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Annual Review of Biophysics

Single-Molecule Studies on the Protein Translocon

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Abstract

Single-molecule studies provide unprecedented details about processes that are difficult to grasp by bulk biochemical assays that yield ensemble-averaged results. One of these processes is the translocation and insertion of proteins across and into the bacterial cytoplasmic membrane. This process is facilitated by the universally conserved secretion (Sec) system, a multi-subunit membrane protein complex that consists of dissociable cytoplasmic targeting components, a molecular motor, a protein-conducting membrane pore, and accessory membrane proteins. Here, we review recent insights into the mechanisms of protein translocation and membrane protein insertion from single-molecule studies.

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INTRODUCTION

Over the past two decades, numerous biological systems have been studied through the use of various single-molecule techniques. One of these crucial cellular processes is protein biogenesis: the synthesis, maturation, and targeting of a protein to specific compartments within a cell. In bacteria, proteins are inserted into or translocated across the cytoplasmic membrane by a highly conserved secretion (Sec) system, centered around a heterotrimeric SecYEG translocon, which is homologous to the eukaryotic Sec61 translocon residing in the endoplasmic reticulum. The translocon works in concert with cytosolic and membrane proteins to form the holotranslocon that facilitates efficient protein insertion and translocation. A highly regulated process of protein targeting directs newly synthesized proteins to the translocon by recognizing and acting on specific inherent signals in these proteins [i.e., signal sequences or hydrophobic transmembrane domains (TMDs)] (19, 25) (**Figure 1**). Secretory proteins are generally targeted posttranslationally to the membrane, whereas membrane proteins utilize the cotranslational targeting route. These two pathways diverge at an early stage when the N-terminus of a nascent protein emerges from the ribosome exit tunnel (58).

At this point, signal recognition particle (SRP) and peptidyl-prolyl *cis-trans* isomerase trigger factor (TF) compete to bind the ribosome nascent chain (10, 36). In the posttranslational route, the N-terminal signal sequence is recognized by TF. During chain elongation and once more than 100 amino acids are exposed from the ribosome exit tunnel, molecular chaperones like SecB bind the nascent chain. SecB stabilizes the newly synthesized preprotein, keeps it in an unfolded translocation-competent state, and directs it to the SecYEG-bound SecA, the motor domain of the holotranslocon (82), where SecB releases the polypeptide to the SecA ATPase for translocation (65). The exact mechanisms by which SecA mediates translocation are still unknown, but several working models have been suggested: (*a*) Brownian ratchet (98); (*b*) power stroke (101, 103); (*c*) peristalsis (25, 101); (*d*) subunit recruitment (11); and (*e*) reciprocating piston model (61). These models all have in common that SecA is needed for the initiation of translocation.

Figure 1

Schematic representation of the bacterial SecYEG pathway. (●) Unfolded secretory proteins are posttranslationally targeted to the SecYEG-bound SecA (green) by the chaperone SecB (blue) and translocated in an ATP-dependent fashion through a membrane pore formed by SecYEG (orange). Translocation is stimulated by the proton motive force (PMF), which involves the SecDF (yajC) (pink) complex. (●) Nascent membrane proteins are bound by signal recognition particle (SRP) (purple) and cotranslationally targeted to the SecYEG translocon via FtsY (dark green) for membrane insertion. (●) YidC (red) is a membrane protein insertase that can insert small hydrophobic proteins into the membrane or work in concert with the SecYEG translocon. Figure adapted from Reference 25. Additional abbreviations: CM, cytoplasmic membrane.

SecYEG-bound SecA is activated for ATPase activity when it binds a preprotein (22, 50, 52, 63). Subsequent binding of ATP results in insertion of the N-terminal signal sequence of the preprotein into the SecYEG pore; concomitantly, SecB is released from the complex (34). Hydrolysis of ATP causes the dissociation of SecA from the preprotein, but SecA remains bound to SecYEG to prevent backsliding of the polypeptide (28, 29, 90). Cycles of ATP binding and hydrolysis drive the slow stepwise translocation of the preprotein through the SecYEG pore, but this process is accelerated by the SecDF complex that pulls proteins in a proton motive force (PMF)-dependent manner through the pore from the periplasmic membrane face (65). With membrane proteins, the first TMD functions as a signal for cotranslational targeting (25). Once the nascent chain emerges from the ribosome exit tunnel and the signal sequence is displayed, SRP binds to the signal on the ribosomal nascent chain (RNC) and targets the complex to the SRP membrane receptor, FtsY (58). FtsY facilitates docking of the RNC-SRP-FtsY complex to the translocon. This results in the formation of a heterodimeric SRP-FtsY complex that is activated for GTP hydrolysis to release the RNC from SRP to the SecYEG complex. Following insertion into the SecYEG pore, the hydrophobic TMDs are partitioned into the lipid bilayer while protein chain elongation continues at the ribosome. Membrane partitioning is possible because of the unique structure of SecY, which forms a lateral gate through which TMDs enter the lipid bilayer (105). In the next sections, we

discuss recent insights into the mechanism of protein translocation and membrane protein insertion in bacteria based on single-molecule approaches.

COTRANSLATIONAL PROTEIN TARGETING

In *Escherichia coli*, the RNC complex is targeted cotranslationally to the SecYEG translocon by SRP and its membrane-bound receptor FtsY (**Figure 2a**). FtsY is a monomeric GTPase that consists of several domains. The A-domain facilitates interactions with the membrane and the translocon (3, 110), while the helical NG-domain contains a Ras-like GTPase subdomain and the binding site for a homologous NG-domain on SRP (30, 37). Structure elucidation of SRP revealed a heterodimeric arrangement, consisting of a single-protein subunit, Ffh, and a 114-nucleotide-long 4.5S SRP RNA. The Ffh protein contains two domains: a methionine-rich M-domain that facilitates high-affinity SRP RNA binding (7) and signal sequence recognition (49) and an NG-domain (**Figure 2a**) that is homologous to the NG-domain on FtsY that facilitates the binding of SRP to FtsY (30, 37). The RNA moiety of SRP is essential and universally conserved. Structural analysis of the RNA molecule suggested large conformational changes upon binding of FtsY that are essential for SecYEG-ribosome interactions (5). The function of this RNA moiety was investigated using single-molecule Förster resonance energy transfer (FRET) (**Figure 2b,c**) to observe conformational dynamics of single SRP molecules (93). To measure conformational changes, the NG-domain of FtsY was labeled with a FRET donor, where the acceptor was located at the distal end of the SRP RNA molecule. In the presence of a nonhydrolyzable GTP analog, stable and functional SRP-FtsY complexes displayed two pronounced FRET states. The low-efficiency FRET state indicates a relative large distance between probes, signifying that the GTPase complex

Figure 2

Structure of RNC-SRP-FtsY complex docked at the SecYEG translocon. (a) A ribosome (gray) exposing a signal sequence (magenta) is bound by SRP (blue), FtsY (green), and the SecYEG translocon (red). (b) Illustration of the principle of Förster resonance energy transfer between two probes. (c) Jablonski diagram of Förster resonance energy transfer. Complex structure in panel a adapted from Reference 51. Abbreviations: RNC, ribosomal nascent chain; S₀, ground state; S₁, excited state; SR, SRP receptor; SRP, signal recognition particle; SS, signal sequence.

residing in the NG-domain of Ffh is located close to the SRP RNA tetraloop. The high-efficiency FRET state corresponds to a state in which the GTPase complex is in close proximity to the FtsY NG-domain on the distal site. By analyzing the dwell times, kinetics of FRET transition states were obtained, revealing two transient intermediate states. Further analysis showed that the conformational changes of the SRP RNA moiety were found to be regulated not only by the GTPase cycle of SRP and FtsY but also by interactions with the SecYEG translocon and translating ribosomes. These single-molecule FRET (smFRET) observations visualized for the first time the movement of the GTPase complex on the SRP RNA moiety and revealed dynamical conformational changes at the single-molecule level with unprecedented detail.

THE SecYEG TRANSLOCON

The bacterial translocon consists of a stable complex of the SecY, -E, and -G proteins (**Figure 3a,b**). The crystal structure of the translocon has provided detailed insights into the structural basis of the translocation mechanism (77, 99, 105, 114). SecY displays a unique

Figure 3

Structure and cellular localization of *Escherichia coli* SecYEG. (a) Structure of SecYEG translocon (PDB ID: 5NCO) with the plug domain (magenta) and the lateral gate flanked by TMD2 and TMD7. SecY TMDs 1–6 (green), TMDs 7–10 (blue), SecE (yellow), and SecG (orange). (b) Structure of SecYEG rotated 90°, top view of the SecYEG translocon visualizing the central pore and lateral gate. (c) Super-resolution imaging of SecE in *E. coli* cells showing stretches of higher fluorescence intensity possibly indicating active sites. Abbreviation: TMD, transmembrane domain.

conformation of 10 α -helical TMDs in which the α -helices 1 to 5 and 6 to 10 are arranged in a structure resembling a bivalve shell (**Figure 3a,b**) (105). Loops between the SecY TMDs 6/7 and 8/9, termed C4 and C5, respectively, extend into the cytosol and are involved in binding of SecA and translating ribosomes (38, 68). SecY is stabilized by SecE, which encompasses the pore in a V-shaped manner (**Figure 3a,b**). The *E. coli* SecE protein consists of two dispensable N-terminal TMDs, an amphipathic helix, which runs parallel to the membrane, and a conserved tilted helix, which contacts the two halves of the bivalve shell formed by SecY. These two helices are connected by the hinge region, which is essential for stability of the pore and provides flexibility to the complex (70, 80, 89). SecG is obsolete for translocation (14) and cell viability but increases the efficiency of translocation at low temperatures or when the PMF is low (45, 46, 72).

SecY creates a characteristic hourglass-shaped channel with an aqueous funnel-like entrance and exit cavity. At its widest point, the pore has a diameter of $\sim 20\text{--}25$ Å from which it narrows down to the central ring with a diameter of ~ 4 Å (105). This pore ring consists of six hydrophobic isoleucine residues that form a seal that prevents leakage of ions in the closed conformation of the pore while maintaining the permeability barrier of the membrane during translocation by forming a tight seal around the polypeptide (44, 78, 87). In addition, a small α -helix domain within the SecY protein functions as a plug to seal the channel at the periplasmic side of the pore (105). During protein translocation, however, some ions pass through the pore (53, 91). To obtain mechanistic insights on how a translocon channel is sealed, single-molecule electrophysiology was employed. Using black lipid membranes, the conductivity of single SecYEG channels was assayed. Early studies using black lipid membranes focused on SecYEG and Sec61 from canine pancreas (96, 97). Remarkably, a similar 115-picosiemens-large conductance was detected for both systems, suggesting that the conductance was facilitated by an open conformation of channels with similar structures. A follow-up study investigated different structural properties of the *E. coli* SecYEG channel (87). Measuring the conductance of wild-type SecYEG, it was found that in the resting state, the channel was impermeable for ions and water molecules. For closer examination of the role of the pore ring sealing properties, a *prlA4* SecY mutant was assayed with the black lipid technique. This mutant belongs to a class of signal-suppressor mutants that allow for translocation of preprotein with defective signal sequences. The *prlA4* mutant contains two mutations, one in the pore ring and another in TMD7 that counteracts the destabilizing mutation in the pore ring. The *prlA4* mutant showed significant conductance in the resting state that arose from intermittent opening of the channel with no apparent cause other than time-dependent structural rearrangements of SecY, supporting the hypothesis that the *prlA4* translocon is less selective and more easily opened. To investigate the role of the plug domain in the ion conductance, mutations were created to investigate crucial amino acid residues or fix the plug in a permanent open position. Mutating the residues in key positions led to a similar intermittent transient opening of the channel akin to the observations with the *prlA4* mutant. By locking the channel in a permanent open position by cross-linking the plug domain to SecE, a massive ion flux was detected. Hardly any channel-closing events were detected in this state. Together, the pore ring and the plug domain effectively seal the channel, wherein the plug domain plays a major role in sealing the channel and the central pore ring forms a gasket for preventing ion leakage during protein translocation. More recent black lipid membrane studies focused on the effect of ligands on the pore conductance (56, 57). Signal peptides and ribosomes change the conductance of the SecYEG pore, implying channel conformations induced by ligands.

Plug domain dynamics were also studied by an in vitro smFRET approach (35). To follow the opening of the channel, the plug and cytosolic loop of TMD2 were labeled with a donor and acceptor fluorophore, respectively. Total internal reflection microscopy (TIRFm) and confocal microscopy were employed to detect the FRET signals. In the presence of a preprotein, SecA,

SecB, and ATP, a bimodal distribution of two plug domain states was obtained. The first state corresponds to a closed conformation of the SecY pore, and the second reflects an open conformation where the plug was displaced from the channel. Although the transition between states seemed instantaneous using TIRFm, the high temporal resolution of confocal microscopy made it possible to resolve fast opening and closing of the channel on the millisecond timescale. Interestingly, initial opening of the channel depends on the hydrolysis of ATP by SecA, while closing of the channel does not. The ATP-driven plug opening produces only a partially opened conformation while complete displacement of the plug additionally requires preprotein insertion into the channel. This observation is in line with the previous black lipid membrane studies and underlines the high dynamical nature of the translocon. The dwell times obtained from the FRET experiments indicate the time the channel was closed or open, and this was related to the length of the preprotein passing through the channel. The duration of the open state was used as a proxy to calculate the translocation rate of substrates with different lengths. A fast rate of ~ 40 amino acids per second was found. In vivo, however, translocation is much faster owing to the PMF (65, 90).

LATERAL GATE-OPENING MECHANISM

The unique structure of the SecYEG channel allows both vectorial protein translocation and lateral insertion of proteins into the membrane. The bivalve shell formed by SecY can on one side open to the lipid bilayer forming the so-called lateral gate. In the structure of the translocon, the lateral gate that localizes between TMDs 2b and 7 is in a closed conformation. The distance between these two helices is ~ 7 Å (105) and must increase to 24 Å (38) to allow passage of TMDs into the lipid bilayer. Biochemical studies indicated a binding pocket for signal sequences and hydrophobic TMDs between TMDs 2b and 7 that triggers the opening of the lateral gate (105). Observing the real-time insertion of a nascent membrane protein is very challenging. To study the fast and transient mechanisms involved in membrane protein insertion, biochemical and single-molecule techniques were employed to monitor and trap the translocon in different intermediate states (2, 41). Herein, the SecM stalling sequence was used to create RNCs displaying different hydrophobicity and lengths of SecYEG substrates (41). One of these substrates is leader peptidase (Lep), which contains two N-terminal TMDs. Stalled RNCs with the first 75 N-terminal residues of Lep display ~ 40 amino acids outside the ribosomal peptide exit tunnel and trap the translocon in a cotranslational intermediate state. This allows for the stable detection of conformational changes of the lateral gate during the protein insertion event, which otherwise would be too transient. Single-molecule FRET (2) and single-molecule photon-induced electron transfer (PET) (41) were used to investigate the gating feature of SecY. Unlike the energy transfer in FRET, an excited state electron is transferred with PET. The process of electron transfer starts when a photon excites an electron. The excitation leaves a vacancy in a ground state orbital that can be filled by an electron from a donor. Additionally, the excited electron can be transferred to an electron acceptor. The two processes generate a charge separation, making the photoexcited molecule a good oxidizing and reducing agent. Moreover, whereas FRET is generally sensitive in the nanometer-scale range, PET can be used to study conformational changes on the angstrom scale. Conformational changes in the SecY structure leading to opening or closing of the lateral gate were investigated using smPET with the fluorophore boron-dipyrromethene dye BODIPY FL (Bpy) and a PET quencher tryptophan attached to TMDs 2b and 7, respectively. In the resting state of SecY, an electron is transferred between the excited Bpy to Trp, resulting in the quenching of Bpy. However, opening of the lateral gate prohibits the electron transfer, and an increased fluorescence is observed. Bpy-labeled SecYEG monomers were integrated into nanodiscs, and

the PET efficiency was monitored in the presence of vacant ribosomes or RNCs, with varying hydrophobicity of the nascent peptides. Remarkably, vacant ribosomes already decreased the PET efficiency, while the PET decreased even further when Lep75 RNCs were introduced. This suggests that vacant ribosomes already induce a partial opening of the lateral gate, while this process is further stimulated by a hydrophobic TMD that likely pries open the lateral gate. Opening of the lateral gate and subsequent insertion of a peptide are dependent on the combined effects of the binding of a ribosome and the insertion of the nascent TMD into the SecY channel.

Another study employing smFRET addresses the conformational changes in SecY in the presence of SecA (2). Like in the smPET study, the TMD-2b and -7 helices on either side of the gate were labeled with donor and acceptor fluorescent probes. A single copy of labeled SecYEG was reconstituted into proteoliposomes, and smFRET efficiency was monitored in the presence of nucleotides and the ATP-dependent motor protein SecA using time-lapse TIRF imaging. In the absence of ligands, a high FRET efficiency was observed, indicating that the probes are in close proximity and signifying a closed state of the lateral gate. Addition of SecA and different nucleotides significantly changed the FRET efficiencies. In the presence of SecA and ATP, three different states of the lateral gate were discerned on the basis of statistical criteria, and these were correlated to a closed, partly open, and open state. In an attempt to obtain a better understanding of these states, nonhydrolyzable AMPPNP was supplied to the sample, trapping SecA in an ATP-bound state. FRET efficiencies measured under these conditions were mainly low, indicating a predominantly open conformation of the lateral gate. In contrast, ADP resulted in a predominantly closed state as high FRET efficiencies were measured. The partly open state might represent an intermediate. However, the broad distributions of the states observed in this study make it difficult to isolate a specific population, and only through extensive data fitting and statistical analysis could evidence for the existence of different populations be obtained. Single-molecule approaches like FRET have the ability to distinguish between subtle conformational changes and should be able to give a more distinct separation between the different conformational states. Overall, it appears that the translocon displays a dynamic structure with large conformational changes between a fully closed and open state of the lateral gate, which is modulated by the various ligands of SecYEG. Although these studies suggest a high conformational plasticity of the translocation pore, the exact mechanism by which hydrophobic segments enter the bilayer remains elusive.

MEMBRANE DYNAMICS OF SecYEG

The localization of the SecYEG translocon has been studied at the single-molecule level in *E. coli* cells (**Figure 3c**) (A.-B. Seinen, D. Sparkman, W. Zhang, I. Prabudishia, A.M. van Oijen, A.J.M. Driessen, unpublished observations). By replacing the chromosomal *secE* gene of *E. coli* with a *yPet-secE* construct, a fluorescently labeled SecE fusion protein was created. Employing a photoactivated localization microscopy (PALM)-type super-resolution approach, the localization of the translocon was resolved with a nanometer-scale resolution at the single-molecule level. Time course imaging of exponentially growing *E. coli* cells resulted in a homogeneous distribution of the SecYEG translocon over the cytoplasmic membrane under native expression conditions. Remarkably, the localization pattern of SecYEG showed regions with an increased detection frequency, indicating possible active sites. Moreover, the localization of the translocon did not follow apparent structures inside the cell or membrane, which is in contrast to previous localization studies using conventional microscopy that depends on fixed cells, protein overexpression, and/or slow acquisition times (12, 32, 43, 94). This underlines the importance of super-resolution imaging at the single-molecule level, giving unprecedented details and insights into the object studied. For efficient membrane protein insertion and/or protein translocation, the PMF and

SecA are essential. These two key mechanisms were selectively disrupted in a study by treating cells with sublethal concentrations of a PMF uncoupler, or the ATPase inhibitor sodium azide (NaN_3), that blocks SecA-mediated protein translocation (A.-B. Seinen, D. Sparkman, W. Zhang, I. Prabudishia, A.M. van Oijen, A.J.M. Driessen, unpublished observations). Although inhibition of SecA did not lead to a significant change of the translocon localization, disruption of the PMF resulted in a more homogeneous distribution of SecE.

Although there is a general agreement in the field about the monomeric state of the functional translocon, some studies employing cross-linkers also indicate the presence of SecYEG oligomers. Fluorescence cross-correlation spectroscopy (FCCS) was employed to visualize the oligomeric state of SecYEG in giant unilamellar vesicles (GUVs) at a lipid-to-protein ratio that is similar to that of the native bacterial membrane (54). Two spectrally nonoverlapping probes were conjugated to single SecYEG translocons, with each translocon labeled once with a certain probe. Codiffusion of the two populations was assessed by the analysis of the fluctuations of the fluorescence intensity. Using GUVs, only a significant monomeric population was observed. To mimic the active translocation state, a proOmpA-Dhfr fusion protein was trapped in the channel owing to the tightly folded Dhfr domain at the carboxyl terminus. Although the majority of translocons were stalled in translocation, no oligomerization was detected using FCCS. These experiments with single-molecule sensitivity suggested that a single copy of SecYEG is sufficient for the interaction with SecA and preprotein translocation. A similar conclusion was reached with SecYEG reconstituted into nanodiscs that were found to be active when only the monomer was reconstituted (100).

Recently, the oligomeric state of the translocon was visualized in living *E. coli* cells under native conditions (A.-B. Seinen, D. Sparkman, W. Zhang, I. Prabudishia, A.M. van Oijen, A.J.M. Driessen, unpublished observations). This approach probed the functional state without artificially induced cross-linking, thus more likely representing the native functional form of the translocon. By correlating the fluorescence intensity of a focus to a single-molecule intensity, the functional state in living cells was found to be monomeric. Additionally, the localization of SecE showed a highly dynamic behavior as indicated by single-particle tracking. From the trajectories of the movement of SecE in a 2D plane through the *E. coli* membrane, diffusion coefficients were calculated using the cumulative probability distribution (CPD) of the step sizes. From this analysis, it followed that the diffusion of SecE was not homogeneous (e.g., the particles did not move with a homogeneous rate). In fact, three different diffusion coefficients were found, correlated to different states of the translocon. The first population likely corresponds to SecYEG translocons diffusing as single complexes in the membrane. The second population displayed a diffusion rate corresponding to a complex of SecYEG with YidC and a large structure, possibly ribosomes or polysomes. The last population was immobile, corresponding to a relatively large complex, possibly the holotranslocon, where the SecYEG translocon is bound to the YidC and SecDF proteins and the large cellular structures indicated before.

THE SecA MOTOR PROTEIN

SecA is a central component of the Sec system. It comprises several highly conserved structural and functional domains involved in cellular localization, nucleotide and preprotein binding, and motor action (**Figure 4a**) (48, 61, 75, 109). The *E. coli* SecA protein is arranged into three main functional domains: the nucleotide-binding domain (NBD), the preprotein cross-linking domain (PPXD), and the C-terminal domain (CTD) (76). The NBD consists of two essential functional nucleotide-binding folds (NBF1 and NBF2) that catalyze the binding and hydrolysis of ATP. Owing to the regulatory function of NBF2, it is also referred to as intramolecular regulator of ATP hydrolysis

Figure 4

The *Escherichia coli* SecA monomer and cellular localization visualized by super-resolution microscopy. (a) Structure of *E. coli* SecA (PDB ID: 2FSF). The nucleotide-binding folds 1 and 2 (NBF1 and NBF2) of the ATPase core, NBD, are shown in blue (residues 1–220 and 377–416) and cyan (residues 417–621), respectively. The preprotein cross-linking domain (PPXD) (residues 221–376) is shown in red. The three subdomains of the C-terminal domain (CTD)—the helical scaffold domain (HSD) (residues 621–669), the intramolecular regulator of ATP hydrolysis 1 (IRA1) (residues 756–829), and the helical wing domain (HWD) (residues 670–755)—are depicted in green, magenta, and yellow, respectively (76). (b) Super-resolution imaging of SecA in *E. coli* cells showing spots of higher fluorescence intensity, possibly indicating active sites.

2 or IRA2 (76, 88). Substrate recognition is mainly regulated by the PPXD and the CTD (18, 76). The latter can be subdivided into three subdomains, termed the C-terminal linker (CTL) or IRA1, the α -helical wing domain (HWD), and the α -helical scaffold domain (HSD), which contacts all the three main domains and contains a two-helix finger motif (31, 76). Besides substrate recognition, the CTD is involved in SecB and phospholipid binding (13). For the interaction of SecA with SecYEG, all SecA (sub)domains, except HWD, are involved (20, 69, 114).

It has been proposed that unfolded preproteins are trapped in a clamp or binding groove that is formed by PPXD, NBD2, and HSD (Figure 4a), while ATP binds at the interface of NBD1 and NBD2. NBD2 controls the ADP release and optimizes ATP catalysis at NBD1, the catalytic subdomain (95). The ongoing ATP binding and subsequent ADP release cause a motion of the motor domain NBD, which is thought to be transmitted to PPXD and CTD, providing the mechanical force necessary for preprotein translocation through the channel (42, 61). To prevent futile ATP hydrolysis cycles in the absence of both substrate and SecYEG, cytoplasmic SecA is

maintained in a thermally stabilized ADP-bound state. This state is accomplished by restricting the activator function of NBD2 owing to physical contact of IRA1 with NBD in the absence of translocation ligands and SecYEG (95). Furthermore, a conserved electrostatic salt bridge located in the HSD, called Gate1, might also function to prevent futile ATP hydrolysis cycles (52). In the presence of preprotein, SecB, and SecYEG, Gate1 is suggested to functionally connect NBD with PPXD, which allosterically stimulates the SecA ATPase activity. Furthermore, the ATPase activity of SecA also depends on the presence of anionic phospholipids (63).

Despite these structural insights, the exact molecular mechanism by which SecA mediates translocation is still poorly understood. On the basis of biochemical assays, a predominantly cytosolic localization has been suggested (1, 15, 16, 50), as SecA is readily isolated from cellular lysates. However, some SecA is tightly bound to the membrane (17), and this likely reflects a population of SecA that is bound to anionic phospholipids via its membrane-penetrating amphipathic N-terminus (8). Anionic phospholipids are essential for protein translocation, but their role has for long been obscure. Recent insights obtained by using microscale thermophoresis on single molecules of SecYEG reconstituted into small and large nanodiscs revealed a surprising role of anionic phospholipids (8). In the presence of a limiting number of lipids (i.e., small nanodiscs), SecA bound with a low affinity to the translocon, while a high affinity was observed when SecA had access to a large lipid surface. Further studies suggested that the insertion of the N-terminus of SecA into the membranes induces a conformational change to SecA to prime it for high-affinity binding to SecYEG. This implies that the lipid-bound form of SecA is an intermediate in the functional cycle.

CELLULAR DYNAMICS OF SecA

In line with this study, super-resolution fluorescence microscopy at the single-molecule level was employed to study the localization of SecA *in vivo* under native conditions and impaired protein translocation conditions (**Figure 4b**) (A.-B. Seinen, D. Spakman, A.M. van Oijen, A.J.M. Driessen, unpublished observations). By replacing the chromosomal *secA* gene of *E. coli* with a *secA-ypet* construct, a fluorescently labeled fusion protein was created. A PALM-type super-resolution microscopy approach resolved the localization of SecA at the single-molecule level. Under native conditions, only a very small cytosolic pool of SecA was observed. Rather, SecA was predominantly bound to and distributed over the cytoplasmic membrane without an apparent preferred cellular location. However, regions of higher detection frequency were detected. These patches possibly indicate active sites, as similar regions were observed for the SecE protein. Remarkably, blocking the ATPase function of SecA using NaN₃ did not result in a change of localization, but a significant amount of SecA molecules were relocated to the cytosol. To observe the dynamical SecA interactions with the SecYEG translocon, a super-resolution dual-color approach was employed utilizing the SecA-Ypet and paTagRFP-SecE fusion proteins (A.-B. Seinen, D. Spakman, W. Zhang, I. Prabudishia, A.M. van Oijen, A.J.M. Driessen, unpublished observations). Kymographs showed a high colocalization in time but also a highly dynamic behavior of SecA. On the basis of CPD analysis, different diffusive populations were found. SecA displays three different states of mobility. In one state, SecA diffuses rapidly along the cytoplasmic membrane ($\sim 2 \mu\text{m}^2/\text{s}$), likely to scan the surface until it binds to a translocon. A further state appears short lived but shows a diffusion characteristic indicative of integral membrane proteins. Furthermore, SecA is also found in an immobile state where it is presumably associated with the translocon in a state that likely represents the holotranslocon, as discussed in the section titled Membrane Dynamics of SecYEG.

The quaternary functional state of SecA has been one of the questions that has raised controversy in the field. Through crystallography, both monomeric and dimeric structures of SecA have been described that differ in conformation and/or dimer interface (48, 75, 76, 108, 113, 114). It is proposed that the native antiparallel dimerization is mediated exclusively by the NBDs of the two monomers (76). SecA is purified from cells in a dimeric form while in vitro translocation studies suggested that SecA is functional as a dimer (22, 24). Mutation-induced monomerization of SecA is associated with a severe loss of activity but can be overcome by a high-overexpression level of SecA, allowing the reformation of dimers (50, 74, 81). To investigate the oligomeric state of SecA in vitro at the single-molecule level, dual-color fluorescence burst analysis (DCFBA) was employed (106). DCFBA relies on the diffusion of particles labeled with two spectrally nonoverlapping probes through the confocal volume. When these particles are binding partners, a co-diffusion through the confocal volume is detected as overlapping fluorescent bursts. To address the oligomeric state of SecA in solvent, proteins were labeled with two spectrally nonoverlapping probes, creating two spectral populations of SecA. Titration experiments with the SecA concentration resulted in a dissociation curve, indicating a K_d of <1 nM for SecA dimerization. The affinity of SecA for SecYEG was also investigated and measured to be ~ 4 nM, in line with previous studies (21). Fluorescence ratio measurements validated that SecA is dimeric when bound to SecYEG. Recently, the oligomeric state of SecA was also assayed in vivo under native conditions using super-resolution fluorescence microscopy (A.-B. Seinen, D. Spakman, A.M. van Oijen, A.J.M. Driessen, unpublished observations). By calculating the number of molecules per focus, by measuring the fluorescence of SecA-Ypet in single foci, the dimeric state of SecA was visualized in living cells. These studies revealed that the majority of the SecA in these foci is dimeric.

THE SecDF COMPLEX

The integral membrane proteins SecD and SecF form an accessory complex (SecDF) that can associate with the translocon. SecDF plays a role in the downstream stages of translocation of secretory proteins as well as in membrane protein biogenesis and stabilization of the SecY protein forming the pore (4, 26, 27, 66). Structure elucidation showed that the SecDF complex consists of 12 TMDs, 6 TMDs per protein, and 6 periplasmic domains (P1–P6) (**Figure 5a**) (79, 86). The transmembrane domains of SecD and SecF form a base substructure that is structurally homologous (102). The SecD P1 and SecF P4 domains, however, form distinct (sub)structures. In contrast to P4, P1 has an additional head substructure covalently linked to the base by a hinge region. Crystal structures showed that this head domain exists in two different conformational states, F and I (102). It has been proposed that these states aid in translocation by interacting with the preprotein at the periplasmic membrane face by pulling the preprotein through the pore (102). The shift between the different states is possibly promoted by the proton-conducting ability of a conserved TMD at the interface of SecD and SecF. The PMF may power the conformational transition, leading to complete translocation of the preprotein (102).

MEMBRANE DYNAMICS OF SecDF

Recently, the localization of the SecDF complex was studied using single-molecule super-resolution microscopy (**Figure 5b**) (A.-B. Seinen, D. Spakman, W. Zhang, I. Prabudishia, A.M. van Oijen, A.J.M. Driessen, unpublished observations). A functional fluorescent SecF-Ypet protein was constructed by integrating the gene of *ypt* into the *secF* locus in *E. coli*, replacing the

Figure 5

Structure of the *Escherichia coli* SecDF complex and cellular localization. (*a*) Structure of SecDF (PDB ID: 3AQP), with the periplasmic domains of SecD and SecF indicated with blue and magenta, respectively. SecD and SecF membrane domains are indicated with green and yellow, respectively. (*b*) Visualization of SecF in *E. coli* cells showing spots of higher fluorescence intensity, possibly indicating active sites.

wild-type gene by a fluorescent fusion construct. Under native conditions, SecF formed highly localized foci in the cytoplasmic membrane of *E. coli*. Uncoupling of the PMF resulted in a more homogeneous distribution through the membrane. This change possibly indicates a disassembly of the holotranslocon, as the function of SecDF depends on the PMF. Furthermore, biochemical, genetic, and single-molecule observations indicate a low abundance of the SecDF complex (67, 79). Studies on the stoichiometry of the holotranslocon suggest that a single SecDF complex associates with a monomeric SecYEG translocon (92). This monomeric state of both SecDF and SecYEG was confirmed using the aforementioned single-molecule microscopy approach. Because of the low abundance of the SecDF complex, not all SecYEG complexes in the membrane will be engaged with SecDF complexes at a given time.

Membrane diffusion analysis of the SecF-Ypet construct indicated that this protein diffuses in two distinct forms. Under native conditions, the majority of SecDF complexes (68%) were immobile, while the remaining 32% showed an apparent diffusion coefficient comparable to that of a membrane protein. Dissipating the PMF resulted in an increase of the immobile population to 79% of all the molecules. Apparently, blockage of protein translocation renders stalled translocons complexed with the SecDF, thereby affecting the dynamics of this complex in the cytoplasmic membrane. These data suggest that the SecDF complex binds in a dynamic manner to

the SecYEG translocon, consistent with its role in the late stages of protein translocation. Thus, the holotranslocon is a dissociable entity that likely forms on demand.

THE MEMBRANE PROTEIN INSERTASE YidC

Another membrane protein that associates with the SecYEG translocon is YidC. It belongs to the universally conserved YidC/Oxa1/Alb3 protein family present in bacteria, mitochondria, and the thylakoid membrane of chloroplasts. Functional studies on YidC suggest that it aids in the insertion, folding, and quality control of membrane proteins (71, 104). Most of the specific YidC substrates are small integral membrane proteins with one or two TMDs, often with one or more polar or charged amino acid residues in a TMD (33, 107). Therefore, YidC may facilitate membrane insertion of proteins that are difficult to insert by SecYEG alone, although the exact molecular basis of substrate specificity remains unresolved. YidC can also function in concert with SecYEG (Figure 1, pathway 9). In the latter *modus operandi*, YidC interacts with the SecDF complex of the holotranslocon, which probably facilitates the association of YidC to SecYEG (73, 112). YidC likely associates near the lateral gate of SecY to have access to nascent TMDs and to aid their membrane partitioning (64, 84, 85). These interactions may create a secluded environment in the membrane where proteins are inserted. The elucidation of the structure of YidC has provided major insights into the mechanisms by which membrane proteins are inserted into the lipid bilayer (Figure 6a,b) (6, 59, 60, 111). YidC homologs share a highly conserved core of five TMDs connected by hydrophilic loops (39). YidC proteins of Gram-negative bacteria contain an additional N-terminal TMD, which is followed by a large periplasmic domain (40, 83). The conserved core of YidC forms a globular structure that contains a hydrophobic groove between TMD3 and TMD5 in which a conserved arginine residue resides. This hydrophobic groove is accessible from the cytoplasmic side and presumably facilitates the passage of membrane segments from the cytoplasm into the lipid bilayer, where the arginine seems to function as a possible selector for insertion.

Although major insights on the YidC structure were obtained using X-ray diffraction from crystals formed in the lipid cubic phase (6, 59, 60, 111), this approach is limited to a nonnative environment. Recently, high-resolution solid-state nuclear magnetic resonance (ssNMR) spectroscopy was used to study the structure of YidC in its native environment (6). NMR-active nuclei were incorporated into YidC *in vivo*. After isolation of the cell envelopes by gentle cell lysis, samples were subjected to ssNMR. NMR spectra of YidC in these native membranes showed a high structural comparison to YidC reconstituted into proteoliposomes. However, significant structural differences were found between the native and reconstituted environments, suggesting different structural dynamics of the YidC protein under native and reconstituted conditions.

MEMBRANE DYNAMICS OF YidC

The YidC crystal structures indicate a monomeric functional state (6, 59, 60, 111), although the protein has a tendency to dimerize. To investigate the functional state of YidC, FCCS was employed to study the native state of YidC upon interaction with ribosomes (55). To this end, solubilized YidC proteins were labeled with two spectrally different fluorophores, creating two pools with each probe. A low cross-correlation for detergent solubilized pools of YidC indicated that under these conditions, YidC remained predominantly monomeric. In the presence of RNCs, this monomeric state was retained. Likewise, binding of stalled RNCs displaying a YidC substrate to nanodiscs containing single YidC proteins further suggests that the monomer is the functional entity (55).

Figure 6

Structure of the *Escherichia coli* YidC insertase and cellular localization in *E. coli*. (a) Crystal structure of YidC (PDB ID: 3WVF), with the periplasmic domain indicated with blue and the membrane and cytosolic domains indicated with green. (b) YidC structure rotated 90°, visualizing the hydrophobic groove, which presumably facilitates the protein insertion into the cytoplasmic membrane. (c) Visualization of YidC in *E. coli* cells showing spots of higher fluorescence intensity, possibly indicating sites for protein insertion.

YidC has recently been visualized in *E. coli* cells, using single-molecule super-resolution microscopy (**Figure 6c**) (A.-B. Seinen, D. Sparkman, W. Zhang, I. Prabudishia, A.M. van Oijen, A.J.M. Driessen, unpublished observations). YidC was fluorescently labeled by integration of the *ypt* gene into the YidC locus, resulting in the labeling of the only *yidC* copy and creating a functional YidC-Ypet fusion protein. Reconstructing the fluorescence detected of natively expressed YidC-Ypet in exponentially growing *E. coli* cells indicated a near-perfect homogeneous distribution of YidC through the cytoplasmic membrane. In vivo single-particle tracking indicated that particles did not move with one diffusion rate. Remarkably, three distinct diffusion coefficients were found comparable to diffusion rates found for SecYEG. Two of these diffusion rates correspond to slow-migrating species, likely representing different compositions of the holotranslocon complex, whereas the fast-moving species likely corresponds to uncomplexed YidC. These data support the notion that the holotranslocon is a dissociable entity.

THE MOLECULAR CHAPERONE SecB

The cytosolic chaperone SecB is a secretion-dedicated chaperone that prevents premature folding of preproteins in the cytosol while facilitating their targeting to the SecA ATPase subunit of the

Figure 7

Structure of the molecular chaperone SecB and optical tweezer experimental setup. (a) SecB quaternary structure (PDB ID: 1QYN), with each monomer indicated with a different color. (b) SecB structure rotated 90° to show the interface of the dimer of dimers. (c) Experimental optical tweezer setup with maltose-binding protein (MBP) tethered between two beads; it is held in place on the left by the optical trap, allowing for force detection, and is attached on the right to a position-controlled micropipette to exert force on MBP.

translocon (34, 62, 82). Ribosome profiling analyses support the notion that SecB recognizes and interacts with regions in the mature part of preproteins (47). This feature makes SecB a unique and intriguing chaperone, as the substrates share no sequence homology apart from a general binding motif consisting of nine charged and aromatic amino acid residues. Structural insights into SecB showed that it is a homotetramer (**Figure 7a,b**) (23) consisting of a dimer-of-dimers arrangement with a 70-Å-long peptide-binding groove on either side of the dimer. These grooves consist of two peptide-binding subdomains: the first, an aromatic deep section of the channel called subsite 1, and the second, a more accessible and shallower hydrophobic subsite 2. The size of the binding groove allows for binding of extended peptide segments of ~20 residues. SecB can bind fragments

of up to 150 residues, suggesting that an unfolded polypeptide may wrap around the tetrameric form, thereby regulating the affinity for substrates (23).

SINGLE-MOLECULE OBSERVATIONS OF THE CHAPERONING FUNCTION OF SecB

To study the folding and unfolding pathway of the SecB substrate maltose-binding protein (MBP) and the influence of SecB on this process, optical tweezers were employed (9). The name is derived from a highly focused infrared laser beam called an optical trap, which is used to capture and manipulate dielectric particles attached to the research object. By changing the distance between the dielectric particles, a pulling or pushing force on the piconewton (pN) scale can be exerted on the object of interest. On the basis of the position of the optical trap and the dielectric particle inside the laser beam, distance and forces are recorded that give, when exerted on a protein, detailed insights into the folding and unfolding forces upon conformational changes. Folding and unfolding events of MBP were assayed using this technique by tethering an MBP molecule between two polystyrene beads. By displacement of the micropipette away from a static trap, a pulling force was applied to MBP, resulting in a force-extension curve. After each extension, the micropipette was moved back toward the static trap, resulting in a relaxation curve. Purified MBP showed a sudden change in this extension curve when a force of 25 pN was applied, indicating that the force applied resulted in the unfolding of MBP to a linear polypeptide. Interestingly, when the distance between the beads was decreased—and hence, the pulling force decreased—MBP folded back into the native conformation, as evident from a subsequent stretching cycle, yielding a similar extension curve. Increasing the number of MBP moieties in the polypeptide between the polystyrene beads from one to four (4MBP) led to the observation of a sawtooth pattern. Interestingly, the unfolding events remained exactly the same as for a single protein, with an average force of 23 pN. However, after allowing for refolding into the native conformation, the second extension curve did not result in a similar curve. Instead, a sudden change was observed when applying 40 pN. Supposedly, the 4MBP molecules close together aggregated to a more stable structure instead of refolding into the native conformation. The effect of SecB on this construct was assayed by the same experimental procedure. When a single MBP molecule was subjected to a pulling force in the presence of SecB, an extension curve similar to that in the absence of SecB was obtained. SecB thus does not influence the unfolding pathway of MBP. However, when the pulling force decreased and MBP was allowed to refold into its native conformation, the subsequent extension curve followed the path of the relaxation curve. This indicated that SecB binds only to the unfolded MBP molecule and prevents its refolding into any stable tertiary conformation. The 4MBP construct showed similar extension and relaxation curves as a single MBP molecule. In the absence of SecB, the 4MBP molecules aggregate, whereas the presence of SecB prevents the formation of aggregates. A folding model, based on the data obtained from the optical tweezer experiments and molecular dynamics simulations, was proposed. First, the peptide is compacted to a molten globule state in either the presence or absence of SecB; however, the presence of SecB prevents the formation of any tertiary conformations and maintains a translocation-competent state. Second, once the core of MBP is formed, SecB fails to bind and the peptide continues to fold, exhibiting similarities with a nucleation-growth mode into the native conformation with the N- and C-terminal α -helices.

FUTURE OUTLOOK ON SINGLE-MOLECULE TECHNIQUES TO INVESTIGATE PROTEIN BIOGENESIS

Here, we have provided an overview of the recent single-molecule insights into the structural and mechanistic aspects of the Sec translocon as well as its cellular distribution and dynamics. The Sec

translocon has been studied by a multitude of genetic and biochemical approaches, mostly bulk assays. Importantly, the structural studies have provided the first glimpse of the mode of action of a molecular machine that performs a dual function of translocation and membrane insertion. In this respect, the new developments in cryogenic electron microscopy will reveal the exact organization of the holotranslocon and will image intermediate stages of protein translocation and membrane protein insertion. The structures of the translocon have inspired biochemical analysis as well as computational studies to further elucidate the complex mechanisms involved in protein biogenesis. Although molecular dynamics can provide deeper insights into local structural dynamics of proteins at very short timescales, it is not possible to mimic the entire process over longer timescales. Also, most of the biochemical techniques cannot faithfully explore the dynamics of this process. Many aspects of protein biogenesis are fast or transient, such that ensemble measurements cannot resolve them. Our knowledge of single-molecule techniques has increased rapidly over the past two decades, and the techniques are more frequently used to study complex biological processes both in vitro and in vivo. Single-molecule techniques will therefore increasingly play a larger role in investigating this process, leading to groundbreaking new insights into the mechanisms and dynamics involved.

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