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Monitoring the Activity of Single Translocons

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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 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 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.

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The molecular mechanism of bacterial protein secretion has been intensively studied since the identification of the sec genes three decades ago [1,2]. 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 [3]. 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 [4]. 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 [5,6] and the integral membrane complex SecD/SecF that is described as a proton-driven “lever” facilitating preprotein transport [7]. The N-terminal signal sequence of the preprotein targets it to the SecYEG translocon and primes the translocon for transport [8]. 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 [9]. A variety of approaches, such as native PAGE, cryo-electron microscopy, and cross-linking, have suggested that the translocon undergoes oligomerization; however, the vast majority of experiments were performed in a
The significance of SecYEG oligomers was challenged by the first crystal structure of SecYE\(_\beta\) homologue of archaea revealing a transmembrane channel formed with increasing translocase protoomer [12]. 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 [13]. 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 cross-linking [14,15] 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) [16,17]. The dimensions of these nanodiscs allow the isolation of either single of 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 [16], 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 [18], SecYEG translocation activity requires the presence of both charged and non-bilayer lipids, such as DOPE, within the membrane [19]. Remarkably, when SecYEG was reconstituted into nanodiscs with the physiologically relevant \textit{Escherichia coli} lipids, no SecA translocation ATPase activity was observed [16], 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. Detergent-solubilized SecYEG is monomeric.

The 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 [10,11]. To characterize the oligomeric state of purified SecYEG prior to reconstitution into nanodiscs, we employed fluorescence correlation spectroscopy (FCS)/fluorescence cross-correlation spectroscopy (FCCS) using SecYEG molecules independently labeled with two different fluorophores [20,21]. 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 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.

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unique cysteine, L148C, within a periplasmic loop that
connects helices 3 and 4 (SecYC148EG) [14]. E. coli
membranes containing over-expressed SecYC148EG
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 [21]. Fluorophore-conjugated
SecYC148EG was purified, and analysis of the labeling
revealed an ≈55% labeling efficiency for both fluor-
ophores, suggesting complete labeling of the single-
cysteine positions but also limited unspecific labeling
[14]. Next, FCCS was used to determine the amount of
oligomeric complexes that bear both fluorophores. Due
to stochastic 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

Fig. 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 the presence of the preprotein proOmpA [25]. 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 [14]. FRET-pair fluorophores Cy3 (Pierce) and Atto 647N were conjugated to preprotein fusion proOmpA<sub>C282</sub>-DhfR and SecYC148,
respectively, at indicated positions. DhfR domain was pre-folded in the presence of ligands MTX and NADPH and thus could not be translocated via the SecYEG pore [27]. 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 (black continuous lines) were within 20% for SecYEG monomers and oligomers. No translocation was observed in the presence of AMP-PNP. For the reaction, 100 nM nanodisc-embedded SecYC<sub>C148</sub>-EG-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 [14]. 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 [28]. Cysteine at the solvent-exposed position 148 was introduced into wild type and PrlA SecYEG over-expression plasmids pET2302 and pET2306, respectively [28], yielding plasmids pET2302c and pET2306c. Expression, purification, and fluorescent labeling were performed according to the established protocol [14]. FRET assay was performed using SLM2 spectrofluorimeter (Aminco Bowmann), as previously described [14].
(DDM), FCCS experiments showed that the cross-correlation signal was within 10% of both autocorrelation traces (Fig. 1B). As the value matched closely the total level of the unspecific labeling, the signal was largely related to single SecYEG proto- mers bearing both fluorophores. Thus, we concluded that the detergent-solubilized SecYEG translocon was present almost exclusively as monomers.

**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) [22]. To achieve the monomorphic state of SecYEG within these nanodiscs (SecYEGmono-Nd), we reconstituted the translocon in the presence of an excess amount of MSP (10-fold) and lipids (500-fold) following established protocols [22,23]. We used lipid bilayers composed of a mixture of DOPE, DOPG, and DOPC that efficiently support efficient SecYEG translocation activity [14]. 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 monomorphic state of SecYEW148EG 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. SecYEGmono-Nd stimulated the SecA translocation ATPase in a conventional ATPase assay in the presence of the preprotein proOmpA [24], while the ATP hydrolysis remained at background levels when assayed in the 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 [14,26].

The translocation activity of SecYEG was assessed in real time using a recently developed fluorescence-based assay [14]. The assay employs Förster 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) [14]. Briefly, membrane-reconstituted SecYEG is incubated at 37°C in the 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 [27]. 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 [14]. Here, the FRET-based assay was conducted with SecYEC148EGmono-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-hydrolyzable 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 [28]. 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 [29]. The additional mutation F286Y at the periplasmic side of helix 7 serves to stabilize the translocon structure [28]. 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 derivative (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 SecYPrlA4EG-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 [30]. 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.

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 [31] or facilitating the internal dynamics of the translocon, such as displacement of the central plug domain [13]. Our experiments on SecYEGmonom-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 (SecYEGoligo-Nd). For this purpose, we increased the amount of SecYEG 10-fold upon the nanodisc reconstitution, reaching a SecYEG:nanodisc ratio of 2:1. The formed nanodiscs contained single and multiple copies of SecYEG [16], and their respective fractions will be determined by the Poisson distribution [21]. 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 SecYC148EG, and the same amount of nanodiscs likely contained equally labeled translocons, which could not be detected by FCCS. Thus, the total number of SecYEG copies did not affect the translocation rate.
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 will only be formed in 50% of nanodiscs containing two copies of SecYEG, and this fraction increases for higher number of copies. Thus, more than 50% of the formed nanodiscs contained dimers of SecYEG.

The functional properties of SecYEG\textsuperscript{ oligo-Nd} were analyzed as described above and compared to SecYEG\textsuperscript{ mono-Nd}, keeping equal concentrations of SecYEG in both samples. Multiple co-reconstituted copies of SecYEG within the SecYEG\textsuperscript{ oligo-Nd} sample stimulated the SecA translocation ATP\textsubscript{ase} activity at the same level as the monomeric SecYEG. Thus, multiple translocons did not favor the functional interactions with the SecA ATP\textsubscript{ase}. When the translocation activity of SecYEG\textsuperscript{ oligo-Nd} was analyzed in the FRET assay, the real-time recording of translocation matched closely the data obtained for the SecYEG\textsuperscript{ mono-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.

### 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 SecY. Using a library of single-cysteine SecYEG, 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 were efficiently labeled 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, that is, at opposite sides of the lateral gate. The liposome-reconstituted SecYEG variant supported translocation and was able to form a translocation intermediate with fluorescent FRET-pair dyes situated within the opening of the lateral gate (Fig. 4B and C). Control experiments with SecY\textsubscript{C148EG-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 was 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 ATP\textsubscript{ase} in its post-hydrolysis state cannot prime the translocon.

Novel robust approaches for membrane protein reconstitution into lipid bilayers of nanodiscs or possibly amphiphilic systems allow biophysical and biochemical studies on membrane protein dynamics in the native state, as well as protein quaternary structure. 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 [30]. 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 [31]. A recent study suggests that SecYEG dimers are recruited upon translocation of SecB-dependent preproteins [38]. 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 the 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 [33,34]. 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 [39], and the impact of periplasmic factors, as well as providing a system amenable for studies on the single-molecule level.

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Conflict of Interest: Authors declare no conflict of interests.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2013.08.012.

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Abbreviations used:
FRET, Förster resonance energy transfer; FCS, fluorescence correlation spectroscopy; FCCS, fluorescence cross-correlation spectroscopy; DDM, n-dodecyl β-d-maltoside; SEC, size-exclusion chromatography.

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