10-14-2013

Sec-Dependent Protein Translocation in Escherichia coli: Biochemical Analysis of Interactions between SecA, Signal Peptidase and Signal Peptides

Meera B. Kolayarattil
University of Connecticut - Storrs, meera.bhanu@uconn.edu

Follow this and additional works at: http://digitalcommons.uconn.edu/dissertations

Recommended Citation
Kolayarattil, Meera B., "Sec-Dependent Protein Translocation in Escherichia coli: Biochemical Analysis of Interactions between SecA, Signal Peptidase and Signal Peptides" (2013). Doctoral Dissertations. 244.
http://digitalcommons.uconn.edu/dissertations/244
Sec-Dependent Protein Translocation in *Escherichia coli*:

Biochemical Analysis of Interactions between SecA, Signal Peptidase and Signal Peptides

Meera Bhanu Kolayarattil

University of Connecticut, 2013

More than 30% of proteins synthesized in the cytoplasm of cells, must be transported into or across the cell membrane to reach their final destinations. These secretory preproteins are synthesized with an amino-terminal signal peptide, which directs their export via specific protein translocation pathways. SecA is a principal component of this export system in bacterial cells, and functions as an ATPase nanomotor that provides energy via ATP hydrolysis for the translocation of preproteins across a membrane-embedded SecYEG translocon channel. The signal peptide is cleaved following translocation, by a membrane-embedded enzyme known as signal peptidase I (SPase I). Analysis of interactions between the signal peptide, SecA and SPase I is of crucial importance towards understanding the bacterial protein transport process, and could eventually aid in the development of antimicrobial drugs.

In this study, we used multiple biochemical and biophysical approaches to probe interactions between different components of the Sec pathway. Using the substituted
cysteine accessibility method (SCAM), we mapped a distinct groove on SecA that binds signal peptides. The accessibility of this binding site is variable, and therefore provides an ideal mechanism for preprotein binding and release. The active oligomeric state and the dimeric interface of SecA are unresolved. We identified a select few residues that lie on the dimer interface of SecA. Our results are consistent with the formation of a parallel dimer. The dimer was found to dissociate upon interaction of SecA with several translocation ligands. These results suggest that monomeric SecA is the translocation-active form of SecA.

Tryptophan fluorescence spectroscopic analysis of a soluble catalytically active form of SPase I (SPase I Δ2-75), indicated that a large hydrophobic region of the periplasmic domain interacts extensively with the membrane, while the rest of the enzyme does not. Studies performed with signal peptide revealed that the peptide positions itself with respect to the membrane and the enzyme, such that the cleavage site is accessible to the enzyme active site. The enzyme also undergoes direct and allosteric structural changes in response to signal peptide binding.

These results provide key insights into fundamental aspects of bacterial protein translocation.
Sec-Dependent Protein Translocation in *Escherichia coli*:

Biochemical Analysis of Interactions between SecA, Signal Peptidase
and Signal Peptides

Meera Bhanu Kolayarattil

B. Sc., St. Joseph’s College, Bangalore University, 2005
M. S. University of Connecticut, 2009

A Dissertation
Submitted in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy
at the
University of Connecticut

2013
APPROVAL PAGE

Doctor of Philosophy Dissertation

Sec-Dependent Protein Translocation in Escherichia coli: Biochemical Analysis of Interactions between SecA, Signal Peptidase and Signal Peptides

Presented by
Meera Bhanu Kolayarattil, B. S., M. S.

Major Advisor
Dr. Debra Kendall

Associate Advisor
Dr. Charles Giardina

Associate Advisor
Dr. Victoria Robinson

University of Connecticut
2013
ACKNOWLEDGEMENTS

I would like to thank a lot of people whom I have had the pleasure to work with over the past several years. Special thanks go to Debbie Kendall for putting up with me patiently, and for never giving up on me. She invested a lot of her time helping me with my research; I really appreciate her kindness, support and guidance over the years. I would also like to thank past and present members of the Kendall lab for always lifting up my spirits, for helping me with my experiments when I was struggling, and for always being super-duper friendly.

I would also like to especially thank Charlie Giardina. He has always been extremely supportive and helpful. I can’t ever remember talking to Charlie about anything under the sun, and not having laughed my heart out! The world really needs more people like him. I would also like to thank Vikki Robinson for being so approachable, patient and helpful.

I also want to thank my family back in India, without their constant support and encouragement, I would never have made it. They have always cheered me on, through both the good and the bad times. I want to especially thank my husband Arun for being my pillar of strength. I am forever grateful to UConn for bringing us together.
CHAPTER I

Introduction

1.1 Historical Background of Protein Translocation ........................................... 1
1.2 Eukaryotic Protein Translocation ................................................................... 2
1.3 The Signal Peptide ......................................................................................... 5
1.4 Prokaryotic Protein Translocation ................................................................ 6
1.5 Modes of Protein Transport .......................................................................... 8
  1.5.1 SRP-mediated Translocation .................................................................. 8
  1.5.2 SecA-mediated Translocation ................................................................ 9
1.6 Overview of the General Secretory Pathway ............................................. 12
1.7 SecA .............................................................................................................. 13
  1.7.1 SecA Structure ...................................................................................... 13
  1.7.2 Different Conformational and Oligomeric States of SecA .................. 14
  1.7.3 Signal Peptide Binding Sites on SecA .................................................. 17
  1.7.4 SecA ATPase Activity .......................................................................... 17
1.8 The SecYEG channel .................................................................................... 19
  1.8.1 SecYEG Channel Structure .................................................................. 19
  1.8.2 Oligomeric States of SecYEG ............................................................... 22
  1.8.3 Factors Responsible for Channel Opening ........................................... 22
1.9 Signal Peptidase .......................................................................................... 23
  1.9.1 SPase I ................................................................................................. 23
  1.9.2 Specificity Determinants ..................................................................... 24
  1.9.3 SPase I Structure and Domain Organization ....................................... 25
CHAPTER II

Analysis of Signal Peptide Interactions with SecA

2.1 Abstract........................................................................................................35
2.2 Introduction..................................................................................................37
2.3 Materials and Methods..............................................................................42
2.4 Results........................................................................................................45
2.5 Discussion..................................................................................................67
2.6 Summary and Future Directions.................................................................73

CHAPTER III

Fluorescence Spectroscopy of Soluble *E. coli* SPase I Reveals Conformational Changes in Response to Ligand Binding

3.1 Abstract........................................................................................................76
3.2 Introduction..................................................................................................78
3.3 Materials and Methods..............................................................................81
3.4 Results........................................................................................................85
3.5 Discussion..................................................................................................103
3.6 Summary and Future Directions
References
LIST OF FIGURES

1.1 Co-translational protein translocation across the ER membrane......................4
1.2 Protein translocation occurs across the bacterial inner and outer membranes.......7
1.3 Bacterial Sec-dependent translocation pathway.............................................10
1.4 Crystal structures of SecA.................................................................16
1.5 Structure of the SecYEG channel...........................................................21
1.6 E. coli SPase I Δ2-75 structure...............................................................27

2.1 Effect of wild-type and mutant PhoA truncated preproteins with signal peptides of
different hydrophobicites on SecA ATPase activity........................................47
2.2 Effect of wild-type and mutant PhoA truncated preproteins with signal peptides of
different hydrophobicites on SecA ATPase activity at different time intervals........50
2.3 Substituted cysteine accessibility strategy..................................................54
2.4 SecA mono-cysteine mutant labeling differences in the presence and absence of
KK-31.................................................................................................57
2.5 Nucleotides affect signal peptide binding to SecA.................................60
2.6 SecA mono-cysteine mutants at the two-helix finger region form disulfide
bonds.................................................................................................62
2.7 SecA dimerizes at the two-helix finger region and various ligands modulate dimer
dissociation..........................................................................................64
2.8 Accessibility of the PPXD-HSD-HWD groove is altered under different conformational states.................................................................71

3.1 Wild-type and single tryptophan SPase I Δ2-75 enzymes.................................87

3.2 In vitro processing of the substrate proOmpA nuclease A by the wild-type and single Trp SPase I Δ2-75 enzymes...............................................................90

3.3 Fluorescence quenching with acrylamide and 10-DN for the wild-type and single Trp SPase I Δ2-75 enzymes in aqueous and lipid environments..........................91

3.4 Signal peptide binding on wild-type SPase I Δ2-75....................................99

3.5 Fluorescence quenching with acrylamide for the wild-type and single Trp SPase I Δ2-75 enzymes in the presence and absence of PhoA WTSP .......................101

3.6 Residues W261, W284 and W300 are affected by ligand binding..............106
LIST OF TABLES

1.1 Species conservation of SPase I.................................................................29
1.2 SPase I substrate binding pockets.............................................................32
3.1 Stern-Volmer constants ($K_{SV}$) derived from acrylamide quenching experiments..96
3.2 Fluorescence quenching data derived from 10-DN quenching experiments………97
Chapter I

Introduction

Parts of this chapter have been incorporated from published material (Auclair, S. M., Bhanu M. K., and Kendall D.A. Signal peptidase I: Cleaving the way to mature proteins. Protein Science, 21: 13–25).

1.1 Historical Background of Protein Translocation

About 20-30% of all proteins synthesized in the cytosol must either reside in extracytoplasmic compartments or become integrated into the cell membrane to perform their function. These proteins are referred to as secretory proteins, and their export is a common phenomenon in both prokaryotes and eukaryotes. Secretory proteins are usually synthesized as preproteins that contain an amino terminal signal peptide, which earmarks the protein for secretion. In 1999, Günter Blobel was awarded the Nobel Prize for his discovery that secretory preproteins have signals that dictate their transport across the cell membrane. Protein translocation has been shown to take place in a few cases, such as for small peptides, in the absence of a dedicated translocation machinery (Hunt et al., 1997), while a majority of secretory preproteins utilize highly specialized and complicated export pathways that comprise several proteinaceous players acting in concert to result in efficient and specific secretory protein export.
1.2 Eukaryotic Protein Translocation

In a eukaryotic cell, protein translocation takes place at the endoplasmic reticulum (ER), the mitochondria, peroxisomes, and the chloroplasts in plant cells. Mitochondrial proteins must cross or may reside in the outer membrane, the intermembrane space, the inner membrane or the mitochondrial matrix. Protein translocation occurs with the help of several chaperones working together with a highly complex translocation machinery known as the translocase of the inner mitochondrial membrane and the translocase of the outer mitochondrial membrane (TIM/TOM) complex (Rehling et al., 2001). In plant cells, chloroplast proteins are synthesized in the cytosol and must be transported across the chloroplast membrane to enter the stroma. This is followed by passage across the thylakoid membrane to enter the thylakoid lumen. The transport process occurs via a proteinaceous translocon in the inner envelope membrane of chloroplasts and the translocon at the outer envelope membrane of chloroplasts (TIC/TOC) complex (Hormann et al., 2007). Peroxisomal proteins are synthesized by free polysomes and are inserted into the peroxisomal membrane with the help of several Pex receptor complexes (Hong 1998, Girzalsky et al., 2010). Unlike the mitochondria, chloroplasts and peroxisomes that strictly translocate and assemble resident proteins, only a few of the ER assembled proteins continue to reside in the ER. Protein transport in the ER is accomplished with the help of a signal recognition particle (SRP) that binds the signal peptide region of preproteins as they are being synthesized in the cytosol by ribosomes (Walter and Blobel 1982) (Fig. 1.1). The SRP bound to the ribosome-nascent chain complex (RNC) is then recognized by the membrane-bound SRP receptor (SR). Once the
ribosome is docked onto the membrane, the preprotein is exported through the Sec61 membrane-embedded protein conducting channel (Osborne et al., 2005).
Figure 1.1. Co-translational protein translocation across the ER membrane. The SRP recognizes the signal peptide of a secretory preprotein and targets the ribosome-nascent chain complex to the SR at the membrane. Synthesis of the preprotein occurs concomitantly with translocation across the Sec61 channel (Rapoport, 2007).
Almost all of the proteins synthesized in the ER are assembled via the secretory (Sec) translocation machinery. These secretory proteins are then incorporated into vesicles that merge with Golgi cisternae; they continue their journey to the plasma membrane and other intra- and extracellular destinations in exocytotic, secretory or transport vesicles (Nickel and Wieland 1998, McNiven and Thompson 2006)

1.3 The Signal Peptide

All secretory proteins share a common feature, called the signal peptide. It acts as the “address tag” of the protein, and therefore, any protein carrying a signal peptide is destined to leave the cytosol and enter the secretory route (von Heijne 1990). A signal peptide is found at the amino terminal region of a preprotein and is 18-30 residues long in Gram negative bacteria. Signal peptides for Sec-dependent transport pathways are divided into three domains; the amino terminal end is made up of one to three positively-charged residues (N domain). The core domain consists of hydrophobic amino acids, referred to as the H domain and can range in length from 10-15 residues. The carboxy terminus (C domain) is made up of polar residues. Residues of the C domain constitute the recognition site for the signal peptidase enzyme (SPase I) (Izard and Kendall 1994). Once the nascent preprotein has been translocated across the membrane, its signal peptide is cleaved off the preprotein by the membrane-bound SPase I to give rise to the mature functional protein (Paetzel et al., 1998). The mature protein folds in the periplasm, and is then exported via the Gsp secretion complex into the extracellular space or via the Omp85 pathway to become an outer membrane protein (Saier 2006).
1.4 Prokaryotic Protein Translocation

In *E. coli* there are 3 different commonly used secretory transport pathways; namely the Sec, TAT and YidC pathways. The TAT (Twin Arginine Translocation) pathway is used to transport proteins that are folded in the cytosol and also may bind cofactors (such as TorA, SufI and HyaA) (Berks et al., 2005) (Fig. 1.2). This system specifically recognizes and binds preproteins that contain the SRRxFLK motif in their signal peptides (Berks 1996). The YidC pathway has been known to play a role in the insertion of membrane proteins (Beck et al., 2001), and is absolutely essential for the insertion of the F\(_1\)F\(_0\) ATPase synthase subunit (van der Laan et al., 2004). YidC is also involved in the lateral integration and folding of membrane proteins as well as in the assembly of certain membrane-protein complexes (Beck et al., 2001). The YidC pathway is known to work in concert with the Sec pathway but may also function independently. The Sec pathway is of utmost importance for the transport of the majority of bacterial secretory proteins. This pathway utilizes a post-translational translocation mechanism. Several components, both cytosolic and membrane-bound, are essential for the transport process, and therefore the key players of this pathway constitute ideal antimicrobial drug targets.
Figure 1.2. Protein translocation across the bacterial inner and outer membrane. Co-translational translocation occurs via the SRP, and post-translational translocation takes place with the help of SecA and SecB for unfolded proteins or via the TAT or ABC pathways for folded proteins. Following translocation the preprotein is cleaved to form the mature protein. The mature protein can then utilize the Omp85 pathway for insertion into the outer membrane, or the Gsp complex to exit into the extracellular space via the Type II export pathway. Similarly, proteins transported via the ABC pathway can enter the extracellular space using the TolC system for Type I export. (Su et al., 2007).
1.5 Modes of Protein Transport

Secretory proteins in bacteria are transported by two separate modes. The co-translational transport pathway utilizes the SRP for the translocation of secretory proteins while they are still being actively synthesized on the ribosome. The post-translational transport pathway on the other hand, uses the SecA ATPase for providing the energy required for translocation after the complete synthesis of the preprotein. Both pathways utilize the SecYEG trimeric transmembranous complex as the channel for exiting the cytosol.

1.5.1 SRP-mediated Translocation

In membrane proteins, the highly hydrophobic patches which are to be embedded within the membrane (called transmembrane segments) often serve as signal peptides. Membrane proteins are targeted largely via the SRP-dependent co-translational pathway (Valent et al., 1995). *E. coli* SRP consists of a 4.5 S RNA component along with the GTPase Ffh (fifty four homolog) (Poritz et al., 1990). The SRP receptor in *E. coli* is called FtsY, which is a prokaryotic homolog of the alpha subunit of the eukaryotic SRP receptor. Once the ribosome nascent chain complex (RNC) is bound by the SRP, it is directed towards the FtsY receptor (Bernstein et al., 1989). Both Ffh and FtsY have nucleotide binding capacities; once the two interact, both bind GTP (Shan and Walter 2005, Reyes et al., 2007). GTP is then hydrolyzed and the complex dissociates; SecYEG then translocates the RNC (Valent et al., 1998).
1.5.2 SecA-mediated Translocation:

In the SecA dependent pathway, the preprotein is entirely synthesized in the cytosol. However, the preprotein is prevented from folding into its native conformation with the help of the cytosolic homotetrameric SecB molecular chaperone, which binds both the signal peptide and the mature region of the preprotein (Kim et al., 2000). SecB interacts with the carboxy terminus of SecA to result in the transfer of the preprotein to SecA (Zhou and Xu 2003). SecA then docks onto the SecYEG channel and undergoes multiple cycles of ATP hydrolysis to translocate the preprotein into the periplasm or into the inner membrane (Rapoport 2007).

My research focuses on two key components of the Sec-dependent post-translational translocation pathway in *E. coli*; SecA, that guides entrance of the preprotein into the translocation pathway and SPase, which cleaves the signal peptide just prior to final localization of the mature protein after exiting the translocation pathway.
Figure 1.3. Bacterial Sec-dependent translocation pathway. A) Components of the Sec system, including the SecYEG channel, YidC and the SecA ATPase nanomotor are illustrated. SecDFYajC forms a heterotrimeric channel that is often found associated with SecYEG. B) Schematic representation of the transport process is depicted, wherein, in the post-translational mode of translocation, SecB transfers the nascent unfolded polypeptide chain to SecA, which undergoes multiple ATP hydrolysis cycles, to provide the energy required for protein translocation into or across the inner membrane. In the case of co-translational translocation, SRP recognizes the signal
peptide as it emerges from the ribosome, and directs the translating ribosome towards SecYEG by interacting with the SRP receptor FtsY. The signal peptide of the preprotein is cleaved by SPase I to yield the mature protein (Papanikou et al., 2007).
1.6 Overview of the General Secretory Pathway

The general Sec (secretory) transport pathway in bacteria comprises the following components: SecA, SecB, SecYEG and SPase I. The SecB cytoplasmic chaperone functions to keep nascent preproteins that have been released from ribosomes, in their unfolded state that is compatible with their transport across the membrane (Fekkes and Driessen 1999). SecB is a homotetramer (Xu et al., 2000), with the monomers arranged such that the preprotein can wrap itself around the chaperone (Crane et al., 2006). SecB utilizes strong electrostatic interactions to bind the carboxy-terminal tail of SecA with a high affinity (Zhou and Xu 2003). SecA receives the preprotein from SecB and then aids in its insertion into and across the SecYEG heterotrimeric transmembrane channel as shown in Fig 1.3. SecA is an ATPase that provides energy for protein translocation via the hydrolysis of ATP (Rapoport 2007). The SecYEG channel is made up of the monomers SecY, SecE and SecG (Brundage et al., 1990). SecY is made up of ten transmembrane segments (TMS), with TMS 1-5 and TMS 6-10 acting as two separate domains, forming a clam shaped structure with a central translocation pore at the mouth of the SecYEG cytosolic funnel (van den et al., 2004). SecE is required for keeping the two SecY domains together, while SecG is known to enhance the efficiency of preprotein translocation through interactions with SecA (Driessen and Nouwen 2008). Once the preprotein has been translocated across the membrane, the signal peptide is cleaved from the preprotein by the SPase I enzyme to give rise to the final mature protein (Paetzel et al., 2002).
1.7 SecA

SecA is a critical component of the Sec-dependent translocation machinery and performs several vital functions such as directing the preprotein into the SecYEG channel as well as generating energy for the actual translocation process by means of ATP hydrolysis. SecA exists as a homodimer in the cytosol with each monomer consisting of about 900 residues (Hunt et al., 2002).

1.7.1 SecA Structure

A SecA monomer contains the following sub-domains: the two nucleotide binding domains NBD I and NBD II, (sometimes referred to as NBD, and the intramolecular regulator of ATP hydrolysis 2 (IRA 2), respectively), the helical wing domain HWD, the helical scaffold domain, HSD, the preprotein cross-linking domain PPXD and the carboxy-terminal linker domain CTL (Sharma et al., 2003) (Fig 1.4). There is also another key domain called IRA 1, which consists of a helix-loop-helix (also known as the 2-helix finger) in the HSD that regulates SecA ATP hydrolysis. IRA1 has been proposed to play a significant role in “pushing” the preprotein into and across the SecYEG channel (Zimmer et al., 2008) as well as in the opening of the channel itself (Whitehouse et al., 2012). The NBDs are responsible for ATP hydrolysis, NBD I binds with a very high affinity to ATP ($K_d$ is 0.13μM) (Sato et al., 1996). NBD II also binds ATP but with a much lower affinity. The two NBDs constitute the “DEAD motor” found in other helicases such as DNA and RNA helicases (Koonin and Gorbalenya 1992). Based on tryptophan fluorescence spectroscopic studies, ATP-bound SecA was found to exist in a more elongated conformation whereas, ADP-bound SecA was
shown to be more compact (den et al., 1996). Hence, it has been postulated that the nucleotide-associated conformational changes of SecA could be responsible for the modulation of structural changes linked to preprotein binding and translocation. Indeed, SecA bound to AMP-PNP, a non-hydrolyzable analog of ATP, was found to crosslink strongly with a preprotein labeled with a photoreactive probe, while the ADP-bound form labeled weakly (van Voorst et al., 2000), indicating that preprotein binding was dependent on the type of nucleotide bound at the NBDs.

The HSD is required for “stapling” together the NBDs and the PPXD, and structural changes at the NBD are likely transmitted to distal regions, such as the PPXD and the HWD (or vice versa) via this large scaffolding alpha-helix (Vrontou et al., 2004). The HSD has also been shown to insert into the SecYEG channel based on protease protection experiments. Hence this region is thought to play a key role in the membrane insertion of SecA.

1.7.2 Different Conformational and Oligomeric States of SecA

SecA is known to exist in two conformations; in the open conformation, a large groove is seen between the PPXD and the HWD which is considered to form an ideal peptide binding site. In the closed conformation, the groove is almost inaccessible, as shown in Fig 1.4. The closed form is found in the cytosol (Driessen 1993). Based on maleimide fluorescein labeling experiments, it was observed that SecA moves into an open conformation in the presence of lipids, which is therefore thought to represent the translocation-active form of SecA (Osborne et al., 2004). This conclusion is also supported by Trp fluorescence studies with SecA in the presence of lipids (Ding et al.,
SecA can associate with the membrane with a low affinity (for anionic phospholipids) (Lill et al., 1990) as well as with a high affinity (to SecYEG) (Hartl et al., 1990). Several crystal structures of SecA from different species have been resolved, both in the monomeric and dimeric forms, as well in the parallel and anti-parallel forms (Hunt et al. 2002, Vassylyev et al., 2006, Papanikolau et al., 2007). Hence, the translocation-active oligomeric state and the dimer interface of SecA are unresolved. The $K_d$ for dimerization of SecA in solution is about 0.1–0.3 µM, while the cellular concentration of SecA is about 5 µM (Wowor et al., 2011). SecA’s monomer-dimer equilibrium has been shown to shift in the presence of signal peptides, phospholipids, nucleotides and at high salt concentrations and high temperature (Or et al., 2002, Benach et al., 2003, Bu et al., 2003, Musial-Siwek et al., 2005, Wowor et al. 2011). The functionally active oligomeric form of SecA is therefore unknown.
Figure 1.4. Crystal structures of SecA. The domain organization of SecA is highlighted, with the PPXD in yellow, HWD in light green, HSD in dark green, NBD I in dark blue, NBD II in cyan and CTL in red. The open and closed forms of SecA are depicted wherein, a large groove between the PPXD, HWD and HSD is accessible or inaccessible, respectively (Hunt et al., 2002 and Osborne et al., 2004).
1.7.3 Signal Peptide Binding Sites on SecA

The exact binding site for the signal peptide on SecA remains unclear despite years of intensive research. Several groups have proposed distinct regions on SecA as potential signal peptide binding sites. Musial-Siwek and colleagues confirmed using FRET and photoaffinity labeling that the preprotein binds in the PPXD domain (Musial-Siwek et al., 2007). This data is in agreement with that obtained by Mizuhsima and colleagues (Kimura et al., 1991) by chemical crosslinking studies with preproteins. Other groups such as Economou and co-workers carried out deletion mutagenesis analysis and found that the PPXD stem contributes to signal peptide binding (Baud et al., 2002, Papanikou et al., 2005). Based on a Thermotoga maritima SecA-SecYEG co-crystal structure, Rapoport and colleagues suggested an alternate binding region, which is likely to involve the groove formed between PPXD and the NBD II domain that is positioned right above the mouth of the SecYEG channel in the open state (Zimmer et al. 2008). NMR studies performed with E. coli SecA and a KRR-LamB signal peptide, identified that the signal peptide binds at a groove region between the PPXD and IRA1 on SecA (Gelis et al., 2007). Hence, the exact signal sequence binding site on SecA is still under examination.

1.7.4 SecA ATPase Activity

It has been well established that SecA possesses a basal level of ATPase activity. SecA’s ATPase activity in the presence of signal peptides or preproteins has been measured under two distinct conditions: in inverted membrane vesicles (IMVs) containing SecYEG to examine the translocation ATPase activity, and SecA
associated with phospholipids in liposomes to examine the lipid ATPase activity (Lill et al., 1990). The ATP hydrolytic process occurs as follows: SecA is bound to the membrane near the SecYEG complex. SecB along with its substrate, the unfolded nascent preprotein chain, reaches the SecA complex and binds to it at the carboxy terminus; the preprotein is transferred to SecA while SecB is released as one ATP molecule binds at the NBD I site on SecA (Fekkes et al., 1997). This ATP binding (hydrolysis not required) plays a key role in the insertion of a 30 kDa segment of SecA along with roughly 20-25 residues of the preprotein chain into the SecYEG complex (Schiebel et al., 1991, van der Wolk et al., 1997).

SecA has been proposed to aid in protein translocation via a Brownian ratchet mechanism, wherein SecA is required for opening the SecYEG channel and the preprotein moves through the pore by diffusion (Simon et al., 1992). Another model, known as the power stroke motor model, postulates that SecA undergoes continuous conformational changes and ATP hydrolysis cycles that constantly provide energy for “pushing” the preprotein across the channel (Schiebel et al. 1991, van der Wolk et al. 1997). Nevertheless, both these models cannot account for the step-wise translocation of 20-25 residues of the preprotein across the channel (Kusters and Driessen 2011). The export process could also involve dimeric SecA bound to dimeric SecYEG, which can together form a large passageway across the membrane. The exact nature of SecA-mediated protein transport remains unresolved, and could perhaps involve a combination of a few or all of the above mentioned mechanisms.
1.8 The Sec YEG Channel

This channel is made up of three subunits, the principal subunit SecY, and the two accessory subunits SecE and SecG (Fig. 1.5) (Brundage et al. 1990). The heterotrimeric composition of this channel is similar to that seen in eukaryotes, termed Sec61αβγ, as well as other organisms, including archaea (Pohlschroder et al., 1997). Three structures, namely, the Sec YEβ channel from Methanococcus jannaschii, the SecA-SecYEG co-crystal from T. maritima and the cryo-EM structure of an E. coli SecYEG channel bound to a translating ribosome have provided important insights into the structure and functioning of this channel (van den et al. 2004, Mitra et al., 2005, Zimmer et al. 2008).

1.8.1 SecYEG Channel Structure

The E. coli SecY protein is comprised of ten transmembrane segments (TMS) and forms the central preprotein conducting channel. These TMSs are divided into two domains, the amino- and carboxy- domains, which contain TMSs 1-5 and 6-10, respectively. The two domains are held in place by a periplasmic loop between TMSs 5 and 6. The protein resembles an hourglass-shaped structure, with a central pore that provides the opening for preprotein translocation from the cytosol, into the membrane to form inner membrane proteins, or across the channel to form periplasmic proteins (van den et al. 2004).

The SecE subunit in E. coli is made up of three TMSs. This protein plays a key role in holding both of the SecY domains together. This is achieved with the help of a cytosolic loop linking TMS2 and 3, and by TMS3 itself (Breyton et al., 2002). Indeed,
in the absence of SecE, SecY can be degraded easily by the protease FtsH (Kihara et al., 1995). The SecG subunit on the other hand, does not play a key structural role, since unlike SecE, it does not contact SecY extensively (Breyton et al. 2002). SecG is also not essential for cell viability. The view from the cis-side of the membrane is that of an “inverted funnel” with a diameter that ranges from 20-25 Å near the cytosol, and constricts to a diameter of roughly 4 Å near the center of the channel, at the middle of the phospholipid bilayer (Driessen and Nouwen 2008). The central narrow pore is formed by several Ile residues that have their side chains positioned towards the interior of the channel, and is referred to as the pore ring. The M. jannaschii structure also revealed that on the periplasmic side, the channel is blocked by what is known as the plug domain; TMS2a forms an α-helix that successfully seals the channel on the trans-side of the membrane. In order for translocation to proceed, three events must occur, namely, the expansion of the pore ring, the displacement of the plug domain, and conformational changes at TMS 5 and 6, which holds the two SecY domains in place. These modifications are thought to take place at the initial stages of protein export, such as upon insertion of the signal peptide. This in turn, results in the formation of a “relaxed” channel that can accommodate large polypeptide chains (Harris and Silhavy 1999, van den et al. 2004, Driessen and Nouwen 2008).
Figure 1.5. Structure of SecYEG channel. Views from the membrane (A) and from the cytosol (B) of the *Methanococcus janaschii* SecYEβ crystal structure (PDB 1RHZ) are presented. SecYEG TMSs 1-5 and 6-10 are shown in red and blue, respectively. A hinge region separating the two halves is highlighted. Sec E and G are colored in yellow and green, respectively. The plug domain that blocks access to the periplasm, and the pore ring made up of Ile residues, comprise the dominant features of the channel’s lateral gate. (Driessen and Nouwen, 2008).
1.8.2 Oligomeric States of SecYEG

The SecYEG complex has also been postulated to exist in monomeric, dimeric and tetrameric states. Monomeric SecYEG was shown to be sufficient for translocation based on fluorescently-labeled SecYEG proteins embedded in lipid vesicles; upon interaction with preprotein and SecA, no oligomerization was detected via fluorescence spectroscopy experiments, indicating that a monomer of SecYEG is functional for protein export (Kedrov et al., 2011). Dimeric SecYEG, on the other hand, was shown to be able to trap arrested preproteins based on cross-linking studies, indicating that SecYEG could function as dimers at the membrane (Bessonneau et al., 2002). Two SecYEG protomers could assemble themselves, such that they form one large continuous channel for translocation of bulkier preproteins (Mitra et al., 2006). However, based on electron and scanning transmission microscopy experiments, it was suggested that SecYEG could function as tetramers (Driessen, 2000). Nevertheless, more recent studies favor the idea that only one channel is sufficient for translocation, as suggested by the *T. maritima* SecA-SecYEG co-crystal structure (Zimmer et al. 2008).

1.8.3 Factors Responsible for Channel Opening

The opening of the channel itself has been postulated to occur via either one of two events. One hypothesis suggests that the signal sequence displaces the plug domain (Harris and Silhavy 1999). An electron cryo-microscopy study of 2D crystals of SecYEG bound to a LamB signal peptide with 15 amino acids of the mature region (Hizlan et al., 2012), indicates that the signal sequence promotes displacement of TMS
2b and 7, and the plug domain; these interactions are thought to be sufficient for opening of the channel. The other hypothesis postulates that SecA itself is actively involved in channel opening, based on the fact that SecA has been shown to insert deeply into the SecYEG channel and also interacts extensively with the SecG subunit (Driessen and Nouwen 2008). Indeed, SecA binds SecYEG with a high affinity ($K_d$ of 10 nM). This interaction is thought to dramatically enhance the rate of ADP release and ATP uptake (Natale et al., 2004). This theory could also explain why the basal ATPase activity of cytosolic SecA is maintained at very low levels (Karamanou et al., 1999).

1.9 Signal Peptidase

Signal peptidases (SPases) cleave signal peptides from nascent preproteins as they are translocated across the membrane from the cytosol. They are integral membrane proteins with their active sites on the trans-side of the inner membrane (Paetzel et al., 2002). SPases have been categorized into several classes; typically non-lipoproteins have their signal peptides cleaved by SPase I, while SPase II recognizes the signal peptides of lipoproteins. SPase I is absolutely essential for cell viability (Date 1983).

1.9.1 SPase I

Bacterial SPase I belongs to the evolutionarily conserved SF serine protease clan that uses the Ser-Lys dyad mechanism, and to the S26 protease family (Rawlings and Barrett 1994). Lys 145 acts as a base and is required for the formation of the Ser nucleophile (Sung and Dalbey, 1992 and Tschantz et al., 1995). Eukaryotic SPase I that resides in the ER belongs to the S27 family which utilizes a Ser-His-Asp triad for
cleavage, and is therefore different from its prokaryotic counterpart (Rawlings and Barrett 1993). This makes bacterial SPase I a viable anti-microbial drug. However, common serine protease inhibitors, that typically inhibit catalytic protease triads, have proven to be ineffective against bacterial SPase I (Kulanthaivel et al., 2004, Smith et al., 2010), hence the development of effective inhibitors is crucial.

SPase I cleaves signal peptides of secretory preproteins that are translocated across the inner membrane via the Sec and TAT pathways (Luke et al., 2009). Following cleavage, the signal peptide itself is further degraded by an enzyme called signal peptide peptidase. The active site of this protease is also located in the periplasm, and catalysis is achieved via a Ser-Lys dyad.

1.9.2 Specificity Determinants

The signal peptide has key recognition elements for SPase I cleavage. The -1 and -3 residues are the most important residues for proteolysis, which gave rise to the so-called -1, -3 rule (von Heijne 1983). Amino acids at these positions are small, neutral residues such as Ala, Gly, Cys and Ser. Since Ala is the most commonly found residue at these positions, this motif is also called the Ala-X-Ala motif (von Heijne 1985). Substitution of the -1 and -3 residues with other amino acids such as Asp, Val and Asn, indicated that these residues could not be recognized and cleaved by SPase I; proteolysis occurred instead at the -3 position since the -4, -5 and -6 residues obeyed the Ala-X-Ala motif rule, indicating the absolute requirement for small, neutral residues at the processing site (Fikes et al., 1990, Shen et al., 1991). The length of the carboxy-terminal end of the signal peptide is also crucial for optimal cleavage.
Experiments carried out with signal peptides with elongated carboxy-terminal regions, revealed that cleavage efficiency was unchanged for preproteins containing nine additional residues, however, a further increase in length was detrimental to proteolytic processing and no cleavage was found to take place for signal peptides that contained 13 carboxy-terminal residues (Jain et al., 1994). Another commonly observed feature of signal peptides is the presence of a small helix-breaking residue at the -6 position, such as Pro and Gly, which may help in the formation of a short β-turn from the -1 to -5 positions that in turn would aid in proper binding and cleavage at the SPase I active site (Paetzel et al. 1998). The early mature region of the preprotein may also play a role in recognition and binding to SPase I, since this region is typically made up of neutral or negatively charged residues (Kajava et al., 2000, Choo and Ranganathan 2008). Indeed, the +1 and +2 residues of the preprotein exhibit conserved sequences in Gram-negative bacteria (Choo and Ranganathan 2008).

1.9.3 SPase I Structure and Domain Organization

SPase I is a membrane-embedded protease with a periplasmic carboxy-terminal catalytic domain. The amino terminus is made up of a short periplasmic region, and two transmembrane domains, separated by a cytosolic loop (von Heijne 1998). The transmembrane domains are essential for anchoring the enzyme to the membrane, such that it can position itself appropriately for cleavage to occur. SPase I Δ2-75, the catalytically active soluble enzyme, lacking the transmembrane segments, has been crystallized in the absence of inhibitors (Fig 1.6) and provides a detailed picture of the catalytic region (Paetzel et al., 2002). Since signal peptides are thought to insert themselves into the phospholipid bilayer of in the inner membrane (Briggs et
al., 1986), the location of the active site of the SPase I enzyme should be either at the membrane surface or embedded in the membrane. The enzyme has also been shown to interact extensively with phospholipids (van Klompenburg et al., 1998).
Figure 1.6. *E. coli* SPase I Δ2-75 structure (PDB 1KN9) (Paetzel et al. 2002). The active site residues (S90 and K145) are highlighted in blue, while residues important for ligand binding and substrate catalysis (S88, G272, S278, D280, R282 and W300) are highlighted in red.
All crystals of SPase I resolved thus far, have been generated using the Δ2-75 mutant, that lacks the transmembrane segments, because it is much easier to purify, and also maintains efficient catalytic cleavage properties (Kuo et al., 1993). The SPase I Δ2-75 mutant consisting of residues 76-323 is divided into two anti-parallel β-sheet domains, a conserved domain I and another non-conserved domain II (Dalbey et al., 1997). Domain II is thought to be essential for structural stability of the enzyme. The key catalytic residues, Ser 90 and Lys 145, are located in the conserved domain I, along with a large exposed hydrophobic region that may interact with the phospholipid bilayer. This hypothesis is further supported by the fact, that SPase I Δ2-75 exhibits enhanced cleavage ability in the presence of phospholipids and Triton X-100 detergent (Tschantz et al., 1995). Similarly, residues W300 and W310 that lie away from the active site, are important for catalysis, indicating the enzyme’s requirement for interaction with lipids for maintaining optimal activity (Kim et al., 1995).

Domain I has been further classified into four separate boxes (B-E) based on sequence conservation between different species (Dalbey et al. 1997) (Table 1.1). Box B consists of residues 88-95, including the catalytic Ser 90, and is located near the trans-side of the inner membrane in the periplasm. This region also contains Ser 88, which plays a key role in enzyme catalysis (Paetzel et al. 2002) by aiding in the formation of an oxyanion hole that is vital for catalysis. Box C is made up of residues 127-134 and Box D is made up of amino acids 142-153, including the catalytic Lys 145. Both these boxes form two anti-parallel β-strands that are thought to form hydrogen bonds with bound signal peptides (Paetzel et al., 2000). A notable feature of Lys 145 is the dramatic reduction of its side chain pKₐ (from a solvent exposed pKₐ of
10.5 to 8.7 in the native enzyme) due to the burial of the side chain in a highly hydrophobic environment formed by several neighboring hydrophobic amino acids (Paetzel et al., 1997). The deprotonated Lys side chain can successfully act as a general base for the catalytic reaction to proceed. Box E is made up of residues 272-282, and contains the invariant Gly 272; the presence of any other residue at this position would likely interfere with the side chain interactions of Lys 145 (Paetzel et al. 2000). Two other amino acids, Asp 280 and Arg 282 are also involved in a conserved salt bridge (Paetzel et al. 1998).

1.9.4 Substrate Binding Pockets

The substrate binding pockets S1 and S3 of SPase bind residues -1 (P1) and -3 (P3) of the preprotein, respectively (Table 1.2). In addition, 11 sites have been identified based on computational modeling studies with SPase I and the E. coli dithiol oxidase (DsbA) peptide containing residues LAFSASAAQYEDG. Together these 13 sites include pockets S7 to S6’ that bind residues -7 to +6, respectively, of the preprotein indicating that the mature region does play a role in cleavage (Choo et al., 2008). As shown in Table 1.2, residues P1 and P3 of the preprotein contact the most number of residues in the enzyme.
### Gram-negative bacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzyme name</th>
<th>MW</th>
<th>% Sequence identity</th>
<th>Box B</th>
<th>Box C</th>
<th>Box D</th>
<th>Box E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>LepB</td>
<td>35,960</td>
<td>100.0</td>
<td>98 *</td>
<td>95</td>
<td>127</td>
<td>134</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>Lep</td>
<td>19,734</td>
<td>37.9</td>
<td>Y KRAAVGLPGD</td>
<td>81</td>
<td>YKRAAVGLPGD</td>
<td>GDNRNSADSRS</td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>Lep</td>
<td>11,710</td>
<td>26.5</td>
<td>RGDIVVF</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photobacter profundus</td>
<td>Lep</td>
<td>22,685</td>
<td>24.9</td>
<td>GDIVVF</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>Lep</td>
<td>31,903</td>
<td>41.8</td>
<td>GDNVVF</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhodobacter capsulatus</td>
<td>Lep</td>
<td>28,873</td>
<td>28.0</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>Lep</td>
<td>35,770</td>
<td>93.5</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>Lep</td>
<td>28,782</td>
<td>38.3</td>
<td>YKRAVGLPGD</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradyrhizobium japonicum</td>
<td>Spf</td>
<td>28,845</td>
<td>30.2</td>
<td>YKRAVGLPGD</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradyrhizobium japonicum</td>
<td>Spf</td>
<td>27,951</td>
<td>29.9</td>
<td>YKRAVGLPGD</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Gram-positive bacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzyme name</th>
<th>MW</th>
<th>% Sequence identity</th>
<th>Box B</th>
<th>Box C</th>
<th>Box D</th>
<th>Box E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>SpfB</td>
<td>21,047</td>
<td>20.0</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>SpfC</td>
<td>21,854</td>
<td>21.5</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>SpfU</td>
<td>21,105</td>
<td>21.5</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>SpfV</td>
<td>18,956</td>
<td>22.2</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>SpfW</td>
<td>26,073</td>
<td>14.4</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>SpfP (pTA1015)</td>
<td>21,252</td>
<td>21.5</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>SpfP (pTA1040)</td>
<td>21,581</td>
<td>22.5</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>SpfA</td>
<td>20,146</td>
<td>17.3</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>SpfB</td>
<td>21,692</td>
<td>23.0</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>SpfS</td>
<td>21,889</td>
<td>20.6</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Endoplasmic reticulum

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzyme name</th>
<th>MW</th>
<th>% Sequence identity</th>
<th>Box B</th>
<th>Box C</th>
<th>Box D</th>
<th>Box E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisae</td>
<td>SecC1</td>
<td>18,762</td>
<td>15.7</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homo sapiens sapiens</td>
<td>SecC16</td>
<td>20,625</td>
<td>17.6</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Archaea

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzyme name</th>
<th>MW</th>
<th>% Sequence identity</th>
<th>Box B</th>
<th>Box C</th>
<th>Box D</th>
<th>Box E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanococcus jannaschii</td>
<td>SpfC</td>
<td>27,724</td>
<td>15.5</td>
<td>GDNRNSADSRS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

30
Table 1.1: Species conservation of SPase I. *E. coli* SPase I. Boxes B-E, located at the enzyme’s periplasmic catalytic region share sequence identity with several other bacterial species, as indicated. The % sequence identity value is relative to that of the *E. coli* enzyme. MW represents the molecular weight of each enzyme. The Ser-Lys catalytic dyad residues of each enzyme are highlighted with an asterisk (Auclair et al., 2012).
Table 1.2: SPase I substrate binding pockets. Residues of SPase I that form subsites S7 to S6’, which have been proposed to bind residues P7 to P6’ of the preprotein are indicated (Auclair et al., 2012).

<table>
<thead>
<tr>
<th>Subsite</th>
<th>Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>S7</td>
<td>Glu 82, Pro 83</td>
</tr>
<tr>
<td>S6</td>
<td>Pro 83, Phe 84</td>
</tr>
<tr>
<td>S5</td>
<td>Phe 84, Gln 85, Asp 142</td>
</tr>
<tr>
<td>S4</td>
<td>Phe 84, Gln 85, Pro 87, Asp 142</td>
</tr>
<tr>
<td>S3</td>
<td>Phe 84, Gln 85, Ile 86, Pro 87, Ile 101, Val 132, Asp 142, Ile 144</td>
</tr>
<tr>
<td>S2</td>
<td>Gln 85, Ile 86, Pro 87, Ser 88, Met 91, Ile 144</td>
</tr>
<tr>
<td>S1</td>
<td>Ile 86, Pro 87, Ser 88, Ser 90, Met 91, Leu 95, Tyr 143, Ile 144, Lys 145</td>
</tr>
<tr>
<td>S1’</td>
<td>Ser 88, Ser 90, Tyr 143, Ala 279</td>
</tr>
<tr>
<td>S2’</td>
<td>Ser 88, Ser 90, Phe 208, Asn 277, Ala 279</td>
</tr>
<tr>
<td>S3’</td>
<td>Tyr 50, Met 249, Asp 276, Asn 277, Ala 279, Arg 282, Tyr 283</td>
</tr>
<tr>
<td>S4’</td>
<td>Gln 244, Asp 245, Asp 276, Asn 277, Arg 282</td>
</tr>
<tr>
<td>S5’</td>
<td>Phe 196, Ser 206, Ala 243, Asp 276, Asn 277</td>
</tr>
<tr>
<td>S6’</td>
<td>Phe 196, Ile 242, Ala 243</td>
</tr>
</tbody>
</table>
1.9.5 Proteolytic Mechanism

The cleavage reaction takes place as follows: once the preprotein substrate binds to the active site, it positions itself such that the P1 and P3 residues become buried inside the S1 and S3 hydrophobic pockets. The Lys 145 side chain amino group is involved in the formation of a hydrogen bond with the Oγ atom of Ser 90. This conformational set-up is facilitated in part by the hydrogen bond formed between the –OH group of Ser 278 and the amino group of Lys 145. The amino group of Lys 145 then acts as the general base and abstracts a proton from the hydroxyl group of Ser 90. The Oγ atom acts as the nucleophile, and attacks the substrate P1 carbonyl group to form a tetrahedral intermediate. This shift of electrons results in the formation of an oxyanion hole, which is stabilized by hydrogen bonds involving the main chain amide group of Ser 90 and the side chain –OH group of Ser 88 (Paetzel et al. 1998, Paetzel et al. 2002). Lys 145 then donates a proton to the amino group of the amide nitrogen of the leaving group, generating an acyl-enzyme intermediate. The deacylating water molecule comes into play, with the loss of one of its protons to the amino group of Lys 145, and the attack of its oxygen atom on the peptide carbonyl group, forming another tetrahedral intermediate. Again, Ser 88 and Ser 90 serve to stabilize the intermediate via hydrogen bonding. Finally, the amide group of Lys 145 donates a proton to the Oγ atom of Ser 90, leading to the breakdown of the tetrahedral intermediate, release of the mature protein, and the restoration of the active site to its apoenzyme form.
1.9.6 Structural Changes in SPase I upon Binding Inhibitors

A significant amount of knowledge about this enzyme has been garnered by analysis resolved crystal structures, both in the absence and presence of inhibitors. To date, the enzyme has been crystallized in the apoenzyme form, and co-crystallized with inhibitors β-lactam 5-S penem, lipopeptide arylomycin A2, and in the presence of both β-sultam and arylomycin A2 (Paetzel et al. 1998, Paetzel et al. 2002, Paetzel et al., 2004, Luo et al., 2009). Nevertheless, efforts at generating co-crystals of the enzyme and signal peptide have not been fruitful.

The resolved crystal structures have shed some light on the subtle structural perturbations that are induced in the enzyme upon ligand binding. Large changes are observed in the positioning of the side chain of W300, indicating that this residue is involved in the formation of van der Waal’s interactions with bound inhibitor (Luo et al. 2009). Ser 88 in the apoenzyme structure, has its side chain positioned closed to that of Ser 90, while in the β-lactam 5-S penem bound inhibitor structure, the side chain is located away from the substrate binding site (Paetzel et al. 1998, Paetzel et al. 2002). Similarly, in the arylomycin A2 bound form, Lys 145 is no longer capable of forming hydrogen bonds with Ser 278 due to steric hindrance from bound inhibitor. A number of residues neighboring the active site have also been shown to form van der Waal’s interactions with bound inhibitors (Luo et al. 2009). However, structural changes resulting from signal peptide and preprotein binding still need to be analyzed in greater detail.
Chapter II

Analysis of signal peptide interactions with SecA

Parts of this chapter have been incorporated from published material (Bhanu, M.K., Zhao, P. and Kendall D.A.: Mapping of the SecA Signal Peptide Binding Site and Dimeric Interface by Using the Substituted Cysteine Accessibility Method. *Journal of Bacteriology* doi:10.1128/JB.00661-13).

2.1 Abstract

SecA is an ATPase nanomotor critical for bacterial secretory protein translocation. Secretory proteins, synthesized as preproteins in the cytosol, carry an amino-terminal signal peptide that is recognized and bound by SecA followed by its transport across the SecYEG translocon and cleavage of the signal peptide by SPase I, to become an inner membrane protein or a periplasmic protein. While the hydrophobic core region of the signal peptide has been shown to be important for translocation (Wang et al., 2000), it is unclear whether parts of the mature region also play a role in recognition and transport. To address this issue, we generated truncated preprotein mutants of the *E. coli* alkaline phosphatase enzyme, wherein crucial hydrophobic residues of the signal peptide were substituted with Ala, and performed ATP hydrolysis assays with wild-type SecA. Our results revealed that the wild-type preprotein maximally stimulated SecA’s ATP hydrolysis property, while the presence of just two Ala substitutions resulted in deleterious effects, reflecting the significance of the
hydrophobic region of the signal peptide for protein export. This data also indicate that the mature region cannot override translocation issues elicited by the presence of defective signal peptides.

The SecA-signal peptide binding process is crucial for the onset of translocation, however, exactly where the signal peptide interacts with SecA is unclear. SecA protomers also interact among themselves to form dimers in solution. However, given that the resolved SecA crystal structures for various bacterial species all have different SecA-SecA interfaces (Hunt et al. 2002, Sharma et al. 2003, Vassylyev et al. 2006, Papanikolau et al. 2007), the orientation of the dimer and the residues involved in dimerization are unclear. To address these issues, we utilized the substituted cysteine accessibility method (SCAM); we generated a library of 23 mono-cysteine SecA mutants, and probed for the accessibility of each mutant cysteine to maleimide-PEG₂-biotin (MPB), a sulfhydryl-labeling reagent, both in the presence and absence of a signal peptide. Dramatic differences in MPB labeling were observed with a select few mutants located at the pre-protein cross-linking domain (PPXD), the helical wing domain (HWD) and the helical scaffold domain (HSD), indicating that the signal peptide binds at the groove formed between these three domains. ADP was found to block productive SecA-signal peptide interactions, while no such effect was observed in the presence of ATPγS, a non-hydrolysable analog of ATP, indicating a key role played by nucleotides in allosterically modulating SecA-signal peptide binding characteristics. The exposure of the signal peptide binding site is also varied under different conditions, such as upon binding membrane phospholipids or the
SecYEG channel, and could therefore provide an ideal mechanism for preprotein transfer into the translocon.

A few residues, namely, G793, A795, K797 and D798 located at the two helix-finger region of the HSD, were identified to be involved in dimerization based on the formation of disulfide-bonded dimers in the absence of reducing agents. ATPγS alone, and more extensively, in conjunction with lipids and signal peptides strongly favored dimer dissociation, while ADP supports dimerization. The former ligands are thought to interact with SecA during translocation at the inner membrane, while the latter is known to bind SecA in the cytosol. This study provides key insight into the structure-function relationships of SecA preprotein binding and dimer dissociation.

2.2 Introduction

About one-third of all proteins synthesized in the bacterial cytosol are destined to reside in the membrane or to be transported through it. A majority of these secretory proteins utilize the bacterial general secretory (Sec) pathway for their translocation. Other pathways include the YidC insertase system, used mainly for membrane protein insertion, and the Tat pathway for secretion of folded proteins into the periplasm [see review (Pohlschroder et al., 2005)]. Secretory proteins are synthesized in the cytosol as preproteins that contain an amino-terminal signal peptide which serves as an “address tag” followed by the mature protein. In the Sec pathway, the molecular chaperone SecB maintains newly synthesized preproteins in their unfolded state, and then transfers them to an ATPase nanomotor known as SecA via the signal peptide region. SecA is a key component of the bacterial Sec pathway that is critical for
membrane protein transport and is crucial for cell viability. The energy required for translocation of the preprotein across the heterotrimeric SecYEG membrane channel is furnished by SecA through ATP hydrolysis (Economou and Wickner 1994).

SecA is a large 102 kDa cytosolic protein. Crystal structures of SecA from different bacterial species have been resolved and the information gleaned from these structures has led to the grouping of SecA regions into the preprotein crosslinking domain (PPXD), the helical wing domain (HWD), the helical scaffold domain (HSD) and the two nucleotide binding domains, the high affinity site NBD I and the low affinity site NBD II (Papanikolau et al. 2007). The structure of the protomer is largely the same in all these structures; however, an opening in the PPXD-HSD-HWD region is present in some of the so-called open forms of SecA, while not in others. SecA has been crystallized both in the monomeric as well as parallel and anti-parallel dimeric forms (Hunt et al. 2002, Vassylyev et al. 2006, Zimmer et al., 2006, Papanikolau et al. 2007). However, there is no general consensus as to the oligomeric form and orientation of the protomers in the homodimer. SecA cycles between its ATP and ADP-bound states to provide energy for translocation. In the ADP-bound form, SecA is thought to exist in a compact form, while ATP binding produces an elongated conformation (den et al. 1996). SecA is known to interact with SecB, the SecYEG channel, and with anionic phospholipids in the membrane bilayer (Papanikou et al. 2007). In addition, SecA specifically interacts with secretory preproteins by recognition of the signal sequence (Akita et al., 1990). Most of these ligands are known to produce conformational changes within SecA, with concomitant changes in its ATPase activity (Vrontou and Economou 2004). Experiments carried out to study
these interactions have provided conflicting results with regard to the alteration of SecA’s monomer-dimer equilibrium (Or et al. 2002, Benach et al. 2003, Bu et al. 2003, Musial-Siwek et al. 2005, Wowor et al. 2011). Exactly how SecA modulates its diverse functions and its association with several ligands is still under examination.

Signal peptides play a key role in the targeting and membrane insertion of preproteins. SecA’s ATPase activity has been shown to increase dramatically in the presence of signal peptides (Miller et al., 1998), indicating that they are responsible in part, for the modulation of the rate of nucleotide hydrolysis by SecA. Indeed, signal peptides with altered amino acid hydrophobicity and length have been shown to dramatically affect translocation (Chou and Kendall 1990). Signal peptides of preproteins that utilize the Sec pathway in Gram-negative bacteria are generally 18-30 residues long and contain three distinct regions; a positively charged amino terminus followed by a central hydrophobic core region and a carboxy terminal polar segment (von Heijne 1985). Residues at the -1 and -3 locations of the signal peptide are crucial for signal peptide cleavage by a membrane-embedded protease, the signal peptidase enzyme (von Heijne 1983, Auclair et al., 2012). Following cleavage, the functional mature protein is released into the membrane or the periplasm, while the signal peptide is cleaved by the signal peptide peptidase enzyme (Paetzel et al. 2002).

Several studies have been carried out to analyze signal peptide binding to SecA. EDAC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) cross-linking studies carried out with multiple truncated SecA mutants and preproteins found that residues 267-340 of the PPXD were involved in preprotein binding (Kimura et al. 1991). The PPXD region was also implicated as the signal peptide binding site in two more
subsequent studies; an NMR study carried out with *E. coli* SecA and a KRR-LamB signal peptide identified the groove formed between the PPXD and the intramolecular regulator of ATP hydrolysis-I domain (IRA1) as a suitable candidate for a signal peptide binding site (Gelis et al. 2007). Similarly, signal peptides with a photoactivatable label were found to cross-link at the PPXD region (Musial-Siwek et al. 2007). However, the hydrophobic cleft formed between the PPXD and NBD II and the two β sheets connecting the PPXD and NBD I have also been suggested (Papanikou et al. 2005, Zimmer et al. 2008). Hence, the region on SecA that binds the signal peptide is still much debated. Although there are significant differences in the approaches utilized for analyzing SecA-signal peptide interactions, a frequently observed site of binding is the PPXD region; interactions with other sites remain controversial. Since this recognition is a crucial prerequisite for preprotein translocation, understanding the mechanism and site of action is of paramount importance for fully comprehending bacterial protein transport.

Since the binding of nucleotides and signal peptides are thought to occur at different locations on SecA, an allosteric mode of translocation has been previously suggested (Karamanou et al. 1999). Trp fluorescence spectroscopic studies on SecA and nucleotide binding suggested that binding of ATP at the NBD produces an elongated conformation of SecA (den et al. 1996), and this could potentially result in the opening of a signal peptide binding groove. ADP binding on the other hand, is thought to produce a more compact form of SecA, and could represent a conformation that represents the signal peptide-free form of SecA (van Voorst et al. 2000). Understanding the influence of nucleotide binding on signal peptide interaction with
SecA is vital for understanding the conformational changes and the “cross-talk” between diametrically opposite regions of SecA.

SecA is thought to likely exist in the cytosol predominantly in a dimeric state, since the $K_d$ of SecA in solution is below 0.3 µM (Wowor et al. 2011), while the total SecA concentration in a bacterial cell is roughly 5 µM (Or et al. 2002). However, the functional oligomeric state of SecA at the SecYEG translocon is unclear, with studies identifying both monomers and dimers as the active form of SecA (Jilaveanu et al., 2005, Or et al., 2005, Or and Rapoport 2007). SecA’s dimer interface and the alignment of its protomers is also unknown, including the identity of the dimeric interface residues.

We have employed the substituted cysteine accessibility method (SCAM) to establish the signal peptide binding site and the dimer interface of SecA (van and Lolkema 2000). For this purpose, we generated a library of mono-cysteine *E. coli* SecA mutants and the accessibility of the single Cys residue on each mutant was probed using maleimide-PEG$_2$-biotin (MPB) in the presence and absence of signal peptide and other ligands. Using this approach, we found that Cys mutants at the PPXD-HSD-HWD region exhibited maximal MPB labeling intensity changes in the presence of a signal peptide. We also found that mono-cysteine mutants at the two-helix finger region of SecA, but not others, formed disulfide-bonded dimeric species, and these dimers could dissociate upon interaction with different ligands. These results are particularly interesting in light of recent studies that point to the two-helix finger playing a significant role in the opening of the SecYEG channel (Whitehouse et al. 2012).
2.3 Materials and Methods

*Generation of SecA mono-Cys and alkaline phosphatase truncated preprotein mutants.* Substitution mutagenesis using the pET-29b-T7 SecA His plasmid, encoding a C-terminal His-tagged SecA, was performed using the QuikChange site-directed mutagenesis kit (Stratagene). All four naturally occurring Cys residues were mutated to Ser to generate a Cys-less SecA template and mono-Cys mutants were generated using this template. Ala substitution mutagenesis using the pGEX-6P-1 alkaline phosphatase truncated preprotein plasmid, encoding an N-terminal glutathione S-transferase (GST) tag and a C-terminal His tag, was performed via QuikChange site-directed mutagenesis kit (Stratagene). All mutations were confirmed via DNA sequence analysis.

*SecA purification.* *E. coli* BL21(DE3) cells harboring the SecA vector were grown in LB medium with 100 µg/ml ampicillin at 37°C until OD$_{600}$ ~0.6. Expression of plasmid-derived SecA was induced with 0.5 mM IPTG, and cells were allowed to grow for 2 hours. Cells were sedimented by centrifugation, resuspended in lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 7.0) followed by sonication on ice for 4 mins. The lysate was centrifuged and the supernatant incubated with washed Talon metal affinity resin beads (Clontech) for 1-2 hours at 4°C. The beads were washed thrice with lysis buffer, followed by elution with 250 mM imidazole. The eluate was dialyzed extensively against PBS buffer (136.89 mM NaCl, 2.68 mM KCl, 10.14 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4). The final protein solution was stored at -80°C in single use aliquots and the working protein stocks used for experiments were stored at
4°C for less than 1 week. Protein concentrations were determined using OD$_{280}$ with a molar extinction co-efficient of 243.6 µM$^{-1}$cm$^{-1}$.

**Alkaline phosphatase truncated preprotein purification.** E. coli BL21(DE3) cells harboring the alkaline phosphatase truncated preprotein vector were grown in LB medium with 100 µg/ml ampicillin at 30°C until OD$_{600}$ ~0.6. Expression of plasmid-derived preprotein was induced with 0.5 mM IPTG, and cells were allowed to grow for 2 hours. Cells were sedimented by centrifugation, resuspended in lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 7.0) followed by sonication on ice for 4 mins. The lysate was centrifuged and the supernatant was incubated with washed Talon metal affinity resin beads (Clontech) for 1-2 hours at 4°C. The beads were washed thrice with lysis buffer, followed by elution with 250 mM imidazole. The eluate was dialyzed into PBS buffer, and incubated with washed glutathione sepharose 4B resin beads (GE Healthcare) for an hour at room temperature, followed by three washes with PBS, and cleaved with PreScission protease (GE Healthcare) overnight at 4°C. The preprotein was collected by running the sample over washed glutathione sepharose 4B resin beads to remove the protease, and purified by HPLC. The preproteins were lyophilized, dissolved in DMSO and stored at -80°C in aliquots.

**ATP hydrolysis experiments.** SecA ATPase assays were conducted in a total reaction volume of 50 µL containing 50 mM HEPES-KOH, pH 7.5., 30 mM KCl, 30 mM NH$_4$Cl, 0.5 mM Mg(OAc)$_2$, 1 mM DTT, 4 mM ATP, 0.5 mg/ml BSA, 0.4 µM SecA, 360 µM E. coli phospholipids and 10 µM of KK-31 or truncated preproteins. Reactions were carried out at 30°C for different time intervals. Release of inorganic phosphate was quantified using the Malachite Green method (Lill et al. 1990).
SCAM experiments. SCAM experiments were carried out in a 20 µL reaction volume with 1 µM SecA protein in PBS buffer, in the presence and absence of 10 µM KK-31 (KKMKQSTIALALLPLLFTPKARTPEKKK-NH₂) signal peptide. The reaction mixture was incubated at 30°C for 20 min. MPB (Thermo Scientific) and L-cysteine stocks were prepared immediately before use. SecA was labeled with 1 µM MPB for 2 min and the labeling reaction was quenched with the addition of 5 µM L-cysteine for 5 min. 10 µl of 6X SDS-PAGE loading dye was added to the reaction followed by heating to 95°C for 5 min. 15 µl of each reaction were run on a 7.5% SDS-PAGE gel, followed by immuno-transfer onto a PVDF membrane (Millipore). The membrane was blocked with Super Block blocking buffer (Thermo Scientific) overnight at 4°C and incubated with a 1:4,000 dilution of HRP-conjugated streptavidin antibody (Thermo Scientific) for 1 hour followed by 3 washes with PBS-Tween, and then incubated with SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) for 5 min. For SecA detection, blots were incubated with a 1:3,000 dilution of monoclonal mouse anti-His antibody (GenScript) for 1 hour, washed with PBS-Tween thrice, and incubated with a 1:6,000 dilution of HRP-conjugated goat anti-mouse antibody (GenScript) for 1 hour. The blots were developed with an X-ray film developer or using the ChemiDoc™ MP System (Bio-Rad). The blot intensities were analyzed using ImageJ and GraphPad software and standardized against BSA, which was also labeled with MPB. Protein structures were presented using PyMol (Schrödinger, LLC) software.

Dimer interface experiments. SecA was reduced with a 1:100 dilution of 50 mM TCEP (Tris(2-carboxyethyl)phosphine) for 30 mins at room temperature. TCEP was
removed using a Zeba desalting spin column (Thermo Scientific). 0.4 µM reduced SecA was incubated in the presence and absence of 4 mM nucleotides, 20 µM KK-31 and 400 µM E. coli polar lipid extract in liposomes (Avanti Polar Lipids) in 20 mM phosphate buffer, pH 7.4, supplemented with 50 mM KCl and 0.5 mM MgCl₂ for 15 mins at 30°C followed by oxidation with 0.5 mM copper phenanthroline for 10 mins at 30 °C and quenching with 5 mM neocuproine for 5 mins at room temperature (de et al., 2005). Samples were run on a non-reducing 7.5% SDS-PAGE gel and analyzed by Coomassie staining.

2.4 Results

*Signal peptide hydrophobicity is vital for maintaining productive interactions of truncated preproteins with SecA.* Studies performed with signal peptides of differing hydrophobicity levels have indicated that the maintenance of an optimal level is crucial for SecA binding (Chou and Kendall 1990, Wang et al. 2000). We wanted to test whether alterations in signal peptide hydrophobicity affect the translocation characteristics of truncated preproteins. Towards this end, we generated mutants of truncated preproteins that contained 21 residues of the signal peptide and 30 residues of the mature region of the E. coli wild-type alkaline phosphatase enzyme. Three such mutants were generated, named 2A swap PhoA, 4A swap PhoA and 6A swap PhoA, which contained two, four and six Ala residues respectively, in lieu of other more highly hydrophobic amino acids (such as Leu and Phe) in the hydrophobic core region of the signal peptide (Fig. 2.1A). Lipid ATPase assays conducted with SecA and the wild-type (WT) and mutant truncated preproteins, indicated that the highest rates of ATP hydrolysis were achieved with the wild-type preprotein. The rates of ATP
hydrolysis were inversely proportional to the number of substituted Ala residues in the preprotein. 2A swap PhoA, 4A swap PhoA and 6A swap PhoA showed a drop in ATP hydrolysis rates of roughly 65%, 96% and >100% respectively, compared to that of the WT (Fig. 2.1 B, C and D). The same trend was similarly observed in a time course experiment, wherein inorganic phosphate release was measured at several time points in the reaction (Fig. 2.2). The data suggest that the WT truncated preprotein is the most effective at enhancing SecA’s lipid ATPase activity, and therefore binds SecA productively. Substitution of just two Leu residues in the hydrophobic core region of the signal peptide resulted in a dramatic decrease in the rate of ATP hydrolysis, with further substitutions exhibiting even lower rates.

These results indicate that the hydrophobic core of the signal peptide is crucial for SecA binding and that the presence of the mature region does not ameliorate the effects induced by a reduction in signal peptide hydrophobicity levels.
(A) Sequence

WT  GPKKSMQKSTIALALLPLLFTPVTKA RTPEMPVLENRAAQGDITAPGGARRLTGDQTKHHHHHH

2A  GPKKSMQKSTIALALPALFTPVTKA RTPEMPVLENRAAQGDITAPGGARRLTGDQTKHHHHHH

4A  GPKKSMQKSTIALAAPALATPVTKA RTPEMPVLENRAAQGDITAPGGARRLTGDQTKHHHHHH

6A  GPKKSMQKSTIALAAAPAAATPVTKA RTPEMPVLENRAAQGDITAPGGARRLTGDQTKHHHHHH

(B) Phosphate released (pmol/µg SecA/min)

- WT PhoA
- 2A swap PhoA
- 4A swap PhoA
- 6A swap PhoA
### Phosphate released (pmol/μg SecA/min)

<table>
<thead>
<tr>
<th>Preprotein</th>
<th>Trial</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>WT PhoA</td>
<td>85.3</td>
<td>58.5</td>
</tr>
<tr>
<td>2A swap PhoA</td>
<td>29.5</td>
<td>20.9</td>
</tr>
<tr>
<td>4A swap PhoA</td>
<td>-0.93</td>
<td>6.70</td>
</tr>
<tr>
<td>6A swap PhoA</td>
<td>-12.1</td>
<td>-12.9</td>
</tr>
</tbody>
</table>

### Relative hydrophobicity (%)

![Graph showing the relationship between relative hydrophobicity and phosphate released.](image)
Figure 2.1. Effect of wild-type (WT) and mutant PhoA truncated preproteins with signal peptides of different hydrophobicity, on SecA ATPase activity.

(A) Sequences of WT and mutant alkaline phosphatase PhoA truncated preproteins are shown, with the alanine substitutions highlighted in bold. The signal peptide sequence begins with M, K, Q … and the preprotein ends with … D, Q, T. The additional residues underlined are for purification purposes. The arrow indicates the site of recognition and cleavage by the SPase I in vivo. (B) Reactions were performed in 50 mM HEPES-KOH buffer, pH 7.0, 30 mM KCl, 30 mM NH₄Cl, 0.5 mM Mg(OAc)₂, 1 mM DTT, 4 mM ATP, 0.5 mg/ml BSA with 0.4 µM SecA, 360 µM E. coli phospholipids and 10 µM of the appropriate preprotein. (C) SecA ATPase activity versus relative hydrophobicity of the different preproteins, with the WT being 100%. (D) The results of panel B are summarized; the amount of phosphate released in each experiment performed in duplicate is given as the difference in the rate of ATP hydrolysis between the signal peptide-induced SecA ATPase activity (SecA+liposome+preprotein) and the endogenous SecA activity (SecA alone). Data points in the “combined” column represent the mean ± SEM of three experiments performed in duplicate.
Figure 2.2. Effect of WT and mutant PhoA truncated preproteins with signal peptides of different hydrophobicity, on SecA ATPase activity.

(A) Reactions were performed as described in Fig. 2.1 except for the indicated reaction times. (B) The results of panel A are summarized; the amount of phosphate released is given as the difference in the rate of ATP hydrolysis between the signal peptide-induced SecA ATPase activity (SecA+liposome+preprotein) and the endogenous SecA activity (SecA alone). Data points represent the mean ± SEM of two experiments performed in triplicate.
**Generation of mono-cysteine SecA mutant library and experimental strategy.** To elucidate the signal peptide binding site and the dimeric interface residues on SecA, we employed the established substituted cysteine accessibility method (SCAM). This method has been used extensively to analyze membrane protein insertion sites (van and Lolkema 2000) and has been utilized to identify regions on SecA that interact with the inner membrane both in vivo and in the presence of right-side out inner membrane vesicles in vitro (Ramamurthy and Oliver 1997, Jilaveanu and Oliver 2007). To exploit this strategy, we generated a Cys-less SecA by mutating all four naturally occurring Cys residues to Ser producing a SecA derivative, which has been shown to have comparable activity to the wild-type (Ramamurthy and Oliver 1997). Using this template, we generated a library of 23 mono-cysteine SecA mutants (Fig. 2.3A) that could be covalently modified with the sulfhydryl-reactive biotinylation reagent MPB (Ramamurthy and Oliver 1997). The residues were chosen such that all five domains of SecA could be investigated, however, residues selected from the two nucleotide-binding domains were kept at a minimum since these two domains are crucial for ATP hydrolysis and are not expected to play a key role in preprotein binding. Some of the residues chosen were also based on previous studies and are in regions that some data suggest may be important for signal peptide interactions (Papanikou et al. 2005, Gelis et al. 2007). The mutant SecA proteins displayed signal peptide-induced ATPase activities comparable to that of the wild-type (Fig. 2.3D) with the exception of T221C, K633C and D654C, that showed activities roughly 70% of that of the wild-type and mutant T109C that was about 250% that of the wild-type; the latter is not surprising, since residue T109 is located close to the site of ATP hydrolysis. If the signal peptide
interaction masks the Cys residue, labeling with MPB would be precluded, whereas if the Cys residue is located outside the region of signal peptide interaction, labeling should occur just as well as in the absence of signal peptide. Towards this end, we used the signal peptide, KK-31 (Fig. 2.3B), that contains 21 residues of the alkaline phosphatase signal peptide plus four residues of the mature region and is flanked at the amino and carboxy termini by three Lys residues to enhance peptide solubility. The alkaline phosphatase signal peptide has been found to be representative of several model Sec pathway peptides (Wang et al. 2000). To ensure Cys specificity of MPB labeling of SecA, we treated wild-type SecA, Cys-less SecA and a mutant M235C with MPB, and found that MPB could successfully label both wild-type and the M235C mutant but was unable to label the Cys-less SecA template (Fig. 2.3C). The MPB reagent includes a maleimide group linked to two PEG groups and a biotin; while we cannot rule out that its length precludes precision mapping, it is nevertheless ideal for interfering with productive signal peptide-SecA interactions that involve extensive surfaces.
<table>
<thead>
<tr>
<th>Mutant</th>
<th>SecA Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>T109C</td>
<td>NBD I</td>
</tr>
<tr>
<td>G151C</td>
<td>NBD I</td>
</tr>
<tr>
<td>F193C</td>
<td>NBD I</td>
</tr>
<tr>
<td>T221C</td>
<td>NBD I</td>
</tr>
<tr>
<td>G227C</td>
<td>PPXD</td>
</tr>
<tr>
<td>M235C</td>
<td>PPXD</td>
</tr>
<tr>
<td>R238C</td>
<td>PPXD</td>
</tr>
<tr>
<td>D242C</td>
<td>PPXD</td>
</tr>
<tr>
<td>D337C</td>
<td>PPXD</td>
</tr>
<tr>
<td>D351C</td>
<td>PPXD</td>
</tr>
<tr>
<td>L353C</td>
<td>PPXD</td>
</tr>
<tr>
<td>N369C</td>
<td>PPXD</td>
</tr>
<tr>
<td>A547C</td>
<td>NBD II</td>
</tr>
<tr>
<td>G615C</td>
<td>NBD II</td>
</tr>
<tr>
<td>K633C</td>
<td>HSD</td>
</tr>
<tr>
<td>E647C</td>
<td>HSD</td>
</tr>
<tr>
<td>D654C</td>
<td>HSD</td>
</tr>
<tr>
<td>P704C</td>
<td>HWD</td>
</tr>
<tr>
<td>E708C</td>
<td>HWD</td>
</tr>
<tr>
<td>N712C</td>
<td>HWD</td>
</tr>
<tr>
<td>V766C</td>
<td>HSD</td>
</tr>
<tr>
<td>S773C</td>
<td>HSD</td>
</tr>
<tr>
<td>S809C</td>
<td>HSD</td>
</tr>
</tbody>
</table>

B
HI-KKMKQSTIALALLPLFFTPVTKARTPEKKK-NH2

C

<table>
<thead>
<tr>
<th>kDa</th>
<th>M</th>
<th>WT</th>
<th>Cys-Less</th>
<th>M235C</th>
</tr>
</thead>
<tbody>
<tr>
<td>116</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>97</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D

![Graph showing the comparison of pmol/min/g SecA for different mutant strains with error bars for each data point.]
Figure 2.3. Substituted cysteine accessibility strategy. (A) *E. coli* SecA mono-cysteine mutants and their locations on SecA are shown. (B) Sequence of KK-31 signal peptide used in this study. (C) Specificity of MPB reagent labeling. 2 µM wild-type SecA, Cys-less SecA and M235C SecA mutant were labeled with 1µM MPB followed by quenching with 5 µM L-cysteine. The samples were run on a SDS-PAGE gel, followed by Western blotting with Streptavidin-HRP antibody as described in materials and methods. (D) Results obtained from ATPase assays performed for the wild-type (WT) and 23 SecA mutants are shown.
Signal peptide binds at the PPXD-HSD-HWD groove. To identify the signal peptide binding site on SecA, we carried out MPB accessibility studies for each of the 23 mutants in the presence and absence of KK-31. We categorized the mutants into three groups, namely strong, medium and weak, based on labeling intensity changes before and after incubation with KK-31, of $\geq 50\%$, 49-25\% and < 25\%, respectively (Fig. 2.4A). As shown in Fig. 2.4B, we found that different mutants exhibited different labeling intensity changes upon incubation with KK-31, with values ranging from 2.16 \% for G227C to 80.32 \% for E708C. We found that maximal labeling intensity changes were elicited in the presence of KK-31 for a select few mutants at the PPXD, HWD and HSD. These include M235C, I242C, N369C, P704C, E708C, S773C and V776C and one with medium labeling intensity changes, R238C (Fig. 2.4C). Together, these residues comprise a region on SecA that is ideally positioned to bind a signal peptide within the PPXD-HSD-HWD groove. Indeed, three additional residues that gave medium intensity changes are neighboring this groove, including F193C, T221C and L353C. The other 12 mono-Cys mutants, showed little to no difference in labeling with and without KK-31. SecA is known to exist in solution in both the open and closed forms, with the accessibility of the PPXD-HSD-HWD groove being enhanced and diminished, respectively (Hunt et al. 2002, Zimmer et al. 2006). Thus, our results suggest that signal peptides could bind SecA in its open form at the PPXD-HSD-HWD groove.
A

<table>
<thead>
<tr>
<th></th>
<th>KK-31</th>
<th>Intensity Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>F708C</td>
<td></td>
<td>STRONG</td>
</tr>
<tr>
<td>R238C</td>
<td></td>
<td>MEDIUM</td>
</tr>
<tr>
<td>T109C</td>
<td></td>
<td>WEAK</td>
</tr>
</tbody>
</table>

B

C

57
Figure 2.4 SecA mono-cysteine mutant labeling differences in the presence and absence of KK-31. (A) Western blots illustrating representative labeling intensity changes. MPB labeling intensity results were classified as strong, medium or weak based on intensity levels of $\geq 50\%$, 49-25\% and < 25\%, respectively. (B) The extent of reduction in labeling intensities of SecA mono-cysteine mutants in the presence of KK-31. Data represent the mean ± S.E. of at least three independent experiments. (C) *E. coli* SecA structure (PDB 2VDA) with mono-cysteine mutants that showed strong, medium and weak labeling intensity differences in the presence of KK-31 are highlighted as red (and residues labeled), orange and yellow spheres, respectively. The different domains are shown with NBD I in cyan, NBD II in blue, PPXD in green, HSD in purple and HWD in pink.
**Nucleotides affect signal peptide binding to SecA.** MPB labeling experiments were performed for SecA in the presence of ADP or ATPγS and in the presence or absence of KK-31 to determine whether nucleotide binding affected signal peptide interactions with SecA. Our results show that mutants M235C, R238C, I242C and V766C that were found to have reduced MPB labeling in the presence of KK-31 and in the absence of nucleotides, could bind KK-31 in the presence of ATPγS, while no binding was observed with ADP (Fig. 2.5). Mutants A547C and N712C on the other hand, did not show reduced labeling with KK-31 in the presence or absence of either nucleotide. These results indicate that ATPγS favors signal peptide binding on SecA, while ADP prevents productive SecA-signal peptide interactions.
Figure 2.5. Nucleotides affect signal peptide binding to SecA. Results from MPB labeling experiments in the presence and absence of ADP or ATPγS, and in the presence and absence of KK-31 are shown. Labeling intensity differences are observed for mutants that lie in the PPXD-HSD-HWD groove (M235C, R238C, I242C and V766C) only in the presence of ATPγS, and not with ADP.
**Residues at the two-helix finger region form disulfide bonds.** While performing our SCAM experiments, we observed that a few mutants, namely G793C, A795C, K797C and D798C, located at the two-helix finger region of the HSD were incapable of labeling with MPB even in the absence of signal peptide. This indicated that the accessibility of the Cys residue in these mutants was dramatically reduced, suggesting that these residues could be buried within the folded SecA structure or could be involved in a disulfide bond. To determine whether lack of labeling with MPB was a result of disulfide bond formation, we used a reducing agent TCEP to ensure complete reduction of disulfide-bonded species, if any. We found that mutants F193C and M235C that lie away from the two-helix finger region labeled well with MPB, irrespective of the presence or absence of TCEP. However, mutants G793C, A795C, K797C and D798C were found to label with MPB only upon reduction with TCEP, indicating that these four mutants formed disulfide bonds (Fig. 2.6). These results indicate that these SecA proteins formed disulfide-bonded dimeric species, suggesting that the two-helix finger region could lie at the dimer interface.
Figure 2.6. SecA mono-cysteine mutants at the two-helix region form disulfide bonds. (A) SecA mono-cysteine mutants L6C, G793C, A795C, K797C, D798C, F193C and M235C were labeled with MPB before and after TCEP treatment, followed by immuno-blotted with anti-biotin antibody. Total SecA concentrations were detected using anti-His antibody as described in experimental procedures. (B) SecA mono-cysteine mutants were analyzed on a 7.5% non-reducing SDS-PAGE gel with no TCEP treatment.
Detection of the dimer interface of SecA. To analyze the dimeric interface of SecA, we examined the molecular weights of the mutants identified earlier, namely G793C, A795C, K797C and D798C, that produced disulfide-linked dimers. Using non-reducing SDS-PAGE, we found that these mutants migrated as dimeric species with a molecular weight of approximately 200 kDa (Fig. 2.6B) while they migrated as monomers with TCEP treatment (data not shown). Since this experiment employs a non-reducing gel, some slight variation in mobility is observed likely representing small differences in folding. Size exclusion chromatography and sucrose gradient density centrifugation experiments showed that the monomer-dimer equilibrium of SecA is substantially shifted towards monomer by the deletion of residues 2-11 of SecA; these residues are therefore thought to lie on the dimer interface (Jilaveanu et al. 2005, Or et al. 2005). We tested the ability of mutant L6C to form dimers, and observed that this protein existed in dimeric states in the absence of TCEP though a small amount of monomer is also observed (Fig. 2.6B). In contrast, consistent with the lack of disulfide-bonded dimers observed, F193C and M235C lie outside the two-helix finger region. The residues that did produce cross-linked dimers are located at the two-helix finger region of the HSD (or lie on the same face of SecA e.g. L6) that is thought to play a key role in the opening of the SecYEG channel (Zimmer et al. 2008).
Figure 2.7. SecA dimerizes at the two-helix finger region and various ligands modulate dimer dissociation. (A) Mono-cysteine mutants G793C, A795C, K797C, D798C, F193C, M235C and L6C were reduced with TCEP, followed by its removal by desalting. Oxidation of 0.4 µM SecA was carried out in the presence of 0.5 mM copper phenanthroline with and without the ligands shown, followed by quenching with 5mM neocuproine. Final concentrations of nucleotides, KK-31 and E. coli phospholipids were 4 mM, 20 µM and 400 µM, respectively. The samples were analyzed on a non-reducing SDS-PAGE gel. Dimer and monomer are indicated as D and M, respectively. (B) SecA monomerization is induced in the presence of ATPγS, signal peptide and lipids. Oxidation experiments with the K797C mono-cysteine SecA mutant were carried out as in (A). The samples were analyzed on a non-reducing SDS-PAGE gel. Dimer and monomer are indicated as D and M, respectively.
**Ligands alter SecA monomer-dimer equilibrium.** To identify ligands that could disrupt the SecA monomer-dimer equilibrium, we reduced the mutant proteins with TCEP, and incubated the proteins with signal peptides, *E. coli* phospholipids, ADP or ATPγS. Copper phenanthroline, a mild oxidizing agent, was then added to enable the reformation of exposed disulfide bonds. If indeed, any of the ligands were able to shift the equilibrium towards the monomeric state, the Cys residues of the two protomers would no longer be in close proximity, hence disulfide bond formation should not occur, and the proteins would migrate as monomers on a non-reducing SDS-PAGE gel. We found that surprisingly, ATPγS had the greatest effect on shifting the equilibrium towards monomer (Fig. 2.7A). Phospholipids could also produce monomers, with the addition of signal peptide leading to a further enhancement of this shift. ADP on the other hand, did not lead to monomerization of SecA. Similar results were also obtained with mutant L6C, although in this case, the dimer was more easily shifted towards monomer in the presence of ligands. Two other mutants, namely F193C and M235C, existed predominantly as monomers both in the absence and presence of all ligands consistent with the location of the Cys residues in these mutants far from the dimer interface precluding disulfide bond formation.

Further experiments were carried out with mutant K797C to analyze the effect of nucleotides in conjunction with other ligands on the alteration of the monomer-dimer equilibrium. Incubation of K797C with ADP and signal peptides did not produce significant monomers, while the addition of phospholipids with and without signal peptide, resulted in the generation of some monomers (Fig. 2.7B). On the other hand, ATPγS was able to form monomers in the presence of signal peptide or phospholipids,
with the greatest shift seen in the presence of all three ligands. These results indicate that binding of ATP leads to the formation of SecA monomers while ADP favors dimerization. The addition of translocation ligands such as phospholipids and signal peptides to ATP-bound SecA further enhances monomer production.

2.5 Discussion

We employed the substituted cysteine accessibility strategy to address the location of the signal peptide interaction with SecA and its oligomeric interface. We utilized 23 mono-cysteine SecA mutants that covered much of the SecA “landscape”, with residues chosen from each of its five domains. The use of a sulfhydryl labeling reagent is advantageous in that our approach is sensitive and specific for binding to Cys residues while also circumventing the common pitfall of false positive results often associated with the use of strong crosslinking reagents such as EDAC. The results from these studies indicate that the signal peptide binds at a groove formed with residues of the PPXD, HSD and HWD. Resolved crystal structures of SecA show that SecA exists in the “open” or “closed” form, where the PPXD maintains tight or loose interactions with the HWD, respectively. Such a swiveling motion of the PPXD involves a 60° rigid body rotation of the PPXD towards or away from NBD II (Osborne et al. 2004). The PPXD has been previously implicated in signal peptide binding (Kimura et al. 1991, Gelis et al. 2007, Musial-Siwek et al. 2007) while other studies have identified different regions on SecA, including the hydrophobic cleft between the PPXD and NBD II, and the β strands connecting the PPXD and NBD I (Papanikou et al. 2005, Zimmer et al. 2008).
Our results identify a region on SecA that could “nestle” a signal peptide such that its hydrophobic core may interact with the predominantly hydrophobic residues of the PPXD while residues of the HWD and the HSD could interact with the amino and carboxy termini of the signal peptide, respectively. Some of the residues of SecA identified include M235, R238, I242, N369, P704, E708, V766 and S773; these results are in close agreement with results obtained from an NMR study carried out in the presence of SecA and a KRR-LamB signal peptide (Gelis et al. 2007). The mature region of the preprotein may interact with the β strands linking PPXD and NBD I (Zimmer and Rapoport 2009). Our results are consistent with proposed mechanisms for preprotein translocation (Gelis et al. 2007); SecA’s signal peptide binding groove may exist in the closed or open form (Fig. 2.8A and B), however, the signal peptide can productively bind SecA only in its open form. Studies carried out with cross-linked SecA-SecYEG proteins (Gold et al., 2013) have indicated that the signal peptide can be released from this site upon further opening of the PPXD-HSD-HWD groove, along with a concomitant tight interaction of SecA with the SecYEG channel (Fig. 2.5C and D). Although two of the structures are from B. subtilis and one is from T. maritima, the opening of the groove is evident from these structures and is likely physiologically relevant. The opening of this groove may also provide a feasible mechanism for trapping the mature region of the preprotein at the clamp between the PPXD and NBD II (Zimmer et al. 2008), followed by its release into the channel.

Our results with nucleotide binding indicate that cytosolic SecA could exist in a closed conformation when bound to ADP. This is especially important in light of the fact that ADP release is the rate-limiting step of protein translocation (Robson et al.,
2009). Perhaps, SecA’s association with ADP in the cytosol prevents opening of the PPXD-HSD-HWD groove, and precludes binding to cytosolic proteins. Once SecA is bound to the membrane, ADP maybe released leading to transformation of SecA to a more elongated conformation that is capable of deep penetration into membrane phospholipids and the SecYEG channel (Ulbrandt et al., 1992), enabling signal peptide binding.

We further established some of the residues on the dimer interface of SecA. Several studies have reported that SecA exists as a dimer in the cytosol (Akita et al., 1991, Woodbury et al., 2002); however, the ligands that alter the monomer-dimer equilibrium, the functional translocation-active oligomeric state of SecA, and the dimer interface of SecA are unclear (Or et al. 2002, Benach et al. 2003, Bu et al. 2003, Musial-Siwek et al. 2005, Jilaveanu and Oliver 2006, Wowor et al. 2011). Monomeric SecA has been shown to be sufficient for maintaining translocation (Or et al. 2002, Or et al. 2005), while experiments carried out with dimeric SecA proteins indicate that monomerization is not required for maintaining translocation activity (Jilaveanu et al. 2005, Jilaveanu and Oliver 2006, Wang et al., 2008). Crystal structures of SecA from different bacterial species have also provided differing reports on the dimeric interface and the orientation of the protomers (Hunt et al. 2002, Sharma et al. 2003, Vassylyev et al. 2006, Zimmer et al. 2006, Papanikolau et al. 2007). We found that mono-cysteine SecA mutants G793C, A795C, K797C and D798C, which lie on the two-helix finger loop region, produced disulfide-bonded dimers in the absence of reducing agents, while mutants F193C and M235C, that are located outside the two-helix finger did not. Our results also indicate that ATPγS and lipids resulted in the partial
dissociation of these dimers while complete monomerization was seen in the presence of ATPγS, lipids and signal peptides, strongly suggesting that monomers are produced when translocation is underway. While we cannot rule out that dimeric SecA may be sufficient for translocation in the cell, based on our experiments, we deduce that monomeric SecA plays an important role in protein export.
Figure 2.8. Accessibility of the PPXD-HSD-HWD groove is altered under different conformational states. (A) Crystal structure of *B. subtilis* SecA (PDB 1M6N) in the closed form. The asterisk indicates the location of the two-helix finger region. (B) Crystal structure of *B. subtilis* SecA (PDB 1TF5) in the open form. (C) Crystal structure of *T. maritima* SecA (PDB 3DIN) in the SecYEG-bound form. (D) Overlay of the three crystal structures of SecA with the closed, open and SecYEG-bound forms colored blue, green and red, respectively, and illustrating the progression in opening the PPXD-HSD-HWD groove. (E) A proposed model that links the changes in
oligomerization of SecA with the translocation of a preprotein is shown. Dimeric cytosolic SecA dissociates into monomers as it engages with SecYEG, and the two-helix finger unlocks the channel. The signal peptide binding groove of SecA is opened, along with concomitant uptake of ATP and signal peptide and preprotein binding.
Our study provides a picture of the dimer interface and describes how the monomer-dimer equilibrium can be altered during translocation. We propose that residues involved in SecA dimerization lie along the two-helix finger region. Our results are in agreement with the *T. thermophilus* parallel dimer crystal structure (Vassylyev et al. 2006). Earlier reports have also suggested that the C-terminal region of SecA plays a key role in dimerization (Hirano et al., 1996, Or et al. 2002, Yu et al., 2013). The two-helix finger is particularly important for interactions with the SecYEG channel, and is thought to be directly involved in channel opening (Whitehouse et al. 2012). As SecA engages the SecYEG channel, SecA could dissociate into monomers, and the two-helix finger would now be available to open the SecYEG channel from the cis-side of the membrane (Fig. 2.8E) (Zimmer et al. 2008, Egea and Stroud 2010). This conformational change may occur in conjunction with the opening of the PPXD-HSD-HWD groove, release of ADP and uptake of ATP, and binding of the preprotein, followed by its successful translocation across the membrane.

2.6 Summary and Future Directions

Our results indicate that the signal peptide binds at the PPXD-HSD-HWD groove on SecA. This groove can bind signal peptides in the open form, which is seen in the ATP-bound state, and when interacting with membranes or SecYEG. In the cytosol, SecA exists as a closed ADP-bound dimer, and therefore does not bind signal peptides. Our findings are also consistent with the formation of a parallel SecA dimer. The residues contributing toward the dimer interface are G793, A795, K797 and D798. The SecA dimer is favored in the presence of ADP, while ATP, lipids and
signal peptides tend to shift the monomer-dimer equilibrium towards the monomer. Hence, we infer that the translocation-active form of SecA is monomeric in nature.

To further understand the interactions occurring between SecA and signal peptides, SCAM experiments can be performed in the presence of SecB, SecYEG and membrane phospholipids to note if there are any differences in labeling patterns in their presence and absence. These studies would require the use of a membrane-permeable reagent. Similarly, one could perform these experiments in the presence of a preprotein, to determine whether the presence of the mature region affects binding at the PPXD-HSD-HWD groove.

It would also be helpful identify binding sites of the mature region of the preprotein on SecA. This could be achieved by performing photocrosslinking experiments with preproteins, wherein, a library of mono-cysteine mutants of the mature region of a preprotein could be generated. These preproteins can be linked to a reagent that contains a photoactivatable label, and a reporter group such as biotin. Similarly, a library of SecA mutants with unique protease recognition sites can be generated. Cross-linking of SecA with preprotein, followed by proteolytic cleavage, and immunoblotting experiments will enable the identification of mature region binding sites on SecA.

It would also be interesting to determine whether SecA proteins from different bacterial species bind signal peptides at the same or different sites on SecA. Similarly, the identification of the binding site of a preprotein that utilizes SecA for protein export on the non-canonical SecA2 (such as in *Mycobacterium tuberculosis*) would be
highly insightful, and help differentiate the transport mechanisms between these two SecA proteins.

The lipid compositions of Gram negative and positive bacterial inner membranes are significantly different. It would be interesting to perform lipid ATPase assays with *E. coli* and *B. subtilis* SecA and signal peptides in liposomes containing different amounts and kinds of phospholipids. Since these two proteins share over 50% sequence similarity, establishing the lipid requirements for their optimal activity would be of great interest, particularly in light of the fact that some lipids, such as cardiolipin, have been recently shown to play a key role in enhancing ATPase activity of *E. coli* SecA (Gold et al., 2010).

Lastly, the resolution of a co-crystal structure of SecA-signal peptide or SecA-preprotein would provide valuable insights into determining the sites of interaction between these two components. Despite providing a static view of the binding process, X-ray crystallographic techniques could answer several key questions regarding the preprotein binding site on SecA.
Chapter III

Fluorescence spectroscopy of soluble *E. coli* SPase I Δ2-75 reveals conformational changes in response to ligand binding

Parts of this chapter have been incorporated from published material (Bhanu, M.K. and Kendall, D.A.: Fluorescence Spectroscopy of Soluble *E. coli* SPase I Δ2-75 Reveals Conformational Changes in Response to Ligand Binding. In press, Proteins: Structure, Function and Bioinformatics).

3.1 Abstract

The bacterial Sec pathway is responsible for the translocation of secretory preproteins. The amino-terminal signal peptide of the preprotein is recognized by SecB in the cytosol, and transferred to the ATPase nanomotor SecA. SecA aids in the translocation of the preprotein into or across the SecYEG translocon. During the later stages of transport, the membrane-embedded signal peptidase I (SPase I) cleaves the signal peptide from a preprotein, to yield the mature inner membrane or periplasmic protein. The sites of interaction of the signal peptide on SPase I are not yet well understood. We used a soluble, catalytically active *E. coli* SPase I Δ2-75 enzyme, which lacks the transmembrane segments and the cytosolic loop of the full-length protease, for our experiments. This truncated enzyme is easier to purify and analyze, exhibits preprotein cleavage activity, and has been used in recent years for X-crystallization and other biochemical studies. In order to study the enzyme’s dynamic conformational changes
while in solution and when interacting with lipids and signal peptides, we utilized tryptophan fluorescence spectroscopy.

The wild-type full length enzyme contains six Trp residues, while the truncated form contains four. We generated four single Trp SPase I Δ2-75 mutants, W261, W284, W300 and W310. Each of the other Trp residues were mutated to Phe. 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 are located at a highly hydrophobic region of the enzyme, however, they are largely exposed to the solvent in solution. W300 and W310 were found to insert into lipids, consistent with their location at the enzyme’s proposed membrane-interfacing region; no such interaction with lipids was observed for W261 and W284, indicating that these residues do not interact with the membrane bilayer.

The solvent accessibilities of W261, W284 and W300 were modified in the presence of signal peptide. These results are intriguing in light of the fact that W261 and W284 are not close to the enzyme active site. Nevertheless, these two Trp residues are located near other amino acids that have been implicated in playing a role in enzymatic structural stabilization and in maintaining enzyme activity. W300 on the other hand, is thought to interact with residues on the amino-terminal end of the signal peptide. These findings suggest that a propagation of structural changes beyond the active site is produced in response to peptide binding.

We measured the signal peptide binding affinity for the enzyme via FRET experiments and the $K_d$ was determined to be 4.4 µM. 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. Our results suggest that the carboxy-terminus of the signal peptide,
which is also the cleavage site of the preprotein, is located near the enzyme active site.
This is brought about by insertion of the hydrophobic core region of the signal peptide
into the membrane bilayer. The positively charged amino-terminus of the signal
peptide can thus interact with the negatively charged phospholipid head group of the
bilayer at the cis-side of the inner membrane. These studies reveal enzymatic
structural changes required for preprotein proteolysis as SPase I interacts with its two
key partners, the signal peptide and membrane phospholipids.

3.2 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
(Driessen and Nouwen 2008). SPase has been shown to be vital for cell survival (Date
1983, Zhbanko et al., 2005, Taheri et al., 2010). Since bacterial SPases vary
considerably from eukaryotes in their proteolytic mechanism, they form an interesting
class of potential anti-microbial drug targets (Paetzel et al., 2002, Smitha Rao and
Anne 2011).
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 (Rawlings and Barrett 1993). The signal peptide substrate is made up of 18-30 residues, and immediately precedes the mature region of the secretory preprotein (von Heijne 1985). The important residues for substrate recognition are the -1 (P1) and the -3 (P3) residues of the preprotein (von Heijne 1983). These residues have been found to specifically bind to the substrate binding pockets S1 and S3, respectively (Paetzel et al. 1998). 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 (Sung and Dalbey 1992, Paetzel et al. 1997, Paetzel et al. 1998). 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 (Paetzel et al. 1998, Paetzel et al. 2002). The signal peptide is further cleaved by the signal peptide peptidase enzyme (Saito et al., 2011).

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 (von Heijne 1998). This issue was partly resolved when a mutant SPase I lacking residues 2-75 was designed, and purified as a soluble protein (Kuo et al. 1993). 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 (Kuo et al. 1993, Tschantz et al. 1995). 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 Δ2-75 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 (Paetzel et al. 1998, Paetzel et al. 2004, Luo et al. 2009). 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 Δ2-75 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 (Fig 3.1A) (Paetzel et al. 2002). Trp fluorescence is highly sensitive to environment, (Chen and Barkley 1998) and can therefore yield vital information regarding its location. We generated four SPase I Δ 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 (van Klompenburg et al. 1998). We further determined the $K_d$ for signal peptide binding to wild-type SPase I Δ2-75, 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.

3.3 Materials and Methods

Construction of the GST-SPase I Δ2-75 vector. The SPase I Δ2-75 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 Δ2-75, and unnecessary for Prescission protease cleavage, were removed via QuikChange mutagenesis (Stratagene), giving rise to the GST-SPase I Δ2-75 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 Δ2-75. E. coli BL21 (DE3) harboring the GST-SPase I Δ2-75 vector were grown in LB medium with 100 µg/ml ampicillin at 37 °C until $OD_{600} \sim 0.7$. Expression of GST-SPase I Δ2-75 was induced with 0.5 mM
IPTG, and cells were grown for 3 hours. The GST-SPase I Δ2-75 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 Δ2-75, 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 degree of labeling was calculated according to the manufacturer’s instructions and was determined to be 100%.

**Signal peptidase labeling.** 10 µM of S302C SPase I Δ2-75 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 ºC.

**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 Δ2-75 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 Δ2-75 and IAEDANS-SP2 were performed at an excitation wavelength of 295 nm and the emission intensities and 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 (Caputo and London 2003).

**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 \( \frac{F_0}{F} = 1 + K_{sv} [Q] \) 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 Δ2-75 were analyzed both in the presence \( (F) \) and absence \( (F_0) \) of quencher (Caputo and London 2003) For binding affinity measurements, the emission intensities at 345 nm were analyzed. \( K_d \) was calculated using the one-site binding model equation: \( \Delta F/F_0 = \Delta F_{max}/F_0[SP2]/(K_d+[SP2]) \), where \([SP2]\) is the concentration of IAEDANS-SP2, \( F \) and \( F_0 \) are the emission intensities in the presence and absence of IAEDANS-SP2, respectively, and \( \Delta F \) represents the change in intensity between \( F \)
and \( F_0 \). For FRET distance calculations, the equation \( E = 1 - \left[ (F_{da} - F_a)/F_d \right] \) 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).

### 3.4 Results

**Characterization of single Trp SPase I Δ2-75 mutants.** *E. coli* SPase I Δ2-75 has four Trp residues, W261, W284, W300 and W310 (Fig. 3.1A). 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. 3.1B). We examined the fluorescence spectra of the wild-type SPase I Δ2-75 as well as the single Trp mutants (Fig. 3.1C). 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.
A

B

<table>
<thead>
<tr>
<th>Name</th>
<th>Residues Changed</th>
</tr>
</thead>
<tbody>
<tr>
<td>W261</td>
<td>W284F/W300F/W310F</td>
</tr>
<tr>
<td>W284</td>
<td>W261F/W300F/W310F</td>
</tr>
<tr>
<td>W300</td>
<td>W261F/W284F/W310F</td>
</tr>
<tr>
<td>W310</td>
<td>W261F/W284F/W300F</td>
</tr>
</tbody>
</table>

C

[Graph showing fluorescence intensity vs. wavelength]
Figure 3.1. Wild-type and single tryptophan SPase I Δ2-75 enzymes. (A) Crystal structure of *E. coli* SPase I Δ2-75 (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 Δ2-75 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.
Local environment of each Trp residue. Of particular interest were the wavelengths of maximum fluorescence emission (\(\lambda_{\text{max}}\)) of each mutant (Fig. 3.1C). 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 \(\lambda_{\text{max}}\) values of 342 and 346 nm, respectively, while W300 and W310 had \(\lambda_{\text{max}}\) 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 Δ2-75 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. 3.2).
Figure 3.2. In vitro processing of the substrate proOmpA nuclease A by the wild-type and single Trp SPase I Δ2-75 enzymes. Cleavage assays were carried out without (-) or with 0.75 µM (1X), 3.75 µM (5X) and 7.5 µM (10X) of the enzyme, and 15 µM 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.
Figure 3.3. Fluorescence quenching with acrylamide and 10-DN for the wild-type and single Trp SPase I Δ2-75 enzymes in aqueous and lipid environments. (A-E) 500 nM SPase I Δ2-75 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 Δ2-75 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.
**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. 3.3A-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; \( \frac{F_0}{F} = 1 + K_{SV} [Q] \), 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. 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. 3.3, a plot of \( \frac{F_0}{F} \) versus acrylamide concentration yields the \( K_{SV} \) value for each mutant from the slope (Table 3.1). 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 Δ2-75 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 \( \lambda_{\text{max}} \) determinations.

*Liposome induced changes in Trp fluorescence.* SPase I Δ2-75 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 (Paetzel et al. 2002). It has also been previously shown that SPase I Δ2-75 exhibits enhanced cleavage ability in the presence of phospholipids and the membrane mimetic detergent Triton X-100 (Tschantz et al. 1995). 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. 3.3A-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 3.1). 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. 3.3F). Using a previously established procedure, (Caputo and London 2003) 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 3.2). 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 (Caputo and London 2003). This suggests that W310 is even more deeply inserted into the membrane than W300.

**Signal peptide binding to wild-type SPase I Δ2-75.** 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 Δ2-75. 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. 3.4A). FRET experiments were performed with the SPase I Δ2-75 S302C mutant labeled with the fluorescent donor dye IAEDANS, and SP2 labeled with the fluorescent acceptor dye IANBD.
Table 3.1: Stern-Volmer constants ($K_{SV}$) derived from acrylamide quenching experiments.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>W261</th>
<th>W284</th>
<th>W300</th>
<th>W310</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>5.34 ± 0.19</td>
<td>2.90 ± 0.10</td>
<td>3.71 ± 0.05</td>
<td>4.46 ± 0.05</td>
<td>4.74 ± 0.09</td>
</tr>
<tr>
<td>Liposomes</td>
<td>3.36 ± 0.15</td>
<td>3.29 ± 0.06</td>
<td>3.28 ± 0.09</td>
<td>3.33 ± 0.08</td>
<td>3.31 ± 0.19</td>
</tr>
<tr>
<td>WTSP</td>
<td>4.47 ± 0.04</td>
<td>1.89 ± 0.05</td>
<td>4.68 ± 0.04</td>
<td>3.28 ± 0.08</td>
<td>4.88 ± 0.04</td>
</tr>
</tbody>
</table>

$^a$ $K_{SV}$ 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 3.2: Fluorescence quenching data derived from 10-DN quenching experiments.

<table>
<thead>
<tr>
<th>Single Trp mutant</th>
<th>$F_0/F^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>W300</td>
<td>1.08 ± 0.01</td>
</tr>
<tr>
<td>W310</td>
<td>1.27 ± 0.01</td>
</tr>
<tr>
<td>WT</td>
<td>1.10 ± 0.02</td>
</tr>
</tbody>
</table>

$^aF_0/F$ 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.
As shown in Fig. 3.4B, 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, (Auclair et al., 2010, Auclair et al., 2013) 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 \textit{E. coli} SPase I Δ2-75 (Paetzel et al. 2002).

Using FRET of the donor Trp residues of wild-type SPase I Δ2-75 and the acceptor IAEDANS-labeled SP2 (Fig. 3.4C) 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 ± 1.6 \( \mu \)M.
A  
MCKQSTIALALLPLLFTPVTKA -NH₂

B

C

1 - (F/F₀) vs [IAEDANS-SP2]μM

Fluorescence Intensity (a.u.)

Wavelength (nm)
Figure 3.4. Signal peptide binding on wild-type SPase I Δ2-75. (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 Δ2-75 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 Δ2-75 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.
Figure 3.5. Fluorescence quenching with acrylamide for the wild-type and single Trp SPase I Δ2-75 enzymes in the presence and absence of PhoA WTSP. (A-E) 500 nM SPase I Δ2-75 wild-type (WT), W261, W284, W300, and W310 in TKE buffer, or in TKE buffer containing 10 μM 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 Δ2-75 in the absence and presence of acrylamide, respectively.
**Effect of signal peptides on the fluorescence emission of SPase I Δ2-75 mutants.** We performed acrylamide quenching experiments for SPase I Δ2-75 in the presence of WTSP to examine its effect on the enzyme’s structure. We compared this data (Fig. 3.5 and Table 3.1) 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 (Luo et al. 2009). These results indicate that signal peptide binding to SPase I Δ2-75 produces conformational changes that are propagated to regions that do not directly contact the peptide.

**3.5 Discussion**

SPase I Δ2-75 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 A$_2$, β-lactam penem and β-sultam inhibitors (Paetzel et al. 1998, Paetzel et al. 2004, Luo et al. 2009). 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 Δ2-75 studies, as it contains four Trp residues (Fig. 3.1 A), 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 (Kim et al., 1995, Kim et al. 1995, Luo et al. 2009). 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 (Paetzel et al. 2002).

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 (Paetzel et al. 2002). 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, (Tschantz et al. 1995) 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 Δ2-75; 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, (Musial-Siwek et al., 2005, Auclair et al. 2010) but SecA has a much greater affinity for the SecYEG channel ($K_d$ = 10 nM) (Driessen and Nouwen 2008).
Figure 3.6. 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 (Klenotic et al., 2000, Paetzel et al. 2002). 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 Δ2-75 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.
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 SecA (Gold et al., 2013) 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, (Van Voorst and De Kruijff 2000) 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 (Wiener and White 1992). This data is supported by modeling experiments of SPase I bound to signal peptide (Paetzel et al. 2002). 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 (van Klompenburg et al. 1998).

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, (Luo et al. 2009) W261 and W284 also underwent solvent accessibility changes. As shown in Fig. 3.6A, 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 (Klenotic et al. 2000, Paetzel et al. 2002). 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 (Klenotic et al. 2000). The latter bond is broken in the aryloymycin A2-bound structure (Paetzel et al. 2004). Similarly, Asn 277 and Ala 279 are in close proximity to a β-sultam inhibitor (Luo et al. 2009). Mutational analysis indicated that Asp 273 and Ser 281 are important for catalysis (Klenotic et al. 2000). 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. 3.6B). W300 has been found to be a residue that is crucial for signal peptidase activity
based on mutation and chemical modification experiments (Kim et al. 1995). 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 (Paetzel et al. 2002, Luo et al. 2009). 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 (Paetzel et al. 2004). 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 (Luo et al. 2009). 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 (Luo et al. 2009). W310 on the other hand, is 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 (Paetzel et al. 2004, Luo et al. 2009). 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 Δ2-75.

3.6 Summary and Future Directions

Our results indicate that a large hydrophobic region of SPase I Δ2-75 is exposed in solution, and can interact extensively with lipids. We also found that the signal peptide
elicits both allosteric and direct structural perturbations in the enzyme. The signal peptide was also found to position itself such that the cleavage region comes in close contact with the enzyme active site to facilitate proteolytic cleavage. This would mean that the hydrophobic core region of the signal peptide must embed itself within the membrane bilayer, suggesting that the signal peptide must exit the hydrophilic SecYEG channel to allow it to gain access to the enzyme.

The interactions of SPase I with signal peptides need to be investigated in further detail in order to fully appreciate the final step of protein translocation. These studies could play a vital role in the development of antimicrobial drugs against pathogenic bacteria. This is particularly true, in light of the fact that multiple inhibitors (β-sultam and arylomycin A2) (Luo et al. 2009) can bind to one molecule of SPase I, indicating that the enzyme-ligand reaction may not be as straightforward as one would imagine.

Toward this end, Trp fluorescence spectroscopy can be further exploited to study SPase I interactions with the WTSP in the presence of lipids. Pre-incubation of the enzyme with lipids, would ensure that the membrane-interfacing region of the enzyme becomes embedded in the liposome. Addition of WTSP, followed by acrylamide quenching experiments can provide a detailed picture of solvent accessibility levels, and therefore, conformational changes that are brought about by the presence of both WTSP and lipids. Alternatively, one can incubate the WTSP with lipids prior to addition of enzyme. The hydrophobic core region of the WTSP should intercalate into the membrane, as shown by previous studies (Briggs et al. 1986). The addition of SPase I, followed by acrylamide quenching will provide valuable data.
regarding enzymatic structural changes. It would be even more interesting to note whether both types of experiments result in the same solvent accessibility changes or not. This may provide us with clues regarding the affinities of the enzyme and WTSP for lipids.

Another set of experiments that may be useful are FRET assays between the enzyme and WTSP in the presence of lipids. The use of multiple variants of the WTSP, each carrying a fluorescent acceptor probe at different locations of the peptide, along with variants of SPase I carrying fluorescent donor probes at different locations, can provide a more comprehensive view of the mode of signal peptide binding. This can also be performed both in the presence and absence of lipids. It would be even more interesting, to use truncated preproteins, or even full length preproteins, in FRET experiments. In such a scenario, the introduction of bulky residues at the -1 and -3 sites would be required to prevent cleavage; this is essential to ensure that the preprotein remains intact for assessing the role of the mature region in SPase I binding. One can also envision similar experiments that could be performed using different signal peptides from preproteins that utilize the Sec pathway, such as the β-lactamase and maltose-binding proteins, as well as peptides or preproteins from the TAT and YidC pathways. These studies would enable us to identify and characterize the signature features of multiple signal peptides that allow it to successfully bind SPase I.

The role of the transmembrane segments of SPase I in cleavage has not yet been well established. It would be very interesting to perform all or some of the above experiments with both the full-length and truncated enzyme to truly appreciate the role
played by residues 2-75. Indeed, the $k_{\text{cat}}$ of the SPase I cleavage reaction is substantially diminished in the absence of the transmembrane segments vs. in its presence (Tschantz et al. 1995). Therefore, these regions must be required for structural stabilization that could dramatically raise the cleavage efficiency of the membrane-embedded enzyme.

Last, but not the least, a co-crystal of SPase I (full-length or truncated) with bound signal peptide, would provide critical information regarding meaningful enzyme-ligand interactions, and would be of great help towards the generation of antibiotics against several disease-causing bacteria.
REFERENCES


recognition by the translocase motor SecA as determined by NMR." Cell 131(4): 756-769.


