli has only four: the cytoplasm, the inner membrane, the periplasm, and the outer membrane (fig 1.1). Proteins which enter the secretion pathway are found in the inner membrane, outer membrane, the periplasm, or the external medium.

Proteins which are sorted from the cytoplasm are typically synthesized with an amino terminal signal or leader peptide. This signal peptide usually consists of the first fifteen to twenty-five amino acids of the precursor protein. Blobel and Dobberstein (1975a,b) hypothesized that the role of the signal peptide was to initiate the translocation of the precursor protein across the membrane into the lumen of the endoplasmic reticulum (ER) in eukaryotic cells or into the periplasm of prokaryotic cells. Once translocation of the exported protein is completed, the signal peptide is
Fig. 1.1. Destinations for bacterial proteins. For gram-negative bacteria, proteins are destined to the cytoplasm, plasma membrane, periplasm and the outer membrane or the outside medium.
proteolytically removed by signal or leader peptidase. Cleavage releases the mature protein into the periplasm of *E. coli* or the ER lumen in eukaryotes allowing it to continue to its final destination.

There are many other factors that are involved in determining the final destination of secreted proteins in addition to the signal peptide. The secretion of proteins also requires a signal peptide, an energy source, chaperones and a protein secretion machinery, termed the translocase complex (Wickner, 1991). The majority of secreted proteins first interact as they are being synthesized with chaperonin proteins, such as secB or groEL. These proteins are then escorted to the translocase machinery, which consists of secA, secY, secE and Band 1 (secG). SecY, secE and secG are integral proteins, while secA is a peripheral membrane protein. SecA is thought to be the first protein which the chaperone-escorted precursor protein encounters at the membrane surface (Hartl, 1990) and it pushes the precursor protein through the membrane using the hydrolysis of ATP as the energy source (Lill et al., 1989). The role of the secY/E/G complex at this point is unknown. Sec D and F are known to be involved with the late stages of translocation (Arkowitz and Wickner, 1994) and are required for preprotein export when the electrochemical gradient is utilized in translocation. In the later steps, the electrical potential of the cell often plays a role to promote translocation of proteins. This occurs as well when the sec apparatus is not utilized for membrane protein assembly (Wolfe et al., 1985; Kuhn et al., 1986). Figure 1.2 shows the translocation and maturation of pro-ompA (Wickner et al., 1991).
Fig. 1.2. Protein Export in *E. coli* (Wickner et al., 1991). The hatched region corresponds to the leader peptide in pro-OmpA.
1.2 Signal or Leader Peptidase I

A. Physiological Role

In *E. coli*, the majority of proteins which are exported are processed by signal peptidase I (also called leader peptidase). The remaining are processed by signal peptidase II (or lipoprotein signal peptidase). Proteins which are processed by signal peptidase II are first modified by a lipid prior to proteolytic processing (Pugsley and Schwatz, 1985). Wickner and colleagues asked whether processing by leader peptidase is important for proper precursor function. And if it is necessary to remove the signal peptide, why?

These questions were answered using a variety of methods. A genetic method was used to show that leader peptidase is needed for proper cell growth. This was accomplished by constructing an *E. coli* strain in which the arabinose promoter was placed upstream from the chromosomally encoded *lepB* gene, which encodes leader peptidase. In this system, leader peptidase is only expressed in the presence of arabinose. In the absence of arabinose, cell death occurs (Dalbey and Wickner, 1985). Signal peptidase II was also shown to be critical for cell viability by adding globomycin, an inhibitor of this enzyme, to the growth media. Under these conditions, precursor proteins accumulate and cell death occurs (Hussain, et al., 1980; Ichihara, et al., 1982). These experiments showed that both signal peptidases are required for normal cell growth.
Since it was found that processing by leader peptidase is essential for normal cell growth, there must be an important physiological reason for the removal of the signal peptide. It was originally thought that the removal of the signal peptide is required for the protein to gain its enzymatic function, but it has been found that many precursor proteins have activity, though, in some cases, to a lesser degree than the mature protein (Haugen and Heath, 1979; Ito, 1987). Another hypothesis was that processing was required for the translocation of the protein through the membrane. Kuhn and Wickner (1985) found that this was not the case, since exported proteins can be inserted across the membrane, but not processed. Further proof of this came from Dalbey and Wickner (1985) who determined that in the absence of a functional leader peptidase, precursors of the maltose binding protein (preMBP), a periplasmic protein, and the outer membrane protein A (pro-OmpA), an outer membrane protein were translocated across the inner membrane into the periplasmic space, but were anchored to the membrane by the signal peptide. Conversely, using a non-cleavable preMBP mutant, preMBP was found to be anchored to the periplasmic face of the inner membrane, indicating that it had translocated but remained unprocessed (Fikes and Bassford, 1987). From these experiments, it has been determined that the physiological role of leader peptidase is to release the mature domain from the periplasmic surface of the inner membrane by removing the membrane-anchoring signal peptide.
B. Membrane Topology

Signal peptidases have been isolated from the ER membrane, the mitochondrial inner membrane, the chloroplast thylakoid in eukaryotes and the inner membrane of prokaryotic organisms. All of these enzymes are known to be integral membrane proteins. They are embedded in the membrane with distinct domains of the protein found on both sides of the membrane. Integral membrane proteins are divided into three classes, as shown in figure 1.3 (Dalbey, 1990). Bitopic membrane proteins span the membrane only once with either their C-terminus facing the cytoplasmic (type I) or periplasmic (type II) side of the membrane. Proteins which span the membrane twice are termed oligotopic and can have both their N- and C-termini exposed to either the cytoplasmic or periplasmic space. Polytopic proteins span the membrane more than two times.

The membrane topology of leader peptidase in *E. coli* is well established and is somewhat unusual in its membrane orientation with both its N- and C-terminal facing the periplasmic side of the membrane (Moore and Muira, 1987) (fig 1.4). This is a rare orientation for membrane proteins which do not have a signal peptide. Leader peptidase has three hydrophobic domains (H1, H2, and H3) where H1 and H2 make up are the transmembrane segments. There are two polar regions found within leader peptidase; P1, a small cytoplasmic loop which connects H1 and H2 together
Fig. 1.3. Possible transmembrane orientations of membrane proteins.
Fig 1.4. Membrane topology of leader peptidase.
and P2 which contains the bulk of the catalytic, periplasmic domain. Bilgin et al. (1990) have recently determined which regions of leader peptidase may be responsible for the activity, by deleting portions of the leader peptidase and measuring processing activity. They determined that H1 and P1 are not required for catalysis. H2 was also not required if it was replaced with unrelated hydrophobic residues, indicating that the catalytic site is localized within the periplasmic domain (P2).

Comparing the membrane topology of the *E. coli* leader peptidase to other prokaryotic signal peptidases suggests that the analogous P2 domain of the peptidases also reside within the periplasmic space of the cell. All of the known prokaryotic proteins range in size from twenty-one to thirty-seven KDa. Based on hydropathy plots, signal peptidases in prokaryotes either span the membrane once or twice (Dalbey and von Heijne, 1992). The first group which consists of the *E. coli* (Wolfe et al., 1983), *Salmonella typhimurium* (van Dijl et al., 1990) and *Pseudomonas fluorescens* (Black et al., 1992) signal peptidases have two transmembrane segments. The second group has one transmembrane spanning segment and so far consists of the *B. subtilis* signal peptidases. The peptidase from *S. typhimurium* and *P. fluorescens* and *B. subtilis* have homology with the *E. coli* signal peptidase, as well as with the yeast homologue, sec11 encoded subunit of the ER signal peptidase (Dalbey and von Heijne, 1992) and the mitochondrial inner membrane proteases (Schneider et al., 1991; Nunnari et al., 1993). In particular, there is high sequence homology over certain regions of the enzyme (see later this chapter) which do not include H1 and
P1 of the *E. coli* enzyme again suggesting that these regions are not critical for activity, (Bilgin et al, 1990).

Eukaryotic signal peptidases have now been purified from a variety of sources, including canine pancreas (Evans et al, 1986; Shellness and Blobel, 1990), hen oviduct (Baker and Lively, 1987), and yeast (YaDeau et al., 1991). The yeast mitochondrial inner membrane protease 1 and 2, exists as a dimer (Schneider et al, 1991; Nunnari et al, 1993). The sec11 and spc18/spc21 subunits of the yeast and the canine signal peptidase, respectively, show homology to the prokaryotic enzymes. The homologous subunits are believed to have only one transmembrane spanning segment (Dalbey and von Heijne, 1992).

1.3 Type 1 Signal Peptidases: A New Proteinase Clan

Much has been determined about the substrate specificity, topology and the membrane assembly properties of leader peptidase, but the catalytic mechanism of this enzyme has not been elucidated. Leader peptidase does not fall into one of the four classical protease groups that include serine, cysteine, metallo, or aspartic acid proteases. Initial results supporting this were obtained by the use of protease inhibitors against these groups of proteases. It was determined by several groups, that leader peptidase is not affected by protease inhibitors against these protease families (Zwizinski et al., 1981; Kuo et al., 1993; Tschantz and Dalbey, 1994). It was proposed that type 1 signal peptidases belong to a new type of serine proteases
(Dalbey and von Heijne, 1992; Tschantz et al, 1993). This was based on the fact that signal peptidases from bacteria, eukaryotic ER and mitochondria have a strictly conserved serine as well as similar specificities and show no sequence homology to other serine proteases.

A. Substrate Specificity

The signal peptides of secreted and membrane proteins have little sequence homology, regardless of their origin, but they do have certain features in common with each other at the cleavage site that have been maintained throughout evolution. An example of this is that a secreted eukaryotic protein can be expressed in *E. coli* and processed correctly by leader peptidase (Talmadge et al., 1980); the converse situation also holds true; the M13 phage procoat protein that infects *E. coli* is processed by the dog microsomal signal peptidase (Roggenkamp et al., 1985).

All signal peptides contain three common features (von Heijne, 1985) (fig 1.5): 1) a short N-terminal region having a net positive charge, 2) a central hydrophobic region containing ten to fifteen residues, and 3) a short typically five to seven residues long C-terminal region which ends in the cleavage site. The cleavage determinants are small uncharged residues (typically alanine) at positions -1 and -3 with respect to the cleavage site, giving rise to the -1,-3 processing rule. Often times there is a helix breaking proline or glycine residue near the -6 position, though this site is not absolutely required for cleavage (Shen et al, 1991).
Fig. 1.5. A typical signal peptide.
Current models of how signal peptidase cleaves substrate hypothesize that the hydrophobic region of the preprotein signal peptide first inserts across the membrane as an α-helix. The helix breaker at the -6 position breaks the helix and presents the cleavage site to leader peptidase at the membrane surface. How can such a simple signal peptide motif direct the selective and efficient process of secretion? It is still not clear. It has been suggested that the mature region of exported proteins plays some role in export. Also, there may have been some evolutionary constraints on proteins which remain cytosolic to avoid signal peptide-like structures (Kaiser et al., 1987). Nevertheless, investigations of how the signal peptide is recognized by signal peptidases are underway in several laboratories.

B. Sequence Homology

The genes coding for signal peptidases from several organisms have now been cloned and sequenced. Alignment of these sequences showed similarities among both prokaryotic and eukaryotic organisms (Dalbey and von Heijne, 1992; van Dijl, 1992). One example, is that the signal peptidases from S. typhimurium, P. fluorescens, and B. subtilis show extensive homologies of 93%, 50% and 31% respectively to the E. coli sequence. The B. subtilis enzyme shows 28% identity to the mitochondrial inner membrane protease 1 and 2 (Dalbey and von Heijne, 1992; van Dijl et al., 1992, 1993; Nunnari et al., 1993) and has some sequence homology to the ER signal peptidases.
There are three strongly conserved regions found in all signal peptidases shown in figure 1.6. Box one which begins at serine 88 and ends at valine 103 of the *E. coli* leader peptidase contains serine 90 which is the only serine conserved throughout the family and has been found to be critical for catalysis (Sung and Dalbey, 1992; Black et al, 1992; Tschantz et al, 1993). The second box begins at glycine 128 and goes to aspartic acid 153; this region contains a strongly conserved basic pair of residues of lysine 145 and arginine 146 in *E. coli*, though in higher eukaryotes the lysine has been replaced with a histidine residue in the ER signal peptidases. The lysine has been shown to be critical for catalytic activity (Black, 1993; Tschantz et al., 1993). The third box begins at glycine 272 with a strictly conserved glycine, aspartic acid and asparagine motif whose importance is unknown and ends at arginine 282. It may be involved in substrate recognition and binding or be necessary for some important structural feature of the signal peptidase. There are also no conserved histidine or cysteine residues which rule out the possibility that the *E. coli* leader peptidase is a classical serine or cysteine protease. Also, the consensus sequence for aspartic acid proteases (Asp-Thr-Gly; Davies, 1990) is not present within the *E. coli* signal peptidase.

C. The Catalytic Mechanism

Until recently, very little was known about the catalytic mechanism of leader peptidase, since protease inhibitors of the four protease groups had little or no effect on activity (Tschantz and Dalbey, 1994; Zwizinski et al.
### Conserved Regions in Signal Peptidases

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| Ec  | SGSMMPTLLICDFILV | GD-IVVFKYPDPKLDYIKRAVGLPDG | GDNRDNSADS R | *
| St  | SGSMMPTLLICDFILV | GD-IVVFKYPDPKLDYIKRAVGLPDG | GDNRDNSADER | 103
| Pf  | SGSMMPTLDVDFILV  | GD-VNVRFRPSDVNHYIKRVGLPDG | GDNRDNSNDS R | *
| Bs  | GDSMYPTLHNRERFV  | GD-IVVL---NGDHDHVKRIGGLPGD | GDNRRNSMDS R | 88 *
| Bs2 | GSKMDPTLVDSERFV  | GDIILN-GKEKSTHYVRGLPDTG   | GDNIQESMDS R | 128
| Ba  | GESNPTLHDRIFV    | GQIVLKN-GEN--EHVIRGGLPGD  | GDNRRNSMDS R | *
| B1  | GTSKDPDLHDGERFV  | GDIVIID-GDEKNVHYVKRLGLPGD | GDNRRSMDS R | 153
| Imp1| GESMLPTLAFNATYVH | GDCIVAL-K-PTDPHRDCKRVTGMPD | GDNLSLSDLS R | 272
| Imp2| GTSMQPTLNPQETLA  | RDIILLFANPRKVCYKVRKGLFD   | GDNYSIDSNS   | 282
| Sec11| SGSMEAPFRQGDLILFL | GD-VVYE-VEQKIPIVHRVLR♀H | GDNNA-GNDIS | *
| Spc18| SGSMEAPHRGDLILFL  | GE-IVVF-IEGRIPIVHRVLIHEK | GDNNA-VEDDR | 88 *
| Spc21| SGSMEAPHRGDLILFL  | GE-IVVF-VEGADIPIVHRVHKHEK | GDNNE-VEDDR | 128

**Consensus**

sgSK-Pt1-gd-### Qd-ivvfr---------#vKrv#-pgd GDN-----D-R

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**Fig. 1.6. Conserved regions in the signal peptidase family.** The asterisk marks proposed catalytic residues.
1981; Kuo et al., 1993). Sequence alignment and site-directed mutagenesis studies on the *E. coli* leader peptidase are consistent with the possibility that signal peptidases belong to a new type of serine protease which do not require a histidine or aspartic acid as a catalytic component in its mechanism. Sung and Dalbey (1992) changed all of the aspartic acid, histidine, and serine residues to alanine, and cysteine residues to serine individually as an initial screen for possible catalytic residues. Several conclusions were drawn from this study. First, leader peptidase does not require any cysteine residues for activity, ruling out the cysteine protease group. Second, leader peptidase is not a metalloprotease, since it lacks zinc which is a required cofactor in this type of protease. Third, leader peptidase is not a classical serine protease because each of the three histidine residues are dispensable for activity. However, it does require a serine residue at position 90 of the protein sequence.

There are several pieces of evidence which indicate that serine 90 is required for catalytic activity. First, this serine is conserved in all signal peptidases (Dalbey and von Heijne, 1992). Secondly when serine 90 is changed to cysteine, which contains the -SH nucleophile instead of the -OH group on the side chain of serine, the enzyme retains much of the wild-type activity. However, when the cysteine specific reagent N-ethyl maleimide is added to this thiol-enzyme, the protein lost its function, while the wild-type does not (Tschantz et al., 1993). From sequence alignment analysis of the signal peptidase family, it was found that there are several basic residues which are strongly conserved within the family. Site-directed mutagenesis
studies of these basic residues found that lysine 145 is essential for activity (Tschantz et al., 1993). Further evidence that lysine 145 is important for activity is that when lysine 145 is changed into cysteine the inactive protein can be reactivated by reacting it with bromoethyl amine, a reagent which converts the cysteine into a lysine analog (Smith and Hartman, 1988; unpublished data, Paetzel, M., Tschantz, W., and Dalbey, R.). Based on these conclusions, leader peptidase bears a strong resemblance to the LexA class of DNA repressor proteins, which undergo autocatalysis and a lesser resemblance to class A β-lactamases.

LexA is a novel serine protease which utilizes a serine and a lysine residue, making up a catalytic dyad (Lin and Little, 1988) and does not utilize a serine-histidine-aspartic acid triad which is found in most serine proteases (Neurath, 1989). This is quite similar to what is found in the E. coli leader peptidase. It is interesting to note that in eukaryotic signal peptidases a histidine has been recruited to replace the lysine, suggesting it may be similar to classical serine proteases. However this is still controversial. As seen in fig. 1.7, our working model of the catalytic mechanism of leader peptidase shows lysine 145 acting as a general base, similar to lexA (Slilaty et al., 1986; Slilaty and Little, 1987). The lysine abstracts a proton from the hydroxyl side chain of serine 90, allowing nucleophilic attack on the scissile bond of the substrate protein. Deacylation occurs in a similar fashion with the lysine abstracting a proton from a water molecule which then allows the hydroxide ion to attack the acyl-enzyme intermediate. At this point presumably the lysine residue donates its
Fig. 1.7. Proposed mechanism for the catalysis of leader peptidase.
proton to the amino group of the peptide dissociating from the enzyme. It was determined that aspartic acid 153 in leader peptidase was not involved in the deacylation step (Tschantz et al., 1993), as is the case with class A β-lactamases (Strynadka et al., 1992; Adachi et al., 1991). Another interesting finding, is that when using a soluble catalytic domain of leader peptidase, lacking the transmembrane domain, phospholipid or detergent is required for optimal activity (Tschantz et al., 1995). One attractive feature of our current model is that serine 90 is located in a weakly hydrophobic region just C-terminal of the transmembrane domain. This places the serine right near the surface of the membrane, the region where leader peptidase is proposed to perform catalysis. Therefore, we believe phospholipid or detergent is required for optimal activity because it mimicks the normal environment near the membrane surface. It is still not clear whether phospholipid binds near the catalytic serine residue causing a local conformational change, which optimizes the active site for proteolytic attack.
Chapter II

Materials and Methods

2.1 Materials

For the growth of bacteria the 20 amino acids, salts, fructose, arabinose and thymine were purchased from Sigma. Yeast extract, bacto-tryptone, bacto-agar and casamino acids were from Difco Laboratory. IPTG was purchased from Boehringer Mannheim.

Enzymes for DNA manipulation, namely Klenow, T4 kinase, T4 ligase, T7 RNA polymerase, alkaline phosphatase and all restriction enzymes were from Bethesda Research Laboratories (BRL). Sequenase version 2.0 and reagents for sequencing were from United States Biochemicals. All oligonucleotides were synthesized in the Biochemical Instrument Center at The Ohio State University.

For SDS polyacrylamide and agarose electrophoresis, fluorography and staining the following reagents were used. Urea and Tris were from ICN. Agarose, SDS, TEMED and ammonium persulfate were purchased from BRL. Bisacrylamide, acrylamide, bromophenol blue, xylene cyanole ff
and Coomassie Brilliant Blue were purchased from Bio-Rad. Sodium salicylate and glycine were from Sigma. Trans-[\textsuperscript{35}S]-methionine was also purchased from ICN. [\textsuperscript{35}S]-dATP was purchased from New England Nuclear.

Deoxynucleotides (dATP, dCTP, dGTP, dTTP) were purchased from Pharmacia. Dithiothreitol, ampicillin, tetracycline and hen egg white lysozyme were purchased from Sigma. The pT7 vector was from BRL, whereas pET-3d was purchased from Novagen.

The chromatographic supports Sephacryl S-100, S-200, Sephadex G-25, G-50 and G-100, SP high resolution, polybuffer exchanger 94 and polybuffer 74 were from Pharmacia. DEAE cellulose was from Whatman. Nickel affinity resin was purchased from Novagen.

Miscellaneous chemicals were from the following companies. Guanidine HCl, glycerol, CAPS, CaCl\textsubscript{2}, TX-100, TX-114, PMSF, KCl, NaEDTA, \(\beta\)-mercaptoethanol, triethanolamine and sodium citrate were from Sigma. \(\beta\)-octyl glucoside and fixed Pansorbin Staph A cells (\textit{S. aureus}) were from Calbiochem. Acetonitrile was purchased from Fisher and sucrose form ICN.
2.2 Bacterial Strains and Plasmids

*E. coli* strains MC1061 [ ΔlacX74, araD139,(ara-leu)7697, galU, galK, hsr, hsm, StrA], HJM114 [ Δ(lac-pro)F’(lac-pro)], JM101 [ Δ(lac-pro) thi/F’, strA, supE, endA, sbcB, hsdR, traD36, proAB, lacIqz, M15], BL21 (DE3) [ F-, ompT, rmb], RZ1032 [ ung-, dut-] and SB221 [ lpp, hsdR, ΔtrpE5, leuB6, lacY, rec1/F’, lacIq, lac+, pro+] were from our collection. IT41, which has a gene encoding a temperature-sensitive leader peptidase, was described by Inada et al. (1989).

The pING plasmid (Johnston et al., 1985) obtained from Dr. Gary Wilcox (Ingene, Inc.), contains part of the arabinose operon that was used for the expression of leader peptidase and its mutants. The pET-3d vector is under control of the T7 promoter as described by Studier et al. (1990), was used for the expression of the Δ2-75 leader peptidase. The pONF1 vector which was used for the expression of the pro-OmpA nuclease A and is IPTG inducible was described by Takahara et al. (1985) and Chatterjee et al. (1995).

2.3 Oligonucleotide-Directed Mutagenesis

A. Preparation of the oligonucleotide

We used oligonucleotide-directed mutagenesis (Zoller and Smith, 1983) to introduce specific mutations in leader peptidase. The
oligonucleotide was synthesized on an Applied Biosystems 320B DNA synthesis instrument. After synthesis, the oligonucleotide was deblocked of the protecting agents by incubating overnight at 55°C. Oligonucleotides were dried in a speed vacuum drier and resuspended in 0.5 ml water. The sample was then loaded onto a 5 ml G-25 sephadex column equilibrated with 50 mM triethanolamine bicarbonate to desalt the oligonucleotide. Fractions were collected and the concentration of DNA was determined by measuring the absorbance at 260 nm (40 µg/ml = 1.0).

B. Preparation of Single-Strand DNA Template

We used a method which incorporates uracil into the template DNA, increasing the efficiency of mutagenesis (Kunkel, 1985). For the preparation of the template DNA, 15 µl of phage (10¹¹ pfu) and 5 ml RZ1032 cells of optical density of 0.2 at 600 nm was added to 1 L LB media containing 0.25 µg/ml uridine and 12.5 µg/ml tetracycline. The cell culture was incubated overnight at 37°C with vigorous shaking. After incubation the cells in 500 ml bottles were pelleted (6000 RPM, 4°C, 10 min) in a JA10 rotor. 250 ml of 20% PEG-2.5 M NaCl was added to the supernatant containing the phage and then incubated 1 hr on ice. The phage precipitate was pelleted by centrifugation (9000 RPM, 4°C, 10 min) in a JA10 rotor. The pellet was resuspended in 15 ml LB media and 5 ml of the 20% PEG-2.5 NaCl was added and incubated on ice for 30 min to reprecipitate the phage. It was then centrifuged (14000 RPM, 4°C, 10 min) in a JA20 rotor. The pellet was resuspended in 6.5 ml TE buffer and 4.5 ml CsCl (n=1.40) was added.
phage solution was then transferred to a Beckman quick seal tube, topped off with mineral oil and balanced. The sample was then centrifuged (40000 RPM, 18°C, 20 hr) in a Ti50 rotor to generate a CsCl gradient to purify the phage. The upper band containing the phage was removed with a syringe and carefully placed into a dialysis tube (12 000 MWCO) and dialyzed against 4 L water for 4 hr. After dialysis 0.7 ml aliquot was added to an equal volume of tris-saturated phenol and vortexed for 10 sec followed by centrifugation in a microfuge for 1 min. The top aqueous phase was extracted two times with diethyl ether to remove traces of phenol. Then 70 μl of a sodium acetate solution (3M sodium 5 M acetate) and 1.5 ml absolute ethanol was added and incubated at -70°C for 20 min. The sample was then microfuged for 10 min and 4°C, the pellet washed with 80% ethanol, and the centrifugation repeated. The pellet was then dried and resuspended in 100 μl TE buffer (10 mM Tris, pH 8.0, 10 mM EDTA). The concentration of the template was measured at 260 nm and sample diluted to give a final concentration 1mg/ml (OD = 20).

C. Oligonucleotide-Directed Mutagenesis: Annealing, Elongation and Ligation

The oligonucleotide was phosphorylated at its 5' end at 37°C for 30 min in the presence of 50 mM Tris, 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine and 0.1 mM EDTA pH 7.5 with T4 kinase and 1mM rATP. The annealing reaction mixture contained 5 μl phosphorylated oligonucleotide (40 mg/ml), 5 μl UTP-incorporated template (1mg/ml) and 1 μl annealing
buffer (0.2 M Tris pH 7.5, 0.1 M MgCl$_2$, 0.5 M NaCl, 0.01 M DTT). This mixture was heated at 65°C for 7 min and allowed to cool slowly to room temperature, so that the oligonucleotide could anneal to the template. To the annealed template, 1 µl each of a solutions containing 10 mM dATP, dCTP, dGTP, dTTP was added to 2 µl of each T4 ligase (2 units) and Klenow fragment of DNA polymerase (7 units), and 2 µl ligation buffer (700 mM Tris pH 7.5, 70 mM MgCl$_2$, 7 mM rATP, 100 mM DTT) and incubated overnight at 14°C.

D. Transfection

To introduce the elongated and ligated DNA into the host *E. coli* cells (JM101), it is first necessary to make the cells competent for transformation by the CaCl$_2$ method (Cohen et al., 1973). 0.8 ml of overnight JM101 cells were back diluted into 80 ml of LB media and incubated at 37°C with vigorous shaking. When the cells reached $A_{600 \text{ nm}} = 0.5$, the cells were centrifuged (6000 RPM, 4°C, 5 min) and the pellet was resuspended in 15 ml ice-cold 50 mM CaCl$_2$. The cells were then incubated on ice for 15 min. After incubation the cells were centrifuged again and resuspended in 6 ml CaCl$_2$ and incubated once again on ice for 15 min. To transflect the cells, 10 µl of the elongated-ligated mutagenized template was added to 300 µl of competent cells and incubated on ice for 5 min. This mixture was then heat shocked at 37°C for 2.5 min and then 0.5 ml LB media was added. To this mixture, 0.8 ml overnight JM101 cells were added and 3 ml top agar (47°C) were added and then the whole mixture was poured onto a prewarmed LB
plate and incubated for 5 hr at 37°C. Single plaques were picked and grown in 2 ml LB media overnight and the phage single-stranded DNA was isolated and screened for mutations by dideoxynucleotide DNA sequencing (Sanger et al., 1977).

2.4 DNA Sequencing

A. Preparation of Single-Stranded DNA

After plaques had formed on a lawn of cells following transfection, single plaques were picked and grown overnight in 2 ml of LB media at 37°C. The next morning, an eppendorf tube was filled to the top with the cell culture and microfuged (12,000xg) for 1 min to pellet the cells. 1 ml of the supernatant was transferred to a fresh tube, while the remaining supernatant was saved as a phage stock. To the 1 ml of phage, 250 μl of 20% PEG-2.5 M NaCl was added. This was vortexed and incubated on ice for 30 min to precipitate the phage. The sample was then microfuged (5 min, RT, 12,000xg) and the supernatant aspirated away. The tube was microfuged again for a few seconds and all traces of the PEG was removed. The phage pellet was resuspended in 100 μl TE buffer. This sample was then treated with 50 μl Tris-saturated phenol by vortexing for 10 sec and then microfuging for 2 min. The top aqueous phase was transferred to a new tube and washed two times with diethyl ether. The single-stranded DNA was concentrated by precipitation with 10 μl 3 M sodium acetate pH 5.5 and 300 μl ethanol, followed by incubation at -70°C for 20 min. The
samples were then microfuged (10 min, 4°C, RT) to pellet the DNA and then washed to remove the salt with ice-cold 80% ethanol followed by microfugation. The supernatant was removed and the DNA dried and then resuspended in 20 µl TE buffer.

B. Dideoxy Sequencing

The dideoxy method of sequencing (Sanger et al, 1977) and the DNA Sequenase 2.0 kit were used to sequence the single-strand DNA. To do this 7 µl template DNA, 1 µl oligonucleotide (A260 = 0.05) and 2 µl annealing buffer (0.2 M Tris pH 7.5, 0.1 M MgCl2, 0.5 M NaCl, 0.01 DTT) were mixed and then incubated at 65 °C for 2 min followed by slow cooling to room temperature. To this annealed mixture, 1 µl 100 mM DTT, 0.5 µl α-[35S]-dATP, 2 µl labeling mix (1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP) and 2 µl Sequenase were added, and this was incubated for 5 min at room temperature. Then 3.5 µl of this labeling mixture was transferred into four separate tubes which contained 2.5 µl ddATP, ddCTP, ddGTP or ddTTP (each 80 µM dNTP, 8 µM respective ddNTP), respectively, and then incubated for another 5 min at 37°C. The reaction was stopped by the addition of 4 µl stop buffer (95% dimethylformamide, 20 mM EDTA, 0.5% bromophenol blue, 0.5% Xylene cyanol ff). The samples were then heated at 95°C for 2 min and transferred to an ice bath and then the samples were loaded onto a sequencing gel.
C. Preparation of Polyacrylamide Gel for DNA Sequencing

DNA polyacrylamide gel electrophoresis was used to separate the DNA fragments. To prepare an 8% gel, 36.04 g urea was dissolved in 15 ml 5X TBE buffer (400 mM Tris, 400 mM boric acid, 10 mM EDTA) and 15 ml of 40:1.33% acrylamide/bisacrylamide solution. Water was added up to a volume of 75 ml. The solution was then filtered through a 5µm filter (Gelman Sciences Acrodisc) and degassed. To this mixture, 200 µl 10% APS and 30 µl TEMED were added, and this was immediately poured between glass plates and allowed to polymerize. After polymerization, the gel was prewarmed for 30 min at 1900 volts and the samples were then loaded onto the gel. After electrophoresis, the gel was dried and exposed to X-ray film overnight.

2.5 Subcloning

A. Preparation of Replicative Form DNA

Once the mutant had been identified by sequencing, the replicative form of the mutant M13 phage DNA was prepared. A large stock of mutant phage was first prepared by infecting 100 µl of JM101 cells (saturated culture) with 50 µl phage (10^{11} pfu) in a 35 ml LB medium. The following morning 20 ml JM101 overnight culture was back diluted into 1 L LB media and grown to A_{600} = 0.5. At this point, 25 ml of phage (10^{11} pfu) was added and allowed to infect the cells for 2 hr. Cells were harvested by
centrifugation (6000 RPM, 4°C, 10 min) in a JA10 rotor. The pellet was resuspended in 8 ml GTE buffer (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA) and incubation continued for 30 min at room temperature for cell lysis to occur. After this step 16 ml of 0.2 M NaOH/1% SDS was added and mixed by vortexing followed by a 10 min incubation on ice. Then 12 ml of 3 M potassium 5 M acetate pH 4.8 was added and incubated for 20 min on ice. The cell debris was pelleted by centrifugation (12000 RPM, 4°C, 15 min) in a JA20 rotor. The supernatant was filtered through course medical gauze and 0.6 volumes of isopropanol was added, followed by vigorous mixing. Samples were incubated at room temperature for 15 min to precipitate the DNA. The DNA was collected by centrifugation (10000 RPM, 20 min, 4°C) in a JA20 rotor. The pellet was dissolved in 2.4 ml TE buffer. Followed by the addition of 4.2 g CsCl, 5 ml CsCl solution (30 g/40 ml TE buffer) and 0.4 ml ethidium bromide (10 mg/ml) followed by vigorous mixing. The solution was placed in 12 ml ultracentrifuge tubes, balanced and centrifuged (45000 RPM, 18°C, 24 hr) in a Ti50 rotor for the purifying the RF form of the DNA. Purified DNA bands were detected by long wave UV light. The lower band, corresponding to supercoiled RF DNA was collected. The ethidium bromide was removed by extracting three times with 3 ml Tris-saturated isobutanol. The DNA was precipitated by adding 15 ml TE buffer, 20 ml isopropanol and 2 ml of 3 M Na Acetate and incubated for 20 min at -70°C. The precipitated DNA was collected by centrifugation (19000 RPM, 45 min, 4°C). The pellet was resuspended in 0.5 ml TE buffer and reprecipitated after the addition of 50 µl 3 M Na Acetate, and 1 ml ethanol and incubated at -70°C for 20 min. The DNA was collected by centrifugation in a microfuge (10 min, 4°C). The
pellet was washed with 80% ethanol to remove the salt and dried in a speed vacuum condenser. The RF DNA was resuspended in 100 μl TE buffer.

B. Restriction Digest and Isolation of Restriction Fragments

Restriction digests of M13 RF or plasmid DNA were performed by mixing 25 μl DNA, 20 μl restriction buffer (BRL optimized buffers), 3 μl each restriction enzyme and water to bring the volume up to 200 μl followed by digestion at 37°C for 4 hours and terminated by the addition of 20 μl agarose loading dye (0.25% bromophenol blue, 0.25% xylene cyanol ff, 15% ficoll type 400 in 1xTBE buffer). After digestion, the DNA was loaded onto a 0.7% agarose gel and electrophoresed until the first dye reached the end of the gel. The restriction fragments were visualized by using low wave ultraviolet light. The gel was cut with a razor blade just in front and in back of the fragment of interest and water-wetted pieces of DEAE paper were placed in the area generated by the cuts. Then the DNA was electrophoresed onto the DEAE paper. The DNA was first washed in low salt buffer (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris pH 8.0) followed by incubation at 65°C for 1 hr in 500 μl high salt buffer (1.0 M LiCl, 0.1 mM EDTA, 20 mM Tris pH 8.0) with vigorous vortexing every 15 min to elute the protein from the paper. The DEAE filter was then discarded and the aqueous solution containing the DNA was phenol-extracted two times and washed with ether 2 times to remove traces of phenol. The DNA was then precipitated by the addition of 1 ml of ice-cold ethanol and incubated for 20 min at -70°C. The DNA was then pelleted by centrifugation for 10 min at
4°C. The pelleted DNA was dried and resuspended in 55 μl TE buffer. This DNA sample was desalted by using a spin column, constructed by punching a small hole with a needle in a 0.5 ml eppendorf tubes and filling them with G-25 medium rehydrated in TE buffer. To desalt the DNA, the small eppendorf tubes were then placed inside a 1.7 ml eppendorf tubes and spun dry, followed by the addition of the DNA sample and microcentrifuging until the sample was collected in the bottom of the tube. The eluate was analyzed on a 0.7% agarose gel for the determination of the yield.

C. Subcloning the Insert DNA into the Vector

After the purification of the insert and vector DNA, the mutant insert was ligated into the expression vector (pING or pET-3d) (Fig 2-1). For the ligation reaction, typically 2 μl restricted vector, 5 μl insert DNA, 2 μl 5X ligation buffer, and 1 μl T4 DNA ligase were mixed and incubated overnight at 14°C.

2.6 Transformation

Competent cells (MC1061 for pING and BL21(DE3) for pET-3d) were made as described in section 2.3.D. 300 μl were mixed with 10 μl ligation reaction or 1 μl of pure plasmid DNA (positive control) and heat-shocked at 37°C for 2.5 min. After the incubation, 0.5 ml LB media was added to the cells and the culture was incubated by shaking at 37°C for 40 min. The
Fig. 2.1. Subcloning the leader peptidase gene (LepB) from M13mp8 into the pING vector. After digestion with Sal I and Sma I, the 1.2 kb fragment containing the LepB gene was isolated from an agarose gel and ligated into the pING vector with T4 DNA ligase.
cells were then pelleted and resuspended in 100 μl and spread on LB/amp plates and incubated overnight at 37°C.

2.7 Minilysate DNA Preparation

To prepare plasmid DNA on a small scale we used an alkaline lysis method. 1.5 ml of overnight grown cells were pelleted to the bottom of a microcentrifuge tube and then resuspended in 100 μl GTE buffer (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA) and incubated at room temperature for 5 min. Then the cells were lysed by the addition of 200 μl of freshly-made 0.2 M NaOH/1% SDS and incubated on ice for 5 min, followed by the addition of 150 μl 5 M potassium acetate pH 4.8 and incubated once again for 5 min. After incubation, the cell debris was pelleted by centrifugation for 1 min. The supernatant containing the DNA was transferred to a new tube and precipitated by the addition of 1 ml ethanol followed by incubation at -70°C for 20 min. The DNA was pelleted by microcentrifugation at 4°C for 10 min. The pellet was then washed with 80% ethanol and the centrifugation was repeated followed by drying of the sample. The DNA was then resuspended in 20 μl TE buffer.
2.8 Screening for Vector Inserts

A. Screening by Immunoprecipitation for Inserts into pING

To determine whether the insert DNA was subcloned into the pING vector, candidate colonies were pulse-labeled and immunoprecipitated. Cells were grown to saturation in 1 ml M9 media containing the 19 amino acids (-met) (Maniatis et al., 1984). The next morning the cells were backdiluted into 1 ml fresh M9 + 19 amino acids media and grown for 3 hours, at which point the cells were induced with 0.2% arabinose (final conc.) and grown for an additional 20 min. The cells were then pulsed-labeled for 1 min with 10 μCi [35S]-methionine. Then 0.5 ml of the labeled cells was removed and transferred into 0.5 ml prechilled 20% trichloroacetic acid and vortexed followed by incubation on ice for 30 min. After this step the samples were microfuged for 5 min at 4°C followed by aspiration of the supernatant, washed with 1 ml ice-cold acetone, and once again microcentrifugation and aspiration were performed. The tubes were then heated at 95°C for 2 min to remove any remaining residual acetone. Then 100 μl 10 mM Tris pH 8.0/2% SDS was added and the tubes were incubated at 95°C for 5 min. These samples were then vortexed and incubated again for 5 min. At this point the samples were microfuged for 5 min at room temperature to remove any remaining cell debris. The supernatant was placed in a new tube and 1 ml immunoprecipitation buffer (10 mM Tris pH 8.0, 5 mM EDTA, 150 mM NaCl, 2.5% TX100) was added to dilute out the SDS and facilitate the immunoprecipitation with antibodies. First, 25 μl of
fixed staph A cells were added to presorb miscellaneous proteins that non-
specifically bind to Staph A and were incubated for 15 min on ice. The
presorbed samples were then microfuged to pellet the staph A cells. The
supernatant was then transferred to a new tube and 7 μl leader peptidase
polycolonial antibody was added. The sample was incubated for 30 min on
ice, after which 25 μl fresh staph A was added to bind the leader peptidase-
antibody complex and incubation was continued for 30 min on ice. The
staph A was then pelleted to the bottom of the tube by microcentrifugation.
The pellet was then washed two times with the immunoprecipitation buffer
and then finally resuspended in 25 μl 2x sample buffer (4% SDS, 160 mM
Tris, pH 6.8, 20% glycerol, 1.5% β-mercaptoethanol, 0.02% bromophenol
blue). The samples were then incubated for 5 min at 95°C to dissociate the
proteins from staph A, then microfuged for 1 min. The supernatant was then
loaded onto a 12% SDS-PAGE gel and electrophoresed. After
electrophoresis, the gel was fixed in 40% methanol/10% acetic acid solution
for 30 min and then incubated in a solution of sodium salicylate (0.9 M) for 5
min. The gel was then dried and exposed to x-ray film overnight. The film
was then developed and the mutant leader peptidase expressing
candidates were determined (fig 2.2).

B. Screening for Δ2-75 inserts into pET-3d

To determine or not the Δ2-75 insert DNA was subcloned into the
pET-3d vector, we induced the proteins from candidate colonies and
assayed the expression of the protein. Candidate colonies were picked
Fig. 2.2. Screening for inserts after ligation into the pING vector by immunoprecipitation. The higher molecular weight bands in lanes 2, 4, 6, 8, 9, and 10 represent insertion of the mutant LepB gene into the pING vector. The Δ4-50 corresponds to a marker protein.
from the LB plate and the cells transferred into 1 ml LB media plus amp and
grown to saturation overnight. Cultures were then back-diluted into 1 ml of
fresh media and grown for 2 hours at 37°C with vigorous shaking. At this
point 200 μl cells were removed and spun down in a microfuge followed by
resuspension in 40 μl 2x sample buffer. These cells represent the
uninduced samples. Cells were then induced with 0.5 mM IPTG and grown
for another 2 hours. Then another 200 μl cells was removed, pelleted and
resuspended as above. These are the induced cells. Then a 25 μl aliquot
of the uninduced and induced cells were loaded onto a 12% SDS-PAGE gel
and electrophoresed. After separating the proteins by electrophoresis, the
gel was stained with 0.25% Coomassie Blue for 15 min and destained with
40% methanol/10% acetic acid solution until protein bands were visualized,
this SDS-PAGE method allowed the Δ2-75 expressing candidates to be
identified (fig 2.3).

2.9 Immunoblotting

Immunoblotting was performed according to the method of Towbin et
al. (1979) to examine the stability of a mutant leader peptidase. Cell lysates,
overproducing leader peptidase were loaded onto a 12% SDS-PAGE gel
and electrophoresed. After electrophoresis the proteins on the gel were
transferred to nitrocellulose paper in a Bio-Rad western blot sandwich
apparatus by performing electrophoresis for 2 hrs at 250 mA using transfer
buffer (20 mM Tris, 150 mM glycine, 20% methanol). The nitrocellulose
Fig. 2.3. Screening for inserts into the pET-3d vector by Coomassie Blue staining. The lower molecular weight band corresponds to the Δ2-75 leader peptidase protein, which are derived from candidates where the Δ2-75 gene has been inserted. The Δ4-50 corresponds to a marker protein.
paper was removed and treated with 1% BSA in TBS buffer (0.15 M NaCl, 0.1 M Tris pH 7.5) plus 0.1% Tween 20 for 1 hr with agitation to block the nitrocellulose. The blot was then transferred to a BSA/TBS solution containing 5 μl leader peptidase antiserum and incubated for 1 hr at room temperature with vigorous shaking. The blot was rinsed and incubated in the BSA/TBS/Tween 20 solution once again for 1 hr at room temperature with agitation. The blot was rinsed again and transferred to a BSA/TBS solution containing 5 μl anti-rabbit IgG conjugated horseradish peroxidase and incubated for 1 hr at room temperature with agitation. The blot was then washed and transferred to a BSA/TBS/Tween 20 solution for the final blocking step and shaken for 1 hr at room temperature. Development was performed by adding 10 mg of 4-chloro-1-naphthol to 4 ml methanol, 16 ml TBS buffer and 70 μl H2O2 and incubating the blot with shaking in this solution until bands were visualized. The development was stopped by rinsing in water and drying.

2.10 SDS-PAGE, Fluorography and Coomassie Blue Staining

A variety of different SDS-PAGE percentage gels were utilized. 12% gels were used for the analysis of leader peptidase proteins, while 17.2% gels were utilized for resolving of pro-OmpA nuclease A proteins and 23% gels for separating the procoat proteins (Ito, 1980). Radiolabelled proteins were visualized by fluorography (Chamberlin, 1979). Just before samples were loaded onto the gel, they were heated at 95°C for 5 min in sample buffer (10 mM Tris pH 8.0, 1 mM EDTA, 2% SDS, 5 mM β-mercaptoethanol
Table 2.1 Preparation of gels for SDS-PAGE

<table>
<thead>
<tr>
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<th>12%</th>
<th>17%</th>
<th>23%</th>
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<tr>
<td><strong>Separating Gel</strong></td>
<td></td>
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<tr>
<td>29:1 Acrylamide:Bis</td>
<td>8 ml</td>
<td>11.5 ml</td>
<td>0 ml</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>5 ml</td>
<td>5 ml</td>
<td>0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Water</td>
<td>6.7 ml</td>
<td>3.2 ml</td>
<td>0 ml</td>
</tr>
<tr>
<td>2 M Tris pH 8.8</td>
<td>0 ml</td>
<td>0 ml</td>
<td>4.0 ml</td>
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<tr>
<td>60% Acrylamide</td>
<td>0 ml</td>
<td>0 ml</td>
<td>7.65 ml</td>
</tr>
<tr>
<td>2% Bis</td>
<td>0 ml</td>
<td>0 ml</td>
<td>0.88 ml</td>
</tr>
<tr>
<td>Urea</td>
<td>0 g</td>
<td>0 g</td>
<td>7.2 g</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>0 ml</td>
<td>0 ml</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>Temed</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.055 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.01 ml</td>
<td>0.01 ml</td>
<td>0.0055 ml</td>
</tr>
</tbody>
</table>

|                |              |              |
| **Stacking Gel (5%)** |            |              |
| 29:1 Acrylamide:Bis | 1.3 ml     |              |
| Water           | 6.1 ml      |              |
| 0.5 M Tris pH 6.8 | 2.5 ml      |              |
| Temed           | 0.1 ml      |              |
| 10% APS         | 0.01 ml     |              |
and 0.01% bromophenol blue) to denature the proteins. Gels were prepared as shown in Table 2.1. Electrophoresis was performed using Tris-glycine running buffer (0.19 M glycine, 0.024 M Tris pH 7.5, 0.0034 M SDS). The gel was either fixed or stained with Coomassie Blue in 40% methanol/10% acetic acid solution for 15 min. For fluorography, the gel was salicylated (0.9 M salicylic acid, 0.9 M NaOH) for 10 min. The gel was dried and exposed to X-ray film or the Coomassie Brilliant Blue stained gel photographed.

2.11 Agarose Gel Electrophoresis

Electrophoresis was performed by dissolving 0.7 g agarose in 100 ml TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) by heating for 5 min in a microwave oven. The agarose was allowed to cool to about 60°C and then poured into a casting tray. After pouring, 3 μl ethidium bromide (10 mg/ml) was added to the gel and the gel was allowed to solidify. The gel was run at 200 mA and the DNA bands were visualized by UV light and then photographed.

2.12 Preparation of Cell Lysates for the Procoat Processing Assay

*E. coli* MC1061 cells harboring the plNG vector with wild-type or mutant leader peptidase were grown in 2 ml M9 media supplemented with the 20 amino acids and 0.5% fructose until A_{600} = 0.5. Then the cells were induced with 0.2% arabinose for 1 hr. The cells were collected by
centrifugation, and the pellet was resuspended in 300 μl of lysis buffer (10 mM EDTA, 10 mM Tris pH 8.0, 20% sucrose, 1% TX-100, 1 mg/ml lysozyme, 5 μg/ml DNase, 1 μg/ml RNase). This was then incubated for 30 min at room temperature and either used directly or diluted with buffer (50 mM Tris pH 8.0, 0.1% TX-100).

2.13 Processing of Procoat

The enzymatic activity of the mutant leader peptidase proteins was determined by the amount of post-translational processing of the substrate procoat. In this assay procoat was synthesized in vitro using an E. coli extract containing the pT712 vector with the procoat gene. The components of the procoat synthesis system are described in Table 2.2 with some modification of the procedure described by Yamane et al. (1987). The mixture containing each of the components was incubated for 30 min at 37°C. The reaction was stopped by the addition of 300 μl of 50 mM Tris pH 8.0/1% TX-100. Leader peptidase activity was measured by adding 10 μl of [35S]-labeled procoat with 2 μl leader peptidase cell extract or dilutions of 1/10, 1/50, 1/200 or 1/400 and incubated at 37°C for 30 min. The samples were then analyzed on a 23% gel, fixed and salicylated and exposed overnight to X-ray film.

2.14 In Vivo Processing of pro-OmpA

Leader peptidase mutant proteins were also tested for activity in a more sensitive in vivo assay. This assay utilizes the temperature-sensitive
Table 2.2 *In vitro* Synthesis of Procoat

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 % Triton X-100</td>
<td>12 µl</td>
</tr>
<tr>
<td>Mix ABC$^a$</td>
<td>9 µl</td>
</tr>
<tr>
<td>$^{35}$S-Methionine</td>
<td>3 µl</td>
</tr>
<tr>
<td>S150-2 (Yamane et al, 1987)</td>
<td>2 µl</td>
</tr>
<tr>
<td>T7 RNA polymerase</td>
<td>1 µl</td>
</tr>
<tr>
<td>pT7 vector with procoat insert</td>
<td>3 µl</td>
</tr>
</tbody>
</table>

$^a$ Mix ABC is composed of 3 ml of each mix A (ratio 41.25:12.5 of mix A1 and mix A2), mix B and mix C (0.07 M Mg(OAc)$_2$:0.074 M Ca(OAc)$_2$ = 1:1).

<table>
<thead>
<tr>
<th>Mix A1</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 M Tris Acetate pH 8.2</td>
<td>8.80 ml</td>
</tr>
<tr>
<td>2.75 M KOAc</td>
<td>4.00 ml</td>
</tr>
<tr>
<td>2.0 M NH$_4$OAc</td>
<td>2.60 ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mix A2</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM amino acids I</td>
<td>2.20 ml</td>
</tr>
<tr>
<td>20 mM amino acids II</td>
<td>2.20 ml</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>0.28 ml</td>
</tr>
</tbody>
</table>

*Amino Acids I* contains all amino acids but met, asn, gln and cys

*Amino Acids II* contains asn, gln and cys

<table>
<thead>
<tr>
<th>Mix B</th>
<th>Components</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_3$PEP</td>
<td></td>
<td>214 mg</td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td>48.5 mg</td>
</tr>
<tr>
<td>CTP</td>
<td></td>
<td>10.6 mg</td>
</tr>
<tr>
<td>GTP</td>
<td></td>
<td>11.5 mg</td>
</tr>
<tr>
<td>UTP</td>
<td></td>
<td>10.7 mg</td>
</tr>
<tr>
<td>Ca-leucovorin</td>
<td></td>
<td>1.1 mg</td>
</tr>
<tr>
<td>TPN</td>
<td></td>
<td>1.0 mg</td>
</tr>
<tr>
<td>tRNA</td>
<td></td>
<td>4.0 mg</td>
</tr>
</tbody>
</table>

Add H$_2$O to a final volume of 4 ml.
leader peptidase strain IT41 (Inada et al, 1989). In this assay the processing of the chromosomally-encoded pro-OmpA protein is analyzed. A 2 ml culture of IT41 which had been transformed with a pING vector expressing leader peptidase was grown to the mid-log phase in M9 media containing all amino acids (50 mg/ml) except methionine and fructose at 32°C. After reaching the mid-log phase, the culture was shifted to 42°C for 1 hr to inactivate the temperature-sensitive chromosomally encoded leader peptidase. Arabinose was then added to 0.2% to induce the pING-encoded leader peptidase for 30 min. The cells were labeled with 250 μCi [35S]-methionine for 15 sec followed by a chase of 500 μg/ml cold methionine. At indicated times, 100 μl aliquots were removed and transferred into 100 μl of 20% ice-cold trichloroacetic acid. Samples were immunoprecipitated with OmpA antiserum. The immunoprecipitates were run on a 12% SDS-PAGE gel, fixed and subjected to fluorography.

2.15 Purification of Leader Peptidase

The purification of leader peptidase utilizes membrane isolation, TX-100 extraction, DEAE chromatography and chromatofocusing. The method used is based on that of Wolfe et al. (1983b). We used the strain MC1061 harboring the plasmid pRD8 which encodes for the wild-type leader peptidase or that of a mutant leader peptidase. Ten liters of MC1061 cells transformed with the pRD8 plasmid were grown at 37°C in M9 media containing 0.5% fructose, 0.2% casamino acids and 100 μg/ml amp. When A600 = 0.5, arabinose was added to a final concentration of 0.2% to induce
the production of leader peptidase and grown for an additional 4 hrs. Cells were then harvested by centrifugation (6000 RPM, 10 min, 4°C) in a JA10 rotor, and resuspended in an equal volume by weight of 50 mM Tris, pH 7.5/10% sucrose. Finally the cells were frozen by adding them slowly to liquid nitrogen and then stored at -80°C until used.

110 g of frozen cell nuggets were added to 250 ml of room temperature buffer (10 mM Tris pH 8.5, 20% sucrose, 5 mM EDTA) and thawed. After thawing, lysozyme and DNase were added (0.15 mg/ml, 0.015 mg/ml, final concentration) to the mixture. The cells were further lysed by freezing the sample in a dry ice/ethanol bath followed by thawing. Magnesium acetate (5 mM final concentration) was added after thawing and the mixture was stirred for 10 min at room temperature. The membrane fraction was collected by centrifugation. The pellet was then resuspended in 25 ml/tube of 10 mM TEA, pH 7.5, 10% glycerol solution and the centrifugation step was repeated. The pellet was once again resuspended 5 times in 10 mM TEA, pH 7.5, 10% glycerol and 1% TX-100 by using a large dounce homogenizer followed by douncing 5 times with a small homogenizer. Centrifugation was repeated as above. The supernatant was loaded onto a DEAE cellulose column (300 ml) being careful not to load the loose opaque white pellet. The column was equilibrated with 10 mM TEA, pH 7.5, 10% glycerol and 1% TX-100. 100 fractions (20 ml each) were collected. Fractions were assayed for leader peptidase by running every fifth fraction on a 12% SDS-PAGE gel and staining by Coomassie Brilliant Blue. Fractions containing leader peptidase were pooled and dialyzed.
overnight in 25 mM imidazole and 1% TX-100 pH 7.4 (9 liters) and dialyzed the next morning in fresh buffer for 4 hours. The dialysate was then loaded by pumping it onto a 5 ml PBE 94 column equilibrated with 25 mM imidazole, pH 7.4 with 1% TX-100. The column was then developed with 100 ml Polybuffer 74 (1:10 dilution) with 1% β-octylglucoside. 2 ml fractions were collected and fractions were assayed by running an aliquot on a 12% SDS-PAGE gel. Fractions containing leader peptidase were pooled and stored at -70°C.

2.16 Circular Dichroism of Leader Peptidase

Circular dichroism was used to determine whether a mutant leader peptidase was in a conformation similar to that of the wild-type protein. The protein concentrations used were 1.7 mg/ml leader peptidase in a 10% polybuffer 74 and 1% β-octylglucoside solution. The concentration of the leader peptidase was determined by using an extinction coefficient of 44 000 cm⁻¹ M⁻¹ at 280 nm. Measurements were made on a Jasco J-500C spectropolorimeter instrument using a 0.5 mm path length jacketed cell. The temperature was held constant at 9°C.

2.17 Leader Peptidase Competition Studies

A more sensitive assay to determine whether an inactive mutant leader peptidase was in a similar conformation as the wild-type protein was to do a competition study and ask whether a mutant competes for substrate
binding with the wild-type enzyme and prevents processing of the procoat protein. The two protein concentrations were equalized to 1.7 mg/ml. Each reaction was held at a total volume of 14 μl, with 7 μl of the reaction being [35S]-procoat, and 1 μl of wild-type enzyme. The mutant leader peptidase was then added incrementally from 1 μl to 6 μl in separate reactions. The difference in volume was made up with 10% polybuffer 74/1% β-octylglucoside. The samples were then incubated at 37°C for 30 min. After incubation, processed and unprocessed procoat proteins were then separated on a 23% SDS-PAGE gel and exposed to x-ray film.

2.18 Purification of Δ2-75 Leader Peptidase

For studies on the soluble leader peptidase, Δ2-75, we purified chemical quantities of the protein, following the purification procedure described by Kuo et al. (1993). 200 ml of BL21 (DE3) cells harboring the pET-3d vector containing the Δ2-75 gene (or other deletion mutant) were grown at 37°C until A600 = 0.5. The cell culture was then induced with 0.5 mM IPTG (final concentration) and grown for another 4 hours. Cells were harvested by centrifugation (6 000 RPM, 10 min, 4°C). Cells were then resuspended in 2 ml of buffer A (20 mM Tris, 5 mM MgCl2, pH 7.4) and lysed by passing through a french press 3 times at 14 000 psi. The lysate was centrifuged at 100 000g for 2 hr and the pellet containing the Δ2-75 was resuspended in 4 ml 4 M guanidine HCl in buffer A. After resuspension, the sample was loaded onto a 100 cm Sephacryl S-100 column equilibrated in the above buffer. Fractions containing the Δ2-75 were pooled and diluted 3
times with dialysis buffer and dialyzed exhaustively at 4°C against buffer A with 0.5 % TX-100. The refolded protein was then concentrated and loaded onto an extractigel-D column (Pierce) equilibrated with buffer B (20 mM Tris, pH 7.4) to remove the detergent. Alternatively the detergent could be removed by loading the concentrated protein onto a Q-Sepharose fast flow column equilibrated with buffer B and then washed extensively, followed by elution with 700 mM NaCl in buffer B.

2.19 TX-114 Extraction of Proteins to Determine Favored Environment

To determine whether leader peptidase is water or detergent soluble we used the method of Bordier (1981), to determine whether a protein is found in a detergent-rich or water phase. In this approach, the detergent TX-114 precipitates at elevated temperatures. 200 µl protein (0.5 mg/ml) in 50 mM Tris pH 8.0, 1% TX-114 was overlaid on 300 µl sucrose cushion (6% sucrose, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.06% TX-114) and incubated at 37°C for 3 min followed by centrifugation in a microfuge for 3 min at 37°C at 2500 RPM. The bottom phase was the first detergent extraction sample. The top phase was removed into a fresh tube and TX-114 was added to 0.5% and resolublized at 4°C for 5 min. The sample was once again overlaid, incubated and centrifuged as above, with the bottom phase being the second detergent extraction sample. The top aqueous phase was transferred to a new tube and TX-114 was added to a final concentration of 2% and resolublized as above. Incubation and centrifugation were repeated, this time without the sucrose cushion. The bottom phase was
discarded and the top phase was the aqueous phase of the extraction. All three samples were brought up to 800 µl with water and the protein was precipitated with an equal volume of ice-cold 20% trichloroacetic acid by incubating on ice for 30 min, followed by microcentrifugation (5 min, 4°C). The pellet was washed with chilled acetone followed once again by microcentrifugation and the acetone was aspirated away and the pellet dried. The pellet was then resuspended in 100 µl 2% SDS/10 mM Tris, pH 8.0 solution by incubating at 95°C for 10 min followed by vortexing. The protein samples were then analyzed on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue.

2.20 Determination of Protein Aggregation

To determine whether proteins were aggregated in an aqueous solution, 50 µg of protein in 2 ml of 20 mM Tris, pH 8.0 were centrifuged 5 hr at 100 000g at 4°C. After centrifugation, the supernatant was transferred to a new tube and the pellet resuspended in a volume equal to the starting volume. Samples were then analyzed on a 12% SDS-PAGE gel, followed by staining with Coomassie Brilliant Blue. The aggregated protein would be in the pellet fraction.

2.21 Peptide Assay for Leader Peptidase Activity

This utilizes a method devised by Dev et al (1990) to assay the activity of leader peptidase using a synthetic peptide and by HPLC. The
peptide used in this assay was Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO\(^{-}\), which corresponds to the -7 to +2 region of the pre-maltose binding protein. Leader peptidase cleaves this substrate between the alanine and lysine residue of this substrate. Either wild-type or Δ2-75 leader peptidase was diluted to various concentrations with 20 mM Tris, pH 7.4 with or without 1% TX-100 to a volume of 95 μl. The volume was brought up to 140 μl with 50 mM Tris, pH 8.0 in the presence or absence of TX-100 (1%), then the peptide (1.8 mM) in 83 mM sodium phosphate (pH 7.7) was added (50 μl). Samples were incubated at 37°C for indicated times. An aliquot (45 μl) was removed and quenched by an equal volume of 0.1% trifluoroacetic acid (TFA). Prior to HPLC analysis, the sample was microfuged for 5 min at room temperature to pellet any particulate matter or detergent present. The elution gradient utilized to separate the cleaved from the uncleaved peptide was as follows: 97%A3%B held constant for 5 min, followed by increasing the gradient linearly to 60%A40%B over a 10 min period, which was then held constant for 5 min, and then brought back down to 97%A3%B over a 5 min period. The solvents used were A = 0.1% TFA and B = 0.1% TFA in acetonitrile. Peptide products were detected spectrophotometrically at 218 nm. The column used was a 25 cm Vydac protein and peptide column. Percent processing of the peptide was determined by quantitation of the 7-mer product peptide peak and the 9-mer substrate peak:

\[
\text{% processing} = \left[ \frac{\text{area 7-mer peak}}{(\text{area 7-mer} + \text{area 9-mer peaks})} \right] \times 100
\]
2.22 Purification of pro-OmpA Nuclease A

To assay the activity of leader peptidase in both crude assays as well as in kinetic experiments, we needed to purify chemical amounts of the substrate pro-OmpA nuclease A. This was done by collecting inclusion bodies of pro-OmpA nuclease A, followed by ammonium sulfate precipitation of the pro-OmpA nuclease A to concentrate the protein. The pro-OmpA nuclease A was resolublized in guanidine HCl. After the solublization of the protein, the pro-OmpA nuclease A was subjected to size exclusion chromatography (Sephacryl S-200), followed by cation exchange (SP high performance) chromatography. The inclusion bodies contain approximately 20% of the pro-OmpA nuclease A that is available for purification. The rest can be easily recovered from the cell lysate supernatant following similar ammonium precipitation and chromatography steps as with the inclusion bodies. Both methods are described below.

10 L of LB media with 100 μg/ml Amp was inoculated with SB221 cells harboring the pONF1 plasmid (Chatterjee et al, 1995). The cell culture was grown to A600 = 1.0 and induced with 2 mM IPTG (final concentration) and growth was allowed to continue for an additional 4 hours. Cells were harvested by centrifugation (6 000 RPM, 10 min, 4°C). The pellet was then resuspended in TEP buffer (25 mM Tris pH 8.0, 5 mM EDTA, 1 mM PMSF) in a ratio of 6 ml/1 g wet cells. The cells were then lysed by passing through a french press 2 times at 14 000 psi. Debris was pelleted by centrifugation (10 000 RPM, 10 min, 4°C) in a JA14 rotor. The supernatant was saved to purify more pro-OmpA nuclease A at a later point. The pellet was
resuspended in 10 ml TEP buffer and centrifugation was repeated as above. The pellet was then resuspended in 22 ml guanidine buffer (7.2 M guanidine HCl, 50 mM sodium citrate, pH 3.0, 5 mM β-mercaptoethanol) and centrifuged for 30 min at 4°C at 56 000 RPM in a Ti70 rotor. The supernatant was dialyzed overnight in 4 L of dialysis buffer 1 (50 mM Tris pH 8.8, 1 M KCl, 10 mM CaCl₂, 20% glycerol) at 4°C to refold the substrate. Dialysis was continued the next day in dialysis buffer 2 (25 mM Tris pH 8.8, 1 M KCl, 10 mM CaCl₂) for 4 hours to remove the glycerol prior to ammonium sulfate fractionation. After dialysis the sample was centrifuged (56 000 RPM, 30 min, 4°C) in a Ti70 rotor to remove the misfolded proteins. The supernatant was then subjected to ammonium sulfate precipitation to concentrate the precursor form of the substrate. Ammonium sulfate was added to 55% saturation and allowed to stir for at least 1 hr at 4°C. Precipitate was collected by centrifugation (11 500 RPM, 30 min, 4°C) in a JA20 rotor. The pellet containing the pro-OmpA nuclease A was resuspended in 4 ml guanidine buffer and loaded onto a Sephacryl S-200 column (120 x 3 cm) equilibrated in guanidine buffer. Fractions were collected (8 ml) and the protein in each fraction was determined by SDS-PAGE. Fractions containing the pro-OmpA nuclease A were pooled and concentrated on an Amicon concentrator using YM10 membranes. The sample was then dialyzed overnight (20 mM Tris, pH 9.2) at 4°C to refold the protein. Dialysis was then continued in 2 L 25 mM Hepes, pH 7.6 with three changes of the buffer to prepare it for further purification by chromatography. The protein was then loaded onto a SP high performance column (15 x 1 cm). The protein was then eluted with a 300 ml gradient of
NaCl (250-350 mM) in 25 mM Hepes, pH 7.6 buffer. Fractions were analyzed for pure protein by SDS-PAGE. Fractions containing pro-OmpA nuclease A were then pooled and dialyzed against 25 mM Tris, pH 8.0/10 mM CaCl$_2$ overnight. Approximately 8-fold more pro-OmpA nuclease A was purified from the saved supernatant (after lysis) by adding ammonium sulfate to 55% to the lysis supernatant as described above and following the above procedure from that point through the SP chromatography step and dialysis.

2.23 pro-OmpA Nuclease A Substrate Cleavage Assay

We used the pro-OmpA nuclease A fusion protein (Chatterjee et al, 1995) as a substrate to assay the activity of wild-type, mutant as well as for Δ2-75 leader peptidase. Leader peptidase was incubated at various concentrations with pro-OmpA nuclease A at concentrations ranging from 10 μM to 30 μM in 50 mM Tris, pH 8.0, 10 mM CaCl$_2$ in the presence or absence of 1% Triton X-100 at 37°C. Reactions were terminated by the addition of 5 μl 5-fold sample buffer, containing 10 mM MgCl$_2$ and frozen immediately. Samples were analyzed by SDS-PAGE using a 17.2% gel and stained by Coomassie Brilliant Blue.

2.24 Kinetic Assay using pro-OmpA Nuclease A

To determine the kinetic constants ($K_{cat}$, $K_m$) of leader peptidase and mutants, we used pro-OmpA nuclease A as a substrate. Substrate concentrations were determined by using a E$^1$% at 280 nm of 8.3
(Chatterjee, et al. 1995). A typical kinetic cleavage reaction (75 µl) containing leader peptidase was in 50 mM Tris, pH 8.0, 10 mM CaCl$_2$ with 1% Triton X-100 containing the substrate typically at 5 different concentrations (35.2, 17.6, 13.2, 8.8, 4.4 µM). In the case of Δ2-75, the kinetic parameters were also measured with the absence of TX-100. The reaction was initiated by the addition of leader peptidase. The concentration of leader peptidase was determined using the Pierce BCA protein assay kit. The concentration of leader peptidase (WT) was $1.37 \times 10^{-4}$ µM, while the Δ2-75 concentration was $2.2 \times 10^{-2}$ µM (+TX-100) or 2.2 µM (-TX-100). The reaction was carried out at 37°C and aliquots of the reaction were removed at various times up to 10 min after initiation of the reaction. The reaction was stopped by the addition of 5 µl 5-fold sample buffer containing 10 mM MgCl$_2$ and the samples were frozen immediately in a dry ice/ethanol bath. The amount of pro-OmpA nuclease A that was processed by leader peptidase was assayed by SDS-PAGE on a 17.2% gel, followed by staining with Coomassie Brilliant Blue. The precursor and mature proteins were quantified by scanning the gels on a Technology Resources, Inc. Line Tamer PCLT 300 scanning densitometer. Percent processing was determined by dividing the area of the mature protein by the sum of mature and precursor peak areas. The initial rates were determined by plotting the amount of product versus time. The V$_{max}$, K$_M$, and K$_{cat}$ values were extracted from a $1/v_i$ versus $1/[S]$ plot. We used Microcal Origins to plot the data and to do linear regression on the data.
3.1 Introduction

The targeting of proteins to various intracellular membrane compartments is typically achieved by an amino-terminal cleavable signal peptide. The signal peptide is subsequently cleaved off by a signal (or leader) peptidase, whose active site is exposed to the lumenal side of the membrane.

Signal peptidases have been purified from *Escherichia coli* (Zwizinski et al., 1980; Wolfe et al., 1982), yeast (YaDeau and Blobel, 1989; Schneider et al., 1991), chicken (Baker and Lively, 1987), and dog (Greenburg et al., 1989) and have been identified in *Salmonella typhimurium* (van Dijl et al., 1990), *Pseudomonas fluorescens* (Black et al., 1992) and *Bacillus subtilis* (van Dijl et al., 1992). Although the eukaryotic and prokaryotic signal peptidases are structurally distinct (Evans et al., 1986; Dalbey and von Heijne, 1992), they have some similar features. First, both the *E. coli* leader peptidase (Zwizinski et al., 1981) and the canine endoplasmic reticulum
(ER) signal peptidase (Jackson and Blobel, 1980) are not inhibited by classical protease inhibitors. Second, there are some conserved sequence patterns among the cloned genes that code for signal peptidases (van Dijl et al., 1992); the *E. coli* leader peptidase has an amino acid homology which ranges from 31% for *B. subtilis* and up to 93% for *S. typhimurium*; there is also weak similarity (20-30%) between the prokaryotic signal peptidases and the yeast Sec11, canine Spc18 and Spc21 subunits of the ER signal peptidases. Third, the substrate specificities of the ER signal peptidase from dog (Folz et al., 1988) and the *E. coli* leader peptidase (Shen et al., 1991; Fikes et al., 1990) are nearly identical. They cleave after the consensus sequence Ala-X-Ala (von Heijne, 1983)

In order to shed light on the mechanism of catalysis of signal peptidases, we have recently used site-directed mutagenesis on the *E. coli* leader peptidase (Sung and Dalbey, 1992) to change amino acids that have been shown to be involved in catalysis in other standard proteases (Thiol, serine, aspartic and metallo proteases) (Dunn, 1989). This work revealed that the substitution of either serine 90 or aspartic acid 153 by an alanine destroyed catalytic function, while other similar mutations at each of the histidine and cysteine residues and changes at the other serine and aspartic acid residues had no marked effect. In addition to the earlier mutagenesis work, it was shown that when serine 90 is replaced with a cysteine it is still active but becomes sensitive to NEM, whereas the wild-type enzyme remains insensitive to NEM, giving further proof that serine 90 is involved in the catalytic mechanism. Based on this and other work, it has been
hypothesized (Dalbey and von Heijne, 1992; Black et al., 1992) that signal peptidases may constitute a new type of serine protease, mechanistically similar to class A β-lactamases. These β-lactamases are serine enzymes that use a lysine residue in the acylation step as a base to deprotonate the hydroxyl group of serine (Strynadka et al., 1992) and a glutamic acid residue (in the deacylation step) as a base to abstract a proton from a water molecule (Adachi et al., 1991; Kelly et al., 1985; Kelly et al., 1986).

In this chapter, it is demonstrated that the replacement of the conserved lysine 145 with either an alanine, asparagine, or a histidine inactivates the enzyme, suggesting that this ionizable residue is a good candidate for an active site residue. Also it was determined that there is no requirement for an acidic residue in catalysis as previously shown (Sung and Dalbey, 1992). The overall study presented here is consistent with the hypothesis that leader peptidase employs a serine-lysine catalytic dyad in its catalytic mechanism.

3.2 Results

A. Role of Basic Amino Acids in Catalysis

It has been previously shown that serine 90 is important for catalytic activity (Sung and Dalbey, 1992; Tschantz et al., 1993) by replacing the serine with an alanine residue. We propose that this serine forms an acyl intermediate with the substrate, but to do this there must be an amino acid which is nucleophilic enough to remove the proton from the serine hydroxyl.
We believe that this may be done by a normally basic residue which has a lowered pKa so that it becomes nucleophilic in nature and is able to abstract a proton from the serine. There are three conserved basic residues in the prokaryotic leader peptidase family which are the arginine 127, lysine 145 and arginine 146 residues. To determine if these basic residues are critical for catalysis, each of them was mutated using site-directed mutagenesis. These mutations were first assayed by an in vitro assay, where $^{35}$S-labeled procoat is used to measure processing activity in a detergent extract. In this assay, procoat is processed by leader peptidase to the coat protein. MC1061 cells bearing the plasmid pRD8, which encodes the wild-type leader peptidase, overproduces the enzyme about 200-fold (fig. 3.1, bottom left panel); Dilution of this extract 200-fold gives roughly the same processing activity as undiluted MC1061 (without plasmid) (fig. 3.1, top left panel). Leader peptidase mutants arginine 127 to alanine and arginine 146 to alanine mutants were found to be partially active in vitro (fig. 3.2a). In contrast, the leader peptidase K145A or K145N mutant was inactive in vitro (fig. 3.2a). When a mutation scores inactive in this assay we also test it in a more sensitive in vivo assay. Briefly, this assay examines the processing of the outer membrane protein A precursor (proOmp A) in IT41, a temperature sensitive leader peptidase strain (Inada et al, 1989). At the nonpermissive temperature of 42°C, processing of proOmp A is slow with a $t_{1/2}$ greater than 90 s (fig. 3.1, top right panel). On the other hand, processing is fast with a $t_{1/2}$ of less than 10 s at 42°C when IT41 bears the plasmid pRD8, encoding the wild-type leader peptidase (fig. 3.1, bottom right panel). As can be seen that both K145A and K145N
Fig. 3.1. *In vitro* and *in vivo* assays for measuring the activity of mutant leader peptidases. For the *in vitro* assay, extracts of MC1061 containing no plasmid or pRD8 (expressing wild-type leader peptidase) were prepared as described in chapter 2.12 and used either directly (without dilution) or after dilutions (1:10, 1:50, 1:200, 1:400). $^{35}$S-labeled procoat, which was synthesized in a transcription/translation system, was added to the extracts and incubated at 37°C for 30 min and subsequently analyzed using a 23% SDS-Polyacrylamide gel to separate the procoat and coat proteins. For the *in vivo* assay, *E. coli* strain IT41, which has a temperature-sensitive mutation in the chromosomal copy lepB gene, was used. IT41 bearing no plasmid or pRD8 plasmid-encoding the wild-type leader peptidase was grown at 30°C to the mid-log phase. After the addition of arabinose (0.2%) to induce leader peptidase, cultures were grown at 42°C for 1 hour. Processing of pro-OmpA was determined using a 12% SDS-Polyacrylamide gel as described in chapter 2.14 and table 2.1.
Fig. 3.1

in vitro

no plasmid

Procoat-Coat-
Dilution: 1 10 50 200 400

pRD8

in vivo

no plasmid

Procoat-Coat-
Dilution: 1 10 50 200 400

Chase time (sec) 10 20 30 40 50 60 90 120

Pro-Omp A
Omp A

Chase time (sec) 10 20 30 40 50 60 90 120

Pro-Omp A
Omp A

PRDB Pro-Omp A
pRD8

PRDB Pro-Omp A
pRD8
Fig. 3.2. Lysine 145 is important for the activity of leader peptidase. A. Leader peptidases R127A, K145A, K145N, K145H, or R146A were assayed for activity in the *in vitro* assay using $^{35}$S-labeled procoat as a substrate. B. The *in vivo* activities of leader peptidases K145A, K145N or K145H were analyzed with IT41, which contains a temperature-sensitive chromosomal copy of leader peptidase, as described in figure legend 3.1.
A. in vitro Assay

R127A
Procoat - Coat -
Dilution: 1 10 50 200 400
R146A
K145A

K145H
Procoat - Coat -
Dilution: 1 10 50 200 400

K145N

B. in vivo Assay

K145A
Pro-Omp A - Omp A -
Chase time 10 20 30 40 50 60 90 120
K145H
K145N

Fig. 3-2
are inactive in this in vivo assay (fig. 3.2b), indicating that lysine 145 is important for catalytic activity of the E. coli leader peptidase. We confirmed using immunoblot analysis that these cells expressed normal levels of the leader peptidase lysine 145 mutant proteins (data not shown). Because a histidine residue is found at this position in the Sec11 yeast subunit, as well as in the spc18 and spc21 subunits of the canine ER signal peptidases (Dalbey and von Heijne, 1992), we tested whether the histidine is functionally interchangeable with the lysine. As can be seen, leader peptidase K145H was inactive both in vitro (fig. 3.2a) and in vivo (fig. 3.2b).

A potential problem using site-directed mutagenesis techniques is that mutations can cause global conformational changes which inactivate the molecule. To rule out that the K145A mutation caused a large conformational change, CD spectroscopy was performed on the purified K145A leader peptidase protein. The far-UV CD spectrum is similar to that of the wild-type protein (fig. 3.3). There are some changes in the 200-220 nm region of the spectra that are attributable to instrument noise as a result of the polybuffer 74 absorbing much of the light in this spectral region as it passes through the sample. Therefore, since the overall shape of the wild-type and K145A leader peptidase spectra appear to be the same, the data suggest that the K145A mutant is not grossly misfolded.

A more sensitive test to show that there has not been a conformational change within the leader peptidase K145A protein is to perform a competition study between the inactive K145A and the wild-type
Fig. 3.3. CD spectra of wild-type and K145A leader peptidase. The CD measurements were made on a solution of 1.7 mg/ml in 10% polybuffer 74 (Pharmacia LKB Biotechnology, Inc.) containing 1% β-octylglucoside at 9°C. The spectra were collected using a Jasco Spectropolarimeter, J-500C, in a 0.5 mm path length cell. The units of $[\Theta]_{\text{MRW}}$ are deg cm$^2$decimol$^{-1}$. 
Fig. 3.3
**Fig. 3.4.** Leader peptidase K145A competes with wild-type leader peptidase for binding procoat. Each reaction (14 μl) contained various amounts of wild-type (1.7 mg/ml) leader peptidase in 10% bolybuffer 74 (Pharmacia), 1% β-octylglucoside, and 7 μl of 35S-labeled procoat in 50 mM Tris, 0.1% Triton X-100, pH 8.0. Reactions in lanes 1-6 each contained 1 μl of wild-type leader peptidase but differing amounts of the K145A protein. Lane 7 contained only K145A leader peptidase. Lane 8 contains only wild-type leader peptidase. Lane 9 contained only the 35S-labeled procoat substrate. All reactions were incubated at 37°C for 30 min and then quenched on ice.
molecule. Figure 3.4 shows there were no structural perturbations at the procoat binding site, since the inactive protein competed with the wild-type leader peptidase for substrate binding. The amount of processing of procoat was measured using different relative amounts of wild-type and K145A proteins. When only wild-type leader peptidase was added to the sample most of the procoat protein was processed to the coat protein (lane 8) whereas no processing of procoat occurred with added purified K145A leader peptidase (lane 7). With equal amounts of wild-type and mutant proteins, the band intensities of procoat and coat protein are equal (fig. 3.4, lane 1). As the amount of K145A leader peptidase was increased, processing was inhibited, with no processing observed at a 6:1 ratio of mutant to protein. This shows that the K145A mutant competes with the wild-type protein for the binding of substrate, and therefore the overall global conformation is not perturbed.

Suggestive evidence of an important lysine residue in the catalytic mechanism is that we were able to inhibit leader peptidase with maleic anhydride, a lysine specific reagent (Habeeb and Atassi, 1970). Figure 3.5 shows the time dependence of inactivation of leader peptidase using a peptide substrate corresponding to the cleavage site of the pre-maltose binding protein (-7 to +2). Though this is indirect evidence because maleic anhydride can react with any free amino group, it is consistant with lysine 145 being critical for activity and also suggests that leader peptidase has a reactive lysine residue, since the loss of activity occurs at a rate which is much faster than a typical reaction using this reagent.
Fig. 3.5 Inhibition of leader peptidase with maleic anhydride. 20 ml of leader peptidase (0.7 mg/ml) was diluted in buffer (10 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 2.5% Triton X-100) to a final volume of 200 ml. Maleic anhydride (dissolved in DMSO) was added to 0.5 mM and incubated at room temperature. Aliquots were removed at 0, 10, 30 and 60 min and added to 12.5 ml of peptide substrate (1.5 mg/ml; Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO⁻) and incubated 3 hr at 37°C. Reactions were terminated by the addition of an equal volume of 0.1% trifluoroacetic acid. Samples were analyzed as described in section 2.21.
Fig. 3.5
B. Role of Acidic Residues in Catalysis

Previously it was determined that aspartic acid 153 was important for activity, possibly as a nucleophile to abstract a proton from water for attack on the acyl enzyme intermediate similar to how the glutamic acid in works in β-lactamase (Sung and Dalbey, 1992). In that previous study the aspartic acid 153 was changed to an alanine residue and there was a total loss of activity in vitro and in vivo. In this study, the aspartic acid was changed to an asparagine and a glutamic acid. Surprisingly, these mutations did not cause a total loss of activity, but caused a decrease in activity in vitro (fig. 3.6a) of about 20-fold and not a total loss as was seen with the D153A mutation. We then tested the D153N leader peptidase in the in vivo assay, and found that it had wild-type leader peptidase activity (fig. 3.6b). These data indicate that aspartic acid 153 is not critical for the catalytic mechanism as was previously suggested.

Aspartic acid 280 is strongly conserved residue within the signal peptidase family which has been suggested to be important for activity (van Dijl et al., 1995). In a previous study it was found that the D280A mutation had a slight effect on the in vitro on processing activity (Sung and Dalbey, 1992). Since it has been suggested that this residue is important for catalytic activity we changed the aspartic acid residue to asparagine, glycine
Fig. 3.6. Aspartic acid 153 is not important for activity. A. Leader peptidases D153N and D153E were assayed for activity in the *in vitro* assay using [35S]-labeled procoat as a substrate. B, the *in vivo* activity of leader peptidase D153N was analyzed with IT41, which contains a temperature-sensitive chromosomal copy of leader peptidase, as described in figure legend 3.1.
A. *in vitro* Assay

<table>
<thead>
<tr>
<th>Procoat - Coat -</th>
<th>D153N</th>
<th>D153E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution:</td>
<td>1 10 50 200 400</td>
<td>1 10 50 200 400</td>
</tr>
</tbody>
</table>

B. *in vivo* Assay

<table>
<thead>
<tr>
<th>Pro-Omp A - Omp A -</th>
<th>D153N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chase time (sec)</td>
<td>10 20 30 40 50 60 90 120</td>
</tr>
</tbody>
</table>

Fig. 3.6
and glutamic acid and reassayed the alanine mutant. We found that each of these changes caused total loss in activity in vitro (fig. 3.7, left panel) but when assayed in the more sensitive in vivo assay, all of these mutations had little or no effect on the processing of the pro-ompA protein (fig. 3.7, right panel). This suggests that aspartic acid 280 has some effect when assayed in a detergent extract, but when tested under physiological conditions had little effect, therefore ruling out the possibility that this residue is absolutely critical in the catalytic mechanism.

3.3 Discussion

It has previously been shown using site-directed mutagenesis techniques that serine 90 is important for the activity of the E. coli leader peptidase (Sung and Dalbey, 1992). Also it was found that a cysteine residue can substitute for this serine residue and give rise to an active leader peptidase variant which can then be inhibited by the cysteine-specific reagent NEM (Tschantz et al., 1993). This is in contrast to the wild-type enzyme which is insensitive to both serine-specific reagents and relatively insensitive to cysteine-specific protease inhibitors (Zwizinski et al., 1981). These studies provided evidence that the serine 90 plays an important role in pre-protein catalysis. We provide evidence that lysine 145 may be directly involved in the catalytic mechanism and also that leader peptidase does not require an acidic residue in the catalytic mechanism. The replacement of the lysine 145 by an alanine, asparagine or histidine residue
Fig. 3.7. Aspartic acid 280 is required in vitro, but not in vivo. Leader peptidases D280A, D280N, D280G and D280E were assayed for activity in the in vitro assay using [35S]-labeled procoat as a substrate as well as being tested for in vivo activity using E. coli strain IT41, which contains a temperature-sensitive chromosomal copy of leader peptidase, as described in figure legend 3.1.
in vitro

D280A
Procoat-Coat-
Dilution: 1 10 50 200 400

D280N
Procoat-Coat-
Dilution: 1 10 50 200 400

D280G
Procoat-Coat-
Dilution: 1 10 50 200 400

D280E
Procoat-Coat-
Dilution: 1 10 50 200 400

in vivo

D280A
Pro-Omp A
Omp A
Chase time 10 20 30 40 50 60 90 120 (sec)

D280N
Pro-Omp A
Omp A
Chase time 10 20 30 40 50 60 90 120 (sec)

D280G
Pro-Omp A
Omp A
Chase time 10 20 30 40 50 60 90 120 (sec)

D280E
Pro-Omp A
Omp A
Chase time 10 20 30 40 50 60 90 120 (sec)

Fig. 37
results in an inactive enzyme both in vivo and in vitro (fig. 3.2). This critical lysyl residue is located in a conserved region (van Dijl et al., 1992), which makes it a very good candidate to be a catalytic residue.

Our data indicate that like class A β-lactamases (Strynadka et al., 1991; Adachi et al., 1991) leader peptidase has an important serine and lysine residue. However, in contrast to class A β-lactamases, which require a glutamic acid residue as a base in the deacylation step (Adachi et al., 1991), no carboxyl side chain amino acid was found to be critical for leader peptidase catalysis in vivo. While we previously reported that the D153A mutation completely inactivates the enzyme (Sung and Dalbey, 1992), we found that a D153N mutation does not have a marked effect on the in vivo activity (Fig. 3.6). This rules out the possibility that aspartic acid 153 plays a direct role in catalysis. We also showed that aspartic acid 280, which has been shown to be important in other signal peptidases, also does not have any effect on the catalytic mechanism when it is changed to an alanine, asparagine, glycine or a glutamic acid residue (Fig. 3.7). On the balance, the data seem to suggest that the catalytic mechanism of leader peptidase is different from that used by class A β-lactamases.

Our current working model is that the E. coli leader peptidase employs a serine-lysine dyad, and not the standard serine-histidine-aspartic acid triad found in most classical serine proteases (Neurath, 1989). Such a novel catalytic dyad involving a serine and a lysine residue has been reported for the catalytic mechanism of autodigestion of the LexA Repressor
(Lin and Little, 1988). It is intriguing that the lysine 145 residue of the *E. coli* leader peptidase is only conserved in prokaryotes. In eukaryotes, it has been replaced by a histidine residue in the yeast Sec11, canine spc18 and spc21 subunits of the ER signal peptidases (Dalbey and von Heijne, 1992), suggesting that these peptidases may use the serine triad mechanism. Since a histidine residue may have been recruited in the evolution of eukaryotes, we also tested whether a histidine residue can substitute for the lysine 145 in the *E. coli* leader peptidase. Figure 3.2 shows that the histidine residue cannot substitute for the lysine residue. Similar results with a histidine substitution of the catalytic lysine were also found for the LexA Repressor (Lin and Little, 1989).

Several lines of evidence suggest that the replacement of lysine 145 with alanine does not result in a grossly misfolded protein. First, the CD spectrum of leader peptidase K145A is similar to the spectrum of the wild-type protein. This shows that there are not any large secondary and tertiary structural changes in the mutant K145A protein. Second, the K145A protein competes with the wild-type leader peptidase for binding of the procoat substrate. This shows that the K145A mutation does not result in large conformational changes at the substrate binding site that would reduce the substrate binding affinity. Thus we feel it is unlikely that leader peptidase K145A is inactive as the result of global conformational changes.

In summary, we believe that lysine 145 acts as a general base catalyst, similar to the way it works in the catalytic mechanism of the LexA
Repressor protein (Slilaty et al., 1986; Slilaty and Little, 1987) and class A β-lactamases (Strynadka et al., 1992). Our data seem to rule out participation of an aspartic acid residue as a base, analogous to the way that glutamic acid functions in the catalytic mechanism of class A β-lactamases. Definitive identification of other active site residues will have to wait until the structure of leader peptidase is solved.
Chapter IV

The Characterization of a Soluble, Catalytically Active Form of *E. coli* Leader Peptidase

4.1 Introduction

In both prokaryotic and eukaryotic cells, proteins destined for the cell surface or for secretion are made with an amino-terminal leader (or signal) sequence that targets the protein into the export pathway. The leader peptide is subsequently removed by a leader (signal) peptidase during or after the exported protein is translocated across the membrane.

To date the *E. coli* leader peptidase is the most studied leader peptidase. This enzyme has been purified (Zwizinski et al., 1981; Wolfe et al., 1982; Wolfe et al., 1983a), its gene cloned and sequenced (Date and Wickner, 1981), and the protein overexpressed under the control of the araB promoter (Dalbey and Wickner, 1985). Gene cloning and mutagenesis techniques have been employed to study the membrane biogenesis of leader peptidase (Wolfe and Wickner, 1984; Dalbey and Wickner, 1986; Dalbey and Wickner, 1987; Dalbey et al., 1987; Zhu and Dalbey, 1989; Laws and Dalbey, 1989; von Heijne, 1989; Nilsson and von Heijne, 1990; Lee et al., 1992) and to determine its physiological role (Dalbey and
Wickner, 1985). While its precise mechanism of action is unknown, recent data suggests that leader peptidase is a member of a novel class of serine proteases, which utilize serine and lysine residues for catalysis (Sung and Dalbey, 1992; Black et al., 1993; Tschantz et al., 1993). The active site of leader peptidase resides in the periplasmic domain (Bilgin et al., 1990), which is anchored to the membrane by two transmembrane segments (fig. 4.1; Wolfe et al., 1983a; Moore and Miura, 1987; San Millan et al., 1989).

Recently, there have been two important developments in the leader peptidase field. First, Kuo et al., (1993) have reported the isolation of a catalytically active, soluble form of leader peptidase, which lacks the membrane anchoring domain. Second, a new substrate (pro-OmpA nuclease A) has become available that has much improved kinetic properties over previously described substrates. The $k_{cat}/K_m$ with the new pro-OmpA nuclease A substrate, a hybrid secretory precursor, is six orders of magnitude greater than with peptide substrates (Chatterjee et al., 1995).

In this chapter, we report on the characterization of the catalytic, periplasmic domain (Δ2-75) of leader peptidase. We find that the truncated leader peptidase has very good catalytic parameters ($k_{cat}$ is 3.0 sec$^{-1}$ and $K_m$ of 32 μM) against the proOmpA nuclease substrate. In contrast to the wild-type enzyme, Δ2-75 is water soluble. No detectable activity is observed for Δ2-75 proteins in which the candidate active site residues are changed to alanine residues. Strikingly, while the Δ2-75 and the substrate are water soluble, the detergent, Triton X-100 is required for optimal activity. Our
Fig. 4.1. Membrane topology of the *E. coli* leader peptidase. H1, H2 and H3 are hydrophobic domains whereas P1 and P2 are polar domains.
results do not agree with recent studies by Kuo et al. (1993) where they find that Δ2-75 lacks a requirement for detergent for optimal catalysis and has the same activity as the wild-type leader peptidase. Possible explanations for this discrepancy in the detergent requirement and the differences in the activity between the wild-type and Δ2-75 are discussed.

4.2 Results

A. Purification and Activity of Truncated Forms of Leader Peptidase

One of the objectives of our research is to isolate a catalytic periplasmic domain of leader peptidase in order to solve its structure. To this end, we have constructed truncated forms of leader peptidase, namely Δ2-75, Δ2-70, Δ2-60. These proteins were overproduced and purified according to Kuo et al. (1993), who reported the procedure for the Δ2-75 mutant, which lacks the membrane-spanning domain. To prepare these deletion mutants of leader peptidase, oligo-directed mutagenesis was used to create a unique Nco I site at various places within the leader peptidase gene (lepB) such that Nco I would cut between the codons of 60 and 61, 70 and 71 or 75 and 76. The genes encoding these truncated proteins were subcloned into the pET-3d vector, which is a T7 polymerase dependent vector often used in the expression of high levels of protein (Studier et al, 1990). We confirmed by coomassie blue staining that the truncated leader peptidase proteins were expressed upon adding IPTG. Briefly, cells (200 ml) were grown, induced and harvested, and the truncated proteins
solublized and purified as described (Kuo et al., 1993). The cells were passed through a french press three times to lyse the cells and a Sephacryl S-100 column was used for gel filtration. Exhaustive dialysis was carried out with 0.5% Triton X-100 in 20 mM Tris, pH 7.4 (without MgCl₂).

After dialysis we analyzed the samples for purity by SDS-PAGE and coomassie blue staining. Figure 4.2a shows that Δ2-75 (lane 6), Δ2-70 (lane 5), Δ2-60 (lane 4) were purified to homogeneity and migrated on the gel faster than the wild-type protein (lane 1, top band). As a control, we confirmed that there is not a protein at this position if we followed the procedure exactly as described above, except that we started with cells bearing the pET-3d vector without the leader peptidase insert. Two fractions were taken from the Sephacryl S-100 column at the same position where the Δ2-75 elutes and, after dialysis, an aliquot of this material was analyzed by SDS-PAGE and coomassie blue staining (lanes 2 and 3).

The activity of the truncated forms of leader peptidase was assayed (fig. 4.2b) using the proOmpA nuclease A, an excellent substrate for the wild-type leader peptidase (Chatterjee et al, 1995). An aliquot (2 μl) containing wild-type (lane 1), Δ2-60 (lane 4), Δ2-70 (lane 5) and Δ2-75 (lane 6) or an aliquot of the control samples (lanes 2 and 3) derived from cells not overexpressing the truncated proteins in the pET-3d vector (see above) was incubated with the proOmpA nuclease A substrate for 60 min. No leader peptidase was added in lane 7. The control samples were derived from a purification of a preparation of cells bearing the pET-3d vector without the
Fig. 4.2. Purification and activity of truncated leader peptidases

**A. Purification of the truncated forms of leader peptidase.** 20 μl of 0.7 mg/ml wild-type leader peptidase (lane 1), and 20 μl of Δ2-60 (lane 4), Δ2-70 (lane 5) and Δ2-75 (lane 6) each of a 0.2 mg/ml solution were applied to a 12% SDS-PAGE gel and the gel stained by Coomassie Brilliant Blue. The concentration of the wild-type and the truncated proteins was determined by the Pierce BCA protein assay kit. As a control, 20 μl of fractions 1 and 2 were analyzed from the sephacryl S-100 column (where the truncated mutants elute) derived from a preparation of cells bearing the pET-3d vector without a leader peptidase insert.

**B. Activity of the truncated proteins.** 2 μl of 0.7 mg/ml wild-type leader peptidase (lane 1), 2 μl of fraction 1 (lane 2), 2 μl of fraction 2 (lane 3), and 2 μl of Δ2-60 (lane 4), Δ2-70 (lane 5) and Δ2-75 (lane 6) each of a 0.2 mg/ml solution, and buffer only (lane 7) were incubated with 15 μl of pro-OmpA nuclease A (29 μM) in 50 mM Tris, 1% Triton X-100, pH 8.0, for 60 min at 37°C. The reaction was terminated by the addition of 5 μl of 5-fold SDS sample buffer and the samples were analyzed by SDS-PAGE with a 17.2% gel and Coomassie Blue staining.

**C. Concentration dependence of Δ2-75 cleavage of proOmpA nuclease A.** An aliquot (1 μl) containing various concentrations of Δ2-75 was incubated with 15 μl of proOmpA nuclease A (29 μM) in 50 mM Tris, 1% Triton X-100, pH 8.0 at 37°C for 60 min. Lane 1, 200 ng; lane 2, 20 ng; lane 3, 4 ng; lane 4, 2 ng; lane 5, 0.8 ng; lane 6, 0.4 ng; lane 7, buffer. The reaction was terminated and analyzed as described in 4.2B.
Fig. 4.2
leader peptidase gene. Figure 4.2b shows that each of the truncated forms of leader peptidase (lanes 4, 5 and 6) has significant activity over background. The low activity of the control samples (lanes 2 and 3) shows that the measured activity of the samples containing the mutant leader peptidase proteins is due to the truncated proteins themselves and not the chromosomally-encoded leader peptidase.

We furthered analyzed Δ2-75 since it had the highest activity of these truncated leader peptidases in this assay. Figure 2c shows that it takes 4 ng of Δ2-75 to see 50% processing of proOmpA nuclease A (lane 4), compared to 0.36 ng for the wild-type leader peptidase (data not shown). Since a 500-fold dilution of Δ2-75 gives equal processing as the background samples in these studies we estimate that the low background activity can account for roughly 1/500th of the activity in the Δ2-75 preparation.

B. Determination of the water solubility of Δ2-75

We evaluated whether Δ2-75 is detergent- or water soluble by determining whether it partitions into the Triton X-114 rich phase (Bordier, 1981). Previously we found that the wild-type leader peptidase partitions into the Triton X-114 phase (Dalbey and Wickner, 1987). Triton X-114 was added to Δ2-75 or, as a control, the wild-type leader peptidase. The samples were mixed at 4°C and then the temperature was raised to 37°C which is above the cloud point of the detergent. At this temperature, the aqueous phase and detergent phases can be separated by centrifugation.
As can be seen in figure 4.3b, Δ2-75 is found in the aqueous fraction (lane 3), with very little protein in the detergent fractions (lanes 1 and 2). In contrast, the wild-type protein is in the detergent-rich phase (fig. 4.3a, lane 1). In addition to these studies, we have found that the detergent Triton X-100 can be removed from the Δ2-75 sample by first absorbing the protein onto a Q Sepharose FF anion exchange resin and then washing the non-ionic detergent away, followed by eluting the protein off the column with 0.7 M NaCl (data not shown). A very concentrated clear solution of Δ2-75 (56 mg/ml) can be prepared in 4 mM Hepes, pH 7.6, without any detergent present (data not shown), again showing that this protein is very water soluble.

C. No Detectable Activity in Δ2-75 with Putative Active Site Mutations

We have used the proOmpA nuclease A processing assay with the Δ2-75 protein to analyze the effects of active site mutations because it is the most sensitive assay to date. Based on a comparison of the activity of Δ2-75 with background activity we estimate that this assay system is 10-fold more sensitive than the in vitro system that was previously used to show that the serine 90 and lysine 145 are possible active site residues (Sung and Dalbey, 1992; Tschantz et al., 1993). Oligo-directed mutagenesis (Zoller and Smith, 1983) was used to mutate either the serine 90 or lysine 145 to alanine. Δ2-75 bearing the S90A or K145A mutation was purified as described above and analyzed by SDS-PAGE and coomassie brilliant blue staining. Figure 4.4a shows only one protein species of the predicted molecular weight was observed (lanes 2 and 3). No detectable processing
Fig. 4.3. Δ2-75 partitions into the aqueous phase in a Triton X-114 extraction study. 200 μl of wild-type (A) or Δ2-75 (B) leader peptidase, each 500 μg/ml was solubilized in 50 mM Tris, pH 8.0, 1% Triton X-114 at 4°C was overlayed on a sucrose cushion (300 μl). After a 3 min incubation at 37°C, the sample was microfuged for 3 min at 37°C at 2,500 rpm to separate the detergent and the aqueous phases. The bottom phase is the first detergent extraction sample. The top phase was removed and Triton X-114 was added again (to a final concentration of 0.5%) and resolubilized by incubating at 4°C. After repeating the incubation and centrifugation steps with the sucrose cushion, the top aqueous phase was transferred to a new tube and Triton X-114 was added once again to a 2% final concentration and resolubilized as above. The bottom phase which remains after the second centrifugation step was the second detergent extraction sample. Finally, the centrifugation step was repeated after incubating the sample at 37°C, and the top phase was saved. This is the aqueous phase sample. The two detergent and one aqueous samples were brought up to 800 μl with water and then the samples were acid-precipitated (Wolfe et al, 1982) and analyzed by SDS-PAGE with a 12% gel (Tschantz et al, 1993) and stained by Coomassie Brilliant Blue. Samples were derived from the first detergent extraction (lane 1), second detergent extraction (lane 2) and the aqueous phase (lane 3). Left panel (A) shows the wild-type leader peptidase (lep); right panel (B) shows Δ2-75 leader peptidase.
Fig. 4.3
Fig. 4.4. Elimination of catalytic activity by active-site mutations in Δ2-75.  

A. Purification of Δ2-75 proteins. Δ2-75 (lane 1), Δ2-75 S90A (lane 2) and Δ2-75 K145A (lane 3) were analyzed on a 12% SDS-PAGE gel and stained by Coomassie Brilliant Blue. 50 μg of each protein was loaded on the gel.  

B. Processing activity by Δ2-75 S90A. Reactions (15 μl) contain proOmpA nuclease A (12 μM) and 1 μl of enzyme at various concentrations were incubated for 1 hr at 37°C. Samples were analyzed by SDS-PAGE with a 17.2% gel and the gel was stained with Coomassie Brilliant Blue. Lane 1 shows the activity with wild-type leader peptidase as a positive control for cleavage. Lane 2 shows the activity of Δ2-75 S90A (100 ng). Lane 3 shows the activity of 10 ng of Δ2-75 S90A. Lane 4 shows the activity in the presence of buffer only.  

C. Processing activity of Δ2-75 K145A. The reactions were carried out as described in B with the same amounts of Δ2-75 K145A in lanes 2 and 3 as indicated for the corresponding lanes for the Δ2-75 S90A protein shown in B.
Fig. 4.4
of the proOmpA nuclease A substrate was observed even at 100 ng of Δ2-75 with either the serine 90 (fig 4.4b, lane 2) or the lysine 145 to alanine mutation (fig 4.4c, lane 2). These results are consistent with the serine 90 and the lysine 145 being active site residues because it is expected that mutating catalytic residues would lower the activity at least 500-fold.

**D. Detergent Requirement for Optimal Activity**

To determine whether detergent is required for optimal activity of Δ2-75, we compared processing with or without the detergent Triton X-100. As described above, the detergent free Δ2-75 was prepared by washing away the detergent after absorbing it onto an anion exchange resin. Figure 4.5 shows that when using the proOmpA nuclease A as a substrate that the addition of Triton X-100 (1%, final concentration) stimulates processing roughly 20-to-50 fold.

While this study shows that the detergent Triton X-100 promotes Δ2-75 cleavage, it is not clear as to how the cleavage is affected. To measure more precisely what effect detergent has on cleavage, we determined the kinetic parameters in the presence or absence of detergent by measuring the initial rates of cleavage at various proOmpA nuclease A concentrations. Table 4.1 summarizes the kinetic constants that were determined from the initial velocity plots. In the presence of detergent, the calculated value of $k_{cat}$ is $3.0 \pm 0.4$ s$^{-1}$ and $K_m$ is $32 \pm 3$ μM. In the absence of detergent, the
<table>
<thead>
<tr>
<th>Dilution</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>250</th>
<th>500</th>
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<tbody>
<tr>
<td>+ TX100</td>
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<td>- TX100</td>
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Fig. 4.5. Δ2-75 requires the detergent Triton X-100 for optimal activity. Processing of proOmpA nuclease A by Δ2-75 at various concentrations was tested by diluting a Δ2-75 stock solution (100 μg/ml) into 50 mM Tris, pH 8.0 with or without Triton X-100 (1%). The reaction contains 15 μl of proOmpA nuclease A (23 μM) in 50 mM Tris, pH 8.0 with or without Triton X-100. The reaction was initiated the addition of Δ2-75 at various dilutions and incubated fro 1 hr at 37°C. The reaction was terminated by the addition of 5-fold sample buffer and the samples were subjected to SDS-PAGE (12%) and stained with coomassie brilliant blue.
Table 4.1.

<table>
<thead>
<tr>
<th>Kinetic constants of Lep Constructs</th>
<th>kcat (sec$^{-1}$)</th>
<th>Km (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT lep</td>
<td>44 ± 9</td>
<td>19.2 ± 4.6</td>
</tr>
<tr>
<td>Δ2-75 + TX-100</td>
<td>3.0 ± 0.4</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>Δ2-75 - TX-100</td>
<td>0.14 ± 0.04</td>
<td>199 ± 50</td>
</tr>
</tbody>
</table>

*aSee Methods for how constants were determined.*
kinetic constants are much poorer. The $k_{cat}$ is $0.14 \pm 0.04 \text{ s}^{-1}$ and $K_m$ is $199 \pm 50 \mu\text{M}$. Therefore, with no detergent present the $k_{cat}$ has decreased roughly 20-fold and the apparent $K_m$ has increased 6-fold. We also measured the kinetic constants for the wild-type leader peptidase with the proOmpA nuclease A substrate, the $k_{cat}$ is $44 \pm 9 \text{ s}^{-1}$ and the $K_m$ is $19.2 \pm 4.6 \mu\text{M}$, indicating that indeed, the Δ2-75 enzyme is very active with a $k_{cat}$ only 15-fold lower than that of the wild-type protein. The $K_m$ values are similar.

We also examined the requirement for detergent with a peptide substrate, Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO⁻, which corresponds to the -7 to +2 region of the maltose binding protein precursor (Dev et al., 1990). This peptide is cleaved by leader peptidase between the alanyl and lysyl residue. We incubated Δ2-75 at various enzyme concentrations with the peptide substrate at 37°C in the presence or absence of 1% Triton X-100. At indicated times (fig 4.6), the % processing was determined by the quantification of the reactant and product peptides which were separated using a reversed phase analytical C18 column utilizing a H₂O/acetonitrile gradient. Figure 4.6 shows the % processing at various times after the addition of leader peptidase or Δ2-75. As observed with the proOmpA nuclease A substrate, the addition of detergent leads to a marked stimulation of processing of the peptide. For example, figure 4-6 shows that 3.5 μM Δ2-75 leads to more peptide processing in the presence of detergent than with 35 μM Δ2-75 without detergent. Moreover, we find that the wild-
Fig. 4.6. The detergent Triton X-100 promotes the activity of Δ2-75 with a peptide substrate. Percent processing of the peptide substrate (Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO⁻) was assayed with Triton X-100 at 3.57 μM Δ2-75 (▼), or at various concentrations (▲, 3.57 μM; ●, 35.7 μM; ■, 178 μM Δ2-75) in the absence of Triton X-100. As a control, the % processing was determined with the wild-type leader peptidase (♦, 0.27 μM) in the presence of Triton X-100. Reactions were initiated by the addition of 50 μl peptide (1.8 mg/ml stock) to 140 μl of the enzyme sample at the indicated concentrations. At the indicated times, aliquots were removed and the % processing was determined (see section 2.21).
type leader peptidase is able to process the peptide substrate approximately 20-fold better than Δ2-75.

One possibility is that the detergent is required to prevent Δ2-75 or the proOmpA nuclease A substrate from denaturing and aggregating. To test this idea, we centrifuged the samples containing either the Δ2-75 or proOmpA nuclease A at 100,000 x g for 5 hours at 4°C and examined whether the protein was found in the pellet. As can be seen in figure 4-7, the majority of the total Δ2-75 protein (T) was located in the supernatant fraction (S) with very little in the pellet fraction (P) after centrifugation. In addition, the proOmpA nuclease A substrate was found exclusively in the supernatant fraction (S), while the wild-type leader peptidase was located exclusively in the pellet fraction (compare total and pellet lanes); the wild-type protein denatures in the absence of detergent because the detergent stabilizes its two transmembrane segments.

4.3 Discussion

We report here the isolation of the soluble, catalytically active form of the *E. coli* leader peptidase and compare its properties to the native membrane bound enzyme. We find that Δ2-75, the periplasmic catalytic domain, retains excellent activity. It has a $k_{cat}$ of 3.0 s$^{-1}$ and a $K_m$ of 32 μM with the proOmpA nuclease A substrate, whereas the wild-type protein has a $k_{cat}$ of 44 s$^{-1}$ and a $K_m$ 19.2 μM (Table 4.1). Additionally, we provide further evidence that serine 90 and lysine 145 are active site residues since their
Fig. 4.7. The majority of the Δ2-75 and the proOmpA nuclease A substrate do not aggregate in the absence of detergent. 50 μg each of wild-type leader peptidase (A), Δ2-75 (B) and proOmpA nuclease A (C) in 2 ml of 20 mM Tris, pH 8.0 buffer were centrifuged 5 hr at 100 000 x g at 4°C. After centrifugation, the supernatant was transferred to a new tube and the pellet was resuspended in a volume equal to the starting volume. Samples were then analyzed on a 17.2% SDS-PAGE gel and then stained with Coomassie Brilliant Blue. Samples were derived from uncentrifuged samples (T), pellet fraction (P) and supernatant fractions (S).
Fig. 4.7

A. Lep

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T S P

B. \( \Delta 2-75 \)

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T S P

C. OmpA-Nuclease

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T S P
replacement with the non-nucleophilic alanine residue results in at least a 500-fold decrease in the activity. Strikingly, we demonstrate that the detergent Triton X-100 promotes Δ2-75 catalyzed cleavage of the pre-protein and peptide substrates. For example, we find in the presence of Triton X-100, the $k_{cat}$ is 20-fold higher and has an apparent $K_m$ that is 6-fold lower than without detergent. Recently, it has been found that *E. coli* phospholipids comprised mostly of phosphotidylethanolamine, phosphotidylglycerol and cardiolipin (Raetz and Dowhan, 1990), stimulate the activity of Δ2-75 (Tschantz et al., 1995). Presently, it is unclear whether the requirement for detergent or phospholipid is a substrate or an enzyme effect. For example, detergent may be required to stabilize the leader peptidase hydrophobic stretch (residues 83-98) within Δ2-75, which contains the catalytic serine 90 residue at its center or it may be needed to stabilize the OmpA leader peptide of the proOmpA nuclease A substrate. However, we favor the hypothesis that the detergent (or phospholipid) requirement is an enzyme effect. First, much larger Δ2-75 crystals are formed when detergent is present in the crystallization buffer (M. Paetzel, M. Tchernaia, N. Strynadka, R. Dalbey, M. N. G. James, unpublished data) and we find that detergent stimulates the activity of Δ2-75 with two different substrates. It is intriguing that detergent is required for optimal activity at concentrations far below the critical micelle concentration of Triton X-100 (Tschantz et al, 1995), where the equilibrium favors the monomer form of the detergent (Helenius and Simons, 1975). This suggests that single detergent molecules, themselves, might be sufficient to enhance the catalytic activity of leader peptidase.
We ruled out that detergent is required to keep the Δ2-75 enzyme, proOmpA nuclease A and the Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile substrates soluble. Both Δ2-75 (fig. 4.3) and proOmpA nuclease A (data not shown) partition into the aqueous phase in a Triton X-114 extraction study and the peptide substrate is quite soluble in aqueous buffer.

Kuo et al. (1993) have reported that Δ2-75 has the same catalytic activity in the presence or absence of detergent and also that Δ2-75 has the same activity as the wild-type leader peptidase. The reason for these discrepancies reported here and Kuo and colleagues is not clear. Some differences in the protocols should be mentioned. Kuo et al. (1993) used the peptide substrate Ac-Trp-Leu-Val-Pro-NLeu-Leu-Leu-Ser-Phe-Ala-Ala-Glu-Gly-Asp-Pro-Ala-NH₂, corresponding to -8 to +7 positions of the procoat protein, to assay for leader peptidase activity. This peptide is a very poor substrate for the wild-type leader peptidase; the kcat is 0.0027 s⁻¹ (Kuo et al., 1993). In the present paper, the kcat is 44 s⁻¹ and 0.027 s⁻¹ for the wild-type enzyme with the proOmpA nuclease A and peptide (Phe-Ser-Ala-Ser-Ala-Leu-Ala-Lys-Ile-COO⁻) substrates, respectively. The peptide substrate and the more physiological pre-protein substrate used in this work may be better at discriminating small changes in leader peptidase activity than the substrate used by Kuo et al. (1993). In addition, Kuo et al. (1993), removed the detergent using an Extract-Gel D column. Although this procedure is useful, it is well known that residual detergent remains even after great efforts have been made to remove the detergent (Allen et al., 1980).
In conclusion, we have determined that the periplasmic domain of leader peptidase retains good catalytic properties of only 15-fold lower than that of the wild-type enzyme. However, we find that the presence of detergent is still required for optimal catalysis, suggesting that phospholipids may play an important role \textit{in vivo} in the catalytic mechanism. This is easy to envision since within the cell the active site serine 90 residue is spatially close to the membrane surface where it could cleave pre-proteins as they are translocated across the membrane. Finally, very recently, we have been able to purify large amounts of homogeneous and concentrated $\Delta2$-75 using a new purification procedure which has allowed us to obtain crystals of $\Delta2$-75 that are suitable for X-ray crystallography (M. Paetzel, M. Tchernaia, N. Strynadka, R. Dalbey, M. N. G. James, unpublished data). Work is now underway to solve the structure of leader peptidase, a member of a new protease family, and this should help in the elucidation of this novel catalytic mechanism.
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