Mechanism of Translocation by the Bacterial ATPase SecA

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Mechanism of Translocation by the Bacterial ATPase SecA

Fully synthesized bacterial secretory proteins are transported across the plasma membrane post-translationally by the ATPase SecA. SecA is a member of the large AAA ATPase family and uses the energy from ATP hydrolysis to push nascent polypeptides through the SecY channel. However, the mechanism by which SecA couples its energy consumption to this translocation activity is unclear. Here, we use single molecule FRET to observe the conformational changes of SecA as it translocates a model polypeptide substrate and ensemble biochemistry experiments, including protease protection assays, to monitor the corresponding movements of the substrate itself. Upon ATP-binding, SecA’s two-helix finger inserts into the SecY channel and pushes the translocating polypeptide chain forward. Subsequently, a clamp domain closes around the substrate and holds it in place as the two-helix finger resets during ATP hydrolysis. The clamp domain re-opens after phosphate release, freeing the polypeptide to passively slide through the SecY channel until the subsequent ATP binding event. The alternating movements of these two domains ensure that translocation is directional and provide a model that may be applicable to a wide range of ATPases that work on polypeptide chains.
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Cherry, this paragraph is just for you. It is short, but it took a long time to write. It may feel simple, but it is not. I struggled to put how much you mean to me in words, and yet, there are words here. They do not do you justice, but they try. Here goes: I love you. You are my partner in everything, equal and appreciated. I don’t how I could have finished this PhD without you. Thank you.

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Chapter 1: Introduction

1.1 Introduction to Protein Translocation

Ribosomes synthesize cellular proteins in the cytosol. However, in order to perform their intended function, as much as 30% of the eukaryotic and prokaryotic proteomes must be inserted into, or trafficked across, various cellular membranes. This process, termed protein translocation, is mediated by the broadly conserved Sec system localized to the eukaryotic endoplasmic reticulum (ER) or prokaryotic plasma membrane (PM) (for review, see Park & Rapoport, 2012; Rapoport et al, 2017). Unfolded proteins are recognized as Sec system translocation substrates on the basis of their hydrophobic transmembrane segments or genetically encoded, cleavable, N-terminal signal sequences (SS).

The core component of the Sec system is the eukaryotic Sec61/prokaryotic SecY integral membrane protein channel. This ER/PM resident protein complex is constricted and plugged when inactive, but can activate to allow the passage of translocation substrates. Upon activating the channel can open bidirectionally: transversally, supporting translocation into the ER lumen or bacterial periplasm, and laterally, mediating the insertion of transmembrane helices (TMs) into the ER/PM. As the channel itself is only a passive pore, accessory proteins must interact with Sec61/SecY to target substrates to the channel and, crucially, to provide energy for the energetically unfavorable process of translocation. Two separate pathways target and translocate substrates either cotranslationally, during their synthesis, or post-translationally, after the nascent polypeptide has been released from the ribosome.

In cotranslational translocation the conserved signal recognition particle (SRP) ribonucleoprotein binds to the ribosome and scans emerging nascent chains for SSs or TMs. Recognition of these translocation signals allows SRP to bind to its cognate receptor on the ER/PM and transfer the ribosome nascent chain complex to the Sec channel. Subsequent rounds of translational
elongation then feed the nascent chain directly into the Sec channel, driven by GTP hydrolysis at the ribosome.

Substrates with signal sequences that evade SRP are targeted to the Sec channel post-translationally through processes that differ in eukaryotes and prokaryotes. In both taxa, a series of chaperones maintain polypeptide substrates in an unfolded conformation after their release from the ribosome and target them to the Sec61/SecY channel. In eukaryotes, the ER luminal Hsp70 chaperone BiP couples its ATP hydrolysis cycle to substrate binding, forming a “Brownian ratchet,” which pulls polypeptides through the membrane (Matlack et al, 1999). In bacteria, the cytosolic ATPase SecA works oppositely, using the energy from ATP hydrolysis to push translocation substrates through the bilayer.

1.2 Structure and Function of the Sec61/SecY Translocon Complex

The heterotrimeric Sec61/SecY complex consists of three subunits: α, β, γ in eukaryotes, corresponding to Y, G, and E in prokaryotes (Fig 1.1A). Whereas the α/Y and γ/E proteins are highly conserved and essential, the β/G subunit is dispensable. In vitro reconstitution has shown the complex to be necessary for both co- and post-translational translocation activity (Brundage et al, 1990; Akimaru et al, 1991; Görlich & Rapoport, 1993). The architecture of the Sec complexes has been determined by both X-ray crystallography and cryogenic electron microscopy (cryo-EM) of homologous complexes from a wide range of species. (Van Den Berg et al, 2004; Zimmer et al, 2008; Egea & Stroud, 2010; Voorhees et al, 2014; Voorhees & Hegde, 2016; Li et
al, 2016; Ma et al, 2019; Itskanov & Park, 2019; Wu et al, 2019). The α/Y subunit is the largest of the three and forms the conduit for the translocating polypeptide (Mothes et al, 1994; Cannon et al, 2005; Li et al, 2016; Ma et al, 2019). The protein has 10 transmembrane helices that each cant in the membrane forming a roughly hourglass shape with both cytosolic and luminal/periplasmic aqueous vestibules (Fig 1.1B). The constriction about the central pore is formed by a ring of six hydrophobic residues that seal the channel to small molecules both when closed and during translocation (Park & Rapoport, 2011). A plug formed by TM helix 2a occupies the luminal/periplasmic cavity and tightly seals the inactive channel (Fig 1.1B).

Viewed from the cytosol, the channel can be subdivided into two halves including TMs 1-5 and 6-10. A hinge between TM5 and TM6 allows these two halves to open relative two each other, analogous to a clamshell, forming a lateral gate between TMs 2b and 7 (Van Den Berg et al, 2004; Egea & Stroud, 2010) (Fig 1.1C). The γ/E subunit includes a long helix that passes through the membrane diagonally, wrapping around the α/Y subunit opposite this gate and supporting the hinge. The β/G, when present, binds alongside TMs 1, 4, and 3 of α/Y, perhaps mediating the interaction with accessory proteins in the bilayer (Fig 1.1C).

The Sec61/SecY channel occupies as many as three distinct conformations, corresponding to different steps of translocation. In its resting state the channel is closed. Its lateral gate is shut and the plug helix is docked directly above the pore constriction (Van Den Berg et al, 2004), sealing the channel from ion and small molecule flux (Park & Rapoport, 2011) (Fig 1.1B-C). The ribosome, SecA, and the eukaryotic post-translational translocation accessory proteins Sec 62/63 all bind to the cytosolic 6/7 and 8/9 loops of Sec61/SecY (Zimmer et al, 2008; Voorhees et al, 2014; Itskanov & Park, 2019; Wu et al, 2019). This binding induces a conformational change in the channel, completely or partially opening the lateral gate and exposing the lipid bilayer to the aqueous cavity of the channel.

The signal sequence of the translocating substrate is then threaded as a loop into the widened pore, displacing the plug from its position (Voorhees & Hegde, 2016; Li et al, 2016; Ma
et al, 2019). The hydrophobic stretch can partition towards the exposed lipid phase and intercalate between the lateral gate TMs 2b and 7, whereas the substrate sequence directly following the signal sequence lies in the pore constriction itself (Fig 1.1D-E). Signal sequence binding also displaces the plug from its position at the luminal side of the pore constriction (Fig 1.1D-E). This movement is necessary to accommodate the translocating polypeptide and has been viewed in cryo-EM structures (Voorhees & Hegde, 2016; Ma et al, 2019), as well as in single molecule Förster Resonance Energy Transfer (smFRET) experiments (Fessl et al, 2018).

The activation of the Sec channel by signal sequence binding is well documented. Several “Prl” mutations that allow the translocation of proteins with defective signal sequences map to the lateral gate and plug domains and would likely destabilize the closed conformation (Li et al, 2007). Furthermore, signal sequence peptides can act in trans, binding to the lateral gate and activating it allosterically for the translocation of proteins lacking signal sequences (Lill et al, 1990; Gouridis et al, 2009).

The signal sequence docking holds the channel open while the polypeptide is threaded through from its N to C terminus. As the channel seal must be preserved during translocation, the pore ring is thought to subtly open and close to allow the passage of sterically large amino acids without compromising the ionic seal. Indeed, mutation of the hydrophobic residues that make up this ring renders the channel permissive to small molecule flux even during translocation (Park & Rapoport, 2011).

After translocation, the plug can return to its position above the pore ring, resealing the channel. The signal peptide is a substrate of the ER/PM resident signal/leader peptidase (Jackson & Blobel, 1977; Dalbey & Wickner, 1985), which cleaves it from the translocation substrate either during or after translocation, allowing it to exit from the lateral gate into the lipid phase and be further degraded (Weihofen et al, 2002). The channel then returns to its fully inactivated state.
1.3 Mechanism of Cotranslational Translocation

Cotranslational translocation occurs similarly in eukaryotes and prokaryotes. Substrate polypeptides are targeted to the Sec61/Y complex during translation and threaded through the channel by the continued addition of amino acids to their C termini (Fig 1.2). The process begins when the highly conserved ribonucleoprotein signal receptor particle (SRP) recognizes a hydrophobic sequence of the nascent chain as it emerges from the exit tunnel of the ribosome (Fig 1.2, Step 1). However SRP will only bind to strongly hydrophobic regions. While TMs are sufficiently hydrophobic to be recognized by SRP, many secretory protein N terminal SSs are more polar...
and evade recognition (Ng et al., 1996; Lee & Bernstein, 2001). Furthermore, SRP can often recognize several of the TMs in a multi-pass membrane protein, allowing it to still target a substrate even if the first hydrophobic sequence evades it (Chartron et al., 2016). Secretory proteins have only a single SS and cannot be targeted by SRP if the SS is not recognized immediately after its translation.

After recognizing a hydrophobic sequence, SRP can bind to the SRP receptor (SR) on the ER/PM, thus bringing the ribosome nascent chain (RNC) complex to the membrane (Fig 1.2, Step 2). The RNC then docks onto the Sec61/Y channel via interactions between the TMs 6/7 and 8/9 loops on Sec61/SecY and the ribosomal 28S/23S rRNA and the ribosomal proteins L23 and L29 (Fig 1.2, Step 3). This docking, and subsequent transfer of the hydrophobic sequence into the lateral gate of Sec61/SecY, fully opens the channel to allow the rest of the polypeptide to pass into the channel as new residues are added to its growing C terminus in the ribosome. As the junction between the ribosome and the Sec channel is fairly tight, GTP hydrolysis in the ribosome is generally sufficient to push the nascent chain into and through the Sec channel (Fig 1.2, Step 4). During translocation, the SS can be cleaved from the translocating protein by signal peptidase, releasing the mature protein on the trans side of the membrane after translation is complete. The ribosome can then recycle from the membrane and dissociate into its subunits (Fig 1.2, Step 5).

In bacteria, the ATPase SecA may also play a role in cotranslational translocation, as it has been identified by ribosome profiling to be proximal to the ribosome during the translation of translocation substrates (Huber et al., 2011). Both in vitro and in vivo, SecA is required for the translocation of a subset of prokaryotic substrates during their translation, in an SRP-independent manner (Neumann-Haefelin et al., 2000; Jan et al., 2014; Wang et al., 2017). One possibility is that these are substrates that are frequently recognized and targeted to the translocon by SecA before their synthesis is complete, leading to their post-translational translocation even as their translation is still being completed. There is some evidence, though, for a direct interaction between
SecA and the ribosome. Photo-crosslinking studies, as well as two cryo-EM structures, suggest an interaction between the ribosome and the N-terminal helix and first nucleotide binding domain (NBD1) of SecA (Singh et al., 2014; Huber et al., 2017; Jamshad et al., 2019; Wang et al., 2019). As the N terminal helix is also responsible for binding anionic lipids, an interaction with the negatively charged ribosome seems plausible. However, this binding mode would prevent SecA from interacting with SecY or the bacterial PM. The purpose and mechanism of this cotranslational role for SecA is therefore quite unclear.

1.4 Mechanism Eukaryotic Post-translational Translocation

Unlike cotranslational translocation, which is a highly conserved process between prokarya and eukarya, post-translational translocation differs significantly between the two taxa and must be covered independently. In eukaryotes, post-translational translocation is mediated by a larger Sec complex consisting of not only the core Sec61α/β/γ complex, but also Sec62/63 and Sec71/72. The Sec63 subunit in this large complex binds to both the N and C terminal halves of Sec61α, prying the lateral gate open to a much greater extent than in the primed states of cotranslational or prokaryotic post-translational translocation (Itskanov & Park, 2019; Wu et al., 2019). Crucially though, the plug domain remains in place above the pore ring to maintain the membrane barrier (Wu et al., 2019).

The unfolded substrate binds to this strongly primed complex, facilitated by the wide open lateral gate which is able to accommodate even the weakly hydrophobic SSs of many secretory proteins (Fig 1.3, Step 1). The interaction is also mediated by interactions between some Hsp70 and Hsp90 proteins (Plath & Rapoport, 2000), which chaperone the translocation substrate from the ribosome, and the TPR domains of Sec72 (Tripathi et al., 2017). The binding of the substrate to the Sec complex then causes the release of all bound chaperones (Plath & Rapoport, 2000), allowing the polypeptide to diffuse both forwards and backwards in the Sec61 channel (Fig 1.3, Step 2).
As the substrate emerges on the luminal side of the membrane it is bound by the Hsp70 BiP (Fig 1.3, Step 3). BiP first weakly binds the polypeptide in its ATP-bound state. Then the J-domain of Sec63 interacts with BiP to stimulate its ATP hydrolysis. In the resulting ADP-bound form, BiP binds strongly to translocation substrates. As it is too large to fit into the channel itself, it prevents substrates from backsliding towards the cytoplasm, while still allowing their forward diffusion into the lumen, forming a “Brownian ratchet” (Matlack et al, 1999). The close proximity of the J domain with the Sec61 cytosolic cavity ensures there is always an ADP-bound BiP near the Sec61 channel. As translocation progresses, new BiP molecules can bind to lumenally exposed substrate sequences until the entire polypeptide has emerged from the channel (Fig 1.3, Step 5). BiP exchanges ADP for ATP, resulting of the release of the polypeptide chain.
**Step 4.** Then, ATP exchange and cleavage of the signal sequence allows the release of the substrate into the lumen (**Fig 1.3, Step 5**). Sec complex translocation can be reconstituted without BiP using antibodies raised against segments of the substrate, providing strong support for this ratcheting model (Matlack *et al.*, 1999).

### 1.5 Mechanism of Prokaryotic Post-translational Translocation

As there is no ATP in the periplasm to support a ratcheting mechanism analogous to that in eukaryotes, bacteria have evolved a separate post-translational translocation mechanism. Syn-
thesized proteins are recognized by the cytosolic ATPase SecA, often via its interaction with the chaperone SecB, which maintains substrates in an unfolded state until their translocation (Collier et al, 1988) (Fig 1.4, Step 1). SecA brings the substrate to the membrane through its interactions with anionic lipids and the SecY channel (Bauer et al, 2014). SecA binding activates the SecY channel in the same manner as ribosome binding does during cotranslational translocation, allowing the signal sequence to bind in the lateral gate (Zimmer et al, 2008; Li et al, 2016; Ma et al, 2019) (Fig 1.4, Step 2). It then uses its ATP hydrolysis cycle to push the substrate through the channel and into the periplasm (Akimaru et al, 1991; Schiebel et al, 1991; Economou & Wickner, 1994) (Fig 1.4, Step 3). Once through the channel, signal sequence cleavage releases the mature secretory protein into the bacterial periplasm (Dalbey & Wickner, 1985) (Fig 1.4, Step 4). While many of the details of this process have been characterized, the exact sequence of conformational changes that couple ATP hydrolysis to the mechanical work required to push a polypeptide chain have been unclear, leading to the evolution of several conflicting models as to the mechanism of this protein.

1.5.1 Architecture of the SecA ATPase

SecA is an AAA ATPase in the RecA family as characterized by its two nucleotide binding domains (NBDs 1 and 2). Connected to these two NBDs, the protein has a long helical scaffold domain (HSD) consisting of a long linker helix extending from the NBDs to a two helix finger (THF) with a conserved Tyrosine reside at its tip. The protein also has a helical wing domain (HWD) and polypeptide crosslinking domain (PPXD) (Hunt et al, 2002) (Fig 1.5A). Structures of the SecY-bound ATPase in the presence of the ATP hydrolysis intermediate analog ADP•BeF₆ show that SecA makes contacts with the TM 6/7 and 8/9 loops of SecY through its PPXD domain in this nucleotide state (Zimmer et al, 2008) (Fig 1.5B). The THF of SecA protrudes into the SecY channel nearly to the pore ring constriction, occupying the cytosolic cavity. A “clamp” formed between the PPXD and NBD2 forms a conduit that aligns with the channel
Figure 1.5. Architecture of SecA/SecY Complex. (A) A cytosolic view of SecA from the X-Ray structure of the complex from Thermotoga maritima. (PDB-ID:3DIN). SecA’s domains are shown in different colors, and ADP•BeFx is shown in its binding site. THF: two-helix finger; PPXD: polypeptide crosslinking domain; NBD1 and NBD2: nucleotide binding domains 1 and 2, respectively; HSD: helical scaffold domain; HWD: helical wing domain. (B) A lateral view from the Cryo-EM structure of the translocating SecA/SecY complex from Geobacillus thermodenitrificans (PDB-ID: 6ITC). The SecA, SecY, SecE, and the translocating polypeptide are shown in
of SecY. Crystal structures of SecA in solution bound to ADP and ADP•BeF\textsubscript{x} show the clamp in two separate “open” and “closed” conformations differing by a large, nearly 80°, rotation of the PPXD (Hunt \textit{et al}, 2002; Osborne \textit{et al}, 2004; Chen \textit{et al}, 2015) (Fig 1.5C). Such a movement in solution is further substantiated by both single molecule FRET and AFM experiments (Ernst \textit{et al}, 2018; Chada \textit{et al}, 2018), though the two differ on how motion correlates with the ATPase cycle. Determining whether the clamp opens and closes while SecA is bound to SecY is a goal of the present study.

Recent crystal and cryo-EM structures of the SecA complex with bound substrate show the path of translocating polypeptides through the ADP•BeF\textsubscript{x}–bound complex (Li \textit{et al}, 2016; Ma \textit{et al}, 2019) (Fig 1.5B). As in the cotranslational translocating channel, the signal sequence binds in the lateral gate of SecY with its N terminus on the cytoplasmic side. The polypeptide then loops back through the SecY pore passing close to the THF. In SecA the substrate lies in the clamp, seen in the closed conformation in these structures. The substrate forms a β-strand to complement a small β-sheet at the back of the clamp (Zimmer & Rapoport, 2009; Ma \textit{et al}, 2019). The position of the substrate in the clamp and near the tip of the THF has been probed with disulfide crosslinks experiments that map the path of the translocating substrate to these domains (Erlandson \textit{et al}, 2008a; Bauer & Rapoport, 2009).

In solution, SecA rests in an equilibrium between dimeric and monomeric forms (Akita \textit{et al}, 1991; Woodbury \textit{et al}, 2002). Several dimer forms have been visualized by X-ray crystallography, including two different anti-parallel dimers (Hunt \textit{et al}, 2002; Papanikolau \textit{et al}, 2007), and a dimer formed by intercalation of the clamps of the two monomers (Zimmer \textit{et al}, 2006). Interestingly, though the clamp is open in this structure, the dimer interface involves the formation of a β-sheet at the back of the clamp similar to the β-sheet formed between translocating
substrates and the closed conformation of the clamp. This would therefore block substrate binding into the clamp and could represent an auto-inhibited dimeric form of SecA. Regardless of the soluble oligomeric state, SecA can only bind SecY as a monomer (Or et al, 2005), as seen in all structures of the complex (Zimmer et al, 2008; Li et al, 2016; Ma et al, 2019). Therefore, only one copy of the ATPase is present during translocation.

1.5.2 ATP Hydrolysis Cycle of SecA

As SecA couples its hydrolysis cycle to its translocation activity, a detailed characterization of its hydrolysis cycle is necessary for understanding possible mechanisms of its function. SecA in solution hydrolyzes ATP at a low basal rate ($k_{\text{cat}} = 0.01 \text{s}^{-1}$ for E.coli SecA at 37°C). Binding to both SecY and anionic lipids increases this hydrolysis rate significantly ($k_{\text{cat}} = 0.27 \text{s}^{-1}$). Substrate further stimulates this rate to a maximal level ($k_{\text{cat}} = 7.6\text{s}^{-1}$) (Robson et al, 2009).

In solution, the cleavage of the γ-phosphate of ATP is comparatively fast ($k_{\text{cleave}} = 6.14\text{s}^{-1}$) when compared to the total hydrolysis rate, meaning ADP release is the rate limiting step in the ATPase cycle (Robson et al, 2009). ATP binding to the Apo enzyme is thought to be extremely fast. In the presence of anionic lipids, SecYEG, and substrate, both ADP release and ATP hydrolysis are accelerated ($k_{\text{cleave}} = 17.9\text{s}^{-1}$), however ADP release is still slower meaning that the protein spends as much as 2/3 of its hydrolysis cycle in the ADP-bound state.

Throughout the ATP hydrolysis cycle, the affinity of SecA for both SecY and substrate changes dramatically. In detergent, SecA can only form a stable complex with SecY in the presence of ATP analogs, such as ADP•BeF$_x$ and ATPγS ($K_d = 0.1 – 0.15\mu\text{M}$). In the ADP-bound and Apo states, SecA affinity for the detergent solubilized translocon complex was unmeasurable (Zimmer et al, 2008). However, once reconstituted into liposomes containing anionic lipids, SecY forms a more stable complex with SecA, even in the presence of ADP, likely because of its additional interactions with the head groups of anionic lipids surrounding SecY (Bauer et al,
The effect of substrate on the stability of the ternary SecA/SecY/substrate complex has been studied using translocation intermediates. These rely on substrates with folded domains which are sterically too large to fit into the SecY channel and thus stall translocation with the N terminal regions of the substrate having passed through the SecY channel, but the C terminal regions remaining on the cytoplasmic side (Schiebel et al., 1991; Erlandson et al., 2008a, 2008b; Bauer et al., 2014). These intermediates persist in the presence of ATP or non-hydrolyzable nucleotide analogs, however, in the presence of ADP, the substrate quickly slides back out of the channel (Erlandson et al., 2008b; Bauer et al., 2014). Therefore, in the presence of ADP, SecA makes weak or no contacts with the substrate, allowing its free diffusion in the SecY channel. Chase experiments measuring the dissociation of labeled SecA from translocation complexes in the presence of these stalled polypeptide substrates show SecA’s affinity for the complex to be increased in all nucleotide states (Bauer et al., 2014). And, while the affinity is still weakest in the presence of ADP, it is unlikely that SecA dissociates upon every hydrolysis cycle, as its dissociation rate while hydrolyzing ATP ($k_{\text{off}} = 0.020 \text{s}^{-1}$) is slower than the hydrolysis rate ($k_{\text{cat}} = 7.6 \text{s}^{-1}$) (Bauer et al., 2014). In fact, a single SecA molecule bound to an active translocation complex likely undergoes hundreds of hydrolysis cycles before dissociating.

1.5.3 Substrate Sequence Specificity of SecA

As much as 10% of the *E. coli* proteome is translocated by SecA. Across this diverse substrate pool there is no obvious sequence similarity that would indicate preferred interaction motifs. SecA is known to contact the substrate in part through its THF (Erlandson et al., 2008a). Several conserved residues at the tip of this finger, including a Tyrosine and two conserved basic residues have been shown to be critical for SecA activity (Erlandson et al., 2008a; Bauer et al., 2014). The exact nature of this contact is not clear as this region of the complex has not yet been well resolved in any structures. Other AAA ATPases that work on polypeptide chains use similar-
ly positioned aromatic residues, in loops at the tips of small finger motifs, to contact their substrates’ backbone (Martin et al., 2008; Rodriguez-Aliaga et al., 2016).

The substrate sequence diversity and comparison to other ATPases led to the hypothesis that SecA might interact with its substrates’ backbone. However, several lines of evidence show that, unlike related ATPases, SecA interacts with the side chains of its substrates through its conserved THF loop residues. The translocation efficiency of engineered substrates with poly-Glycine stretches is much lower than wild-type proteins (Bauer et al., 2014). In fact, if the finger encounters a poly-G stretch it makes hardly any contact at all, allowing the substrate to slide freely in the pore, even in the ATP-bound state (Bauer et al., 2014). Several other amino acids seem to interact weakly with SecA. If A,V,I,L,S,T,N, and Q are all deleted from a model substrate, its translocation becomes less efficient, suggesting that the remaining amino acids interact weakly with the THF (Bauer et al., 2014). Surprisingly, this includes charged residues, which one might expect to interact with the conserved basic amino acids at the tip of the THF, but not necessarily aromatic residues, which were not contained at all in the studied sequence. In fact, aromatic residues likely make strong contacts with SecA, as crosslinking a sequence of aromatic residues near, or even to, the THF finger accelerates the release of ADP from SecA (Allen et al., 2016).

### 1.5.4 Models for SecA-Mediated Translocation

Despite the plethora of data, a consensus model of SecA’s mechanism is lacking. This is in part because each of the structures of the SecA/SecY translocation complex have been acquired in the presence of the same nucleotide, and therefore look quite similar, despite the presence or absence of a substrate. It is unclear therefore, whether or not SecA moves significantly during translocation. The differences in affinity of SecA for the rest of the complex at different states of its hydrolysis cycle suggests that some conformational changes must occur to disrupt the interfaces between SecA and SecY or the substrate. However, as alternate conformations have not yet been observed directly, the nature and magnitude of these movements is unclear.
Two contrasting models, termed here the “Push and Slide” and “Brownian Ratchet” models, have been proposed to explain how SecA uses the energy from ATP hydrolysis to produce the mechanical work necessary to move polypeptides. The models explain how translocation may occur by invoking either a conformationally dynamic or a more static version of the ATPase. Much of this study has been designed to differentiate between and refine them.

1.5.4.1 The Push and Slide Model

In the ‘Push and Slide’ model, SecA moves the substrate through power strokes of its two helix finger (Zimmer et al., 2008; Erlandson et al., 2008a; Bauer et al., 2014) (Fig 1.6). Upon ATP binding, small conformational changes between the two NBDs are transferred and amplified through the long linker helix of the HSD to the THF (Hunt et al., 2002). The THF then contacts the substrate polypeptide chain and inserts into the SecY channel, pushing the substrate with it. This inserted state is likely close to the state captured in each structure to date, as all the imaged complexes are formed in the presence of ADP•BeF₆ (Zimmer et al., 2008; Li et al., 2016; Ma et al., 2019). Following ATP hydrolysis, the THF disengages from the polypeptide and retracts from SecY. The efficiency of this power stroke is dependent on the sequence of amino acids near the tip of the THF when ATP binds (Bauer et al., 2014). During the ensuing ADP-bound phase the polypeptide is free to slide within the SecY channel (Erlandson et al., 2008b; Bauer et al., 2014). If a non-interacting amino acid happened to sit near the tip of the THF during the ATP bound phase, it may diffuse, either forwards or backwards, away from this position after ATP hydrolysis. This would give a stronger interacting amino acid a chance to occupy the region near the THF, preventing translocation from completely stalling at stretches of non-interacting residues.

This model successfully explains how SecA can process a diverse array of substrates and is consistent with the observation of polypeptide sliding during the ADP-bound state. However, it is inconsistent with the experiments showing that immobilizing the THF through disulfide crosslinks does not impair translocation (Whitehouse et al., 2012). The extent to which these
crosslinks impair THF movements, though, is unclear. Crosslinks between the SecA THF and a clearly structured and inflexible region of SecY (residue 183) abolish all translocation activity (Whitehouse et al, 2012). SecA activity is only preserved when the THF is crosslinked to an unstructured region of SecY in the long flexible loop between TMs 6 and 7 (residue 268) (Whitehouse et al, 2012). This crosslink would likely not completely impair THF mobility, and the loss of activity upon crosslinking to more structured regions of the channel indicates that some THF movement is required for translocation.

Figure 1.6. The Push and Slide Model of Translocation. (A) A schematized version of the SecA/SecY complex. The component proteins are colored and labeled, as are the two helix finger (THF) and clamp of SecA. The area inside the dashed box is enlarged in (B), as well as in Fig. 1.7. (B) In the ADP-bound state (1-2), the polypeptide substrate (green circles), including any amino acids that interact with SecA’s THF (yellow circles), is free to slide forwards and backwards through the SecY channel. Upon exchange of ADP for ATP (3-4), the THF inserts into the SecY channel. (3) If it interacts weakly with the substrate amino acids in its vicinity, the polypeptide chain is not pushed forwards. (4) However, if the THF interacts with the amino acids near its tip, it pushes the polypeptide further into the SecY channel.
The model also does not explain how the progress of translocation achieved by the power stroke is maintained as the THF retracts. One possibility is that the clamp tightens to hold the substrate in place during ATP hydrolysis. Fixing the clamp in an open conformation impairs SecA activity (Gold et al, 2013), but, prior to the present study, there was little evidence for motion away from the closed state during translocation.

1.5.4.2 The Brownian Ratchet Model

The “Brownian ratchet” model addresses how translocation could occur if the THF were static throughout the ATPase cycle, as the disulfide immobilization experiments suggest (Allen et al, 2016; Corey et al, 2019) (Fig 1.7). Here, SecA contacts SecY through the THF and modulates the channel opening and closing. When in its ATP bound state SecA holds the channel open. Upon ATP hydrolysis the channel narrows until the resulting ADP is released. Such expansion and contraction of the SecY channel has been observed through single molecule FRET experiments as well as Hydrogen Deuterium Exchange (HDX) Mass Spectrometry and could allow or restrict the sliding of the polypeptide chain (Allen et al, 2016; Corey et al, 2019; Ahdash et al, 2019). If the conversion from a closed to an open state was coordinated with the steric size of amino acids on the cytosolic side of the channel, it would permit their diffusion across the membrane, only to trap them on the periplasmic side by closing after their passage. Indeed, as mentioned earlier, the presence of aromatic residues in the cytosolic vestibule stimulated the release of ADP from SecA, which would result in SecY channel opening. As long as the SecY channel only widens when it senses a steric blockage in its cytoplasmic vestibule, and not in the periplasmic cavity, the resulting ratcheting mechanism would be sufficient to provide directed translocation.

This model is supported by steered molecular dynamics (MD) experiments, which use the structure of the SecA-SecY complex undergoing translocation of a model substrate as an initiation condition and observe the dynamics of the complex in varying ATP states for as long as
The SecY channel width does correlate with the SecA ATP state as predicted, though 2μs is much slower than the ATP hydrolysis rate ($k_{cat} = 7.6s^{-1}$) so conversions between states were rarely seen. However, as the initial structure used in the simulations does not contain a full length substrate, but rather a substrate analog fused to the THF, the path of the full substrate was modeled *in silico* for the MD experiments. The resulting modeled substrate does not engage SecA in an identical manner as that seen in the structure of the A/Y complex with a real substrate polypeptide (Ma *et al*, 2019). Notably, the β-sheet formed between

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**Figure 1.7. The Brownian Ratchet Model of Translocation.** Panels are enlargements of the area defined in Fig 1.6A (1) In SecA's ADP-bound state, the SecY channel is constricted, allowing the polypeptide chain only limited freedom to slide backwards and forwards. Bulky substrate side chains (yellow circles) or short α-helices are sterically too large to pass through the constriction. (2) If a bulky side chain passes near the tip of the THF and encounters the SecY pore constriction, it induces SecA to exchange its bound ADP for ATP. (3) ATP binding to SecA causes the SecY channel to open. (4) The open channel can accommodate bulky side chains and α-helices, which slide backwards and forwards through SecY until SecA hydrolyzes ATP and the pore constricts.
the substrate and the SecA clamp in its closed state is missing. This allows the substrate to slide much more permissively than it would in the actual closed state.

1.5.5 Other Energetic Contributions to Prokaryotic Translocation

Translocation activity can be reconstituted \textit{in vitro} with only SecA, SecY, and a polypeptide substrate indicating that this is the minimal necessary complex. \textit{In vivo}, though, other factors contribute to the activity of this core complex to increase the efficiency of translocation.

1.5.5.1 Passive Ratcheting Mechanisms

Ratcheting mechanisms, like the one above, occur whenever the forward progress of translocation is due to Brownian diffusion of the polypeptide chain. Generally, such diffusion would be bidirectional and would produce no net movement. However, energetic contributions to the system can produce directional bias, allowing the resulting Brownian ratchet to support translocation activity. Whether or not the SecA/SecY system functions via a ratcheting model is addressed in the present study. However, other factors likely contribute to translocation through different ratcheting mechanisms \textit{in vivo} (Liebermeister \textit{et al}, 2001). While these hypothetical ratcheting mechanisms are labeled here as “passive ratchets,” that is just to clarify that the source of energy is not the SecA ATPase. Energy input is obligatory to produce directional transport, and if it is not provided by SecA, it would have to be sourced elsewhere.

Direct observations of these effects are lacking in bacteria, but, their likely existence can be surmised based on observations in eukaryotes. For instance, the Hsp70 chaperone BiP functions by a coordinated ATP-dependent Brownian ratchet mechanism to promote translocation into the eukaryotic ER (Matlack \textit{et al}, 1999). Binding of the chaperone creates a steric block on the polypeptide substrate that prevents it from backsliding through the channel. Prokaryotes lack chaperone ATPases in the periplasm, so an identical mechanism does not exist in bacteria. How-
ever, there are a number of energy independent periplasmic chaperones that could bind emerging translocation substrates and bias their diffusion away from backsliding towards the cytoplasm (Schäfer et al, 1999; Antonoaea et al, 2008). The energy in this system would come from the previous translocation of the chaperones themselves. Energy expended in the translocation of periplasmic chaperones would then be recouped when they bind and bias the diffusion of future substrates through the SecY channel.

Furthermore, even the folding of the polypeptide chain as it exits from SecY could provide a ratcheting mechanism. Folded domains are too large to pass through SecY. On the cytoplasmic side of the channel, post-translational translocation substrates are often maintained in an unfolded state by the chaperone SecB. However, once across the membrane, they are free to fold into their native domains, which would prevent backsliding. The Ca\(^{2+}\) - dependent folding of *bordetella* leukotoxin RTX domains drives their transport through the Type 1 Secretion System (T1SS), proving that protein folding alone can provide the necessary energy for translocation (Bumba et al, 2016). Co-translocational folding could be driven in part by the oxidizing environment of the periplasm. The presence of the thiol oxidoreductases DsbA/B in the periplasm allows disulfide bond formation specifically in this compartment. Loops formed by intramolecular disulfide bonds are too large to fit through SecY and would create another ratcheting opportunity for translocating polypeptides. Co-translocational folding could even be induced by the environment within the periplasmic vestibule of SecY. Molecular dynamics studies suggest that this cavity may rearrange upon ATP-binding to SecA in order to drive the folding of elongated polypeptide stretches into more compact secondary structure elements (Corey et al, 2019). The energy required to bias translocation via co-translocational folding could be provided by the previous translocation of the Dsb proteins, in a manner similar to the chaperones discussed above. Energy is also expended maintaining translocation substrates in an unfolded state in the cytoplasm. This energy is released once the polypeptides are allowed to spontaneously fold in the periplasm.

A recent study proposes a third ratcheting mechanism for post-translational translocation
substrates dependent on the electric potential across the plasma membrane (Allen et al, 2019). The bacterial membrane is polarized, with a lower charge inside the cell than outside, resulting in a potential of roughly -150mV across the membrane. This potential produces a proton motive force (PMF), which would support the transport of negative residues from the cytosol to the periplasm. Positively charged amino acids would be more difficult to translocate against PMF. The model contends, however, that local pH environments within the SecA clamp or cytosolic vestibule of SecA promote the deprotonation of Lysine residues rendering their charge neutral. These can then more easily pass through the membrane and are re-protonated on the periplasmic side. As the channel is thought to present a larger energetic barrier to charged than uncharged residues (Dalal & Duong, 2009; Nouwen et al, 2009), this re-protonation would prevent backsliding, even though the PMF would favor the return of these Lysines to the cytosol.

Mutation of Arginine residues in model substrates to Lysine, which has a lower pK$_a$ than Arginine and would be more easily deprotonated, increases translocation efficiency in a manner dependent on buffer pH and PMF. This observation would explain why secreted bacterial proteins have a higher usage rate of Lysine versus Arginine when compared to the rest of the proteome (Allen et al, 2019).

### 1.5.5.2 The Membrane Potential-Dependent SecDF Translocation Complex

This “proton ratchet” remains speculative and mostly untested. Whether or not the SecY/SecA environment truly promotes the deprotonation of Lysines is unclear. However, the PMF has long been known to play a role in translocation through the protein complex SecDF (Pogliano & Beckwith, 1994). While SecDF cannot translocate substrates in vitro by itself, it can complete translocation that has been initiated by SecA without further ATP hydrolysis using only the membrane potential as an energetic source (Schiebel et al, 1991).

SecDF has been crystallized in several conformations (Tsukazaki et al, 2011; Furukawa
et al., 2017, 2018). The overall complex consists of two 6 TM proteins that come together to form a proton conducting tunnel at their interface. Several conserved Arginine residues within this tunnel provide a putative path for ions to flow across the membrane. Three large periplasmic “P” domains have been observed in two conformations: “I” and “F” forms, in which the most distal P1 “head” pivots on a flexible linker to either move close to the membrane or move away from it, respectively.

Both the I and F forms were crystallized with a PEG molecule bound in a cavity in the P1-head domain (Tsukazaki et al., 2011; Furukawa et al., 2017), possibly indicating the substrate binding site in vivo. Interconversion between these two forms would likely produce a pulling force on the bound substrate (Tsukazaki, 2018). But how the proton flux is coupled to this movement is still unclear. It seems unlikely that the flow of a single ion would be sufficient to cause the conformational change observed between the I and F forms. Perhaps the movement is instead coupled to a local membrane depolarization that accumulates upon the opening of the conducting tunnel, but this model has yet to be tested.

1.6 About this Work

It is clear that translocation in vivo employs a wide range of mechanisms to move a polypeptide from inside the cell to the outside. Ribosomes, ATP hydrolysis, PMF, and passive Brownian ratchets could all collaborate to make this process maximally efficient. But even the simplest of these mechanisms: the ATP-dependent translocation of a substrate through SecA through the SecY channel, is incompletely understood. Despite sophisticated biochemical reconstitutions and detailed structural characterizations of the complex, the mechanism by which SecA produces mechanical work from its ATP hydrolysis cycle remains unclear.

One difficulty has been the inability to determine the structure of the translocation complex in the presence of nucleotides other than ADP•BeF₆⁻. As such, each of the published struc-
tures to date look remarkably similar, leading to the conclusion that SecA may remain static throughout its hydrolysis cycle. In this work, we use single molecule techniques (Chapter 2) to demonstrate that SecA is remarkably dynamic during translocation and ensemble biochemical methods (Chapter 3) to show that these dynamics directly lead to the translocation of model substrates.

In Chapter 2 we use smFRET experiments on reconstituted translocation complexes to show that SecA’s THF makes large movements in and out of the translocon, consistent with the pushing power-stroke of the previously proposed “Push and Slide” model. Furthermore, we show that the clamp of SecA opens and closes around the translocating substrate independently of the THF. By aligning each of these two movements with stages of the ATPase cycle we construct a conformational model explaining how SecA couples its ATP hydrolysis to polypeptide translocation.

In Chapter 3 we use ensemble biochemical techniques to monitor the dynamics of the polypeptide chain immediately after ATP binding to SecA. The incubation of stalled translocation complexes with non-hydrolyzable ATP analogs results in a large movement of the substrate into the channel. This is dependent on the conserved residues at the tip of the THF and is likely the result of the THF power-stroke demonstrated in Chapter 2. These data confirm that the conformational changes of SecA are directly coupled to movements of the translocating polypeptide.
Chapter 2: Protein translocation by the SecA ATPase occurs by a power-stroke mechanism

2.1 Foreword

The following chapter was published in (Catipovic et al, 2019) and has been reproduced, with minor changes, for inclusion in this thesis. The project was conceived by Marco Catipovic, Benedikt Bauer, Joseph Loparo, and Tom Rapoport. All experiments were performed by Marco Catipovic. The manuscript was written by Marco Catipovic and Tom Rapoport.

2.2 Introduction

Many processes in the cell involve AAA family ATPases that perform mechanical work to remodel or relocate proteins. Examples include hexameric ATPases, such as the p97 ATPase (Cdc48 in yeast), which extracts proteins from membranes or tight complexes, the Clp’s and the ATPases of the 26S proteasome, which push polypeptides into a proteolytic chamber, and the NSF protein, which disassembles SNARE complexes involved in membrane fusion (for review, see Bodnar & Rapoport, 2017; Ye et al, 2017; Yedidi et al, 2017; Zhao et al, 2007). Another important member of this ATPase family is SecA, which translocates polypeptides through the plasma membrane in bacteria (for review, see Corey et al, 2016; Cranford-Smith & Huber, 2018; Rapoport et al, 2017). SecA acts a monomer (Or et al, 2005) and uses the energy of ATP hydrolysis to move its substrates through the protein-conducting SecY channel (Economou & Wickner, 1994). How any of these ATPases perform mechanical work is poorly understood.

SecA is a multi-domain protein (Fig 1.5) with two nucleotide-binding, RecA-like domains (NBD1 and NBD2), which bind the nucleotide at their interface and move relative to one another during the ATP hydrolysis cycle (Hunt et al, 2002). A two-helix finger, consisting of two helices connected by a loop, inserts into the cytoplasmic opening of the SecY channel (Fig 1.5) (Li et al, 2016; Zimmer et al, 2008). A conserved Tyr residue within the loop contacts the trans-
locating polypeptide chain (Bauer et al., 2014; Erlandson et al., 2008a), which is positioned above the SecY channel by a clamp formed by rotation of the polypeptide-crosslinking domain (PPXD) towards NBD2 (Fig 1.5).

The SecY channel is formed from three polypeptide chains (SecY, SecE, and SecG). The large SecY subunit consists of N- and C-terminal halves and forms an hourglass-shaped pore. The cytoplasmic cavity is empty, while the extra-cellular cavity is filled with a plug domain. At the constriction in the middle of the membrane is a pore ring of amino acids. During translocation, the plug is displaced (Li et al., 2016; Fessl et al., 2018), and the polypeptide chain moves through the pore ring across the membrane.

Several models have been proposed to explain SecA function. In a ratcheting model (Allen et al., 2016; Corey et al., 2019), the finger serves as a sensor for bulky amino acid residues of the substrate. When such a residue is encountered, SecA converts from the ADP-bound to the ATP-bound state and the SecY channel opens, allowing the residue to diffuse through the pore. Following ATP hydrolysis, the channel closes, trapping the bulky residue on the other side of the membrane. In this model, the finger does not move relative to the channel. By contrast, in a power-stroke model (Bauer et al., 2014), ATP binding at the NBDs would cause the two-helix finger to interact with the polypeptide chain and push it into the channel; following ATP hydrolysis, the finger would disengage and allow free diffusion of the chain in either direction. Here, the finger would undergo large movements towards and away from the channel. In one extreme version of this model, the “plunging model”, large domains of SecA would reach entirely through membrane to deliver the substrate to the other side (Banerjee et al., 2017; Economou & Wickner, 1994). As proposed, however, the power-stroke models fail to explain how a SecA domain would return to its starting position without erasing the work done during its power stroke. One possibility is that the clamp holds the polypeptide chain when the two-helix finger resets (Zimmer et al., 2008), but this model seems to be in contradiction with the observation that the polypeptide chain can slide back and forth through the SecA-SecY complex (Bauer et al., 2014; Erlandson et
Recent single-molecule experiments support the idea that the clamp of SecA undergoes nucleotide-dependent conformational changes (Ernst et al., 2018; Vandenberk et al., 2018; Chada et al., 2018), but it remains unclear whether they occur during translocation, as the studies were performed in the absence of SecY and translocation substrate.

Here, we use single-molecule FRET experiments to follow conformational changes of SecA during protein translocation. Single-molecule experiments are required because the ATP hydrolysis cycles of all SecA molecules cannot be synchronized in traditional biochemical assays. Our results show that, upon ATP-binding to SecA, the two-helix finger undergoes a large conformational change that pushes the polypeptide into the SecY channel. When the finger resets, the clamp tightens around the polypeptide, thus preserving the progress of translocation. Passive sliding of the polypeptide chain occurs after ATP hydrolysis, when the clamp opens. Our results lead to a comprehensive model for SecA function, which may also be applicable to hexameric ATPases.

2.3 Results

2.3.1 Experimental Design

We used single-molecule Förster resonance energy transfer (FRET) in combination with a reconstituted translocation system (Fig 2.1A). A translocation intermediate was generated, using purified SecA, SecYEG, and substrate. SecA and SecY were labeled with different fluorophores and the translocation complex was immobilized on a glass surface via the substrate. This strategy ensured that all components were present in each observed complex. In contrast, if two different dyes are placed into the same protein (Allen et al., 2016; Fessl et al., 2018; Ernst et al., 2018; Vandenberk et al., 2018), one cannot exclude that the unlabeled components are missing and that FRET changes are caused by the dissociation or association of the complex, rather than
Figure 2.1. SecA’s two-helix finger makes large movements during the ATP hydrolysis cycle. (A) Experimental setup to measure single-molecule FRET in translocation complexes immobilized on a surface. (B) Cy5 and Cy3 fluorophores were introduced into the two-helix finger of SecA (PDB 3DIN; red space filling model; helices highlighted) at position 809 and into SecY (blue) at position 394, respectively. (C) Representative traces obtained with ADP•BeFx. The upper FRET trace was calculated from the middle traces obtained by exciting the donor fluorophore and measuring both donor (green) and acceptor (red) fluorescence. The lowest trace was obtained by exciting the acceptor fluorophore directly. The arrow indicates a bleaching event. (D) Distribution of FRET values determined from 97 traces as in (C) fit with a Gaussian model.
by conformational changes within the complex. It should be noted that attaching complexes to a glass surface via the SecY channel or lipids yielded very few FRET traces, likely because substrate was absent from many complexes. Also, complexes assembled in vivo required ADP•BeFx during purification, which could not be substituted with other nucleotides in the FRET experiments.

In our specific experimental setup, we introduced single cysteines at different positions into cysteine-lacking E. coli SecA and labeled them with the acceptor fluorophore Cyanine 5 (Cy5). The donor fluorophore (Cy3) was attached to a single cysteine introduced at different positions into cysteine-free E. coli SecY. All SecA and SecY mutants retained translocation and ATPase activity after labeling (Fig S2.1, Fig S2.2). Proteoliposomes were then reconstituted with labeled SecYEG complex and mixed with labeled SecA, ATP, and substrate. The substrate consisted of a fusion of the first 175 amino acids of proOmpA, including the N-terminal signal sequence (SS), a dihydrofolate reductase (DHFR) domain, and a biotinylation tag. The proteoliposomes were then attached to a coverslip by neutravidin, which interacted with both the biotinylated C-terminus of the substrate and biotinylated polyethylene glycol (PEG) molecules at the surface.

In the presence of methotrexate, the DHFR domain of the substrate is tightly folded and too large to move through the SecA-SecY complex, therefore preventing complete translocation of the fusion protein (Bauer & Rapoport, 2009). Essentially all channels were occupied with translocation intermediate (Fig S2.3). In the presence of ATP, the substrate is constantly sliding out of the proteoliposomes and is then pushed back into the SecY channel (Bauer et al, 2014). Thus, despite the fact that, on average, the DHFR domain is abutting the channel, the polypeptide chain is undergoing continuous translocation.

FRET was monitored in a flow chamber with wide field total internal reflection fluores-
cence (TIRF) microscopy. As expected from our setup, fluorescent spots were only detected on the surface in the presence of all components (Fig S2.4). Alternating excitation of Cy3 and Cy5 allowed for measurement of both FRET between SecY and SecA, as well as direct detection of SecA. Our experimental design ensured that both partners are present and allowed single SecA molecules to be monitored through many hydrolysis cycles over a period as long as 30 s, i.e. observation times far longer than those allowed by solution FRET experiments. Although the time resolution was limited to 33 ms per frame, this is about 20 times faster than the duration of an ATP hydrolysis cycle measured in bulk (Fig S2.2).

FRET traces were either obtained in the presence of ATP or the nucleotide was exchanged in the flow chamber to either ADP·BeF₃, which mimicks the transition state of ATP hydrolysis, or ATPγS, a slowly hydrolyzing ATP analog. Complexes could not be imaged in the presence of ADP alone, as SecA binds only weakly to SecY in the presence of this nucleotide (Bauer et al, 2014). While the fluorescent spots were stable with nucleotide analogs, they rapidly disappeared in the presence of ATP, likely because SecA dissociates in its ADP-bound state, allowing the substrate to slide backwards in the SecY pore until the entire proteoliposome is released from the glass surface. We therefore added unlabeled SecA when imaging with ATP, keeping the total concentration below the Kₐ of SecA dimerization (Woodbury et al, 2002) to ensure that only active SecA monomers are observed. The increased concentration allowed SecA to rebind abandoned complexes before they dissociated. Although most rebinding SecA molecules were unlabeled, some were labeled and allowed the observation of FRET over extended time periods.

2.3.2 Movement of the Two-helix Finger of SecA During Protein Translocation

We first analyzed movements of the two-helix finger of SecA. To this end, the donor fluorophore was placed into a periplasmic loop of E. coli SecY (position 394) and the acceptor fluorophore into position 809 of the two-helix finger (Fig 2.1B). The probes are predicted to be about 50 Å apart according to crystal structures obtained in the presence of the transition state analog.
Consistent with the observation that ADP•BeFx allows stable binding of SecA to the SecY channel, a static FRET signal between SecA and SecY was observed in all traces (Fig 2.1C; top trace). Direct excitation of the SecA-bound fluorophore...
showed that SecA remained bound to the channel (bottom trace). At the end of a trace, the acceptor fluorescence bleached in one step, and the donor fluorescence was de-quenched (see arrow), as expected for a FRET signal. In no case did acceptor fluorescence return, confirming that, in the presence of ADP•BeF₆, SecY-bound SecA is not exchanged with SecA in bulk solution. The analysis of many traces showed that the FRET ratios had a Gaussian distribution with a mean value of 0.60 +/- 0.12 (Fig 2.1D). While distance estimates based on FRET probes in a proteinaceous environment are unreliable due to orientation restrictions of the fluorophores, a naïve estimate using the standard FRET equation gives a distance of 51 Å, in close agreement with the structural prediction.

In the presence of ATP, SecA repeatedly bound and dissociated from the SecY channel, as demonstrated by direct excitation of the acceptor fluorophore (Fig 2.1E; bottom trace). While bleaching and dissociation cannot be distinguished a priori in individual traces, the imaging lifetime of individual SecA molecules in the presence of ATP was generally shorter than in the presence of ADP•BeF₆ (Fig S2.5), suggesting that SecA does indeed dissociate in these traces. The FRET signal was highly dynamic when SecA was bound (top trace), alternating between high and low states with mean FRET ratios of 0.90 +/- 0.09 and 0.11 +/-0.08 (Fig 2.1F; additional examples of traces are shown in Fig S2.6). The high and low FRET states likely correspond to states in which the two-helix finger is either inserted into or withdrawn from the SecY channel. The large FRET difference indicates that the finger undergoes a substantial conformational change, although its precise movement cannot be deduced from the FRET values. The low FRET state shows a significantly higher occupancy than the high FRET state (Fig 2.1F). When the donor fluorophore was placed at a different position in SecY (position 103), a markedly similar behavior was observed (Fig 2.2A-C). Again, a constant FRET level was observed in the presence of ADP•BeF₆, which matched well the estimated inter-fluorophore distance derived from the crystal structures. As before, in the presence of ATP, the FRET traces were dynamic during SecA-bound periods (Fig 2.2D). Histograms derived from these traces also showed two populations at low and high FRET levels, with a higher occupancy in the low FRET state (Fig 2.2E).
Figure 2.3. The two-helix finger switches between two states. (A) A representative FRET trace (blue line) was obtained as in Fig 1, and fit with a hidden Markov model (black dashed line). (B) Traces as in (A) obtained in the presence of different nucleotides, were used to determine the number of states best fit by the Markov model. (C) Transition density plot of idealized ATP FRET states obtained in (B). (D) The distributions of dwell times of the low FRET states observed in ATP were fit with a single exponential (1500 low FRET states). The inset shows average dwell time and error, defined as the standard error based on the number of traces. (E) As in (D), but with high FRET (1656 high FRET states). (F) Representative traces obtained with ATPγS by exciting either the donor (middle trace) or acceptor (bottom trace) fluorophore and calculating FRET (upper trace). The arrow indicates a bleaching event. (G) Distribution of FRET values determined from 168 traces as in (D) fit with a Gaussian model (black curve).
Figure 2.4. Representative FRET data for SecA conformations before Pi release. (A) Representative traces for the two-helix finger (positions 809 in SecA and 394 in SecY) obtained with...
To connect these FRET changes to the ATP-hydrolysis cycle of SecA, we fit the FRET traces with a hidden Markov model (Bronson et al, 2009; McKinney et al, 2006; van de Meent et al, 2014) (Fig 2.3A). These models employ a maximum evidence approach to find the most likely number of structural conformations that underlie the observed data. Individual traces were fit with an increasing complexity of models, which were scored positively for the closeness of their fit to the data and negatively for the number of discrete FRET states included. In this way, the most parsimonious model was selected that reproduces the data without evoking extraneous conformations (Bronson et al, 2009). This analysis confirmed that in the presence of ADP•BeF\textsubscript{3} only one conformational state exists, while in the presence of ATP, and with both donor positions, the majority of traces showed two states (Fig 2.3B, Fig 2.2F). Transitions between these idealized FRET states can also be plotted as transition density plots (TDPs) to show how these FRET states connect to each other (McKinney et al, 2006). TDPs of idealized FRET states obtained in the presence of ATP showed symmetry across the principle diagonal, indicating cycling between only two FRET states (Fig 2.3C, Fig 2.2G). Thus, the high and low FRET states simply interchange with each other. The distribution of dwell times for the two FRET states observed with ATP could each be fit with a single exponential and demonstrated that the mean life time for the low FRET state is about twice as long as that of the high FRET state (Fig 2.3D,E, Fig 2.2H,I). The low and high FRET states likely correspond to ADP- and ATP- bound states, respectively, as previous experiments showed that SecA spends most of its time during the ATP hydrolysis cycle in the ADP-bound state (Robson et al, 2009). This assumption is consistent with the relatively high intermediate FRET signal observed with the transition state mimic ADP•BeF\textsubscript{3} (Fig 2.1C,D). Furthermore, the sum of the high and low FRET life-times gives an estimate of the overall ATP
hydrolysis rate that agrees with bulk measurements performed at the same temperature (Fig S2.2). Finally, FRET experiments with ATPγS, a slowly hydrolyzing analog, also showed two conformational states (Fig 2.3B), but the high FRET state now lasted as long as the low FRET state (Fig 2.3F,G). Interestingly, the high FRET value was close to that measured in the presence of ADP•BeF₆ (0.67 +/- 0.11 versus 0.6 +/- 0.11), suggesting that ATPγS extends the duration of the transition state of ATP hydrolysis.

Given that the average FRET efficiency observed for the two-helix finger is different in the transition state of ATP hydrolysis (ADP•BeF₆) and the ADP-bound state (0.6 versus 0.1), we asked if the transition between them happens before or after Pᵢ-release. We therefore measured FRET in the presence of ADP and Pᵢ, as well as ADP and vanadate (Vᵢ), a phosphate analog that binds more stably. In both conditions, the two-helix finger was primarily in the low-FRET state (Fig 2.4A-D), indicating that it withdraws before Pᵢ release. The two-helix finger was more dynamic in the presence of ADP•Pᵢ and ADP•Vᵢ than with ADP•BeF₆, either because of increased conformational flexibility or frequent dissociation of Pᵢ/Vᵢ.

Taken together, these results show that, during protein translocation, the two-helix finger of SecA undergoes a large conformational change. It alternates between two conformations during ATP hydrolysis: in the short-lived ATP-bound state, the finger inserts deeply into the SecY channel and gives a high FRET signal, and in the longer ADP-bound state, it withdraws from the pore and produces low FRET. In the transition state, mimicked by ADP•BeF₆, the finger is in an intermediate position, but it retracts completely following completion of ATP hydrolysis, before Pᵢ release. Movement of the two-helix finger into the channel would push the polypeptide forward, and movement away would reset the finger for the next cycle.
2.3.3 Movement of the Clamp of SecA

The observation of only two states of the two-helix finger during ATP hydrolysis requires a mechanism that prevents the finger from dragging the polypeptide backwards when the finger moves away from the channel. A likely candidate for holding the polypeptide during finger resetting is the clamp, a groove formed by the rotation of the PPXD towards NBD2 (Zimmer et al, 2008) (Fig 1.5). Rotation of the PPXD can be inferred from crystal structures of soluble SecA that show this domain at different distances from NBD2 (Hunt et al, 2002; Osborne et al, 2004; Chen et al, 2015), and movement of the translocating polypeptide chain through the clamp is indicated by crosslinking experiments (Bauer & Rapoport, 2009). However, it remained unclear whether the clamp simply forms a conduit for the translocating polypeptide chain or cyclically binds and releases it during ATP hydrolysis. To test whether the clamp undergoes nucleotide-dependent movements, we placed the acceptor fluorophore into the PPXD (position 233) and the donor fluorophore at position 103 in the N-terminal half of SecY (Fig 2.5A,B). In the presence of ADP•BeF₃, a static FRET signal of 0.60 +/- 0.08 was observed (Fig 2.5C,D, Fig 2.6A). Again, the distance estimated with the standard FRET equation agreed well with those measured in crystal structures (Li et al, 2016; Zimmer et al, 2008). In the presence of ATP, we once again observed exchange of SecA molecules on the SecY channel, and changes between two conformations when SecA was bound to the channel (Fig 2.5E,F, Fig 2.6A,B; additional examples of traces are shown in Fig S2.7). Similar results were obtained when the donor fluorophore was moved to position 336 in the C-terminal half of SecY (Fig 2.7A-G), demonstrating that FRET changes are due to conformational changes of the SecA clamp, rather than the channel. Experiments with the slowly hydrolyzing ATP analog ATPγS showed an increase in the occupancy of the high FRET state (Fig 2.6C,D), consistent with the clamp movements being linked to ATP hydrolysis. The predominance of the high FRET state in ATPγS indicates that clamp is closed during ATP hydrolysis, though it is unclear whether the initial closure occurs either during ATP binding or hydrolysis.
Figure 2.5. SecA’s clamp opens and closes during the ATPase cycle. (A) Cy5 and Cy3 fluorophores were introduced into the clamp of SecA (PDB 3DIN; red space filling model) at position 233 and into the N-terminal half of SecY (blue) at position 103, respectively. The PPXD and NBD2 making up the clamp are shown as a violet and magenta ribbon models, respectively. (B) Rotated view of (A) with SecY masked except for the labeled residue 103. (C) Representative traces obtained with ADP•BeFx by exciting either the donor (middle trace) or acceptor (bottom trace) fluorophore and calculating FRET (upper trace). The arrow indicates a bleaching event. (D) Distribution of FRET values determined from 197 traces as in (B) fit with a Gaussian model (black curve). (E) As in (C), but with ATP. Periods in which a fluorescently labeled SecA molecule is bound are indicated by grey shading. (F) As in (D), but with ATP (228 traces).
Figure 2.6. Clamp dynamics and comparison with the two-helix finger. (A) FRET traces of clamp movements in the presence of different nucleotides were used to determine the number of states best fit by a Markov model. (B) Transition density plot of idealized ATP FRET states obtained in (A). (C) Representative traces obtained with ATPγS by exciting either the donor (middle trace) or acceptor (bottom trace) fluorophore and calculating FRET (upper trace). The arrow indicates a bleaching event. (D) Distribution of FRET values determined from 315 traces as in (C) fit with a Gaussian model (black curve). (E) Comparison of high and low FRET state occupancy in ADP·Pi and ADP·Vi for the clamp and THF. (F) The distributions of dwell times of the low FRET states observed in ATP were fit with a single exponential (1539 low FRET states). The inset shows average dwell time and error, defined as the standard error based on the number of traces. (G) As in (F), but with high FRET (1773 high FRET states). (H) Comparison of dwell times of the high FRET states for the two-helix finger (THF) and clamp for different fluorophore positions. Errors as in (G) with significance based on two sample Kolmogorov-Smirnov tests with a 1% threshold. n.s. P = 0.012 (left), P = 0.681 (right); * P < 1*10-35 (between each pair of THF/clamp mutants).
Interestingly, whereas the two-helix finger adopted different conformations in the ATP-bound state and in the transition state of ATP hydrolysis, the clamp did not change much, as the high FRET signal for the clamp was similar in ATP and ADP•BeF$_x$ (0.49 +/- 0.09 and 0.60 +/- 0.08). Data taken in the presence of ADP and either P$_i$ or V$_i$ show that the clamp remains closed even when ATP hydrolysis is completed, as there was a clear bias towards the higher FRET state in both conditions (Fig 2.4E-H). The clamp remains closed while the two-helix finger undergoes a transition to the low FRET state, as the ratio of low to high FRET occupancy was significantly higher for the finger than for the clamp, both with ADP•P$_i$ and ADP•V$_i$ (Fig. 2.6E). Thus, it seems that the two-helix finger starts moving away from the channel during ATP hydrolysis, while the clamp remains closed and only opens after P$_i$ is released.

Support for this model comes from comparing the kinetics of the conformational changes of the clamp and two-helix finger. The sums of the lifetimes of the high and low FRET states observed for each domain were about the same (~500 ms), suggesting that they are measurements of the same hydrolysis cycle. However, the division of this cycle between the high and low FRET states was different. The high FRET state of the clamp lasted about one fifth as long as the low FRET state (Fig 2.6F,G, Fig 2.7I,J) and only half as long as the high FRET state of the two-helix finger (Fig 2.6H), supporting the idea that the two-helix finger and clamp move during different stages of the ATP hydrolysis cycle (Fig 2.8A). Our data not only suggest that the clamp remains closed when the finger starts moving away from the channel, but also that the clamp does not close until the finger has moved all the way into the channel. The latter conclusion is based on the fact that the clamp has a shorter high FRET lifetime than the two-helix finger and that it transitions to the low FRET state later, so that the period the clamp spends in the high FRET state is shifted relative to that of the two-helix finger (Fig 2.8A). Taken together, these data lead to a model in which the clamp is open during the power-stroke of the two-helix finger, but then closes to hold the polypeptide chain while the finger resets. However, the exact point of clamp closure remains uncertain; the data cannot discern between closure during ATP binding or hydrolysis.
Figure 2.7. Clamp movements observed by alternative fluorophore positions. (A) Cy5 and Cy3 fluorophores were introduced into the clamp of SecA (PDB 3DIN; red space filling model) at position 233 and into the C-terminal half of SecY (blue) at position 336, respectively. The PPXD and NBD2 making up the clamp are shown as a violet and magenta ribbon models, respectively. (B) Rotated view of (A) with SecY masked except for the labeled residue 336. (C) Representative traces obtained with ADP•BeFx. The upper FRET trace was calculated from the middle traces obtained by exciting the donor fluorophore and measuring both donor (green) and acceptor (red) fluorescence. The lowest trace was obtained by exciting the acceptor fluorophore directly. The arrow indicates a bleaching event. (D) Distribution of FRET values determined from 163 traces as in (B), fit with a Gaussian model (black curve). (E) As in (C), but in the presence of ATP. Periods in which a fluorescently labeled SecA molecule is bound are indicated by grey shading. (F) As in (D), but with ATP (178 traces). (G) Traces obtained in the presence of different nucleotides were used to determine the number of states best fit by the Markov model. (H) Transition density plot of idealized ATP FRET states obtained in (F). (I) The distribution of dwell times of the low FRET states observed in ATP was fit with an exponential (1550 low FRET states). The inset shows average dwell times and errors, defined as the standard error based on the number of traces. (J) As in (I), but with high FRET (1778 high FRET states).
2.4 Discussion

Our results lead to a new model for how the SecA ATPase moves polypeptides into the SecY channel. The model combines features of the previously proposed “push-and-slide” mechanism (Bauer et al, 2014) with an essential role for the clamp to hold the polypeptide chain during resetting of the finger. Without this additional mechanism, the bidirectional movement of the two-helix finger towards and away from the channel would result in no net translocation, as the finger would drag the polypeptide with it when it retracts. Thus, clamp tightening is essential to preserve progress of the substrate when the finger moves away from the channel.

Our single-molecule FRET results, together with previous bulk solution experiments (Robson et al, 2009; Bauer et al, 2014) lead to a model for the coupling of ATP hydrolysis by
SecA with polypeptide translocation (Fig 2.8B). When ATP binds to SecA, the two-helix finger inserts deeply into the SecY channel, pushing the polypeptide substrate towards the extracellular side of the membrane (stage 1). Next, the clamp closes around the polypeptide chain, with SecA’s PPXD contacting the NBD2 through the conserved C-loop segment (Chen et al., 2015; Zimmer et al., 2008). Contact between the C-loop and NBD2 could trigger ATP hydrolysis, i.e. clamp closure would occur before ATP hydrolysis (stage 2). Alternatively, the clamp could close only during ATP hydrolysis. In the transition state of ATP hydrolysis (stage 3), the two-helix finger has started to retract, while the clamp is closed. After ATP hydrolysis, but before P$_i$ release, the two-helix finger has retracted all the way, while the clamp remains closed (stage 4). This allows the two-helix finger to reset without dragging the polypeptide chain backwards. After P$_i$ release, the clamp reopens, allowing the passive sliding of the polypeptide in either direction (stage 5).

Our model is based on independent observations of the movements of the two-helix finger and clamp, aligned by using complexes trapped in the transition state of ATP hydrolysis (ADP•BeF$_4$) or after ATP hydrolysis, but before P$_i$-release (ADP•P$_i$ or ADP•V$_i$). Linking the FRET changes of the two domains was also facilitated by measuring the kinetics during ATP hydrolysis and by performing experiments in ATPγS, which biases the system towards an ATP-bound or ATP-hydrolysis intermediate state. However, future experiments with three-color FRET will be required to simultaneously follow the movements of both domains.

Passive sliding of the polypeptide chain remains a major part of the translocation mechanism (Bauer et al., 2014; Erlandson et al., 2008b), as SecA spends most of its time in the ADP-bound state (stage 5), in which the two-helix finger is disengaged and the clamp is open. Indeed, ADP release is rate-limiting in the ATP-hydrolysis cycle and the apo-state is exceedingly transient (Robson et al., 2009). Furthermore, in the ATP-bound state, the two-helix finger does not interact strongly with all amino acids encountered (Bauer et al., 2014), so that a power stroke does not always result in active pushing. But, passive sliding in the ADP-bound state allows translocation of any polypeptide segment encountered by SecA. Although we employed a stalled translocation intermediate in our study, the polypeptide chain is continuously sliding backwards.
and must be re-inserted by SecA into the SecY channel, thus mimicking real translocation, even if the C-terminus of the polypeptide does not enter the channel. Backsliding in vivo might be reduced by the membrane potential across the inner membrane, folding of the polypeptide in the periplasm, or by binding of the polypeptide to periplasmic proteins.

Our results argue against a proposed ratcheting model, in which the two-helix finger makes only small movements relative to the channel and the polypeptide chain is free to slide in the ATP-bound state (Allen et al., 2016; Corey et al., 2019). The FRET data indicate that the finger makes in fact very large movements, alternating between a withdrawn conformation and one in which it inserts into the channel. The large movements of the two-helix finger must originate from much smaller conformational changes at the interface between the NBDs, which are propagated and amplified through a long linker helix (Hunt et al., 2002). Whether the two-helix finger, or any other SecA domain, moves all the way through the membrane, as previously proposed and supported by recent crosslinking experiments (Banerjee et al., 2017; Economou & Wickner, 1994) requires further investigation, as it is difficult to see from the available crystal structures how the finger could pass the SecY pore constriction. Our in vitro experiments did not include SecDFYajC, components that facilitate protein secretion in vivo (Pogliano & Beckwith, 1993). Although these components are not essential, they might modify the movements of SecA during translocation (Economou et al., 1995). Surprisingly, the two-helix finger of SecA can be crosslinked to a cytosolic loop in SecY without abolishing translocation activity (Whitehouse et al., 2012). However, this loop is the longest and most flexible on the cytosolic face of SecY and might therefore not arrest the finger. Crosslinking to a more rigid position in SecY’s cytosolic cavity does in fact eliminate translocation activity (Whitehouse et al., 2012).

Hexameric ATPases that move polypeptides, such as the 19S subunit of the proteasome, the Cdc48 ATPase, and the Clp proteins, may use a similar mechanism as SecA. In this case, each of the six subunits has a loop analogous to the two-helix finger of SecA, which pushes the polypeptide chain through the central pore (Hinnerwisch et al., 2005; Martin et al., 2008; Han et al., 2008).
Because it is difficult to separate the movements of the six loops during the ATP hydrolysis cycles, even with single-molecule experiments (Aubin-Tam et al., 2011), monomeric SecA provides a unique, tractable model to determine the mechanism by which ATPases move polypeptides.

In fact, recent structures of the *Plasmodium* translocon of exported proteins (PTEX) suggest an analogous model for the Hsp101 ATPase (Ho et al., 2018). Here, a set of three pore loops undergo similar movements as the two-helix finger of SecA during the ATPase cycle, pushing the polypeptide chain forward. When the loops release the substrate, other loops assume the role of SecA’s clamp, holding the polypeptide in place and thus preventing its backward movement. It is therefore possible that the proposed SecA mechanism is generally employed by polypeptide-moving ATPases.

2.5 Materials and Methods

2.5.1 Protein Expression and Purification

SecA and SecY were expressed and purified as previously described (Bauer et al., 2014). Cysteine-free SecA N95 (lacking the non-essential C-terminus (Matsuyama et al., 1990)) with a C-terminal His-6 tag and 3C protease cleavage site was cloned into a pET30b (EMD Millipore, Burlington, Massachusetts) vector and expressed in BL21(DE3) E. coli (New England Biolabs, Ipswich, Massachusetts) for 4 hrs at 37 °C after induction at OD$_{600}$ 0.8 with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were collected by centrifugation for 10 min at 4000 x g, resuspended in buffer A (50 mM HEPES/KOH pH 7.5, 300 mM NaCl, 15 mM imidazole, 20 mM b-mercaptoethanol (BME)), and lysed by two passes through an EmulsiFlex-C3 (Avestin, Ottawa, Canada) at 20,000 psi. Soluble components were separated from the membrane fraction by centrifugation at 110,000 x g for 45 min. The supernatant was bound to 2 ml Ni$^{2+}$ resin,
washed with 50 ml buffer B (50 mM HEPES/KOH pH7.5, 100 mM NaCl) and incubated overnight in 5 ml buffer B at 4 °C with 5 μM 3C protease. The flow-through was then collected and subjected to anion exchange chromatography (HiTrap Q FF, GE Healthcare Life Sciences, Marlborough, Massachusetts) followed by size exclusion chromatography (Superdex 200 10/300GL, GE Healthcare Life Sciences) in buffer C (50 mM HEPES/KOH pH 7.5, 50 mM KCl).

The three E. coli SecYEG protein components, with an N-terminal His-6 tag on SecE, were cloned into a pBAD22 vector (ATCC, Manassas, Virginia) under a single L-arabinose-inducible promoter. The cells were grown to OD₆₀₀ 0.6 and induced for 4 hrs at 37 °C by addition of 10 ml 20 % L-arabinose. The cells were collected, lysed, and fractionated in the same manner as for SecA. The membrane fraction was solubilized for 90 min in buffer D (buffer A, 10 % glycerol) with 1 % n-dodecyl β-D-maltoside (DDM, Anatrace Inc., Maumee, Ohio). The extract was subjected to high speed centrifugation at 110,000 x g for 45 min. Subsequent steps were carried out in buffers containing 0.03 % DDM. The protein was bound to 1ml Ni²⁺ resin in buffer D, washed with 50 ml buffer D, and eluted in 5 ml buffer E (buffer B, 10 % glycerol) with 250 mM imidazole. The eluate was then subjected to cation exchange chromatography (HiTrap SP FF, GE Healthcare Life Sciences) and size exclusion chromatography (Superdex 200 10/300, GE Healthcare Life Sciences) in buffer F (buffer C, 10% glycerol).

proOmpA(1-175)-DHFR-Avitag (proOmpA-DHFR) was cloned into a pET30b vector and co-expressed with the biotin ligase BirA encoded on the pBirAcm plasmid (Avidity, LLC., Aurora, Colorado). Both vectors were transformed into BL21(DE3) E. coli and the cells grown to OD₆₀₀ 0.6. Expression was induced by the addition of 1 mM IPTG and the media was further supplemented with 20 μM D-biotin. After 2 hrs at 37 °C, cells were collected by centrifugation and lysed by two passes through an EmulisFlex-C3 at 20,000 psi in Buffer A. The lysate was centrifuged for 45 min at 110,000 x g and the insoluble pellet containing proOmpA was collected. The pellet was incubated in buffer A with 6 M urea for 1 hr at 23 °C and then centrifuged again at 110,000 g for 45 min. The supernatant was collected and diluted with buffer A to 2 M
urea before being mixed with 1 ml High Capacity Streptavidin Agarose (Thermo Fisher Scientific, Waltham, Massachusetts) for 1 hr at 4 °C. The beads were then washed with 50 ml buffer C with 2 M urea before the protein was eluted by addition of 10 ml buffer C with 6 M urea.

2.5.2 Protein Labeling

Both SecA and SecY were labeled using the same protocol. 500 μl of 10 μM purified protein was incubated with 40 μM of tris(2-carboxyethyl)phosphine (TCEP) for 20 min on ice. 100 μM maleimide-conjugated Cyanine3 (Cy3) or Cyanine5 (Cy5) (Lumiprobe, Hunt Valley, Maryland) was added to the SecY and SecA, respectively, from 10 mM stock in dimethyl sulfoxide (DMSO) and rotated overnight at 4 °C. Labeling was quenched by the addition of 10 mM 1,4-dithiothreitol (DTT). Dye excess was then removed via gel filtration through a 30 cm column packed with Superfine G50 Sephadex (GE Healthcare Life Sciences) equilibrated in buffer C for SecA or buffer F with 0.03 % DDM for SecY. The first visible dye peak was collected and further purified by size exclusion chromatography (Superdex 200 10/300, GE Healthcare Life Sciences). Labeling efficiencies were generally around 80% for SecA mutants and 60% for SecY mutants.

2.5.3 Liposome Preparation and Membrane Protein Reconstitution

Liposomes were prepared from E. coli Polar Lipid Extract (Avanti Polar Lipids, Alabaster, Alabama). 2 mg of lipids from 25 mg/ml chloroform stock were dried under nitrogen stream, resuspended in 500 μl diethyl ether, dried again, and stored under vacuum overnight to remove all solvent traces. The resulting lipid film was hydrated in 500 μl of buffer C by vortexing, followed by shaking for 1 hr at 750 rpm at 23 °C. This suspension was then sonicated in a bath sonicator (Branson Ultrasonics, Danbury, Connecticut) for 30 min and subjected to 5 freeze/thaw cycles. Finally, the liposomes were passed 21 times through a 50 nm polycarbonate filter (Avestin) in a Mini-Extruder (Avanti Polar Lipids).
To reconstitute SecYEG into these liposomes, 50 µl of 4 mg/ml liposomes were mixed with 0.4 % Triton X-100 and 20 pmol (single molecules experiments) or 200 pmol (bulk experiments) of purified protein. The reconstitution volume was brought to 100 µl by addition of buffer C and the mixture was incubated for 30 min at 4 °C. Detergent was then removed by 4 sequential batches of SM-2 biobeads (Bio-Rad Laboratories, Hercules, California) for 1 hr, 4 hrs, 12 hrs, and 2 hrs. The final proteoliposomes were centrifuged for 5 min at 14,000 x g to remove any insoluble material before use.

2.5.4 Microscope Setup

The microscope used was a through-objective TIRF microscope built on an inverted microscope body (Olympus IX71, Olmypus, Tokyo, Japan) as described elsewhere (Graham et al, 2016). Samples were illuminated by 532 nm and 641 nm lasers (Coherent Sapphire 532, and Cube 641, Coherent, Santa Clara, California). The beams were expanded and focused through the rear window into the back aperture of an oil immersion objective (Olympus UPlanSApo, 100x, NA 1.40). A suspended lens in front of the back microscope port allowed for adjustment of the TIRF angle. Cy3 and Cy5 emission was separated by a dichroic mirror (Chroma) and projected onto two halves of an EM-CCD camera (Hamamatsu, ImageEM 9100-13, Hamamatsu Photonics, Hamamatsu City, Japan). The field of view was manipulated by a digitally-controlled, automated microstage (Mad City Labs Inc., Madison, Wisconsin) and the microscope focus was manually adjusted.

2.3.5 Flow Chamber Preparation

Cover glasses (22 x 60 mm No. 1.5; Fisher Scientific, Hampton, New Hampshire) were cleaned by four alternating 30 min washes in ethanol and KOH, with intervening rinses in de-ionized water, followed by a 10 min sonication in acetone. The glasses were silanized by a 2 min
treatment with 2 % (3-aminopropyl)triethoxysilane (APTES, Sigma-Aldrich, St. Louis, Missouri) in acetone, thoroughly washed with deionized water, and heated for 30 min at 110 °C. 150 mg 5 kDa methoxypolyethylene glycol–succinimidyl valerate (mPEG-SVA) and 2.5 mg 5kDa biotin-PEG-SVA (both Laysan Bio, Arab, Alabama) were dissolved in 1 ml of 100 mM NaHCO$_3$ pH 8.2. 100 μl of the PEG solution was sandwiched between pairs of cleaned cover glasses and incubated at room temperature for 4 hrs. Glasses were then separated, washed thoroughly with deionized water, and stored under vacuum.

Flow chambers were constructed by sandwiching rectangles of double sided Kapton tape (Bertech, Torrance, California) with 5 x 15 mm slits cut into them between cleaned cover glasses and 2.5 x 2.5 x 0.5 cm quartz glass blocks (Quartz Scientific Inc., Fairport Harbor, Ohio). To allow perfusion of the chambers, tubing (PFTE #24; Cole-Parmer, Vernon Hills, Illinois) was inserted through holes drilled 10 mm apart in the quartz and sealed by epoxy. Before use, flow chambers were flushed with 500 μl of buffer G (50 mM Hepes/KOH pH 7.5, 50 mM KCl, 5 mM MgCl$_2$, 8 mM protocatechuic acid (PCA, Santa Cruz Biotechnology, Dallas, Texas), 200 nM protocatechuate 3,4-dioxygenase (PCD, Sigma-Aldrich), 20 mM BME, 0.2 mg/ml bovine serum albumin (BSA, New England Biolabs), 10 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich), and then incubated with 0.5 mg/ml neutravidin (Thermo Fisher Scientific) in buffer G for 30 min. Unbound neutravidin was removed by a second wash with 500 μl of buffer G.

2.5.6 Single Molecule FRET Complex Assembly and Surface Tethering

Translocation intermediate complexes were assembled in bulk by mixing 20 nM Cy3-labeled SecYEG proteoliposomes, 20 nM Cy5-labeled SecA, 1 μM proOmpA(1-175)-DHFR-biotin with 5 mM ATP and 50 μM methotrexate (MTX, Sigma-Aldrich) in 50 μl buffer H (50 mM Hepes/KOH pH 7.5, 50 mM KCl, 5 mM MgCl$_2$, 20 mM BME, 0.2 mg/ml BSA). Reactions were incubated for 10 min at 37 °C while shaking at 650 rpm in a thermomixer (Eppendorf, Germany).
ny). When indicated, 1mM ADP•BeF₆, 1mM ADP and Vᵢ (sodium orthovanadate, New England Biolabs), 5mM ATPγS (Jena Bioscience, Jena, Germany), or 1mM ADP and Pᵢ were added after the initial 10 min, along with 1 U of hexokinase (Roche Applied Science, Germany) and 20mM glucose, and incubated for an additional 5 min. Assembled complexes were diluted 1:20 in buffer G supplemented with 50 μM MTX and 5 mM ATP, 1 mM ADP•BeF₆, 1mM ADP•Vᵢ, 5mM AD-P•Pᵢ or 5 mM ATPγS. 20 nM unlabeled SecA was added at this point during ATP μexperiments. The diluted reactions were added to flow cells and incubated for 5 min at 23 °C. Unbound protein was then washed out with 500 μl of buffer G containing 50 pM Cy5-labeled SecA, 50 μM MTX, and 5 mM ATP, 1 mM ADP•BeF₆, 1mM ADP•Vᵢ, 5mM ADP•Pᵢ, or 5 mM ATPγS. ATP reactions also contained 1 nM unlabeled SecA and an ATP regeneration system consisting of 8 mM phosphocreatine and 50 ng/ml creatine kinase (Sigma-Aldrich).

2.5.7 Single Molecule Imaging

The 532 nm and 641 nm lasers were set to 45 W/cm² and 35 W/cm² surface density, respectively. The camera EM gain was set to its maximal setting. The camera integration time was set to 33 ms and the illumination was switched between the two lasers by Uniblitz VS14 shutters (Vincent Associates, Rochester, New York) synchronized to the camera. Alternating excitation (AlEx) illumination was used, where single frames switched between direct excitation of the Cy3 molecules and direct excitation of the Cy5 molecules. 1000 frames were recorded for each video, consisting of 33 total seconds. The stage was translated and refocused between each video to find previously unexposed, unbleached regions of the cover glass. Data for each mutant/nucleotide combination was aggregated from multiple videos taken from at least four separate immobilizations collected over at least two different days.
2.5.8 Extraction of FRET Data from Videos

Initial analysis was performed in the iSMS single molecule FRET software suite (Preus et al, 2015) in MATLAB (Mathworks, Natick, Massachusetts). The Cy3 and Cy5 channels of each video were aligned by maximizing the co-localization of fluorescence spots projected on each half of the camera field of view. Raw fluorescence traces were extracted from co-localized spots by fitting a five-pixel diameter circular aperture function to each spot and integrating the intensity within the circle. A mean background signal calculated from a ring of pixels with a 10 pixel diameter centered on each fit spot was subtracted from the integrated intensity. These raw traces were filtered to select complexes with 1:1 SecA:SecY stoichiometry as evidenced by single-step photobleaching, as well as clear anti-correlated FRET behavior either within a trace or in response to acceptor bleaching. In general, each video had roughly 25 monomeric SecY spots, about 15 of which co-localized with a monomeric SecA. Of these co-localized spots, about 10-20% exhibited anti-correlated FRET activity (Table S2.1). Traces were manually segmented to account for multiple SecA molecules binding and dissociating within one video. Raw fluorescence was then corrected for Cy3 bleed-through into the Cy5 channel as well as direct excitation of Cy5 by the 532 nm laser. These correction factors were calculated independently for each imaging session based on the measured fluorescence in the Cy5 channel after Cy5 and Cy3 photobleaching, respectively. A γ-factor correction was also calculated for each session based on total fluorescence in both channels before and after acceptor bleaching. FRET was then calculated from these corrected fluorescence traces (Lee et al, 2005).

2.5.9 Estimation of Inter-Fluorophore Distances

FRET histograms were compiled by a non-weighted aggregation of FRET values calculated from each frame prior to photobleaching/dissociation of all traces for a given mutant/nucleotide combination. These histograms were fit with Gaussian Mixture Models using the fitgmdist() function in MATLAB. The mean and standard deviation of Gaussian components
aligned to prominent peaks were used to calculate the estimated distances between fluorophores, using 55 Å as a value for the Förster radius between Cy3 and Cy5 (Yu et al., 1994). Calculated values were compared to inter-Cα distances measured between corresponding amino acids in published structures of the SecA/SecY complex (PDBs 3DIN, 5EUL) using PyMol (Schrödinger LLC., New York, New York). The number of traces included in each histogram is indicated in the corresponding figure legends.

2.5.10 Hidden Markov Analysis

Hidden Markov Model analysis of individual FRET traces was performed in the vbFRET software suite in MATLAB (Bronson et al., 2009). This program finds the idealized parameters, including the number, value, and transition probabilities, of FRET states for each trace using a maximum evidence approach. The program was run with up to 5 discrete FRET states allowed per trace using default parameters, including 10 fitting attempts per trace, a maximum of 100 iterations per VBEM, and a convergence threshold of $10^{-5}$. The most likely number of states represented in each FRET trace was reported. The number of traces analyzed in this manner is the same as the number reported for the corresponding FRET histograms. Transition density plots were constructed from these idealized states for ATP data by plotting the FRET value fit by vbFRET one frame before each predicted transition against the FRET value one frame after each prediction (McKinney et al., 2006). Transition density plots for data in the presence of ADP•BeF$_x$ could not be plotted as the model predicted no transitions in most traces.

2.5.11 Dwell-time Analysis

FRET states from individual traces were then grouped into consensus high and low FRET states using ebFRET (van de Meent et al., 2014), again using default parameters except for a precision of $10^{-5}$. The mean dwell times of these high and low FRET states were found by fitting
single exponential functions to the ensembles of individual states grouped into each FRET. Since events that terminate within the integration time of a given frame often get attributed the duration of the full frame, each data point was treated as left-censored by up to one frame while fitting. Also, the first and last FRET state of each trace was excluded as the state was not necessarily observed for its full duration. A small population of long-lived, non-exponentially distributed, low FRET states was truncated by limiting the analysis to states with durations less than 2 seconds. Error on these mean lifetimes was the standard error of the mean (SEM) based on the number of individual traces fit by ebFRET for the given mutant/nucleotide combination. The number of FRET states from which the dwell times were measured is indicated in the corresponding figure legends.

2.5.12 Translocation Assays

Bulk translocation assays were performed on wild type and labeled SecA and SecYEG purified in the same manner as for single molecule experiments. 35S-Met-labeled proOmpA was generated by in vitro translation. mRNA was transcribed from linearized template with an SP6 promoter followed by the Kozak consensus ribosome binding site directly 5’ to the proOmpA gene start codon using a RiboMax SP6 in vitro transcription kit (Promega, Madison, Wisconsin). 2 μg of transcription product RNA was mixed with 35 μl of nuclease treated rabbit reticulocyte lysate (Promega), 1 μl of 1 mM amino acid mixture minus methionine (Promega), and 2 μl (.022 μCi) of EasyTag Express S35 Labeling Mix (PerkinElmer, Waltham, Massachusetts). Translation product was precipitated by the addition of 150 μl saturated ammonium sulfate and re-suspended in 50 μl 6 M urea pH 6.8.

Reactions were assembled in buffer H. 100 nM SecA and 100 nM SecYEG proteoliposomes were mixed with 1 μl of the in vitro translation products in a 50 μl total volume. Reactions were initiated by the addition of 5 mM ATP and incubated for 10 min at 37 °C while shaking at 650 rpm. For the channel saturation experiments, 1 μl of purified proOmpA-DHFR was added.
at varying concentrations along with 50 μM MTX before the addition of the in vitro translation product and incubated for an additional 10 min at 37 °C. Reactions were terminated by transfer to ice and addition of 0.4 mg/ml proteinase K, and, where indicated, 0.2 % Triton X-100. Digests were continued for 45 min and quenched with 2 mM phenylmethylsulfonyl fluoride (PMSF). Reactions were then precipitated in 10 % trichloroacetic acid (TCA), and resuspended in 1X Laemmli buffer with 300 mM Tris base. Samples were then analyzed by SDS-PAGE. The gels were vacuum dried, exposed to autoradiography film overnight, and imaged by a Personal Molecular Imager (Bio-Rad).

2.5.13 ATPase Assays

ATP activity was measured with an EnzChek Phosphate Assay kit (Thermo Fisher Scientific). Activity was measured with both wildtype proteins and fluorophore-labeled mutants. 100 μl reactions were prepared in 1X EnzChek assay buffer (Thermo Fisher Scientific) with 200 μM 2-amino-6-mercapto-7-methylpurine riboside (MESG), 100 nM SecA, 100 nM SecYEG proteoliposomes, 0.2 mg/ml BSA, 20 mM BME, 50 μM MTX, and 1 μM proOmpA-DHFR. 0.1 U of purine nucleoside phosphorylase (Thermo Fisher Scientific) was added and the reactions were incubated for 30 min at 23 °C. 5 mM ATP was then added and the change in absorbance of 360 nm light (OD₃₆₀) was monitored over 90 min at 15 s intervals in a M5 plate reader (Molecular Devices, San Jose, California). Reactions were run in quadruplicate together with a control reaction lacking ATP. The dependence on SecA concentration was measured similarly with 0, 1, 5, 10, 25, 50, 75, 100, 150, or 200 nM SecA. A standard curve was generated by measuring the absorbance of P₃ standards (Thermo Fisher Scientific). Average ATP consumption rates were converted to a per molecule SecA rates using the SecA concentrations and ATPase rates measured from the SecA titration experiment.
Chapter 3: Forward Movement of the Translocating Polypeptide through the SecY Channel

3.1 Foreword

The data included in this section are preliminary and unpublished. The single molecule FRET experiments are a continuation of a collaboration with the lab of Joseph Loparo. The ensemble biochemistry experiments, including both the protease protection and crosslinking assays, were conceived of by Marco Catipovic and Tom Rapoport using several protocols developed by Benedikt Bauer. Photocrosslinking mass spectrometry experiments are planned in collaboration with the lab of Jarrod Marto. Mass spectrometry will be performed by Scott Ficarro. All other experiments were performed by Marco Catipovic.

3.2 Introduction

In Chapter 2 we observed the movements of the two-helix finger (THF) and clamp domains of SecA as the ATPase hydrolyzed ATP to push a polypeptide substrate through the SecY channel. This led to the conclusion that SecA uses a power-stroke mechanism to produce from the chemical energy released during ATP hydrolysis the mechanical work needed for translocation (Catipovic et al., 2019). ATP binding to the nucleotide binding domains causes a large movement of the two-helix finger into the SecY pore, which would push the polypeptide substrate with it. Subsequently, the clamp domain closes and the THF retracts during ATP hydrolysis. Release of the resulting inorganic phosphate (P\textsubscript{i}) allows the clamp to return to its initial open state, freeing the substrate to slide forwards and backwards through the SecY channel.

This model is in direct contrast with an alternate ratcheting model (Allen et al., 2016; Corey et al., 2019), in which communication between the nucleotide binding domains of SecA and the SecY channel causes the SecY pore to open and close as SecA hydrolyzes ATP (see Section 1.5.4 for details of both models). Here, the translocating polypeptide can slide during
both SecA’s ATP and ADP–bound states, though sterically large side chains or small segments of secondary structure can only pass through the widened channel triggered by ATP binding.

The single molecule FRET experiments presented in Chapter 2 seem to discriminate between these models. In the ratcheting model, there is no predicted movement of the THF. However, it is now clear that the THF makes large movements in and out of the channel with every ATP hydrolysis cycle. Crucially though, the single molecule experiments provide direct observations of only SecA’s THF and clamp, not of the translocating polypeptide chain. Demonstration that SecA does in fact use the power-stroke mechanism requires evidence that ATP-binding to SecA causes the substrate to make the predicted large movement into the SecY channel, and that ATP hydrolysis and the return to the ADP-bound state allows permissive sliding of the polypeptide.

The sliding phase of polypeptide translocation is well documented. Translocation intermediates can be formed with an \textit{in vitro} system using a substrate that includes a domain sterically too large to fit into the SecY channel. After establishing these intermediates, the ATP in the reaction can be scavenged and any movement of the intermediate can be assayed by measuring the amount of substrate remaining resistant to protease degradation. As predicted, without continued ATP hydrolysis the translocation intermediates slide back out of the SecY channel in minutes (Erlandson \textit{et al}, 2008b; Bauer \textit{et al}, 2014). However, incubation with non-hydrolyzable analogs such as ATPγS or ADP•AlF$_4^-$ do not allow the polypeptide to slide backwards (Erlandson \textit{et al}, 2008b; Bauer \textit{et al}, 2014), indicating that it is only in the ADP-bound state of SecA that the polypeptide is free to slide.

Whether the polypeptide moves forward upon ATP-binding to SecA is less clear. When translocation intermediate complexes are incubated with ATPγS or ADP•AlF$_4^-$, the resulting protease-protected fragments are slightly larger than the protected fragments in the presence of hydrolysable ATP (Schiebel \textit{et al}, 1991; Erlandson \textit{et al}, 2008b). This is consistent with ATP-binding causing the stalled polypeptide to be pushed forward and then held in this position until after
hydrolysis. However, it is possible that such a shift could be due to tight binding of SecA protecting a segment of the substrate outside of the SecY channel from proteolytic degradation.

Furthermore, there is no direct evidence that this shift is due to SecA’s THF as the power-stroke model predicts. Disulfide crosslinks can be formed between the THF and the translocating polypeptide substrate, indicating they are in close proximity during translocation (Erlandson et al, 2008a). Also, mutation of a conserved Tyrosine or two basic residues at the THF tip abolishes the de novo translocation activity of SecA and impairs its ability to complete the translocation of pre-formed intermediates (Bauer et al, 2014). But, whether or not these THF mutants can in fact produce a forward step of the translocating substrate upon incubation with non-hydrolyzable ATP analogs is unknown.

To study the movements of the polypeptide substrate during translocation we employed a combination of single molecule FRET, protease protection, and cross-linking assays. Results from the single molecule and cross-linking experiments are preliminary and do not yet provide positive support for our hypothesis. However, the protease protection experiments provide clear evidence that ATP-binding to SecA pushes the substrate into the SecY channel, and that this movement is dependent on the THF of SecA.

3.3 Results

3.3.1 Single Molecule FRET Assays

In our previous single molecule experiments, we placed a donor fluorophore on SecY and an acceptor fluorophore on the THF or PPXD of SecA to monitor the distance changes between the labeled residues as the THF moved or the clamp opened and closed. Both these components were immobilized near the glass coverslip imaging surface as long as they remained engaged with the translocating polypeptide substrate. This substrate was a fusion of proOmpA with a
DHFR domain and a C-terminal biotinylation tag. SecA binds the substrate and begins translocating it through SecY until it reaches the DHFR domain (position 352). This domain folds tightly in the presence of methotrexate and creates a steric block to translocation, stalling the substrate in the SecY pore (Bauer & Rapoport, 2009). The C-terminal biotin can then interact with neutravidin on the imaging surface to immobilize the entire complex.

We reasoned that minor modification of this setup could allow us to observe movements of the substrate relative to SecY. By moving the acceptor fluorophore from SecA to the substrate, we could observe FRET between any position on the polypeptide chain and our established reference positions on SecY. We therefore created single Cysteine mutants of our substrate construct and labeled them with a maleimide-Cy5 fluorophore (Fig S3.1).

The Cysteine mutations were positioned along the substrate to introduce fluorophores at positions relative to the donor fluorophore on SecY expected to produce a diversity of FRET values based on their positions in the ADP•BeF$_x$—stabilized complex. The regions of the translocating polypeptide that lie in SecA’s clamp or in the SecY channel can visualized in the cryo-EM structure of the translocating SecA/SecY complex, and have also been mapped using disulfide cross-linking (Ma et al, 2019; Bauer & Rapoport, 2009). Roughly 20 amino acids directly to the N-terminus of the folded domain contact SecA, and further 10-15 residues lie in the SecY channel. Accordingly, we first mutated residues 296, 311, 317, 325, and 337 of the substrate to introduce fluorophores at these positions, which are 56, 41, 35, 27, and 15 residues N-terminal, respectively, to the DHFR domain (Fig 3.1). Based on the cryo-EM structure, this should place two fluorophores in the SecA clamp (position 337 and 325), two in the SecY channel (positions 317 and 311), and one inside the lumen of the liposome (positions 296) (Fig 3.1A).

We used two donor positions on SecY to observe FRET with these different acceptor positions in the substrate, though not every acceptor position was imaged with each donor. In the presence of ADP•BeF$_x$, the substrate should be firmly held by SecA and the entire complex static in the same conformation as that captured by cryo-EM. As such, we can use the structure
to predict the relative FRET intensities of the 5 acceptor fluorophores with the 2 donor positions. Donor position 295, on the luminal face of SecY, was expected to give high FRET with acceptor positions 311 and 317, lower FRET with positions 296 and 325, and likely no FRET with position 337 (Fig 3.1B). Donor position 103, on the cytosolic face of SecY, should have high FRET with positions 311, 317, and 325 and lower FRET with positions 296 and 337 (Fig 3.1C).

The experiments were carried out in much the same way as the FRET experiments in Chapter 2. Labeled SecY proteoliposomes, labeled and biotinylated substrate, and unlabeled SecA were mixed in bulk and incubated with ATP and methotrexate. ADP•BeF₆ could be added to the resulting complexes, which were then diluted and allowed to bind to a PEGylated coverslip containing 2% biotin-PEG pre-incubated with neutravidin. After washing out unbound material, the resulting complexes could be viewed using TIRF microscopy with alternating excitation of the Cy3 (donor) and Cy5 (acceptor) fluorophores.
Both labeled components could be detected immobilized on the imaging surface with a high degree of co-localization (Fig S3.2A,B). The majority of Cy3-SecY proteoliposomes aligned with a Cy5-substrate spot, though there were also a number of isolated Cy5-substrate spots, indicative of the substrate’s ability to bind to the surface through its biotin moiety without contributions from the other components (Fig S3.2C). However, despite co-localization of the donor and acceptor fluorophores, very few spots yielded signal from the Cy5 channel when excited at the Cy3 excitation wavelength, indicating that there is little FRET occurring between the fluorophores (Fig S3.2D).

Only 1-2% of co-localized spots yielded measurable FRET signals. These FRET traces were highly heterogeneous. Even in the presence of ADP•BeF₆, many traces demonstrated dynamic behavior, switching between several FRET values (Fig 3.2A-C). For the traces that did exhibit more static FRET, the FRET value did not match well with expectations based on the predicted position of the substrate label relative to the reference points on SecY (Fig 3.1B,C). When dynamic, most positions had low FRET states close to 0 FRET and high FRET values spread between 0.4 and 0.8. Some of this heterogeneity could be due to the low number of traces analyzed. But, over 100 traces were analyzed with substrate labeled at position 317 and SecY labeled at position 295 in the presence of ADP•BeF₆. Even in this larger data set, roughly half of all traces were dynamic, occupying more than one FRET state, and half were static (Fig 3.2B-D).

It is unlikely that the substrate would be moving through the channel when SecA is bound to ADP•BeF₆. Ensemble biochemistry protease protection experiments have shown that the substrate is only free to slide when SecA is in the ADP-bound state or if there is a long stretch of Glycine residues contacting SecA (Bauer et al., 2014). Therefore, the FRET we observed with these constructs is only artefactual. The fact that the vast majority of co-localized spots displayed no FRET activity suggests that, when properly assembled, energy transfer is inhibited between the two fluorophores, perhaps because of rotational restrictions on the substrate fluorophores buried in the SecA clamp or inside the SecY pore. The FRET we did observe may have been from
Figure 3.2. Heterogeneity in smFRET data of substrate movements. (A) FRET histograms for data taken with a donor fluorophore at position 103 on the cytosolic face and an acceptor at the indicated position in the proOmpA-DHFR substrate in the presence of ADP•BeFx or ATP. For ADP•BeFx, from left to right, N = 8, 25, 17, 10 traces. For ATP, from left to right, N = 6, 18, 17, 8 traces. (B) Representative FRET traces for experiments with the donor fluorophore at position 295 on the periplasmic side of SecY and acceptor at position 317 in the substrate. The upper set of plots shows data from a static molecule, the lower set from a dynamic molecule. For each set, the upper FRET trace was calculated from the middle traces obtained by exciting the donor fluorophore and measuring both donor (green) and acceptor (red) fluorescence. The lowest trace was obtained by exciting the acceptor fluorophore directly. The gray box indicates the
the minority of complexes that did not assemble properly or were just co-localized via non-specific interactions, which brought the two fluorophores in close enough proximity to induce energy transfer.

Single molecule FRET remains a promising assay for directly observing the movement of the translocating polypeptide substrate. The technique is sensitive to motion over small distances and has already been employed to monitor the conformational dynamics of both SecA and SecY during translocation (Allen et al., 2016; Fessl et al., 2018; Ernst et al., 2018; Catipovic et al., 2019). However, we have yet to successfully repurpose the method in order to observe substrate movements. It is unclear why the labeling positions on SecY and the substrate we have tried to date have failed to generate FRET, but it is likely that a large array of positions may need to be screened before appropriate labels can be identified.

A bulk assay to measure FRET in biochemical ensembles, rather than with single molecules, could be a more efficient way to screen for FRET-competent label positions. These experiments are complicated by the low efficiency of complex formation. As much as 90% of added polypeptide substrate does not get incorporated into translocation intermediate complexes, creating a significant background fluorescence signal. However, in the presence of ADP•BeFx, the complexes should be stable enough to be re-purified after formation (Zimmer et al., 2008; Li et al., 2016; Ma et al., 2019). Sequentially affinity purification steps using tags on SecA’s C-terminus, followed by a tag on the substrate’s C-terminus should yield a homogenous population of translocation intermediate complexes. FRET in these complexes can then be measured in a plate reader, with a higher throughput than in a single molecule setup. This method would give no information about the movement of the polypeptide chain, but could provide a valuable tool for identifying labeling positions that produce a FRET signal worth studying in the microscope.

(Figure 3.2 continued) period before either fluorophore bleaches. (C) Distribution of FRET values determined from 104 traces as in (B). (D) Traces as in (B) were fit with a hidden Markov Model to determine the number of FRET states in each.
3.3.2 Protease Protection Assays

While designing substrates for single-molecule FRET experiments, we realized that these substrates could also be used in ensemble protease protection assays to generate data about the movements of the polypeptide chain during translocation. Protease protection has long been a staple method to study translocation activity with in vitro reconstituted systems (Watanabe et al., 1990; Brundage et al., 1990; Driessen & Wickner, 1990). In the assay’s simplest form, SecA and a model substrate are mixed with SecY proteoliposomes. When ATP is added to the reaction, the ATPase can translocate the substrate through SecY into the lumen of the liposome, where it is protected from subsequent protease degradation. The addition of sterically large obstructions to the C-terminus of the substrate, either folded protein domains, or polypeptide loops formed by intramolecular disulfide loops can provide a blockage to stall translocation and create intermediate protected species (Schiebel et al., 1991; Arkowitz et al., 1993; Bauer et al., 2014) (Fig 3.3A). ATP is required for translocation, and the addition of detergent during protease treatment negates any protection the liposome offered to translocated species (Fig 3.3A).

Using the labeled substrate from the single molecule experiments, the substrate remaining after protease treatment can be detected specifically by monitoring the signal from the Cy5 fluorophores incorporated at specific Cysteine residues in the polypeptide chain. This fluorophore is easily detected on a fluorescence gel scanner. When these substrates are incubated with liposomes and SecA, the substrate can pass completely into the liposome yielding a full-length protected species (Fig 3.3A). However, if methotrexate is added to the reaction, the DHFR domain folds stably and stalls translocation. In this case, only an N-terminal segment of the polypeptide is protected from degradation by SecY and the liposome. If the fluorophore-labeled Cysteine is in this region, protease treatment yields a smaller, labeled, protected fragment (Fig 3.3A). If the label is on a segment of the substrate exposed to the proteinase in the intermediate complex, no protected species will be seen. Though, if the label is close to the DHFR, a labeled fragment representing this domain will appear, as the DHFR is too tightly folded to be proteolytically...
susceptible (Arkowitz et al., 1993) (Fig 3.3B).

Because the N-terminal protected fragment is only visible if it contains the labeled Cysteine, moving this Cysteine throughout the sequence provides a method for mapping the exact boundary between the proteolytically protected and unprotected regions of the substrate. Using a series of substrates containing Cysteines at various positions between residue 296 and 342, we
Figure 3.4. Position of MTX-stalled intermediate in the presence of ATP and ATPγS. (A) DHFR fusion substrates with labels at the indicated positions were incubated with SecY.
mapped the boundary to a position between residue 334 and 337, in the presence of ATP (Fig S3.3A,B). Each of the label positions N-terminal to residue 334 yield a band corresponding to the intermediate fragment, while each of the positions more C-terminal to 334 produce a band corresponding to the DHFR domain when digested (Fig S3.3A). This folded region, therefore, likely protects the segment of polypeptide about 10-15 amino acids directly preceding it from degradation. Digestion of a construct with a fluorophore species at residue 334, 18 residues N-terminal to the DHFR domain, yielded both fragments: the N-terminal intermediate and the DHFR domain (Fig S3.3A). This position is near the boundary between the region of substrate protected from degradation by SecY and the region protected by degradation by the stably folded DHFR.

To test if ATP binding caused the translocation substrate to move further into the SecY channel, we repeated the previous experiment, with the same fluorophore positions, after adding the slowly hydrolysable analog ATPγS to the preformed translocation intermediate complexes. As before, in the presence of ATP, the digestion product corresponding to the N-terminal protected fragment was visible with substrates labeled through residue 334 (Fig 3.4A,B). However, the addition of ATPγS shifted the boundary of the protected fragment to residue 342 (Fig 3.4A,B). Furthermore, the molecular weight of the N-terminal fragment protected after ATPγS addition was slightly higher (Fig 3.4A) than with ATP. Together, these data are consistent with a larger segment of the substrate becoming protease protected after the addition of ATPγS than in the presence of ATP.

The shift in the size of the protected species on an SDS-PAGE gel offers a more direct opportunity to study the movement of the stalled substrate upon addition of non-hydrolyzable
analogs. It can be visualized with only a single labeled substrate, eschewing the need for a whole array of Cysteine mutants. To verify that the previously seen shift is due to ATPγS binding, we added a titration of the nucleotide to preformed complexes using substrate labeled at position 266, well inside the liposome. As the concentration of ATPγS added increased, the fraction of the intermediate substrate shifted up in the gel from its initial position grew correspondingly.

Figure 3.5. Shift of MTX-stalled intermediate after the addition of ATPγS and WB mutant SecA. (A) DHFR fusion substrates with a label at position 266 was incubated with SecY proteoliposomes and SecA in the presence MTX and ATP. After formation of intermediate, ATPγS was added at the indicated concentration and incubated for the indicated period before proteolysis. Samples were analyzed by SDS-PAGE and fluorescence scanning. (B) As in (A), but with Walker B mutant SecA (WB) in place of ATPγS. (C) Intermediates were formed as in (A) with ATP or ATPγS as indicated. Proteolysis was then carried out at 4°C (lanes 1, 2) or at 22°C (lanes 3-14) for the indicated time.
(Fig 3.5A). Also apparent was the increase in a fragment slightly smaller than the full length fusion substrate as the ATPγS concentration increased (Fig 3.5A). This fragment was at times visible in the presence of ATP alone (Fig 3.4A), but its prevalence increased with ATPγS.

We hypothesized that this fragment was due to tight binding of SecA to the substrate blocking proteinase K access to the polypeptide segment between SecY and the DHFR. This would allow proteolysis to only occur at the very C-terminal tail of the substrate where the disordered biotinylation tag can be degraded. Indeed, unlike the full length substrate, this fragment cannot be detected by blotting with fluorescent streptavidin (Fig S3.4). Also, digestion of intermediate complexes incubated with ADP•BeF₆, rather than ATPγS, which stabilizes the interaction between SecA and SecY, yielded only the C-terminally truncated product (Fig S3.4). If ADP•BeF₆ does move the polypeptide forward, the shift is not visible as tight SecA binding obscures any potential cleavage sites at the entrance of the SecY channel.

Walker B ATPase mutants present a second method to monitor the effect of a single SecA ATP-binding event on the movement of the translocating polypeptide chain. These mutations in the nucleotide binding domain allow the ATPase to bind, but not hydrolyze ATP, in contrast to Walker A mutations which abolish ATP binding. The D209N mutation of *E. coli* SecA is a well-characterized Walker B mutation that functions as a dominant negative ATPase *in vitro* (Mitchell & Oliver, 1993; Economou *et al.*, 1995; van der Wolk *et al.*, 1995; Bauer *et al.*, 2014). The ATP-bound mutant binds tightly to SecY. However, as it cannot hydrolyze and release the nucleotide, it is unable to disengage the translocon, blocking the binding of wild-type proteins and inhibiting translocation.

Though they cannot initiate translocation, Walker B (WB) SecA mutants efficiently replace wild-type SecA at stalled translocation intermediate complexes (Bauer *et al.*, 2014). As they cannot hydrolyze their ATP, this effectually captures the translocation complexes in the ATP-bound state. Incubation of stalled intermediate complexes with WB mutant SecA before protease treatment slightly shifts a population of the protected substrates towards higher molecu-
lar weights, resulting in a doublet band just above the initial stalled position (Fig 3.5B). Increasing the concentration of WB SecA improves the efficiency of this shift, as well as produces the higher molecular weight band seen with ATPγS and ADP•BeF₆, which corresponds to digestion of just the substrate’s C-terminal tag (Fig 3.5B).

Strangely, the shift produced by the binding of WB SecA is not the same as that formed by incubation of preformed complexes with ATPγS (Fig 3.5A,B). One potential reason for this is that the change in the size of the protected species in the presence of ATPγS is only partially due to an actual movement of the polypeptide substrate. Tight binding of the SecA to the substrate could be protecting several additional residues just outside of the SecA channel. In support of this hypothesis, protease digestion of the ATPγS-bound complex at room temperature for increasing durations yields less of both the C-terminally truncated band and the shifted fragment seen previously after incubation with ATPγS (Fig 3.5C). In place of these fragments, a new fragment appeared just above the initial stalled position producing a doublet band nearly identical to that seen in the presence of the WB SecA mutants (Fig 3.5C). Protease treatment of the ADP•BeF₆-stabilized complex at room temperature for 45 min also produced less of the C-terminally truncated fragment and revealed fragments similar to both the ATPγS and WB mutants—protected substrate species (Fig S3.5A). The WB mutant protected fragments were unchanged after increased protease incubation time and temperature (Fig S3.5B). Together, these data suggest that only the shift in the size of the stalled substrate visible after the addition of WB mutant SecA is caused by a movement of the polypeptide. The non-hydrolyzable ATP analogs ATPγS and ADP•BeF₆ also produce this movement when added to translocation intermediate complexes, but it’s masked by tight binding of the SecA extending the region of substrate protected from protease digestion beyond the entrance to the SecY channel.

The presence of the well-folded DHFR domain, which was fused to the C-terminus of the substrate to stall translocation, contributed to the difficulty in measuring substrate movement by protease protection. When the complex was incubated with ADP•BeF₆, the en-
tire substrate, except for the C-terminal tag, was protected from degradation. Therefore, we switched to an alternate method of stalling the translocating polypeptide in the SecY channel. Two Cysteines inserted into the C terminus of a truncated proOmpA substrate form a disulfide when oxidized. The resulting loop, much like the folded DHFR domain, is too sterically large to fit through the SecY pore. By oxidizing or reducing the buffer reaction, translocation of this model substrate can be alternatingly stalled and released within the same experiment (Bauer et al., 2014). Subsequent protease treatment shows a full length substrate gets translocated under reducing conditions, while only an N-terminal fragment is protected in oxidizing conditions (Fig S3.6). As before, ATP is required for translocation and detergent compromises the liposomes’ ability to exclude protease from their lumen (Fig S3.6). Unlike the fluorophore-labeled substrates, the disulfide looping substrates can be generated via *in vitro* translation in the presence of S\(^{35}\)-methionine, yielding radio-labeled protein that can be visualized using autoradiography.

These stalled substrates formed in oxidizing conditions are stable over several minutes if provided hydrolysable ATP (Fig 3.6). However if the ATP is scavenged by the addition of hexokinase and glucose, the substrates will slide backwards out of the channel (Fig 3.6). As before, non-hydrolyzable ATP analogs or WB mutant SecA added to the intermediate translocation complexes produce apparent forward shifts when added to preformed complexes (Fig 3.6). However, unlike the shifts generated with the DHFR fusion substrate, the shifted fragments generated by ATP\(^{\gamma}\)S and ADP\(^{\cdot}\)BeF\(_{\delta}\) seem resistant to protease treatment at room temperature for up to an hour (Fig S3.7). Some degradation happens after 45-60 minutes, possibly because the liposomes become leaky, but the degradation is of the entire stalled fragment. No new bands are revealed smaller than those initially observed.

The shifted fragments generated by the addition of ATP\(^{\gamma}\)S or WB mutant SecA are qualitatively different than those generated by ADP\(^{\cdot}\)BeF\(_{\delta}\) and seem to contain several protected species of similar, but not identical, sizes rather than one clear band (Fig 3.6). We hypothesized this may be due to differences in nucleotide hydrolysis rate between the three analogs. ATP\(^{\gamma}\)S is
a slowly hydrolyzable, rather than non-hydrolyzable nucleotide, and the D209N WB mutant is only partially, not entirely, incapable of ATP hydrolysis (Mitchell & Oliver, 1993; van der Wolk et al, 1995). ADP•BeFx, though, is strictly non-hydrolyzable. During the ADP-bound phase with the slowly-hydrolyzing ATPγS or WB mutant SecA the substrate can slide back slightly, possibly smearing the observed shifted band.

To test this, we reduced the disulfide after forming stalled complexes, releasing the substrate and allowing translocation to be completed if the complex is supplied with ATP (Fig S3.8). However, translocation does not complete in the presence of ADP and the substrate slides back out of the channel (Fig S3.8). The addition of ADP•BeFx concurrent with the reduction of the disulfide, permits neither translocation to complete nor the substrate to slide backwards (Fig S3.8). If the disulfide is reduced at the same time that ATPγS or WB mutant SecA are added, translocation proceeds, but at a much slower rate (Fig S3.8), consistent with slow nucleotide hydrolysis.

Figure 3.6. Shift of disulfide-stalled substrates after addition of different nucleotides or ATP-hydrolysis mutant SecA. Radio-labeled disulfide looping substrates were incubated with SecY proteoliposomes and SecA in the presence of NaTT and ATP forming a translocation intermediate (lane 1). Subsequently hexokinase and glucose, to scavenge ATP, or ADP•BeFx, ATPγS, or Walker B mutant SecA (WB) were added and incubated for the indicated period before proteolysis (lanes 2-6). After proteolysis, samples were analyzed by SDS-PAGE and autoradiography.
Interestingly, in the presence of ATPγS, the substrate accumulates at a single band, of similar size to that protected in the presence of ADP•BeF₆⁻, before translocation can complete, indicating that these two nucleotides two produce the same initial shift of the substrate upon binding to SecA. Thus, the variance in nucleotide hydrolysis likely accounts for the difference in the size and sharpness of the protected species seen after the addition of ATPγS, WB mutant SecA, and ADP•BeF₆⁻.

We next wondered whether the movements of the polypeptide chain seen upon ATP binding to SecA were truly due to the two-helix finger as our single molecule data predicted. Two-helix finger mutants, much like the WB mutant, cannot initiate translocation, although they are capable of completing translocation with reduced efficiency in the presence of ATP (Erlandson et al., 2008a; Bauer et al., 2014). Therefore, stalled intermediate complexes were formed with WT SecA before substituting it for a mutant copy with a defective two-helix finger. One method for introducing THF mutants was to carry them as additional mutations on D209N WB mutant SecA. As the WB SecA is a dominant negative, which has already been shown to efficiently replace WT SecA (Bauer et al., 2014), it can introduce a defective THF into the stalled complex. As before, the WB mutant with a wild-type THF caused the substrate to move forward slightly when added to stalled intermediate complexes (Fig 3.7). However, when WB mutants with a single point mutation at the conserved Tyr located at the tip of the THF were added to the stalled complexes, no shift was observed. The substrate did not slide back out of the channel, consistent with it having a functional clamp to hold the polypeptide in place, but it could not push it forward into SecY (Fig 3.7). Conversely, the addition of a WB mutant with a point mutation in the clamp, believed to lock the clamp in an open conformation (Chen et al., 2015), allows the substrate to readily slide out of the channel (Fig 3.7). Together these data support a model where the THF is responsible for the forward movement of the translocating polypeptide, and the clamp prevents the substrate from backsliding during the ATP-bound phase.

An alternative method to introduce mutant SecA to preformed translocation intermediate
complexes makes use of SecA proteins whose N-terminal lipid binding domain is replaced by a Histidine tag (SecA dN20::H6) (Bauer et al., 2014). This N-terminal helix is necessary to target SecA to the plasma membrane, and mutants without it lack in vitro translocation activity. However, if this domain is substituted by a H6 tag the resulting SecA can be specifically targeted to lipid bilayers that contain NTA-Ni modified lipids, rescuing their translocation activity (Fig S3.9A,B). This interaction is easily disrupted by the addition of high concentrations of imidazole, which competes with the His-tagged SecA for Ni binding (Fig S3.9A,B).

Accordingly, translocation intermediate complexes were formed by SecA dN20::H6, which was then washed off and replaced with wild-type or mutant SecA. We found the washout of HisTagged SecA to be less efficient than previously reported (Bauer et al., 2014). A population of translocation competent SecA dN20::H6 remained for up to 6 minutes at room temperature after the addition of imidazole, even though most of the substrate slid out of the channel during this period (Fig S3.10). Therefore, only mutations that prevented the polypeptide from backsliding after addition of imidazole could be studied with this assay. When replacing SecA dN20::H6 after the addition of imidazole, only WT SecA and SecA with a single THF point mutation
Figure 3.8. Shift of disulfide-stalled substrates after addition of SecA with THF or clamp mutations. (A) Radio-labeled disulfide looping substrates were incubated with SecY proteoliposomes containing Ni-NTA lipids and dN20::H6 SecA in the presence of NaTT and ATP forming a translocation intermediate. Imidazole and the indicated SecA mutants were added and incubated for an additional 10 minutes (lane 1). ATPγS was then added and incubated for the indicated period before proteolysis (lanes 2-6). After proteolysis, samples were analyzed by SDS-PAGE and autoradiography. (B) As in (A), but DTT was added to the reaction at the same time as ATPγS.
(Y794G) could keep the intermediate from backsliding out of the channel in the presence of ATP long enough to fully wash off the His-tagged copy (Fig S3.11). More severe THF mutants and clamp mutants could not restrain the substrate (Fig S3.11) for long enough.

The single point mutation at the tip of the two-helix finger had been previously shown to be capable of both maintaining the intermediate and completing translocation in the presence of ATP after reduction of the disulfide, though with reduced efficiency (Bauer et al., 2014). However, unlike wild-type SecA substituted for SecA dN20::H6, this mutant was unable to produce any shift of the substrate after the addition of ATPγS (Fig 3.8). Furthermore, once the disulfide was reduced in the presence of ATPγS, the THF mutant was unable to complete translocation of the released substrate, whereas the wild-type SecA completed translocation, through the shifted fragment, with kinetics similar to those seen earlier (Fig 3.8). This single point mutation is, therefore, defective in converting the THF power stroke into a forward movement of the translocating polypeptide.

3.3.2 Crosslinking Assays

These protease protection data, including experiments with both DHFR fusion substrates as well as with disulfide looped substrates, suggest that the SecA ATPase does in fact push the translocating substrate into the SecY channel with its THF upon each ATP-binding event. However, the assay is incapable of offering more precise information as to the size of this movement. The conformational change of the two-helix finger seen by single molecule FRET was quite large, but could not be accurately measured as both the high and low FRET states occurred in the less sensitive regions of the FRET curve. From the gels, we could estimate the shift in size of the protected fragment to be a kDa or more. However, this size change is not an accurate proxy for the movement of the substrate. While it is a highly promiscuous protease, proteinase K cleaves preferentially at the carboxyl side of aromatic and aliphatic residues. A large observed shift in the gel could result from a small movement of the polypeptide if that movement happened to protect
a preferred cleavage site on the substrate.

Therefore, we turned to an alternate method for quantifying the magnitude of the poly-peptide movement after ATP binding. Cysteines placed in the substrate can be crosslinked to a Cysteine introduced into the pore of SecY (position 282) (Bauer & Rapoport, 2009). We introduced single Cysteines into a Cysteine-free in vitro translated substrate containing the first 177

Figure 3.9. Disulfide crosslinking efficiency as a function of substrate Cysteine position in the presence of ADP•BeFx or WB mutant SecA. (A) Radio-labeled DHFR-fusion substrates with Cysteines at the indicated positions were incubated with SecY-282C proteoliposomes containing in the presence of MTX and ATP forming a translocation intermediate. WB mutant SecA was added to the reaction along with CP3, to induce disulfide formation between the substrate and SecY. Crosslinking was quenched by the addition of NEM and samples were analyzed by SDS-PAGE and autoradiography. (B) Quantification of (A). Crosslinking efficiency is the fraction of total substrate in each lane found in the crosslinked band. The black circles and diamonds are data points from two replicates. The black line is the mean. The gray line is the data from crosslinks formed in the presence of ATP alone (Fig S3.12). (C) As in (A), but with ADP•BeFx in place of WB mutant SecA. The positions of the uncrosslinked and SecY-crosslinked substrates are indicated. (D) Quantification of (C).
amino acids of proOmpA fused with a DHFR domain through a short G-S linker. As before, folding of the DHFR domain in the presence of methotrexate caused a translocation blockage, stalling the substrate in the SecY channel (Bauer & Rapoport, 2009). Once oxidized, disulfides form between the stalled substrate and the SecY channel (Fig S3.12A). The efficiency of the crosslinking depends on the proximity of the two Cysteines in the stalled complex (Fig S3.12B). In the presence of ATP, the maximal crosslinking efficiency occurred with the substrate Cysteine positioned at residue 152 (Fig S3.12B,C): a replication of published results (Bauer & Rapoport, 2009). However, the crosslinking efficiencies observed were roughly half those previously reported. Also, a large percentage of the substrate material aggregated and stayed in the stacking gel during SDS-PAGE.

The addition of ATPγS or WB mutant SecA would be expected to move the position of maximal crosslinking efficiency towards the C-terminal of the substrate as more of the polypeptide chain is pushed into the SecY channel. However, this was not observed. Strangely, the addition of WB mutant SecA caused the crosslinking peak to shift to residue 140, towards the polypeptide’s N terminal, suggesting the substrate slid backwards (Fig 3.9A,B). The addition of ADP•BeF₆ seemed to partially inhibit crosslinking: disulfide formation was much less efficient in the presence of the nucleotide. Despite the lower signal, the general profile of the crosslinking efficiency as a function of Cysteine position within the substrate was similar in the presence of ADP•BeF₆ as with ATP (Fig 3.9C,D). It’s possible that the polypeptide slips backwards over 10 amino acids in the presence of WB SecA or that it moves less than the 3 residue distance between adjacent inserted Cysteines with ADP•BeF₆. However, given the low rates of disulfide formation and substrate aggregation problem, the data are not of high enough quality to make such statements.

In lieu of disulfide crosslinking, we turned to photo-crosslinking to monitor substrate movements in the SecY pore. Single 4-Benzoyl-L-phenylalanine (Bpa) residues were incorporated into SecY using amber suppression at the same position where we had previously inserted a
Cysteine (SecY-282Bpa) (Chin et al, 2002). When irradiated with UV light, this non-natural amino acid forms a covalent linkage with any C—H groups in its immediate proximity (Kauer et al, 1986). These crosslinked species can then be analyzed by tandem mass spectrometry to identify the location of the bonded residues on either protein, with potentially single amino acid resolution, though the resolution can be sequence sensitive (Leitner et al, 2016; O’Reilly & Rappsilber, 2018).

Translocation intermediates formed with SecY-282Bpa and in vitro translated S\(^5\)-Me-thionine labeled substrates containing C-terminal disulfide loops to stall polypeptide in the SecY pore formed crosslinks when irradiated with UV light (Fig S3.13). Crosslinking efficiency improved with increased irradiation time and was dependent on the addition of ATP during translocation (Fig S3.13A,B). Crosslinks failed to form when the substrate disulfide was reduced, likely because the occupancy of the in vitro translated polypeptide chain in the SecY channel is low unless it is stalled during translocation (Fig S3.13B). When the disulfide loop was oxidized, crosslinks were able to form after the addition of ATP\(\gamma\)S, WB mutant SecA, and ADP•BeF\(_x\) (Fig S3.13B). Several other crosslinked bands were also visible after irradiation (Fig S3.13B). How these species may have formed and be visible is unclear, as only the SecY has a Bpa residue, and only the substrate should be detectable via autoradiography of its the radio-labeled Methionine residues.

In vitro translation, however, does not produce enough substrate to provide adequate material for mass spectrometry experiments. We therefore overexpressed and purified the C-terminal disulfide looping substrate from E. coli. The inclusion of a Myc epitope in the N-terminal intermediate allowed the detection of the protease protected or crosslinked substrate by Western blot. This substrate could be crosslinked to Bpa-derivatized SecY in the same manner as the in vitro translated substrate (Fig 3.10A). Unlike the in vitro translation substrate, however, the crosslinking of the chemically purified substrate was not sensitive to the redox state of the buffer. Crosslinks could be formed in the presence of both NaTT and DTT (Fig 3.10A). This is likely
Figure 3.10. Photocrosslinking of purified substrates to SecY channel. (A) Disulfide looping substrates containing an interior myc epitope in the intermediate were incubated with SecY-282Bpa proteoliposomes and SecA in the presence ATP (lane 2). Where indicated, the reaction was performed in the absence of ATP (lane 3) and proteolysis in the presence of Triton X-100 (TX-100) (lane 4). The addition of methotrexate (NaTT) caused the two substrate Cysteines to form a disulfide, stalling translocation (lane 6,7,8), whereas the addition of DTT inhibited disulfide formation and allowed complete translocation (lanes 5,9,10). Proteinase was added where indicated to determine the translocated species (lanes 2-6). UV irradiation allowed photo-crosslinks to form between the Bpa residue in SecY and nearby substrate residues (lanes 7,9). Lane 1 is 10% of the input substrate sample. After proteolysis or crosslinking, samples were analyzed by SDS-PAGE and Western blotting for the myc epitope. The sizes of the translocation intermediate (IM), the full length substrate (pOA FL) and the SecY-crosslinked substrate (pOAxY) are indicated. (B) Stalled translocation complexes were formed as in (A) in the presence (lane 2,3) or absence (lane 4) of ATP. Irradiation with UV light (lanes 2,4,5,6,7) formed crosslinks between SecY and the substrate. Where indicated WB mutant SecA, ATPγS, or ADP•BeFx were added before irradiation. Samples were analyzed by SDS-PAGE and stained with coomassie blue. The sizes of SecA (A), hexokinase (HK), SecY (Y), SecE (E), and SecG (G) are all indicated. Lane 1 is 10% of the input substrate sample.

because the higher concentration of substrate in the reaction ensures the channel is nearly saturated, even though individual substrates pass through the SecY channel rapidly when reduced. The crosslink species could also be detected without blotting as faint coomassie-stained bands (Fig 3.10B). These crosslinks could be formed in the presence of WB SecA mutants, as well as ATPγS and ADP•BeF₆ (Fig 3.10B).

Comparison of the residues crosslinked to the SecY channel with each of these conditions should provide some insight into the magnitude of the polypeptide movements. However, these crosslinked species have not yet been identified by mass spectrometry, so the resolution of the assay is not yet clear.
3.4 Discussion

Structural and single molecule experiments have converged on a model for SecA function where the ATPase makes large conformational changes to push the translocating polypeptide through the SecY channel (Bauer et al., 2014; Catipovic et al., 2019; Ma et al., 2019). Specifically, ATP binding to the NBDs causes the THF to insert into the channel, pushing the polypeptide with it. The clamp between the PPXD and NBD2 then closes to hold the polypeptide in place while the THF retracts during ATP hydrolysis. Finally, upon release of the inorganic phosphate, the clamp reopens, releasing the polypeptide chain and allowing it to slide freely through the SecY channel until the next ATP-binding event.

Existing biochemical data supports parts of this model. It was clear that the polypeptide could diffuse forwards and backwards during the ADP-bound phase (Erlandson et al., 2008b; Bauer et al., 2014). The importance of the THF finger was also well established: its tip makes close contact with the substrate and several conserved residues at this interface are immutable (Erlandson et al., 2008a; Bauer et al., 2014). However, direct evidence that the THF’s conformational changes are responsible for pushing the polypeptide across the membrane were lacking. In this Chapter, we’ve addressed this deficiency in the model by using a series of assays to observe the movement of the polypeptide in the SecY channel after ATP binding to SecA. While data from single molecule experiments and crosslinking experiments have yet to shed new insight on whether the polypeptide does in fact move through the channel in large ATP-dependent steps, protease protection experiments provide clear evidence that ATP-binding to SecA causes the polypeptide to make a large movement forwards through SecY. This movement is dependent on the two-helix finger, while the clamp domain plays a role in keeping the substrate from sliding when SecA is in its ATP-bound state.

We repurposed the single molecule FRET assay used in Chapter 2 to observe the conformational dynamics of SecA and monitor the position of the translocating substrate in the SecY pore. In principle, we expected the FRET measured with different Cysteine mutant substrates to
correlate with estimates based on the positions of the labeled residues, in the presence of ADP•BeF₆, in the Cryo-EM structure of the complex. However, this was not observed. There seemed to be no correlation between the measured FRET and the position along the substrate of the acceptor fluorophore. Beyond that, many of the substrate unexpectedly demonstrated dynamic FRET in both the presence of ATP and ADP•BeF₆. While this is potentially an intriguing observation, the efficiency of formation of FRET-competent complexes in our single molecule assay was so low as to put in question the validity of any data collected. We may have only observed FRET from improperly assembled or aggregated complexes, which would explain the lack of consistency between these FRET data and the structural model.

It’s also possible the additional stabilization agents in the cryo-EM structure affected the observed position of the substrate. The introduction of a disulfide crosslink between the translocating substrate and the plug domain, as well as a nanobody fused to SecA with affinity for the stalling GFP sequence, may have moved the substrate by about 10 residues from its steady state position (Ma et al., 2019). The position of the maximal disulfide crosslinking efficiency between stalled translocation intermediate substrates and the pore ring of SecY was 30 residues N-terminal to the folded DHFR (Bauer & Rapoport, 2009), whereas the substrate residue proximal to this SecY position in the structure is 45-50 amino acids before the folded GFP. This might also explain why our FRET did not match well with the expected values generated from the structural data. Protease protection assays agree more closely with the disulfide measurement, as the position where protection by SecY begins, is 18-23 residues N-terminal to the DHFR. The discrepancy between the crosslinking and protease protection data is to be expected, as the channel likely protects a portion of the translocating polypeptide on the cytosolic side of the pore ring.

In principle, further optimization could still resolve the issues with this single molecule approach. However, an alternative ensemble biochemistry approach proved to be a more accessible tool for monitoring substrate movements. By incubating stalled intermediate complexes with non-hydrolyzable ATP analogs we could synchronize, and thus observe in bulk, the shift of the
translocating polypeptide upon binding of the ATPase to the nucleotide as a change in the size of the protease protected substrate species. This shift could be induced by adding non-hydrolyzable ADP•BeF$_x$, slowly hydrolysable ATPγS, or slowly hydrolyzing dominant negative Walker B SecA mutants with ATP. Furthermore, the motion of the substrate is dependent on the residues at the tip of the two-helix finger. Mutation of a conserved Tyrosine produced a SecA mutant that could interact with substrate and prevent it from backsliding, but could not push it further into the SecY channel.

The apparent shift in the size of the proteinase-protected fragment as analyzed by SDS-PAGE is roughly 1 kDa, corresponding to a step size of roughly 8-12 amino acids. While such a large shift seems consistent with the large magnitude of the FRET change observed between the THF’s inserted and withdrawn states, such an estimation using a proteinase protection assay is extremely approximate. For one, SDS-PAGE separation is an inexact method for measuring protein mass, as charge and folding state can also affect migration. More troublesome, though, is that the proteinase protection of the substrate does not necessarily correlate exactly with its position in the SecY channel. Proteinase K, while a relatively promiscuous enzyme, has preferred cleavage motifs at the C-terminal peptide bond of aromatic or aliphatic residues. The occlusion of such a cleavage site by a small movement could appear as a much larger step if the next available cleavage site is several residues distant.

To develop a more quantitative assay to measure the step size of the translocating polypeptide as it is pushed by the SecA THF, we are turning to cross-link mass spectroscopy. Cross-links can form between the stalled polypeptide chain and photosensitive amino acids inserted into the SecY channel in the presence of ATP or non-hydrolyzable nucleotide analogs. Analyzing the position at which crosslinks form most efficiently should provide a more rigorous approach to answer this question.

The size of these steps is of general interest, as there is a discrepancy in existing data on step sizes of protein-translocating AAA ATPases. Single molecule studies, generally using optical
tweezers, have observed substrates moving through the central pores of hexameric ATPases such as ClpX and ClpB with step sizes upwards of 10 amino acids (Aubin-Tam et al, 2011; Sen et al, 2013; Olivares et al, 2014; Rodriguez-Aliaga et al, 2016; Olivares et al, 2017; Avellaneda et al, 2020). However, cryo-EM structures of these same proteins, as well as several other ATPases, often with bound substrates, show a distance of only 6 to 7 Å between adjacent ATPase pore loops, suggesting a step size of only 2 residues (Dong et al, 2019; De la Peña et al, 2018; Puchades et al, 2017; Gates et al, 2017; Twomey et al, 2019; Fei et al, 2020).

Whether or not SecA is representative of these hexameric ATPases is unclear. Like many hexameric ATPases, it contacts the translocating polypeptide through an aromatic residue position at the tip of loop, suggesting some level of conservation. However, it is only a monomer, and may therefore have evolved a unique mechanism for substrate processing. The fact that it is a monomer though, presents an opportunity to measure biochemically a single step of the motor caused by a lone ATP hydrolysis event. Both our single molecule FRET data, as well as our protease protection data, suggest that this step size may be closer to that seen for other ATPases using optical tweezers, than predicted from structures, but more quantitative data are necessary before such a claim can be made.

Despite their limitations in estimating the step size, the protease protection data provide a strong validation for the SecA translocation model presented in Chapter 2. The binding of ATP to SecA, captured in our assays by non-hydrolyzable nucleotide analogs, or Walker B SecA mutants clearly pushes the substrate into the SecY channel in a manner dependent on the THF. Once pushed, the substrate is held by the clamp while the THF resets. Even with a bound ATP, SecA cannot prevent the substrate from sliding if the clamp is held in an open conformation. While the power-stroke resulting from the THF movement is likely a mechanism conserved in other ATPases that move polypeptide chains, the magnitude of this movement may vary from ATPase to ATPase. Further development of both the cross-linking and single molecule FRET assays presented here from their current preliminary stage will be necessary to better answer this question.
3.5 Material and Methods

3.5.1 Protein Expression and Purification

SecA, along with all SecA mutants excepting Walker B mutants, SecYEG derivatives (excepting SecY-282Bpa), and proOmpA(FL)-DHFR-Avitag were expressed as described in Section 2.5.1. Walker B SecA (WB SecA), along with WB SecA containing THF and clamp mutations, were expressed as previously described. Cysteine-free WB SecA N95 (lacking the non-essential C-terminus (Matsuyama et al., 1990)) with a C-terminal His-6 tag was cloned into a pET30b (EMD Millipore, Burlington, Massachusetts) vector and expressed in BL21(DE3) E. coli (New England Biolabs, Ipswich, Massachusetts) for 4 hrs at 37 °C after induction at OD₆₀₀ 0.8 with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were collected by centrifugation for 10 min at 4000 x g, resuspended in buffer A (50 mM HEPES/KOH pH 7.5, 300 mM NaCl, 15 mM imidazole, 20 mM b-mercaptoethanol (BME)), and lysed by two passes through an EmulsiFlex-C3 (Avestin, Ottawa, Canada) at 20,000 psi. The resulting lysate was centrifuged for 45 min at 110,000 x g and the insoluble pellet containing WB SecA was collected. The pellet was incubated in buffer A with 6 M urea for 1 hr at 23 °C and then centrifuged again at 110,000 g for 45 min. The supernatant was bound to 2 ml Ni²⁺ resin, and washed 4 times with 10ml of buffer A containing 6, 4, 2, and finally 0 M urea. The protein was eluted in 5ml of buffer B (50 mM HEPES/KOH pH7.5, 100 mM NaCl) containing 250mM imidazole. The eluate was collected and subjected to anion exchange chromatography (HiTrap Q FF, GE Healthcare Life Sciences, Marlborough, Massachusetts) followed by size exclusion chromatography (Superdex 200 10/300GL, GE Healthcare Life Sciences) in buffer C (50 mM HEPES/KOH pH 7.5, 50 mM KCl).

The three E. coli SecYEG protein components, with an N-terminal His-6 tag on SecE, were cloned into a pBAD22 vector (ATCC, Manassas, Virginia) under a single L-arabinose-in-
ducible promoter. An amber stop codon (TAG) was included at position 282 in SecY to allow incorporation of the non-native amino acid L-4-Benzoylphenylalanine (Bpa). The plasmid carrying a conjugate tRNA for this amino acid and its tRNA synthetase (pEVOL-pBpF, ), as well as the pBAD22 vector carrying SecYEG were cotransformed in BL21(DE3) E. coli (New England Biolabs, Ipswich, Massachusetts). The cells were grown to OD_{600} 0.4 and 1mM Bpa (ChemPep Inc., Wellington, FL) was added to the media. Cells were further grown to OD_{600} 0.8 and induced for 4 hrs at 37 °C by addition of 10 ml 20 % L-arabinose. The cells were collected, lysed, and fractionated in the same manner as for SecA. The membrane fraction was solubilized for 90 min in buffer D (buffer A, 10 % glycerol) with 1 % n-dodecyl β-D-maltoside (DDM, Anatrace Inc., Maumee, Ohio). The extract was subjected to high speed centrifugation at 110,000 x g for 45 min. Subsequent steps were carried out in buffers containing 0.03 % DDM. The protein was bound to 1ml Ni^{2+} resin in buffer D, washed with 50 ml buffer D, and eluted in 5 ml buffer E (buffer B, 10 % glycerol) with 250 mM imidazole. The eluate was then subjected to cation exchange chromatography (HiTrap SP FF, GE Healthcare Life Sciences) and size exclusion chromatography (Superdex 200 10/300, GE Healthcare Life Sciences) in buffer F (buffer C, 10% glycerol).

proOmpA_A21Y_myc_174/212C_H6 as cloned into a pET30b vector and expressed BL21(DE3) E. coli grown to OD_{600} 0.6 for 1 hrs at 37 °C. Cells were collected by centrifugation and lysed by two passes through an EmulisFlex-C3 at 20,000 psi in Buffer A. The lysate was centrifuged for 45 min at 110,000 x g and the insoluble pellet containing proOmpA was collected. The pellet was incubated in buffer A with 6 M urea for 1 hr at 23 °C and then centrifuged again at 110,000 g for 45 min. The supernatant was bound to 0.5 ml Ni^{2+} resin, and washed 4 times with 2ml of buffer A containing 6M urea. The protein was eluted in 2ml of buffer B (50 mM HEPES/KOH pH7.5, 100 mM NaCl) containing 250mM imidazole and 6M urea. The eluate was collected and subjected size exclusion chromatography (Superdex 200 10/300GL, GE Healthcare Life Sciences) in buffer C (50 mM HEPES/KOH pH 7.5, 50 mM KCl) containing 6M urea.
3.5.2 Protein Labeling

SecY was labeled using the protocol described in Section 2.5.2. Single Cysteine derivatives of proOmpA(FL)-DHFR-Avitag were also labeled with a Cy5-maleimide fluorophore using the protocol described in Section 2.5.2, with the exception that all steps were carried out in buffers containing 6M urea.

3.5.3 Liposome Preparation and Membrane Protein Reconstitution

Liposomes were prepared as described in Section 2.5.3, with the exception that liposomes for ensemble biochemical assays were extruded to 400nm, rather than 50nm, and liposomes intended for use with dN20::H6 SecA included 10 %, by mass, of 18:1 DGS-NTA(Ni) (Avanti Polar Lipids, Alabaster, Alabama) as well as 200mM sucrose in all buffers.

3.5.4 Microscope setup

Microscope setup was identical to that described in Section 2.5.4.

3.5.5 Flow Chamber Preparation

Flow chambers were prepared as described in Section 2.5.5.

3.5.6 Single Molecule FRET Complex Assembly and Surface Tethering

Single Molecule FRET complexes were assembled as described in section 2.5.6, with the exception that 1 μM unlabeled proOmpA(1-175)-DHFR-biotin was replaced with 100nM Cy5-labeled proOmpA(FL)-DHFR-biotin and 20 nM Cy5-labeled SecA was replaced with 1 μM unlabeled SecA N95.
3.5.7 Single Molecule Imaging

Single molecule imaging was performed as described in Section 2.5.7.

3.5.8 Extraction of FRET data from videos

Data extraction was performed as described in Section 2.5.8. FRET histograms were compiled from extracted data by a non-weighted aggregation of FRET values calculated from each frame prior to photobleaching/dissociation of all traces for a given mutant/nucleotide combination.

3.5.9 Hidden Markov Analysis

Hidden Markov Model analysis of individual FRET traces was performed in the vbFRET software suite in MATLAB (Bronson et al., 2009). This program finds the idealized parameters, including the number, value, and transition probabilities, of FRET states for each trace using a maximum evidence approach. The program was run with up to 5 discrete FRET states allowed per trace using default parameters, including 10 fitting attempts per trace, a maximum of 100 iterations per VBEM, and a convergence threshold of $10^{-5}$. The most likely number of states represented in each FRET trace was reported.

3.5.10 DHFR-Stalled Translocation Assays

Translocation assays were performed using proOMPA(FL)-DHFR-Avitag(riothin) purified and labeled as described in sections 3.5.1 and 3.5.2 along with WT unlabeled SecYEG proteoliposomes and SecA. Reactions were assembled in buffer H (50 mM Hepes/KOH pH 7.5, 50 mM
KCl, 5 mM MgCl₂, 20 mM BME, 0.2 mg/ml BSA). 100 nM SecA and 100 nM SecYEG proteoliposomes were mixed with 100nm proOmpA-DHFR in a 50 μl total volume. When indicated, 50μM Methotrexate hydrate (MTX, Sigma-Aldrich) was added to the reaction. Reactions were initiated by the addition of 5 mM ATP and incubated for 10 min at 37 °C while shaking at 650 rpm. ATP-free reactions were performed in the presence of 1 U of hexokinase (Roche Applied Science, Germany) and 20mM glucose in place of ATP. For specified reactions, 5mM ATPγS, 1mM ADP•BeFₓ, or 500nM WB SecA were added and incubated for an additional 5min at 37°C. For specific experiments the concentration added or the incubation was varied, as noted in the corresponding figures. Reactions were terminated by transfer to ice and addition of 0.4 mg/ml proteinase K, and, where indicated, 0.2 % Triton X-100. Digests were continued for 45 min on ice, unless otherwise noted, and quenched with 2 mM phenylmethylsulfonyl fluoride (PMSF). Reactions were then precipitated in 10 % trichloroacetic acid (TCA), and resuspended in 1X Laemmli buffer with 300 mM Tris base. Samples were then analyzed by SDS-PAGE. The gels were imaged an Odyssey CLx fluorescence scanner (Li-Cor, Lincoln, Nebraska) using the 700 nm excitation channel with auto-exposure.

3.5.11 Western Blotting

For Western blotting, gels were transferred to a 0.2 μm nitrocellulose membrane using a Trans-Blot Turbo System (Biorad) for 30 min at 25 V and 1 A. The membrane was washed 3 times with TBST (500 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1 % Tween 20) and with blocked with 3% nonfat milk in TBST. Gels were blotted for biotin over night with 100 ng/ml Streptavidin-Dylight800 (ThermoFisher) and for the C-myc epitope over night at 4°C with 100ng/ml THE™ c-Myc Tag Antibody (Mouse, GenScript, Piscataway, NJ). After overnight blotting primary antibodies removed with 3 TBST washes, and for the c-myc blots, the membranes were incubated for 1 hour at 22°C with 100 ng/ml goat anti-mouse IgG-Dylight680 (Invitrogen). Secondary antibody was washed off with three TBST washes and the membranes were imaged using
an Odyssey CLx fluorescence scanner (LiC-Cor) using the 700 nm channel (c-myc blots) and 800 nm channel (biotin blots) with auto-exposure.

### 3.5.12 Disulfide-Stalled Translocation Assays

$^{35}$S-Met-labeled proOmpA containing a truncation from position 176 to 294, 3 additional Methionine residues at positions 47-49, and Cysteines at the resulting positions 167 and 205 (proOmpA-3M-167/205C) was generated by *in vitro* translation. mRNA was transcribed from linearized template with an SP6 promoter followed by the Kozak consensus ribosome binding site directly 5' to the proOmpA gene start codon using a RiboMax SP6 *in vitro* transcription kit (Promega, Madison, Wisconsin). 2 μg of transcription product RNA was mixed with 35 μl of nuclease treated rabbit reticulocyte lysate (Promega), 1 μl of 1 mM amino acid mixture minus methionine (Promega), and 2 μl (.022 μCi) of EasyTag Express S$^{35}$ Labeling Mix (PerkinElmer, Waltham, Massachusetts). Translation product was precipitated by the addition of 150 μl saturated ammonium sulfate and re-suspended in 50 μl 6 M urea pH 6.8.

Reactions were assembled in buffer I (50 mM Heps/KOH pH 7.5, 50 mM KCl, 5 mM MgCl$_2$, 0.2 mg/ml BSA). 100 nM SecA and 100 nM SecYEG proteoliposomes were mixed with 1 μl of the *in vitro* translation products in a 50 μl total volume. Reactions were initiated by the addition of 5 mM ATP and incubated for 10 min at 37 °C while shaking at 650 rpm. When indicated, 400 μM sodium tetrathionate (NaTT, Sigma-Aldrich) or 10 mM Dithiothreitol (DTT, GoldBio, St. Louis, MO) was added to the reaction. Reactions were initiated by the addition of 5 mM ATP and incubated for 10 min at 37 °C while shaking at 650 rpm. ATP-free reactions were performed in the presence of 1 U of hexokinase (Roche Applied Science) and 20mM glucose in place of ATP. For specified reactions, 5mM ATP$_7$S, 1mM ADP•BeF$_x$, or 500nM WB SecA were added and incubated for an additional 5min at 37°C. For specific experiments the concentration added or the incubation was varied, as noted in the corresponding figures. In some cases, 10 mM DTT was added after the initial incubation with NaTT to release the disulfide and observe the
completion of translocation.

For experiments performed with dN20::H6 SecA, reactions were assembled with SecYEG proteoliposomes containing 10 % DGS-NTA(Ni) lipids. After incubation for 10 min at 37°C, 250 mM imidazole, along with 500 nM of the indicated SecA mutants, was added to the reaction. At this point, either 5 mM ATPγS or 1 mM ATP was also added and incubations were continued for the indicated time. In some cases, 10 mM DTT was added prior to additional nucleotides and incubated for additional 10 min.

Reactions were terminated by transfer to ice and addition of 0.4 mg/ml proteinase K, and, where indicated, 0.2 % Triton X-100. Digests were continued for 45 min and quenched with 2 mM phenylmethylsulfonyl fluoride (PMSF). Reactions were then precipitated in 10 % trichloroacetic acid (TCA), and resuspended in 1X Laemmli buffer with 300 mM Tris base. Samples were then analyzed by SDS-PAGE. The gels were vacuum dried, exposed to autoradiography film overnight, and imaged by a Personal Molecular Imager (Bio-Rad).

3.5.13 Floatation Assay

40 μg 400 nm liposomes containing either 100 % E. coli polar lipids (ecPL, Avanti Polar Lipids) or 90 % ecPL and 10 % DGS-NTA(Ni) (Avanti Polar Lipids) was mixed with either 2.5 μM WT SecA or 2.5 μM dN20::H6 SecA in buffer C, including 250 mM imidazole where indicated, in a 20 μl volume. This sample was mixed with 20 μl 80 % Nycodenz (Alere Technologies AS, Oslo, Norway) in buffer C in a 175 μl open top polypropylene ultracentrifuge tube (Part #: 342630, Beckman Coulter, Indianapolis, IN). Above this 40 μl layer was stacked 4 more 40 μl layers containing 30, 20, 10, and 0 % Nycodenz in buffer C, either with or without imidazole. The tubes were centrifuged at 200,000 x g for 1 hour at 25°C in a TLS-55 rotor (Beckman Coulter). After centrifugation, 5 40 μl fractions were taken from each tube. 15 μl of each fraction was mixed with 5 μl of 4X reducing Laemmli buffer, heated to 65°C for 10 min, and
analyzed by SDS-PAGE with coomassie staining.

3.5.14 Disulfide Crosslinking

$^{35}$S-Met-labeled proOmpA (177)-DHFR was generated by *in vitro* translation as described in Section 3.5.13. Reactions were assembled in buffer I. 500nM SecY-282C proteoliposomes, 1 μM SecA, and 1μl of *in vitro* translated substrate were mixed with 1 μl of the *in vitro* translation products in a 20 μl total volume. Reactions were initiated by the addition of 5 mM ATP and incubated for 10 min at 37 °C while shaking at 650 rpm. ATP-free reactions were performed in the presence of 1 U of hexokinase (Roche Applied Science, Germany) and 20mM glucose in place of ATP. Oxidation was performed by incubation for 10 min at 37°C with 50 μM Copper Phenathroline (CP3, Sigma), unless other concentrations are indicated. Cross-linking was quenched by the addition of 10 mM N-ethylmaleimide (NEM, Sigma) for 5 min on ice. 7 μl 4X non-reducing Laemmli buffer was added to the samples, which were then heated to 65°C for 10 min. Samples were then analyzed by SDS-PAGE. The gels were vacuum dried, exposed to autoradiography film overnight, and imaged by a Personal Molecular Imager (Bio-Rad).

3.5.15 Photo-Crosslinking

Reactions were assembled in buffer J (50 mM Hepes/KOH pH 7.5, 50 mM KCl, 5 mM MgCl$_2$). 100 nM SecA and 100 nM SecYEG proteoliposomes were mixed with 200nM of proOmpA_A21Y_myc_174/212C_H6 in a 50 μl total volume. Reactions were initiated by the addition of 5 mM ATP and incubated for 15 min at 37 °C while shaking at 650 rpm. When indicated, 400 μM sodium tetrathionate (NaTT, Sigma-Aldrich) or 10 mM Dithiothreitol (DTT, GoldBio, St. Louis, MO) was added to the reaction. Reactions were initiated by the addition of 5 mM ATP and incubated for 10 min at 37 °C while shaking at 650 rpm. ATP-free reactions were performed in the presence of 1 U of hexokinase (Roche Applied Science) and 20mM glucose in
place of ATP. For specified reactions, 5mM ATPγS, 1mM ADP•BeF₆, or 500nM WB SecA were added and incubated for an additional 5min at 37°C. After incubation the samples were placed on an ice-cold metal block, and a long-wave UV lamp (365 nm, Blak-Ray) was positioned 5 cm from the block. The samples were irradiated for 45 min. Reactions were then precipitated in 10 % trichloroacetic acid (TCA), and resuspended in 1X Laemmli buffer with 300 mM Tris base. Samples were then analyzed by SDS-PAGE stained with coomassie or probed by Western blotting with antibodies against the c-myc epitope.
Chapter 4: Summary and Perspectives

4.1 Mechanism of SecA-Mediated Post-Translational Translocation

The experiments described here suggest a mechanism for how SecA uses energy from ATP hydrolysis to perform the mechanical work necessary for protein translocation. Upon ATP binding to the NBDs, the THF inserts into the channel of SecA. Such an inserted state had been previously visualized in the context of translocating SecA in the presence of ADP•BeF$_{x}$, but we now show that this state is only intermediate, and that the THF moves beyond the observed conformation. The full extent of this movement is not yet clear, but it may insert as far as the pore ring residues of the SecY channel, roughly half way across the membrane. Movement beyond this constriction point seems unlikely as the hydrophobic gasket residues would likely provide a steric blockage halting the THF’s movement. During this movement, the THF makes contact with the translocating polypeptide and pushes it into the channel.

Subsequently, before the hydrolysis of the bound ATP, the PPXD of SecA moves from an open conformation to a closed position, forming a clamp with NBD2 that grips the substrate tightly. Once the substrate is restrained, the THF can reset to its initial withdrawn position without dragging the polypeptide out of the SecY channel with it. Mutations to the PPXD that fix the clamp in an open conformation allow the polypeptide to slide in the channel even in the ATP-bound state of SecA, when the clamp would normally be closed. In the hydrolysis intermediate represented by ADP•BeF$_{x}$, the THF passes through the partially inserted position seen in structures while the clamp is closed. After hydrolysis, the THF is full retracted, but the clamp remains closed until the release of the resultant inorganic phosphate. Finally, the clamp can reopen, releasing the polypeptide chain and allowing it to slide freely until another ATP molecule binds SecA.

This research answers several previously outstanding questions in the field of bacterial
post-translational translocation. For one, it is now clear that the THF is dynamic during translocation. Previous models had proposed that the THF does not move as SecA hydrolyzes ATP (Whitehouse et al, 2012; Allen et al, 2016; Corey et al, 2019). Consistent with this view, all the existing structural models of SecA bound to SecY have captured the ATPase in identical conformations (Zimmer et al, 2008; Li et al, 2016; Ma et al, 2019). However, our single molecule experiments now show that this was a consequence of the ADP•BeF₄ nucleotide used in each purification. The THF, in fact, makes large conformational changes, inserting and withdrawing from the SecY pore as it binds and hydrolyze ATP.

Furthermore, we have shown that these conformational changes of the SecA THF actively push the translocating polypeptide into the SecY channel. Even if the THF is dynamic, it was possible that the translocation of the substrate was only indirectly related to this movement. Other domains of the ATPase could push the substrate forward in response to different stages of the ATP hydrolysis cycle. However, it is now clear that the substrate takes a large step forward into the channel only upon ATP-binding to SecA: the same point at which the THF inserts. Furthermore, while neither the exact size of the THF movement nor the polypeptide step have been measured, their general magnitudes seem of similar scale. Most importantly, though, we have shown that the conserved Tyrosine at the tip of the THF, which has been shown to make close contacts with the substrate (Erlandson et al, 2008a), is crucial for its forward motion. Together, these data provide strong evidence that the motion of the THF provides the power-stroke to translocate the substrate across the membrane.

Finally, we now propose an important role for the clamp domain of SecA during translocation. Previously, it had been thought that once bound to SecY with a polypeptide in its grasp, the clamp remained stationary and merely kept the translocating substrate aligned with the SecY channel (Bauer et al, 2014). Again, this evidence was supported by the similarities in clamp conformations seen between the three structures of SecA bound to SecY. However, the recent cryo-EM structure shows the substrate peptide forms a β-strand to complement a small β-sheet
in the hinge region of the clamp (Ma et al, 2019). This mode of binding would likely hold the translocating polypeptide quite firmly and not allow forward translocation of the substrate, never mind the Brownian diffusion previously observed. Rectifying this contradiction, our single molecule data show that the clamp opens and closes over the course of the ATPase hydrolysis cycle. Crucially, it is open when the THF first engages the substrate upon ATP-binding. This explains why the observed mode of substrate binding in the cryo-EM structure does not hinder the THF’s ability to push the polypeptide into SecY. The clamp then closes after the THF inserts into SecY and remains closed while the finger resets. This presumably holds the substrate in place and prevents the THF from undoing the work it has achieved. Without the clamp, the THF would likely erase the progress of translocation each time it resets during ATP hydrolysis. Finally, the clamp opens again upon inorganic phosphate release, allowing the polypeptide to slide in the channel and making sure it is unhindered and can be moved again by the THF upon the next ATP-binding event.

Together, these new data explain how SecA can function as a processive translocase, even as a monomer. Many AAA ATPases work as hexamers. This ensures that some subset of subunits are holding the substrate in place when others reset. SecA accomplishes this same effect by handing the substrate off between the THF and clamp. If the clamp were not gripping the polypeptide chain tightly while the THF retracts, the finger would likely undo the translocation progress it had made upon insertion, resulting in large number of futile ATP hydrolysis cycles. Our data show how these two domains cooperate to produce processive mechanical work.
4.2 Outstanding Questions in Post-Translational Translocation

4.2.1 Structural Biology

While our data have clarified some of the uncertainty surrounding the SecA mechanism, several other questions remain, or have been newly raised by our results. One important deficit is the lack of structural information regarding the SecA-SecY complex bound to nucleotides other than ADP•BeF$_6$. Our single molecule data suggest that SecA has at least several unseen conformations, but two are crucial for understanding the system. When bound to ATP, we would expect the THF to be inserted further into the SecY channel than previously observed. This conformation could likely be visualized by determining the structure of the complex with a bound Walker B mutant SecA, rather than wild-type, SecA. This mutation impedes ATP hydrolysis, locking the SecA ATPase in the ATP-bound state, rather than the transition state visualized in the presence of ADP•BeF$_6$.

A more difficult conformation to observe would be the ADP-bound state when both the THF and clamp have released the substrate. In this state, the affinity of SecA for SecY is low, preventing co-purification of the complex (Zimmer et al, 2008). However, the structure may be observable as a minor class if pre-established *in vitro* translocation intermediate complexes were incubated briefly with hexokinase and glucose before being quickly frozen onto EM grids. Together these structures would provide a strong test of the model proposed based on single molecule and biochemical data.

4.2.2 Translocation Initiation

The model proposed here only explains how SecA uses ATP hydrolysis to work on substrates already engaged with the SecY translocon. How SecA and SecY cooperate to initiate translocation is less clear. Several mutants that are incapable of translocating substrates *de novo*
can complete the translocation of previously established intermediates, suggesting that the two processes utilize different mechanisms (Bauer et al., 2014). Even basic questions such as the order of interaction between the translocation components remain ambiguous. For instance, does SecA bind substrate when it is in solution (Lill et al., 1990; Akita et al., 1990; Kimura et al., 1991; Gelis et al., 2007; Gouridis et al., 2009), or only after it has bound the membrane through its N-terminal helix (Bauer et al., 2014)? In support of this second hypothesis, most post-translation-al substrate signal sequences contain a basic motif at their N-termini that could potentially recruit them to negatively charged membranes. It’s possible that both SecA and substrate interact with the lipid bilayer independently before binding each other or SecY.

It is also possible that substrates can dock into the SecY lateral gate without the involvement of SecA at all. In eukaryotic post-translational translocation, there is no energy input from the luminal ATPase BiP until after a loop of the substrate has penetrated through the SecY channel and is exposed on the luminal side (Matlack et al., 1999). The initial insertion likely happens through thermal diffusion, supported by the energetic gain of partitioning the hydrophobic signal sequence into the membrane environment outside the lateral gate (Wu et al., 2019). As equilibrium favors this inserted state, energy would only be required to improve the kinetics of insertion. Therefore, many substrates in prokaryotes could potentially insert into the SecY channel spontaneously. In this case, the mutations in SecA that prevent translocation initiation would have some role other than substrate recruitment and insertion in the establishment of translocation complexes.

**4.2.3 Other Energetic Contributions to Post-translational Translocation**

While SecA and SecY are the minimal components necessary for the translocation of model substrates *in vitro*, other factors associate with the translocon *in vivo* that likely improve the efficiency and specificity of translocation in cells. The SecDF complex uses the proton motive force (PMF) generated by the hydrogen gradient across the inner membrane to support
translocation (Schiebel et al., 1991; Pogliano & Beckwith, 1994; Tsukazaki et al., 2011). How the complex couples the flow of protons through the membrane to translocation is unclear. Crystal structures suggest a model where ion flux is coupled to conformational changes of the proteins complex’s periplasmic domain, which binds substrate and could potentially pull the polypeptide chain through SecY (Tsukazaki et al., 2011; Furukawa et al., 2017, 2018). This model has not been sufficiently tested yet, however, and it seems unlikely that the flux of a single proton could induce the conformational changes needed. As such, there may be some mechanism to count or store the energy of multiple ion passages before triggering the movements necessary for translocation activity.

The folding of the translocation substrate on the trans side of the SecY channel, or its binding to periplasmic chaperones, could produce a Brownian ratchet to further drive translocation of diverse substrates. These effects have not been well studied in bacteria, though they are likely to contribute since eukaryotic post-translational translocation is driven entirely by a ratcheting mechanism (Matlack et al., 1999). More comprehensive reconstitutions, using liposome-encapsulated chaperones or substrates that could fold stably only after passage through the SecY channel would provide a valuable tool for the in vitro study of the energetic contributions of ratcheting to bacterial translocation.

### 4.3 Implications for Other Translocating AAA ATPases

Because it is a monomer, SecA provides a unique tool for studying the mechanism of polypeptide translocation by AAA ATPases that share features with SecA. For instance, many AAA ATPases contact their substrates through conserved aromatic residues at the tip of polypeptide loops similar to SecA’s THF (Martin et al., 2008; Rodriguez-Aliaga et al., 2016). This suggests that conformational changes analogous to SecA’s THF movement could also be used to move substrates by these other ATPases. In hexamers, the movements of these individual loops and their contributions to the translocation of the substrate are not easily separated. But, as we
have shown, these movements can be directly visualized in monomeric SecA. Measuring the size of the THF movement in SecA, as well as the resulting movement of the polypeptide, could provide information as to how single subunits of hexameric AAA ATPases process their diverse substrates.

Whether or not the mechanism of SecA is a perfect model for hexameric ATPases is unclear, though. SecA contacts the translocating polypeptide chain through the substrate’s side chains, whereas many hexameric ATPases, such as the proteasomal ATPases and Cdc48/p97 make contacts with the backbone of the substrate (De la Peña et al, 2018; Dong et al, 2019; Twomey et al, 2019). Furthermore, SecA is a relatively weak ATPase, as it cannot translocate tightly folded protein domains. Several hexameric ATPases have powerful unfolding activity or are able to extract proteins directly from cellular membranes or protein aggregates (Aubin-Tam et al, 2011; Bodnar & Rapoport, 2017b). This difference in force could simply be a function of the proteins’ oligomeric states, or it could represent a fundamental difference in their mechanisms.

Despite these caveats, SecA still represents an attractive model for mechanistic studies of AAA ATPases because, as we have shown, single proteins can be labeled and observed in isolation. A wealth of structural knowledge exists on static conformations of ATPases. They have been visualized with varying nucleotide states, cofactors, and substrates. This study, however, represents one of the first real-time observations of conformational changes in a substrate-bound AAA ATPase undergoing hydrolysis, as well as a direct read-out of the resulting movements of the translocating substrate. As such, we think it an important contribution to both the fields of translocation and the study of molecular mechanisms of ATPases in general.
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Appendix

Supplemental Table to Chapter 2

Table S2.1. Sample Sizes in Analysis

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**Figure S2.1. Translocation activity of fluorescently labeled SecA and SecY.** ProOmpA was synthesized in reticulocyte lysate in the presence of 35S-methionine. Translocation was tested with either wild type SecA or SecA in which single cysteines were introduced and labeled with Cy5. Proteoliposomes contained either SecYEG with wild type SecY or SecY labeled at the indicated positions with Cy3. After incubation in the presence of ATP, the samples were treated with proteinase K to degrade any non-translocated material. Where indicated, the reaction was performed with wild type components in the absence of ATP and proteolysis in the presence of Triton X-100 (TX-100). All samples were analyzed by SDS PAGE followed by autoradiography. Lane 1 shows 10% of the input sample.
Figure S2.2. ATPase activity measured with fluorescently labeled SecA and SecY. (A) Wild type SecA and SecYEG complex reconstituted into proteoliposomes was incubated at 24 °C with a fusion of proOmpA with DHFR and ATP. The release of inorganic phosphate (P_i) over time was measured with an enzymatic assay. The grey curves show the actual data of four experiments and the black curve the average. (B-E) As in (A), but with single-cysteine mutants of SecA and SecY labeled with fluorophores at the indicated positions. (F) The slopes in (A-E) and the dependence of ATPase activity on SecA concentration were used to calculate the hydrolysis rates per SecA molecule. The error bars show standard deviations of four replicates.
Figure S2.3. Saturation of translocation sites with proOmpA-DHFR. ProOmpA was synthesized in reticulocyte lysate in the presence of 35S-methionine and incubated with proteoliposomes containing SecYEG in the presence of increasing concentrations of unlabeled proOmpA-DHFR and methotrexate (MTX), a substrate analog of DHFR. The fusion protein generates a stalled translocation intermediate with the MTX-stabilized DHFR domain remaining outside the vesicles. After incubation, proteinase K was added to digest all non-translocated material, and the samples were analyzed by SDS PAGE and autoradiography. As a control, MTX was omitted (lane 14), so that proOmpA-DHFR was completely translocated into the liposomes and did not plug the translocation sites. Lanes 2-4 show samples in the absence of proOmpA-DHFR. In lane 4, proteolysis was performed in the presence of Triton X-100 (TX-100). Lane 1 shows 10% of the input material. Note that proOmpA-DHFR was used for the single-molecule experiments at 1 μM concentration, which is more than sufficient to saturate all translocation sites.
Figure S2.4. Assembly of translocation complexes for single molecule imaging. (A) Translocation intermediates were assembled with proOmpA-DHFR, Cy5-labeled SecA, and proteoliposomes containing SecYEG labeled with Cy3 on SecY. The complexes were immobilized via a biotin-tag at the C-terminus of the substrate to a glass surface containing biotinylated PEG, which was pretreated with neutravidin. Individual SecA and SecY molecules were visualized by TIRF via direct excitation of their fluorophores (left and right halves of the image). (B) As in (A), but without neutravidin preincubation. (C) As in (A), but without substrate. (D) As in (A), but without ATP, so that no translocation intermediate is generated.
Figure S2.5. Binding lifetime of SecA in ATP and ADP•BeF₅ₓ. Survival plot of all SecA binding events delimited by SecA dissociation or bleaching. Binding lifetimes in the presence of ADP•BeF₅ₓ are in plotted red and lifetimes in the presence of ATP in blue.
Figure S2.6. Representative FRET traces for two-helix finger movements in the presence of ATP. (A-C) Representative traces obtained with ATP. The upper FRET trace was calculated from the middle traces obtained by exciting the donor fluorophore and measuring both donor (green) and acceptor (red) fluorescence. The lowest trace was obtained by exciting the acceptor fluorophore directly. Periods in which a fluorescently labeled SecA molecule is bound are indicated by grey shading.
Figure S2.7. Representative FRET traces for clamp movements in the presence of ATP (A-C) Representative traces obtained with ATP. The upper FRET trace was calculated from the middle traces obtained by exciting the donor fluorophore and measuring both donor (green) and acceptor (red) fluorescence. The lowest trace was obtained by exciting the acceptor fluorophore directly. Periods in which a fluorescently labeled SecA molecule is bound are indicated by grey shading.
Figure S3.1. Purified and labeled translocation substrates. (A) Full length proOmpA fused with DHFR containing a C-terminal biotinylation tag was purified from E. coli, labeled on Cysteine residues introduced at the indicated positions with a maleimide-Cy5 fluorophore, analyzed by SDS-PAGE, and stained with coomassie blue. (B) As in (A), but the gel is imaged with a fluorescence scanner detecting the Cy5 fluorophore.
Figure S3.2. Single Molecule imaging of fluorescent translocation complexes. (A) Translocation intermediates were assembled with Cy3-labeled proOmpA-DHFR, SecA, and proteoliposomes containing SecYEG labeled with Cy3 on SecY. The complexes were immobilized via a biotin-tag at the C-terminus of the substrate to a glass surface containing biotinylated PEG, which was pretreated with neutravidin. Individual SecY molecules were visualized by TIRF via direct excitation of their fluorophores. (B) Same field of view as in (A), but with direct excitation of the Cy5 fluorophores on the proOmpA-DHFR substrate. (C) Overlay of images from (A) and (B), with a five pixel offset for clarity. (D) Same field of view as (B), but emission from Cy5 proOmpA-DHFR was collected after direct excitation of the Cy3 on SecY.
Figure S3.3. Position of substrate in translocation intermediate complexes. (A) DHFR fusion substrates with labels at the indicated positions were incubated with SecY proteoliposomes, SecA, and ATP in the presence or absence of MTX before digestion with proteinase K. After proteolysis, samples were analyzed by SDS PAGE and fluorescence scanning. (B) Quantification of the liposome-protected species from (A) in the presence of MTX relative to the protein protected in the absence of MTX. Cyan and magenta traces are two replicates.
Figure S3.4. Substrate C-terminus is exposed to protease in translocation intermediate. (A) DHFR fusion substrate labeled at position 266 with Cy5 was incubated with SecY proteoliposomes, SecA, and ATP in the presence or absence of MTX. ATPγS or ADP•BeFx was added to some samples (lanes 4,5) before digestion with proteinase K. Lane 1 is 10% of the input substrate sample. Samples were analyzed by SDS-PAGE and fluorescence scanning. (B) As in A, but samples were analyzed by Western blot with Dylight800 – labeled streptavidin.
Figure S3.5. Protease resistance of shifted intermediate fragments formed with DHFR fusion substrate. (A) DHFR fusion substrates with a label at position 266 was incubated with SecY proteoliposomes and SecA in the presence of MTX and ATP. After formation of intermediate, WB mutant SecA, ATPγS, or ADP•BeFx was added to some samples (lanes 2,3,4) before proteolysis at 22°C. Samples were analyzed by SDS-PAGE and fluorescence scanning. (B) Intermediates were formed as in (A) with ATP or WB mutant SecA as indicated. Proteolysis was then carried out at 4°C (lanes 1, 2) or at 22°C (lanes 3-11) for the indicated time.
Figure S3.6. Protease Protection Assay Using *in vitro* translated, radio-labeled substrate. Substrate consisting of a truncated proOmpA lacking all but two Cysteines near its C-terminus was *in vitro* translated from rabbit reticulocyte lysate in the presence of S35 Methionine. This substrate was mixed with proteoliposomes containing SecYEG (blue) and SecA (not shown). After incubation in the presence of ATP (lane 2), the samples were treated with proteinase K to degrade any non-translocated material. Where indicated, the reaction was performed in the absence of ATP (lane 3) and proteolysis in the presence of Triton X-100 (TX-100) (lane 4). Under reducing conditions, the Cysteines remain unreacted and the entire substrate translocates into the liposome (FL, lane 5). The addition of oxidant (NaTT) causes the two Cysteines to form a disulfide-bonded loop, stalling translocation and producing an intermediate fragment after protease treatment (IM, lane 6). All samples were analyzed by SDS PAGE followed by autoradiography. Lane 1 shows 10% of the input sample.
Figure S3.7. Protease resistance of shifted intermediate fragments formed with disulfide looping substrate. In vitro translated substrates with C-terminal Cysteine residues were incubated with SecY proteoliposomes and SecA in the presence of NaTT and ATP. After formation of translocation intermediates ATPγS or ADP•BeF₆, was added to the samples as indicated. Proteolysis was then carried out at 4°C (lane 1) or at 22°C (lanes 2-6) for the indicated time.
Figure S3.8. Completion of translocation of disulfide-stalled substrates after addition of different nucleotides or ATP-hydrolysis mutant SecA. Radio-labeled disulfide looping substrates were incubated with SecY proteoliposomes and SecA in the presence of NaTT and ATP forming a translocation intermediate (lane 1). Subsequently hexokinase and glucose, to scavenge ATP, or ADP•BeFx, ATPγS, or Walker B mutant SecA (WB) were added, as indicated, along with DTT to reduce the disulfide and release the intermediate. Complexes were incubated for an additional indicated period before proteolysis (lanes 2-6). After proteolysis, samples were analyzed by SDS-PAGE and autoradiography.
Figure S3.9. Lipid binding and translocation activity of dN20::H6 SecA. (A) Floatation assays with WT and dN20::H6 SecA. The SecA was mixed with the indicated liposomes in the presence or absence of imidazole in the bottom layer of a Nycodenz stack containing, 40, 30, 20, 10, and 0% Nycodenz. After centrifugation, 5 equal fractions were collected and analyzed by SDS-PAGE and coomassie staining. SecA which binds to lipids floats towards the top fraction (T), while SecA that cannot bind remains in the bottom fraction (B). (B) Substrate consisting of a truncated proOmpA lacking all but two Cysteines near its C-terminus was in vitro translated from rabbit reticulocyte lysate in the presence of S35 Methionine. This substrate was mixed with SecYEG proteoliposomes including 10% Ni-NTA lipids and SecA. After incubation in the presence of ATP (lane 2), the samples were treated with proteinase K to degrade any non-translocated material. Where indicated, the reaction was performed in the absence of ATP (lane 3) and proteolysis in the presence of Triton X-100 (TX-100) (lane 4). Under reducing conditions, the Cysteines remain unreacted and the entire substrate translocates into the liposome (FL, lane 5). The addition of oxidant (NaTT) causes the two Cysteines to form a disulfide-bonded loop, stalling translocation and producing an intermediate fragment after protease treatment (lane 7). Imidazole was included in the reactions as indicated (lanes 6,8). All samples were analyzed by SDS PAGE followed by autoradiography. Lane 1 shows 10% of the input sample.
Figure S3.10. Completion of translocation after imidazole dissociation of dN20::H6 SecA from liposomes. (A) Substrate consisting of a truncated proOmpA lacking all but two Cysteines near its C-terminus was in vitro translated from rabbit reticulocyte lysate in the presence of S35 Methionine. This substrate was mixed with SecYEG proteoliposomes including 10% Ni-NTA lipids and SecA. Samples were incubated with ATP and NaTT to form translocation intermediates. Imidazole was then added to these complexes and incubated for the indicated time before proteolysis. All samples were then analyzed by SDS PAGE followed by autoradiography. (B) DTT was added to samples from (A) after incubation for the indicated time with imidazole. Incubation was continued for the indicated times before proteolysis. All samples were then analyzed by SDS PAGE followed by autoradiography.
Figure S3.11. Backsliding of the substrate in the presence ATP with SecA THF and clamp mutants. Substrate consisting of a truncated proOmpA lacking all but two Cysteines near its C-terminus was *in vitro* translated from rabbit reticulocyte lysate in the presence of S35 Methionine. This substrate was mixed with SecYEG proteoliposomes including 10% Ni-NTA lipids and SecA. Samples were incubated with ATP and NaTT to form translocation intermediates (lane 1). Subsequently, imidazole and the specified SecA mutants were added, and incubation was continued for the indicated times before proteolysis (lanes 2-6). All samples were then analyzed by SDS PAGE followed by autoradiography.
Figure S3.12. Disulfide Crosslinking Stalled Translocating Polypeptides to the SecY channel pore. (A) Substrate consisting of the first 177 residues of proOmpA, with a single Cysteine at position 152, fused to a DHFR domain was in vitro translated in the presence of S35 Methionine. This substrate was incubated with SecY 282C proteoliposomes, SecA, and ATP in the presence of MTX to fold the DHFR domain and produced stalled intermediate complexes. To these complexes, the indicated concentration of Copper Phenanthroline (CP3) was added to oxidize the Cysteines and form disulfide crosslinked complexes. Crosslinking was quenched by the addition of NEM and samples were analyzed by SDS-PAGE and autoradiography. (B) SecA, SecY-282C proteoliposomes, and substrates with single Cysteines at the indicated positions were mixed in the presence or absence of ATP. CP3 was added to form crosslinks as in (A). Crosslinking was quenched by the addition of NEM and samples were analyzed by SDS-PAGE and autoradiography. (C) Quantification of (B). Crosslinking efficiency is the fraction of total substrate in each lane found in the crosslinked band. The black line is the mean and error bars are S.D. of 4 replicates.
Figure S3.13. Photocrosslinking of \textit{in vitro} translated substrates to SecY channel. (A) Disulfide looping substrates were \textit{in vitro} translated in the presence of S35 Methionine and incubated with SecY-282Bpa proteoliposomes and SecA in the presence ATP and NaTT. The resulting stalled translocation complexes were UV-irradiated for the indicated period of time. Samples were then analyzed by SDS-PAGE and autoradiography. (B) Intermediate complexes were formed as in (A) in the presence (lane 2) or absence (lane 4) of ATP. The addition of methotrexate (NaTT) caused the two substrate Cysteines to form a disulfide, stalling translocation (lane 2,3,5,6,7,8), whereas the addition of DTT inhibited disulfide formation and allowed complete translocation (lanes 4). Irradiation with UV light (lanes 2,4,5,6,7,8) formed crosslinks between SecY and the substrate. Where indicated, WB mutant SecA, ATPγS, or ADP•BeF were added before irradiation. Samples were analyzed by SDS-PAGE and autoradiography. The sizes of uncrosslinked (pOA) and SecY-crosslinked (xY) substrate is indicated.