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Fluorescence spectroscopy of soluble *E. coli* SPase I $\Delta 2-75$ reveals conformational changes in response to ligand binding

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Abstract

The bacterial Sec pathway is responsible for the translocation of secretory preproteins. During the later stages of transport, the membrane-embedded signal peptidase I (SPase I) cleaves the signal peptide from a preprotein. We used tryptophan fluorescence spectroscopy of a soluble, catalytically active *E. coli* SPase I $\Delta 2-75$ enzyme to study its dynamic conformational changes while in solution and when interacting with lipids and signal peptides. We generated four single Trp SPase I $\Delta 2-75$ mutants, W261, W284, W300 and W310. Based on fluorescence quenching experiments, W300 and W310 were found to be more solvent accessible than W261 and W284 in the absence of ligands. W300 and W310 inserted into lipids, consistent with their location at the enzyme's proposed membrane-interface region, while the solvent accessibilities of W261, W284 and W300 were modified in the presence of signal peptide, suggesting propagation of structural changes beyond the active site in response to peptide binding. The signal peptide binding affinity for the enzyme was measured via FRET experiments and the K_d determined to be 4.4 μ M. The location of the peptide with respect to the enzyme was also established; this positioning is crucial for the peptide to gain access to the enzyme active site as it emerges from the translocon into the membrane bilayer. These studies reveal enzymatic structural changes required for preprotein proteolysis as it interacts with its two key partners, the signal peptide and membrane phospholipids.

Keywords

signal peptide; protein transport; preprotein cleavage; acrylamide quenching; signal peptidase

INTRODUCTION

The bacterial secretory pathway consists of the following key components: the membrane anchored SecYEG channel that provides passage for protein transport, the soluble ATPase SecA that is instrumental in providing energy for the translocation of a secretory preprotein through the membrane, and the signal peptidase enzyme (SPase), which cleaves the signal peptide to yield the functional mature protein.¹ SPase has been shown to be vital for cell survival.^{2–4} Since bacterial SPases vary considerably from eukaryotes in their proteolytic mechanism, they form an interesting class of potential anti-microbial drug targets.^{5,6}

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Bacterial SPase I, which cleaves the signal peptides of secretory non-lipoproteins, utilizes a serine-lysine catalytic dyad, unlike the eukaryotic enzymes, that generally use a serine-histidine-aspartate triad.⁷ The signal peptide substrate is made up of 18–30 residues, and immediately precedes the mature region of the secretory preprotein.⁸ The important residues for substrate recognition are the -1 (P1) and the -3 (P3) residues of the preprotein.⁹ These residues have been found to specifically bind to the substrate binding pockets S1 and S3, respectively.¹⁰ The most important residues on SPase I for catalysis are Ser 90 which acts as the nucleophile and Lys 145 which forms the general base.^{10–12} Several residues at or near the active site (for example, Ser 88 and Ser 278) play crucial roles in cleavage, such as, in the formation of hydrogen bonds to stabilize enzyme transition states. Once the substrate is bound to the enzyme, Ser 90 is poised to attack the carbonyl carbon of the P1 residue, and the reaction proceeds to subsequently release the mature protein, and the signal peptide.^{10,13} The signal peptide is further cleaved by the signal peptide peptidase enzyme.¹⁴

Traditionally, SPase I studies were rendered difficult because the enzyme is embedded in the membrane. It has a short periplasmic amino terminal domain, followed by two transmembrane segments and a large periplasmic region containing the catalytic active site.¹⁵ This issue was partly resolved when a mutant SPase I lacking residues 2–75 was designed, and purified as a soluble protein.¹⁶ This mutant contains only the C-terminal periplasmic domain, carrying residues important for catalysis. It exhibits efficient protease activity providing an ideal experimental substitute for the full-length enzyme.^{16,17} However, the mutant is considerably hydrophobic, necessitating purification from inclusion bodies, thereby significantly reducing its yield due to aggregation and refolding issues.

We have successfully developed a fusion protein that can be isolated from the soluble cell fraction. Our method involves the generation of a protein carrying a glutathione S-transferase (GST) tag fused to the $\Delta 2-75$ *E. coli* SPase I. This protein can be purified from the soluble lysate, refolding is not required, and the GST tag is cleaved off resulting in high yields of the enzyme.

To date, crystal structures of SPase I bound to inhibitors have helped our understanding of how the enzyme may bind preproteins.^{10,18,19} We utilized tryptophan fluorescence as a means of studying SPase I structure and its interactions with the membrane and signal peptide. Unlike crystallography, which is an excellent method for examining protein 3D structures, this approach is ideal for analyzing dynamic enzyme-ligand interactions and conformational changes under several parallel conditions. SPase I contains six Trp residues, while the $\Delta 2-75$ mutant has four. Two of these residues (W261 and W284) flank a conserved region of the enzyme that is thought to be essential for catalysis, while the other two (W300 and W310) are found at the extreme C-terminal end of the enzyme.¹³ Trp fluorescence is highly sensitive to environment,²⁰ and can therefore yield vital information regarding its location. We generated four SPase I $\Delta 2-75$ mutants, each carrying only one Trp residue and determined the fluorescent yield and solvent accessibility in both aqueous and lipid environments for each using acrylamide quenching. Our results are consistent with the hypothesis that the carboxy-terminal residues of the enzyme interact with the membrane.²¹ We further determined the K_d for signal peptide binding to wild-type SPase I $\Delta 2-75$, the impact of signal peptide binding on Trp solvent accessibility and the location of the signal peptide with respect to the enzyme. These results are consistent with the active site becoming embedded in the bilayer upon signal peptide binding and cleavage. These studies reveal the importance of the dynamic conformational changes that the enzyme must undertake in order to cleave the signal peptide as it emerges from the translocon into the phospholipid bilayer.

MATERIALS AND METHODS

Construction of the GST-SPase I $\Delta 2-75$ vector

The SPase I $\Delta 2-75$ gene (generously provided by Dr. Ross Dalbey) was subcloned into pGEX-6P-1. Three amino acids (Leu, Gly and Ser) directly upstream of SPase I $\Delta 2-75$, and unnecessary for Prescission protease cleavage, were removed via QuikChange mutagenesis (Stratagene), giving rise to the GST-SPase I $\Delta 2-75$ vector. Trp to Phe and Cys to Ser substitutions for fluorescence studies were introduced via QuikChange. All mutations were confirmed by DNA sequence analysis.

Expression and purification of SPase I $\Delta 2-75$

E. coli BL21 (DE3) harboring the GST-SPase I $\Delta 2-75$ vector were grown in LB medium with 100 $\mu\text{g/ml}$ ampicillin at 37 °C until $\text{OD}_{600} \sim 0.7$. Expression of GST-SPase I $\Delta 2-75$ was induced with 0.5 mM IPTG, and cells were grown for 3 hours. The GST-SPase I $\Delta 2-75$ fusion was purified on a Glutathione Sepharose 4B column and cleaved with PreScission Protease according to the manufacturer's instructions (GE Healthcare) to remove the GST tag. Protein concentrations were determined by the Bradford assay method (Pierce).

Cleavage Assays

Cleavage assays were carried out with 0.75 μM to 7.5 μM of the wild-type and mutant SPase I $\Delta 2-75$, and 15 μM of the substrate proOmpA nuclease A in 50 mM Tris-HCl buffer at pH 8.0 containing 1% Triton X-100 at 37 °C for 2 hours. Samples were run on a 12.5% SDS-PAGE gel, and analyzed by Coomassie Brilliant Blue staining.

Signal peptide labeling

The fluorescent dyes IAEDANS and IANBD (Invitrogen) were dissolved in DMSO to a final concentration of 10 mM. The peptide SP2 was dissolved in DMSO to a final concentration of 3 mM. The labeling reaction was carried out in 20 mM phosphate buffer, pH 7.0, with 2 mM fluorescent dye and 200 μM SP2 peptide in the dark at room temperature for 4 hours with shaking. The reaction was terminated with the addition of 30 μM β -mercaptoethanol. The labeled peptide was purified by HPLC, lyophilized and dissolved in DMSO to a final concentration of 3 mM and stored in aliquots at -70 °C. The labeling was calculated according to the manufacturer's instructions and was determined to be 100%.

Signal peptidase labeling

10 μM of S302C SPase I $\Delta 2-75$ was labeled with 200 μM IAEDANS in TKE buffer (25 mM Tris-HCl, pH 7.5, 25 mM KCl, 1 mM EDTA) for 4 hours with shaking in the dark at room temperature. The reaction was stopped with the addition of 30 μM β -mercaptoethanol. Free IAEDANS dye was removed with an Amicon Ultra 50K MWCO centrifugal filter (Millipore) by repeated washes with TKE buffer, until the absorbance of the filtrate at 336 nm was zero. The labeled protein was stored in aliquots at -70 °C.

Fluorescence Measurements

Steady-state fluorescence spectra were obtained on a Fluoromax-3 spectrofluorometer (Jobin Yvon, Inc.) equipped with Glen-Thompson polarizers and a programmable Thermo Neslab water bath. Samples were placed in 3 mm path length quartz cuvettes (Starna Cell Inc.). 500 nM of SPase I $\Delta 2-75$ in TKE buffer at 20°C was used for obtaining data. The excitation and emission slits were both set to give a 3nm band-pass. The excitation wavelength was set to 295 nm, and the emission intensities were measured from 310–400 nm at a rate of 0.5 nm/s. For IAEDANS-S302C and IANBD-SP2 energy transfer experiments, the excitation wavelength was set to 336 nm and the emission intensities were read from 346–660 nm.

Binding affinity measurements for wild-type SPase I $\Delta 2-75$ and IAEDANS-SP2 were performed at an excitation wavelength of 295 nm and the emission intensities were read from 310–530 nm. Polarizer angles were maintained at 0° for excitation and 55° for emission, except in the case of experiments involving liposomes where the excitation and emission angles were set to 90° and 0°, respectively, in order to reduce light scattering effects.

Preparation of Liposomes

E. coli phospholipids (Avanti Polar Lipids) were dried under nitrogen to remove chloroform, and were subsequently kept under vacuum for at least one hour. Lipids were resuspended in buffer containing 50 mM HEPES-KOH, pH 7.0, 30 mM KCl, 30 mM NH₄Cl, 1 mM DTT, and sonicated using a Branson probe-tip sonicator until optical clarity was achieved. The sample was spun down in a table-top centrifuge for 30 minutes at 14,000 rpm and the supernatant containing liposomes was stored in the dark at 4°C and used within 24 hours.

Acrylamide Quenching

Aliquots of a 4 M acrylamide (Fisher Scientific) stock were used to provide a concentration series for the sample and the buffer blank. Sample scans were corrected for acrylamide and buffer fluorescence contributions. Experiments were carried out under both aqueous and lipid environments.

10-doxylnonadecane (10-DN) Quenching

Liposomes were prepared as described above, except that 5.5 mol % of the *E. coli* lipids were replaced with the quencher 10-DN (Avanti Polar Lipids) prior to sonication.²²

Data Processing

All fluorescence emission spectra were analyzed using Origin 6.0 software. For quenching data, emission intensities at 340 nm were analyzed, in order to eliminate interference from the Raman scattering. The Stern-Volmer equation $F_0/F = 1 + K_{SV} [Q]$ was used for curve fitting where F_0 and F are the fluorescence intensities in the absence and presence, respectively of quencher Q and K_{SV} is the Stern-Volmer constant. For quenching experiments with 10-DN, emission intensities at 330 nm for SPase I $\Delta 2-75$ were analyzed both in the presence (F) and absence (F_0) of quencher.²² For binding affinity measurements, the emission intensities at 345 nm were analyzed. K_d was calculated using the one-site binding model equation:

$$\Delta F/F_0 = \frac{\Delta F_{\max}/F_0 [\text{SP2}]}{K_d + [\text{SP2}]}$$

Wherein, [SP2] is the concentration of IAEDANS-SP2, F and F_0 are the emission intensities in the presence and absence of IAEDANS-SP2, respectively, and ΔF represents the change in intensity between F and F_0 . We assume that the [SP2] free = [SP2] total for this reaction. For FRET distance calculations, the equation $E = 1 - [(F_{da} - F_a)/F_d]$ was used, where E represents the energy transfer efficiency, F_{da} , F_a and F_d represent the fluorescence intensity of the sample containing both donor and acceptor, acceptor alone and donor alone, respectively, at 490 nm. The distance between the donor and acceptor (R) was calculated using the equation $E = R_0^6 / (R_0^6 + R^6)$ where R_0 represents the distance between donor and acceptor at which energy transfer efficiency is 50%. All protein structures were presented using PyMol software (Schrödinger).

RESULTS

Characterization of single Trp SPase I $\Delta 2-75$ mutants

E. coli SPase I $\Delta 2-75$ has four Trp residues, W261, W284, W300 and W310 (Fig. 1A). We generated four mutants by site-directed mutagenesis, each of which contains a single Trp residue. The remaining three Trp residues in each mutant were substituted with Phe, in order to maintain aromaticity at each site, thereby minimizing structural perturbations that may arise due to such mutations. Each mutant was named after the residue number of the respective Trp residue remaining (Fig. 1B). We examined the fluorescence spectra of the wild-type SPase I $\Delta 2-75$ as well as the single Trp mutants (Fig. 1C). To study the contribution of fluorescence from each of the single Trp mutants, the samples were excited at 295 nm; at this wavelength, native Phe and Tyr residues exhibit minimal fluorescence. The wild-type enzyme exhibited the highest fluorescence intensity, which is as anticipated since it contains four Trp residues. Each of the four mutants showed sizeable fluorescence emission yields and, therefore, all four non-interacting Trp residues are major contributors to the overall fluorescence emission yield of the wild-type, with W284 and W310 contributing marginally more than W261 and W300 to the overall quantum yield.

Local environment of each Trp residue

Of particular interest were the wavelengths of maximum fluorescence emission ($\lambda_{\max}$) of each mutant (Fig. 1C). The higher the wavelength of maximum emission, the more solvent accessible the Trp is expected to be. W300 and W310 emitted maximally at wavelengths more red-shifted than those of W261 and W284. W261 and W284 had $\lambda_{\max}$ values of 342 and 346 nm, respectively, while W300 and W310 had $\lambda_{\max}$ values of 351 and 353 nm respectively. These results indicate that in aqueous solution W300 and W310 are more solvent accessible than W261 and W284. In vitro cleavage studies with each of these mutant enzymes and the wild-type SPase I $\Delta 2-75$ indicated that all mutants were functional and capable of cleaving the signal peptide of the proOmpA nuclease A preprotein, albeit with somewhat different efficiencies (Fig. 2).

Fluorescence quenching experiments

To understand the native environmental conditions of each of these Trp residues, we performed fluorescence quenching experiments with the well-known collisional quencher acrylamide (Fig. 3A–E). The more solvent exposed a Trp residue is, the more accessible it is to externally added acrylamide; therefore, treatment with acrylamide would result in a decrease in fluorescence emission intensity with a concomitant increase in fluorescence quenching. We employed the Stern-Volmer equation for studying acrylamide quenching; $F_0/F = 1 + K_{SV} [Q]$, where F_0 and F are the fluorescence emission intensities in the absence and presence of acrylamide, respectively, and Q is the quencher acrylamide concentration. K_{SV} denotes the Stern-Volmer quenching constant. K_{SV} is a product of t_0 , the fluorescence lifetime of the excited Trp residue and k_q , the collisional quenching constant. A high K_{SV} value is indicative of a solvent exposed Trp residue that was accessible and hence well quenched by acrylamide, whereas a low K_{SV} value indicates that the Trp residue is in a hydrophobic environment and not exposed to acrylamide for quenching. As shown in Fig. 3, a plot of F_0/F versus acrylamide concentration yields the K_{SV} value for each mutant from the slope (Table I). The fact that the K_{SV} value of the WT is higher than each of the mutants, could be a result of conformational changes that may have occurred upon mutation of the three Trp residues in each mutant to Phe. This could also be responsible for the somewhat different cleavage activities observed for each of the four mutants. W261 and W284 had low K_{SV} values of 2.90 and 3.71 respectively, while W300 and W310 had higher values of 4.46 and 4.74, respectively. These results indicate that for SPase I $\Delta 2-75$ in aqueous solution, the

former two Trp residues are more buried while the latter two are accessible to acrylamide for quenching, in agreement with the relative $\lambda_{\max}$ determinations.

Liposome induced changes in Trp fluorescence

SPase I $\Delta 2-75$ is thought to interact with membranes since a large part of the crystal structure consists of exposed hydrophobic residues; this region has been proposed to form the membrane interacting, or membrane penetrating interface.¹³ It has also been previously shown that SPase I $\Delta 2-75$ exhibits enhanced cleavage ability in the presence of phospholipids and the membrane mimetic detergent Triton X-100.¹⁷ We employed two different fluorescence quenchers to study quenching in the presence of *E. coli* phospholipids, to measure the extent of interaction of the wild-type and single Trp mutants, with liposomes. Quenching experiments were performed with the collisional quencher acrylamide (Fig. 3A–E) and compared with the results in aqueous solution. The results indicated that W300 and W310, that form a part of the proposed membrane interacting surface, were found to be less solvent accessible and less exposed to acrylamide in the presence of liposomes than in their absence, with K_{SV} values of 3.33 and 3.31, respectively, a drop from aqueous K_{SV} values of 4.46 and 4.74, respectively (Table I). We also observed an increase in fluorescence emission intensities for W300 and W310 in lipids (data not shown), when compared to that in aqueous solution, which is indicative of movement of the Trp residue to a more hydrophobic environment. Therefore, the changes we see in K_{SV} likely reflect changes in k_q . W261 and W284 on the other hand, underwent substantially less changes in their solvent accessibility levels.

To ascertain the extent of interaction of W300 and W310 with lipids, we used the intrinsic fluorescence quencher 10-DN (Fig. 3F). Using a previously established procedure,²² we produced 10-DN embedded lipid vesicles. We measured fluorescence intensities of proteins in liposomes in the absence (F_0) and presence (F) of 10-DN and observed that W300 and W310 underwent quenching in the presence of 10-DN with F_0/F values of 1.08 and 1.27, respectively (Table II). No fluorescence quenching was observed for W261 and W284 (data not shown). The greater the penetration of the Trp residue into the liposome, the higher the tendency for its fluorescence to undergo quenching as it comes in contact with the lipid embedded 10-DN.²² This suggests that W310 is even more deeply inserted into the membrane than W300.

Signal peptide binding to wild-type SPase I $\Delta 2-75$

We performed Förster resonance energy transfer (FRET) experiments to determine the orientation of the *E. coli* wild-type alkaline phosphatase signal peptide (WTSP) when bound to wild-type SPase I $\Delta 2-75$. We engineered a variant of the WTSP, named SP2, which contains a Cys residue at position two of the WTSP for labeling with thiol-reactive fluorescent dyes (Fig. 4A). FRET experiments were performed with the SPase I $\Delta 2-75$ S302C mutant labeled with the fluorescent donor dye IAEDANS, and SP2 labeled with the fluorescent acceptor dye IANBD. As shown in Fig. 4B, upon incubation of the enzyme with SP2, the fluorescence emission intensity of IAEDANS-S302C dropped, while the emission intensity of IANBD-SP2 increased, indicative of efficient energy transfer between the two groups. We determined the FRET efficiency value E to be 0.44. Using an R_0 value of 33 Å for the IAEDANS-IANBD dye pair,^{23,24} we calculated the R value, or the distance between the acceptor and the donor, to be 32.3 Å. This experimentally determined value is consistent with results from modeling studies of an OmpA signal peptide bound to the *E. coli* SPase I $\Delta 2-75$.¹³

Using FRET of the donor Trp residues of wild-type SPase I $\Delta 2-75$ and the acceptor IAEDANS-labeled SP2 (Fig. 4C) we measured the equilibrium binding affinity of the SP2

peptide to the enzyme. The FRET was signal peptide dose dependent and saturable. We calculated the K_d of this reaction to be $4.36 \pm 1.6 \mu\text{M}$.

Effect of signal peptides on the fluorescence emission of SPase I $\Delta 2-75$ mutants

We performed acrylamide quenching experiments for SPase I $\Delta 2-75$ in the presence of WTSP to examine its effect on the enzyme's structure. We compared this data (Fig. 5 and Table I) with the data obtained from experiments conducted in the absence of the signal peptide. We found that W300 and W261 became less solvent accessible. K_{SV} values for W261 dropped from 2.90 to 1.89 in the presence of WTSP, while W300 values dropped from 4.46 to 3.28. W284 became more solvent exposed (K_{SV} value increased from 3.71 to 4.68 in the presence of WTSP), while W310 remained more or less unaffected by the absence or presence of signal peptide (K_{SV} values of 4.74 and 4.88, respectively). The structural changes observed near W261 and W284 are consistent with their location being proximal to residues directly involved in enzyme stabilization (such as R282 and D280), while W300 has been shown to interact with a bound inhibitor in crystal structures.¹⁸ These results indicate that signal peptide binding to SPase I $\Delta 2-75$ produces conformational changes that are propagated to regions that do not directly contact the peptide.

DISCUSSION

SPase I $\Delta 2-75$ has been difficult to analyze because of inherent issues with protein stability, purification and crystallization. Insight into the structural aspects of this enzyme have been gained by examining crystal structures formed in the presence of inhibitors such as arylomycin A₂, β -lactam penem and β -sultam inhibitors.^{10,18,19} While these studies have revealed information about the residues involved in catalysis, they do not reveal dynamic changes in the enzyme under different conditions. Furthermore, any information gathered thus far, has been based on inferences drawn from inhibitor-bound structures that may not provide an accurate representation of the enzymatic changes induced in the presence of signal peptide. It is vital to understand the enzyme's conformational changes upon interaction with signal peptides and membrane lipids that play key roles in the proteolysis mechanism at the later stages of protein export. We employed Trp fluorescence spectroscopy for SPase I $\Delta 2-75$ studies, as it contains four Trp residues (Fig. 1A), two of which (W300 and W310) are located at the C-terminal region of the enzyme, at the face thought to be proximal to the membrane, and are therefore valuable for studying potential membrane lipid interactions. W300 is also thought to play a role in substrate binding based on inhibitor-bound crystal structures, chemical modification and mutagenesis studies.^{18,25,26} The other two Trp residues (W261 and W284) are found immediately adjacent to the conserved Box E domain of the enzyme, which harbors several important amino acids involved in the structural stabilization of the enzyme-substrate complex.⁵

Trp residues W261 and W284 were found to exist in a relatively non-polar environment and did not undergo major changes in their solvent accessibility levels in the presence of lipids, indicating that they do not form a part of the membrane-interacting surface of the enzyme. Of these two residues, W261 is thought to be buried inside the protein based on crystal structures of the apoenzyme.¹³ On the other hand, W300 and W310 were the most solvent exposed residues; they both moved into a more hydrophobic environment in the presence of lipids, consistent with these residues being a part of the membrane-interfacing region of the enzyme. This membrane association property is thought to be vital for catalysis,¹⁷ since the enzyme is required to position itself such that it gains access to the P1 and P3 residues of the signal peptide for cleavage as it emerges from the inner membrane SecYEG translocon.

We report here the binding constant of the wild-type alkaline phosphatase signal peptide to *E. coli* SPase I $\Delta 2-75$; our experiments indicate that the signal peptide binds the enzyme

with a K_d of 4.4 μM . The signal peptide is known to bind the ATPase nanomotor SecA with a K_d of about 1–3 μM ,^{24,27} but SecA has a much greater affinity for the SecYEG channel ($K_d = 10 \text{ nM}$).¹ The signal peptide has to interact with SecA and SPase I for its successful entry and exit from the translocon, respectively. The affinity that the signal peptide exhibits for both of these key components is similar; therefore, directionality of the signal peptide is not achieved via a “baton relay” mechanism wherein progressively tighter binding plays a key role. Instead, the directional movement of the preprotein probably occurs due to several other factors, including tight association between SecA and SecYEG. This interaction results in conformational changes within SecA²⁸ such that the signal peptide binding groove opens to facilitate signal peptide release and mature region binding. The signal peptide may then traverse the channel and transition into the inner membrane, since its hydrophobic region is thought to insert into the bilayer,²⁹ for cleavage to proceed. This hypothesis is further supported by our FRET experimental data, which suggests that the amino-terminus of the signal peptide (residue P20) and the membrane-interfacing region of the enzyme (residue S302) are separated by 32.3 Å, in agreement with the combined depth of the hydrophobic core of the inner leaflet’s fatty acyl chain region (15 Å) and the glycerol head group (15 Å) on the cis-side of the membrane.³⁰ This data is supported by modeling experiments of SPase I bound to signal peptide.¹³ This distance would allow the carboxy terminal end of the signal peptide to position itself at the enzyme active site, while the hydrophobic region of the signal peptide intercalates with the inner membrane fatty acyl region and the positive charge of the N-terminus interacts with the negatively charged phospholipid head group at the cytosolic side of the inner membrane. This result would also suggest that the enzyme undergoes significant insertion into the membrane, consistent with fluorescence quenching data and previous studies using membrane phospholipids.²¹

We conducted fluorescence quenching experiments with the four Trp mutants to analyze changes that take place within the enzyme upon binding signal peptide. Surprisingly, we found that in addition to W300 that has previously been implicated in inhibitor binding studies,¹⁸ W261 and W284 also underwent solvent accessibility changes. As shown in Fig. 6A, the side chains of residues Asp 280 and Arg 282 are about 3 Å apart, and have been observed to form a salt bridge in the crystal structure.^{5,31} Asp 280 also forms hydrogen bonds with Ser 278, that could in turn, aid in the hydrogen bonding observed between Ser 278 and Lys 145.³¹ The latter bond is broken in the arylomycin A₂-bound structure.¹⁹ Similarly, Asn 277 and Ala 279 are in close proximity to a β -sultam inhibitor.¹⁸ Mutational analysis indicated that Asp 273 and Ser 281 are important for catalysis.³¹ This suggests that the conformational changes elicited in the presence of bound signal peptide are propagated to residues in the immediate vicinity of the active site, and can therefore lead to an alteration in the solvent accessibility levels of the W261 and W284 residues.

The decrease in solvent exposure for W300 could be a result of rotation of the aromatic side chain towards the interior of the protein, and away from the solvent, as a result of peptide-binding induced structural changes, or due to the signal peptide sterically hindering the exposure of the aromatic side chain towards the solvent (Fig. 6B). W300 has been found to be a residue that is crucial for signal peptidase activity based on mutation and chemical modification experiments.²⁵ Crystal structure analysis revealed that W300 is approximately 20 Å away from the active site, and is therefore thought to play an indirect role in enzyme activity.^{13,18} Proposed hypotheses include the formation of a groove along with P83, F84, Q85 and F100 that may accommodate side chain residues of the signal peptide.¹⁹ Indeed, binding studies carried out with arylomycin A₂ by the Paetzel group, identified a van der Waal’s interaction between W300 and a methylene group of the arylomycin fatty acid chain.¹⁸ W300 has also been postulated to lie close to the S7 binding site within the enzyme, which binds the P7 residue of the signal peptide.¹⁸ The same crystal structure also has a β -sultam inhibitor bound, which lies close to residue Glu 307 of the enzyme. Nevertheless,

both W300 and W310 do not interact with this inhibitor, since β -sultam is positioned close to the enzyme active site, which would likely be occupied by the cleavage region of a preprotein at the inner membrane. W310 is also located away from both the active site and the substrate binding pockets, although its side chain position is different for two different inhibitor bound structures.^{18,19} Taken together, these results indicate that local allosteric structural changes caused by signal peptide binding at the enzyme active site lead to a propagation of conformational changes (a “domino-effect”) to the residues in the immediate vicinity of the active site, as well to residues that could interact with amino acids on the signal peptide that lie farther away from the cleavage site. These studies provide insight into signal peptide interactions with SPase I Δ 2-75.

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References

1. Driessen AJ, Nouwen N. Protein translocation across the bacterial cytoplasmic membrane. *Annu Rev Biochem.* 2008; 77:643–667. [PubMed: 18078384]
2. Date T. Demonstration by a novel genetic technique that leader peptidase is an essential enzyme of *Escherichia coli*. *J Bacteriol.* 1983; 154(1):76–83. [PubMed: 6339483]
3. Zhibanko M, Zinchenko V, Gutensohn M, Schierhorn A, Klosgen RB. Inactivation of a predicted leader peptidase prevents photoautotrophic growth of *Synechocystis* sp. strain PCC 6803. *J Bacteriol.* 2005; 187(9):3071–3078. [PubMed: 15838034]
4. Taheri T, Salmanian AH, Gholami E, Doustdari F, Zahedifard F, Rafati S. *Leishmania* major: disruption of signal peptidase type I and its consequences on survival, growth and infectivity. *Exp Parasitol.* 2010; 126(2):135–145. [PubMed: 20417202]
5. Paetzel M, Karla A, Strynadka NC, Dalbey RE. Signal peptidases. *Chem Rev.* 2002; 102(12):4549–4580. [PubMed: 12475201]
6. Smitha Rao CV, Anne J. Bacterial type I signal peptidases as antibiotic targets. *Future Microbiol.* 2011; 6(11):1279–1296. [PubMed: 22082289]
7. Rawlings ND, Barrett AJ. Evolutionary families of peptidases. *Biochem J.* 1993; 290 (Pt 1):205–218. [PubMed: 8439290]
8. von Heijne G. Signal sequences. The limits of variation. *J Mol Biol.* 1985; 184(1):99–105. [PubMed: 4032478]
9. von Heijne G. Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem.* 1983; 133(1):17–21. [PubMed: 6852022]
10. Paetzel M, Dalbey RE, Strynadka NC. Crystal structure of a bacterial signal peptidase in complex with a beta-lactam inhibitor. *Nature.* 1998; 396(6707):186–190. [PubMed: 9823901]
11. Paetzel M, Strynadka NC, Tschantz WR, Casareno R, Bullinger PR, Dalbey RE. Use of site-directed chemical modification to study an essential lysine in *Escherichia coli* leader peptidase. *J Biol Chem.* 1997; 272(15):9994–10003. [PubMed: 9092541]
12. Sung M, Dalbey RE. Identification of potential active-site residues in the *Escherichia coli* leader peptidase. *J Biol Chem.* 1992; 267(19):13154–13159. [PubMed: 1618816]
13. Paetzel M, Dalbey RE, Strynadka NC. Crystal structure of a bacterial signal peptidase apoenzyme: implications for signal peptide binding and the Ser-Lys dyad mechanism. *J Biol Chem.* 2002; 277(11):9512–9519. [PubMed: 11741964]
14. Saito A, Hizukuri Y, Matsuo E, Chiba S, Mori H, Nishimura O, Ito K, Akiyama Y. Post-liberation cleavage of signal peptides is catalyzed by the site-2 protease (S2P) in bacteria. *Proc Natl Acad Sci U S A.* 2011; 108(33):13740–13745. [PubMed: 21810987]
15. von Heijne G. Life and death of a signal peptide. *Nature.* 1998; 396(6707):111, 113. [PubMed: 9823886]

16. Kuo DW, Chan HK, Wilson CJ, Griffin PR, Williams H, Knight WB. Escherichia coli leader peptidase: production of an active form lacking a requirement for detergent and development of peptide substrates. *Arch Biochem Biophys*. 1993; 303(2):274–280. [PubMed: 8512314]
17. Tschantz WR, Paetzel M, Cao G, Suci D, Inouye M, Dalbey RE. Characterization of a soluble, catalytically active form of Escherichia coli leader peptidase: requirement of detergent or phospholipid for optimal activity. *Biochemistry*. 1995; 34(12):3935–3941. [PubMed: 7696258]
18. Luo C, Roussel P, Dreier J, Page MG, Paetzel M. Crystallographic analysis of bacterial signal peptidase in ternary complex with arylomycin A2 and a beta-sultam inhibitor. *Biochemistry*. 2009; 48(38):8976–8984. [PubMed: 19655811]
19. Paetzel M, Goodall JJ, Kania M, Dalbey RE, Page MG. Crystallographic and biophysical analysis of a bacterial signal peptidase in complex with a lipopeptide-based inhibitor. *J Biol Chem*. 2004; 279(29):30781–30790. [PubMed: 15136583]
20. Chen Y, Barkley MD. Toward understanding tryptophan fluorescence in proteins. *Biochemistry*. 1998; 37(28):9976–9982. [PubMed: 9665702]
21. van Klompenburg W, Paetzel M, de Jong JM, Dalbey RE, Demel RA, von Heijne G, de Kruijff B. Phosphatidylethanolamine mediates insertion of the catalytic domain of leader peptidase in membranes. *FEBS Lett*. 1998; 431(1):75–79. [PubMed: 9684869]
22. Caputo GA, London E. Using a novel dual fluorescence quenching assay for measurement of tryptophan depth within lipid bilayers to determine hydrophobic alpha-helix locations within membranes. *Biochemistry*. 2003; 42(11):3265–3274. [PubMed: 12641458]
23. Auclair SM, Oliver DB, Mukerji I. Defining the solution state dimer structure of Escherichia coli SecA using Forster resonance energy transfer. *Biochemistry*. 2013; 52(14):2388–2401. [PubMed: 23484952]
24. Auclair SM, Moses JP, Musial-Siwiek M, Kendall DA, Oliver DB, Mukerji I. Mapping of the signal peptide-binding domain of Escherichia coli SecA using Forster resonance energy transfer. *Biochemistry*. 2010; 49(4):782–792. [PubMed: 20025247]
25. Kim YT, Muramatsu T, Takahashi K. Identification of Trp300 as an important residue for Escherichia coli leader peptidase activity. *Eur J Biochem*. 1995; 234(1):358–362. [PubMed: 8529665]
26. Kim YT, Muramatsu T, Takahashi K. Leader peptidase from Escherichia coli: overexpression, characterization, and inactivation by modification of tryptophan residues 300 and 310 with N-bromosuccinimide. *J Biochem*. 1995; 117(3):535–544. [PubMed: 7629019]
27. Musial-Siwiek M, Rusch SL, Kendall DA. Probing the affinity of SecA for signal peptide in different environments. *Biochemistry*. 2005; 44(42):13987–13996. [PubMed: 16229488]
28. Gold VA, Whitehouse S, Robson A, Collinson I. The dynamic action of SecA during the initiation of protein translocation. *Biochem J*. 2013; 449(3):695–705. [PubMed: 23126322]
29. Van Voorst F, De Kruijff B. Role of lipids in the translocation of proteins across membranes. *Biochem J*. 2000; 347(Pt 3):601–612. [PubMed: 10769162]
30. Wiener MC, White SH. Structure of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of x-ray and neutron diffraction data. III. Complete structure. *Biophys J*. 1992; 61(2):434–447. [PubMed: 1547331]
31. Klenotic PA, Carlos JL, Samuelson JC, Schuenemann TA, Tschantz WR, Paetzel M, Strynadka NC, Dalbey RE. The role of the conserved box E residues in the active site of the Escherichia coli type I signal peptidase. *J Biol Chem*. 2000; 275(9):6490–6498. [PubMed: 10692453]

FIG 1.

Wild-type and single tryptophan SPase I $\Delta 2-75$ enzymes. (A) Crystal structure of *E. coli* SPase I $\Delta 2-75$ (PDB 1T7D) is shown with the location of tryptophan residues highlighted in yellow and the serine-lysine catalytic dyad (S90 and K145) labeled in magenta. The enzyme active site is circled. (B) The name of the four single tryptophan mutants generated for this study and the residues substituted with phenylalanine in each are shown. (C) Fluorescence emission spectra of the wild-type and four single tryptophan SPase I $\Delta 2-75$ mutants are shown. The proteins were excited at 295 nm, and the emission spectra were measured from 310–400 nm as described in Materials and Methods.

FIG 2.

In vitro processing of the substrate proOmpA nuclease A by the wild-type and single tryptophan SPase I Δ 2-75 enzymes. Cleavage assays were carried out without (-) or with 0.75 μ M (1X), 3.75 μ M (5X) and 7.5 μ M (10X) of the enzyme, and 15 μ M of the substrate in 50 mM Tris-HCl, pH 8.0 and 1% Triton X-100 at 37 °C for 2 hours. Samples were run on a 12.5% SDS-PAGE gel followed by Coomassie Brilliant Blue staining. MW represents the molecular weight standard. The fusion preprotein proOmpA nuclease A and the nuclease A product are shown as preprotein (P) and mature (M), respectively.

FIG 3.

Fluorescence quenching with acrylamide and 10-DN for the wild-type and single Trp SPase I $\Delta 2-75$ enzymes in aqueous and lipid environments. (A–E) 500 nM SPase I $\Delta 2-75$ wild-type (WT), W261, W284, W300, and W310 in TKE buffer, or in TKE buffer containing 0.24 mM *E. coli* lipids in liposomes, were quenched with increasing amounts of acrylamide at 20 °C. The reaction mixtures were excited at 295 nm and read at emission wavelengths of 310–530 nm. Stern-Volmer plots are shown wherein, F_0 and F represent the fluorescence emission intensities at 340 nm of SPase I $\Delta 2-75$ in the absence and presence of acrylamide, respectively. (F) Results from fluorescence quenching experiments of WT, W300 and W310 in liposomes in the presence (F) and absence (F_0) of 10-DN are shown.

FIG 4.

Signal peptide binding on wild-type SPase I $\Delta 2-75$. (A) Sequence of the SP2 peptide used in the study is shown. A Cys residue was introduced at position two of the *E. coli* alkaline phosphatase signal peptide. (B) Results from FRET experiments between 500 nM IAEDANS-S302C SPase I $\Delta 2-75$ and 15 μM IANBD-SP2 are shown. The reaction mixture was excited at 336 nm and the emission spectrum was read from 346–660 nm as described in Materials and Methods. (C) The binding affinity of IAEDANS-SP2 to 500 nM wild-type SPase I $\Delta 2-75$ was determined via FRET experiments. The reaction mixture was excited at 295 nm and emission intensities were measured from 310–530 nm. The K_d for the reaction was calculated using the one-site binding model equation as described in Materials and Methods.

FIG 5.

Fluorescence quenching with acrylamide for the wild-type and single Trp SPase I $\Delta 2-75$ enzymes in the presence and absence of PhoA WTSP. (A–E) 500 nM SPase I $\Delta 2-75$ wild-type (WT), W261, W284, W300, and W310 in TKE buffer, or in TKE buffer containing 10 μ M PhoA WTSP, were quenched with increasing amounts of acrylamide at 20 °C. The reaction mixtures were excited at 295 nm and read at emission wavelengths of 310–530 nm. Stern-Volmer plots are shown wherein, F_0 and F represent the fluorescence emission intensities at 340 nm of SPase I $\Delta 2-75$ in the absence and presence of acrylamide, respectively.

FIG 6.

Residues W261, W284 and W300 are affected by ligand binding. (A) Amino acids from the conserved Box E domain located near W261 and W284 (in red) that are crucial for ligand binding, structural stabilization and enzyme activity are highlighted in blue.^{5,31} The active site residues S90 and K145 are colored magenta, and the bound inhibitors (β -sultam and part of arylomycin A₂) are shown in yellow (PDB 3IIQ). (B) W300 forms van der Waal's interactions with bound inhibitor. An overlay view of the crystal structures of *E. coli* SPase I Δ 2-75 in the apoenzyme form (PDB 1KN9) in red and when bound to arylomycin A₂ and a β -sultam inhibitor (PDB 3IIQ) in blue is presented, illustrating the movement of the aromatic side chain of W300 towards a fatty acid methylene group of the arylomycin A₂ inhibitor. The distances between the active site and W300 (23 Å), and between W300 and the bound inhibitor (4.7 Å) are highlighted.

Table I

Stern-Volmer constants (K_{SV}) derived from acrylamide quenching experiments.

	K_{SV} (M ⁻¹) ^a				
	WT	W261	W284	W300	W310
Aqueous ^b	5.34 ± 0.19	2.90 ± 0.10	3.71 ± 0.05	4.46 ± 0.05	4.74 ± 0.09
Liposomes ^c	3.36 ± 0.15	3.29 ± 0.06	3.28 ± 0.09	3.33 ± 0.08	3.31 ± 0.19
WTSP ^d	4.47 ± 0.04	1.89 ± 0.05	4.68 ± 0.04	3.28 ± 0.08	4.88 ± 0.04

^a K_{SV} values were determined from the linear plots for acrylamide quenching, using the equation $F_0/F = 1 + K_{SV} [Q]$ where F_0 and F represent fluorescence intensities in the absence and presence of acrylamide, and $[Q]$ represents the concentration of acrylamide used.

^b Experiments were carried out in TKE buffer.

^c Experiments were carried out after 30 min at RT with 0.24 mM *E. coli* lipids in liposomes in TKE buffer.

^d Experiments were carried out after 30 min at RT with 10 μM PhoA WTSP in TKE buffer.

Table II

Fluorescence quenching data derived from 10-DN quenching experiments.

Single Trp mutant	F_0/F^a
W300	1.08 ± 0.01
W310	1.27 ± 0.01
WT	1.10 ± 0.02

^a F_0/F refers to fluorescence emission intensities of samples in the presence and absence of 10-DN incorporated liposomes. Experiments were carried out after 30 min at RT with 5.5% 10-DN quencher in *E. coli* lipids in liposomes in TKE buffer.