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MECHANISTIC AND STRUCTURAL STUDIES OF PROKARYOTIC TYPE 1 SIGNAL (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
1997

Dissertation Committee:
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Prof. Patrick E. Ward

Approved by

Adviser
Department of Chemistry
ABSTRACT

Proteins which are translocated across the bacterial inner membrane are initially tethered to the membrane by an amino-terminal signal peptide. Type 1 leader (signal) peptidase, an integral membrane endopeptidase, plays an essential role of cleaving off signal peptides, thereby releasing exported protein from the lipid bilayer.

Bacterial leader peptidase is worthy of intense study due to its potential as a novel target for antibiotics as well as its unique mechanism. All evidence to date is consistent with leader peptidase utilizing a serine/lysine catalytic dyad mechanism, understanding this unique active-site arrangement will lead to a deeper understanding of the full structural and functional repertoire of the hydroxyl/amine interactions in proteins.

The goals of this research were to investigate the mechanism and structure of the Escherichia coli leader peptidase.

In chapter 2 of this work we review and discuss in detail the evidence for catalytic hydroxyl/amine dyads within serine proteases.

Chapter 3 contains a report on the investigation into the structural and chemical flexibility of the essential Lys145 within E. coli leader peptidase. In this study we have combining site-directed mutagenesis and chemical modification to produce lysine analogs at the 145 position. We have found that partial activity can be restored to an inactive K145C mutant by reacting this mutant with the reagents 2-bromoethylamine•HBr, 3-bromopropylamine•HBr, and 2-mercaptopethylamine, yet activity is not restored when reacted with the reagent
(2-bromoethyl)trimethylammonium·Br. These results are consistent with Lys145 of the
*E. coli* leader peptidase serving as a general base or as a critical hydrogen bond acceptor
in the deprotonated state. We also provide evidence that maleic anhydride inactivates
leader peptidase due to its modification of lys145. In addition, we have modeled the
active-site of *E. coli* leader peptidase based on the x-ray crystal structure of the *E. coli*
UmuD'. This model reveals a shallow hydrophobic cleft adjacent to the catalytic center.
We present a proposed mechanism for leader peptidase.

Chapter 4 describes the expression, purification, and characterization of a
catalytically active, soluble fragment of leader peptidase (Δ2-75). Reported here is an
improved purification procedure which has made possible the search for conditions for
the preparation of the crystalline form of this enzymes (chapter 5). We show that Δ2-75
leader peptidase has an isoelectric point of 5.6 and that it has optimal activity in the
presence of detergent or phospholipid even though it is lacking its transmembrane
segments.

Chapter 5 is a report of the first crystallization of a leader (or signal) peptidase.
Crystals of *E. coli* Δ2-75 leader peptidase were formed by the sitting drop vapor
diffusion technique using ammonium dihydrogen phosphate as the precipitant. The
detergent Triton X-100 was required to obtain crystals sufficiently large enough for x-
ray data collection and characterization. These crystals belong to the tetragonal space
group P4<sub>2</sub>2<sub>1</sub>2 with unit cell dimensions of \(a = b = 115\ \text{Å}\) and \(c = 100\ \text{Å}\), and contain
2 molecules per asymmetric unit. The limits of diffraction of these tetragonal crystals
varied from 3.6 Å to 2.5 Å. We have discovered a new crystal form of leader peptidase
(orthorhombic, P222<sub>1</sub>. \(a = 101.0\ \text{Å}, b = 112.5\ \text{Å}, c = 115.2\ \text{Å}\), 4 molecules per
asymmetric unit) which diffracts past 2.3 Å.

Chapter 6 discusses the data collection statistics on the orthorhombic crystal
form of *E. coli* Δ2-75 leader peptidase and the efforts to solve its structure by multiple
isomorphous replacement (MIR).
Dedicated to
Sandra Irene Paetzel and to
the memory of Charles William Paetzel
ACKNOWLEDGMENTS

I wish to thank my advisor, Professor Ross E. Dalbey, for providing me the opportunity to work on such an interesting enzyme. This work would not have been possible without his incredible enthusiasm and encouragement.

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I am forever thankful to Michael Dunne for his scientific advise as well as his friendship.

I am sincerely grateful to Professor Ming-Daw Tsai and Professor Lawrence J. Berliner for serving on my dissertation committee.

I would like to thank Proctor & Gamble for a 1 year graduate fellowship.

Lastly, I would like to thank my family; Natalie, Paul, Stephen and Sandra. Thank you for your love and encouragement.
VITA

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Emphasis in Biological Chemistry

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<td>2BEA</td>
<td>2-bromoethylamine·HBr</td>
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<tr>
<td>2BETMA</td>
<td>(2-bromoethyl)trimethylammonium·Br</td>
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<td>2MEA</td>
<td>2-mercaptopropanethiylamine</td>
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<td>3BPA</td>
<td>3-bromopropylamine·HBr</td>
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<tr>
<td>Å</td>
<td>ångstroms (10⁻¹⁰ meters)</td>
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<td>Ala (A)</td>
<td>Alanine</td>
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<td>Amp</td>
<td>ampicillin</td>
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<td>ammonium persulfate</td>
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<td>Asp (D)</td>
<td>aspartic acid</td>
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<td>B. subtilis</td>
<td>Bacillus subtilis</td>
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<tr>
<td>Bis</td>
<td>bisacrylamide</td>
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<tr>
<td>βME</td>
<td>β-mercaptoethanol</td>
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<td>CAPS</td>
<td>3-[cyclohexyl(amo)no]-1-propanesulfonic acid</td>
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<td>cysteine</td>
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<td>Da</td>
<td>Daltons</td>
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<td>DFP</td>
<td>di-isopropyl fluorophosphate</td>
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<td>dimethyl sulfoxide</td>
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<td>DTNB</td>
<td>5,5'-dithiobis(2-nitrobenzoic acid)</td>
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<td>ethylenediaminetetraacetic acid</td>
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<td>HEPES</td>
<td>4-[2-hydroxyethyl]-1-piperazine ethane sulfonic acid</td>
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<td>intensity of a x-ray reflection</td>
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<td>Isoelectric focusing</td>
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<td>isopropyl-β-D-thiogalactopyranoside</td>
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<tr>
<td>LB</td>
<td>Luria broth</td>
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<tr>
<td>Lep</td>
<td>Leader peptidase</td>
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<td>Leu (L)</td>
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<td>MA</td>
<td>Maleic anhydride</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamps</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein</td>
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<tr>
<td>Met (M)</td>
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<td>min.</td>
<td>Minute</td>
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<td>MPD</td>
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<td>Polyacrylamide gel-electrophoresis</td>
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<td>Polyethylene glycol</td>
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<tr>
<td>pI</td>
<td>Isoelectric point</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>Proline</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
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<td>Room temperature</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
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<td>Sec</td>
<td>Seconds</td>
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<td>Bacillus subtilis type 1 signal peptidase</td>
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<td>Tris(hydroxymethyl)aminomethane</td>
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<td>Wild-type enzyme</td>
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<td>°C</td>
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<tr>
<td>Kcat</td>
<td>Turnover number, the maximum number of substrate molecules converted to product per active site of the enzyme per unit time. (units = Sec$^{-1}$).</td>
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<td>Km</td>
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</table>
V_{max} \quad \text{Maximum velocity or rate of the reaction, occurs when the enzyme is saturated with substrate (units = moles/second)}

K_{cat}/K_{m} \quad \text{specificity constant, a rate constant that refers to the properties and reactions of the free enzyme and free substrate (unit = Sec^{-1}M^{-1})}

Mutation nomenclature used: e.g. K145C, lysine at position 145 is changed to cysteine.

The cleavage-site nomenclature: The substrate cleavage-site for leader peptidase is the amide bond linking the -1 residue (corresponding to the signal peptide residue) and the +1 residue (corresponding to the mature protein residue or the new amino-terminal residue). The -1 residue is also called the P1 site and the +1 residue is also called the P1' site in the Schechter and Berger (1967) nomenclature.
CHAPTER 1

INTRODUCTION

1.1 Signal peptidase, its role in the signal peptide strategy of sorting newly synthesized proteins

Both prokaryotic and eukaryotic cells are made up of different biochemically distinct compartments. For example gram negative bacteria such as *Escherichia coli* contain four distinct compartments; the cytoplasm, the inner membrane, the periplasm, and the outer membrane. One of the elements that make these compartments distinct from each other is the proteins that reside in these compartments. Newly synthesized proteins need to be sorted from their place of synthesis, usually the cytoplasm, to their final working environment.

The signal peptide strategy of sorting newly synthesized proteins into the different cellular compartments was discovered in 1972 by Milstein and coworkers. They observed that a secretory protein, immunoglobin light chain, was slightly larger in molecular weight when synthesized in an *in vitro* system lacking cell membranes. Further *in vitro* studies by Blobel and Dobberstein (1975) confirmed that secretory polypeptides contain an additional hydrophobic sequence of amino acids at their amino-terminus. Blobel and Dobberstein (1975) hypothesized that the role of the signal
peptide was to initiate the translocation of a precursor protein across the membrane into the lumen of the endoplasmic reticulum in eukaryotes or into the periplasm of prokaryotes. After the protein is translocated across the membrane, a protease (signal peptidase) cleaves off the hydrophobic signal peptide releasing the mature protein from the membrane and allowing it to move on to its ultimate destination.

Signal peptidases have been found in the secretory pathway of prokaryotes and eukaryotes as well as in organelles such as the mitochondria and the chloroplast.

1.2 Signal peptides

Signal peptides have very little sequence homology, but they do have some common features (von Heijne, 1990). Signal peptides typically contain a positively charged amino-terminal region (n-region), a central hydrophobic core region (h-region), and a specific signal peptidase recognition cleavage site (c-region). The determinants of the cleavage are a small uncharged residue (usually alanine) at positions -1 and -3 with respect to the cleavage site. Signal peptides often have a turn inducing amino acid (proline or glycine) approximately 6 residues amino-terminal to the cleavage site. (see Fig. 1.1).

1.3 Bacterial signal (leader) peptidase

The majority of proteins exported from bacteria contain a signal peptide which is processed by the the type 1 signal peptidase (also called leader peptidase). Type 2 signal peptidase cleaves the signal peptide from proteins which are first modified by a lipid (Pugsley and Schwartz, 1985). To date, fifteen bacterial type 1 signal peptidases have been sequenced (Cregg et al., 1996). The most extensively studied example is the *Escherichia coli* leader peptidase. It has been shown that leader peptidase is an essential enzyme for the viability of the *E. coli* cell (Dalbey and Wickner, 1985; Date, 1983).
Figure 1.1: General features of a typical bacterial signal (or leader) peptide.
This has lead to the possibility that bacterial signal peptidases may be a potential target for future antibiotics.

The goal of the work described in the following pages is to understand the mechanism and structure of leader peptidase and therefore set the stage for the rational design of novel antibiotics. It has been proposed that type 1 signal (leader) proteases utilize a serine/lysine catalytic dyad mechanism instead of the classical serine/histidine/aspartic acid catalytic triad. Chapter 2 discusses the evidence for this interesting mechanism. Chapter 3 presents further experimental evidence that is consistent with leader peptidase utilizing a serine/lysine dyad mechanism. To obtain information on the structure of leader peptidase we have purified, characterized (chapter 4), and crystallized (chapters 5) a soluble, catalytically active fragment of leader peptidase and we are presently working on a solution to this x-ray crystal structure by multiple isomorphous replacement (MIR) (chapter 6).
CHAPTER 2

CATALYTIC HYDROXYL/AMINE DYADS WITHIN SERINE PROTEASES

The "catalytic triad" mechanism which involves a serine, histidine, and aspartic acid, has become synonymous with serine proteases. However, recently, mechanistically novel serine proteases have been discovered. These proteases use hydroxyl/ε-amine or hydroxyl/α-amine "catalytic dyads" as their reactive centers.

2.1 Proteases are ubiquitous

Proteases are ubiquitous in nature and play pivotal roles in both intracellular and extracellular processes, as well as in the regulation of physiological pathways, including the degradation of misfolded proteins, processing short-lived signaling proteins, control of apoptosis and signal peptide cleavage. Most of the proteases fall within four groups: the serine, cysteine, aspartic acid and metallo proteases.
Figure 2.1: A. The classical “catalytic triad”. B. The Ser/Lys “catalytic dyad”. C. The hydroxyl/α-amine “catalytic dyad”.

A.

Asp ←——— O ———— H ———— N6 ———— H ———— Ser

B.

Lys ←——— N ———— H ———— O ———— Ser

C.
2.2 Catalytic triad mechanism

The standard mechanism for the serine proteases, which have evolved both by convergent and divergent evolution (Neurath, 1989), involves the catalytic triad. The catalytic triad consists of a histidine general base, which abstracts the proton from the serine hydroxyl sidechain, allowing the serine to act as a nucleophile and attack the carbonyl group of the amide bond within the protein substrate. The third player in the triad, an acidic residue, acts to orient the histidine residue and neutralize the charged histidine intermediate (Fig. 2.1a.). Deacylation of the enzyme involves an activated water acting as the nucleophile (Perona & Craik, 1995).

2.3 Catalytic triad dissected

Extensive site-directed mutagenesis studies have been carried out on the classical serine proteases to decipher the importance of each member of the Ser/His/Asp "catalytic triad". These investigations indicate that substitution of any of the catalytic triad residues results in large effects on catalysis, but the histidine and serine residues are the most important for catalysis. For instance, mutation of the catalytic serine or histidine residue within subtilisin (Carter & Wells, 1988) and trypsin (Corey & Craik, 1992) results in a $10^6$-fold decrease in activity. By contrast, mutation of the catalytic aspartate residue within subtilisin (Carter & Wells, 1988) or trypsin (Corey & Craik, 1992; Craik et al, 1987) results in a $10^4$-fold reduction in activity.

Despite being one of the most thoroughly studied class of enzymes, there still remains controversy over the "catalytic triad" mechanism proposed for the classical serine proteases such as subtilisin and trypsin.
2.4 Are some catalytic triads actually dyads?

It is striking that there is a proteolytic catalytic antibody in which the active site contains serine and histidine residues, but not an equivalent aspartic acid residue (Zhou et al., 1994). Similarly, the structure of an esterase from *S. scabies* reveals a serine and histidine residue, but instead of the aspartic acid, there is an aromatic tryptophan residue which cannot function as an acid. The authors suggest that this is an example of a catalytic dyad (Wei et al., 1995).

Another example of a potential catalytic dyad has been observed in the wheat serine carboxypeptidase; here, the carboxylate of the active site aspartate is not colinear with the imidazole of the histidine, which led Liao and coworkers to suggest that the catalytic triad of all serine proteases should be regarded as two dyads, a Ser/His dyad and a His/Asp dyad (Liao, et al., 1992).

Cysteine proteases, which have been the focus of considerable recent interest owing to their involvement in apoptosis, usually contain a Cys/His/Asn catalytic triad, which is analogous to the Ser/His/Asp triad in serine proteases. However, the asparagine is not absolutely critical: in papain, if it is replaced with alanine, only a 150-fold reduction in activity is observed (Vernet et al., 1995). Thus, even with proteases that possess a catalytic triad, the dyads Ser/His (serine proteases) and Cys/His (cysteine proteases) seem to be the most critical residues.

2.5 Hydroxyl/£-amine dyad catalysis

There is a growing list of serine proteases which contain an essential lysine, but no essential histidines (see Table 2.1). These proteases have been grouped into their own ancestral clan of serine peptidases (Rawlings & Barretts, 1994). It has been proposed that this group of proteases utilizes a “Ser/Lys dyad” mechanism, whereby an
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Biological function</th>
<th>Catalytic mechanism</th>
<th>Essential residues</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> Leader peptidase</td>
<td>signal peptide cleavage</td>
<td>Ser/Lys dyad</td>
<td>S90, K145</td>
<td>Tschantz et al., 1993</td>
</tr>
<tr>
<td><em>E. coli</em> LexA</td>
<td>repressor of SOS regulon</td>
<td>Ser/Lys dyad</td>
<td>S119, K156</td>
<td>Little et al., 1994</td>
</tr>
<tr>
<td><em>E. coli</em> UmuD</td>
<td>SOS mutagenesis response</td>
<td>Ser/Lys dyad</td>
<td>S60, K97</td>
<td>Peat et al., 1996</td>
</tr>
<tr>
<td><em>E. coli</em> Tap protease</td>
<td>tail specific protease</td>
<td>Ser/Lys dyad</td>
<td>S430, K455</td>
<td>Keller &amp; Sauer, 1995</td>
</tr>
<tr>
<td><em>E. coli</em> β-lactamase</td>
<td>antibiotic resistance</td>
<td>Ser/Lys dyad</td>
<td>S70, K73</td>
<td>Strynadka et al., 1992</td>
</tr>
<tr>
<td><em>Streptomyces</em> D-Ala-D-Ala peptidase</td>
<td>cell wall biosynthesis</td>
<td>Ser/Lys dyad</td>
<td>S62, K65</td>
<td>Kelly et al., 1989</td>
</tr>
<tr>
<td><em>Psuedomonas</em> Asparaginase</td>
<td>asparagine deamidation</td>
<td>Thr/Lys dyad</td>
<td>T100, K173</td>
<td>Lubkowski et al., 1994</td>
</tr>
<tr>
<td><em>E. coli</em> Penicillin acylase</td>
<td>unknown</td>
<td>Ser/α-amine dyad</td>
<td>S1, α-amine</td>
<td>Duggleby et al., 1995</td>
</tr>
<tr>
<td><em>T. acidophilum</em> 20S Proteasome</td>
<td>degradation of short-lived proteins</td>
<td>Thr/α-amine dyad</td>
<td>T1, α-amine</td>
<td>Lowe et al., 1995</td>
</tr>
</tbody>
</table>

**Table 2.1** Proteases and amidases which utilize a catalytic hydroxyl/amine dyad mechanism
Enzyme Residues Evidence

**Hydroxyl/(ε-Amine) Dyads**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Residues</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> Leader Peptidase</td>
<td>S90, K145</td>
<td>SDM₁,₂, CM²-₃, t⁴, pKa = 8.7³</td>
</tr>
<tr>
<td><em>E. coli</em> Repressor LexA</td>
<td>S119, K156</td>
<td>SDM⁵, t⁶, pKa = 9.3⁷,₈</td>
</tr>
<tr>
<td><em>E. coli</em> UmuD</td>
<td>S60, K97</td>
<td>SDM⁹, XS¹⁰</td>
</tr>
<tr>
<td><em>E. coli</em> Tsp protease</td>
<td>S430, K455</td>
<td>SDM¹¹, CM¹¹, t¹¹</td>
</tr>
<tr>
<td><em>E. coli</em> β-lactamase</td>
<td>S70, K73</td>
<td>SDM¹², CM¹³, t¹⁴, XS¹⁵</td>
</tr>
<tr>
<td><em>Streptomyces</em> D-Ala-D-Ala peptidase</td>
<td>S62, K65</td>
<td>SDM¹⁶, XS¹⁶</td>
</tr>
<tr>
<td><em>Psuedomonas</em> Asparaginase</td>
<td>T100, K173</td>
<td>SDM¹⁷, 1₈, XS¹⁹</td>
</tr>
</tbody>
</table>

**Hydroxyl/(α-Amine) Dyads**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Residues</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> Penicillin acylase</td>
<td>S1, α-amine</td>
<td>SDM²⁰, CM²¹, t²², XS²³</td>
</tr>
<tr>
<td><em>T. acidophillum</em> 20S Proteasome</td>
<td>T1, α-amine</td>
<td>SDM²⁴, XS²⁵</td>
</tr>
</tbody>
</table>

**Others Lysine General Bases**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Residues</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas putia</em> Mandelate Racemase</td>
<td>K166</td>
<td>SDM²⁶, XS²⁶, pKa = 6²⁶</td>
</tr>
<tr>
<td>yeast Enolase</td>
<td>K345</td>
<td>SDM²⁷, XS²⁸</td>
</tr>
<tr>
<td><em>E. coli</em> Aspartate Aminotransferase</td>
<td>K258</td>
<td>SDM²⁹, CM²⁹, XS³⁰, pKa = 7.3²⁹</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em> Leucine Dehydrogenase</td>
<td>K80</td>
<td>SDM³¹, XS³², pKa = 8.9³¹</td>
</tr>
</tbody>
</table>

**Table 2.2:** Evidence for enzymes which utilize an amine general base.

e-amino group of a lysine side chain acts as the general base to increase the nucleophilicity of the active site serine (see Fig. 2.1b.) (Slilaty & Little, 1987; Black, 1993). The best-characterized enzyme within this group are those from *Escherichia coli*: leader peptidase (Tschantz et al., 1993), LexA repressor (Slilaty & Little, 1987), and most recently Tsp protease (Keiler & Sauer, 1995). Generally, it appears that the Ser/Lys dyad proteases are less efficient than the triad proteases, although leader peptidase, which is very efficient against pre-protein substrates is an exception to this rule. Among the many interesting unanswered questions about the function of these enzymes, perhaps two of the most critical are (1) do they contain an "oxyanion hole" like the more classical proteases, and (2) how is the lysine maintained in the unprotonated state so that it is able to function as the general base? Answers to these questions will hopefully be provided by future experiments.

2.6 Leader peptidase

Although leader peptidase, which removes signal peptides from exported proteins is certainly a serine proteases, it is not possible to classify it as such by use of classical protease inhibitors. For instance, leader peptidase is not sensitive to serine protease inhibitors such as diisopropyl fluorophosphate (DFP) and phenyl methyl sulfonyl fluoride (PMSF) (Zwizinski et al., 1981). However, it is inhibited by certain 
β-lactams, which are believed to react with the catalytic serine residue (Kuo et al., 1994). Site directed mutagenesis studies have shown that within *E. coli* leader peptidase, Ser90 (Sung & Dalbey, 1992) and Lys145 (Black, 1993; Tschantz et al., 1993) are essential for activity. When Ser90 is replaced with Cys, the resulting active thiol90 leader peptidase can be inactivated by the cysteine-specific reagent N-ethyl maleimide (Tschantz et al., 1993). Interestingly, the lysine residue proposed to be involved in the catalytic dyad is conserved only within prokaryotes and the mitochondrial leader peptidases. The homologous subunits within the endoplasmic
reticulum signal peptidase do not contain the conserved lysine; rather, they contain a histidine residue at this position (van Dijl, et al., 1992).

Leader peptidase and the LexA-like enzymes share some sequence similarity in their active-site region, as well as similarity in their cleavage sites. Furthermore, they all show a preference for alanine at the P1 position (Rawlings & Barrett, 1994).

2.7 LexA

LexA, a repressor which can cleave itself in response to activation by the regulatory protein RecA, contains two essential residues, Ser119 and Lys156, which are proposed to be active-site residues (Slilaty & Little, 1987). The main evidence that Lys156 functions as the general base comes from pH-rate profile studies. The pH-rate profile of LexA, in the absence of its activator protein RecA, reveals that a basic group with a pKa of about 10 is important for catalysis (Slilaty et al., 1986). When Lys156 is changed to Arg, the optimal pH for autocatalysis increases to above 11. These results support the hypothesis that the deprotonation of a basic residue at this position is critical for activity (Lin & Little, 1989).

Interestingly, the crystal structure of the protein UmuD', which shares significant sequence homology with LexA, revealed that the side chains of its proposed active-site residues (Ser60 and Lys97) were positioned correctly to form a hydrogen bond (Peat et al., 1996). The active-site residues of UmuD' are also superimposable onto the active site residues of the class A ß-lactamase (see Fig. 2.2). Although the identity of the general base in class A ß-lactamase is still a controversy, there is strong crystallographic evidence for a serine/lysine dyad mechanism in these enzymes, which hydrolyze ß-lactam rings in a manner similar to peptide bond hydrolysis by proteases (Strynadka et al., 1992). These authors propose that the buried Lys73 is kept in the neutral state by association with two proximal carbonyl oxygens. These interactions help to align the lysine side-chain amine with the nucleophilic Ser70 hydroxyl group.
Figure 2.2: The backbone structure of UmuD' showing the active-site residues, Ser60 and Lys97. Superimposed are two segments of the TEM1 β-lactamase which contain the active-site residues Ser70 and Lys73.
(Strynadka et al, 1992). Conversely, NMR and chemical modification studies by the Frere laboratory support the theory that Glu166 working with an intervening water molecule acts as the general base (Damlon et al., 1996). The significance of the connection between the Ser/Lys dyads and the binding of β-lactam type inhibitors and substrates is still unknown. A model showing the initial step of the novel Ser/Lys catalytic dyad mechanism for leader peptidase and the LexA repressor is shown in Fig. 2.3.

2.8 Tsp protease

Recent studies with the Tsp protease have revealed that alanine substitutions of Ser430 and Lys455 inactivate the enzyme, suggesting that the serine and lysine are involved in the mechanism of this protease. Similar to leader peptidase, substitution of Ser430 with Cys results in a partially active thiol-protease that can be inhibited with cysteine-specific modifying reagents (Keiler & Saurer, 1995).

Other enzymes that appear to use a hydroxyl/amine dyad are highlighted in Table 2.1. These include D-Ala-D-Ala transpeptidase from Streptomyces (Kelly et al, 1989) and Pseudomonas 7A glutaminase-asparaginase (Lubkowski et al., 1994).

2.9 Hydroxyl/α-amine dyad catalysis: variations on a theme

Another type of hydroxyl/amine catalytic dyad, similar to the Ser/Lys dyad, is found at the active-site of penicillin acylase (Duggleby et al., 1995). This enzyme contains an amino-terminal serine residue which appears to use its own α-amino group as a general base (Fig. 2.1c.). Although not a protease, penicillin acylase hydrolyzes an amide bond in substrates such as penicillin G.
Figure 2.3: A. A model of the membrane topology of the *Escherichia coli* leader peptidase and its first step in the cleavage of a leader peptide from an exported protein. The ε-amino group of Lys145 serves as a general base to abstract the proton from the hydroxyl sidechain of Ser90, which then acts as the nucleophile to attack the scissile peptide bond of the translocated preprotein substrate. Leader peptidase has a substrate specificity for preproteins that contain a small uncharged residue at the P1 position (seen as a black dot in the figure) and a small uncharged or aliphatic residue at the P3 position (seen as a black dot in the figure) in the leader sequence. B. The first step in the intramolecular cleavage of the *E. coli* LexA repressor. The ε-amino group of Lys156 serves as a general base to abstract the proton from the sidechain of Ser119, which then acts as the nucleophile to attack the scissile peptide bond in this intramolecular cleavage reaction. The cleavage site for LexA is the peptide bond between Ala84 and Gly85, which lie in the hinge region (red). The amino-terminal region (green) is the DNA binding domain. The carboxy-terminal region (blue) contains the active-site residues involved in the specific cleavage.
Figure 2.3:
The 20S proteasome from *Archaeon T. acidophilum* also appears to employ an 
α-amino group as a general base in its proteolytic mechanism (Lowe et al, 1995). The 
proteasome is unique in that instead of a serine hydroxyl it uses a threonine hydroxyl as 
the nucleophile. Huber and coworkers (Lowe et al, 1995) propose, from their crystal 
structure of the proteasome, that Thr1 utilizes its own α-amino group in a hydrogen 
bonded five-membered ring structure to activate its own side-chain hydroxyl group (see 
Fig. 2.1c.). Alternatively they propose that Thr1 uses Lys33 as a general base.

2.10 How lysine can function as a general base in catalytic dyads

The ε-amino group of lysine must reside in the deprotonated state for lysine to 
act as a general base in catalytic dyads. The pKa of free lysine in water is 10.5. Therefore, the enzyme must provide a local environment for the lysine which will enable 
it to remain deprotonated.

There are many examples in the literature of enzymes which contain lysine 
residues with significantly depressed pKa values. These low pKa values are usually 
explained from an electrostatic or polarity view point. In the electrostatic situation, a 
lysine with a low pKa is generated when a positive charge, such as another lysine or an 
arginine, is immediately proximal to the lysine. This electrostatic effect in enzymes was 
first investigated in detail by Westheimer’s laboratory in their study of the enzyme 
acetoacetate decarboxylase (Westheimer, 1995).

An increase in a lysine side chain pKa can occur when there is a carboxylate 
nearby. In this scenario, the pKa of the ε-amino group increases, as a result of its 
greater affinity for protons in order to neutralize the interaction with the negatively 
charged carboxylate. Slilaty & Vu have shown that one of the two conserved negative 
charges within the LexA repressor family (Glu152) may be positioned near the 
proposed lysine general base (Slilaty & Vu, 1991). They demonstrate that removal of
this negatively charged residue actually increases the rate of the LexA cleavage reaction, and propose that a negative charge in the active site would impede the activity of the LexA reaction by stabilizing the transition state’s positively charged lysine, and as a result slow the protonation of the leaving amino group. They also point out that the ε-amino group of lysine, serving as a general base, is different from the imidazole side chain of histidine in that it does not require a counter charge to effect a proton transfer (Slilaty & Vu, 1991). Therefore, in the absence of the histidine general base, an active site negative charge appears to hinder the catalytic efficiency, and thus a dyad should be sufficient for catalysis. This was first observed when a 10-fold increase in activity was seen upon removing a negative charge (Asp32) from the active site of a subtilisin mutant which lacked the histidine general base (Carter & Wells, 1988).

A hydrophobic microenvironment is another situation which produces a lowered lysine pKa. There are examples of buried lysines with pKa values as low as 6.5 (Dao-pin et al., 1991).

In the case of the LexA repressor, the binding of the regulatory protein RecA is required in vivo for the intramolecular proteolysis to occur. In the absence of RecA, proteolysis will occur only at elevated pH (>10). It is proposed that the RecA reduces the pKa of the Lys156 so that it is deprotonated at physiological pH (Little et al., 1994). There is no evidence yet to show whether the effect that RecA has on the pH at which autocatalysis occurs is due to electrostatic, polar, or steric effects.

As the serine (hydroxyl) and lysine (amine) interactions are being investigated in the biochemical field, the physical organic chemists are exploring with great interest the triple hydrogen bond arrangement that can occur between these functional groups (Borman, 1995) (Fig. 2.1b.). The additional stability of this hydrogen bonding arrangement may contribute to low lysine pKa values in some cases. But it is possible that such hydrogen bonds would form only upon binding of the substrate, if they are similar at all to the classical serine proteases. It has been shown for the classical serine proteases that a strong hydrogen bond between the catalytic serine hydroxyl group and the catalytic histidine imidazole group usually only forms upon binding of the substrate.
Conversely, the proposed catalytic residues of the free RTEM-1 β-lactamase (Lys73 and Ser70), as well as the catalytic residues of UmuD' (Lys97 and Ser60), have been shown to be within hydrogen-bonding distance (Strynadka et al., 1992; Peat et al., 1996). Investigation into the mechanism of intramolecular general base catalysis is still underway at the small-molecule level and might lead to insights regarding these reactions within enzymes (Kahn & Arifin, 1996).

2.11 Concluding remarks

Recent developments have shown that serine proteases can function without the landmark Ser/His/Asp "catalytic triad". It has been proposed that these proteases carry out catalysis, in some instances, employing an active-site Ser/Lys dyad. This novel mechanism, where lysine is believed to function as a general base, may help to explain why such proteases are resistant to inhibitors against the classical serine protease group. Other variations from the classical catalytic triad theme exist, such as that found in the penicillin acylase, which seems to utilize just one residue, the amino-terminal serine, as its active center.

So far, catalytic hydroxyl/amine dyads are found predominantly in enzymes from prokaryotic sources or sources that are believed to have evolved from prokaryotes. Thorough study of these enzymes will lead to a deeper understanding of the full structural and functional repertoire of the hydroxyl/amine interactions in proteins and will be instrumental in the rational design of novel antibiotics.
CHAPTER 3

USE OF SITE-DIRECTED CHEMICAL MODIFICATION TO STUDY AN ESSENTIAL LYSINE IN *ESCHERICHIA COLI* LEADER PEPTIDASE

3.1 Introduction

*E. coli* leader (signal) peptidase is an integral membrane serine protease which functions to cleave off the amino-terminal leader (signal) sequence from proteins which are targeted to the cell surface of bacteria. Leader peptidase has been cloned (Date & Wickner, 1981), sequenced (Wolfe et al., 1983), overexpressed (Dalbey & Wickner, 1985; Wolfe et al., 1982), and purified (Wolfe et al., 1982; Tschantz & Dalbey, 1994). The use of protease inhibitors to classify leader peptidase into a specific protease class has failed (Allsop et al., 1995; Zwizinski et al., 1991). Site-directed mutagenesis studies have demonstrated that there is an essential serine 90 (Sung & Dalbey, 1992) and lysine 145 (Black, 1993; Tschantz et al., 1993), yet no essential histidines or cysteines (Sung & Dalbey, 1992). The most convincing evidence for serine 90 being the nucleophile in the proteolytic reaction was provided by the work of Tschantz et al.
(Tschantz et al., 1993) which showed that when serine 90 is replaced with a cysteine, leader peptidase is still active and that this thiol-leader peptidase could then be inhibited by reacting it with the cysteine specific reagent N-ethylmaleimide (Tschantz et al., 1993).

The essential serine and lysine are fully conserved within the type 1 prokaryotic and mitochondrial signal peptidases (Dalbey & von Heijne, 1992; van Dijl et al., 1992). There are 19 lysines in the *E. coli* leader peptidase and only lysine 145 is conserved. Interestingly, the essential lysine is replaced by a histidine in the homologous yeast, chicken, and canine endoplasmic reticulum signal peptidase subunits (Dalbey & von Heijne, 1992; van Dijl et al., 1992).

All evidence to date points towards leader peptidase utilizing a serine/lysine dyad mechanism. With this mechanism, the lysine 145 would act as the general base to abstract the proton from the hydroxyl group of the serine 90 side chain, thereby allowing for the nucleophilic attack on the scissile peptide bond of the translocated preprotein substrate. Leader peptidase along with LexA (Roland & Little., 1990), UmuD (Peat et al., 1996), and most recently, Tsp protease (Keiler & Sauer, 1995) represent the most thoroughly characterized members of the class (Clan) of serine proteases which contain an essential lysine, yet no essential histidine residue (Rawlings & Barretts, 1994).

For lysine to act as a general base its side chain amine must be unprotonated. For this to be possible an enzyme must provide an environment for the lysine in which its pKa would be depressed. The microenvironment near the lysine would include either a local positive charge or a hydrophobic surrounding. There are many examples of lysine residues which have significantly lower pKa’s as compared to the pKa of 10.5 for lysine in solution (Dao-pin et al., 1991; Planas & Kirsch, 1991). One of the most carefully studied active site lysines is that of acetoacetate decarboxylase, which has a pKa of 6.0 (Highbarger & Gerlt, 1996; Westheimer, 1995). Moreover, there is crystallographic evidence that lysine is capable of serving as a general base for a serine
residue in β-lactamase (Strynadka et al., 1992), and most recently in the structure of the *E. coli* UmuD' protein (Peat et al., 1996). These two enzymes have catalytic-sites which are superimposable (Peat et al., 1996).

To further investigate the structural and chemical flexibility of the essential lysine 145 in the catalysis of leader peptidase, we have combined site-directed mutagenesis with chemical modification to introduce unnatural amino acids ("lysine-like analogs") at this position. To simplify the interpretation of the results we have used an active mutant in which all native cysteine residues have been replaced with serine residues. This cysteine-less variant of leader peptidase will be referred to as No Cys leader peptidase. The K145C mutant in the No Cys leader peptidase background presents a single cysteine within the enzyme for modification. This inactive K145C mutant, which will from now on be referred to as K145C, No Cys, regains activity after being reacted with the reagent 2-bromoethylamine•HBr to form a lysine analog (γ-thia-lysine or S-(aminoethyl)-cysteine) at the 145 position. We also show that leader peptidase is inhibited by the lysine modifying reagent maleic anhydride, and we provide evidence that the inactivation of leader peptidase upon reaction with maleic anhydride is due to its modification at lysine 145. In addition, we have modeled the active-site of *E. coli* leader peptidase based on the structure of *E. coli* UmuD'(Peat et al., 1996), and proposed a mechanism for leader peptidase. The study reported here is consistent with leader peptidase utilizing a serine/lysine dyad mechanism in its catalysis.

### 3.2 Experimental procedures

#### 3.2.1 Materials

The 2-Bromoethylamine•HBr (2BEA), 3-Bromopropylamine•HBr (3BPA), (2-bromoethyl)trimethylammonium•Br (2BETMA), 2-mercaptoethylamine (2-MEA), and
(S)-2-aminoethyl-L-cysteine·HCl were purchased from Sigma. The maleic anhydride was from Matheson Coleman & Bell.

3.2.2 Bacterial strains and plasmids

The leader peptidase proteins were expressed in MC1061 *E. coli* cells harboring the pING plasmid carrying the mutant leader peptidase gene. These proteins contained six consecutive histidine residues engineered into the P1 (cytoplasmic) domain of leader peptidase which allowed for the purification of the mutants from the chromosome-expressed wild-type leader peptidase. Briefly, amino acid residues 35 to 40 were substituted with histidine residues. The No Cys variant of *E. coli* leader peptidase had the three cysteines at positions 21, 170, and 176 replaced with serine residues.

The pro-OmpA nuclease A gene was in the IPTG-inducible plasmid pONF1 (Takahara et al., 1985).

3.2.3 Purification of the 6-His tagged leader peptidase proteins

The 6-His tag / nickel affinity chromatography method (Smith et al., 1988) was used to purify the overexpressed leader peptidase mutants away from the wild-type chromosome-expressed copies of leader peptidase. All leader peptidase proteins used in this study, except the wild-type, contained the 6-His tag. *E. coli* MC1061 cells containing the pING plasmid encoding the mutant leader peptidase protein were grown in M9 minimal media (1-8 liters) containing 100 μg/ml ampicillin until an absorbance of 0.5 at 600 nm was reached. Expression was induced by the addition of arabinose to a final concentration of 0.3%, and the incubation of the cultures were continued for 4 hours. The cells were pelleted and then resuspended in an equal weight of 50 mM Tris, pH 7.5, 10% sucrose. The cells were frozen by dropping them into liquid nitrogen and stored at -80 °C until needed. 10 g of frozen cell nuggets were added to 25 ml of thaw buffer (50 mM Tris, pH 7.5, 20% sucrose) and thawed. Lysozyme (6 mg) and DNase
(60 µl at 10 mg/ml) were added to the thawed cells and then stirred for 10 minutes. The mixture was then freeze/thawed in a dry ice/ethanol bath. 200 µl of 1M Magnesium acetate was added and the solution was allowed to stir for 15 minutes at room temperature. The solution was then centrifuged at 18,000 rpm (4°C, 30 minutes) and the pellet was resuspended in 25 ml of 10 mM triethanolamine, 10% glycerol, pH 7.5. The centrifugation step was then repeated once more. The pellet was resuspended by douncing in binding buffer (5mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 8.0, 1% Triton X-100, 10 mM β-mercaptoethanol). The suspension was then centrifuged again at 18,000 rpm. The supernatant was loaded onto a 1 ml nickel column (Novagen resin) which was equilibrated with the same buffer. The column was then washed with 20 ml of binding buffer followed by a second wash with wash buffer (60 mM imidazole, 0.5 M NaCl, 10 mM Tris, pH 8.0, 1% Triton X-100, 10 mM β-mercaptoethanol). The 6-His tagged leader peptidase was eluted by using an imidazole step gradient from 100 to 500 mM imidazole. Eluted fractions were assayed for protein by SDS-PAGE followed by Coomassie staining. Fractions containing 6-His leader peptidase were dialyzed against 50 mM Tris-HCl pH 8.0, 1% Triton X-100, 10 mM β-mercaptoethanol and then stored at -80°C.

3.2.4 Purification of the pro-OmpA nuclease A

The *E. coli* strain SB211 containing the plasmid pONF1 was used to overexpress the pro-OmpA nuclease A substrate, a hybrid of the signal peptide of the outer membrane protein A (OmpA) fused to *staphylococcal* nuclease A (Takahara et al., 1985). The pro-OmpA nuclease A was expressed and purified as described by Chatterjee et al. (1995).
3.2.5 Kinetic assay using pro-OmpA nuclease A

To determine the kinetic constants (Vmax, kcat, and Km) of the chemically modified K145C leader peptidase proteins, we used pro-OmpA nuclease A as a substrate. Substrate concentrations were determined by using an E1%o at 280 nm of 8.3 (Chatterjee et al., 1995). The cleavage reactions (75 μl) were run in TGC buffer (50 mM Tris, 50 mM Glycine, 50 mM CAPS, 10 mM CaCl2, 1% Triton X-100) at pH 9.0, unless indicated otherwise, containing the substrate at 5 different concentrations (35.2, 17.6, 13.2, 8.8, 4.4 μM). The reaction was initiated by the addition of leader peptidase at a final concentration of 1.37 x 10^-4 μM, which was determined by the Pierce BCA protein assay kit. The reaction was carried out at 37°C and aliquots of the reaction were removed at various times such that less than 7% processing of the substrate was achieved. The reaction was stopped by the addition of 5 μl of 5X sample buffer containing 10 mM MgCl2 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 band by the sum of the mature and precursor band areas. The initial rates were determined by plotting the amount of product versus time. The Vmax, Km and kcat values were calculated from a 1/vi versus 1/[s] plot. We used the computer programs KaleidoGraph and CricketGraph to plot the data and for linear regression analysis of the data. All values are from at least 2 different experiments.

3.2.6 Chemical modification of the K145C, No Cys leader peptidase

The reactions were carried out basically as described by Smith and Hartman (1988). Briefly, leader peptidase at a concentration of 1 - 4 mg/ml in 50 mM Tris-HCl,
pH 8.5, 1% Triton X-100 was treated with a sufficient amount of freshly prepared 2M 2-bromoethylamine·HBr (2BEA) (Planas & Kirsch, 1991; Smith and Hartman, 1988; Gloss and Kirsch, 1995a; Messmore et al, 1995; Itano & Robinson, 1972; Gloss and Kirsch, 1995b; Lorimer et al., 1993; Raftery and Cole, 1966; Cole, 1967; Lindley, 1956), 2M 3-bromopropylamine·HBr (3BPA) (Gloss and Kirsch, 1995a; Messmore et al, 1995), 2M 2-bromoethyl)trimethylammonium·Br (2BETMA) (Messmore et al, 1995; Itano & Robinson, 1972), or 2M 2-mercaptoethylamine (2MEA) (Gloss and Kirsch, 1995a) such that the final concentration of reagent was 100 mM. All reagents were titrated to pH 8.5 before adding them to the enzyme. The reaction solution was then covered with nitrogen and incubated at room temperature overnight. The reaction mixture was then dialyzed against 2 volumes of 4.5 liters of 50 mM Tris-HCl pH 8.0, 1% Triton X-100 at 4 °C. All buffers and solutions were purged with nitrogen. The extent of reaction was assayed by quantification of cysteine residues with DTNB before and after reaction with the reagents. The extent of the 2BEA reaction was also quantified directly by amino acid composition.

3.2.7 Quantifying cysteines with DTNB

A solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, 100 µl at 10 mM) in 0.1 M phosphate, pH 7.28, 1 mM EDTA was added to 275 µl of 4 mg/ml leader peptidase and 625 µl of 6.4 M guanidine-HCl. This mixture was incubated for 15 minutes at room temperature, and then its absorbance was measured at 412 nm. A molar absorbance coefficient of 13,700 M⁻¹ cm⁻¹ was used to calculate the moles of cysteine present in the samples (Riddles et al, 1979). All solutions were made up fresh and purged with nitrogen. The concentration of the protein was determined using the Pierce BCA method. The K145C, No Cys leader peptidase was stored in reducing conditions. Before the DTNB reaction, or other modifications, the β-mercaptoethanol
was dialyzed away in nitrogen purged buffer (2 volumes of 4.5 liters of 50 mM Tris-HCl pH 8.0, 1% Triton X-100) at 4 °C.

3.2.8 Amino acid compositional analysis

To directly quantify the extent of aminoethylation of the cysteine 145 we measured the appearance of a γ-thia-lysine residue within the leader peptidase protein by using (S)-2-aminoethyl-L-cysteine·HCl (Sigma) as a standard in the amino acid compositional analysis. The amino acid analysis was performed at the W.M. Keck Foundation Biotechnology Research Laboratory in New Haven CT.

3.2.9 Combined maleic anhydride and 2-bromoethylamine reaction

A total of 0.007 μmoles of the No Cys or the K145C, No Cys mutant leader peptidase was first reacted with 0.7 μmoles of DTNB and then dialyzed against 50 mM Tris-HCl, 0.5% Triton X-100, pH 8.5 (buffer A). The mutants were then reacted with 2.3 μmoles of maleic anhydride, incubated at room temperature for 30 minutes, and then dialyzed against buffer A. The cysteine in the K145C, No Cys mutant was then deprotected by reaction with β-mercaptoethanol followed by dialysis. These samples (60 μl) were then reacted overnight at room temperature with 10 μl of freshly prepared 2M 2-bromoethylamine·HBr. This product was then reacted with maleic anhydride again. All reagents were made up fresh in nitrogen purged buffer. After each reaction the samples were dialyzed extensively (overnight against 9 liters of nitrogen purged buffer A). The activity of the leader peptidase enzymes were assayed before and after each chemical modification step.
3.2.10 Alignment of leader peptidase with UmuD' and modeling the leader peptidase active-site

We have aligned the amino acid sequence of the catalytic region from the solved structure of UmuD' (residues 40-139, the total length of the UmuD protein is 218 residues) with the corresponding proposed catalytic region of Leader peptidase (residues 75-202, the total length of Leader peptidase is 323 amino acid residues). The alignment protocol XALIGN (Wishart et al., 1994a,b) used is a derivative of the NW_ALIGN program originally developed for SEQSEE (Wishart et al., 1994b). The pairwise alignment module implemented in the comparison of UmuD' and leader peptidase is based on the Needleman-Wunsch dynamic programming algorithm (Needleman & Wunsch, 1970) and the sequence/structure alignment algorithms are based loosely on the protocols described by Lesk et. al. (1986). Residue anchoring and residue clustering features are analogous to the gap and extension penalties incorporated into regular dynamic programming schemes (Wishart et al., 1994a). The appropriate substitutions of the UmuD' residues as well as manual manipulations were done using the program TOM-frodo (Jones, 1985). The coordinates for the crystal structure of UmuD' were kindly provided by T. Peat and W. Hendrickson (Peat et al., 1996). The rendering of the modeled active-site region and hydrophobic cleft adjacent to the active-site of E. coli leader peptidase was created using the program Raster3D (Merrit & Murphy, 1994).

3.3 Results

3.3.1 Purification of the 6-His tagged mutants

The use of the 6-His tag / nickel affinity chromatography method (Smith et al., 1988) of purification has allowed us to purify the overexpressed mutants of leader peptidase away from the wild-type leader peptidase expressed by the E. coli
chromosome. Due to leader peptidases excellent kinetic properties, background activity from the chromosomal wild-type copies of leader peptidase was always a concern when assessing the activity of overexpressed mutants of leader peptidase in the past. A mock purification run was performed to demonstrate that there was no detectable background wild-type leader peptidase activity from this purification procedure. Cells containing the expression vector with no leader peptidase gene insert were lysed and brought through the nickel affinity column procedure. All fractions eluted from the column showed no detectable activity (data not shown). The yield of each purified mutant was approximately the same. We obtained typically 4.6 mg of purified K145C, No Cys from 10 grams of frozen cell nuggets (See experimental procedure).

3.3.2 Restoration of activity to the inactive leader peptidase mutant K145C, No Cys

We were able to restore partial activity to an inactive K145C, No Cys mutant of leader peptidase by reacting it with the reagent 2-bromoethylamine•HBr (2BEA, Fig 3.1A). Figure 3.1 A shows significant processing of the pro-OmpA nuclease A substrate at 1 and 10-fold dilutions of the 2BEA modified leader peptidase. This chemically modified K145C, No Cys mutant has a kcat value which is approximately 100-fold lower than that of the wild-type leader peptidase (see Table 3.1). The control experiment in which we have used the No Cys mutant, containing the native lysine at the 145 position, showed no change in the activity upon incubation with the 2BEA (Fig. 3.1B).

Modification of the K145C, No Cys mutant with the reagents 3-bromopropylamine•HBr (3BPA) and 2-mercaptoethylamine (2MEA) also restored activity to this inactive mutant, although to a slightly lower extent (Fig. 3.2, 3.3 and Table 3.1). The latter recovery in activity by 2-mercaptoethylamine is most likely due to formation of a lysine analog at cysteine 145 as reaction of the modified leader peptidase
Figure 3.1: Restoring activity to the leader peptidase mutant K145C, No Cys via modification with 2-bromoethylamine·HBr (2BEA).

\[
\text{Br}
\quad \text{NH}_3^+ 
\quad + \quad \text{K145C} \xrightarrow[2\text{BEA}]{\text{K145C-EA, No Cys Lep}} \quad \text{K145C, No Cys Lep}
\]

A. A 17.2% SDS-PAGE gel stained with Coomassie Brilliant Blue shows the processing of the pro-OmpA nuclease A pre-protein substrate (10 µl at 11.75 µM) (P) to the mature form (M) after a 1 hour incubation with 1 µl of each dilution (starting at 2.7 µM) of the K145C, No Cys mutant of leader peptidase before and after modification with 2-bromoethylamine·HBr (2BEA). B. As a control the same experiment was run with the No Cys leader peptidase mutant containing the native lysine at the 145 position. All leader peptidase samples were purified by the 6-His tag method (see experimental procedures). The extent of the aminoethylation reaction was accessed by quantitation of free cysteines by DTNB (see Table 3.2), and by direct amino acid compositional analysis using (S)-2-aminoethyl-L-cysteine as a standard. See experimental procedures for more details on the reactions and the analysis of the results. See Table I for results of kinetic reactions.
Figure 3.1:
Figure 3.2: 2-bromoethylamine·HCl does not restore activity to the K145H mutant of leader peptidase. A 17.2% SDS-PAGE gel stained with Coomassie Brilliant Blue shows no processing of the pro-OmpA nuclease A pre-protein substrate (10 µl at 11.75 µM) (P) to the mature form (M) after a 1 hour incubation with 1 µl of each dilution (starting at 2.7 µM) of the K145H mutant of leader peptidase before and after modification with 2-bromoethylamine·HBr (2BEA).
Figure 3.3: Restoring activity to the leader peptidase mutant K145C, No Cys via modification with 3-bromopropylamine•HBr (3BPA).

A 17.2% SDS-PAGE gel stained with Coomassie brilliant blue shows the processing of the pro-OmpA nuclease A pre-protein substrate (10 μl at 11.75 μM) (P) to the mature form (M) after a 1 hour incubation with 1 μl of each dilution (starting at 2.7 μM) of the K145C, No Cys mutant of leader peptidase before and after modification with 3-bromopropylamine•HBr (3BPA). The extent of modification was assessed by quantifying the cysteine residues with DTNB before and after the reaction with 3BPA (see Table 3.2). See experimental procedures for details of the analysis.
**Figure 3.4:** Restoring activity to the leader peptidase mutant K145C, No Cys via modification with 2-mercaptoethylamine (2MEA).

A 17.2\% SDS-PAGE gel stained with Coomassie brilliant blue shows the processing of the pro-OmpA nuclease A pre-protein substrate (10 \( \mu l \) at 11.75 \( \mu M \)) (P) to the mature form (M) after a 1 hour incubation with 1 \( \mu l \) of each dilution (starting at 2.7 \( \mu M \)) of the K145C, No Cys mutant of leader peptidase before and after modification with 2-mercaptoethylamine (2MEA). The extent of modification was assessed by quantifying the cysteine residues with DTNB before and after the reaction with 2MEA (see Table 3.2). Other details of the modification of the K145C, No Cys mutant are described in the experimental procedures.
A positively charged, nontitratable lysine analog at the 145 position does not restore activity to K145C, No Cys leader peptidase.

A 17.2% SDS-PAGE gel stained with Coomassie Brilliant Blue shows the processing of the pro-OmpA nuclease A pre-protein substrate (10 µl at 11.75 µM) (P) to the mature form (M) after a 1 hour incubation of the K145C, No Cys mutant of leader peptidase (1 µl at 2.7 µM) reacted with either 2-bromoethylamine·HBr (2BEA) or (2-bromoethyl)trimethylammonium·Br (2BETMA) for various lengths of time. The extent of reaction was assessed by quantifying the cysteine residues with DTNB before and after the reaction with 2BETMA or 2BEA (see Table 3.2).
Table 3.1: Kinetic parameters for the cleavage of the preprotein substrate proOmpA nuclease A by the wild-type, mutant, and chemically modified *E. coli* leader peptidase. All kinetic parameters were measured at pH 9.0. All enzymes, except the wild-type, contain the 6-His tag and were purified by the 6-His tag / nickel affinity chromatography method (Smith et al., 1988). The K145C mutant had all other cysteine residues mutated to serine (K145C, No Cys). The following mutants showed no detectable activity: K145C, K145C-ETMA, K145A, K145R, K145H.
<table>
<thead>
<tr>
<th>Residue 145</th>
<th>Side Chain</th>
<th>Lnp Nomenclature</th>
<th>kcat (sec⁻¹)</th>
<th>Km (µM)</th>
<th>kcat/Km (sec⁻¹ M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>-CH₂CH₂CH₂NH₃⁺</td>
<td>WT</td>
<td>120.0 ± 10.7</td>
<td>10.9 ± 2.8</td>
<td>(1.1 ± 0.2) x 10⁷</td>
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<tr>
<td>(Wild-Type)</td>
<td></td>
<td>No Cys</td>
<td>110.2 ± 0.1</td>
<td>20.6 ± 6.8</td>
<td>(5.4 ± 1.0) x 10⁶</td>
</tr>
<tr>
<td>(R146A)</td>
<td></td>
<td>R146A</td>
<td>36.8 ± 11.0</td>
<td>29.0 ± 3.0</td>
<td>(1.3 ± 0.2) x 10⁶</td>
</tr>
<tr>
<td>Cysteine</td>
<td>-CH₂SH</td>
<td>K145C, No Cys</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>S-(aminooethyl)cysteine</td>
<td>-CH₂SCH₂CH₂NH₃⁺</td>
<td>K145C-EA, No Cys</td>
<td>1.2 ± 0.1</td>
<td>20.1 ± 2.1</td>
<td>(6.1 ± 1.1) x 10⁴</td>
</tr>
<tr>
<td>(γ-thia-lysine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-(aminopropyl)cysteine</td>
<td>-CH₂SCH₂CH₂NH₃⁺</td>
<td>K145C-PA, No Cys</td>
<td>0.8 ± 0.1</td>
<td>21.2 ± 5.1</td>
<td>(4.2 ± 0.5) x 10⁴</td>
</tr>
<tr>
<td>(γ-thio-homo-lysine)</td>
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<td></td>
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</tr>
<tr>
<td>S-(aminothiol)cysteine</td>
<td>-CH₂SSCH₂CH₂NH₃⁺</td>
<td>K145C-MEA, No Cys</td>
<td>0.3 ± 0.1</td>
<td>21.1 ± 2.4</td>
<td>(1.5 ± 0.6) x 10⁴</td>
</tr>
<tr>
<td>(γ-dithio-homo-lysine)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-[(trimethylammonio)ethyl] cysteine</td>
<td>-CH₂SCH₂CH₃N(CH₃)⁺</td>
<td>K145C-ETMA, No Cys</td>
<td>a</td>
<td>a</td>
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Table 3.1
<table>
<thead>
<tr>
<th>reagent</th>
<th>moles of Cys / moles of Lep before reaction</th>
<th>moles of Cys / moles of Lep after reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-bromoethylamine·HBr</td>
<td>0.95 +/- 0.11</td>
<td>0.09 +/- 0.07</td>
</tr>
<tr>
<td>3-bromopropylamine·HBr</td>
<td>1.13 +/- 0.09</td>
<td>0.16 +/- 0.11</td>
</tr>
<tr>
<td>2-mercaptoethylamine</td>
<td>0.89 +/- 0.14</td>
<td>0.11 +/- 0.07</td>
</tr>
<tr>
<td>(2-bromoethyl)trimethylammonium·Br</td>
<td>0.97 +/- 0.17</td>
<td>0.07 +/- 0.04</td>
</tr>
</tbody>
</table>

**Table 3.2:** Extent of reaction assayed by quantifying cysteines with DTNB
See experimental procedures for specifics on the analysis.
with a large excess of β-mercaptoethanol resulted in the disappearance of the restored activity (data not shown). The 2MEA reaction goes nearly to completion (see Table 3.2). The disulfide bond formation between the reagent and the free cysteine would most likely occur during the dialysis step when the reagent would not be in great excess (see experimental procedures).

Modification of the K145C, No Cys leader peptidase mutant with the reagent (2-bromoethyl)trimethylammonium•Br to form the non-titratable quaternary amine lysine analog (4-thialaminine) at the 145 position showed no detectable restoration of activity (Fig. 3.5). No recovery in activity of the K145C, No Cys mutant is seen even after treating the protein for 24 hours with 2BETMA. In contrast, recovery is seen after two hours of treatment with 2BEA. This is consistent with lysine 145 being involved in a critical hydrogen bond or serving as a general base in the catalysis.

To assess the extent of reaction between the K145C, No Cys mutant of leader peptidase with each of the above reagents we used DTNB to quantify the number of cysteines with and without the modification reaction. We have found that all of these reactions went nearly to completion (Table 3.2). The extent of modification by 2BEA was also directly measure by amino acid compositional analysis using the standard (S)-2-aminoethyl-L-cysteine•HCl (Sigma). We saw the appearance of 1.1 residues of aminoethylated cysteine (γ-thia-lysine) upon reaction with 2BEA.

3.3.3 Inhibition of leader peptidase activity by modification of lysine 145 with maleic anhydride

In prior work it was found that maleic anhydride inhibits leader peptidase in a concentration dependent and time dependent manner (Tschantz, 1995 dissertation). To investigate whether this inhibition with maleic anhydride was due to the modification of lysine 145, we reacted the K145C, No Cys mutant with maleic anhydride to modify all accessible lysines. We then attempted to react the cysteine at the 145 position with
Figure 3.6: Modification of Lysine 145 with maleic anhydride inhibits leader peptidase. Protection of Cys 145 in the K145C, No Cys mutant of leader peptidase with DTNB allows for the modification of lysines with Maleic anhydride (MA) followed by deprotection with \( \beta \)-mercaptoethanol (\( \beta \)ME) and then restoration of activity by reaction with 2-bromoethylamine•HBr (2BEA). This restored activity can then be inhibited by maleic anhydride. A 17.2% SDS-PAGE gel stained with Coomassie Brilliant Blue shows the processing of the pro-OmpA nuclease A pre-protein substrate (P) to the mature form (M) after a 1 hour incubation with dilutions of the K145C, No Cys mutant of leader peptidase before and after the final maleic anhydride modification. See experimental procedures for the details of each of the above reactions.
2BEA to restore activity and also produce a single accessible amine which then could be modified by maleic anhydride with subsequent inhibition of activity. Our preliminary experiments had shown that after treatment with maleic anhydride the activity of K145C, No Cys leader peptidase was no longer recoverable (data not shown). Maleic anhydride is known to react with thiol groups as well as amino groups (Means & Feeney, 1971). Therefore we protected the cysteine by reacting it with DTNB to form the TNB protected cysteine before reacting it with maleic anhydride. We were then able, after deprotection with βME, to recover the activity of the K145C, No Cys Leader peptidase by aminoethylation, and then finally inhibit this recovered activity with maleic anhydride (Fig. 3.6). The recovered activity from the DTNB/maleic anhydride/β-mercatoethanol/2BEA reaction was approximately 33 % of that seen from the 2BEA reaction alone (data not shown). Maleic anhydride is so far the only lysine specific reagent we have found which inhibits leader peptidase to a significant extent.

3.3.4 Sequence alignment of leader peptidase with UmuD' and modeling of the *E. coli* leader peptidase active-site

As a first step toward obtaining an idea of what the active-site of leader peptidase may look like, we have modeled the active-site region of *E. coli* leader peptidase based on the x-ray crystal structure of *E. coli* UmuD' (Peat et al., 1996). Previously, van Dijl and colleagues have shown that these proteins are structurally and functionally related (van Dijl et al., 1995). The residue anchoring feature of the alignment program XALIGN proved to be particularly important in the alignment of UmuD' and leader peptidase. High scoring pairwise alignments were only achieved when the two catalytic residues of the Ser/Lys dyad in each of the proteins were constrained to match. There is a 23.4 % sequence identity and 37.2 % sequence similarity (conservative substitutions) between the UmuD' (residues 40-139) and leader peptidase (residues 75-202) (Fig. 3.7). The regions of highest homology surround the putative catalytic residues.
Figure 3.7: The sequence alignment of *E. coli* leader peptidase (residues 75-202) and *E. coli* Umu D' (residues 40-139). The protocol XALIGN (Wishart et al., 1994a,b) was used to perform this alignment. The proposed active-site residues of leader peptidase are marked by asterisks.
Figure 3.8: Ball-and-stick rendering (Merrit & Murphy, 1994) of the modeled active site region of *E. coli* leader peptidase based on the structure of *E. coli* UmuD' (Peat et al., 1996).
Figure 3.9: Space-filling representation (Merrit & Murphy, 1994) of the hydrophobic cleft adjacent to the active site of *E. coli* leader peptidase, modeled from the structure of *E. coli* UmuD' (Peat et al., 1996). See experimental procedures and results for details of the alignment and modeling programs and procedures.
(Ser90/Lys145 in leader peptidase and Ser60/Lys97 in UmuD'). There is a 31.1% identity and 53.3% sequence similarity (conservative substitutions) between UmuD' and leader peptidase when comparing these aligned sequences between Pro48 and Lys98 (in UmuD'). Prior studies comparing the sequences of UmuD and *Bacillus Subtilis* signal peptidase (SipS) revealed a 25% identity and 42% similarity between these proteins (van Dijl et al., 1995). A model of the leader peptidase active-site was built by replacing the residues within the UmuD' structure with the corresponding aligned residue from leader peptidase. The model of the Leader peptidase active site reveals a shallow hydrophobic cleft just adjacent to the catalytic site. This hydrophobic cleft involves Ile144, Leu95, Phe192, Ile101, Ile86, Val103, Val131, and Phe84 (Fig. 3.9). Similar to UmuD', the proposed catalytic serine and lysine residues appear to be buried within a hydrophobic environment (Fig. 3.8, Fig. 3.9).

3.4 Discussion

3.4.1 Site-directed chemical modification

Although site-directed mutagenesis has become the principle technique for investigating the role of individual amino acid residues in an enzymatic mechanism, the choice of side chains is limited to those specified by the genetic code. Expanding the choice of side-chain functionalities allows one to examine in greater detail the chemical and structural nature of specific amino acid residues within a protein. The two methods currently available for the introduction of unnatural amino acids into proteins are; *in vitro* translation (Ellman et al., 1992; Chung et al., 1993) and chemical modification (Means and Feeney, 1971; Lundblad, 1995). The use of site-directed mutagenesis in combination with chemical modification (site-directed chemical modification) is a very powerful technique for introducing unnatural amino acids at specific sites within a protein (Gloss and Kirsch, 1995a,b). In this study we have used the method of
site-directed chemical modification to investigate the structural and chemical flexibility of an essential Lys residue within *E. coli* type 1 signal (leader) peptidase.

The inactive leader peptidase mutant K145C, No Cys showed significant restoration of activity upon reaction with 2-bromoethylamine•HBr.

The fact that the functionally restored leader peptidase K145C-EA, No Cys (lysine 145 changed to aminoethyl cysteine) shows a reduced kcat value and yet a normal Km value (as compared to the No Cys mutant, Table 3.1) is consistent with the Lysine 145 being directly involved in catalysis. In these studies we have removed the other cysteines in leader peptidase to simplify the interpretation of the aminoethylation results. The cysteine-less (No Cys) leader peptidase had almost wild-type activity (Table 3.1).

As a control, we confirmed that the 2BEA reagent had no effect on the activity of the No Cys mutant (Fig. 3.1B.) showing that the restoration of the activity upon the reaction with 2BEA is from the aminooethylation of the cysteine 145 residue. If the restoration of the activity in the K145C, No Cys mutant were due to modification of some other residue other than the cysteine 145, the addition of 2BEA to the No Cys mutant would have increased the activity in this enzyme as well. The inactive K145H mutant also showed no recovery of activity when treated with 2BEA (Fig. 3.2).

To assess length requirements of the side chain at the the 145 position, we reacted the K145C, No Cys mutant with 3-bromopropylamine•HBr and 2-mercaptoethylamine which generates a side chain amine which is 5 atoms from the Cα instead of 4 atoms like lysine. These modifications also restored activity to the K145C, No Cys mutant, although slightly less than that seen with 2-bromoethylamine (Fig 3.3, 3.4 and Table 3.1), revealing that there may be significant flexibility in the active-site.

Reacting the K145C, No Cys mutant with the reagent (2-bromoethyl)trimethylammonium bromide (2BETMA), which puts a positively charged, nontitratable lysine analog (4-thialaminine) at the 145 position, resulted in no restoration of activity (Fig. 3.5). This is consistent with the hypothesis that lysine 145 is essential
due to a critical hydrogen bond or its role as a general base, and not because of its charge. It is possible that the additional bulkiness of the methyl groups on the e-amino group within the lysine analog (4-thialaminine) is responsible for the lack of activity.

3.4.2 Why does S-(aminoethyl)-cysteine not fully substitute for lysine?

S-(aminoethyl)-cysteine would appear to be a good substitute for a lysine residue. The only difference between the two residues would be the difference between a thioether group and a methylene group. The difference between the C-S and C-C bond lengths (1.807Å vs. 1.532Å) would be compensated by the more acute angle of the C-S-C bond (99° vs. 112°) (Messmore et al., 1995). Molecular modeling studies predict that the primary amine group in S-(aminoethyl)-cysteine and lysine can be superimposable to within 0.1 Å (Messmore et al., 1995).

In this study we have seen that aminoethylation of the inactive K145C mutant of type 1 signal peptidase leads to only partial restoration of activity (approximately 1 % of the wild-type activity). Similar studies with other enzymes have also shown less than complete restoration of activity. Aspartate aminotransferase (Planas and Kirsch, 1991), ribonuclease A (Messmore et al., 1995), and ribulosebisphosphate carboxylase/oxygenase (Smith and Hartman, 1988) showed 7%, 8%, and 60% restoration of activity, respectively, upon aminoethylation of an inactive Lys to Cys mutant.

Less than ideal substitution of lysine by S-(aminoethyl)-cysteine, and therefore less than complete restoration of activity to inactive Lys to Cys mutants, might be explained by the unusual properties of the CS-CC torsional unit (see Figure 3.10). Gas phase studies on model compounds show that the CS-CC torsional unit prefers the gauche over the anti conformation (-0.05 to -0.2 Kcal/mol), whereas the CC-CC torsional unit prefers the anti conformation (+0.8 Kcal/mol) (Gellman, 1991).
Figure 3.10: The anti and gauche conformations of the lysine CC-CC and S-(aminoethyl)-cysteine CS-CC torsional units as viewed in a Newman projection down the C4-C5 bond and S-C4 bond, respectively. The favored conformations are based on the x-ray crystal structures of S-(aminoethyl)-cysteine and lysine (Ammon et al., 1991), and gas phase model compound studies (Gellman, 1991).
The CS-CC tortional unit is also present in the amino acid methionine. A survey of x-ray crystal structures show that the CS-CC torsional unit within methionine shows no apparent conformational preference (Gellman, 1991; Janin et al., 1978). The x-ray crystal structure of S-(aminoethyl)-cysteine is consistent with a preference for the gauche conformation (Ammon et al., 1991). It was found that S-(aminoethyl)-cysteine contained a CS-CC side-chain torsion angle of 67.8° as compared to the lysine side-chain CC-CC torsion angle of 173.1°. The C2 to C5 distance for the S-(aminoethyl)-cysteine was 4.209 Å, whereas that for lysine was 5.118 Å (Ammon et al., 1991). Therefore, the thioether side-chain at the 145 position of K145C-EA signal peptidase maybe shorter and more flexible than the alkyl side-chain of lysine within wild-type signal peptidase. This may help to explain why the propylamine side chain is almost as effective as the ethylamine.

Aminoethylation has been used to introduce new cleavage-sites for trypsin digestion of proteins (Itano and Robinson, 1972; Cole, 1967; Raftery and Cole, 1966). It has been observed that these S-(aminoethyl)-cysteiny1 bonds are cleaved much more slowly than the lysyl bonds (Cole, 1967; Raftery and Cole, 1966). This may reflect S-(aminoethyl)-cysteine’s preference for the gauche rather than the anti side chain torsion angle (Fig. 3.10).

Other important differences between S-(aminoethyl)-cysteine and lysine include the effect of the thioether group on the pKa of the ε-amino group, and the larger polarizability of the sulfur atom. The pKa of the ε-amino group of S-(aminoethyl)-cysteine is 1.1 pH units lower than that for lysine (Gloss & Kirsch, 1995a).

### 3.4.3 Maleic anhydride inhibits leader peptidase

We have shown evidence that maleic anhydride inhibits leader peptidase by reacting with the proposed catalytic residue lysine 145 (Fig 3.6). Kim et al. found only low level inhibition of leader peptidase upon the addition of the lysine modifiers succinic
anhydride and TNBS (Kim et al., 1995). It is not yet clear why only maleic anhydride was successful in modifying lysine 145. Other lysine modifying reagents we have tried without any effect on activity include potassium cyanate, TNBS, succinic anhydride, acetic anhydride, and pyridoxyl 5-phosphate. It may be that these other reagents were not able to gain access to the active-site region due to the active-site's proposed hydrophobic environment. Another possibility is that maleic anhydride is the only reagent tried so far that can bind and induce a conformation within leader peptidase that allows lysine 145 to be reactive (lowered lysine pKa). Lysine 145 may be less reactive to modification by lysine specific reagents because of its possible hydrogen bond to serine 90 and the potentially buried nature of lysine 145.

3.4.4 The mechanism of *E. coli* leader peptidase

Taken together the results from this study as well as others (Black, 1993, van Dijl et al., 1995) are consistent with the hypothesis that leader peptidase utilizes a lysine as its general base. We have proposed a mechanism for leader peptidase whereby the deprotonated e-amine of lysine 145 abstracts the proton from the $\gamma$ of serine 90 making it nucleophilic enough to attack the scissile bond of the pre-protein substrate (Fig. 3.1 A). The tetrahedral intermediate I could be stabilized by a yet unidentified oxyanion hole, and the breakdown of the tetrahedral intermediate I to form the acyl-enzyme intermediate would be accelerated by the protonation of the leaving amine (mature protein) via lysine 145 (Fig. 3.11B). It is possible that the lysine 145 could also act as the general base in the formation of tetrahedral intermediate II, whereby lysine 145 would activate a water which would attack the ester carbonyl of the acyl-enzyme intermediate (Fig. 3.11C).

There are many unanswered questions regarding the mechanism of leader (signal) peptidase. From site-directed mutagenesis and chemical modification studies it is clear that serine 90 and lysine 145 are critical residues, but it is not yet clear whether there exists an oxyanion hole similar to the classical serine proteases. Another
unanswered question is whether lysine 145 serves as the general base in both activation steps of the reaction. The forthcoming x-ray crystal structure of the soluble fragment of *E. coli* leader peptidase (Paetzel et al., 1995) may help to answer these questions regarding the mechanism of this very unique serine protease.
Figure 3.11: The proposed mechanism of *E. coli* leader peptidase based on site-directed mutagenesis and chemical modification studies.
CHAPTER 4

CHARACTERIZATION OF A SOLUBLE, CATALYTICALLY ACTIVE FORM OF
ESCHERICHIA COLI LEADER PEPTIDASE:
REQUIREMENT OF DETERGENT OR PHOSPHOLIPID FOR OPTIMAL ACTIVITY

4.1 Introduction

One approach to obtaining structural information on a membrane protein is to purify a soluble fragment lacking the transmembrane segment(s) and attempt to crystallize it and solve its structure by x-ray crystallographic methods. The fragment can be generated either by limited proteolysis or by deletion of the portion of the gene corresponding to the transmembrane segments. We have used the later approach to initiate our efforts to solve the structure of the periplasmic portion of *Escherichia coli* leader peptidase. This soluble fragment lacks residues 2 through 75, the residues corresponding to the two transmembrane domains, and is referred to as Δ2-75 leader peptidase. Before embarking on the sometimes long journey to x-ray diffraction quality
crystals, it is best to know as much as one can about the protein in which one is attempting to crystallize. The purpose for the purification of Δ2-75 leader peptidase, as well as its biochemical characterization, is to obtaining structural as well as mechanistic information on the catalytic portion of this integral membrane serine protease. The exact location where signal peptide cleavage takes place is still under investigation. Its membrane topology within the lipid bilayer is well characterized (Wolfe et al., 1983; Bilgin et al., 1990; Moore & Miura, 1987; and Whitley et al., 1993) and extensive site-directed mutagenesis studies along with chemical modification studies are consistent with it’s catalytic residues being identified as Ser90 and Lys145 (Sung & Dalbey, 1992; Tschantz et al., 1993; Paetzel et al., 1997). The first step in the proposed mechanism of leader peptidase (see Fig. 3.11) involves Lys145 acting as the general base to abstract the hydroxyl hydrogen from Ser90 making it nucleophilic enough to attack the scissile bond of the pre-protein which has translocated across, but still anchored to, the lipid bilayer (Fig. 3.11A). The two transmembrane segments of leader peptidase correspond to residues 1-22 (H1) and 59-76 (H2) (see Fig.4.1). This puts the catalytic Ser90 relatively close to the lipid bilayer. From the primary sequence of leader peptidase it can be seen that Ser90 is in the middle of a stretch of mildly hydrophobic residues near the membrane surface. This may play an instrumental role in the activity of leader peptidase. It is possible that leader peptidase catalysis takes place within the lipid bilayer. This would provide an interesting environment for catalysis of an enzyme which appears to utilize a lysine as it’s general base. It is well known that a hydrophobic environment is one scenario which allows for a depressed lysine e-amino pKa (see section 2.10).

In these studies we have developed an efficient Δ2-75 purification procedure which has allowed us to purify large amounts of this soluble leader peptidase in the total absence of detergent. In contrast to the wild-type leader peptidase (pI = 6.8), Δ2-75 has an acidic isoelectric point of 5.6. We find that the catalysis of Δ2-75 is accelerated by
Figure 4.1: The segment of leader peptidase corresponding to the soluble Δ2-75 or P2 domain. The residues 2 through 75, corresponding to the two transmembrane segments (2-22 and 59-75) as well as the P1 cytoplasmic domain (23-58) have been deleted in the Δ2-75 leader peptidase.
the presence of the detergent Triton X-100. Triton X-100 is required for optimal activity of Δ2-75 at a level far below the critical micelle concentration. Moreover, we find that *E. coli* phospholipids stimulate the activity of Δ2-75, suggesting that phospholipids may play an important physiological role in the catalytic mechanism of leader peptidase.

4.2 Experimental procedures

4.2.1 Purification of Δ2-75 leader peptidase

The Δ2-75 leader peptidase was overexpressed in *E. coli* BL21 (DE3) containing the gene encoding Δ2-75 subcloned into the pET-3d vector. The cultures (1 liter) were grown in LB media containing 100 μg/ml ampicillin and 10 ml of overnight starter culture. The cells were grown to an optical density of 0.5 at 600 nm. Expression was induced with isopropylthiogalactoside (IPTG, 0.5 mM final concentration). After the induction with IPTG, the Δ2-75 accumulated in inclusion bodies within the cytoplasm of the cells. The cells were lysed by passing them five times through a French pressure cell at 16,000 psi. The inclusion bodies were then pelleted away from the cell lysate by centrifugation at 12,000 g for 5 minutes, and washed at least 4 times with 0.5 % Triton X-100, 10 mM EDTA, 20 mM Tris-HCl pH 7.4 (buffer A) (Marston et al., 1984) (Fig. 4.2). The solubilization of Δ2-75 was achieved by dissolving the washed inclusion bodies in 6 M guanidine-HCl in 20 mM Tris-HCl pH 7.4 (100 mL per liter of cell culture), followed by incubation at room temperature for 1 hour. This solution was then added slowly to 2 volumes of buffer A or a similar buffer lacking detergent, put into dialysis tubing, and dialyzed 5 times with the same buffer; while changing the dialysis buffer every 12 hours. After dialysis, the
protein solution was centrifuged at 15,000 g for 1 hour to pellet any misfolded protein. The supernatant was applied to a Q Sepharose FF anion exchange column equilibrated with 20 mM Tris-HCl pH 7.4 (buffer B). The column was washed with 1 liter of buffer B to remove the Triton X-100. The Δ2-75 was then eluted from the column with 0.7 M NaCl in buffer B, and then dialyzed extensively against buffer B. The protein was then stored in buffer B at -70 °C.

4.2.2 Isoelectric focusing

The Δ2-75 (1 μL) was loaded onto an isoelectric focusing gel using the Pharmacia Phast Gel system, Phast gel IEF 3-9 media, and Pharmacia broad-range pI standards. 0.2 μg of standards was applied to the gel. The gel was visualized by Phast Gel Blue R Coomassie staining. All samples were run from both the anode and the cathode side of the gel to ensure that the proteins focused to the same point in the gradient.

4.2.3 The dependence of Δ2-75 activity on the Triton X-100 critical micelle concentration (CMC)

Δ2-75 was incubated at various concentrations at 37 °C for 1 hour in the presence of pro-OmpA nuclease A and buffer (50 mM Tris-HCl, pH 8.0) or Triton X-100 (see below for concentrations). Briefly, reactions contained 15 μl of pro-OmpA nuclease A (21 mM) and 1 μl of Δ2-75 at a concentration of 0.1 (lane 1), 0.01 (lane 2), or 0.001 mg/ml (lane 3) and 1 μl of buffer (50 mM Tris-HCl, pH 8.0) or detergent at the different concentrations. The detergent levels (final concentrations) were 10-fold
below the CMC (0.024 mM Triton X-100), at the CMC (0.24 mM Triton X-100), and 10-fold above the CMC (2.4 mM Triton X-100). The processing of pro-OmpA nuclease A (p) to nuclease A (m) was analyzed by SDS-PAGE using a 17 % polyacrylamide gel and stained by Coomassie blue staining.

4.2.4 The effect of phospholipids on the activity of Δ2-75

Δ2-75 was incubated at various concentrations at 37 °C for 1 hour in the presence of pro-OmpA nuclease A. Briefly, reactions contained 10 μl of pro-OmpA nuclease A (31 μM), 1 μl of Δ2-75 at concentrations of 0.1 mg/ml (lane 1), 0.01 mg/ml (lane 2), or 0.001 mg/ml (lane 3), and 1 μl of phospholipid mixture or buffer (50 mM Tris-HCl, pH 8.0). The lipid concentrations were 2.1 μg/ml, 21 μg/ml, 210 μg/ml, or 2.1 mg/ml. Pro-OmpA nuclease A (p) and nucleaseA (m) were resolved on a 17.2 % polyacrylamide gel and stained by Coomassie brilliant blue. E. coli total lipid extract was purchased from Avanti Polar Lipids, Inc. 100 μl of the lipids (25 mg/ml) was transferred to a glass vial and dried under nitrogen. 100 μl of 50 mM Tris-HCl, pH 8.0, was added to the lipid and vortexed to make the phospholipid mixture (Tanford, 1973).
4.3 Results

4.3.1 Improved Δ2-75 purification procedure

Following the isolation of inclusion bodies procedure of Marston et al. (1984) has allowed us to eliminate the messy and inefficient denaturing gel filtration step initially proposed by Kuo et al. (1993). A comparison of these procedures is made in Table 4.1. We now can obtain relatively pure (95%) inclusion bodies (seen as a white pellet) which can be solubilized and the refolding initiated in less than one day.

We obtain approximately 40 mg of pure Δ2-75 from one liter of cell culture. The refolded Δ2-75 is seen as a single band on both SDS-PAGE (Fig. 4.3) and IEF (Fig. 4.4) electrophoresis gels stained with Coomassie brilliant blue. We are also able to refold this protein in the total absence of detergent which has allowed us to measure with greater certainty the effect of detergent or phospholipid on the activity of this enzyme.

4.3.2 Determination of the isoelectric point of Δ2-75

The isoelectric point of Δ2-75 was determined by isoelectric focusing electrophoresis. Figure 4.4 shows that Δ2-75 runs as a single band on this gel system with a pI of 5.6. We also obtained the same pI for Δ2-75 when determined by Pharmacia chromatofocusing chromatography (data not shown).
Figure 4.2: Isolation and washing of Δ2-75 leader peptidase inclusion bodies. L = lysate, S = supernatant, P = pellet. The cells were lysed by passing them 5 times through a French Pressure cell at 16,000 psi. The inclusion bodies were isolated by centrifugation at 12,000 x g for 5 minutes. Contaminating proteins were washed away from the Δ2-75 inclusion bodies by resuspending them in 0.5 % Triton X-100, 10 mM EDTA, 20 mM Tris-HCl pH 7.4 followed by centrifugation at low speed (12,000 x g for 5 minutes). This washing procedure was repeated 5 times.
Figure 4.3: SDS-PAGE gel of purified Δ2-75 leader peptidase. The gel was stained with Coomassie blue. Molecular weight standards are run in the left lane.
**OLD** (Kuo et al., 1993) | **NEW** (Paetzel et al., 1995)
--- | ---
1. cell culture | 1. cell culture
2. induction (IPTG) | 2. induction (IPTG)
3. cell lysis (French press) | 3. cell lysis (French press)
4. centrifugation (100,000 g) | 4. centrifugation (12,000 g)
5. solubilization (4 M guanidine-HCl) | 5. washing of inclusion bodies
6. S-100 guanidine-HCl column | 6. solubilization (6 M guanidine-HCl)
7. refold (Triton X-100 / dialysis) | 7. refold (Triton X-100 / dialysis)
8. concentration (amicon) | 8. anion ex. (concentrates/removes detergent)
9. removal of Triton X-100 (Extracti-gel D) |

**Table 4.1**: Δ2-75 leader peptidase purification methods
4.55 — — — — — — — — — — — — A 2-75 pi = 5.6

Figure 4.4: The isoelectric focusing of A2-75 leader peptidase. 1 µL of A2-75 was loaded onto an isoelectric focusing gel using the Pharmacia Phast Gel system, Phast gel IEF 3-9 media, and Pharmacia broad-range pI standards. The gel was visualized by Phast Gel Blue R Coomassie staining. The left lane shows the pI standards (0.2 µg of standards was applied to the gel).
4.3.3 Detergent requirement for optimal activity

We asked the question whether a detergent micelle is required by measuring pro-OmpA nuclease A processing at different concentrations of Triton X-100 at levels far above and far below the critical micelle concentration (CMC). The CMC of Triton X-100 is 0.24 mM (Chattopadhyay & London, 1984). In the absence of detergent, there is a low level of pro-OmpA nuclease A cleavage (Fig. 4.5, far left panel). The addition of detergent at levels 10-fold lower than the CMC results in strong stimulation of processing (Fig. 4.5, middle left panel). A further increase in processing is observed when the detergent concentration was raised to the CMC level (Fig. 4.5, middle right panel), but no additional increase was seen when the detergent concentration was increased to 10-fold higher than the CMC (Fig. 4.5, far right panel).

4.3.4 Phospholipids stimulate the activity of Δ2-75 leader peptidase

Since the detergent Triton X-100 enhanced the activity of Δ2-75, we asked the question whether phospholipids play a role in catalysis. E. coli lipid extract was purchased from Avanti Polar lipids, Inc., and used to prepare a mixed phospholipid bilayer system by vortexing following established techniques (Tanford, 1973; see section 4.2.4). We then tested the ability of Δ2-75 to cleave pro-OmpA nuclease A in the presence of no phospholipid, or 2.1 μg/ml, 21 μg/ml, 210 μg/ml, or 2.1 mg/ml phospholipid. Figure 4.6 shows that the addition of phospholipid stimulates the activity of Δ2-75. There is an increase in the amount of pro-OmpA nuclease A converted to the mature nuclease A as the concentration of the phospholipid is increased from 2.1 μg/ml to 2.1 mg/ml.
Figure 4.5: Δ2-75 leader peptidase requires the detergent Triton X-100 for optimal activity. The processing of the pre-protein substrate pro-Omp A nuclease A (p) to the mature nuclease A (m) by successive dilutions of Δ2-75 (lanes 1-3) in the absence or presence of Triton X-100 below, at, or above the CMC is analyzed by SDS-PAGE. (see section 4.2.3 for experimental details).
Figure 4.6: *E. coli* phospholipids stimulate the activity of Δ2-75 leader peptidase. The processing of the pre-protein substrate pro-Omp A nuclease A (p) to the mature nuclease A (m) by successive dilutions of Δ2-75 (lanes 1-3) in the absence or presence of increasing concentrations of mixed *E. coli* phospholipids is analyzed by SDS-PAGE. (see section 4.2.4 for experimental details).
4.3 Discussion

Initially while studying Δ2-75, we followed the purification procedure of Kuo et al., 1993. This procedure involved a high speed centrifugation spin (100,000 g), solubilization, gel filtration under denaturing conditions, a concentration step, and then removal of detergent by a hydrophobic matrix column. This procedure produced relatively pure Δ2-75 but the procedure took at least 5 days to complete and the yield was limited by the amount one could load onto the gel filtration column. Only 2 ml of crude solubilized extract could be loaded onto the column at a time. This denaturing column was messy as well as inefficient. Our new purification procedure eliminates this step by taking advantage of a slow centrifugation step after the lysis of the cell. This slow centrifugation pellets mainly the most dense matter in the lysate, the inclusion bodies. The improved purification procedure has allowed use to purify sufficient amounts of this soluble enzyme to characterize its properties and compare them to the wild-type enzyme (Table 4.2) as well as initiate the screening for crystallization conditions (see chapter 5).

Strikingly, we demonstrate that the detergent Triton X-100 promotes Δ2-75 catalyzed cleavage of the pre-protein pro-OmpA nuclease A. We also find *E. coli* phospholipids, comprised of mainly phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (Raetz & Dowhan, 1990), stimulate the activity of Δ2-75. Presently it is not clear 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 (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 pro-OmpA nuclease A substrate. However we favor the idea that the detergent (or phospholipid) requirement is an enzyme effect.
<table>
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<tr>
<th></th>
<th>Δ2-75</th>
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<tr>
<td>Mr</td>
<td>27,952</td>
<td>35,988</td>
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</tr>
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<tr>
<td>Km (µM)</td>
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<td>19</td>
<td></td>
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<tr>
<td>kcat/Km (sec⁻¹ M⁻¹)</td>
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<td>2.3 x 10⁶</td>
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Table 4.2: Properties of Δ2-75 and wild-type *E. coli* leader peptidase. Kinetic constants were assayed at pH 8.0 in the presence of 1% Triton X-100 with the pre-protein substrate pro-OmpA nuclease A (Tschantz et al., 1995).
Much larger Δ2-75 crystals are formed when detergent is present in the crystallization buffer (Paetzel et al., 1995; see chapter 5). It is intriguing that detergent is required for optimal activity at concentrations far below the critical micelle concentration of Triton X-100, where the equilibrium between monomer and micelle strongly favors the monomer form of the detergent (Helenius & Simons, 1995). This suggests that single detergent molecules, themselves, might be sufficient to enhance the catalytic activity of leader peptidase.

This requirement for the presence of detergent or phospholipid for optimal activity of a soluble catalytically active form of leader peptidase has lead us to investigate further the nature of this requirement. We have now purified an active Δ2-75 as well as an inactive Δ2-98 leader peptidase in the total absence of detergent (no Triton X-100 present during the refolding step). This has allowed our collaborators, van Klompenburg and de Kruiff, to show that Δ2-75 binds to inner and outer membranes of E. coli. The Δ2-75 also was shown to bind to inner membrane vesicles and vesicles composed of purified membrane lipids with comparable efficiencies. Since Δ2-98 lacks the hydrophobic stretch (H3), which surround the catalytic serine 90, it was possible from surface tension experiments to show that the interaction is caused by penetration of part of the catalytic domain between the lipids. The Δ2-75 had a much great affinity for the membranes than did the Δ2-98. They found that penetration into the bilayer was mediated by the phospholipid phosphatidylethanolamine and that it does not seem to be an electrostatic interaction (van Klompenburg, W., Paetzel, M., de Jong, J.M., Dalbey, R.E., Demel, R.A., von Heijne, G., de Kruiff, B. (1997) submitted for publication). These experiments, along with our experiments showing the requirement of detergent or phospholipid for optimal activity of Δ2-75, are consistent with leader peptidase having some requirement for a hydrophobic environment. It may be that leader peptidase is
similar to other enzymes which catalyze a reaction at the membrane surface, such as the lipases, and undergo an interfacial activation (Davis et al., 1990; Jennens and Lowe, 1994; Derewenda et al., 1994a; Derewenda et al., 1994b). Further experiments will be needed to test these interesting mechanistic possibilities.
CHAPTER 5

CRYSTALLIZATION OF A SOLUBLE, CATALYTICALLY ACTIVE FORM OF
ESCHERICHIA COLI LEADER PEPTIDASE
(THE TETRAGONAL CRYSTAL FORM)

5.1 Introduction

Leader (signal) peptidase is an essential protein in both prokaryotic and eukaryotic cells. It functions to remove amino-terminal leader (signal) sequences from proteins that are exported to the cell surface. To date, the leader peptidase from Escherichia coli has been the most thoroughly characterized. Its gene has been cloned (Date et al., 1981) and sequenced (Wolfe et al., 1983), and it has been overexpressed (Dalbey and Wickner, 1985), and purified (Wolfe et al., 1982; Tschantz and Dalbey, 1994). Leader peptidase is located in the inner membrane and contains two transmembrane segments with its large carboxy-terminal segment protruding into the
periplasmic space (Bilgin et al., 1990; Moore and Miura, 1987; Wolfe et al., 1983; Whitley et al., 1993). It consists of a single polypeptide chain with a molecular weight of 35,988 Da (323 amino acid residues), contains one disulfide bond (von Heijne, unpublished data), and has an isoelectric point of 6.9 (Wolfe et al., 1983). The primary sequence of leader peptidase shows that there is a third hydrophobic region in addition to the two transmembrane segments, that resides roughly six residues from the region where the surface of the membrane bilayer is likely located (Moore and Miura, 1987).

Leader peptidase has a substrate specificity for pre-proteins which contain a small uncharged residue at the P1 site (nomenclature according to Schechter and Berger, 1967) and a small uncharged or large aliphatic residue at the P3 position in the leader sequence (von Heijne, 1990; Fikes et al., 1990; Shen et al., 1991). There have been various peptide substrates constructed (Dev et al., 1990) in order to measure the catalytic parameters of leader peptidase, but the pre-protein substrates such as pro-OmpA nuclease A (Chatterjee et al., 1995) have proven to give the most reliable catalytic constants.

The catalytic mechanism for leader peptidase is so far unknown, yet all available evidence is most consistent with leader peptidase using a serine/lysine dyad to catalyze the cleavage of leader peptides from exported proteins. Extensive site-directed mutagenesis studies have shown that serine 90 and lysine 145 are required for activity (Tschantz et al., 1993; Black, 1993), whereas no histidine nor cysteine residues were found to be essential (Sung & Dalbey, 1992; Black et al., 1992). Additional evidence that serine 90 and lysine 145 play key roles in the catalytic activity of leader peptidase was revealed when site-directed mutagenesis was combined with chemical modification. When serine 90 was changed to a cysteine residue, leader peptidase was still active. This thiol-leader peptidase was then inhibited by N-ethyl maleimide, a cysteine-specific reagent (Tschantz et al., 1993). In a recent study, lysine 145 was changed to a cysteine residue to produce an inactive leader peptidase. Partial activity in this mutant was restored by reacting this inactive leader peptidase with 2-bromoethylamine to produce an ethylamine lysine analog at the 145 position (Paetzel et al., 1997, see chapter 3).
From the primary sequence, one can see that serine 90 could be positioned near the surface of the membrane, where it could attack the peptide bond of the pre-protein as it inserts across the membrane. The fact that serine 90 is in the middle of a stretch of hydrophobic residues near the membrane surface may also play an instrumental role in the activity of leader peptidase. Notably, the serine 90 residue is conserved in prokaryotes and eukaryotes whereas the lysine 145 is conserved only within the prokaryotes and the mitochondrial leader peptidases. In the yeast and canine endoplasmic reticulum signal peptidase subunits the lysine 145 is replaced by a histidine (van Dijl et al., 1992; Dalbey & von Heijne, 1992). This observed conservation of residues among the leader (signal) peptidases would suggest that the more evolved peptidases may use a more conventional proteolytic mechanism. The serine/lysine dyad mechanism has been proposed for the lexA repressor (Roland et al., 1990) and there is crystallographic evidence for a lysine serving as the general base in β-lactamase (Strynadka et al., 1992).

Recently, Kuo, et al. (1993) reported the isolation of Δ2-75, a soluble and catalytically active fragment of leader peptidase (M_r 27,952) lacking the two transmembrane segments. We followed their procedure to clone and express Δ2-75, and characterize its activity more thoroughly (Tschantz et al., 1995). Presented here is an improved large scale purification procedure along with the results of the crystallization trials of the soluble fragment of leader peptidase (Δ2-75).

5.2 Material and methods

The Δ2-75 was overexpressed in E. coli BL21 (DE3) containing the gene encoding Δ2-75 subcloned into the pET-3d vector. After the induction with isopropylthiogalactoside, the Δ2-75 accumulated in inclusion bodies within the
cytoplasm of the cells. The cells were lysed by passing them five times through a French pressure cell at 16,000 psi. The inclusion bodies were then pelleted away from the cell lysate by centrifugation at 12,000 g for 5 minutes, and washed at least 4 times with 0.5% Triton X-100, 10 mM EDTA, 20 mM Tris-HCl pH 7.4 (buffer A) (Marston et al., 1984). The solubilization of Δ2-75 was achieved by dissolving the washed inclusion bodies in 6 M guanidine-HCl in 20 mM Tris-HCl pH 7.4 (100 mL per liter of cell culture), followed by incubation at room temperature for 1 hour. This solution was then added slowly to 2 volumes of buffer A, put into dialysis tubing, and dialyzed 5 times with the same buffer; while changing the dialysis buffer every 12 hours. After dialysis, the protein solution was centrifuged at 15,000 g for 1 hour to pellet any misfolded protein. The supernatant was applied to a Q Sepharose FF anion exchange column equilibrated with 20 mM Tris-HCl pH 7.4 (buffer B). The column was washed with 1 liter of buffer B to remove the Triton X-100. The Δ2-75 was then eluted from the column with 0.7 M NaCl in buffer B, and then dialyzed extensively against buffer B. The refolded Δ2-75 was concentrated to 10 mg/mL by pressurized ultrafiltration using an Amicon YM10 filter. The protein was then stored in buffer B at -70°C.

The sparse matrix method (Jancarik & Kim, 1991) was used for initial screening of crystallization conditions. The trials were performed at room temperature using the sitting-drop vapor diffusion technique with a drop consisting of 5 μL of Δ2-75 (10 mg/mL) and 5 μL of reservoir solution.

The native Δ2-75 crystals were characterized using an Enraf-Norius precession camera mounted on a high-intensity Rigaku Rotaflex RU-200 rotating anode X-ray generator operating at 40 KV and 150 mA (6 KW) with graphite monochromatized CuKα radiation.
5.3 Results and discussion

The purification procedure described above produced approximately 40 mg of pure Δ2-75 per liter of cell culture. The largest gain in purification was achieved in the initial pelleting and washing of the inclusion bodies to give approximately 95% pure Δ2-75. The refolded Δ2-75 was seen as a single band on both SDS-PAGE (Fig. 4.3) and IEF (Fig. 4.4) electrophoresis gels stained with coomassie brilliant blue.

Within 5 days, small crystals formed from a heavy precipitate in the condition which contained 1.0 M ammonium dihydrogen phosphate and 0.1 M sodium citrate, pH 5.6. These crystals were too small for x-ray analysis, but appeared to be protein from their ability to absorb dyes and their sensitivity to mechanical stress (McPherson, 1982). These crystals were unusually stable in low ionic strength solutions and showed relatively high birefringence. Although Δ2-75 is active and completely soluble in the absence of detergent (concentrations of 56 mg/mL are easily achievable), it was found that the presence of Triton X-100 was required for the production of Δ2-75 macrocrystals. Interestingly, it has recently been shown that Δ2-75 requires detergent or phospholipid for optimal activity (Tschantz et al., 1995; see chapter 4). Less nucleation was achieved and larger crystals were obtained by the addition of 2-methylpentane-2,4-diol and the use of macroseeding. The sitting-drop contained 4 μL of Δ2-75 (10 mg/mL, 0.1% Triton X-100, 20 mM Tris-HCl pH 7.4), 1 μL of 0.1% nonidet P-40, and 2 μL of reservoir solution containing 0.7 M ammonium dihydrogen phosphate, 0.1 M sodium citrate pH 5.6, and 5% 2-methylpentane-2,4-diol. The maximum size of these crystals is approximately 0.3 mm x 0.1 mm x 0.1 mm (Fig. 5.1).
Figure 5.1: Photomicrographs of the tetragonal crystal form of *E. coli* Δ2-75 leader peptidase.
<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
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<td>Typical dimensions</td>
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</tr>
<tr>
<td>Unit cell dimensions</td>
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</tr>
<tr>
<td>Interaxial angles</td>
<td>α = β = γ = 90°</td>
</tr>
<tr>
<td>Space group</td>
<td>Tetragonal $P4_{21}2$</td>
</tr>
<tr>
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</tr>
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<td>Molecules per asymmetric unit</td>
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</tr>
<tr>
<td>Molecules per unit cell</td>
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</tr>
<tr>
<td>$V_m$</td>
<td>2.95 Å³/Da</td>
</tr>
<tr>
<td>Limits of diffraction</td>
<td>3.6 Å - 2.5 Å</td>
</tr>
</tbody>
</table>

**Table 5.1:** The characteristics of the tetragonal crystal form of *E. coli* Δ2-75 leader Peptidase.
Figure 5.2: Precession photograph showing the X-ray diffraction pattern seen from the crystals of Δ2-75. Shown here is the $hk0$ zone. This diffraction pattern was collected from an Enraf-Norius precession camera mounted on a high-intensity Rigaku Rotaflex RU-200 rotating anode X-ray generator operating at 40 kV and 150 mA (6 kW) with graphite monochromatizing CuKα radiation.
Figure 5.3: Washed crystalline $\Delta 2-75$ leader peptidase runs at the same molecular weight as non-crystalline $\Delta 2-75$ leader peptidase when analyzed by 12\% SDS-PAGE. Five crystals approximately 0.1 x 0.1 x 0.1 mm in size were washed extensively with fresh mother liquor and then dissolved in 2 x sample buffer and applied to the gel (this lane is designated Xtl.). The lane corresponding to the non-crystalline $\Delta 2-75$ is designated $\Delta 2-75$. The last crystal wash was run in the lane designated by W. The gel was silver stained using the BioRad silver staining kit.
Figure 5.4: The effect of pH on the rate of crystallization and quality of Δ2-75 crystals. The crystallization solution for Δ2-75 includes ammonium dihydrogen phosphate and sodium citrate. One approach to obtaining more ordered crystals is to lower the precipitant concentration in order to form the crystals more slowly. In our system lowering the precipitant concentration would also effect the pH of our system. Therefore we have setup a grid of crystallization drops where we have varied the concentration of the precipitant and the pH of the buffer (sodium citrate). After mixing the buffer (sodium citrate) and the precipitant solution (ammonium dihydrogen phosphate), the actual pH of each well solution was measured with a micro pH probe. The crystal trials were monitored every 12 hours for crystal growth. The enclosed areas indicate region of pH values in which crystal nucleation occurred within a given time frame. The upper right hand area contained pH values from 4.40 to 3.97. At these pH values small crystals can be seen within 12 hours of setup. In the middle region (pH 4.77 to 4.06) small crystals are seen in less than 36 hours. The upper left region (pH 4.91 to 4.69) gives larger single crystals in 3 to 11 days.
A careful study of the effect of pH on the nucleation rate of these crystals helped in the optimization of the crystallization conditions (Fig. 5.4).

Although there is precedent for small nonionic detergents being helpful in the crystallization of soluble proteins (McPherson, 1986), as well as membrane protein crystals forming in the presence of the large detergent Triton X-100 (Gros et al., 1988), it is interesting that this soluble leader peptidase should require Triton X-100 for optimal crystallization and activity.

On the basis of systematic absences and diffraction symmetry, the diffraction patterns demonstrate that these Δ2-75 crystals belong to the tetragonal space group P4_2_1_2 with unit cell dimensions of a = b = 115 Å and c = 100 Å. Taking 27,952 daltons as the molecular mass (electrospray ionization mass spectrometry, Kuo et al., 1993) and two Δ2-75 molecules per asymmetric unit, the V_m value for these Δ2-75 crystals was calculated to be 2.95 Å³/Da, which lies within the normal range observed for globular proteins (Matthews, 1968). The crystal used for the precession camera work showed resolution to 3.6 Å, yet preliminary data collected on twin San Diego multiwire detectors showed strong reflections to 2.5 Å. Auto-indexing of these data confirmed the unit cell dimensions and P4_2_1_2 space group determined from the precession photos. The quality and resolution of these data were limited by the small size of the crystals, therefore data collection at a synchrotron radiation source will be helpful in these studies. A crystal structure solution will be pursued by the multiple isomorphous replacement method. The search for heavy atom derivatives may be helped by the fact that the crystals seem to be very stable in a variety of solutions.

The crystallization of the active, soluble fragment of leader peptidase presents the opportunity to gain insight into the catalytic mechanism of this remarkable serine protease. In addition, leader peptidase is a target for new antibiotics. The forthcoming three-dimensional structure of leader peptidase will be helpful in the rational design of these antibiotics.
6.1 The orthorhombic crystal form of Δ2-75 leader peptidase

We have discovered an orthorhombic crystal form of Δ2-75 which diffracts to high resolution (1.8 - 2.3 Å). Because leader peptidase is a potential target for novel rationally designed antibiotics, it is essential that we obtain as high a resolution structure as possible. This chapter reports on the data collection statistics for both native and potential heavy atom derivative data sets on this new crystal form. To date, a native data set complete to 2.3 Å, and thirteen potential heavy atom derivative data sets have been collected.
Figure 6.1: A photomicrograph of the orthorhombic crystal form of A2-75 leader peptidase. The orthorhombic crystal form of A2-75 has an irregular shape with typical dimensions of approximately 0.3 x 0.2 x 0.2 mm. The unit cell dimensions are a = 101.0 Å, b = 112.5 Å, c = 115.2 Å. These crystals belong to the orthorhombic space group P222₁.
Figure 6.2: A diffraction pattern from the orthorhombic crystal form of Δ2-75 leader peptidase. The numbers inside the rings define the resolution in Angstroms. This image was collected at the EMBL. DESY synchrotron in Hamburg Germany.
<table>
<thead>
<tr>
<th></th>
<th>Tetragonal</th>
<th>Orthorhombic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical dimensions</td>
<td>0.3 x 0.1 x 0.1 mm</td>
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</tr>
<tr>
<td>Unit cell dimensions</td>
<td>(a, b = 115 \text{ Å}, c = 110 \text{ Å})</td>
<td>(a = 101.0 \text{ Å}, b = 112.5 \text{ Å}, c = 115.2 \text{ Å})</td>
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<td>Interaxial angles</td>
<td>(\alpha = \beta = \gamma = 90^\circ)</td>
<td>(\alpha = \beta = \gamma = 90^\circ)</td>
</tr>
<tr>
<td>Space group</td>
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<td>Orthorhombic P22_1</td>
</tr>
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<td>1,308,960 Å(^3)</td>
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<td>Molecules per unit cell</td>
<td>16</td>
<td>16</td>
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<tr>
<td>(V_m)</td>
<td>2.95 Å(^3)/Da</td>
<td>2.92 Å(^3)/Da</td>
</tr>
<tr>
<td>Limits of diffraction</td>
<td>3.6 Å - 2.5 Å</td>
<td>2.3 Å - 1.8 Å</td>
</tr>
</tbody>
</table>

**Table 6.1:** A comparison of the crystal forms of Δ2-75 leader peptidase.
6.2 Characterization of the orthorhombic crystal form of Δ2-75 leader peptidase

As can be seen from Table 6.1, the characteristic of the orthorhombic crystal form are in some ways similar to that of the tetragonal form. These two crystal forms have similar Matthew's coefficients (Vm) as well as the same number of molecules per unit cell. The orthorhombic crystal form is different from the tetragonal form in that it contains four molecules per asymmetric unit instead of the two molecules per asymmetric unit seen in the tetragonal form. This would suggest that the molecules pack differently, possibly more tightly, in the orthorhombic form. The greater number of molecules per asymmetric unit could prove to be helpful in the refinement of the structure. As can be seen from the spot profile in Figure 6.3, these crystals are very ordered. This is a big improvement over the tetragonal crystal form which was sometimes very mosaic. The symmetry and systematic absences in the pseudo-precession photos (Fig. 6.4) are consistent with the orthorhombic space group P222₁. The intensities of systematic absences in Figure 6.5 confirm this space group.

6.3 Data collection on the native orthorhombic Δ2-75 leader peptidase crystals

A native data set complete to 2.3 Å was collected on beam line X12C at the National Synchrotron Light Source, Brookhaven National Laboratory. The data set was collected from a single crystal. To prevent free radical decay within the crystal due to the intensity of the x-rays, we froze the crystal at 100 °K using the Oxford cryosystem. The diffraction intensities were recorded on a Mar image plate detector, using a 180 mm plate. The crystal to detector distance was 174.824 mm. Phi rotations were made with 0.80 degree increments every 60 seconds. Over 90° of data was collected. The wavelength of the x-rays used was 1.09000 Å. The crystal was still diffracting nicely at
the end of the data set. Reflections could be seen out to 1.8 Å when using the 300 mm plate. The data was processed using the computer programs Denzo and Scalepack. The data was 97.3 % complete over all, with an overall $R_{\text{merge}}$ of 7.6 % (see Table 6.2). This data has nearly 4-fold redundancy.

<table>
<thead>
<tr>
<th>Resolution Shell (Å)</th>
<th>No. of measurements</th>
<th>No. of unique reflections</th>
<th>Completeness (%)</th>
<th>$R_{\text{merge}}$ *</th>
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<td>97.3</td>
<td>0.076</td>
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Table 6.2: Statistics for native data collection on the orthorhombic Δ2-75 leader peptidase crystals. This data was collected on beam line X12C at the National Synchrotron Light Source, Brookhaven National Laboratories. The computer programs Denzo and Scalepack were used to process this data (developed by Zbyszek Otwinowski).

* $R_{\text{merge}} = \Sigma_{h,i} \| I_{h,i} - <I_h> \| / \Sigma_{h,i} I_{h,i}$ where $<I_h>$ is the average intensity of the i observations of the reflection $h$. 
Averaged spot profile in sector 3, 3 (x,y) # of spots 120
Weighted position of the spots 129.399, 128.401 (x,y)

Figure 6.3: The spot profile for a typical reflection taken from a Δ2-75 leader peptidase orthorhombic crystal form data set. The narrow and symmetrical shape of the spot is an indication that these are very ordered crystals. This profile was generated using the program Scale pack (developed by Zbyszek Otwinowski).
Figure 6.4: Pseudo-precession photos from the reflection files of the native data set on the orthorhombic crystal form of Δ2-75 leader peptidase. The symmetry and systematic absences in the 0kl, h0l, and hkd levels are consistent with the orthorhombic space group P2221. The program HKLPLT from the CCP4 suite of protein crystallography programs was used to generate these pictures.
### Intensities of systematic absences

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<th>l</th>
<th>Intensity</th>
<th>Sigma</th>
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<td>55.8</td>
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<td>0</td>
<td>39</td>
<td>127.4</td>
<td>137.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>

### h00 if P2221, should be present, if P212121, every n+1 is missing

| 5  | 0  | 0  | 21.3      | 4.4   | 4.9     |
| 7  | 0  | 0  | 441.6     | 31.2  | 14.1    |
| 9  | 0  | 0  | -0.8      | 9.7   | -0.1    |
| 11 | 0  | 0  | 9.7       | 8.4   | 1.2     |
| 13 | 0  | 0  | 347.2     | 32.0  | 10.9    |
| 15 | 0  | 0  | 972.9     | 64.6  | 15.1    |
| 17 | 0  | 0  | 3.9       | 12.0  | 0.3     |
| 19 | 0  | 0  | 2451.8    | 144.7 | 16.9    |
| 21 | 0  | 0  | 344.4     | 27.7  | 12.4    |
| 23 | 0  | 0  | 4391.8    | 329.4 | 13.3    |
| 25 | 0  | 0  | 254.0     | 24.2  | 10.5    |
| 27 | 0  | 0  | 184.8     | 23.8  | 7.8     |
| 29 | 0  | 0  | 244.0     | 33.7  | 7.2     |
| 31 | 0  | 0  | 635.7     | 61.0  | 10.7    |
| 33 | 0  | 0  | 324.2     | 42.1  | 7.7     |

I.E. SPACE GROUP HAS TO BE P2221 as h00 are present.

**Figure 6.5:** The intensities of systematic absences from a data set collected on a Δ2-75 leader peptidase crystal at the EMBL synchrotron in Hamburg Germany. The systematic absences confirm that this crystal form of Δ2-75 leader peptidase belongs to the orthorhombic space group P222₁. These values were generated using the program Scale pack (developed by Zbyszek Otwinowski).
6.4 Data collection on potential heavy atom derivatives of the orthorhombic Δ2-75 leader peptidase crystals.

So far there has not been a protein structure solved which has a sufficient enough sequence homology to leader peptidase to give a successful molecular replacement solution for leader peptidase. Therefore we need to pursue a solution to our structure using the technique of multiple isomorphous replacement (MIR).

Although our crystallization conditions are not ideal for the solubility and stability of many of the heavy atom compounds used for MIR, we have managed to collect thirteen potential heavy atom derivative data sets using ten different heavy metal compounds.

Three potential heavy atom derivative data sets were collected at the Brookhaven National Laboratory synchrotron and ten more potential heavy atom derivative data sets were collected at the DESY EMBL synchrotron in Hamburg Germany. The statistics for these data sets are listed in Table 6.3 and Table 6.4.
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<tr>
<th>Abbrev.</th>
<th>K₂Pt(NO₂)₄</th>
<th>K₂PtCl₄</th>
<th>methylHgAcetate</th>
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</thead>
<tbody>
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<td>274.6</td>
</tr>
<tr>
<td>Conc. (mM)</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Soak time (hours)</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Resol. (Å)</td>
<td>2.5</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>λ (Å)</td>
<td>1.09</td>
<td>1.09</td>
<td>1.09</td>
</tr>
</tbody>
</table>

**Table 6.3:** Potential heavy atom derivative data sets of Δ2-75 leader peptidase crystals. This data was collected at the National Synchrotron Light Source, Brookhaven National Laboratory beam line X12C July 19-22, 1996. The diffraction intensities were recorded on a Mar image plate detector, using a 180mm plate. Each data set was collected from 1 crystal soaked in the specified concentration of heavy atom compound dissolved in the cryosolvent. The Oxford cryosystem was used to maintain the crystal at 100°K.
Table 6.4: Potential heavy atom derivative data sets of Δ2-75 leader peptidase crystals. This data was collected at the Hamburg EMBL DESY synchrotron beam line BW7A and BW7B, Nov. 1-4, 1996. The diffraction intensities were recorded on a Mar image plate detector, using a 180mm plate. Each data set was collected from 1 crystal soaked in the specified concentration of heavy atom compound dissolved in the cryosolvent. The Oxford cryosystem was used to maintain the crystal at 100°K. * Data not yet processed.
<table>
<thead>
<tr>
<th>Compound</th>
<th>abbrev.</th>
<th>M.W (Da)</th>
<th>Conc. (mM)</th>
<th>Soak time (hours)</th>
<th>resol. (Å)</th>
<th>beamline</th>
<th>λ (Å)</th>
<th>completeness (%)</th>
<th>R_merpe</th>
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</thead>
<tbody>
<tr>
<td>MethylHgAc</td>
<td>mma</td>
<td>275</td>
<td>16.8</td>
<td>6</td>
<td>2.7</td>
<td>BW7A</td>
<td>0.990</td>
<td>*</td>
<td>*</td>
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<tr>
<td>MethylHgAc</td>
<td>mma2</td>
<td>275</td>
<td>6.1</td>
<td>12</td>
<td>2.9</td>
<td>BW7A</td>
<td>0.990</td>
<td>76.3</td>
<td>0.103</td>
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<tr>
<td>EthylHgPO₄</td>
<td>emp</td>
<td>325</td>
<td>4.9</td>
<td>6</td>
<td>2.9</td>
<td>BW7A</td>
<td>0.990</td>
<td>99.6</td>
<td>0.069</td>
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<tr>
<td>NaAuCl₂</td>
<td>aucl</td>
<td>291</td>
<td>7.6</td>
<td>8</td>
<td>2.9</td>
<td>BW7A</td>
<td>0.990</td>
<td>84.1</td>
<td>0.141</td>
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<tr>
<td>K₂PtCl₄</td>
<td>ptc1</td>
<td>415</td>
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<td>9</td>
<td>2.9</td>
<td>BW7A</td>
<td>0.990</td>
<td>91.5</td>
<td>0.115</td>
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<tr>
<td>K₂Pt(CNS)₄</td>
<td>cns</td>
<td>505</td>
<td>5.7</td>
<td>12</td>
<td>2.9</td>
<td>BW7A</td>
<td>0.990</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>K₂PdCl₄</td>
<td>pdcl</td>
<td>326</td>
<td>9.2</td>
<td>6</td>
<td>2.9</td>
<td>BW7A</td>
<td>0.990</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>C₁₃H₁₆HgNO₆·Na</td>
<td>c13hgb</td>
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<td>5.5</td>
<td>10</td>
<td>2.6</td>
<td>BW7B</td>
<td>0.889</td>
<td>*</td>
<td>*</td>
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<tr>
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<td>6.5</td>
<td>12</td>
<td>2.6</td>
<td>BW7B</td>
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<td>*</td>
<td>*</td>
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<tr>
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<td>5.9</td>
<td>15</td>
<td>2.6</td>
<td>BW7B</td>
<td>0.889</td>
<td>*</td>
<td>*</td>
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</tbody>
</table>

Table 6.4


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Paetzel, M. and Dalbey, R.E.(1997b) “Investigating the structural and chemical flexibility of the essential lysine in Escherichia coli type 1 signal peptidase by the use of site-directed chemical modification.” The proceedings of the 11th International Conference on Proteolysis and Protein Turnover.


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