A single copy of SecYEG is sufficient for preprotein translocation

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The heterotrimeric SecYEG complex comprises a protein-conducting channel in the bacterial cytoplasmic membrane. SecYEG functions together with the motor protein SecA in preprotein translocation. Here, we have addressed the functional oligomeric state of SecYEG when actively engaged in preprotein translocation. We reconstituted functional SecYEG complexes labelled with fluorescent markers into giant unilamellar vesicles at a natively low density. Förster's resonance energy transfer and fluorescence (cross-) correlation spectroscopy with single-molecule sensitivity allowed for independent observations of the SecYEG and preprotein dynamics, as well as complex formation. In the presence of ATP and SecA up to 80% of the SecYEG complexes were loaded with a preprotein translocation intermediate. Neither the interaction with SecA nor preprotein translocation resulted in the formation of SecYEG oligomers, whereas such oligomers can be detected when enforced by crosslinking. These data imply that the SecYEG monomer is sufficient to form a functional translocon in the lipid membrane.

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Introduction

A major share of the bacterial proteome localizes to the cell surface or is targeted to the periplasm. Most of these proteins are synthesized in the cytosol with an N-terminal signal sequence (preproteins) and are transported across the bacterial cytoplasmic membrane by the Sec translocon (Driessen and Nouwen, 2008). In *Escherichia coli*, nascent preproteins are captured by the chaperone SecB (Driessen, 2001), maintained in their unfolded state and delivered to the

Sec complex (Figure 1A). Next, preproteins are actively translocated via the SecYEG channel in a process that is fuelled by ATP hydrolysis by the motor protein SecA (Vrontou and Economou, 2004). During the last two decades, a multitude of biochemical data collected on various aspects of the translocon function has led to a detailed insight in the mechanism of preprotein translocation. The structure of the SecYEG channel in several conformations, including a free closed state and a SecA-bound state has been elucidated by X-ray crystallography (van den Berg *et al*, 2004; Tsukazaki *et al*, 2008; Zimmer *et al*, 2008). Recent cryo-EM studies visualized monomeric Sec61p and SecYEG complexes bound to a ribosome translating a secretory protein (Becker *et al*, 2009). The structural data suggest that a single copy of the SecYEG complex is sufficient to form a pore in the membrane, but several biochemical studies indicate that the translocon is a highly dynamic structure and that oligomers of SecYEG are formed during the catalytic cycle. SecYEG dimers were shown to be ubiquitous in native and synthetic membranes when overexpressed (Meyer *et al*, 1999; Bessonneau *et al*, 2002; Scheuring *et al*, 2005), but also at endogenous levels (Boy and Koch, 2009). A dynamic equilibrium between SecYEG monomers and dimers in the membrane has been suggested, that is shifted towards SecYEG dimers or higher oligomers upon the interaction with SecA and preproteins (Manting *et al*, 2000; Bessonneau *et al*, 2002; Scheuring *et al*, 2005). Two-dimensional crystals of the *E. coli* SecYEG showed a dimeric architecture with the C-terminal helix of SecE forming the dimerization interface (Breyton *et al*, 2002), also termed as the 'back-to-back' dimer. On the other hand, tag-based co-purification experiments suggested that SecYEG forms a monomeric complex with the preprotein in native membranes (Yahr and Wickner, 2000), though also dimers were suggested to interact with the preprotein (Bessonneau *et al*, 2002). In a complementary approach, monomers of SecYEG reconstituted in nano-discs were shown to be competent in SecA binding and SecA ATPase activity, but no translocation activity was determined (Alami *et al*, 2007). Since many of these experiments studied the SecYEG complex in the detergent solubilized or an otherwise restricted state, it has remained unclear if the monomer represents the functional state in translocation. Recently, an effort has been made to investigate the SecYEG oligomeric state at the single-molecule level in lipid membranes (Deville *et al*, 2011). Based on semiquantitative results, it was stated that dimerization is required for efficient translocation, but only minor differences in translocation efficiency between SecYEG monomers and crosslinked dimers were demonstrated. In order to resolve this question, a quantitative approach is required that thoroughly tests the various hypotheses.

Here, we establish a novel quantitative *in vitro* assay for protein translocation with single-molecule sensitivity using fluorescence (cross-) correlation spectroscopy (FCS/FCCS) and Förster's resonance energy transfer (FRET) measure-

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Figure 1 Purification and membrane reconstitution of SecYEG translocon. (A) Scheme of the Sec translocase of bacteria, with SecYEG as membrane-embedded protein-conducting channel, and an unfolded preprotein that is actively translocated by the SecA ATPase. The SecB chaperone prevents the preprotein from folding or aggregation after leaving the ribosome and delivers it to the SecYEG:SecA complex. (B) Coomassie-stained SDS-PAGE of purified SecYEG reconstituted into liposomes made of synthetic lipids. (C) SecYEG translocation activity in model membranes. SecYEG reconstituted into liposomes composed of synthetic lipids (10 mol % cardiolipin, 20 mol % DOPG, 30 mol % DOPE and 40 mol % DOPC) showed a faster rate of translocation as compared with SecYEG reconstitution into *E. coli* polar lipids, as monitored by the translocation of proOmpA_{C282}-fluorescein into the membrane vesicles. (D) Sucrose protects the SecYEG functional state upon dehydration. SecYEG-containing proteoliposomes (protein-to-lipid molar ratio 1:1000) were desiccated overnight under vacuum and rehydrated prior the translocation reaction. The SecYEG level was identical in intact and rehydrated proteoliposomes as judged from SDS-PAGE (upper panel). The activity of SecYEG was tested by conventional translocation assay (de Keyzer *et al*, 2002) and compared with the untreated controls (lower panel). For each sample, 10% of proOmpA used in the translocation reaction is indicated as a reference. No decrease in the SecYEG translocation activity was observed upon the dehydration/rehydration step, suggesting that SecYEG retained its functional state.

ments. FCS analyses temporary fluctuations in SecYEG and preprotein fluorescence within the femtoliter-sized observation volume of a confocal microscope. Molecular diffusion, a primary source of these fluctuations, is quantified by FCS, and FCCS employs the fluctuations information from molecules bearing spectrally separated fluorophores, so their co-diffusion can be detected upon the complex formation. FRET is a conventionally used approach to study molecular interactions and structural dynamics both in large molecular ensembles and at the single-molecule level, which is used here to detect SecYEG:preprotein complexes and to probe SecYEG oligomerization. Here, we describe the properties of functional translocons in a non-invasive manner at the single-molecule level, and address the oligomeric state of SecYEG in the lipid environment at different stages of the functional cycle. Our results strongly suggest that a single copy of the SecYEG complex is sufficient both for SecA binding and for preprotein translocation.

Results

Formation of SecYEG-containing giant unilamellar vesicles

Giant unilamellar vesicles (GUVs) are well suited for a range of *in vitro* studies, including microscopy and electrophysiology (Walde *et al*, 2010). Here, we aimed to establish a GUV-based system for studying the SecYEG translocon by means of ultrasensitive microscopy, in particular FCS (Garcia-Saez *et al*, 2010). An FCS experiment on integral membrane proteins generally requires planar lipid bilayer, so that the protein diffusion occurs in a two-dimensional space. GUVs of tens of microns in diameter represent a suitable system ensuring both the relevant environment for the protein and a flat surface within the observation volume of a confocal

microscope. We screened different mixtures of synthetic phospholipids and probed their propensity to form stable GUVs (Walde *et al*, 2010). Lack of surface undulations, homogeneity and the ubiquity in the preparation were the primary criteria. The SecYEG activity is critically dependent on the content of anionic lipids in the membrane (van Voorst and de Kruijff, 2000; van der Does *et al*, 2000; Gold *et al*, 2010), so we supplied liposomes with DOPG and cardiolipin. Mixing DOPG (20 mol %), cardiolipin (10 mol %), DOPE (30 mol %) and DOPC (40 mol %) allowed for efficient GUV formation both from empty liposomes and after SecYEG reconstitution. This lipid composition was selected for further experiments. The activity of the reconstituted translocons was probed by measuring accumulation of fluorescently labelled preprotein proOmpA inside SecYEG proteoliposomes and its protection from added protease (de Keyzer *et al*, 2002). The purified SecYEG reconstituted into synthetic lipid membranes (Figure 1B) showed a high translocation activity, which even exceeded the activity of SecYEG reconstituted in the polar lipid extract of *E. coli* (Figure 1C). Stimulation of the translocon activity by certain lipid compositions has been previously described (van der Does *et al*, 2000), although no comparison between synthetic and native lipids has been carried out. The improved activity could be due to a more efficient reconstitution but this phenomenon was not further studied. Replacing the cardiolipin fraction with DOPG did not affect the translocation efficiency (Supplementary Figure S1).

For fluorescence measurements we used the variant, SecY_{C295}EG, that contained a single cysteine residue in the periplasmic loop connecting transmembrane segments 7 and 8 of SecY. This residue could be efficiently conjugated with maleimide-derivative fluorophores, such as AlexaFluor 488-C₅-maleimide or Atto 647N-maleimide with a labelling

efficiency of about 90%. Prepared proteoliposomes contained fluorophore-labelled SecY_{C295}EG at a molar protein-to-lipid ratio of 1:30 000. This ratio is substantially lower than those used in conventional bulk experiments and it allowed eliminating effects of unspecific interactions due to molecular crowding. To form GUVs, we used a modified protocol for gentle hydration of desiccated proteoliposomes (Reeves and Dowben, 1969) that allowed for spontaneous and efficient GUV formation. The protein translocation activity of the SecYEG-containing proteoliposomes after the dehydration/rehydration cycle was retained at essentially the same level as that of untreated proteoliposomes (Figure 1D). Thus, SecY_{C295}EG was functionally reconstituted in GUVs and these were further used to examine SecYEG dynamics.

After 10 min rehydration, GUVs of different sizes ranging up to 50 µm in diameter could be observed by means of phase-contrast and scanning confocal microscopy (Figure 2A–C). We tested the permeability of the GUVs for large molecules using fluorescently labelled SecA-Atto 647N as a reporter. SecA-Atto 647N was added to the solution after GUV formation and this resulted in an intensive fluorescence signal in the GUVs exterior only (Supplementary Figure S2). The concentration of SecA-Atto 647N was measured inside the GUVs by FCS after 20 min, and no signal was detected in at least 90% of the vesicles (Supplementary Figure S2). This implied that the SecYEG complexes exposing their cytoplasmic surface towards the GUVs interior were not accessible for SecA binding and thus do not participate in translocation.

To monitor the membrane-embedded SecY_{C295}EG, the laser beam was focused on the GUV surface (Figure 2C and D). The fluorescence intensity of AlexaFluor 488-labelled SecY_{C295}EG molecules diffusing through the focal volume was recorded and further used to build the autocorrelation curve (Figure 2E). The autocorrelation amplitude declined rapidly at the millisecond time range due to diffusion

processes, and the midpoint provided an estimate for the average residence time of SecYEG molecule within the observation volume (τ_D). The curves were fitted assuming two-dimensional diffusion within the planar bilayer providing a diffusion coefficient of $(2.6 \pm 0.6) \times 10^{-8} \text{ cm}^2/\text{s}$. The measured diffusion coefficient of SecY_{C295}EG was within the range typical for multi-spanning membrane proteins (Ramadurai *et al*, 2009). It was also close to the value of $3.1 \times 10^{-8} \text{ cm}^2/\text{s}$ that was theoretically calculated using Saffman–Delbrück model (Saffman and Delbrück, 1975) assuming the SecYEG radius of 2 nm, the height of transmembrane domains of 4.5 nm, and the lipid bilayer viscosity around 0.1 Pa·s. The autocorrelation function $G(t)$ yielded the concentration of SecY_{C295}EG complexes reconstituted in the lipid membranes, since the amplitude $G(0)$ equals the reciprocal of the particles number in the focal volume (Equation (1); Figure 2E). The reconstitution procedure ensured a low density of SecY_{C295}EG in the membranes, so that normally about 10 molecules were observed within the observation volume. This value was optimal for FCS measurements, as it allowed for both sufficient statistics and sensitivity in the fluorescence fluctuations (Krichinsky and Bonnet, 2001). An accurate measure of the SecY_{C295}EG surface density by FCS provided the unique opportunity to determine the actual molar protein-to-lipid ratio in the membranes. The observation volume radius around 200 nm corresponded to the membrane area of $\approx 120\,000 \text{ nm}^2$ that accommodated in total $\approx 350\,000$ lipid molecules of the bilayer. Thus, the actual protein-to-lipid ratio was 1:35 000 in agreement with the ratio used for reconstitution, and the SecYEG surface density was 100 molecules/µm². Importantly, the SecY_{C295}EG density in the GUVs correlated well with that in *E. coli* membrane considering 300 SecYEG copies per bacterium (Matsuyama *et al*, 1990) with dimensions of $1 \times 3 \text{ µm}$ and a corresponding surface area of 2.5 µm^2 .

Figure 2 Fluorescence correlation spectroscopy on SecYEG-containing vesicles. (A) Scheme of the home-built dual-laser confocal microscope used to study GUV-incorporated SecYEG. Dual-laser excitation was used for FCCS measurements, and the argon laser was used for FRET studies. Synthetic lipids allow for GUV formation in presence of reconstituted SecYEG complexes as monitored in phase-contrast (B) and fluorescent modes (C). A selected GUV (white asterisk) was scanned through its centre in Z-direction parallel to the optical axis to locate lower and upper membrane planes (D). The fluorescence signal was measured on the upper plane (black asterisk) and used to calculate the autocorrelation curve (E). The protein concentration N and the diffusion time τ_D were extracted from the experimental data and used for further analysis (see text for details).

Monitoring SecYEG:preprotein complex formation

A fusion protein proOmpA-DhfR that consists of the unfolded OmpA protein precursor (proOmpA) and dihydrofolate reductase (DhfR) domain was used to study the translocation reaction (Arkowitz *et al*, 1993). This protein cannot be fully translocated through the SecYEG channel when the folded Dhfr is stabilized by its ligands methotrexate and NADPH. Under those conditions, the preprotein remains trapped in the SecYEG channel leaving the Dhfr domain exposed at the cytoplasmic side (Arkowitz *et al*, 1993) while the

unfolded proOmpA is largely translocated through the pore thus forming a stable translocation intermediate (Figure 3A; Supplementary Figure S3). A fluorophore conjugated to the translocated proOmpA domain and a spectrally separated fluorophore bound at a loop on the periplasmic side of SecY should allow for FRET. Hence, the translocation of proOmpA through SecY_{C295}EG can be directly observed as an increase in the FRET efficiency between the donor (Cy3) and the acceptor (Cy5) markers conjugated to proOmpA and SecYEG, respectively (Förster's distance for the Cy3/Cy5

Figure 3 Formation of SecYEG:preprotein complex. **(A)** The proOmpA preprotein is trapped in the SecYEG channel by the presence of a folded Dhfr domain fused to the C-terminus. A single cysteine residue was introduced at different positions within the proOmpA domain (numbers and red circles) and fluorescently labelled. Complex formation was probed in bulk by measuring FRET efficiency between fluorophores Cy3 and Cy5 conjugated to proOmpA-DhfR variants and SecY_{C295}EG in IMV, respectively. **(B)** Trapping the preprotein within SecYEG increases the acceptor fluorescence intensity due to FRET. The FRET efficiency depended on the fluorophore position along the proOmpA polypeptide chain (numbers aside). No change in fluorescence was observed in absence of the acceptor fluorophore on SecY_{C295}EG. **(C)** FRET-FCS recordings on the SecYEG:preprotein complex. GUV-embedded SecYEG complexes were monitored using the confocal microscope set-up. When both SecA and ATP were present, FCS recordings on the GUV surface showed a dramatic change in proOmpA-DhfR time-correlated fluorescence. Autocorrelation traces were fitted to two-dimensional diffusion model (black lines; equation (1)). **(D)** A 4-fold increase in the fluorescence intensity of proOmpA-DhfR-Atto 647N was observed upon trapping within SecYEG. At least 20 GUVs were examined at each condition to calculate the average fluorescence intensity; error bars present s.e.m. values. **(E)** The molecular brightness of the donor SecYEG-AlexaFluor 488 decreased upon formation of the translocation intermediate (average $\pm$ s.e.m. with $n > 20$). **(F)** The fraction of active translocons in GUV membranes. Approximately 80% of the correctly oriented SecYEG complexes were involved in the translocation reaction in presence of 500 nM SecA. Reducing the SecA concentration 10-fold caused a decline in SecYEG:preprotein interaction. Solid lines present fits with normal distributions.

donor-acceptor pair > 5 nm). Bulk measurements showed an up to 30% increase in the acceptor fluorescence intensity when the proOmpA-DhfR-Cy3 was trapped in the SecY_{C295} EG-Cy5 channel (Figure 3B), and the FRET signal was strictly ATP dependent. Labelling the translocated polypeptide chain at different positions modulated the FRET efficiency as this determined the distance of the donor fluorophore to the SecY_{C295}EG-linked acceptor (Figure 3B). The maximum signal was observed when labelling proOmpA at residues 282 and 290, suggesting that these residues are in close vicinity to the SecYEG periplasmic interface. Placing the fluorophore either near the DhfR domain (residue 315) trapped at the cytoplasmic side of SecYEG or within the translocated proOmpA region (residue 245) led to a minimal FRET efficiency due to their large distance from the acceptor fluorophore at the periplasmic exit of SecYEG channel. Residue 315 of the preprotein resides at the cytoplasmic interface of the membrane, thus being separated from the SecY_{C295} residue by almost 10 nm, which is the sum of the membrane-embedded SecYEG (~5 nm) and bound SecA (~3 nm) (Figure 3A). Residue 245 lies within the translocated part of proOmpA and thus it is distanced from the SecYEG pore.

FRET-FCS-based translocation assay

We further extended the approach to study translocation in GUVs using FCS and FRET to detect SecYEG:preprotein complexes. The AlexaFluor 488 fluorophore introduced into the SecY_{C295} subunit served as a donor in the designed FRET assay and allowed to monitor the surface of a GUV during the experiment. GUVs were supplied with the proOmpA_{C282}-DhfR conjugated with the Atto 647N fluorophore (acceptor; Förster distance for the donor-acceptor pair ~4 nm), SecB and 5 mM ATP. Recordings in the blue channel of the SecY_{C295} EG-AlexaFluor 488 were used to determine an autocorrelation curve as described above to monitor the diffusion of the SecY_{C295}EG protein within the membrane (Figure 3C). The autocorrelation curve of the acceptor fluorophore in the absence of SecA was dominated by the cross talk between the channels (Figure 3C, dashed red line), that was minimized due to large spectral separation of used fluorophores. Also, the corresponding fluorescence intensity remained at background levels, below 1000 photons/s (Figure 3D). After the sample was incubated for 10 min at 37°C in the presence of 0.5 µM SecA the fluorescence intensity of the acceptor fluorophore increased ~4-fold and the autocorrelation curve acquired a characteristic shape (Figure 3C and D). The average molecular brightness of the donor SecY_{C295} EG-AlexaFluor 488 decreased by about 20% when proOmpA_{C282}-DhfR-Atto 647N was trapped in the SecYEG channel (Figure 3E). The substantial increase in the acceptor fluorescence was in agreement with the bulk translocation experiments (Figure 3B), but now observed at a much lower protein concentration. The enhanced FRET signal (400% versus 30%) is likely due to better signal-to-noise ratio when using a confocal microscope instead of the fluorescence spectrophotometer, as well as larger spectral separation of the fluorophores used.

The fluorescence signal recorded in the acceptor channel in the presence of SecA and ATP corresponded to the partially translocated and trapped proOmpA_{C282}-DhfR-Atto 647N molecules. The contribution of fluorescence from the membrane-bound proOmpA_{C282}-DhfR-Atto 647N to the

fluorescence correlation function could be neglected, as their molecular brightness was at least 10-fold lower than that of SecYEG-trapped preproteins. If ATP was replaced by the non-hydrolysable analogue AMP-PNP or if no SecA was added, we did not observe either an increase in the acceptor fluorescence (Figure 3D) or a time-correlated signal. Thus, the approach discriminated stable translocation intermediates from the partially inserted or non-specifically bound preprotein molecules. Decreasing the translocation time down to 3 min caused an increase in the amplitude of proOmpA-DhfR-Atto 647N autocorrelation function, as the number of DhfR-trapped intermediate complexes decreased (Supplementary Figure S4).

SecYEG:preprotein complexes are ubiquitous in GUVs

The autocorrelation curves recorded in both channels upon the translocation reaction contained detailed and quantitative information about the protein diffusion rates and the stoichiometry of the interactions. Since the amplitude of the autocorrelation function yields an accurate measure of the number of fluorescent particles (Figure 2E), the technique could be used to directly quantify the efficiency of translocation intermediate formation expressed as the ratio of the total number SecYEG complexes and trapped proOmpA-DhfR molecules. The most probable SecY:preprotein ratio measured on individual GUVs was 2.8 (Figure 3F, grey bars; $n = 45$). The vesicles manifesting higher ratios, that is, lower translocation efficiency, typically showed a high SecYEG content of 20–30 molecules in observation volume. They likely reflected a minor non-unilamellar fraction comprising <10% of the vesicle ensemble. Accounting for the dual membrane topology of the reconstituted SecYEG complexes and the limited labelling efficiency of the preprotein (~90%), we concluded that at least 76%, that is, the vast majority of correctly oriented SecYEG complexes was functionally active assuming a stoichiometric complex of proOmpA-DhfR and SecYEG. Reducing the SecA concentration down to 50 nM, that is, below the K_M value of ~100 nM (Kusters *et al*, 2011), led to a lowered translocation efficiency (Figure 3E, red bars).

Diffusional mobility of SecYEG and the preprotein

Remarkably, we observed a decrease in the SecYEG mobility in the membrane upon the formation of a proOmpA-DhfR translocation intermediate with a reduction in the SecYEG diffusion coefficient from $(2.6 \pm 0.6) \times 10^{-8} \text{ cm}^2/\text{s}$ to $(2.1 \pm 0.7) \times 10^{-8} \text{ cm}^2/\text{s}$ (Figure 4A). A similar decrease in the mobility was observed if only SecA was added to GUVs. Solely, the binding of the large extramembrane protein SecA may affect the mobility of the protein embedded into the highly viscous membrane ($\mu_{\text{membrane}} \sim 100 \times \mu_{\text{water}}$). However, the observed change may also occur due to increased molecular crowding and local distortion of the lipid environment upon SecA binding, a SecYEG conformational change (Zimmer *et al*, 2008) or the hypothetical SecYEG oligomerization. We also characterized the mobility of the preprotein proOmpA_{C282}-DhfR-Atto 647N depending on its localization, that is, in solution, membrane-bound, and in the translocation intermediate state (Figure 4B). The first two states were studied using the direct excitation with the He-Ne laser (633 nm), whereas the third state was analysed using FRET-FCS measurements, when exciting the SecY_{C295} EG-conjugated AlexaFluor 488 donor fluorophore with the

Figure 4 The mobility of SecYEG in the membrane depends on the functional state of the translocon. **(A)** The lateral diffusion coefficient of SecY_{C295}EG-AlexaFluor 488 in GUV membranes measured using FCS is reduced in presence of the SecA motor protein and when trapping the preprotein proOmpA-DhfR. At least 20 GUVs were examined at each condition to calculate average diffusion coefficients; error bars present s.e.m. values. **(B)** The mobility of the preprotein proOmpA_{C282}-DhfR-Atto 647N depends on its localization. The diffusion coefficient is maximal for three-dimensional diffusion in solution ($n = 10$) and minimal for SecYEG-trapped form ($n = 77$). Membrane-bound proOmpA-DhfR is characterized by the marginal lateral mobility ($n = 12$). Average values for diffusion coefficients are shown; error bars present s.e.m. values. **(C)** SecY_{C295}EG-trapped proOmpA_{C282}-DhfR demonstrates the same diffusional mobility as the translocon. The ratio of their diffusion times was calculated for individual GUVs ($n = 77$) based on the FCS-FRET assay. The solid line presents fit with the normal distribution.

Argon-ion laser (488 nm). SecB-bound proOmpA-DhfR in solution showed the highest mobility ($D = (51 \pm 19) \times 10^{-8} \text{ cm}^2/\text{s}$). Using the Einstein-Stokes equation, this diffusion coefficient could be assigned to a particle of $\sim 4 \text{ nm}$ radius. The size estimation agrees well with the radius of the SecB tetramer of 3 nm (Dekker *et al*, 2003) extended by the preprotein coiled around, and it is further supported by the recent EM imaging of SecB:OmpA complex (Tang *et al*, 2010). ProOmpA-DhfR localized to the membrane surface of the GUVs in absence of SecA showed a 3-fold lower diffusion coefficient $(16 \pm 6) \times 10^{-8} \text{ cm}^2/\text{s}$ that is similar to the lipid mobility within the bilayer (Doeven *et al*, 2005). This implied that most preprotein molecules were bound non-specifically to the lipid bilayer. Even lower diffusion coefficient, $(2.1 \pm 0.8) \times 10^{-8} \text{ cm}^2/\text{s}$, was measured for the SecYEG-trapped preprotein. Here, the preprotein molecule should remain fluorescent over the time the stable complex was diffusing through the excitation volume of the Argon-ion laser. Indeed, the ratio of the corresponding diffusion times of SecY and proOmpA-DhfR was 0.99 ($n = 77$, Figure 4C) supporting the notion that they co-diffused.

Probing the oligomeric state of SecYEG in the membrane

The diffusion characteristics determined by FCS provide a measure for the size of the target molecules, since the diffusion coefficient in solution scales inversely with the molecular radius, as described by the Einstein-Stokes relation. In contrast, diffusion of particles embedded in highly viscous lipid membranes is strongly affected by interactions with the membrane environment, while the dependence on the particle dimensions is logarithmical, that is, rather weak, according to the Saafman-Delbrück theory (Saffman and Delbrück, 1975). Thus, using the FCS approach alone would not allow unambiguous discriminating between monomeric and dimeric species of SecYEG in the ensemble. As a control, we prepared a SecY_{C50}E_{C106}G dimer, where

SecE_{C106} subunits were crosslinked in presence of copper phenanthroline (Kaufmann *et al*, 1999), and the SecY_{C50} subunits were labelled with AlexaFluor 488. The diffusion coefficient of this covalently stabilized dimeric SecYEG was $(2.4 \pm 0.4) \times 10^{-8} \text{ cm}^2/\text{s}$, so the mobility decreased only by 10% as compared with the non-crosslinked SecYEG complex.

The confocal microscope can be used for FCCS measurements when extended with a second, spectrally separated laser beam (Figure 2A) that is aligned with the 488 nm laser to a high degree of spatial overlap (Schwille *et al*, 1997; Doeven *et al*, 2008). Fluorescence fluctuations of differently labelled diffusing species are monitored and cross-correlated over the measurement time to detect molecular binding/dissociation processes. This method has been recently applied to study interactions on the membrane interface and within the membrane (Doeven *et al*, 2008; Garcia-Saez *et al*, 2009). Here, we employed the approach to probe the SecYEG oligomeric state. We tested the sensitivity of the FCCS set-up by monitoring the lateral diffusion of the variants, SecY_{C148C313}EG and the disulphide-bonded covalent dimer SecY_{C50}E_{C106}G, which bear both the AlexaFluor 488 and Atto 647N fluorophores. In these cases substantial cross-correlation signal was measured, up to 50% of the autocorrelation amplitude of Atto 647N-labelled species (Supplementary Figure S5). Also, a non-zero cross-correlation signal was measured for SecYEG:proOmpA-DhfR complexes in which the SecY_{C295}EG and proOmpA_{C282}-DhfR units were labelled, as described above (Supplementary Figure S5).

We co-reconstituted the SecY_{C295}EG complexes individually labelled with AlexaFluor 488 and Atto 647N fluorophores into GUVs (Figure 5A), and recorded both the autocorrelation and cross-correlation spectra. SecYEG alone did not show a substantial cross-correlation signal ($< 5\%$ of the autocorrelation amplitude), suggesting that only monomeric SecYEG complexes were present in the membrane (Figure 5B). Even though the dynamic equilibrium between SecYEG monomers and dimers has been described earlier (Manting *et al*, 2000;

Figure 5 Probing the oligomeric state of SecYEG. **(A)** Schematic representation of the studied translocon states. Differently labelled SecY_{C295}EG complexes were co-reconstituted into GUV membranes and SecYEG oligomerization was probed under described conditions. **(B)** FCS/FCCS analysis of SecY_{C295}EG diffusion. Autocorrelation curves revealed the diffusion of differently labelled SecY_{C295}EG species, while cross-correlation curves reflected presence/absence of dual-labelled complexes. At various functional states of SecYEG, no cross-correlation signal was detected suggesting that no dual-labelled SecYEG complexes were formed and no oligomerization occurred. **(C)** Studying the oligomeric state of SecYEG by FRET. No FRET was detected between SecYEG protomers at different functional states, as the average fluorescence intensity remained at the background level. FRET between AlexaFluor 488-conjugated SecYEG variants and the preprotein proOmpA_{C282}-Dhfr-Atto 647N confirms the SecYEG functionality. Error bars present s.e.m. values with $n > 20$. **(D)** FRET-FCS analysis of SecYEG oligomerization. If only SecY_{C295}EG-AlexaFluor 488 was excited, its fluorescence fluctuations resulted in a characteristic autocorrelation trace, while recordings on the acceptor, SecY_{C295}EG-Atto 647N, were dominated by the cross talk at all studied conditions. SecY_{C148C313}EG conjugated with both AlexaFluor 488 and Atto 647N was used as a reference control, and the corresponding FRET-FCS recording resulted in a sigmoid autocorrelation trace in the acceptor channel.

Scheuring *et al*, 2005), the low protein concentration in GUVs may favour the monomeric form for the resting SecYEG complexes. Stable association of SecYEG with SecA was triggered by saturating SecA with the non-hydrolysable nucleotide AMP-PNP (5 mM) (Economou and Wickner, 1994). Under these conditions also no increase in the SecYEG FCCS signal was detected, supporting previous observations that SecA can interact with the monomeric SecYEG (Alami *et al*, 2007). When proOmpA-Dhfr was added and translocation was initiated at 37°C in presence of ATP, again no change in cross-correlation signal was observed. Since almost all correctly oriented SecYEG was involved in translocation as shown above, the lack of a cross-correlation signal suggests that the translocon is formed by a single SecYEG copy.

To verify these FCCS results, we designed an additional FRET-based experiment to probe the oligomeric state of SecYEG. If differently labelled SecYEG complexes formed oligomers, it would result in a substantial FRET between fluorophores. The FRET efficiency depends strongly on the distance between donor and acceptor fluorophores and

reduces dramatically at separations above 5–6 nm. Since the lateral dimensions of a single SecYEG complex are ~4 nm (Zimmer *et al*, 2008), and several models of the dimer organization exist (Breyton *et al*, 2002; Mitra *et al*, 2005), we had to ensure that the fluorophores would come in proximity upon putative oligomerization. Several variants of SecYEG were designed bearing single cysteine residues at different locations along the periplasmic side of the channel (Supplementary Figure S6). Dual-labelled SecY_{C148C313}EG molecules were used to control the method sensitivity. Within the SecYEG structure conjugated AlexaFluor 488 and Atto 647 were positioned within 3.6 nm (Supplementary Figure S6) and resulted in an intensive FRET-FCS signal (Figure 5B). The acceptor fluorescence intensity measured on GUV membranes was 9.4 ± 0.6 kHz (average $\pm$ s.e.m., $n = 7$).

Designed SecYEG variant was fully functional as measured in the bulk translocation assays (de Keyser *et al*, 2002; Supplementary Figure S1). The AlexaFluor 488-conjugated forms were effective in the formation of a proOmpA_{C282}-Dhfr

intermediate as monitored via FRET in GUVs at low protein-to-lipid ratios (Figure 5C). Efficient FRET between SecYEG and the preprotein-bound fluorophores was observed if the donor fluorophore was placed on the SecE_{C120} subunit, suggesting that SecYEG subunits remain assembled during the translocation cycle. For each SecYEG variant, we prepared GUVs bearing both SecYEG complexes labelled with AlexaFluor 488 and Atto 647N and studied their interactions at the listed conditions (Figure 5A and C). No FRET signal was detected either upon SecA binding or the translocation reaction, as the fluorescence intensity remained at the background level and the FCS recordings were dominated by the cross talk (Figure 5C and D). Taken together, the data strongly suggest that SecYEG actively engaged in translocation is monomeric.

Discussion

The organization of membrane proteins in biological membranes remains an intriguing question in modern cell biology. As an example, the oligomeric state of the translocation pore SecYEG has remained in the focus of intensive debates for two decades (Rusch and Kendall, 2007). Evidence has been provided for the presence of dimers and higher oligomers of SecYEG in cellular and model membranes but their biological role has remained elusive. Conflicting results from different studies could be reconciled by the dynamic nature of the translocon (Bessonneau *et al*, 2002; Scheuring *et al*, 2005), but they also suggest that the experimental outcome may be determined by the conditions employed. In the experiments presented here, we developed an *in vitro* approach to study protein translocation by the bacterial Sec translocon down to single-molecule resolution using fluorescence microscopy. The method is based on the well-established *in vitro* translocation assay routinely used in the field, but expanded to GUVs in order to observe the diffusion of single SecYEG complexes in the membrane. It allowed not only for the detailed and quantitative description of the translocation reaction, but also provided new insights on the oligomeric state of the active SecYEG complex. Only a minor modification of SecYEG was needed to make it amenable for single-molecule fluorescence detection. Neither the introduced cysteine mutations in periplasmic loops nor the conjugated fluorophores at those positions affected the SecYEG activity. SecYEG complexes were embedded into GUVs at natively low density (Matsuyama *et al*, 1990) and the membrane environment was thoroughly adjusted to contain all components essential for the SecYEG activity. In particular, GUVs contained a substantial amount of cardiolipin, a lipid that has recently been implicated in SecYEG dimer formation (Gold *et al*, 2010). Importantly, all measurements have been performed on functional SecYEG complexes in the membrane environment and did not require additional sample treatment, such as detergent solubilization or staining, thus avoiding possible artifacts. The dynamics of individual components of the reaction (preprotein, SecYEG subunits) could be studied at various stages of the functional cycle. As expected, a reduced mobility of the proOmpA-DhfR preprotein was observed upon its binding to the membrane surface and when it was trapped within the SecYEG channel. While a previous study on SecYEG reconstituted in nanodiscs was limited to a demonstration of the interaction of

SecYEG with the SecA motor protein (Alami *et al*, 2007), our approach allowed monitoring the preprotein translocation reaction for the SecYEG complex. Quantitative analysis of SecYEG:proOmpA-DhfR interactions suggested that the majority of SecYEG was actively involved in translocation. Under none of the experimental condition employed we detected oligomers of SecYEG, while using two different fluorescence-based studies based on either FCCS or FRET approaches.

As a conventional method for studying macromolecular interactions, bulk FRET measurements have been employed previously to probe SecYEG oligomerization. Two different studies were performed on SecYEG complexes labelled at nearby positions either at SecY or at SecE subunits and reconstituted into lipid vesicles at protein-to-lipid ratio of at least 1:5000 (Mori *et al*, 2003; Scheuring *et al*, 2005). Though the presence of SecYEG oligomers was reported in both cases, some results remained difficult to interpret. No SecYEG complex dynamics was revealed in FRET experiments in bulk: thus, either no subunit exchange between SecYEG oligomers was observed (Mori *et al*, 2003), or the SecYEG complex remained static upon the functional cycle, in contrast to what was suggested from electron microscopy experiments (Scheuring *et al*, 2005). Limited protein aggregation upon the reconstitution procedure, which is difficult to assess in bulk assays, might at least partially account for those results. To validate a positive FRET signal, one may test its dependence on the fluorophore positions on SecYEG, expecting that the FRET efficiency is dependent on the oligomer architecture.

Our data strongly suggest that a single copy of SecYEG is sufficient to conduct the translocation reaction. Oppositely, a recent model on how the translocon may function includes two copies of SecYEG arranged in a 'back-to-back' orientation (Deville *et al*, 2011). SecYEG dimers were reported as minimal functional units based on studies employing genetically engineered SecY–SecY fusion proteins (Osborne and Rapoport, 2007). Such a covalently fused dimer was designed to compensate a point mutation at residue 357 of the SecY subunit that normally renders SecYEG non-functional. When introduced into only one of the two copies of the fused dimer, the point mutation did not inactivate the other SecYEG copy, thus presumably leaving one channel active in translocation. This lack of a dominant-negative effect on the neighbouring SecYEG channel in this fused dimer suggests that each channel functions on its own. This notion that preproteins pass the membrane through a single pore is also supported by our recent studies showing that a preprotein derivatized to a large bulky molecule can be translocated by a SecYEG pore in which the lateral gate is fixed with a flexible chemical cross-linker (du Plessis *et al*, 2009; Bonardi *et al*, 2011). The hypothesis that one SecYEG protomer would form a translocating pore for the preprotein, while the other would serve for SecA binding (Osborne and Rapoport, 2007), predicts that there must be a strong functional cooperativity in SecYEG oligomerization as each of these functions specify a critical activity of the SecYEG complex. However, such cooperativity was not observed in a recent study on the functional oligomeric state of SecYEG. Herein, the translocation efficiencies of SecYEG monomers and a covalently crosslinked SecYEG_{C106G} dimer were compared (Deville *et al*, 2011). Only a 2-fold increase in the translocation efficiency for the

covalently crosslinked SecYEG dimer, consistent with each protomer being independently functional. The relevance of these results for the functional oligomeric state remains uncertain as dimerization in such studies is induced by crosslinking while the exact geometry of individual SecYEG protomers within a hypothetical oligomer is unknown. Moreover, the structure of the SecYEG:SecA complex suggests that a single SecYEG channel provides sufficient interface for docking a single SecA molecule above the translocating pore (Zimmer *et al*, 2008). As it was noted therein, such complex contains a SecYEG monomer and is equipped with all crucial interactions previously determined by crosslinking studies (Mori and Ito, 2006; van der Sluis *et al*, 2006).

While a single SecYEG complex may serve as a minimal translocon, our study does not exclude the possibility of oligomerization *in vivo*. Different roles may be attributed to its oligomerization in bacterial membranes. The SecYEG:SecA interaction may be modulated by another copy of SecYEG present and the kinetics of the preprotein translocation may be influenced by SecYEG oligomeric state. While the current study is limited to steady states of the translocon, monitoring its dynamics in real time in future will demonstrate if transient oligomerization is needed for the translocation. *In vivo* clustering of translocons within the protein-rich bacterial membrane may promote their oligomerization due to molecular crowding, while SecYEG was highly diluted within the lipid bilayer in our study. Future research should also be focused on the interaction of SecYEG with accessory proteins such as the SecDF complex and YidC that modulate the translocon activity in native cellular membranes and should examine their potential role in SecYEG oligomerization.

Materials and methods

SecYEG purification and labelling

PCR-mediated mutagenesis was used to introduce codons for cysteine residues into the cysteine-less SecYEG gene cluster. Positions for cysteine residues were selected based on the *M. jannaschii* SecY ϵ structure (van den Berg *et al*, 2004), and evenly distributed along the protein perimeter on the translocon periplasmic side (Supplementary Figure S6). SecYEG variants were cloned in the pET20 vector (Table I), and overexpressed in *E. coli* SF100 as described elsewhere (van der Does *et al*, 2003). Membrane fractions were isolated and inner membrane vesicles (IMVs) were

purified by sucrose gradient centrifugation followed by incubation with 0.18 M Na₂CO₃, pH 11 for 30 min on ice (van der Does *et al*, 2003). Harvested IMVs were solubilized with 2% DDM for 30 min in presence of Complete protease inhibitor cocktail (Roche), incubated with Ni²⁺-NTA agarose beads (Qiagen) for 1 h and extensively washed using a Bio-Spin Micro column (Bio-Rad) with the buffer solution containing 50 mM Tris-HCl pH 8, 100 mM NaCl, 0.05% DDM, 20% glycerol and 10 mM imidazole. The fluorophore-maleimide conjugation was performed at pH 7.0 to ensure specific labelling of the cysteine residues. Ni²⁺-NTA agarose-bound SecYEG complex was incubated with 100 μ M of either AlexaFluor 488-C₅-maleimide (Invitrogen, USA) or Atto 647N-maleimide (Atto-Tec, Germany) for 2 h at 4°C on a rolling bank, followed by extensive washing and elution with the buffer solution containing 300 mM imidazole. For the SecY_{C148C313}EG variant, both fluorophores were added to achieve dual labelling. The estimated purity of SecYEG was above 90%, as judged from SDS-PAGE. The amount of free fluorophore that remained during the last washing step never exceeded 5% of the total fluorescence in the elution fraction. SecYEG and fluorophore concentrations were estimated spectrophotometrically. The extinction coefficient for SecYEG at 280 nm was calculated as 71,000/M/cm. Extinction coefficients for fluorophores were as provided by manufacturers. For all SecYEG constructs labelling efficiencies reached up to 90%, while un-specific labelling remained below 5% for AlexaFluor 488 and below 10% for Atto 647N as tested using the cysteine-less SecYEG variant. To obtain a dimeric SecYEG complex, the SecY_{C50}E_{C106}G variant was crosslinked in IMVs in presence of 1 mM Cu-phenanthroline as previously described (Kaufmann *et al*, 1999). No crosslinking occurred via SecY subunit, as tested using the SecY_{C50}EG variant. Fluorescent labelling was performed as described above, either with AlexaFluor 488 alone or together with Atto 647N. Labelling efficiency for the crosslinked SecY_{C50}E_{C106}G was substantially reduced, and did not exceed 70%.

The activity of the reconstituted SecYEG complexes was confirmed by *in vitro* translocation assays as described (de Keyser *et al*, 2002), using the fluorescently labelled precursor of outer membrane protein A (proOmpA) as a substrate (0.5 μ M). The reaction was carried out in presence of the chaperone SecB (4 μ M) and motor protein SecA (0.5 μ M). The N-terminal end of the SecY subunit contained an enterokinase cleavage site DDDDK followed by the hexa-histidine tag, so its accessibility to externally added enterokinase could be detected by SDS-PAGE as a shift in the apparent molecular mass (van der Does *et al*, 2000). Only SecYEG exposing the cytoplasmic domain out of proteoliposomes was subjected to the cleavage. The N-terminus of the oppositely oriented SecYEG was protected by the lumen of the liposome where it is inaccessible for the enterokinase. Fractions of cleaved/non-cleaved SecY were quantified from SDS-PAGE using AIDA software (raytest Isotopenmessgeräte GmbH, Germany).

Preprotein preparation

ProOmpA and its derived fusion with dihydrofolate reductase (proOmpA-Dhfr) were overexpressed in *E. coli* DH5 α , purified from

Table I List of strains and plasmids used

Strain/plasmid	Short description	Source
<i>E. coli</i> SF100	<i>F</i> -, Δ lacX74, <i>galE</i> , <i>galK</i> , <i>thi</i> , <i>rpsL</i> , <i>strA</i> , Δ phoA (<i>pvuII</i>), Δ ompT	Baneyx and Georgiou (1990)
<i>E. coli</i> DH5 α	<i>supE44</i> , Δ lacU169 (Δ 80lacZ α M15) <i>hsdR17</i> , <i>recA1</i> , <i>endA1</i> , <i>gyrA96 thi-1</i> , <i>relA1</i>	Hanahan (1983)
pEK20	Cysteine-less SecYEG	van der Sluis <i>et al</i> (2002)
pET84	SecY (G295C)EG	Kusters <i>et al</i> (2011)
pEK20-C50	SecY (A50C)EG	This study
pEK20-C148	SecY (L148C)EG	This study
pEK20-C215	SecY (L215C)EG	This study
pEK20-C313	SecY (G313C)EG	This study
pET2523	SecYE (S120C)G	Scheuring <i>et al</i> (2005)
pEK20-C148-C313	SecY (L148C, G313C)EG	This study
pEK20-C50-EC106	SecY (A50C)E (L106C)G	This study
pET627	SecYE (L106C)G	Kaufmann <i>et al</i> (1999)
pET501	proOmpA (S245C;C290S;C302S)-Dhfr (C334S)	Bol <i>et al</i> (2007)
pET504	proOmpA (S282C;C290S;C302S)-Dhfr (C334S)	Bol <i>et al</i> (2007)
pET505	proOmpA (C302S)-Dhfr (C334S)	Bol <i>et al</i> (2007)
pET507	proOmpA (S315C;C290S;C302S)-Dhfr (C334S)	Bol <i>et al</i> (2007)

inclusion bodies, and stored in 6 M urea (van der Does *et al*, 2003). Mutants bearing single cysteine residues in proOmpA domain (Table 1) were fluorescently labelled with Cy3- or Atto 647N-maleimide with an efficiency of ~90%. To achieve the folded form of the Dhfr domain, the fusion protein was incubated in the presence of methotrexate and NADPH prior the translocation reaction (Arkowitz *et al*, 1993).

Bulk FRET measurements on SecYEG:preprotein complex

Variants of proOmpA-Dhfr containing single cysteine residues within proOmpA domain were labelled with Cy3-maleimide dye (donor) and whole cells overexpressing SecY_{C295}EG were labelled by Cy5-maleimide (acceptor) as described elsewhere (Slotboom *et al*, 1999). Inside-out IMVs were generated upon disrupting cells in French press, so the SecY_{C295}EG-conjugated Cy5 fluorophore was located in the vesicle lumen. Fluorescence measurements upon the translocation intermediate formation were performed using an Aminco Bowman spectrofluorometer. Donor fluorophore was excited at 525 nm, and FRET efficiency was measured as a change in the acceptor fluorescence at 670 nm.

Confocal microscopy

A home-built confocal microscope (Doeven *et al*, 2005) was used for FCS/FCCS measurements. An argon-ion laser at 488 nm was used to excite the AlexaFluor 488 fluorophores, and He-Ne laser at 633 nm—to excite Atto 647N. All measurements were carried out at room temperature. The calibration procedure was carried out as previously described (Doeven *et al*, 2005, 2008). To determine the excitation volumes for both lasers, diffusion of AlexaFluor 488 and AlexaFluor 633 was measured in solution and corresponding autocorrelation curves were fitted using diffusion coefficients of 300×10^{-8} and 198×10^{-8} cm²/s, respectively (Hutschenreiter *et al*, 2004; van den Bogaart *et al*, 2007). Fluorescence fluctuations on GUV membranes were recorded in series, each containing 10 measurements over 8 s. Autocorrelation traces were built for individual measurements within a series, and non-disturbed measurements were averaged and used for further analysis. FCS data acquired on SecYEG within the GUV membranes were analysed assuming one-component two-dimensional protein diffusion, and autocorrelation traces were fitted to equation:

$$G(t) = \frac{1}{N} \left(1 + \frac{t}{\tau_D} \right)^{-1} \quad (1)$$

where $G(t)$ is the amplitude of the autocorrelation function, N —average number of fluorescent particles in the laser focus and τ_D —diffusion time. We excluded fast intramolecular dynamics and

occasional fluorophore photoconversion from analysis by limiting FCS data to the time range from 10^{-1} to 10^3 ms. The measured number of particles and the fluorescence intensity were used to estimate the molecular brightness of fluorophores. To assess the co-diffusion of differently labelled proteins, the fluorescence intensity in blue and red channels was cross-correlated (Schwille *et al*, 1997). The amplitude of the resulting FCCS function is proportional to the concentration of dual-labelled molecular complexes and could be used to determine the protein oligomerization level. Laser beam alignment was controlled using dual-labelled λ -DNA as a reference sample for the FCCS.

Since the detection volumes of the blue and the red channels differ due to the diffraction limitation, we determined the ratio of these volumes to calculate the concentration ratios from the autocorrelation amplitudes for FRET-FCS translocation assay. Herein, we performed FCS measurements on membrane-incorporated fluorescent lipid analogue 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide, DiA (Invitrogen, USA). Due to its spectral properties the dye could be efficiently excited at 488 nm, and its fluorescence was recorded in both the blue and red channels. The ratio of particle numbers detected in both channels ($N_{\text{blue}}/N_{\text{red}} = 0.95$) was used for correcting the FRET-FCS data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: AK designed and performed the experiments, analysed the data and wrote the paper; IK designed and performed the experiments and wrote the paper; VVK designed technical aspects of the experiments and analysed the data; AJMD designed the experiments, supervised the work and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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