Chapter 4

Monitoring the Activity of Single Translocon

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Abstract – Recent studies introduced a novel view that the SecYEG translocon functions as a monomer and interacts with the dimeric SecA ATPase, which fuels the preprotein translocation reaction. Here, we used nanodisc-reconstituted SecYEG to characterize the functional properties of single copies of the translocon. Using a method based on intermolecular Förster’s resonance energy transfer, we show for the first time that isolated nanodisc-reconstituted SecYEG monomers support preprotein translocation. When several copies of SecYEG were co-reconstituted within a nanodisc, no change in translocation kinetics was observed, suggesting that SecYEG oligomers do not facilitate enhanced translocation. In contrast, nanodisc-reconstituted monomers of the PrlA4 variant of SecYEG showed increased translocation rates. Experiments based on intramolecular Förster’s resonance energy transfer within the nanodisc-isolated monomeric SecYEG demonstrated a nucleotide-dependent opening of the channel upon interaction with SecA. In conclusion, the nanodisc-reconstituted SecYEG monomers are functional for preprotein translocation and provide a new prospect for single-molecule analysis of dynamic aspects of protein translocation.
1. Introduction

The molecular mechanism of bacterial protein secretion has been intensively studied since the identification of the sec genes three decades ago\textsuperscript{27,330}. Multidisciplinary efforts have led to a rigorous characterization of the essential components of the pathway, such as the membrane-embedded protein-conducting channel, or translocon, SecYEG, and the ATPase SecA\textsuperscript{131}. Biochemical and biophysical data, together with the recently available molecular structures of individual components and their complexes, imply a functional model of protein translocation in bacteria\textsuperscript{130}. In the current view, a single heterotrimer SecYEG forms a narrow pore within the membrane and interacts at its cytoplasmic membrane face with the motor protein SecA. The SecA ATPase directs the unfolded, translocation-competent preprotein into the SecYEG pore and employs the energy of ATP hydrolysis to facilitate preprotein transport. Other components involved in the pathway include a small ATP-independent chaperone SecB that prevents the preprotein from premature folding in the cytoplasm due to competing hydrophobic interactions\textsuperscript{107,164}, and the integral membrane complex SecD/SecF that is described as a proton-driven “lever” facilitating preprotein transport\textsuperscript{241}. The N-terminal signal sequence of the preprotein targets it to the SecYEG translocon and primes the translocon for transport\textsuperscript{147}. During translocation, the signal sequence of the preprotein is removed from the translocating preprotein by the leader peptidase and released to the lipid bilayer.

Although the overall organization of the Sec pathway is generally accepted, several specific issues remain the focus of intense discussion, such as the functional oligomeric state of the SecYEG translocon upon SecA binding and preprotein translocation\textsuperscript{331}. A variety of approaches, such as native PAGE, cryo-electron microscopy and crosslinking, have suggested that the translocon undergoes oligomerization, however the vast majority of experiments were performed in a non-physiological detergent environment\textsuperscript{332,333}. The significance of SecYEG oligomers was challenged by the first crystal structure of SecYE\textsubscript{β} homologue of archaea revealing a transmembrane channel formed within a single translocon protomer\textsuperscript{10}. Since the channel was captured in its closed idle state, it was further suggested that the second protomer may be essential for opening the pore for translocation\textsuperscript{84}. More recently, two independent functional studies demonstrated that a single SecYEG monomer is required for binding and translocation of the preprotein. Experiments based on \textit{in vitro} fluorescence spectroscopy and \textit{in vivo} chemical crosslinking\textsuperscript{110,334} investigated the membrane-embedded translocon during its functional cycle including its association with the SecA ATPase and preprotein. The active translocon appeared largely monomeric and neither SecA binding, nor preprotein transport, led to the formation of oligomeric SecYEG complexes. However, an analysis of the translocation dynamics has not been performed so far, and thus transient SecYEG oligomerization at an intermediate stage of the translocation reaction could not be excluded.
In an alternative approach, analysis of the SecYEG oligomeric state within the lipid membranes was conducted on translocons reconstituted into nanodiscs, small lipid patches of defined size (~10 nm)\textsuperscript{335,336}. The dimensions of these nanodiscs allow isolating either single or several copies of the translocon within single nanodiscs, such that their functional properties may be measured in a direct way. It was suggested that only the dimeric form of SecYEG embedded into pure DOPG lipids supports the ATPase activity of SecA\textsuperscript{335}, but in that study, translocation was not addressed experimentally. Although anionic lipids, such as DOPG, are required for translocon activity and DOPG-formed membranes alone have a strong propensity to stimulate the SecA ATPase activity\textsuperscript{170}, SecYEG translocation activity requires the presence of both charged and non-bilayer lipids, such as DOPE, within the membrane\textsuperscript{337}. Remarkably, when SecYEG was reconstituted into nanodiscs with the physiologically-relevant \textit{E. coli} lipids, no SecA translocation ATPase activity was observed\textsuperscript{335} which suggested that technical issues interfered with this analysis.

Here, we aimed to investigate the functional properties of nanodisc-reconstituted SecYEG within a native-like lipid bilayer using a direct translocation assay. Our results demonstrate that monomers of SecYEG conduct preprotein translocation. The rate of translocation is not stimulated further when several SecYEG molecules are reconstituted into the nanodisc, demonstrating that the monomeric state of SecYEG is necessary and sufficient for protein translocation.

2. Detergent-solubilized SecYEG is monomeric

Nanodisc-reconstituted translocon represents an attractive lipid-based system that allows isolation of a single copy of SecYEG and testing it directly for its functional properties. To control the number of SecYEG translocons reconstituted into these nanodiscs, it is important to ensure that the detergent-solubilized SecYEG is in a monodispersed state. Previous reports suggested that the oligomeric state of SecYEG in detergent is dynamic and that the subtle equilibrium between monomers and dimers can be fine-tuned by different physicochemical factors, such as the detergent concentration\textsuperscript{332,333}. To characterize the oligomeric state of purified SecYEG prior to reconstitution into nanodiscs, we employed fluorescence correlation spectroscopy/fluorescence cross-correlation spectroscopy (FCCS) using SecYEG molecules independently labeled with two different fluorophores\textsuperscript{338,339}. This high-sensitivity technique analyzes fluorescence fluctuation of spectrally separated fluorophores and their temporal correlation when illuminating those within two aligned laser confocal volumes (Fig. 1A). A positive correlation between fluorescence levels may reflect co-diffusion of fluorescently labeled species, for example, as a result of binding or oligomerization, while independent diffusion results in zero cross-correlation. For the fluorescence experiments we used a SecYEG mutant containing a unique cysteine L148C within a periplasmic loop that connects helices 3 and 4 (SecY\textsubscript{C148EG})\textsuperscript{110}.
Figure 1. SecYEG is monomeric in detergent. (A) The oligomeric state of DDM-solubilized and fluorescently-labeled SecY C148EG was analyzed by means of fluorescence cross-correlation spectroscopy (FCCS). AlexaFluor 488- and Atto 647N-labeled translocons diffuse through the aligned laser excitation volumes, and corresponding fluorescence fluctuations are cross-correlated if SecYEG form dual-labeled oligomers. (B) FCS/FCCS analysis on SecY C148EG diffusion in detergent. Temporal auto-correlation in fluorescence in individual blue (505-570 nm) and red (640-700 nm) channels reported on the translocon diffusion speed, and the cross-correlation between these channels indicated the fluorophore coupling, thus described SecYEG oligomerization. FCS analysis provided a diffusion coefficient for SecYEG of 28.3±1.6 cm²/s (mean ± S.D., n = 10) as determined in the blue channel, in good agreement with previous measurements. The cross-correlation level was within 10% of both auto-correlation traces that matched closely the unspecific labeling of SecYEG. The fluorophores AlexaFluor 488-C₅-maleimide and Atto 647N-maleimide were purchased from Life Technologies/Molecular Probes and Atto-Tech, respectively. FC(C)S data was recorded using LSM 710 inverted confocal microscope equipped with a Confocor 3 unit (Carl Zeiss GmbH, Germany). Data were fitted within 20 µs to 500 ms range assuming normal three-dimensional diffusion of molecules (shown in thin black lines) using ZET 2010 software (Carl Zeiss GmbH, Germany). SecYEG was present at 100 nM total concentration in 0.1% DDM, 100 mM KCl, 10% glycerol, and 50 mM KPi pH 7.4.

E. coli membranes containing over-expressed SecY C148EG translocons were incubated in the presence of two maleimide-containing fluorophores, AlexaFluor 488 and Atto 647N, simultaneously; thus, the individual protomers of SecYEG could be stochastically labeled with the fluorophores. Fluorophore-conjugated SecY C148EG was purified, and analysis of the labeling revealed an ≈55% labeling efficiency for both fluorophores, suggesting complete labeling of the single cysteine positions but also limited unspecific labeling. Next, FCCS was used to determine the amount of oligomeric complexes that bear both fluorophores. Due to stochastical labeling and fluorophore distribution, the value could be also used...
to estimate the amount of equally labeled oligomers. When conducted in a solution containing 0.1% n-dodecyl β-D-maltoside (DDM) FCCS experiments showed that the cross-correlation signal was within 10% of both auto-correlation traces (Fig. 1B). As the value matched closely the total level of the unspecific labeling, the signal was largely related to single SecYEG protomers bearing both fluorophores. Thus, we concluded that the detergent-solubilized SecYEG translocon was present almost exclusively as monomers.

3. Nanodisc-isolated SecYEG monomers support translocation

To investigate the role of the SecYEG oligomeric state in the translocation cycle, we reconstituted SecYEG into nanodiscs constricted by the MSP1E3D1 scaffold protein (MSP) \(^{340}\). To achieve the monomeric state of SecYEG within these nanodiscs (SecYEG\(_{\text{mono}}\)-Nd), we reconstituted the translocon in presence of an excess amount of MSP (10-fold) and lipids (500-fold) following established protocols \(^ {90,340}\). We used lipid bilayers composed of a mixture of DOPE, DOPG and DOPC that efficiently support efficient SecYEG translocation activity \(^ {110}\). The formed nanodiscs were fractionated by size-exclusion chromatography (SEC) and their assembly was verified by SDS-PAGE and negative stain electron microscopy (Fig. 2A and B). The monomeric state of SecYC\(_{148}\)EG labeled with two fluorophores and reconstituted into nanodiscs was confirmed by FCCS, as the cross-correlation signal remained below 10% in the peak SEC fractions (Fig. 2C). When the amount of SecYEG in the reconstitution reaction was increased, multiple copies of the translocon could be reconstituted into single nanodiscs that resulted in high cross-correlation signal (Fig. 2D; see below).

To characterize the interaction of the nanodisc-isolated translocon with the SecA motor protein we analyzed its stimulatory effect on the SecA ATPase hydrolysis activity. SecYEG\(_{\text{mono}}\)-Nd stimulated the SecA translocation ATPase in a conventional ATPase assay in presence of the preprotein proOmpA \(^ {69}\), while the ATP hydrolysis remained at background levels when assayed in presence of nanodiscs loaded with pure lipids (Fig. 3A). Thus, a single membrane-embedded copy of SecYEG was sufficient to interact with the cytosolic motor protein SecA and stimulate its ATPase activity in a preprotein-dependent manner \(^ {65,110}\).
Figure 2. Preparation of SecYEG-containing nanodiscs. (A) Detergent-solubilized SecYEG was reconstituted into the lipid bilayer consisting of DOPG, DOPE, and DOPC at 1:1:1 molar ratio in the presence of the major scaffold protein MSP1E3D1 (MSP) resulting in nanodiscs formation. Reconstituted molecular complexes were subjected to SEC90, with a maximum elution near fraction #14. Coomassie-stained SDS-PAGE shows content proteins (indicated on the left). Positions for molecular weight markers (PageRuler Prestained Protein Ladder, Thermo Scientific) are shown on the right. (B) Individual nanodiscs (encircled in red) as visualized by uranil acetate negative-stain electron microscopy. (C and D) FCCS analysis of the SecYEG oligomeric state within nanodiscs. Using an excess of MSP and lipids relative to SecYEG during the reconstitution (molar ratio SecYEG:MSP:lipids of 1:10:500) allowed trapping single copies of the translocon within nanodiscs, as confirmed by low cross-correlation level, probably arising from dual-labeled SecYEG protomers (see Fig. 1B). If SecYEG and MSP were used at stoichiometric amounts upon the reconstitution (molar ratio SecYEG:MSP:lipids of 10:10:500), nanodiscs largely contained multiple copies of SecYEG that resulted in a high cross-correlation level (D). The fraction of “oligomers” was estimated around 80%. The same color coding for FCS/FCCS data was used for panels C and D, that is described in the panel C. SecYEG-Nd were present in 100 mM KCl, 5% glycerol, and 50 mM Tris-HCl pH 7.5. FCS/FCCS data were fitted within 20 μs to 500 ms range assuming normal three-dimensional diffusion of molecules (shown in thin black lines). Deviations from the diffusion model at time range 5-10 ms probably arise from large particles, such as occasionally formed proteoliposomes.
Figure 3. SecYEG is functional as a monomer. (A) Single and multiple copies of SecYEG support ATPase activity of SecA. Functional interactions of SecA with nanodisc-reconstituted SecYEG were assayed in presence of the preprotein proOmpA. Either form of the translocon, but not empty nanodiscs, supported the ATPase activity of SecA in the translocation reaction. (B) Translocation activity of SecYEG-Nd probed in real-time FRET-based assay. FRET-pair fluorophores Cy3 (Pierce) and Atto 647N were conjugated to preprotein fusion proOmpA-C282-DhfR and SecY-C148, respectively, at indicated positions. DhfR domain was pre-folded in presence of ligands MTX and NADPH and so could not be translocated via the SecYEG pore. Formation of the stable translocation intermediate was followed as a change in FRET signal. (C) SecYEG monomers and dimers are active in translocation. Either form of the translocon could associate with the preprotein and translocate the unfolded proOmpA domain upon adding ATP giving rise to FRET-based acceptor fluorescence. The translocation rates estimated from single-exponential fitting (solid black lines) were within 20% for SecYEG monomers and oligomers. No translocation was observed in presence of AMP-PNP. For the reaction 100 nM of nanodisc-embedded SecY-C148EG-Atto 647N was incubated at 37°C.
with 200 nM pre-folded proOmpA<sub>C282</sub>-DhfR-Cy3 and 1.8 μM SecA as described before<sup>110</sup>. Reactions were initiated by adding 5 mM ATP or AMP-PNP. (D) Translocation rate depends on the structural features of SecYEG. The translocation-enhanced mutant SecYEG PrlA4 reconstituted into nanodiscs as monomers demonstrated faster kinetics and higher total translocation efficiency than wild-type SecYEG that agreed with previous data<sup>342</sup>. Cysteine at the solvent-exposed position 148 was introduced into wild-type and PrlA SecYEG over-expression plasmids pET2302 and pET2306, respectively<sup>342</sup>, yielding plasmids pET2302c and pET2306c. Expression, purification and fluorescent labeling were performed according to the established protocol<sup>110</sup>. FRET assay was performed using SLM2 spectrofluorometer (Aminco Bowmann), as previously described<sup>110</sup>.

The translocation activity of SecYEG was assessed in real-time using a recently developed fluorescence-based assay<sup>110</sup>. The assay employs Förster’s resonance energy transfer (FRET) between fluorophores conjugated to the substrate preprotein and the translocon that occurs upon the formation of a translocation intermediate (Fig. 3B)<sup>110</sup>. Briefly, membrane-reconstituted SecYEG is incubated at 37°C in presence of the SecA ATPase, the chaperone SecB and the preprotein proOmpA fused to a folded domain of dihydrofolate reductase (DhfR). Upon addition of ATP, the proOmpA-DhfR substrate is partially translocated until the globular DhfR domain blocks further transport and traps it within the SecYEG pore, thus forming a stable translocation intermediate<sup>341</sup>. Upon formation of the translocation intermediate, the fluorescence donor (i.e., Cy3) within the translocated proOmpA domain comes into close proximity to the acceptor fluorophore (i.e., Atto 647N or Cy5) conjugated at the periplasmic side of SecY, and thus FRET between the fluorophores occurs (Fig. 3B). As the donor fluorophore is conjugated at the C-terminal end of proOmpA at position 282, the measured FRET signal implies that a large part of the preprotein has been translocated. Formation of SecYEG:preprotein translocation intermediate complexes is monitored in time as an increased emission of the acceptor fluorophore and the validity of this assay was previously established with membrane-embedded SecYEG<sup>110</sup>. Here, the FRET-based assay was conducted with SecY<sub>C148EGmon</sub>-Nd in the presence of an excess of SecA and proOmpA-DhfR to analyze whether the SecYEG monomer is able to translocate preproteins. Upon addition of ATP we observed an increase in fluorescence of the translocon-conjugated acceptor dye Atto 647N (Fig. 3C). The effect was ATP-dependent, as the non-hydrolysable ATP analogue AMP-PNP did not cause an increase in the dye emission. Taken together, since the nanodiscs support the SecA translocation ATPase activity and preprotein translocation, these results strongly imply that a single SecYEG copy is sufficient to form a functional translocon. Importantly, as SecYEG monomers are isolated within individual nanodiscs, this assay excludes the possibility that transient oligomerization of SecYEG is required for the activity.
To further validate that the observed translocation represents an authentic activity of the membrane-reconstituted translocon, we employed a mutant of SecYEG that displays the prlA phenotype. The point mutation N408I within the transmembrane domain 10 of the SecY subunit resulted in a relaxation of the central constriction pore and favors a tight association with the SecA motor protein. Due to these properties, the SecYEG prlA4 mutant displays enhanced preprotein translocation rates and is capable of translocating preproteins with a defective signal sequence, or even lacking one. The additional mutation F286Y at the periplasmic side of helix 7 serves to stabilize the translocon structure. To analyze the effect of the prlA4-mutations on the translocation kinetics in nanodiscs, we introduced a solvent-exposed cysteine residue at position 148 into the wild-type SecYEG (SecYWTEG) and its PrlA4 derivate (SecYPrlA4EG). Both translocon variants were purified, labeled with Atto 647N-maleimide fluorophore, and reconstituted into nanodiscs as monomers. Translocation was assayed as described above using the FRET-based assay. Indeed, the SecY PrlA4EG-Nd translocon showed an enhanced activity in comparison to the wild-type variant, as stable translocation intermediates were formed much faster upon the addition of ATP (Fig. 3D). Also, the maximum FRET signal approached a significantly higher level for the PrlA4 translocon suggesting more efficient formation of the translocation intermediate. Previously, we have shown that the PrlA4 mutant also exhibits a lower level of preprotein rejection at the initiation stage, allowing it to achieve a higher translocation yield as compared to the wild-type. Therefore, we conclude that the elevated FRET level observed in SecYPrlA4EG-Nd is due to the more efficient formation of translocation intermediates, while the fast kinetics arise from a tighter interaction with the SecA ATPase.

4. Multiple SecYEG copies do not affect the translocation rate

Previous reports suggested that oligomeric species of SecYEG are required for the translocation reaction by either ensuring docking of the SecA ATPase or facilitating the internal dynamics of the translocon, such as displacement of the central plug domain. Our experiments on SecYEGmono-Nd demonstrate that a single translocon is sufficient for translocation, but a beneficial effect of the oligomerization on the translocation kinetics cannot be ruled out. To investigate the role of the SecYEG oligomeric state in translocation we prepared nanodiscs containing multiple copies of SecYEG (SecYEG oligo-Nd). For this purpose we increased the amount of SecYEG 10-fold upon the nanodisc reconstitution, reaching the SecYEG:nanodisc ratio of 2:1. The formed nanodiscs contained single and multiple copies of SecYEG, and their respective fractions were determined by the Poisson distribution. The presence of multiple SecYEG complexes within nanodiscs was confirmed by FCCS (Fig. 2D) showing high levels of fluorescence cross-correlation when the dual-labeled SecYEG complexes were reconstituted at high density into the nanodiscs. From the cross-correlation
value it appeared that about 50% of nanodiscs contained differently labeled SecY_{C148EG}, and the same amount of nanodiscs likely contained equally labeled translocons, which could not be detected by FCCS. Thus, the total number of SecYEG oligomers was estimated at 80-90%. Since both orientations of SecYEG within the lipid bilayer were possible, only co-oriented protomers within a disc might be able to form a dimer. Assuming stochastic orientation of protomers upon reconstitution, dimers could be formed in 50% of nanodiscs containing two copies of SecYEG, and the fraction increased for higher number of copies. Thus, more than 50% of formed nanodiscs contained dimers of SecYEG.

The functional properties of SecYEG_{allgado-Nd} were analyzed as described above and compared to SecYEG_{monogo-Nd}, keeping equal concentrations of SecYEG in both samples. Multiple co-reconstituted copies of SecYEG within the SecYEG_{allgado-Nd} sample stimulated the SecA translocation ATPase activity at the same level as the monomeric SecYEG (Fig. 3A). Thus, multiple translocons did not favor the functional interactions with the SecA ATPase. When the translocation activity of SecYEG_{allgado-Nd} was analyzed in the FRET assay, the real-time recording of translocation matched closely the data obtained for the SecYEG_{monogo-Nd} (Fig. 3C). The apparent translocation rates estimated from a single-exponential fit matched within 20%, implying that multiple copies of SecYEG did not manifest faster intermediate formation, demonstrating that there is no catalytic advance in translocation upon SecYEG oligomerization.

5. Structural dynamics of isolated SecYEG

Next, we aimed to examine the conformational change of single SecYEG complexes upon translocation intermediate formation in particular focusing on the opening of the “lateral gate” formed by transmembrane domains 2 and 8 of SecY88. Using a library of single-cysteine SecYEG110 we observed that cysteine residue at position 313 was accessible for the Atto 647N-maleimide dye, but could barely be labeled with Cy3-maleimide, while both dyes efficiently conjugated to cysteine at position 148 (Fig. S1A). Thus, a double-cysteine mutant of SecYEG was designed, which harbors cysteines in positions 148 and 313 at periplasmic loops 3-4 and 7-8, respectively, i.e. at opposite sides of the lateral gate. The liposome-reconstituted SecYEG variant supported translocation and was able to form a translocation intermediate with fluorescein-labeled proOmpA_{C282-DhfR} (Fig. S1B). The translocon was dual-labeled by incubating it step-wise with the Cy3 and Atto 647N maleimide derivates. The procedure allowed specific conjugation of fluorophores at the pre-defined positions, i.e. Cy3 at cysteine 148 and Atto 647N at cysteine 313 (Fig. 4A and Fig. S1A). We achieved similar labeling efficiencies of approximately 80% for each fluorophore with unspecific labeling at levels well within 10% as assessed with the cysteine-less SecYEG variant. Limited proteolysis by the outer membrane protease OmpT was employed to verify the specific labeling of SecYEG88. Cleaving SecY_{C148-C313EG-Cy3-Atto 647N} within the loop between helices 6-7 by the OmpT protease
resulted in two defined fragments. The Cy3 dye was almost exclusively found at the N-terminal fragment of SecY, while the majority of Atto 647N was conjugated to the C-terminal fragment (Fig. S1C).

**Figure 4. Conformational dynamics of SecYEG.** (A) Site-specific labeling of SecY N-terminal and C-terminal fragments with Cy3 (green star) and Atto 647N (red star) fluorophores, respectively, allowed the monitoring of dynamics of lateral gate opening (indicated with the arrows) of the nanodisc-isolated translocon. **(B)** Binding of SecA to dual-labeled SecY$_{C148,C313}$EG caused pre-opened state of the translocon that was characterized by enhanced donor fluorescence due to separation of N- and C-terminal fragments of SecYEG and opening the gate (panel above). No change in fluorescence of SecY$_{C148}$EG-conjugated Cy3 was observed in absence of the acceptor dye (panel below). Fluorescence emission spectra were recorded using SLM2 spectrofluorometer. Reaction was carried out at 25°C using 100 nM nanodisc-embedded SecYEG, 500 nM SecA, 200 nM pre-folded proOmpA-DhfR and 2 mM AMP-PNP. The donor fluorophore Cy3 was excited at 520 nm. **(C)** SecA binding alters SecYEG conformation. Changes in the FRET donor fluorescence reflected dynamics of the translocon. The conformational change of SecY$_{C148,C313}$EG was only triggered by SecA complexed with AMP-PNP that stably mimics the ATP-bound state of the ATPase (panel above). Other nucleotides barely affected the donor fluorescence. No nucleotide-specific changes in donor fluorescence were observed for single-labeled SecY$_{C148}$EG-Cy3 (panel below). For sensitivity considerations, change in FRET was measured as a change in the donor (Cy3) fluorescence. Reaction was carried out at 25°C using 100 nM nanodisc-embedded SecYEG, 500 nM SecA, 200 nM pre-folded proOmpA-DhfR and 2 mM of respective nucleotide. The conformational changes occurred independently of the presence of a preprotein (proOmpA-DhfR; Fig. S2). Fluorescence spectra were recorded before adding nucleotide and after 5 min incubation of the reaction. Spectra were recorded 3 times, and independent triplicate measurements were performed to estimate standard deviations. Prolonged incubation of nanodiscs in the presence of ADP caused decrease in the overall SecYEG fluorescence after 20 min. Partial protein aggregation at these conditions might be a potential reason that would also explain variations between individual recordings and associated high standard deviations (Fig. 4C).
Next, the dual-labeled SecY$_{C148,C313}$EG-Cy3-Atto 647N was reconstituted into nanodiscs constricted by MSP1E3D1 to obtain the monomeric state of the translocon. When the dual-labeled SecY$_{C148,C313}$EG was excited at 520 nm, emission from both Cy3 and Atto 647N fluorophores was observed demonstrating FRET between these dyes, as no direct excitation of the acceptor fluorophore Atto 647N alone was detected at 520 nm (Fig. S1d). As the fluorescent FRET-pair dyes are situated within the same SecY$_{C148,C313}$EG molecule at opposite sides of the lateral gate, conformational changes within the translocon, such as lateral gate opening can be monitored via intramolecular FRET. The nanodisc-reconstituted system allows the background from protein:protein interactions and associated intermolecular FRET to be excluded, even at low lipid:protein ratios. We analyzed different conformational states of nanodisc-reconstituted SecY$_{C148,C313}$EG-Cy3-Atto 647N by monitoring changes in the fluorescence emission spectrum that contained characteristic peaks for the FRET donor and acceptor. In the presence of proOmpA-DhfR and SecA the translocon should be primed for preprotein transport that is assumed to pre-open the lateral gate$^{175,201}$. When using AMP-PNP to stabilize the complex, we reproducibly observed a significant increase in donor fluorescence indicating separation of two dyes, as expected for the opening of the lateral gate (Fig. 4B, C). Control experiments with SecY$_{C148}$EG-Cy3 complexes labeled with the donor fluorophore alone revealed no change in donor fluorescence under the conditions that resulted in the altered FRET. The change in FRET for the dual-labeled SecYEG was nucleotide-specific and SecA-specific (Fig. 4C) but preprotein-independent (Fig. S2). As the structure of AMP-PNP is almost identical with that of ATP, the formed complex may mimic a naturally occurring state when ATP-bound SecA primes the translocon. As AMP-PNP cannot be hydrolyzed by SecA, this conformational state is stable. ADP-bound SecA did not change the emission spectra, suggesting that the ATPase in its post-hydrolysis state cannot prime the translocon.

Novel robust approaches for membrane protein reconstitution into lipid bilayers of nanodiscs$^{340}$ or possibly amphiphilic systems$^{345}$ allow biophysical and biochemical studies on membrane protein dynamics in the native state, as well as protein quaternary structure$^{346,347}$. Here, we used the nanodisc-reconstituted SecYEG translocon to investigate its functional properties and the potential effect of translocon oligomerization on protein translocation. Preprotein translocation by monomeric SecYEG could be measured directly for the first time by employing a FRET-based method to detect the formation of a translocation intermediate. Our data support recent findings that the monomeric SecYEG is functional for translocation but importantly now excludes the possibility of transient oligomerization during the functional cycle. The presence of multiple SecYEG complexes within a nanodisc had little effect on the translocation kinetics, whereas translocation could be greatly enhanced when employing the monomeric SecYEG PrlA4 mutant$^{304}$. Thus, we conclude that multiple copies of SecYEG do
not provide a catalytic advantage relative to the monomer. Therefore, these data do not support the hypothesis that SecYEG functions as a dimer with one protomer exclusively functioning as a SecA binding site and the other protomer functioning as a protein conducting channel\textsuperscript{344}. The most recent study suggested that SecYEG dimers are recruited upon translocation of SecB-dependent preproteins\textsuperscript{348}. However, the general significance of those findings remains to be determined as SecB is not an essential component of the translocase and is even absent in most bacterial species. Obviously, the specificity of particular oligomeric states of SecYEG for different substrates can be investigated in future using nanodisc-based experiments. We also demonstrated that the nanodisc-reconstituted monomeric SecYEG undergoes a conformational change upon binding of SecA trapped in an AMP-PNP-bound state. The decrease in FRET efficiency between fluorophores introduced in the N- and C-terminal domains of SecY is consistent with an increased distance between these two domains as expected upon the opening of the lateral gate and central pore\textsuperscript{175,201}. The active monomeric SecYEG reconstituted into nanodiscs constitutes a novel system for studying further aspects of protein translocation, such as kinetics and translocon dynamic assembly\textsuperscript{109}, and the impact of periplasmic factors, as well as providing a system amendable for studies on the single-molecule level.

6. Acknowledgements

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7. Supplementary Data

Figure S1. (A) Position-specific labeling of SecYEG with FRET-pair fluorophores. Cy3-maleimide fluorophore could be conjugated at high efficiency to the cysteine residue at position 148 within SecY. Low Cy3 labeling was observed for position 313. The double-cysteine mutant SecY(C148,C313)EG was pre-bound to Ni²⁺-NTA agarose beads and incubated for 2 h with excess of Cy3-maleimide to achieve labeling of the residue C148. After the non-reacted fluorophore was removed, the protein was incubated with an excess of Atto 647N-maleimide that could occupy available cysteine residues. Unreacted dye was removed upon extensive washing of the column-bound SecYEG, and the protein complex was eluted in presence of 300 mM imidazole. Light absorbance was recorded using Cary 100 UV-Vis spectrophotometer. Labeling efficiency was calculated using following extinction coefficients: SecYEG ε₃₈₀ = 70.000 cm⁻¹ M⁻¹, Cy3 ε₅₆₀ = 150.000 cm⁻¹ M⁻¹, and Atto 647N ε₆₅₀ = 150.000 cm⁻¹ M⁻¹. (B) Double-cysteine mutant SecY(C148,C313)EG-Cy3-Atto 647N could efficiently form the translocation intermediate of proOmpA-DhfR. Fluorescein-labeled substrate protein proOmpA-C282-DhfR was completely degraded in absence of SecA, but became protected as full-length (proOmpA-DhfR) and cleaved (proOmpA) forms upon the translocation reaction. In-gel fluorescence was recorded using LAS 4000 fluorescence imager (Fuji) with appropriate excitation and emission filter settings. (C) Specific localization of fluorophores within SecY(C148,C313)EG. Site-specific proteolysis of SecYEG by OmpT protease resulted in two distinct N- and C-terminal fragments of SecY. Fluorescence imaging demonstrated the specific localization of conjugated fluorophores Cy3 at the N-terminal fragment SecY-N (position 148), and Atto 647N at the C-terminal fragment SecY-C (position 313). (D) Intramolecular FRET between Cy3 and Atto 647N. Fluorescence excitation of the donor fluorophore Cy3 within the double-labeled SecY(C148,C313)EG-Cy3-Atto 647N protein resulted in two emission peaks.
at 570 nm (Cy3) and 665 nm (Atto 647N). No direct excitation of Atto 647N was observed at 520 nm for single-labeled SecYC31EG-Atto 647N protein. Thus, dual-labeled SecYEG allowed for intra-molecular FRET. Fluorescence emission spectra were recorded using QuantaMaster 40 spectrofluorometer (Photon Technology International).

Figure S2. SecA binding alone triggers the lateral gate opening in SecYEG. FRET between fluorophores Cy3 and Atto 647N conjugated to nanodisc-embedded SecYC148,C31EG was analyzed upon binding of SecA in the absence of the preprotein proOmpA-DhfR as described in the caption of Figure 4C. When the SecYEG:SecA complex was stabilized by AMP-PNP, an increase in donor fluorescence was observed. No significant changes in fluorescence were detected when using other nucleotides or AMP-PNP alone (upper panel). No change in Cy3 fluorescence was observed in absence of the acceptor fluorophore (lower panel). It was concluded that SecA binding in presence of AMP-PNP stabilized the pre-open state of the translocon even in absence of the preprotein.