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Single-Molecule Studies of Bacterial Protein Translocation

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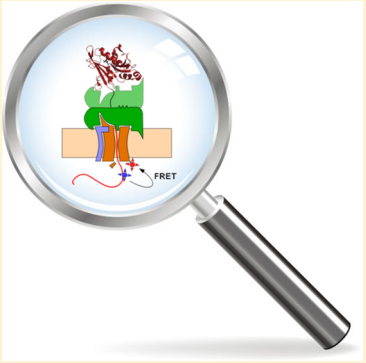
ABSTRACT: In prokaryotes, a large share of the proteins are secreted from the cell through a process that requires their translocation across the cytoplasmic membrane. This process is mediated by the universally conserved Sec system with homologues in the endoplasmic reticulum and thylakoid membranes of eukaryotes. The Sec system also facilitates the membrane insertion of integral membrane proteins, an essential step along their folding pathway. In bacteria, the Sec system consists of the protein-conducting channel (SecYEG) that associates with soluble components, such as the motor protein SecA or translating ribosomes, and with integral membrane proteins, such as the heterotrimeric complex termed SecDFyajC and the YidC insertase. Over the past three decades, biochemical and structural studies have provided a comprehensive view of protein translocation, but the exact mechanistic details of this process remain to be resolved. For a number of other biomolecular systems, single-molecule biophysical analysis has efficiently complemented the conventional biochemical studies conducted in bulk, with high-sensitivity measurements probing the structure and dynamics of individual molecules *in vitro* and *in vivo*. Here, we review recent advances in studies of protein translocation employing single-molecule techniques with the aim of resolving molecular mechanisms, thereby providing a new and detailed view of the process.



Over the past two decades, we have witnessed a new era in biological research in which the visualization of a cellular process at the molecular level has become possible using methodologies transferred from physics laboratories. Systems with different complexities, from tissues and isolated cells to biomolecular complexes and individual molecules, are now amenable to having their structure and dynamics studied in detail. One of these cellular processes is the translocation of proteins across cellular membranes. Protein translocation has been intensively investigated in the bacterium *Escherichia coli* since the discovery of the *sec* genes encoding a protein complex that mediates the translocation of secretory proteins (also termed preproteins) across the cytoplasmic membrane.[1,2] The corresponding proteins of this system are termed Sec (secretion) proteins, and they cooperate to drive the translocation of unfolded secretory proteins across the membrane as well as the integration of hydrophobic membrane proteins into the lipid bilayer (Figure 1A).[3]

As a central component, the membrane integral heterotrimeric SecYEG complex (also termed translocon) constitutes a protein-conducting channel in the cytoplasmic membrane. The translocon is universally conserved, and similar systems mediate the translocation of proteins into the lumen of the endoplasmic reticulum (Sec61 complex) and the thylakoid of plant chloroplasts.[4,5] Depending on the mode of action, the bacterial SecYEG complex can associate with different ligands (Figure 1A). During the co-translational insertion of membrane proteins into the cytoplasmic membrane, SecYEG interacts with translating ribosomes in a process mediated by the ribonucleoprotein signal recognition particle (SRP) and the SRP receptor (FtsY).[6] The hydrophobic signal anchor of a nascent polypeptide chain emerging from the ribosome is recognized by SRP whereupon the entire ribosome−nascent chain complex (RNC) is guided to the membrane-associated SRP receptor, FtsY (Figure 1A). FtsY forms a heterodimer with SRP, and upon GTP hydrolysis, the RNC is released to SecYEG to proceed with membrane protein insertion. At that stage, the signal anchor inserts into the channel, and upon continued chain elongation at the ribosome, the signal anchor is released into the lipid bilayer, most likely via the opened lateral gate in SecY. On the other hand, during the post-translational translocation of secretory proteins, synthesized proteins are targeted to SecYEG via the SecB/SecA pathway that utilizes cycles of ATP binding and hydrolysis to drive unfolded preproteins across the membrane. In *E. coli*, many newly synthesized preproteins first associate with the molecular chaperone SecB[7,8] that maintains preproteins in a so-called translocation-competent state, which corresponds to a loosely folded, nonaggregated state.[9] Together with an N-terminal signal sequence of the preprotein, SecB also contributes to the targeting of the preprotein to the SecYEG−SecA complex via binding to SecA,[10,11] a step that is followed by the dissociation of the preprotein from SecB and its transfer to the SecA protein.[12] SecYEG can also interact with another heterotrimeric integral membrane protein complex, termed SecDFyajC, that likely promotes protein translocation by binding translocating polypeptides at the *trans* side of the translocon in a process that requires proton motive force (pmf).[13,14] Another membrane

Received: July 10, 2013
Revised: September 4, 2013
Published: September 11, 2013

   dx.doi.org/10.1021/bi400913x | *Biochemistry* 2013, 52, 6740−6754

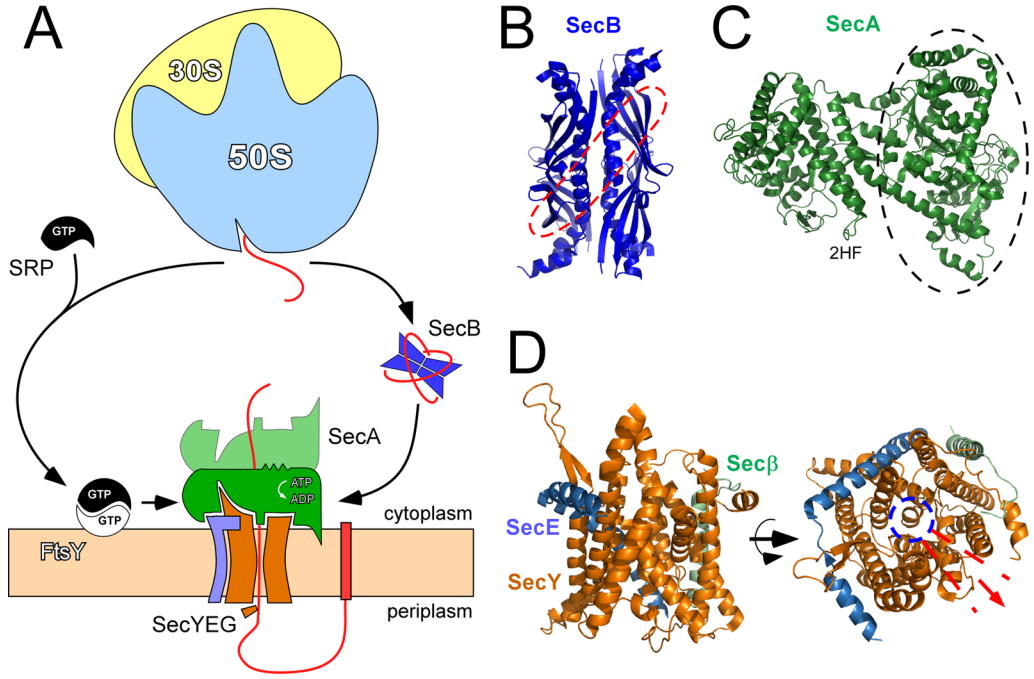


Figure 1. Protein translocation and membrane protein insertion via the translocon. (A) Scheme of primary pathways. Newly synthesized preproteins are translocated post-translationally via SecB/SecA (right) routes, while nascent inner membrane proteins are targeted co-translationally via the SRP/FtsY (left) route to insert at the translocon. (B) Ribbon diagram of the structure of the bacterial molecular chaperone SecB [Protein Data Bank (PDB) entry 1QYN].[17] The position of the substrate-binding groove is colored red. (C) Ribbon diagram of the structure of the bacterial SecA ATPase (PDB entry 1M74).[19] The ATPase domains are encircled. The "two-helix-finger" domain is indicated as 2HF. (D) Structure of the archaeal SecYEβ translocon with side and top views (PDB entry 1RH5).[31] The central protein-conducting pore is colored blue. In the idle state, the pore is closed by the short "plug" domain. The presumable path for membrane-inserted domains via the lateral gate is colored red.

protein YidC has been shown to interact with SecYEG and assist in the folding and membrane integration of a subset of membrane proteins.[15,16]

In recent years, the atomic structures of most Sec proteins have been determined in their isolated states, as well as in translocation-active complexes. The soluble components SecA and SecB were the most amenable for structural studies, allowing broad insights into their organization, dynamics, and interactions. The SecB chaperone is a homotetramer that is assembled as a dimer of dimers (Figure 1B).[8,17] A long hydrophobic groove on both sides of the SecB tetramer constitutes a possible binding site for an unfolded preprotein. SecB interacts with a preprotein polypeptide stretch of 150−200 amino acids; this is a cooperative event involving all four subunits of SecB, so high-affinity binding is achieved.[9] The architecture of the SecB−preprotein complex was revealed via negative stain electron microscopy (EM) at low resolution, and a certain asymmetry of the complex was reported.[18] Furthermore, biochemical, crystallization, and nuclear magnetic resonance (NMR) studies defined the SecA−SecB interaction site, suggesting that a tetramer of SecB binds to the zinc-stabilized carboxyl termini of a homodimeric SecA.[11,12] However, further analysis of the architecture awaits higher-resolution information about the SecB−preprotein and SecB−SecA complexes.

X-ray structures of the ATPase SecA isolated from various bacteria have been presented (Figure 1C). These differ in both the tertiary and the quaternary organization. A majority of the structures demonstrate a dimeric SecA in which the protomers are arranged in an antiparallel orientation.[19−21] For the *Thermus thermophilus* SecA, a parallel dimer was observed.[22] Binding of nucleotides and substrates triggers conformational changes within the SecA protein as observed in crystal structures and in NMR and biochemical studies.[19,23,24] However, how the energy from ATP hydrolysis is converted into mechanical work to drive preprotein translocation is still poorly understood. From a crystal structure of a complex of SecA bound to SecYEG, a prominent domain, the so-called "two-helix-finger" of SecA (2HF), was implicated in driving protein transport by repetitive up and down movements (Figure 1C).[25] The hypothesis was supported by a cross-linking study that show that residues at the tip of this "finger" cross-link to translocating preproteins.[26] However, a movement of the 2HF domain may not extend far enough to translocate polypeptide segments of 2−2.5 kDa as observed in biochemical studies.[27,28] A recent biochemical study revealed that cross-linking of the 2HF domain to the SecY subunit did not abolish polypeptide transport, indicating that this polypeptide pushing function of the 2HF is unlikely.[29] In the co-structure of SecA bound to SecYEG, the 2HF domain is located between transmembrane segments (TMS) 6 and 7 of SecY. An alternative hypothesis is that the 2HF domain may directly trigger the opening of the SecYEG channel, thus allowing the preprotein to pass through the central aqueous pore. Such a mechanism is also supported by a recent fluorescence-based study.[30]

The SecYEβ translocon from the archaeon *Methanocaldococcus jannaschii* was crystallized in the closed state (Figure 1D).[31] The crystal structure revealed that a single complex accommodates an hourglasslike membrane pore in the center of the largest subunit, SecY. In the closed state of the channel, the pore is sealed by a "constriction ring" built of aliphatic amino acids and by a short α-helical domain of TMS 2a, called a "plug", at the *trans* side. The N- and C-terminal parts of the

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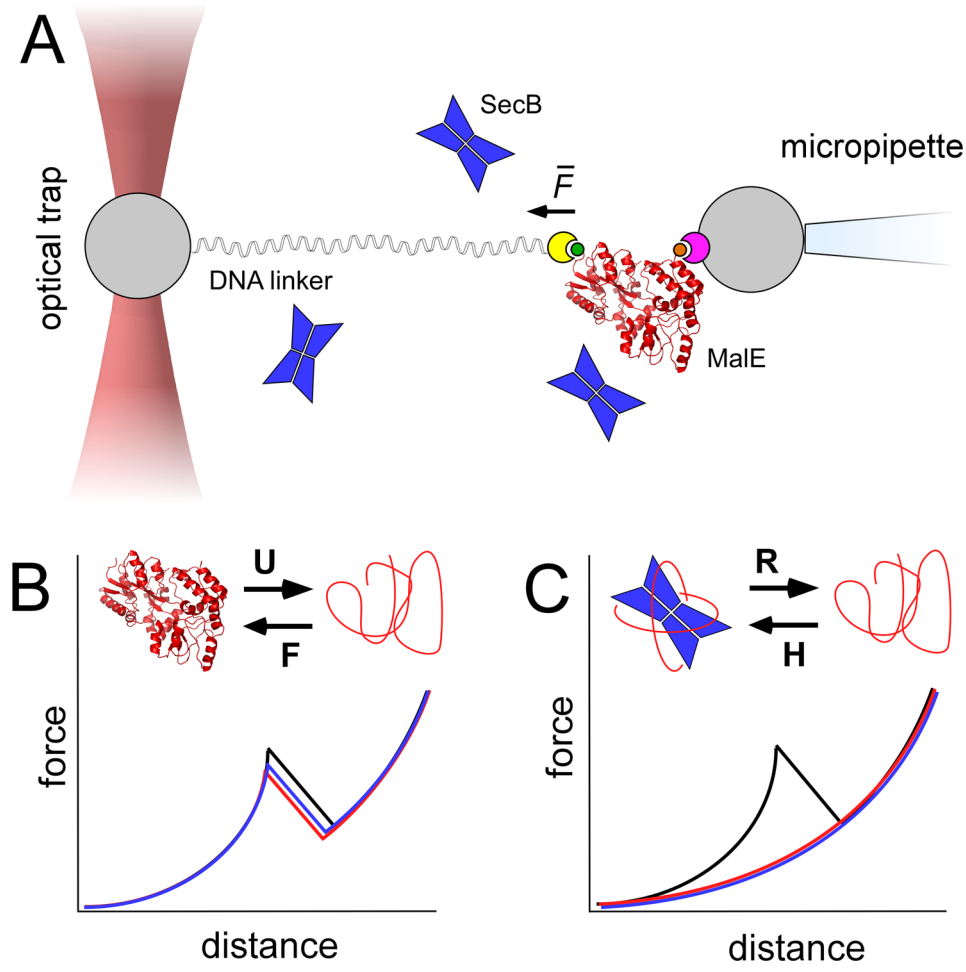

Figure 2. Single-molecule force spectroscopy on the SecB–substrate complex. (A) Design of the optical tweezers setup. Mechanical force *F* is applied to a single molecule of MalE protein to trigger its directional unfolding (B and C, black lines), followed by the relaxation of the polypeptide chain.[53] (B) MalE protein rapidly folds into its native structure (transition F), so it can be repeatedly unfolded (transition U), resulting in a characteristic force peak in force–distance spectra (red and blue lines below). (C) In the presence of the SecB chaperone, MalE does not acquire its stable folded structure but remains loosely folded when held by SecB (transition H). Force exerted by optical tweezers causes uncoiling of the polypeptide chain and release from SecB (transition R), while no stable interactions are detected in the force–distance spectra (red and blue lines below). For the sake of simplicity, the relaxation curves in the optical tweezers experiments are not indicated in the force–distance spectra.

SecY subunit that each consist of five TMSs are pseudosymmetric and connected through a hinge loop that may provide interdomain flexibility, such as to allow the opening of a "lateral gate" between TMS 2 and 7 upon translocation or membrane protein integration (Figure 1D). The peripheral subunit SecE embraces the SecY subunit and contains a conserved TMS that localizes at the back of SecY, while an amphiphilic α-helix lies on the plane of the membrane. The nonessential SecG/Secβ subunit is bound at the periphery of the N-terminal part of SecY, and the structural data do not provide specific clues about its function. Further crystallization studies involving SecYE(G) complexes from different organisms have shown a highly conserved fold and small deviations in tertiary contacts that may reflect the naturally occurring dynamics of the translocon.[25,32,33] Finally, the structure of the accessory SecDF complex has also been resolved by X-ray crystallography.[14] SecD and SecF are both polytopic membrane proteins with a large periplasmic domain. The structure revealed that the periplasmic domains of SecD and SecF form a kind of headpiece that may undergo a large movement during translocation. This domain may bind the unfolded preprotein once it emerges from the translocon at the *trans* side, whereupon a pmf-dependent movement of the headpiece may impose a pulling force on the translocating preprotein and prevent it from backsliding into the translocation pore.[34]

Complementary to X-ray crystallography, single-particle analysis of cryo-EM data has been employed to study the translocon structure in association with its largest ligand, the ribosome.[35−37] This approach was used to study the interaction of the bacterial and eukaryotic translocon with ribosomes and demonstrated for the first time that a single detergent-extracted SecYEG/Sec61 complex is sufficient for binding a translating ribosome.[36,37] More recently, the membrane-embedded translocon has been studied using nanodisc technology (see the section on SecYEG oligomeric state).[38] These studies showed the SecYEG–ribosome complex assembled in a physiologically relevant environment and in particular revealed a remodeling of the lipid surface by the ribosome upon interactions with SecYEG.

Protein translocation relies on dynamic interactions between the various Sec proteins, and the efficiency of this process is thus largely defined by the affinities of those proteins in forming functional complexes. Measuring the interaction and oligomerization of proteins with affinities in the nanomolar

dx.doi.org/10.1021/bi400913x | *Biochemistry* 2013, 52, 6740−6754

range is hampered by the detection limit of conventional biochemical methods, while minor subpopulations and intermediate steps are unlikely to be resolved in bulk measurements. As an alternative, the advanced level of understanding makes protein translocation a system that is particularly amenable for single-molecule investigation for resolving key questions about its structure, function, and dynamics. Here, we summarize the various single-molecule approaches adopted for this system in recent years. We also discuss how those studies contributed to a deeper mechanistic insight that would have been difficult to obtain by classical biochemical assays.

PROTEIN TARGETING AND DELIVERY TO THE TRANSLOCON

Preprotein−SecB Interactions during Post-Translational Targeting. Since a large pool of ribosomes resides within the bacterial cytoplasm, targeting newly synthesized polypeptide chains to the membrane represents an essential step prior to translocation or membrane integration. This targeting is achieved via the SecB or SRP/FtsY pathway, respectively. The chaperone SecB is recruited for the post-translational delivery of preproteins to the translocon. The exact effect of SecB on the structure of bound preproteins has remained obscure. Early studies based on bulk assays suggested that the SecB-bound precursors of the outer membrane protein (proOmpA) and maltose-binding protein (preMalE) maintain significant amounts of tertiary and secondary structure,[39,40] which may be further unfolded by SecA prior to translocation.[41,42] On the other hand, the translocon can also translocate partially folded structures,[42] and the SecY pore exhibits an unexpectedly high plasticity that allows it to transport bulky and rigid molecules.[43] Together with molecular modeling of folded helical domains entering the translocon,[44] those observations question the need for a completely unwound polypeptide chain prior to translocation.

To elucidate the SecB−substrate interactions in the most direct manner, single-molecule force measurements have been applied. Mechanical force has been recently recognized as a major factor in biological reactions ranging from cellular adhesion and intracellular transport by cellular motors to protein unfolding and protein translocation through membranes.[45,46] Atomic force microscopy (AFM) and optical tweezers techniques have been used extensively to examine forces that act on single molecules and are commonly determined by weak noncovalent interactions of a few k_BT.[47,48] Forces measured in AFM studies typically range from tens to several hundreds of piconewtons for noncovalent interactions, thus making it possible to measure stability and folding mechanisms of soluble and membrane proteins, interactions that drive protein−protein or protein−DNA recognition, and the binding of ligands and inhibitors to individual biomolecules.[47,49,50] The force exerted in optical tweezers can be as weak as a few piconewtons, which is sufficient to resolve the stability of individual molecular bonds or to measure forces generated by cellular machines, such as the ribosome or kinesin.[51,52] Because of this advanced force sensitivity, optical tweezers were employed to characterize the interaction of SecB with its natural substrate, MalE (Figure 2).[53] In the optical tweezers setup, the examined molecule (MalE) is tethered between two optically transparent polystyrene or composite beads.[54] These beads can be trapped within laser focal volumes, as the flow of photons in the laser beam generates a force toward the center of the beam that effectively positions the beads inside. Alternatively, only one optically controlled bead is employed, while another end of the examined molecule is immobilized on the surface or being held by a micropipet (Figure 2A). In contrast to fluorescence spectroscopy methods, no labeling or staining of molecules is required for such force measurements. Via the implementation of polymer spacers, such as polyethylene glycol or double-stranded DNA between the instrumental surfaces and the examined molecule, the effects of immobilization and photo-optical damage are reduced, so the molecule may follow its natural dynamics. The structural stability of MalE against the external force was used as a reporter for its interactions with SecB, and the effect of SecB on folding was analyzed by measuring the stability of MalE molecules. To exert a mechanical force, supporting beads were retracted by changing the position of the laser focal volume, thus stretching on the DNA tethers and the MalE molecule. The recorded force−distance spectrum described the response of the tethered molecules to the mechanical stress. After the initial DNA stretching phase, the applied force caused unfolding of MalE with a characteristic force peak of ∼20 pN in the force−distance spectrum, followed by unwinding of the polypeptide chain (Figure 2B). In the absence of SecB, subsequent relaxation of the unfolded protein led to the complete refolding of single MalE proteins, and a repeated stretching phase resulted in the characteristic unfolding peak. Unlike single MalE proteins, multiple fused MalE domains underwent irreversible aggregation upon unfolding. However, when the experiment was conducted in the presence of SecB, the initial unfolding event was not affected but the following relaxation did not cause either refolding or aggregation of MalE (Figure 2C). The corresponding force−distance spectrum showed a gradual increase in length with an increasing force, but no unfolding events that would indicate the presence of stable structured domains. This implies that SecB transfers completely unfolded polypeptides to the SecA ATPase domain, thus abrogating the need for energy-dependent protein unfolding.

Introduction of a charged arginine residue into the hydrophobic moiety of the SecB substrate-binding surface led to dramatic destabilization of the SecB−preprotein complex.[55] Although the affinity of SecB for substrates was barely affected, the mutated chaperone lacked the ability to prevent aggregation and supported translocation only weakly. Optical tweezers were employed to analyze the folding state of the protein in the presence of the malfunctional chaperone. In contrast to wild-type SecB, the mutant could barely prevent MalE from folding, because unfolding peaks were repeatedly observed in unfolding−relaxation cycles. As their position and intensity varied, MalE bound to the mutated SecB could achieve only partially folded structures characterized by low stability, which could relate to either on-pathway folding intermediates or non-native structural elements. These observations emphasized the role of tight hydrophobic contacts in the assembly of the SecB−preprotein complex and their role in the holdase activity of SecB.[9] Recently, the tweezers approach was also applied to examine the effect of the trigger factor on protein folding. The data showed that this molecular chaperone stabilizes intermediate folds of the MalE protein and that it prevents unproductive interactions between unfolded regions of multi-domain proteins.[56]

Targeting of Ribosomes to the Translocon. The SRP/FtsY targeting pathway fulfills an essential role in the co-

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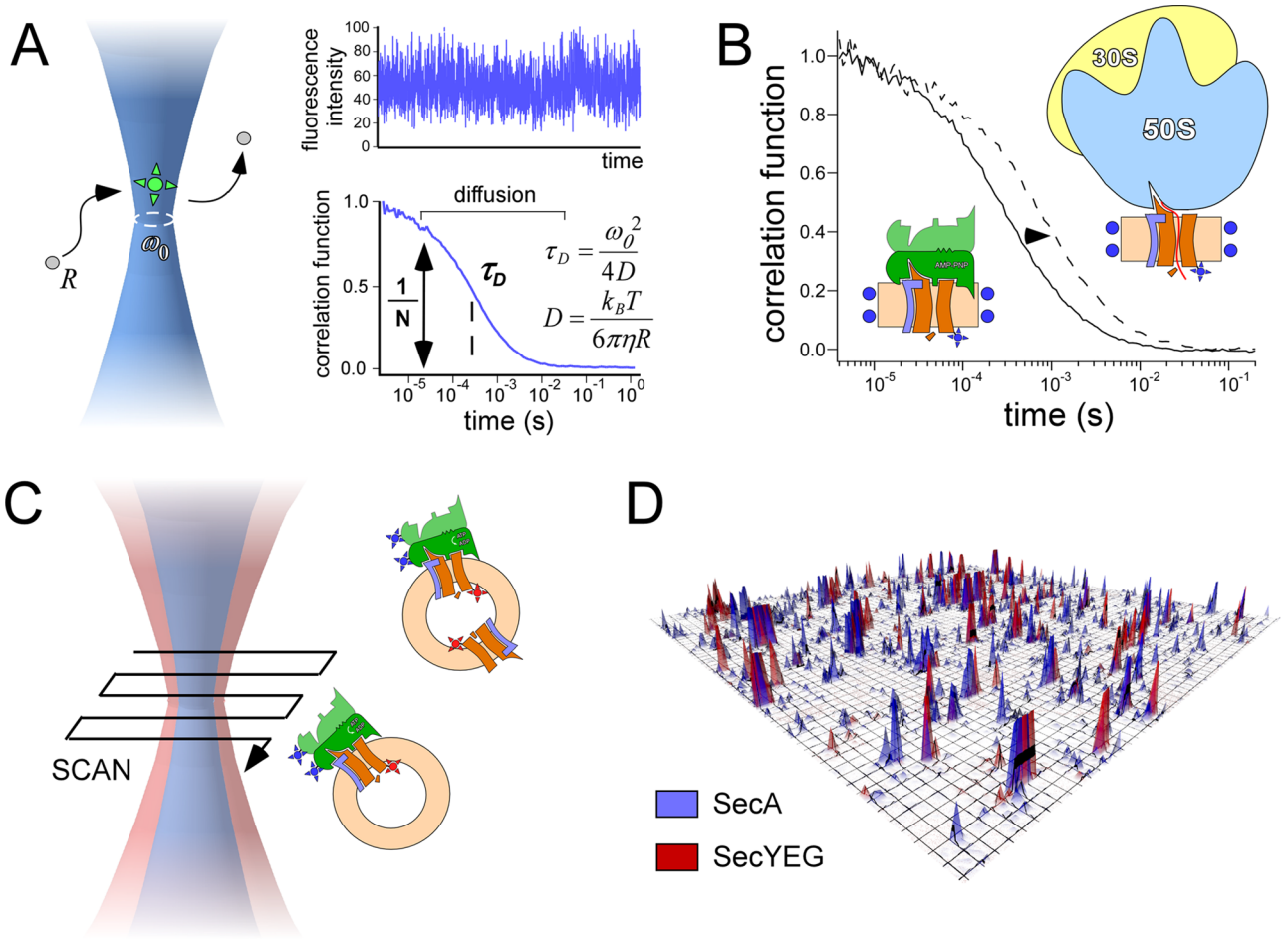

Figure 3. Fluorescence spectroscopy used to assess the SecYEG–SecA and SecYEG–ribosome interaction. (A) FCS employs fluctuations in fluorescence intensity upon diffusion of molecules through the laser focus of diameter ω_0. The calculated correlation trace provides the average number of molecules within the confocal volume (N) and the average residence time within (τ_D). Calculated diffusion coefficient D allows an assessment of molecular radius R of examined molecules via the Einstein–Stokes relation. (B) The interaction of SecYEG with ribosomes increases its apparent molecular radius and causes slow diffusion as manifested by the rightward shift in the FCS trace.[62] (C) Scanning DCFBA records fluorescence intensities of interacting molecules, such as SecYEG and SecA.[72] (D) The ratio of fluorescence for comigrating molecules is analyzed to determine the stoichiometry of formed molecular complexes.

translational targeting of RNCs to the translocon (Figure 1A). *E. coli* SRP consists of a 4.5S RNA and the Ffh protein.[6] Structural analysis suggested a large conformational change within SRP upon binding of FtsY that appears to be essential for the SecYEG–ribosome interaction.[57] The dynamics of this process has recently been investigated using the single-molecule fluorescence assay based on Förster's resonance energy transfer (FRET) between fluorophores conjugated within the *E. coli* SRP.[58] Individual SRP molecules bearing fluorophores at the Ffh protein and at the distal end of the 4.5S RNA were specifically immobilized on a surface and imaged by a total internal reflection fluorescence (TIRF) microscope. The background fluorescence is strongly reduced in the TIRF microscope, as the formed evanescence excitation wave illuminates the volume only within 100 nm of the surface, so detection of single surface-bound molecules can be achieved. Both donor and acceptor fluorescence signals were recorded in real time for individual SRP molecules, so their temporary correlation could be monitored and expressed as the change in FRET efficiency. The observed changes were assigned to the conformational dynamics of SRP, such as the displacement of the Ffh–FtsY complex from its resting position toward the RNA distal end and the return to its initial position either directly or via defined intermediates. A detailed analysis of the occurring transitions was conducted. Remarkably, the dynamics of the protein domains and associated GTP hydrolysis were critically dependent on the structure of the RNA. Adding ribosomes or SecYEG complexes strongly affected the FRET distribution and stabilized certain SRP conformations. Both the GTPase activity and shuttling of the Ffh–FtsY complex along the RNA scaffold were abolished in the SRP complexed with translating ribosomes. Formation of a quaternary complex with detergent-solubilized SecYEG strongly promoted binding of the Ffh–FtsY complex to the distal end of the RNA that is essential for a direct SecYEG–ribosome interaction at the ribosomal tunnel exit and the transfer of the protein substrate to the translocon. While a detailed model of the SRP targeting cycle had been proposed on the basis of structural insights,[59] the single-molecule analysis allowed measurements of the lifetimes of each conformation, the occupancy of transient states, and the effect of ribosomes and the translocon on SRP dynamics.

Co-translational membrane protein insertion requires that the ribosome remain associated with the translocon. The process is largely directed and powered by the protein elongation forces generated at the ribosome and by hydrophobicity-based membrane partitioning of the inserting trans-

dx.doi.org/10.1021/bi400913x | *Biochemistry* 2013, 52, 6740–6754

membrane domains. However, a number of membrane proteins additionally require the activity of SecA during insertion, as large hydrophilic loops need to be translocated to the *trans* side of the membrane. Biochemical analyses have revealed that the interaction sites for SecA and the ribosome overlap on the cytosolic surface of SecY, including the long structured loops C4 and C5. Therefore, a competitive binding mechanism was proposed,[60] although biochemical bulk assays suggested that SecA and ribosomes can bind SecY simultaneously.[61] Recently, the interaction of SecYEG with SecA and with RNCs was analyzed by fluorescence spectroscopy and surface plasmon resonance (SPR) that is a bulk biophysical method for detecting ligand interactions.[62] In a conventional setup, inner membranes bearing overexpressed levels of SecYEG were immobilized on a detection chip, and upon a flush of SecA or ribosomes along the detection surface, high-affinity binding to SecYEG could be detected. Importantly, the interaction with ribosomes was strongly stimulated by the emerging nascent chain and was maximal when the ribosome exposed a hydrophobic polypeptide segment at the exit tunnel. When RNCs were added to the preformed SecYEG−SecA complex, RNCs appeared no longer to bind to membranes.

Although this method indicated that binding of SecA and RNCs to SecYEG is mutually exclusive, the exact identity of the molecular species that bind cannot be determined in SPR measurements. Therefore, this analysis was extended by the use of high-sensitivity fluorescence correlation spectroscopy (FCS).[63] FCS is based on the temporal analysis of fluctuations in the fluorescence intensity of a protein-conjugated fluorophore, which originate to a large extent from the diffusion of proteins through the confocal detection volume of a laser beam (Figure 3A). Measuring the fluorescence intensity continuously at the submicrosecond bit rate allows building of a correlation function that describes the system dynamics and the relaxation rate. The major change in the correlation function commonly caused by molecular diffusion typically occurs at a 100 μs to 1 ms time scale. The characteristic decay time τ provides an average time that molecules spend inside the focal volume. As the mobility of the macromolecules is related to their dimensions via the Einstein−Stokes equation (Figure 3A), their size can be estimated from FCS measurements. Thus, the method can be used to monitor the assembly of large macromolecular complexes based on the mobility of participating components. It is in particular suited to the analysis of the binding of ribosomes to other molecular assemblies. Because of the large size, the diffusion of ribosomes is relatively slow, and thus, the diffusion of a fluorescently labeled ligand is dramatically slowed once it binds to the ribosome.[62] For such experiments, the SecY subunit was specifically labeled with a maleimide-functionalized fluorophore at a unique cysteine in a periplasmic loop. The mobility of labeled SecYEG present either in detergent or in small lipid patches of nanodiscs was measured in its free state and in the presence of ribosomes. In detergent, ribosome-bound SecYEG shows an up to 3-fold slower diffusion than free SecYEG (Figure 3B), in agreement with the molecular radii of the translocon (~3 nm) and the ribosome (~10 nm). Remarkably, detergent-solubilized SecYEG could interact both with RNCs and empty ribosomes, while SecYEG reconstituted into the nanodiscs showed a high specificity for RNCs, suggesting that the native lipid environment renders specificity to these interactions. In agreement with biochemical and SPR studies, modifications within the C4 and C5 loops of SecY interfered with ribosome binding. Introduction of SecA on the other hand restored the high mobility of the SecYEG nanodiscs as it caused the dissociation of the SecYEG−RNC complex. Interestingly, no competition was observed for detergent-solubilized SecYEG, suggesting that SecA binding was strongly impaired in the nonphysiological detergent environment. Essentially, the single-molecule experiments showed that SecA and ribosomes bind to SecYEG in a mutually exclusive manner. A similar FCS-based experimental scheme was recently employed to study the interaction between the YidC protein and ribosomes, showing that the complex assembly is critically dependent on the presence of an emerging nascent chain and the lipid environment of YidC.[64]

■ ASSEMBLY OF THE FUNCTIONAL SECYEG−SECA TRANSLOCASE

Oligomeric State of SecA and Interactions with SecYEG. SecA is a soluble ATPase that exists as a homodimer in the cytosol.[65,66] It binds with high affinity (K_D ~ 3−4 nM) to SecYEG during protein translocation. A variety of biochemical approaches have led to conflicting data with regard to whether SecA forms monomers during protein translocation[67,68] or needs to remain dimeric to be active.[69−71] To address the oligomeric state of SecA in solution as well as its functional oligomeric state when it is bound to SecYEG, a fluorescence study has been conducted employing dual-color fluorescence burst analysis (DCFBA). Herein, two populations of SecA, labeled with spectrally separated fluorophores, were monomerized by treatment with high salt and then allowed to dimerize by being desalted. Dilution of the SecA concentration resulted in a dissociation curve that displayed a K_D for SecA dimerization of <1 nM. Because the cellular SecA concentration has been estimated to be around 5−8 μM, this would imply that cytosolic SecA is predominantly dimeric. The binding of SecA to proteoliposomes containing fluorescently labeled SecYEG was assayed using DCFBA,[72] a technique that determines the comigration of particles by detecting overlapping fluorescent bursts that occur when the binding partners codiffuse through the confocal laser volume (Figure 3C,D).[73] Using this technique, an affinity of SecA for SecYEG of ~4 nM was measured, which is in good agreement with bulk cosedimentation assays and SPR studies.[74] To determine the oligomeric state of SecA bound to the SecYEG proteoliposomes, fluorescently labeled SecA was covalently cross-linked through its carboxyl-terminal cysteine residues, and the SecA:SecYEG fluorescence ratio for the covalently cross-linked SecA dimer was compared to the non-cross-linked SecA:SecYEG fluorescence ratio using the DCFBA methodology.[72] Under low-salt conditions, no difference in the fluorescence ratio could be detected, while under high-salt conditions, the non-cross-linked SecA bound with a much lower ratio, indicating binding of the SecA monomer. Because SecA in solution forms monomers under high-salt conditions and the affinity of binding of the SecA monomer to SecYEG was equal to that observed with the SecA dimer, the data suggest that SecA binds SecYEG in an asymmetric manner. The concentration dependence of the binding and oligomerization of SecA at SecYEG could be correlated with the translocation of the preprotein proOmpA. While binding and dimerization are saturated in the low nanomolar concentration range, the translocation rate only saturates at a SecA concentration of ~100 nM. These results imply that SecA is dimeric during protein translocation with one SecA protomer interacting with SecYEG directly and the other binding to the prebound SecA

dx.doi.org/10.1021/bi400913x | *Biochemistry* 2013, 52, 6740−6754

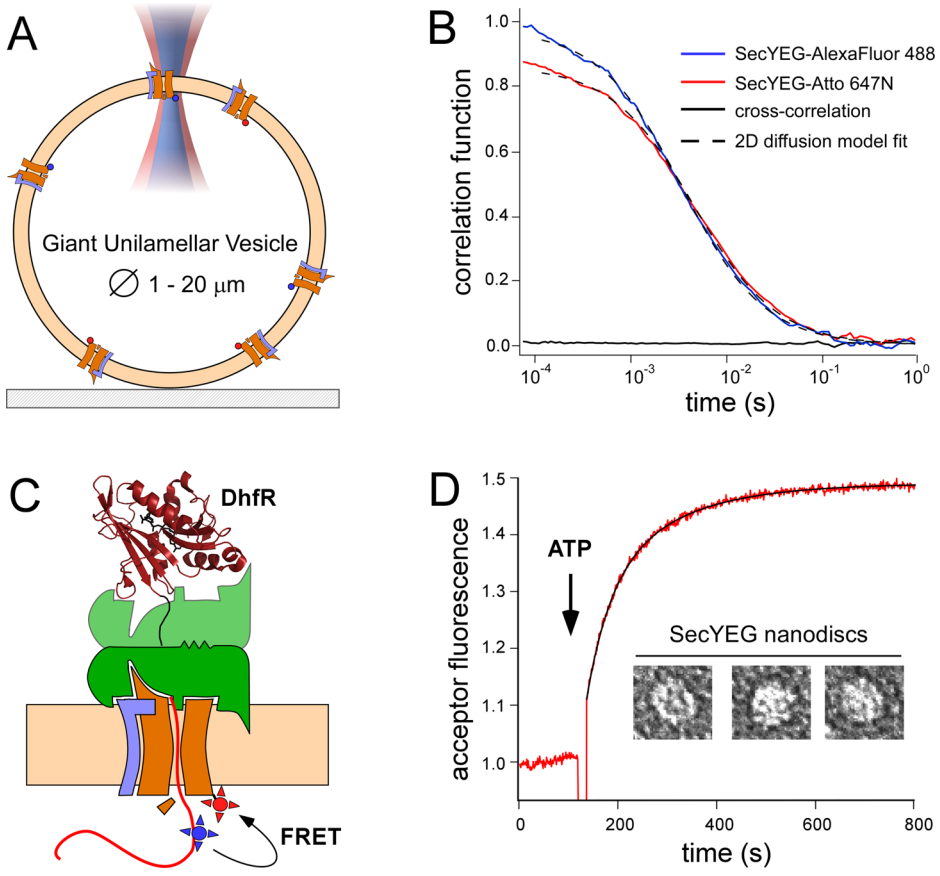

Figure 4. Oligomeric state of the translocon in membranes. (A) Analysis of translocon diffusion in the free-standing membrane of giant unilamellar vesicles by means of FCCS. (B) The absence of the cross-correlation signal for dual-labeled SecYEG implies a monomeric state of the translocon along its functional cycle.[90] (C) FRET-based translocation assay. A folded DhfR domain conjugated to the preprotein traps the protein in the SecYEG pore as a stable translocation intermediate. FRET-paired fluorophores at the preprotein and *trans* side of SecYEG allow the study of the kinetics of translocation. (D) FRET-based assay demonstrating that a single nanodisc-isolated translocon is competent for the preprotein translocation as the acceptor fluorescence increases upon triggering of translocation of proOmpA-DhfR with ATP. Individual nanodiscs imaged via negative-stain EM are shown in the inset.

(Figure 1A). To consolidate results from previous studies, a reciprocating piston model of protein translocation was proposed that describes membrane cycling of SecA at the SecYEG complex with one SecA copy staying in contact with SecYEG and a preprotein and another SecA copy cycling in an ATP-dependent manner.[75] Because other assemblies formed by dimers of SecYEG and either monomers or dimers of SecA have been also proposed[76,77] and because the functional dimerization interface of SecA has not been firmly established,[78] further studies should elucidate the structure of active assembled translocase.

Functional Oligomeric State of the Translocon. The functional quaternary structure of the translocon has been widely debated since the discovery of the SecYEG complex. The elucidation of its functional form is a prerequisite for understanding protein translocation. Crystal structures demonstrated that a single translocon may form a pore within the membrane.[31] On the other hand, a variety of biochemical studies suggest that the SecYEG complex readily assembles into oligomers. As the visualization of SecYEG *in vivo* remains challenging and has been barely addressed thus far,[79] the vast majority of biochemical and biophysical methods used to study the translocon oligomeric state are indirect. Intermolecular cross-linking and protein mobility analysis in polyacrylamide gels were extensively applied to study SecYEG oligomerization but also demonstrated serious drawbacks. In spite of being efficient, cross-linking of individual SecYEG complexes is strongly dependent on the molecular environment, such as the lipid bilayer or detergent micelles, and also different arrangements of the SecYEG protomers have been proposed.[80,81] The migration of SecYEG on polyacrylamide gels under nondenaturing conditions revealed distinct species of the translocon that differ in their apparent molecular masses. Such species were assigned to oligomeric complexes built of one, two, and four SecYEG protomers.[82−84] As "dimeric" SecYEG was the most ubiquitous complex in the presence of SecA and a preprotein, it was believed to represent the functional translocon. However, diverse detergents were employed to extract the translocon from membranes prior to the mobility analysis. These detergents not only influence the aggregation behavior of membrane proteins[85] but also contribute a significant share to the apparent protein mass and dimensions.[86] Therefore, it remained possible that SecYEG migration was determined by the micelle size instead of a different number of SecYEG copies. Detergent molecules could also alter native interactions within the translocon and either disrupt naturally occurring complexes or trigger nonphysiological oligomerization.

To analyze SecYEG oligomerization in lipid bilayers, bulk FRET spectroscopy and single-particle EM have been

dx.doi.org/10.1021/bi400913x | *Biochemistry* 2013, 52, 6740−6754

employed, yielding contradictory information about the membrane-embedded oligomeric state of the translocon.[82,87,88]

Most recently, high-resolution AFM imaging has been conducted on membranes that contained SecYEG reconstituted at a low density.[89] Nanometer-sized irregularities protruding above the lipid bilayer were assigned to individual SecYEG molecules, and a comprehensive analysis of their dynamics and dimensions was conducted. It was concluded that SecYEG was present predominantly as monomers, although dimers and tetramers were also detected. However, only the resting state of SecYEG was analyzed in this study, so no conclusion about the quaternary structure of functional translocons could be reached.

To address the functional SecYEG oligomeric state in lipid membranes, a fluorescence correlation-based approach was used.[90] As described above, three-dimensional diffusion of molecules in solution greatly depends on their molecular size, and the characteristic diffusion time measured in FCS is linearly proportional to the molecular radius. However, the mobility of membrane-embedded molecules as described by the Saffman−Delbrück law is largely determined by the viscosity of the membrane, while there is only a weak logarithmic dependence on their molecular size.[91] Thus, the SecYEG oligomeric state was examined using fluorescence cross-correlation spectroscopy (FCCS) that analyzes fluctuations in fluorescence intensity coming from two spectrally different fluorophores conjugated to individual translocons thereby reporting information about the degree of comigration.[92] FCCS analysis performed on SecYEG incorporated into giant unilamellar vesicles (GUVs) at a lipid:protein ratio that matches the density of SecYEG in native membranes did not reveal significant amounts of translocon dimers (Figure 4A,B).[90] The interaction with the SecA motor protein did not induce oligomerization, although previous cross-linking experiments suggested that a SecYEG dimer is required to form a binding interface for SecA.[76] Because oligomerization may occur during protein translocation, this process was mimicked by the use of the unfolded preprotein proOmpA fused to the folded dihydrofolate reductase domain (proOmpA-DhfR).[41] In the presence of ATP, this protein is partially translocated but becomes trapped within the SecYEG pore because of the tightly folded DhfR moiety, thus yielding a stable translocation intermediate. Formation of this translocation intermediate was monitored in real time using FRET between fluorophores at SecYEG and the preprotein (Figure 4C).[90] Although a majority of the translocons (>80%) were trapped in this preprotein-bound state, no oligomerization was detected on the basis of the FCCS analysis of SecYEG diffusion. Complementary FRET measurements between SecYEG molecules within the GUVs also did not reveal oligomers, even though different models for the architecture of the putative SecYEG oligomers were tested by placing fluorophores at different potential binding interfaces. Thus, we concluded that a single copy of SecYEG is sufficient for the interaction with SecA and for preprotein translocation. This model of SecYEG functioning as a monomer was recently confirmed by cross-linking experiments in which translocation intermediates were formed within living bacteria.[93] Although both monomers and oligomers of SecYEG were detected in the membranes, only the monomeric form was found to be active in protein translocation.

Although there is agreement about the monomeric state of the functional translocon in the independent approaches discussed above, evidence of functional oligomers of SecYEG was also implied from single-molecule studies employing site-specific cross-linked SecYEG dimers.[77] This work was inspired by an earlier hypothesis about the functional asymmetry in a SecYEG dimer, with one protomer dedicated to SecA binding and the other protomer functioning as a protein-conducting pore.[76] Remarkably, this functional asymmetry seemed to occur with only a genetically fused SecY dimer and could not be demonstrated with native SecY. TIRF microscopy was employed to visualize SecYEG-containing liposomes immobilized on a surface and to assess the function of the SecYEG monomers and chemically cross-linked dimers. The number of fluorescently labeled SecYEG molecules per proteoliposome was determined by counting their discrete photobleaching steps. At a sufficiently high lipid:protein ratio, mostly single SecYEG channels could be reconstituted into the proteoliposomes. Unfortunately, no control experiment was conducted on the cross-linked SecYEG to validate the presence of single dimers in the liposomes; thus multiple reconstituted translocons could be present because cross-linked SecYEG appears to be highly prone to aggregation.[77,80] Addition of fluorescently labeled proOmpA together with SecA and ATP resulted in the appearance of fluorescent spots. When employing tryptophan quenching, the counts of proOmpA fluorescence spots at the monomeric SecYEG decreased below an arbitrary offset, and this proOmpA was assigned as being bound to the outer vesicle surface, but not translocated. For proteoliposomes containing dimeric SecYEG, the remaining counts appeared to be higher than with the monomeric SecYEG, and this led the authors to conclude that dimeric SecYEG is obligatory for protein translocation.[77] However, several pitfalls can be recognized in this study. Tryptophan suppresses the fluorescence via static quenching and dynamic quenching that require short-range interaction on the order of 1−2 nm[94,95] and so demonstrates limited quenching efficiency.[77] Thus, counting of the fluorescent spots remaining after this incomplete quenching does not record the translocation activity as nontranslocated proOmpA molecules may not be completely quenched and partially quenched states will still be counted as fluorescent events. As the study lacks evidence that translocation is recorded with single-molecule sensitivity, it is uncertain whether the method was sufficiently sensitive to detect the less frequent translocation events expected with the monomeric SecYEG proteoliposomes. In a follow-up study employing another cross-linking analysis, it was concluded that the functional translocon is formed by a single SecYEG copy, thus abandoning the previous conclusions based on the TIRF experiments.[96]

In another approach, lipid-containing nanodiscs have been used to isolate different oligomeric states of SecYEG, so their functional properties could be probed.[30,97,98] Nanodiscs are built of the major scaffold protein (MSP) that forms a belt around a lipid bilayer 10−13 nm in diameter, and 100−200 lipid molecules can be entrapped within a single nanodisc depending on the MSP size.[99] The lipid bilayer of the nanodisc is sufficiently large for incorporating single copy or multiple copies of a membrane protein, thus ensuring a nativelike environment that supports protein activity. Initial experiments showed that nanodisc-reconstituted SecYEG in the presence of *E. coli* lipids supports the ATPase activity of the SecA motor protein, and it was suggested that monomeric SecYEG was sufficient for these interactions.[97] Follow-up research showed that multiple copies of SecYEG can be reconstituted within larger nanodiscs, allowing the properties of translocon dimers and monomers to be studied separately.[30,98] A recent study of

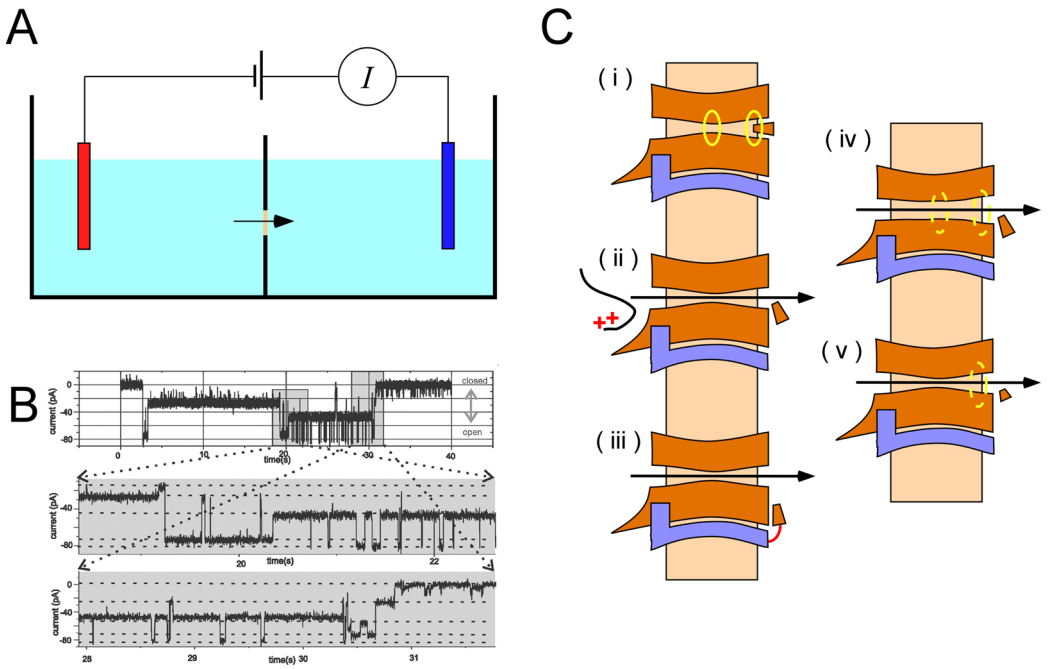

Figure 5. Single-molecule conductivity measurements on the protein-conducting channel. (A) Design of an electrophysiology black-membrane setup. Native or reconstituted membranes containing SecYEG/Sec61p translocons seal the diaphragm between the two chambers, and transient electric currents via single-membrane channels are recorded under a constant applied voltage. (B) Opening and closing of a single membrane-reconstituted Sec61p channel under the applied voltage display as steps in the current recording.[108] (C) The conductivity of the translocon varies upon the functional cycle and is tuned by mutations. The intact channel remains impermeable for ions in its resting state (i), while different levels of conductivity are observed upon the interaction of the translocon with isolated synthetic signal sequences (ii), covalent immobilization of the plug domain in a completely open conformation (iii), *prlA* mutations (iv), and the deletion of the plug domain (v).

nanodisc-reconstituted SecYEG showed that a translocation intermediate can be efficiently formed in nanodiscs that contained single translocons surrounded by a nativelike lipid bilayer (Figure 4C).[30] The translocation rate was barely affected by multiple co-reconstituted copies of the translocon, but it was greatly enhanced for PrlA SecYEG (Figure 4D).[30] These data validate previous results for monomeric SecYEG as a functional unit.

Remarkably, an alternative study reported no ATPase activity for SecA in the presence of monomeric SecYEG, while multiple copies of SecYEG seemed to support this activity.[98] As the ATP hydrolysis rate was dependent on the presence of the preprotein, this phenomenon was assigned to translocation activity exclusively restricted to SecYEG dimers. For the analysis, SecYEG was reconstituted into nanodiscs in pure phosphatidylglycerol lipid bilayers. Anionic lipids, such as phosphatidylglycerol, strongly stimulate the interaction of SecA with membranes, and increased levels of ATP hydrolysis can be observed even in the absence of the SecYEG complex.[100,101] Because the translocon requires at least 10% nonbilayer lipids, such as phosphatidylethanolamine, to be active,[102] it is likely that the translocation activity was highly impaired in this system.

STRUCTURAL DYNAMICS OF THE TRANSLOCON

The cytoplasmic membrane of bacteria is an energy-transducing membrane, and thus, it is of critical importance that during protein translocation, the membrane remain sealed for small ions and protons. Because SecYEG forms a pore within the membrane, the question of what mechanisms for sealing exist to prevent leakage of ions has been raised. Moreover, early reports of protein translocation in bacterial membranes demonstrated a translocation-associated membrane permeability of chloride ions resulting in a reduction in the transmembrane electrical potential ($\Delta\psi$).[103,104] Although also a certain level of ion conductivity is supported by the SecYEG-associated SecDF complex,[14] the intrinsic ion conducting properties of the translocon have been examined in great detail. In early studies, direct conductivity recordings were performed to associate protein-conducting channels in the endoplasmic reticulum and *E. coli* membranes with large membrane pores.[105−107] More recently, single-molecule electrophysiology techniques have been employed to characterize the ion conductance of the purified translocon and describe their structural dynamics.

Common for electrophysiological recordings, a lipid bilayer is formed over a millimeter-sized pore in the wall that separates two chambers (Figure 5A). Electrodes are immersed into a buffer solution on each side of the pore and kept under a constant voltage, while the electrical current level is monitored. Opening and closing of individual channels are detected as changes in the ion current, and the associated conductivity level is used to estimate the pore size of the channel. Aiming for single-molecule resolution, one needs to incorporate examined protein channels into the pore-filling bilayer at a sufficiently low density, so that the probability of detecting currents through multiple channels is avoided. A representative single-channel conductivity recording of the Sec61 translocon is shown in Figure 5B.[108] In the early studies, native membranes extracted from either *E. coli* or canine pancreas were added to the solution in the presence of calcium to induce membrane fusion.[105] Both ER and bacterial membranes showed opening of channels of similar conductivities of 115 pS that were attributed to protein translocons.[105,106] Remarkably, the initial

dx.doi.org/10.1021/bi400913x | *Biochemistry* 2013, 52, 6740−6754

experiments demonstrated that ER channel opening was dependent on the presence of GTP, which was readily explained by the involvement of the GTPase SRP in protein transport via the Sec61 translocon.[109,110] Both the ER and *E. coli* translocons were described as anion-selective channels in agreement with indirect membrane permeability measurements.[103,111,112] However, this view has been challenged by recent electrophysiology experiments, suggesting that the eukaryotic translocon Sec61 has no selectivity for the ionic charge.[108]

Follow-up studies using the purified and membrane-reconstituted translocons have revealed that the conductivity depends on the translocation state as a ribosome-bound polypeptide chain that inserts into the translocon efficiently seals the channel. Binding of ribosomes to the purified bacterial SecYEG complex resulted in ion conductivity, suggesting that this interaction alone primes the translocon for further substrate insertion.[113] When the nascent chain was released from the Sec61−ribosome complex by the addition of puromycin, the polypeptide chain escaped from the translocon likely, leaving it open, and a large conductivity of 220 pS was observed.[106] Closing of the single ER channels could be triggered by removing the bound ribosomes,[106] by the ATP-dependent release of the chaperone BiP,[108] or upon calcium-dependent binding of calmodulin molecules.[114] These different mechanisms of sealing the channel may occur via the plug, the constriction ring, or by capping the channel at its lumenal or cytoplasmic sides. This highlights the importance of maintaining the closed conformation of the idle translocon that might be essential to avoid leakage of calcium ions from the ER lumen.

The interaction of the translocon with polypeptide substrates has also been assayed in the absence of ribosomes. In the presence of a synthetic signal peptide, a channel conductivity of 220 pS has been reported that was attributed to the opening of the bacterial translocon (Figure 5C).[107] This led to the conclusion that binding of the signal sequence at the *cis* side of the translocon is sufficient to trigger the open state. However, this phenomenon occurred only with a *trans* negative $\Delta\psi$, which is the opposite of its polarity *in vivo*. Similarly, ER secretion proteins that contained the signal sequence were capable of triggering the opening of Sec61 channels.[108] As the bacterial translocon remains impermeable for ions in the absence of the signal sequence,[115] the single-molecule current measurements suggest a model of SecYEG translocon gating by its substrate. However, the structural data discussed above suggest that even in the absence of substrate, SecA is critically involved in channel opening.[25,30]

An effort was taken to investigate the role of the SecA protein in translocon channel opening using electrophysiological measurements.[116] When SecA is injected into *Xenopus* oocytes in the absence or presence of liposomes, patch-clamp measurements recorded a transient ATP-dependent change in membrane permeability. This was attributed to the opening and closing of membrane pores built of SecA alone. Interestingly, opening of these putative channels was dependent on the presence of a substrate protein but was not affected by a synthetic signal peptide. Remarkably, the observed conductivity was independent of the SecYEG channel and significantly lower (50 pS) than the value measured for purified SecYEG present in black lipid membranes. This implies a different pore architecture and/or gating mechanism. As SecA has been shown to interact strongly with the lipid membranes even in the absence of SecYEG and has a strong propensity to insert into the lipid bilayer while forming high-order structures at the lipid interface,[117,118] it may well be that SecA causes membrane destabilization resulting in these low-conductivity events.[100,101,119] It is unclear, however, how this SecA feature relates to native protein translocation that strictly requires the translocon.

In the closed state, the central pore of the SecYEG/Sec61 translocon is sealed by the narrow constriction ring and the plug domain.[14,31] During the translocation cycle, the plug has to vacate the central position to accommodate a translocating polypeptide chain. Immobilization of the plug by cross-linking indeed abolishes translocation[120] unless the cross-linker allows a limited degree of movement (2−5 Å),[121] in line with the modest dislocation of the plug observed in the SecYEG−SecA crystal structure.[25] Cross-linking studies suggested a large conformational change during channel opening during which the plug would move all the way to the peripheral helix 3 of the SecE subunit, shifting by at least 2 nm from its idle position.[122,123] When the cross-linking was performed *in vivo* to generate a permanently open channel, this state appeared to be lethal for the cells.[122] Single-channel electrophysiology showed that chemical cross-linking of the plug all the way up to the cytosolic C-terminus of SecE yielded a constantly open channel with a remarkably broad conductivity range (Figure 5C).[115] These observations suggest a high plasticity of the translocon with the presence of multiple ion-conducting conformations as previously described for the native Sec61 complex.[108] The conductivity measurements on SecYEG were used to estimate a pore size, providing a broad range of radii between 0.4 and 1.2 nm. Although the crystal structure of the translocon shows a rather narrow central pore, efficient SecYEG-mediated translocation of a substrate protein conjugated with bulky synthetic molecules suggests that the channel can even open up to 1.1−1.2 nm.[43] The latter represents a functional pore size that likely includes space provided by an open lateral gate.

Though a completely opened channel would allow the unrestricted movement of ions across the membrane, deletion of the plug domain is not lethal to cells but renders the translocon with a *prl* phenotype while the structural stability is reduced.[120,124,125] The hydrophobic constriction ring may act as a seal as long as the channel is in the idle state,[31,126] while during translocation, the constriction ring may function as a gasket surrounding the translocating polypeptide chain and preventing leakage of ions.[112,115] Indeed, fluorescent studies have demonstrated that preproteins encounter a hydrophobic region while passing through the SecY pore.[127] However, recent crystallographic analysis of the archaeal SecYEβ complex containing plug deletions revealed an unexpected structural rearrangement within the channel, suggesting the formation of a new plug domain built by neighboring residues.[125] Although the *de novo* formed plug appears to be sufficient to seal the channel and support cellular viability, such translocons also exhibit a *prl* phenotype because a number of the stabilizing interactions are lost upon the deletion of the original plug domain. These structural findings for the plug domain were extended by single-molecule conductivity experiments.[115] The electrophysiology setup followed a conventional scheme, though the probed membrane area was reduced 50−100 times compared to previous measurements.[105] While wild-type SecYEG remained impermeable for ions, plugless mutants demonstrated multiple opening and closing events. The

conductivity level increased with the extent of plug domain truncation, but even when the entire domain was deleted, the channel was still able to attain its closed state (Figure 5C).[115] The PrlA4 mutant of SecYEG demonstrated a similar behavior, as it oscillated between the open and closed conformations, suggesting a leaky channel. Unlike overexpression of wild-type SecYEG, overexpression of the transiently open PrlA4 and plugless SecYEG mutants causes a depolarization of the $\Delta\psi$.[111] It should be noted that recent electrophysiology measurements on a plugless variant of SecYEG reconstituted into synthetic lipid bilayers did not reveal such transient openings of the channel, and its properties remained identical to those of the wild-type translocon in the idle state.[128]

■ OUTLOOK

Our current understanding of the protein secretion mechanisms arises largely from bulk biochemical analysis, structural studies, and thorough single-molecule investigations. As described above, modern biophysical methods have allowed comprehensive analysis of the Sec system at the level of individual molecules, including conformational dynamics of individual proteins, protein−protein interactions, and assemblies of functional translocons. Although single-molecule analysis of protein secretion is still in the early stages, the current studies have already demonstrated their great potential by answering primary questions about the functional mechanisms of the Sec translocon, such as the quaternary state of the SecYEG channel and the dynamics of the translocon.

A number of future research directions for single-molecule studies can be envisioned. Investigating kinetic aspects of the translocation for individual translocons would be one of the main challenges in the field. These studies are difficult to conduct in bulk as they require temporal synchronization of multiple translocons along the functional cycle. In contrast, no synchronization is needed when monitoring the reaction at individual translocons. Kinetic analysis performed in real time will not only complement existing bulk translocation assays but also reveal the actual translocation rates, the translocation step size, and possible kinetically limiting steps. It may also be used to assess the effect of mutations in SecA or alteration of the physicochemical properties of translocating polypeptide chains. Once established on a minimal translocon system, additional factors, such as the SecDF complex, the YidC insertase,[129] or the pmf, can be introduced into the assays, expanding the analysis toward single-molecule studies of membrane protein biogenesis. With a broad selection of fluorescent probes, diverse colocalization experiments can be performed focusing on more than two binding partners to assess the dynamics of the translocon and its association with its binding partners along the functional cycle. Here, visualization of single molecules not only will provide the most direct view of the process but also may probe transient complexes formed along the translocation, their architecture, and characteristic lifetimes.

Another perspective in single-molecule analysis is based on recent developments in super-resolution fluorescence microscopy. Several approaches established in the field, such as photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and stimulated emission depletion microscopy (STED), allow visualization of cellular organization and dynamics at the spatial resolution below the diffraction limits, down to tens of nanometers.[130] Such approaches will be useful for investigating various aspects of protein translocation, such as translocon localization and assembly *in vivo*. Going beyond the diffraction limit will also allow *in vivo* translocation studies in the relatively small bacterial cells, whose dimensions are otherwise comparable with those of the confocal laser volume.[131] Single-particle tracking has served to monitor the dynamics of twin-arginine (Tat) translocons within the bacterial membrane.[132] The Tat translocon is dedicated to secretion of folded protein domains, and recently, cooperation between Sec and Tat systems has been shown by biochemical methods.[133] Thus, single-molecule methods may provide further information about these intriguing interactions.

Still, several bottlenecks should be overcome upon designing single-molecule experiments. One of those is the lack of a single-molecule "quality control" procedure that would evaluate the functionality of individual molecules. Introducing such procedures will discriminate individual properly folded and reconstituted translocons from occasional aggregates to refine the data collection. Another important task is to establish nonhindered protein translocation in supported or semi-supported planar lipid bilayers that will open broad prospects for TIRF microscopy and single-particle tracking applications that allow single translocons in lipid membranes to be monitored in real time. These will be important challenges for the future.

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Funding

This work was financially supported by The Netherlands Foundation for Scientific Research, Chemical Sciences (NWO-CW) and Earth and Life Sciences (NWO-ALW), and the Foundation for Fundamental Research on Matter (FOM).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Eli van der Sluis for valuable comments on the manuscript.

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