Signal peptidase, which removes signal peptides from preproteins, has a substrate specificity for small uncharged residues at −1 (P1) and small or larger aliphatic residues at the −3 (P3) position. Structures of the catalytic domain with a 5S-penem inhibitor and a lipoprotein inhibitor reveal candidate residues that make up the S1 and S3 pockets that bind the P1 and P3 specificity residues of the preprotein substrate. We have used site-directed mutagenesis, mass spectrometric analysis, and in vivo and in vitro activity assays as well as molecular modeling to examine the importance of the substrate pocket residues. Generally, we find that the S1 and S3 binding sites can tolerate changes that are expected to increase or decrease the size of the pocket without large effects on activity. One residue that contributes to the high fidelity of cleavage of signal peptidase is the Ile-144 residue. Changes of the Ile-144 residue to cysteine result in cleavage at multiple sites, as determined by mass spectrometry and Edman sequencing analysis. In addition, we find that signal peptidase is able to cleave after phenylalanine at the −1 residue in a double mutant in which both Ile-86 and Ile-144 were changed to an alanine. Also, alteration of the Ile-144 and Ile-86 residues to the corresponding residues found in the homologous Imp1 protease changes the specificity to promote cleavage following a −1 Asn residue. This work shows that Ile-144 and Ile-86 contribute to the signal peptidase substrate specificity and that Ile-144 is important for the accuracy of the cleavage reaction.

In all living cells, proteins that are exported to the cell surface are synthesized with an N-terminal, cleavable signal peptide. The signal peptides are then proteolytically removed by a type I signal peptidase (SPase) that has its catalytic domain on the trans side of the membrane.

SPases have been identified and characterized in a wide variety of Gram-negative and Gram-positive bacteria, mitochondria, and the endoplasmic reticulum (for reviews, see Refs. 1 and 2). Except for the SPase found in the endoplasmic reticulum, the SPases are proteases that use a serine-lysin catalytic dyad mechanism (3–9). The active site serine residue, Ser-90 in Escherichia coli SPase, is conserved in the homologous subunit within the endoplasmic reticulum signal peptidase complex. However, it differs from the bacterial and organelar SPases by the substitution of a histidine in place of the catalytic lysine. The endoplasmic reticulum signal peptidase uses either a serine-histidine dyad mechanism or the classical serine-histidine-aspartic acid triad mechanism (4).

The crystal structure of the E. coli signal peptidase with covalently attached inhibitor proved that SPase is a serine protease and is consistent with Lys-145 being involved in the activation of the nucleophilic Ser-90 (10). The structure provided a picture of how SPase can bind and cleave signal peptides from exported proteins. Fig. 1 shows a structural model of the E. coli SPase (shown in a light gray ribbon diagram) with a theoretical model of a signal peptide (yellow) docked into its substrate binding sites (displayed as a molecular surface, green, red, cyan). One of the striking features of the protein structure is a large hydrophobic patch comprised of an antiparallel β-strand domain (residues 81–85, 99–105, 292–307, and 312–314) that we believe allows the catalytic domain to interact with the membrane during catalysis. Fig. 1A shows the proposed orientation of SPase relative to the membrane surface with the hydrophobic domain (indicated in yellow) of the signal peptide extending downwards into the lipid bilayer.

In both prokaryotic and eukaryotic organisms, SPase exhibits similar substrate requirements. With the exception of mitochondrial Imp1, type 1 SPase specifically recognizes substrates with small amino acid residues at the −1 (P1) position or small uncharged residues at the −3 (P3) position of the signal peptide relative to the cleavage site (11–14). The identity of determinants within SPase that control cleavage of the substrate at the correct site and not at other nearby potential sites remains unknown. The substrate specificity determinants within SPase that allow it to cleave signal peptides following the −3, −1 rule are also undefined. One clue comes from the structure. The structure of E. coli SPase with a bound inhibitor indicates that nonpolar atoms from the residues Ile-86, Pro-87, Ser-88, Gly-89, Ser-90, Met-91, Leu-95, Tyr-143, Ile-144, and Lys-145 form the S1 pocket. In addition, modeling studies suggest that nonpolar atoms from the residues Phe-84, Ile-86, Ile-101, Val-132, Asp-142, and Ile-144 comprise the S3 pocket. The identity of determinants within SPase that control cleavage of the substrate at the correct site and not at other nearby potential sites remains unknown. The substrate specificity determinants within SPase that allow it to cleave signal peptides following the −3, −1 rule are also undefined. One clue comes from the structure. The structure of E. coli SPase with a bound inhibitor indicates that nonpolar atoms from the residues Ile-86, Pro-87, Ser-88, Gly-89, Ser-90, Met-91, Leu-95, Tyr-143, Ile-144, and Lys-145 form the S1 pocket. In addition, modeling studies suggest that nonpolar atoms from the residues Phe-84, Ile-86, Ile-101, Val-132, Asp-142, and Ile-144 comprise the S3 pocket (10). The residues Ile-144 and Ile-86 (colored red in Fig. 1) bridge the S1 and S3 binding pockets.

As a first step to identify residues that control cleavage fidelity, and substrate specificity, we mutated residues in the E. coli SPase substrate binding site and examined the effects of...
those changes on the cleavage of various substrates. We found that substitution of SPase Ile-144 by a cysteine residue results in cleavage of the proOmpA nuclease A substrate at multiple sites. Cleavage at multiple sites is in contrast to the action of wild-type SPase, which cleaves exclusively at the correct site. Cleavage at multiple sites is in contrast to the action of wild-type SPase, which cleaves exclusively at the correct site. In addition, wild-type SPase, which cleaves exclusively at the correct site.

In Vitro and in Vivo Assay of Signal Peptidase Cleavage—The in vitro activity of wild-type and mutant SPases was determined using various proOmpA nuclease A substrates. Cleavage of the substrate was performed at different dilutions of SPase, as described (16). To determine the activity of SPase in vivo, we used the temperature-sensitive IT41(DE3) strain. The IT41(DE3) strain was modified from the original temperature-sensitive SPase I strain, was obtained from Dr. Yoshikazu Nakamura (15). The IT41 cell line was modified by incorporation of the T7 RNA polymerase gene using λDE3 phage supplied in the λDE3 lysogenization kit (Novagen) to produce IT41(DE3). Lysogens successfully expressing the T7 RNA polymerase were verified using the included T7 tester phage in addition to verifying temperature-sensitive growth. The pET 23b vector, which contains isopropyl-1-thio-β-D-galactopyranoside-inducible SPase with a His 6 tag, was described previously (16). The pONF1 vector overexpressing proOmpA nuclease A was described in Ref. 17. This nuclease A substrate has a six-histidine sequence at codons 2–7 (18).

**DNA Methods**—The QuickChange mutagenesis method (Stratagene Inc.) was used to make oligonucleotide-directed mutations within the SPase and proOmpA nuclease A proteins. Mutations were verified by gene sequencing. The calcium chloride and electroporation methods were used for DNA transformation into E. coli strain, DH5α.

Purification of Signal Peptidase and proOmpA Nuclease A—BL21(DE3) cells containing His 6-tagged SPase encoded by the pET23b vector were used for overexpression of SPase. SPase was expressed and purified using ion exchange and nickel affinity chromatography, as described by Ref. 16. ProOmpA nuclease A containing a His 6 tag was purified over a Ni 2+-nitrilotriacetic acid-agarose (Qiagen, Inc.) column, as described in Ref. 18, with modifications to utilize denaturing conditions. Following ammonium sulfate precipitation, proteins were dissolved in 6 M guanidine buffer and loaded on 1-mL bed volume of nickel-nitrilotriacetic acid resin. Resin was washed with 8 M urea buffer (pH 8.0) to remove guanidine, and elution of bound protein was accomplished using 8 M urea buffers of reducing pH (pH 6.3, 5.9, 4.5). Following elution, proteins were assayed for purity using 17% SDS-PAGE, and selected fractions were refolded slowly by dialyzing against increasing volumes of 25 mM HEPES (pH 7.5) with 1% Triton X-100 utilizing a total of 4 liters of buffer.

**In Vitro and in Vivo Assay of Signal Peptidase Cleavage**—The in vitro activity of wild-type and mutant SPases was determined using various proOmpA nuclease A substrates. Cleavage of the substrate was performed at different dilutions of SPase, as described (16). To determine the activity of SPase in vivo, we used the temperature-sensitive IT41(DE3) strain. The IT41(DE3) strain was modified from the original temperature-sensitive SPase I strain by introducing the T7 RNA polymerase gene, allowing expression of the pET 23b encoded SPases. The procedure for measuring in vivo activity of the SPase mutants is the same as that described by Ref. 16. Single colony transformants of IT41(DE3) were transferred into M9-glucose media and grown to a density of ~0.3 A max. The cells were then shifted to 42°C and incubated for an additional 1.5 h. The cells were pulsed with [35S]methionine for 10 s and then chased with non-radioactive methionine for the indicated times.

**Complementation of the Growth Defect**—The processing of proOmpA was assayed in the temperature-sensitive SPase strain, IT41(DE3). All medium used to culture IT41(DE3) were prepared with a low salt concentration (2.5g NaCl/liter). Competent cells of IT41(DE3) were prepared using the CaCl2 method and transformed with a plasmid expressing the SPase mutant to be studied. The transformed IT41(DE3) cells were plated on solid medium with 100 μg/ml ampicillin and grown at 30°C until colonies reached 1–2 mm in size. Single transformants were selected and resuspended in fresh medium and streaked in duplicate on fresh plates for incubation at 30°C and 42°C to assay for complementation of growth at the nonpermissive temperature.

**Automated Edman Degradation**—For N-terminal protein sequence analysis, enzyme reaction mixtures were separated by SDS-PAGE and then transferred electrothermally to polyvinylidene difluoride membranes. Transferred proteins were visualized on the polyvinylidene difluoride blots by staining with Coomassie Blue then excised for sequence analysis. N-terminal Edman degradations were performed using an Applied Biosystems Model 492 automated protein sequencer.

**Matrix-assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry**—SPase cleavage of proOmpA nuclease A was also analyzed using mass spectrometry. Small aliquots of reaction mixtures (10–20 μl) were precipitated by the addition of 10 volumes of ice-cold acetone. After standing on ice for 10 min, the acetone-precipitated proteins were sedimented by centrifugation in an Eppendorf centrifuge at 13,000 rpm for 10 min. The supernatant was carefully removed by aspiration, and the protein pellets (usually not visible) were redissolved by the addition of 5 μl of 10% formic acid. The dissolved proteins were mixed with the matrix solution: 5 μl of saturated solution of a-cyano-4-hydroxycinnamic acid (10 mg dissolved in 500 μl of 0.1% trifluoroacetic acid and 500 μl of CH3CN). The stainless steel MALDI-TOF target plate was spotted with 1 μl of each reaction sample and analyzed using a Bruker Daltonics Autoflex mass spectrometer in the...
The instrument was calibrated with a mixture of protein standards including insulin (5,734.6 Da); cytochrome c (12,361.1 Da), and myoglobin (16,952.6 Da). The mass accuracy in the 17 kDa mass range was ± 11 Da.

**Prediction of Theoretical Mass for Cleavage Products**—To predict the size of postulated cleavage products to match with the mass spectrometry data (see Table III), various portions of the sequence of the mature domain of proOmpA-Nuclease A were entered into the PeptideMass tool on the ExPASy Proteomics Server (us.expasy.org/tools/peptide-mass.html). Singly charged average masses were calculated without enzymatic cutting to obtain the total mass of the predicted protein products of cleavage by signal peptidase.

**Molecular Modeling**—Models of each of the SPase mutants were made using the atomic coordinates from the recent crystal structure of signal peptidase in complex with a lipopeptide inhibitor (Protein Data Bank code IT7D) as the template (19). The mutations were made with the program XtalView (20). The model for the proOmpA nuclease A (PONA) substrate was built into the active site of SPase using the acyl-enzyme penem inhibitor (10) and noncovalently bound lipopeptide inhibitor complexes (19) as a guide to position the P1 and P3 residues into the S1 and S3 binding pockets. The paths taken by the signal peptide along the surface of SPase and the parallel β-sheet type hydrogen bonding interactions with SPase are basically the same as those observed in the lipopeptide inhibitor complex structure. The models of

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**FIG. 2.** Effect of mutations that are predicted to increase or decrease the size of the binding pocket on SPase activity. A, mutations that are predicted to increase the size of the binding pocket. P indicates the position of the uncleaved preprotein, and M indicates the position of the mature protein. B, mutations that are predicted to decrease the size of the binding pocket. Wild-type PONA was digested by serial dilutions of stock wild-type and mutant forms of signal peptidase to study the effect of changing the size of the substrate binding pockets. The 0 dilution lane in the upper gel is a control in which no enzyme has been added and indicates the position of the unprocessed form of PONA. Processing of PONA was initiated by the addition of 1 μl of signal peptidase stock (0.1 mg/ml) or a corresponding serial dilution of the stock to 10 μl of PONA reaction mixture (0.04 mg/ml in 50 mM Tris, 10 mM CaCl₂, 1% Triton X-100, pH 8.0). Reactions were incubated for 1 h at 37 °C and then run on 17% SDS-PAGE.
In vitro Analysis of SPase Binding Site Mutants with the Wild-type Preprotein Substrate—To test the importance of amino acids that comprise the S1 and S3 pockets for SPase catalysis, site-directed mutagenesis was used to alter substrate binding residues predicted to increase or decrease the size of the S1 and S3 pockets. The pocket residues were changed to alanines to increase the pocket size (Fig. 2). As can be seen, these mutations that resulted in a smaller pocket size impaired roughly 10–20-fold when compared with the wild-type enzyme, whereas the V132A mutant exhibited a 100-fold decrease in activity. In contrast, the other mutants had near WT activity. To decrease the size of the S1 and S3 pockets, the predicted pocket size had only a modest effect on the activity.

<table>
<thead>
<tr>
<th>Mutant enzyme</th>
<th>Proposed pocket</th>
<th>Activity relative to 100% WT</th>
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</thead>
<tbody>
<tr>
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<td>S3</td>
<td>50</td>
</tr>
<tr>
<td>F84W</td>
<td>S3</td>
<td>10</td>
</tr>
<tr>
<td>I86A</td>
<td>S1/S3</td>
<td>0.5</td>
</tr>
<tr>
<td>G99A</td>
<td>S1</td>
<td>100</td>
</tr>
<tr>
<td>M91A</td>
<td>S1</td>
<td>5</td>
</tr>
<tr>
<td>L95A</td>
<td>S1</td>
<td>10</td>
</tr>
<tr>
<td>L95R</td>
<td>S1</td>
<td>0</td>
</tr>
<tr>
<td>I130A</td>
<td>S1</td>
<td>100</td>
</tr>
<tr>
<td>V132A</td>
<td>S3</td>
<td>1</td>
</tr>
<tr>
<td>V132I</td>
<td>S3</td>
<td>100</td>
</tr>
<tr>
<td>D142E</td>
<td>S3</td>
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<td>D142I</td>
<td>S3</td>
<td>100</td>
</tr>
<tr>
<td>Y143A</td>
<td>S1/S3</td>
<td>10</td>
</tr>
<tr>
<td>Y143W</td>
<td>S1/S3</td>
<td>100</td>
</tr>
<tr>
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</tr>
<tr>
<td>I144C</td>
<td>S1/S3</td>
<td>50</td>
</tr>
<tr>
<td>I144S</td>
<td>S1/S3</td>
<td>10</td>
</tr>
<tr>
<td>I144A/I86A</td>
<td>S1/S3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The Signal Peptidase Binding Site

Activities of mutant SPases were determined from dilution studies shown in Figure 2. Dilution of WT and mutant necessary to achieve 50% processing was determined and comparison yielded the relative activities.

Rats and mice treated with poly I:C demonstrated an increase in the amount of IFN-α mRNA and protein released in the supernatant of cultured cells treated with poly I:C. This increase in IFN-α production was correlated with an increase in the expression of IFN-α mRNA and protein. The results suggest that poly I:C treatment activates the production of IFN-α in vivo and in vitro.

The signal peptidase-plex (PONAs) are energy-minimized using the program CNS (21). Figures were prepared using the program PyMol (22).

RESULTS

In vitro Analysis of SPase Cleavage of Preprotein Substrate Mutants—As a first step to examine the substrate specificity of the different SPase mutants, we examined the ability of the WT and selected SPase mutants to process PONA substrates with different –1 or –3 residues (Fig. 3A). The WT SPase cleaved the WT PONA (with a –1 and –3 alanine) to generate the mature nuclease A domain. In contrast, the WT enzyme cleaved PONA with phenylalanine at the signal peptide –1 position to produce a product running slower on SDS-PAGE than the mature nuclease A domain. Alternatively, a PONA substrate with a –3 phenylalanine is cleaved by the WT SPase to produce a cleavage product of a similar size as that observed with the WT substrate. A similar cleavage pattern is observed for the –3 Phe mutant with the other SPase mutants assayed. In contrast, the SPase I144A, I144C, and I144S mutants can cleave the –1 Val PONA to a rapidly migrating OmpA nuclease A like that observed when the WT SPase processes WT PONA. Only a small amount of cleavage of the –1 Val PONA mutant is observed with the double mutant I144A/I86A. With the –3 phenylalanine proOmpA nuclease A variant, all the I144 mutants produce a processed band similar in size to the wild-type. However, almost no processing of the –1 phenylalanine mutant is observed for any of these mutants.

We followed the time course of –1 Phe PONA processing using higher enzyme concentrations since very little processing of the PONA was observed for the Ile-144 mutants in lower enzyme concentrations. Indeed, cleavage is observed at these higher SPase levels for all of the mutants (Fig. 3B). Interestingly, a lower molecular weight processed band is observed when cleavage is performed with the WT, I144A, and I144S SPase enzymes. These studies showed that the WT and SPase mutants cleaved the PONA mutant substrates, but it was not clear where cleavage was occurring.

Mass Spectrometric Analysis of Signal Peptide Cleavage—To identify the sites of cleavage of the various substrates by the SPase enzymes, mass spectrometry was utilized to measure the masses of the major (Table II, major peak (*)) and minor products. The mass data are shown in Table II, and Table III shows the predicted theoretical masses corresponding to cleavage of the PONA substrates at various positions from –11 to +6. By comparing the mass data (Table II) with the theoretical masses (Table III) for processing at various sites, we determined whether processing had occurred. For selected samples, Edman degradation was performed on the cleavage products to confirm the processing site. Edman degradation showed that the WT enzyme cleaved the WT PONA substrate exclusively at the predicted site. In addition, mass spectrometry showed that a peak at 16,812 ± 11 Da (16,812 Da theoretical) was produced by SPase cleavage (Fig. 4A), indicating cleavage at the predicted site. In contrast, WT SPase processed the –1 Phe mutant eight residues toward the N terminus from the wild-type cleavage site following the Ala-Leu-Ala sequence. Edman degradation yielded the sequence, GFATVAQ. MALDI-TOF analysis determined a mass of 17,637 Da (17,634 Da theoretical) (Fig. 4B). Processing of the –1 Val substrate by WT SPase shows the majority of cleavage occurring at the same Ala-Leu-Ala sequence, in this case producing a peak of mass 17,601 Da (17,586 Da theoretical). For this digest, other minor cleavage products are observed as well with masses: 17,052, 17,810 and 16,651 corresponding to cleavage following –3 Ala (17,040 Da theoretical), –11 Ala (17,770 Da theoretical), and +2 Thr (16,640 Da theoretical).

In contrast to the fidelity seen when the WT SPase cleaves the wild-type PONA, the I144C mutant cleaved the same substrate at a number of sites, as determined by mass spectrometry. Although the major peak is at the normal cleavage site, significant cleavage was also observed at other sites (Fig. 4A). Mass analysis revealed a major product of 16,819 Da as well as alternate cleavage products of mass 17,028, 16,751, 16,646, and 16,559 Da (compare this with the WT, where only a 16,812 Da cleavage product is observed). Analysis of these data with the theoretical masses of various PONA cleavage products (Table III) revealed that cleavage occurred after the –3 Ala (17,011 Da theoretical), +1 Ala (16,741 Da), +2 Thr (16,640 Da), and +3 Ser (16,553 Da). A second trial digestion confirmed the results from the first trail, producing the same cleavage products (Table II). Multiple cleavage sites occurred as well with the –1 Phe PONA substrate by the I144C mutant (Table II). The first trial shows the following
peaks: 17,075, 16,808, 16,547, and 17,628 Da. Analysis of these masses showed corresponding cleavage sites after $\text{Ile}^{[144]}\text{Ala}$ (17,088 Da theoretical), $\text{Ile}^{[144]}\text{Phe}$ (16,812 Da), $\text{Ile}^{[144]}\text{Ser}$ (16,553 Da), as well as the upstream $\text{Ile}^{[144]}\text{Ala}$ (17,634 Da) site seen with the WT enzyme. The second trial showed a different pattern: 16,550, 16,637, and 16,738 Da with major cleavage occurring after $\text{Ile}^{[144]}\text{Ser}$ (16,553 Da theoretical), $\text{Ile}^{[144]}\text{Thr}$ (16,640 Da), and $\text{Ile}^{[144]}\text{Ala}$ (16,741 Da). Taken together, the results demonstrate that the Ile-144 residue is important for determining the accuracy of SPase cleavage.

Although the I144C mutant looses its fidelity of cleavage, the I144A/I86A mutant cleaved the WT PONA substrate only at

**TABLE II**

**Mass spectral data of PONA cleavage products**

Mass data for the major and minor peaks determined by mass spectrometry is tabulated below. The first entry in the column indicates the major peak (*) followed by any other minor peaks observed. Predicted masses for theoretical cleavage are indicated in parentheses.

<table>
<thead>
<tr>
<th>PONA</th>
<th>WT SP</th>
<th>I144C SP</th>
<th>I144A/I86A SP</th>
<th>I144C/I86C SP</th>
<th>I144C/I86T SP</th>
<th>I144C/I86F PONA</th>
<th>I144A/I86A SP</th>
<th>I144C/I86N PONA</th>
<th>I144A/I86F PONA</th>
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<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
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<td>1</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>*16812 (16812)</td>
<td>*16815 (16812)</td>
<td>*16819 (16812), 16646 (16640), 16751 (16741), 16559 (16553), 17028 (17012)</td>
<td>*16812 (16812)</td>
<td>*16812 (16812)</td>
<td>*16819 (16812)</td>
<td>*16822 (16812), 17033 (17012)</td>
<td>*16812 (16812)</td>
<td>*16812 (16812)</td>
</tr>
</tbody>
</table>
the correct site producing a mass of 16,812 Da (theoretical 16,812 Da) (Table II). Strikingly, this double mutant also cleaves the –1 Phe PONA at the correct cleavage site position (following the –1 Phe), generating a 16,808 Da product in our first trial and a 16,817 Da product in the second trial, both closely matching the predicted mass of 16,812 Da within the error range of ± 11 Da. Due to the fact that the –1 Phe PONA is a poor substrate, some of the uncleaved preprotein of theo-

TABLE III

Predicted theoretical masses of various PONA cleavage products

<table>
<thead>
<tr>
<th>AA Sequence</th>
<th>Name</th>
<th>WT</th>
<th>–IV</th>
<th>–IF</th>
<th>–IN</th>
<th>–SF</th>
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<td>19,691</td>
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<tr>
<td>L</td>
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<td>16323</td>
<td>16,323</td>
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</table>

-PONA | 16,195 | 16,195 | 16195 | 16195 | 16,195 |

The theoretical masses of possible PONA cleavage products were predicted using the PeptideMass tool on the ExPASy Proteomics Server. Average masses are reported as [M + H]+.

The Signal Peptidase Binding Site

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FIG. 4. Mass analysis demonstrates altered cleavage profile for SPase mutants. Selected mass spectra are paired to illustrate alteration in PONA processing in the various SPase mutants. A, processing of WT PONA by WT SPase yields a single peak corresponding to the WT cleavage product (17,023 is the peak due to the matrix adduct). In the lower panel, digestion by I144C SPase shows multiple peaks, demonstrating a loss of fidelity. B, in the upper panel, processing of the −1 Phe PONA by the WT enzyme yields peaks, which are heavier than the product corresponding to cleavage at the −1 site, indicating selection of alternate processing sites. In contrast, the I144A/I86A mutant shows cleavage following the −1F residue (lower panel). C, mass spectra data from digestion of −1 Asn PONA by the WT and I144C/I86T SPase are shown. Processing is observed following the −1 Asn and −3 Ala residues in both cases. In the lower panel, the shift in relative intensity of the peaks indicates a change in preference for the −1 Asn cleavage site for the I144C/I86T mutant.
In Vivo Analysis of Signal Peptidase Binding Site Mutants—To investigate how detrimental the SPase mutations would be with the proteins in the natural membrane environment, we examined processing of proOmpA in vivo using the temperature-sensitive IT41 strain (15), modified by the incorporation of the T7 RNA polymerase as described above. IT41(DE3) was grown in M9/glucose medium at 30 °C to a density of 0.3 $A_{600}$ and then shifted to 42 °C for 1.5 h. The cells were pulsed with $[^{35}S]$methionine for 10 s and then chased with nonradioactive methionine for the indicated times. Fig. 7 shows that the processing of proOmpA is dramatically delayed. However, IT41(DE3) bearing a plasmid encoding WT SPase shows rapid processing. I86A SPase, which showed 1/1000 the activity as WT enzyme in a detergent extract with proOmpA nuclease A, was active in vivo. Processing was completely normal at the nonpermissive temperature when IT41(DE3) was transformed with a plasmid expressing I86A SPase. No in vivo activity was observed for L95R and active site residue mutant, K145A. Except for the L95R SPase mutant, all of the SPase mutants with substrate binding pocket mutations showed activity in vivo (Table IV).

Complementation of the growth defect of IT41(DE3) at 42 °C was assayed for all the SPase mutants. Most mutants clearly showed complementation in this experiment with equivalent colonies on both 30 and 42 °C plates. Other mutants showed a reduced number of colonies on the 42 °C plate, whereas still
TABLE IV

<table>
<thead>
<tr>
<th>Lep Mutant</th>
<th>Time point of 50% processing</th>
</tr>
</thead>
<tbody>
<tr>
<td>No plasmid control</td>
<td>120 s</td>
</tr>
<tr>
<td>WT (+control)</td>
<td>&lt;10 s</td>
</tr>
<tr>
<td>I144A/I86A</td>
<td>10 s</td>
</tr>
<tr>
<td>L95R</td>
<td>50 s</td>
</tr>
<tr>
<td>K145A (−control)</td>
<td>70 s</td>
</tr>
</tbody>
</table>

![Fig. 7](Image)

**Fig. 7. In vivo complementation of OmpA processing defect in IT41(DE3) at 42 °C.** Spase mutants were studied for their ability to complement for the temperature-sensitive Spase defect of IT41(DE3) at 42 °C. Only fresh transformants of Spase and Spase mutants into IT41(DE3) were used. Colonies incubated at 30 °C (see “Experimental Procedures”) were transferred to 2 ml of M9 + glucose medium and incubated at 30 °C until a density of 0.3 A_won was reached. The cultures were then shifted to a 42 °C incubator and incubated for an additional 1.5 h. Cell cultures were labeled with [35S]methionine for 10 s, at which time the 0 time point was taken. After the 0 time point was taken, the chase was begun with the addition of excess nonradioactive methionine with additional time points taken at indicated times. At each time point, 250 μl of culture was quenched by addition to 250 μl of ice-cold 20% trichloroacetic acid. These were then subjected to immunoprecipitation with OmpA antiserum (see “Experimental Procedures”) and run on 15% SDS-PAGE. P indicates the position of the uncleaved proOmpA protein, and M indicates the position of mature OmpA.

**TABLE IV**

**In vivo processing of proOmpA in the temperature-sensitive strain IT41(DE3) at the nonpermissive temperature 42 °C**

The following mutants exhibited 50% processing in <10s like WT Sp: F84A, F84W, G89A, I86A, M91A, M91I, L95A, V103I, I30A, V132A, L95A, V132I, D142E, D142L, Y143A and I144A. This table indicates the amount of chase time required to observe 50% processing for the WT and mutant Spases. Times are indicated in seconds of chase.

![Fig. 8](Image)

**Fig. 8. Molecular modeling of binding site mutants within E. coli signal peptidase.** The amino acid residues of signal peptidase that contribute to the binding pocket surface are shown in van der Waals spheres. The side chains from those residues that contribute to the S1 binding pocket (Pro-87, Ser-88, Gly-89, Ser-90, Met-91, Leu-95, Tyr-143, Lys-145) are colored green. The side chain atoms from those residues that contribute to the S3 binding pocket (Phe-84, Ile-101, Val-132, Asp-142) are colored cyan. The side chain atoms from those residues that contribute both to the S1 and the S3 binding pockets (Ile-86 and Ile-144) are colored hot pink. The main chain atoms are colored white. The P3 to P5 residues of the PONA substrate are shown in yellow (carbon), blue (nitrogen), and red (oxygen). A, the wild-type signal peptidase with wild-type proOmpA nuclease A. B, the I144C mutant of signal peptidase. C, the I86A/I144A mutant of signal peptidase with the bound P1 Ala to P1 Phe mutant of proOmpA nuclease A. D, the 186C, 1144C mutant of signal peptidase.

Others exhibited poorly reproducible growth. Throughout this study, the binding pocket mutant, L95R, and active site mutant, K145A, negative controls exhibited no growth at 42 °C in any of the trials conducted. These results indicate that the mutants, although impaired to varying degrees in their in vitro activities, can still complement in vivo in most cases, whereas the negative controls clearly cannot.

**Molecular Modeling of Binding Site Mutants of Signal Peptidase Binding in Complex with Signal Peptides—**To help in the interpretation of the mutations made both within the binding pockets of Spase and within the recognition residues of the PONA substrate, we have modeled the Spase-PONA complex. The models use the recently solved crystal structure of the complex between signal peptidase and the lipopeptide Arylopyruvate A2 as the template (Protein Data Bank code 1T7D) (19). As described under “Experimental Procedures,” the model for PONA was built into the active site of Spase using the acyl enzyme penem inhibitor crystal structure and noncovalently bound lipopeptide inhibitor complex crystal structure as a guide to position the P1 and P3 residues into the S1 and S3 binding pockets. The path taken by the substrate along the surface of Spase and the nonspecific binding interactions with Spase are basically the same as those observed in the lipopeptide inhibitor complex structure. The ribbon diagram of WT Spase in complex with the WT PONA signal peptide is shown Fig. 1. PONA makes parallel β-sheet interactions with the strands that line the binding sites (residues 83–90 and 142–145) (Fig. 1B). As can be seen from Figs. 1B and 8A, the P1 Ala residue points into the S1 pocket, and the P3 Ala points into the shallow S3 pocket. The residues Ile-86 and Ile-144 sit just at the borderline between the S1 and S3 pockets and contribute atoms to both pockets. The change in the volume of the binding pockets can be seen when the residues contributing to the binding site are depicted in van der Waals spheres (Fig. 8). The ability of the double mutant I86A/I144A to cleave a PONA substrate with a −1 Phe can be rationalized from the modeling, which shows the increased volume within the S1 binding pocket provided by the isoleucine to alanine substitutions, allows the phenyl side chain to fit into the pocket without any van der Waals clashes (Fig. 8C). A similar reasoning based on steric effects can be used to explain the ability of the I86C/I144C to bind and cleave PONA substrates with a −1 Asn (Fig. 8D). It is not clear from the molecular modeling why the mutant I144C (Fig. 8B) has the ability to cleave at multiple sites
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**DISCUSSION**

Here we investigated which residues within the substrate binding pocket of SPase are responsible for the high fidelity of SPase and contribute to the substrate specificity of SPase. Toward this objective, we changed all the candidate S1 and S3 residues within SPase that are believed to bind to the −1 and −3 residues of the precursor substrate. Changes were made that were designed to either increase or decrease the size of the binding pocket. With the exception of the ΔS6A SPase mutant, the results of increasing the size of the pocket showed that most of the mutants were still quite capable of cleaving the substrate, with only a few mutants demonstrating a 10–20-fold reduction in activity. These results suggest that there is plasticity in the substrate binding pocket. The mutants that were designed to decrease the size of the substrate binding pocket again only had modest effects. This is probably due to the fact that the pockets are already quite small within the WT SPase and that the enzyme is most likely able to make the necessary structural adjustments required to bind the mutant substrates. A comparison of the three crystal structures so far available for the E. coli signal peptidase (the apo-enzyme (23), the covalently bound acyl-enzyme inhibitor complex (10), and the noncovalently bound peptide inhibitor form (19)) show that adjustments in both the main chains and side chains surrounding the binding site are observed (19).

Our results showed that mutation of isoleucine 144 to cysteine alters the fidelity of the enzyme. The I144C SP cleaved not only at the normal processing site but also at other sites: after the −3 Ala, −1 Ala, +1 Ala, +2 Thr, and +3 Ser with respect to the normal cleavage position (Fig. 9 shows a cleavage site summary). The proximal sequences including the cleavage sites are: TVA-Q for the −3 Ala; QAA-A for the −1 Ala; QAA-T for the +1 Ala; AAT-S for the +2 Thr; and ATS-T for +3 Ser, where the - indicates the position of cleavage. One possible explanation for this lack of fidelity may be that the cysteine mutation in the binding pocket may lead to sliding of the prebound substrate within the active site region. Sliding of the substrate once prebound would lead to a cleavage profile with sites clustered around the canonical site as observed in our experiments. The WT leucine in the substrate binding pocket leads to optimal binding, which prevents sliding, and thereby cleavage occurs at only the normal site. These results support the idea that the isoleucine 144 residue is important for SPase to cleave its substrate accurately at the proper site.

In addition to identifying a residue contributing to the accuracy of SPase cleavage, we identified residues that contribute to the substrate specificity of SPase. As mentioned in the introduction, the substrate specificity determinants of this enzyme are at the −1 and −3 position of the substrate protein. Signal sequences within preproteins contain a −1 Ala, Gly, Ser, and Thr and a −3 Ala, Val, Ser, Thr, Gly, and Leu (24). The E. coli SPase has been shown to cleave precursor substrates with a −1 Ala, Gly, Ser, Cys, Pro and, in some cases, Thr (12–14). In addition, it can cleave substrates with a −3 Gly, Leu, Val, Thr, Cys, Ser, Ile, Pro, and Asn. In bacteria, Phe is never found at the −1 or −3 position (25). Out of 334 Gram-negative signal peptide sequences in the Swiss-Prot data set, there are two Val and one Asn at the −1 position (25). However, they are believed to be incorrectly assigned signal peptide cleavage site residues (25). Strikingly, the substrate specificity of SPase is changed with the mutation of the Ile-144 and Ile-86 residues to alanine. Although the WT enzyme cannot cleave the proOmpA nuclease substrate after the −1 Phe, the ΔS6A/I144A mutant cleaved the substrate after the −1 Phe with no other cleavage products observed by mass spectrometry (Figs. 4B and 9). On the other hand, the WT SPase cleaved the −1 Phe substrate at an alternative site eight residues upstream (following the −9 Ala) from the normal cleavage site, following the standard processing rules. This suggests that residues well into the proposed signal peptide hydrophobic helical region of these mutant substrates are able to melt into the appropriate β-strand conformation needed to make the intermolecular contacts with the SPase binding site. Previously, processing at an alternative site has been observed when processing at the normal site was prevented by mutating the −1 residues (12). The I144C mutant cleaves the −1 Phe PONA substrate at multiple sites, again showing inaccuracy in cleavage of the substrates.

Although ΔS6A/I86A SPase can cleave after −1 Phe, it is a very poor enzyme. Its activity is around 1/10,000 when compared with the WT enzyme processing the WT proOmpA nuclease A substrate. A similar large reduction in activity was found with trypsin when the active site Gly-226 was changed to an Ala, resulting in increased preference for cleaving substrates with lysines relative to arginines (26). In addition, the substrate specificity of granzyme B can be extended at the P4 position (but the activity is lowered dramatically from 10- to 100-fold) by mutating the active site residues Ile-99 and Asn-218 to Ala residues (27). Further changes in the SPase protein, both in the active site and out of the active site region, may be necessary to increase the catalytic potency toward the −1 Phe substrate.

Our results indicate that the −1 and −3 residues within the PONA substrate strongly influence where the WT SPase cleaves the substrate. Although the WT SPase cleaves the −1 Val and −3 Phe substrates downstream within the mature domain at the cleavage sites, VAT-S and AAT-S, respectively...
(Fig. 3A and Table II), it cleaves the −1 Phe PONA substrate eight residues upstream (from the normal cleavage site) within the leader sequence at ALA-G. For this substrate, WT SPase cannot cleave after the +2 Thr because the F is an inappropriate −3 residue (FAT-S), thereby preventing cleavage at this site (24).

A similarly strong influence of the −1/−3 residue is seen with the I144C and I144A/I86A SPases. The −1 Val PONA is processed by the SPase mutants mainly after the +2 Thr residue (following the cleavage sequence VAT-S), although I144C processing yielded at least one other minor peak, indicating cleavage after the −1 Val. Cleavage of the −1 Val PONA by the I144A/I86A mutant occurs at the VAT-S site exclusively over the normal cleavage position at −1. This is in contrast to the AQF-A cleavage site observed with the −1 Phe PONA where SPase I144A/I86A cleavage does occur after the −1 position. It appears that in this case as well, the binding of valine by the S1 pocket is highly unfavorable, causing the shift to the VAT-S site. In addition, the SPase pocket is highly unfavorable, causing the shift to the VAT-S position. In general, whereas both Imp 1-type double mutants and other Ille-144 mutants (data not shown) demonstrated a preference for cleavage following −1 Asn, WT SPase cleaves WT PONA 10,000 times better than the −1 Asn PONA. In contrast, the SPase Ille-144/Ile-86 mutants mimicking the Imp1 binding residues cleave WT PONA only 100 times better. Taken together, these observations strengthen the body of evidence that these residues, in particular, hold a special importance in determining the −1, −3 substrate recognition sequence.

Future studies will have to examine the substrate specificity of the SPase with the Imp1 Ille-144/Ile-86 residues. Chen et al. (9) have shown that, in addition to cleaving its endogenous substrate, i-cyt b2, with a −1 Asn, Imp1 can process substrates with a −1 Ser, Cys, Met, Ala, and Leu. In addition, we will analyze the SPase S1 and S3 mutants, utilizing a library of peptide substrates with random sequence surrounding the OmpA cleavage site. These experiments hold the promise of identifying differences in the preferred cleavage sequence for each of the mutants, which is not possible using designed substrate sequences alone. These future data could help in clarifying the effects on substrate selection and fidelity observed in the current studies. In addition, crystallographic and spectroscopic analysis of these binding site mutants in complex with substrate mutants will help in the understanding of this interesting molecular recognition event that most likely occurs at or within the membrane surface.

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The Identification of Residues That Control Signal Peptidase Cleavage Fidelity and Substrate Specificity
Andrew Karla, Mark O. Lively, Mark Paetzel and Ross Dalbey

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