UNIVERSITY OF GRONINGEN

Dynamics within the Sec translocon

Binding of Ribosome Nascent Chain complexes to SecYEG

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8/30/2016

Research project I

Molecular Biology and Biotechnology

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SecYEG is one of the main components of the Sec-translocon in Escherichia coli. Via two targeting routes, this system is responsible for translocating proteins across or incorporate proteins into the cytoplasmic membrane. The latter is done through the association of a ribosome nascent chain complex to the SecYEG channel. To gain more insight, both SecYEG and the RNCs were purified from E. coli expression strains. SecYEG was reconstituted into proteoliposomes and nanodiscs to test its translocation activity and the binding ability to RNCs. Through these experiments a K_D in the nanomolar range was determined for the binding of RNCs to SecYEG nanodiscs.

Contents

INTRODUCTION

The bacterial cytosol is densely packed solution containing a huge crowd of proteins which all have their distinct destination and function. Among these particles, new proteins are synthesized by transcribing information from the bacterial DNA, which can be translated to an amino acid sequence by a ribosome. Once the N-terminal signal sequence of a new protein exits the ribosomal polypeptide exit tunnel, there are several signals which correspond to different locations to which proteins can be transported and potentially incorporated[1]. Each of these different signals is specified in this N-terminal sequence, which favors the correct interaction with the corresponding initial binding partner. There are several environments which are the final location of a newly translated protein. If the proteins function does not contribute to a process within the cytoplasm, it will be inserted into or translocated across the cytoplasmic membrane. The main machinery to facilitate transport of proteins to their final destination in *Escherichia coli* is the Sec machinery. There are two major routes to recognize and target proteins towards the Sec translocon.

In general, most pre-proteins are targeted to the Sec translocon post-translationally (fig 1b). Thereby the N-terminal signal sequence is bound by the trigger factor (TF). Compared to the signal sequences for the co-translational route, these sequences have a less hydrophobic character, which initiates transport across the cytoplasmic membrane. Once the translation reaction is finished, the peptide is kept unfolded and prevented from degradation by the molecular chaperone SecB via a long hydrophobic groove on both sides of the tetramer. The unfolding of the precursor protein avoids

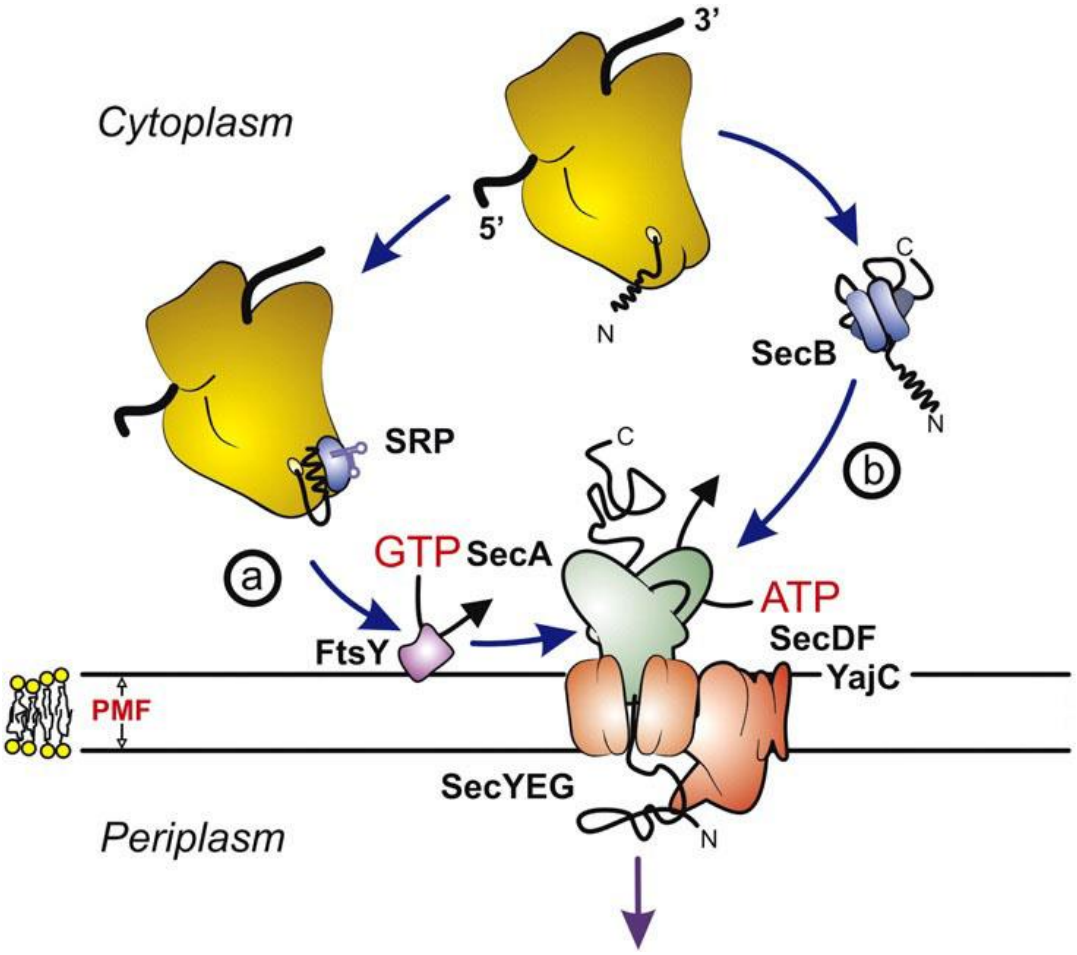

Figure 1: Schematic overview of the *Escherichia coli* Sec translocase. (a) Co-translational and (b) post-translational targeting routes and translocation of newly synthesized proteins[37].

misfolding and ensure a translocation-competent conformation[2]. The pre-protein is then transferred to SecYEG, where it interacts with the motor ATPase SecA upon ATP binding. Translocation is then conducted in a step-wise mode through multiple cycles of ATP binding and hydrolysis. During this hydrolysis, SecA is bound to SecYEG by a mutual binding site, which can be also used by FtsY, and additional sites located across the cytosolic loops C2-C6 of the SecY subunit[3].

In contrast, the co-translational route is mainly used for membrane protein targeting (fig 1a). When emerging from the ribosomal exit tunnel, nascent peptide chains are bound by the ribonucleoprotein signal recognition particle (SRP) via a hydrophobic SRP-specific signal-anchor sequence. Subsequently, SRP transfers the recruited ribosome-nascent chain complex (RNC) to FtsY, a receptor located at the cytoplasmic side of the bacterial membrane[4]. This interaction is facilitated by the binding with the cytosolic loops of the SecY subunit and the negatively charged phospholipids in the inner membrane[5]. Especially, the binding to the phospholipids is crucial for this interaction[6]. Next, the nascent chain is transferred to the main complex of the Sec translocon, SecYEG. This channel then guides the nascent chain into the inner membrane to be incorporated or translocated across into the periplasm. These reactions rely on the driving force of the translating ribosome.

In both routes, proteins are targeted to the heterotrimeric SecYEG complex, which forms the protein conducting channel. SecY forms the actual channel and consists of 10 trans-membrane segments. A cross section of the channel shows an hourglass shape[7]. This shape is created by the pore ring around the middle of the channel. The resulting pore creates a strong seal between the cytoplasm and the periplasm to ensure that the channel is closed until new pre-proteins require translocation. To ensure that translocated pre-proteins cannot return to the cytoplasm after translocation, the *trans*-side of the SecY channel is occupied by a plug domain. This domain folds back into the periplasmic side of the hourglass to block the channel after translocation is finished. SecE connects the symmetrical sides of SecY using a tilted and amphipathic helix. SecG is associated with SecY[1,8]. Both SecE and SecG are connected to the SecY channel. Together with SecA, a SecYE complex forms the minimal translocase[9]. SecG is not essential, but translocation is significantly less efficient in the absence of this subunit[10]. It has been shown that the deletion of SecG causes an accumulation of pre-proteins, which leads to a cold-sensitive phenotype and influences SecA[11,12]. SecA is a soluble motor protein which generates the driving force for protein translocation by ATP hydrolysis. The proton motive force may stimulate the translocation reaction during several stages[13]. SecA contributes in both the co- and post translational route. It can recognize both the SecB chaperone loaded with a pre-protein and signal sequences from individual pre-proteins. It has also been shown that SecA participates in other reactions like folding proteins without a signal sequence[14]. SecA forms homo-dimers and it is likely that it is mostly dimeric in the cytosol[15]. Although many studies agree on the dimeric state of this complex, the provided structures contradict on the dimerization interface and the orientation of the second promotor. In most structures, SecA is organized in an antiparallel manner, however author et al show a parallel SecA-SecA organization. Although different structures leave potential for multiple populations, only functional conformations bound to SecYEG will contribute to the translocation process[8].

Multiple models have been proposed to depict the mechanism of translocation via the Sec machinery. From a biochemical study, it was deducted that this system relied on multiple complexes to function and the subunits of SecYEG were not the sole power behind the translocation. Even though locking the 2HF domain of SecA did not influence the translocation rate[16]. The function of SecA in this machinery was originally described in the Brownian ratchet mechanism. This model described a

fluctuating back and forth movement in which there was no actual displacement to the SecYEG channel. This random movement would be irreversibly halted when the first part of the substrate would emerge from the channel by ATP dependent trapping. But this model did not fit with the observations of a step-wise translocation[17,18]. Later on, the power stroke model was suggested. This model was based on the insertion or conformational change of SecA to facilitate translocation[19]. Subsequently a more refined model was proposed: the reciprocating piston model. This model consist of two phases where either SecA of ATP binds to the translocon.

Even though such models purpose mechanisms which are backed up by properly conducted experiments, the functional state of SecYEG remains to be elucidated. Studies have shown that this highly dynamic system could function as a monomer or as a back to back orientated dimer[20,21]. Most studies were performed using detergents or other restricting states for the SecYEG complex. Novel studies described the functional properties of the translocon in an unrestricted manner using either giant unilamellar vesicles or nanodiscs, analyzed by fluorescence (cross-) correlation spectroscopy and Föster resonance transfer energy measurements. This data strongly suggest that a single monomeric copy of SecYEG is sufficient to conduct a translocations reaction[22,23].

Different studies to increase insight into the Sec-translocon were based on bulk experiments. To give more insight in the machinery behind the Sec-translocon, a single molecule set-up could be created. This involves the usage of ribosome nascent chain complexes (RNCs) which bind to SecYEG These RNC complexes are composed of a temporally stalled ribosome containing the coding mRNA for the membrane protein and the partially translated nascent amino acid chain. After it binds to the SecYEG protein conducting channel, the translation of the nascent chain resumes until the SecM stalling sequence is translated, which halts the translation. This fixes the process and binds the RNC to the SecYEG channel. This will cause a significant decrease in diffusion speed of the pair of complexes due to the size of the RNC. This would have been analyzed by TIRF microscopy by exciting the fluorescent label attached to SecYEG.

RESULTS

Overexpressing SecYEG in crude membranes

To overexpress SecYEG an *E. coli* pEK20-C148 culture was grown in LB medium. Throughout the growth, the OD_{600} was measured (fig 2). The starting OD_{600} was 0.05 and the culture was induced at an OD_{600} of 0.68. After the induction, the cultures grew with a linear growth rate for one hour at a rate of 0.0108 min^{-1}. In the second hour the bacteria stopped growing, which could indicate that their internal energy distribution was more committed towards protein production than to cellular growth.

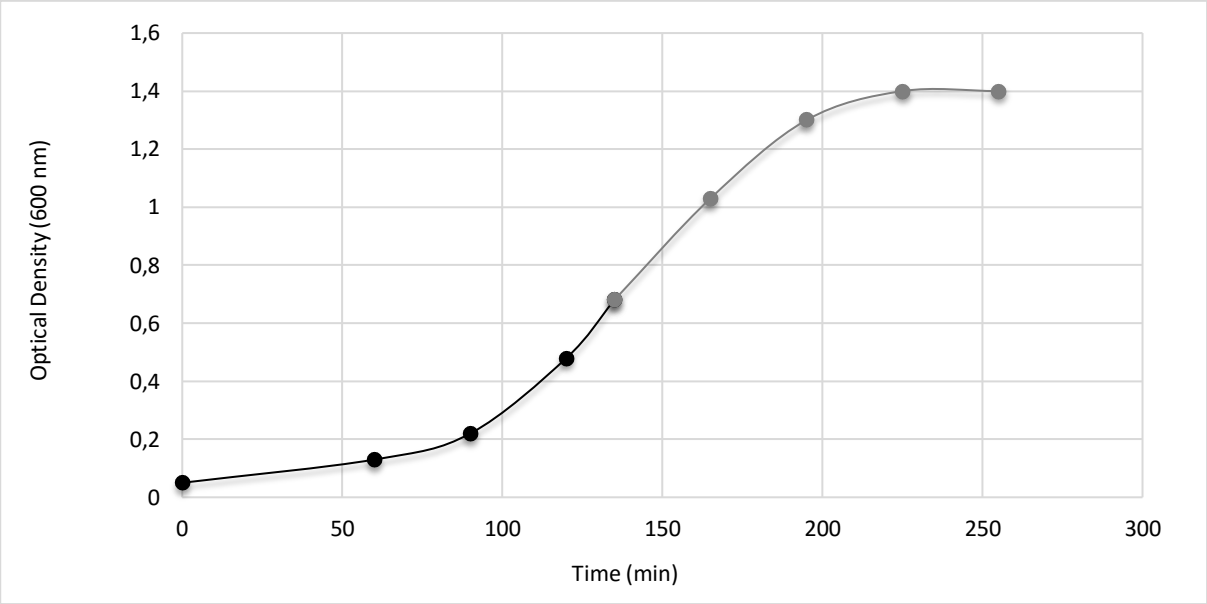

Figure 2: Growth of *E. coli* SF100 pEK20-C148 before and after induction with 0.5 mM IPTG. The culture showed exponential growth before the protein expression was induced. After induction growth stopped gradually over time. This indicates a redistribution of energy from cellular expansion toward protein synthesis. Black; Pre IPTG induction. Gray; post IPTG induction.

The cells containing the overexpressed SecYEG were disrupted and crude membranes were isolated. To verify the overexpression of SecYEG, the final product from the isolation process was analyzed by SDS-PAGE. It was shown that the isolated crude membranes contained high quantities of SecYEG as seen by high intensity bands at 37 kDa for the SecY and at 15 kDa for the SecEG subunit (fig 3).

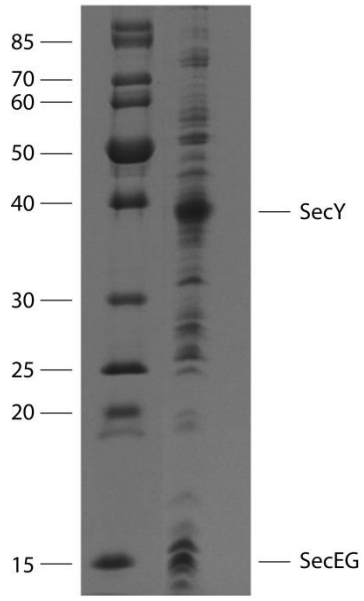

Figure 3: SDS-PAGE analysis on protein composition of *E. coli* crude membranes. The crude membranes show overexpression of both SecY at 37 and SecEG at 15 kDa.

To determine the total protein concentration in the crude membranes, a Lowry assay was performed. This assay is based on the reaction between the protein, in particular tyrosine and tryptophan, and copper, which reduces Cu (II) to Cu (I). Subsequently, Cu (I) reduces foline to molybdenum blue, which can be detected photo metrically. Using a BSA standard curve, the unknown protein concentration can be determined. Through this method an average total protein concentration of 65 mg/ml was determined. This value was used to set the amount of starting material in the SecYEG purifications conducted during this project.

Purifying, labelling and reconstituting SecYEG

Furthermore, purifications were conducted using Atto647N and Cy5 maleimide conjugated fluorophores. These fluorophores function as a marker for the SecYEG complexes for experiments which require protein visualization e.g. microscopy, FRET. The Atto647N labelling procedure resulted in an average labelling efficiency of 102%. This resembles the labelling of every SecY subunit of the cysteine at position 148 in addition to a minor amount of unspecific labelling. The labelling with Cy5 was less efficient with an average labelling efficiency of 37%. This resembles a mixture of labeled and unlabeled SecY subunits. The protein composition of the elutions, together with the specificity of the labelling, were analyzed using SDS-PAGE (fig 4). It shows the protein composition of different unlabeled samples from the purification process. Little protein is lost during this process and the elutions mostly contained pure of SecYEG. On the image, this is visualized as 2 protein bands caused by the addition of the denaturizing agent SDS, which results in a disassembly of the SecYEG complex into SecY, running at 37 kDa, and SecEG running at 16 kDa (fig 4a). Subsequently it shows the purification process of SecYEG combined with labelling procedure of Atto647N. The elutions of this process show a comparable composition and purity compared to the purification process without any fluorophore present. The protein bands show a different running behavior due to the increased hydrophobicity from the attached Atto647N fluorophore (fig 4b). The next panel shows a

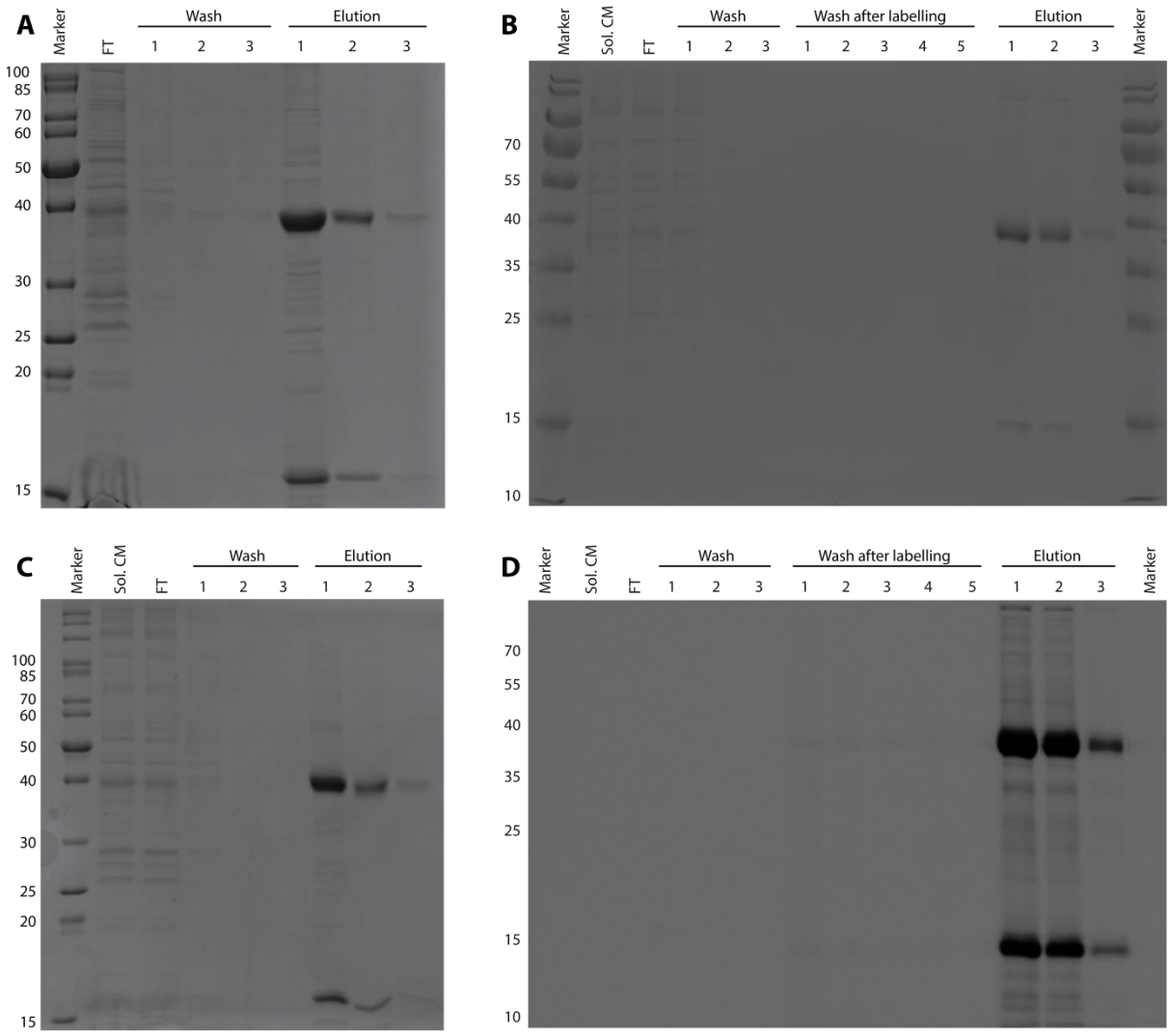

Figure 4: SDS-PAGE analysis on protein composition of SecYEG purification. Sol. CM; Solubilized crude membranes, FT; Flow-through. (a) Coomassie stained SDS-PAGE gel of the purification of unlabeled SecYEG. Elutions show mostly SecYEG and no impurities. (b) Coomassie stained SDS-PAGE gel of the purification of Atto647N-labeled SecYEG. Elutions shows comparable yield and purity as the unlabeled SecYEG. Size-shift due to hydrophobicity of the fluorophore. (c) Coomassie stained SDS-PAGE gel of the purification of Cy5-labeled SecYEG. Elutions shows comparable yield and purity as the unlabeled SecYEG. (d) Fluorescence image with a red filter of panel B. SecYEG specifically labeled with the fluorophore. Little to no unspecific labelling apart from SecEG, which did not contain a maleimide group.

comparable composition and purity of the purification process in the presence of Cy5 fluorophores. The presence of Cy5 did not influence the running behavior of SecYEG (fig 4c). Finally, a fluorescence image is shown to visualize proteins which are labeled with a fluorophore Atto647N from the analysis in panel b. The labelling reaction mainly occurred on the SecYEG subunits. There was a small amount of unspecific labelling with the Atto647N fluorophore, which is represented by the fluorescence of SecEG, which do not harbor a cysteine residue, in elutions 1,2 and 3 (fig 4d).

After purifying and contingent labelling of SecYEG, the complex was reconstituted into proteoliposomes to maintain its native activity. Detergent-solubilized lipids and SecYEG were mixed and the formation of proteoliposomes was initiated by slow removal of detergent. Proteoliposomes were collected by centrifugation and the presence of SecYEG was confirmed by SDS-PAGE (fig 5). The left panel shows the SDS-PAGE analysis of unlabeled SecYEG proteoliposomes. (fig 5a). The middle panel shows the identical analysis for Atto647N labeled SecYEG proteoliposomes, irrespectively from the bound fluorophores (fig 5b). The right panel shows the remainder of the fluorescent signal when excited with red light, after the reconstitution procedure (fig 5c).

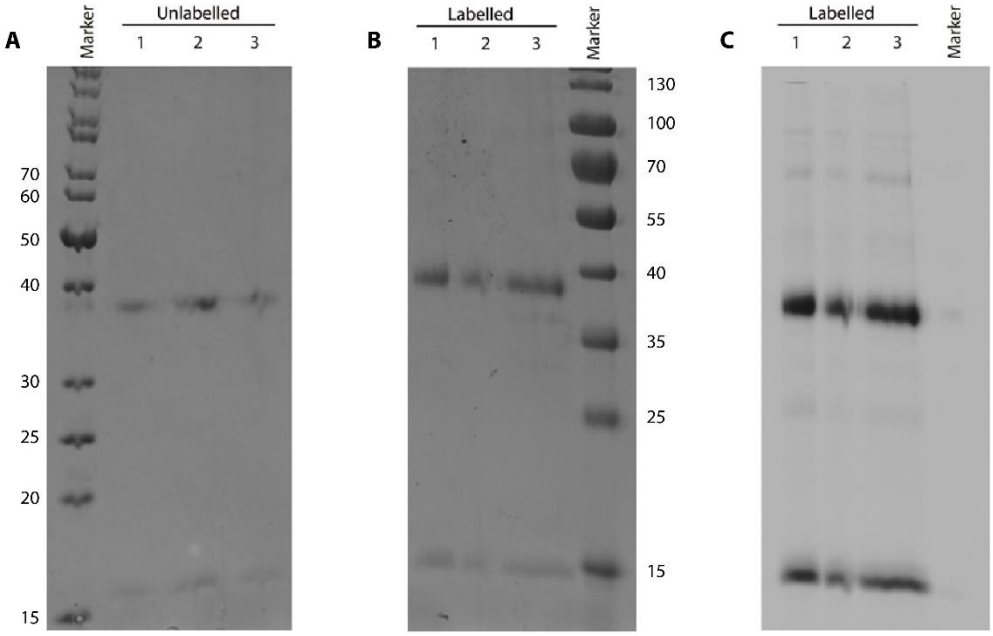

Figure 5: SDS-PAGE analysis of SecYEG proteoliposomes. (a) Coomassie stained SDS-PAGE gel containing unlabeled SecYEG proteoliposomes, (b) Coomassie stained SDS-PAGE gel containing Atto647N labeled SecYEG proteoliposomes, (c) Fluorescence image with a red filter of the SDS-PAGE gel containing Atto647N labeled SecYEG proteoliposomes. The labeled proteoliposomes show a fluorescent signal specific to the incorporated labeled SecYEG.

Translocation assay SecYEG proteoliposomes

SecYEG requires to be in a lipid based environment to maintain its native activity[24]. The protein was purified in a detergent based solution and labeled with fluorophores by a maleimide-cysteine binding reaction. These factors could influence the native translocation ability of the transporter, which could result in false data interpretation. Therefore the native activity of purified and labeled SecYEG was tested in a translocation assay containing a native substrate of the post-translational route: Outer Membrane Protein A in its pre-protein state (proOmpA). This assay is based on the natural affinity of SecYEG for this substrate. By fluorescently labelling the substrate and allowing translocation into proteoliposomes, a percentage of the total fluorescence can be determined after all the non-translocated fluorescent substrate is cleaved. This is done over a titration of SecA to ensure proper functionality of the reaction. The percentage of proOmpA translocation into unlabeled, Cy5 labeled and Atto647N labeled SecYEG proteoliposomes are plotted against the SecA concentration (fig 6). The unlabeled SecYEG shows an increase of translocation which saturates at 25% at a SecA concentration of 200 nM. Cy5 labeled SecYEG shows a similar increasing trend of SecA-dependent proOmpA translocation comparable to unlabeled SecYEG activity. However, the trend of translocation shows no saturation at higher SecA concentration. In contrast, the translocation activity of Atto647N labeled SecYEG was significantly lower compared to the unlabeled complex. On average the Atto647N labeled SecYEG shows half the activity of the unlabeled translocon.

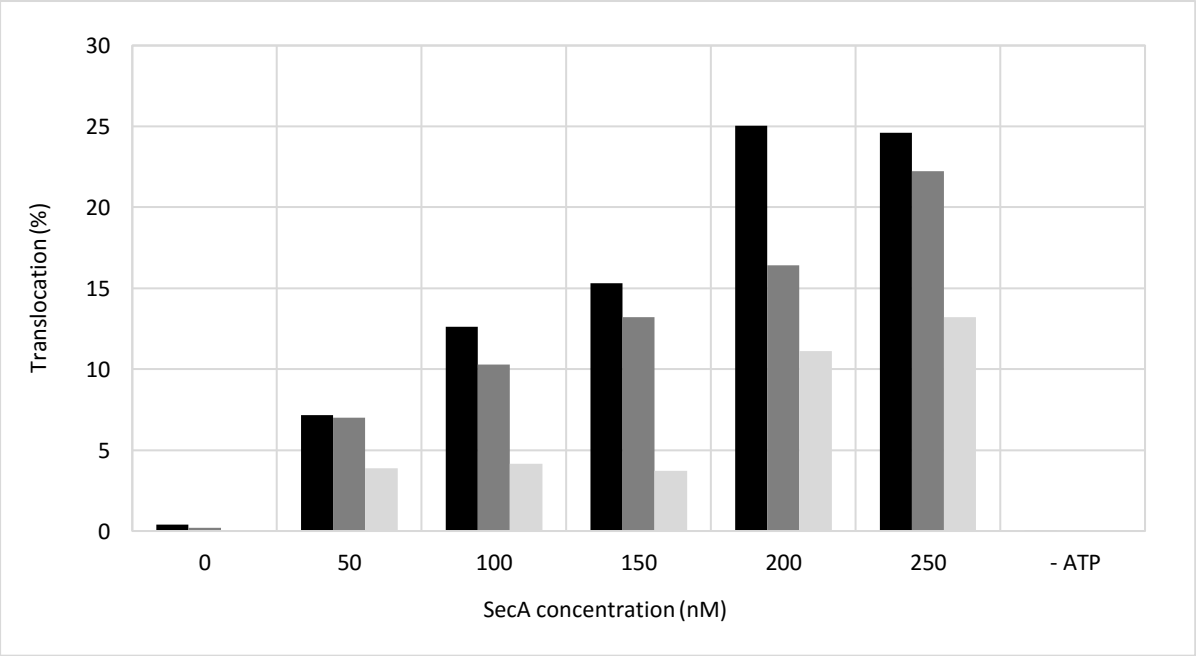

Figure 6: Translocation of proOmpA into SecYEG proteoliposomes. Unlabeled SecYEG proteoliposomes show increased translocation correlated to the SecA concentration. The Cy5-labeled SecYEG proteoliposomes show a similar increasing percentage of translocation compared to the unlabeled complexes. The Atto647N-labeled SecYEG proteoliposomes show a similar increasing trend as the other complexes, although the absolute percentages of translocation are considerately lower. Black: Unlabeled SecYEG. Gray: SecYEG-Cy5. White: SecYEG-Atto647N.

Overexpressing, purifying and detecting RNCs

Translocation by the co-translational route is conducted through transferring a whole ribosomal complex the SecYEG by the membrane receptor FtsY. Even though, ribosomes are relatively large complexes, these translocation processes occur quickly. This makes them hard to detect and record. To slow down this fast movements, they are blocked by using RNCs. These complexes consist of a stalled ribosome containing the coding mRNA for a membrane protein and the partially translated nascent amino acid chain. The stalling of the translation is achieved through the interaction of a SecM stalling motif, which is incorporated in the nascent chain sequence. SecM is a regulatory protein for SecA expression. It contains an arrest sequence that interacts with the ribosomal protein exit tunnel through its proline-166 residue. In this study, the nascent chain sequence also contained FtsQ residues with its transmembrane domain as a substrate for the translocation process and an N-terminal streptavidin3-tag for the purification process.

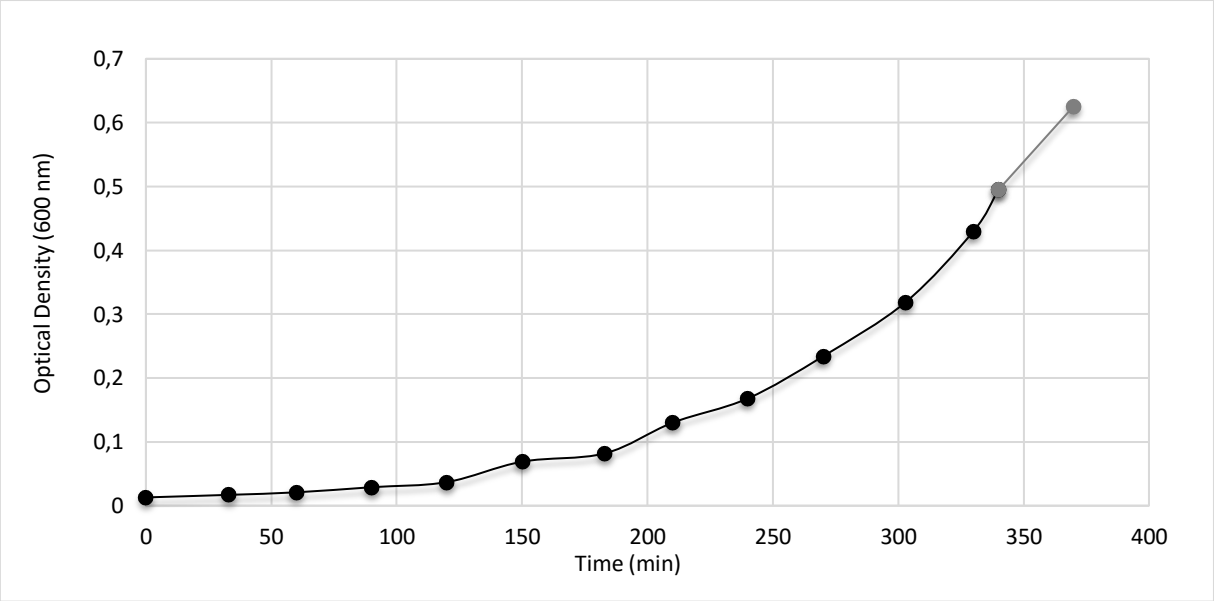

Figure 7: Initial growing of *E. coli* BL21Δtig pUC19Strep$_3$FtsQSecM. The culture showed exponential growth before the RNC expression was induced. After induction growth slowed down to a linear rate. This indicates that the cells were still able to grow even though the presence of the RNCs. Black; Pre IPTG induction. Gray; post IPTG induction.

To purify RNCs, *E. coli* BL21Δtig pUC19Strep$_3$FtsQSecM was grown until OD_{600} 0.495 and RNC overexpression was induced. After the induction, the cultures grew with a linear growth rate for 30 minutes (fig 7). The first attempts to isolate and purify RNCs from cultures grown did result in a ribosome concentrations in the range of 50 nM in every elution. This was done by isolating the ribosomes through a sucrose cushion and purifying the RNCs using streptavidin-affinity chromatography. The protein and ribosome composition from the samples of the RNC purification were analyzed by SDS-PAGE and western blot (fig 8). The presence of native protein and free ribosomes in the flow-through and in several washing steps of the purification process was detected in the Coomassie stained SDS-PAGE analysis (fig 8a). However, no RNCs were eluted from the column in this process. This was caused by the absence of nascent chains in the stalled ribosomes. The immunoblot against the streptavidin-tag, which is fused to the nascent chain (approximate height of 15 kDa) shows no presence of the nascent chain in the isolated ribosomes (fig 8b).

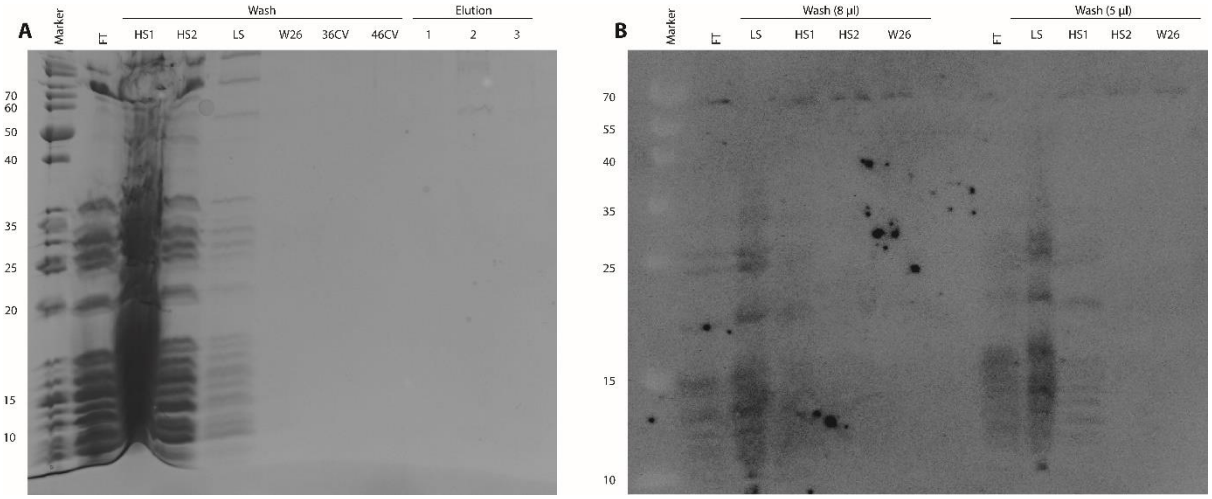

Figure 9: Analysis of protein composition of RNC purification process. FT; Flow-through, HS1; High salt 1, HS2; High salt 2, LS; Low salt, W26; Washing fraction 26. (a) Coomassie stained SDS-PAGE gel RNC purification process. Both the flow-through and several washing samples contained ribosomes. There were no ribosomes present in the elutions. (b) Western blot of flow-through and washing fractions of the RNC purification process. Although ribosomes were present in these solution, no nascent chains were visible on the blot.

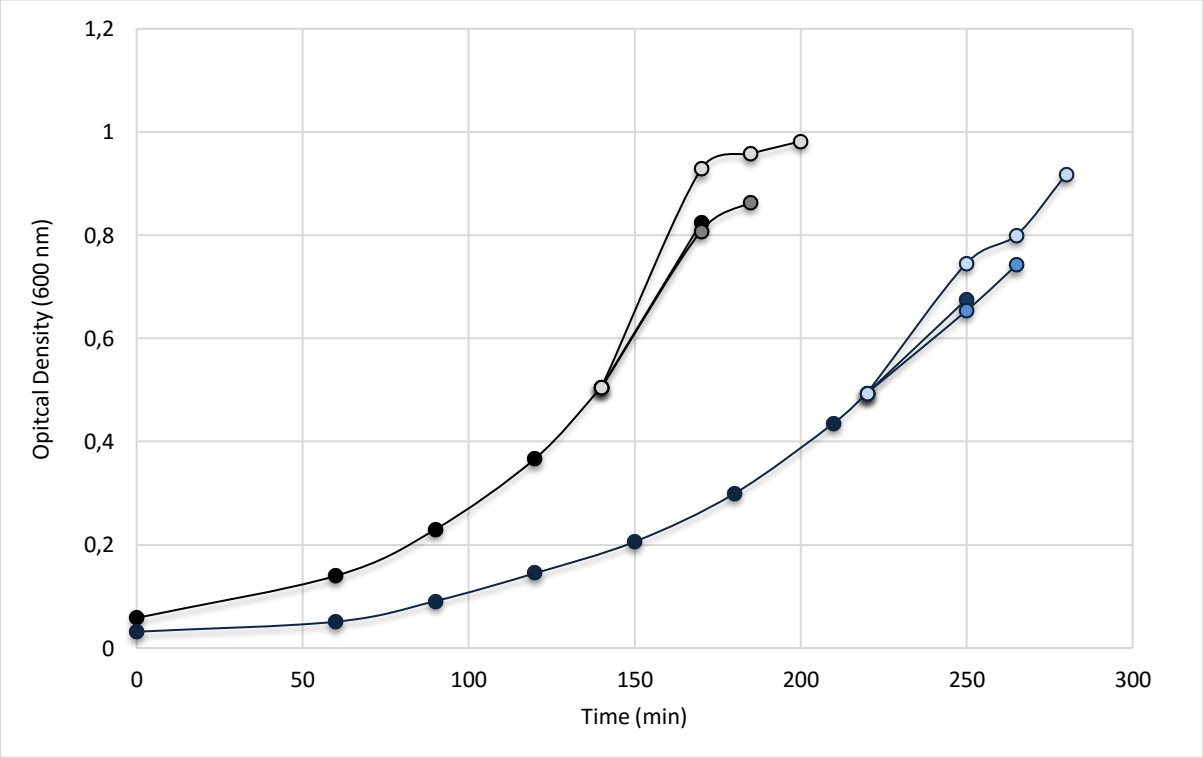

Figure 8: Growth of *E. coli*-DE3-Δtig and *E. coli* KC6-DE3-ΔSmpBΔSsrA when overexpressing RNCs under variable lengths of induction time. Growth of *E. coli* KC6-DE3-ΔSmpBΔSsrA was inhibited by the large amount of stalled ribosomes. Both strains showed decreased growth rates after induction of the RNC expression compared to the early growth phase. Black; BL21 pre IPTG induction. Black: BL21 post IPTG 30 min induction. Gray; BL21 post IPTG 45 min induction. White: BL21 post IPTG 60 min induction. Dark Blue; KC6 pre IPTG induction. Dark blue; KC6 post IPTG 30 min induction. Light blue; KC6 post IPTG 45 min induction. White blue; KC6 post IPTG 60 min induction.

To increase the yield of RNCs, the expression was tested in two different bacterial strains *E. coli* BL21(DE3)Δtig and *E. coli* KC6(DE3)ΔSmpBΔSsrA. The *E. coli* BL21(DE3)Δtig strain is a standard expression strain where the trigger factor has been deleted from the genome to ensure the formation

of the stalled RNCs[25]. The *E. coli* KC6(DE3)ΔSmpBΔSsrA strain is an expression strain where two ribosome rescuing proteins, SmpB and SsrA, were deleted, which could benefit the RNC overexpression[26]. The influence of the overexpression time was tested in both strains containing the pUC19Strep$_3$FtsQSecM plasmid. A higher growth rate was observed for the BL21(DE3)Δtig strain compared to the KC6(DE3)ΔSmpBΔSsrA strain (fig 9). This is due to the inability of the KC6(DE3)ΔSmpBΔSsrA strain to rescue stalled ribosomes, which inactivates them irreversibly. This hinders growth significantly, hence the slower growth during the entire experiment. Further, increasing the induction time resulted in a linear growth rate for most conditions. When an induction time of 60 minutes was applied to both strains, their growth plateaued for a short time, after which they tended to return to their linear growth rate. This shows that both strains are able to take the additional stress of an increased induction time.

Cells were lysed and the soluble components were isolated. SDS-PAGE analysis was conducted on these components (fig 10). The coomassie stained gel shows an increasing amount of ribosomes correlated with an increased induction time in both strains (fig 10a). The absolute amounts of product are present in comparable amounts. The immunoblot against the streptavidin-tag, which is fused to the nascent chain, shows that the amount of nascent chain increased as the induction time is prolonged (fig 10b). The BL21(DE3)Δtig shows a slightly higher expression of the nascent chain over 60 minutes. This strain was used in combination with the 60 minutes of induction time for future RNC purifications. Here, the elutions now contain more ribosomes. These condition resulted in a RNC yield of 300-450 nM of ribosomes and contained 6-7 µg/ml total protein per elution.

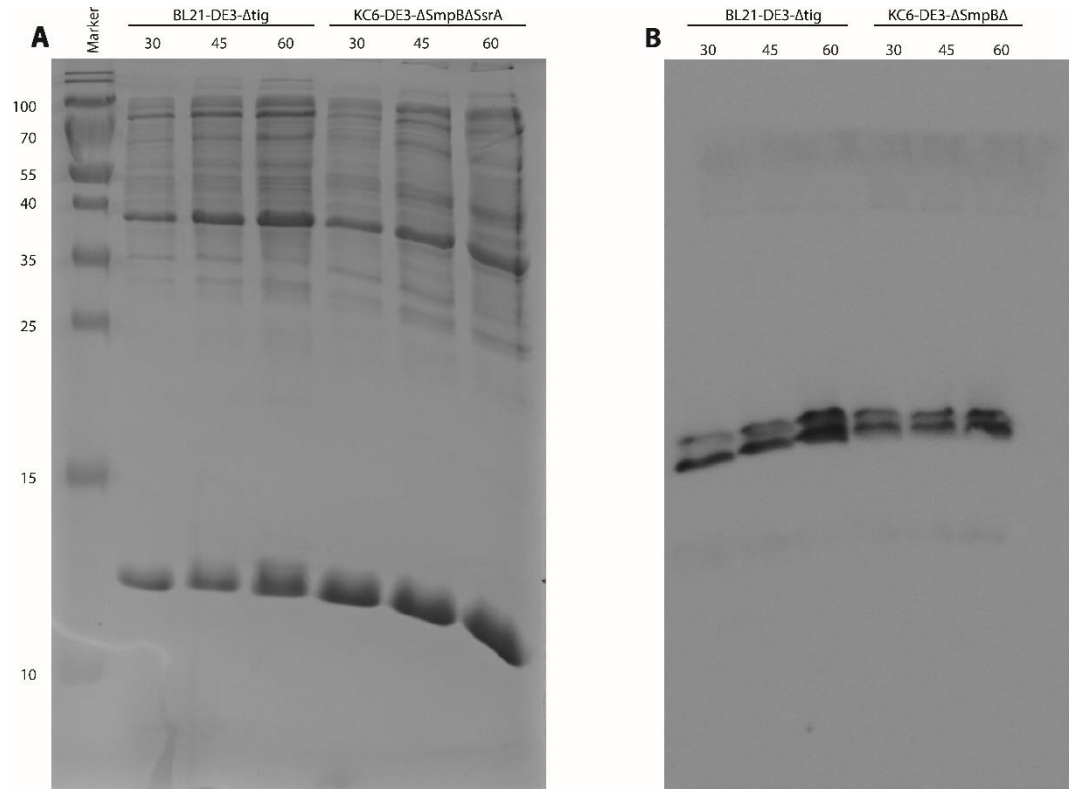

Figure 10: Analysis on protein composition of RNC overexpression. (a) Coomassie stained SDS-PAGE gel RNC expression in different strains over a variable induction time (30, 45 or 60 min). Both strains showed an increased RNC expression upon increased induction time. (b) Western blot nascent chain expression in different strains over a variable induction time. Both strains showed an increased nascent chain expression upon increased induction time (30, 45 or 60 min).

Micro Scale Thermophoresis RNCs

To test the activity of RNCs, Micro Scale Thermophoresis (MST) was used to analyze the binding ability of purified RNCs. This technique is based on the difference of speed of particles when moving along a temperature gradient.

Although it was shown by the *in vitro* translocation assays (fig 6) that the Cy5-SecYEG proteoliposomes were active, the early MST measurements did not give a steady fluorescent signal due to a non-uniform size of the proteoliposomes. When a mixture of proteoliposomes with different sizes was used for MST, the fluorophores, bound to SecYEG, would not be distributed evenly throughout all the samples and moreover, there even could be an uneven distribution within one sample. To create more uniform SecYEG proteoliposomes, they were extruded in two steps through a 200nm and 50nm filter. By pressing the proteoliposomes through these filters, their size became more uniform. This improved the fluorescent signal, although the traces still had a low quality. An additional problem in combining proteoliposomes with MST is the presence of aggreagation in the samples. This aggreagation was formed even though the buffers contained 0.5 mg/ml BSA, which should prevent the formation of aggreagates. To further prevent aggragation, two more modifications were made to the procedure. To the existing buffer, 50 mM of L-arginine was added, which waspreviously shown to prevent aggreagation[27]. Additionally, the MST measurments were performed at 37°C.. The increase in temperature could increase the efficiency of the binding reaction, which could improve the MST traces. After implementing these changes in the protocol, the MST measurement were conducted (fig 11a). Although a binding curve cold be acquired with these MST traces, several traces were excluded due to fluorescence signals outside the range (fig 11b). Moreover, the remaining traces still contained

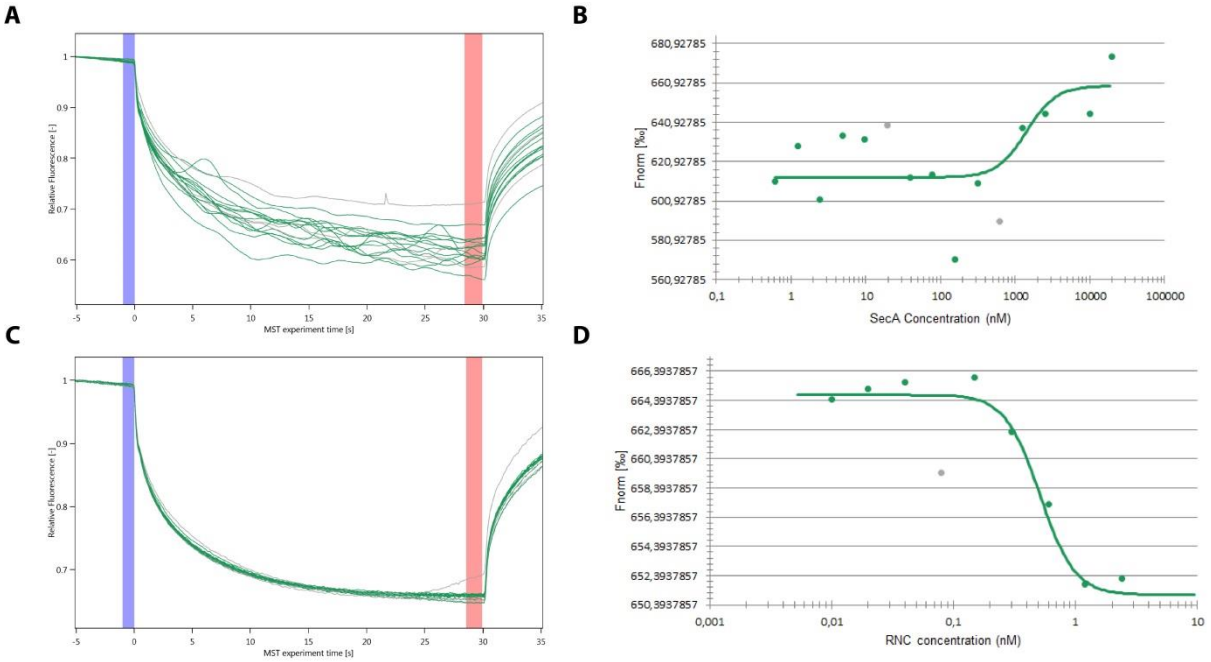

Figure 11: MST binding experiments of SecYEG proteoliposomes to SecA and SecYEG nanodiscs to RNCs. A) MST traces extruded Cy5 SecYEG-proteoliposomes with 50 mM L-arginine at 37°C plotted as normalized fluorescence over time. The traces were of a poor quality due to aggregate formation. B) MST binding curve generated from the traces in panel A plotted as hot-fluorescence/cold-fluorescence ratio against SecA concentration. After excluding some outliners, a binding curve could be formed with an K_D of 1.4 µM C) MST traces of Cy5 SecYEG-nanodiscs at 37°C plotted as normalized fluorescence over time. The traces were of a good quality. D) MST binding curve generated from the traces in panel C plotted as hot-fluorescence/cold-fluorescence ratio against RNC concentration. After excluding some outliners, a binding curve could be formed with an K_D of 0.5 nM Green; trace/data point. Gray; outlier. Blue/red; measuring area fluorescence without MST(cold) and with MST(hot).

peaks, which indicates that aggreagations were still present dispite all the precautions. This showed that proteoliposomes were not suitable to be used as a support system for these MST experiment including SecYEG.

The proteoliposomes were replaced by nanodiscs. These complexes are small, lipid based bilayers enwrapped by a hydrophilic scaffold protein, mostly containing single membrane transporters. Nanodiscs do not form aggreagates as fast as the proteoliposomes because of the hydrophilic character of their scaffold protein.. The nanodiscs were formed usingApoE422k as a scaffold protein. This scaffold protein is the N-terminal 22kDa fragment derived from the human apolipoprotein E4. ApoE422k is. It contains of a four-helix bundle, where each helix faces the lipids of the nanodiscs with its hydrophobic face. The hydrophilic tail faces outwards, which creates a lipid-base highly soluble complex, to prevent aggregation in a water based solution[28,29]. The conducted nanodiscs based experiments were done under the same conditions as the proteoliposome experiments with one alteration. The buffers did not contain 50 mM L-arginine because the combination of the BSA and the character of the scaffold protein would prevent aggregation. The resulting traces did not contain any peaks, which indicated that there were no aggregates present in the solution (fig 11c). This resulted in a good quality binding curve, without many outliers (fig 11d). The RNCs bound to the SecYEG nanodiscs with a K_D of 0.5713 nM. This experiments demonstrates that the RNCs were able to bind to the SecYEG channel.

Purifying additional components Sec-pathway: SecA

SecA is the motor protein that powers the post translational route of the Sec-system and is therefore an essential component for *in vitro* binding and translocation assays conducted in this study. SecA was purified through a cation exchange. The grown material shows a clear overexpression of SecA at approximately 100 kDa (fig 12a). After the overexpressed SecA was bound to the column , it was washed with a low salt buffer and some SecA was lost in the process. Eluting SecA from the column, resulted in 20 fractions containing SecA (fig 12a/b). This was this was concentrated to a concentration of 10 µM.

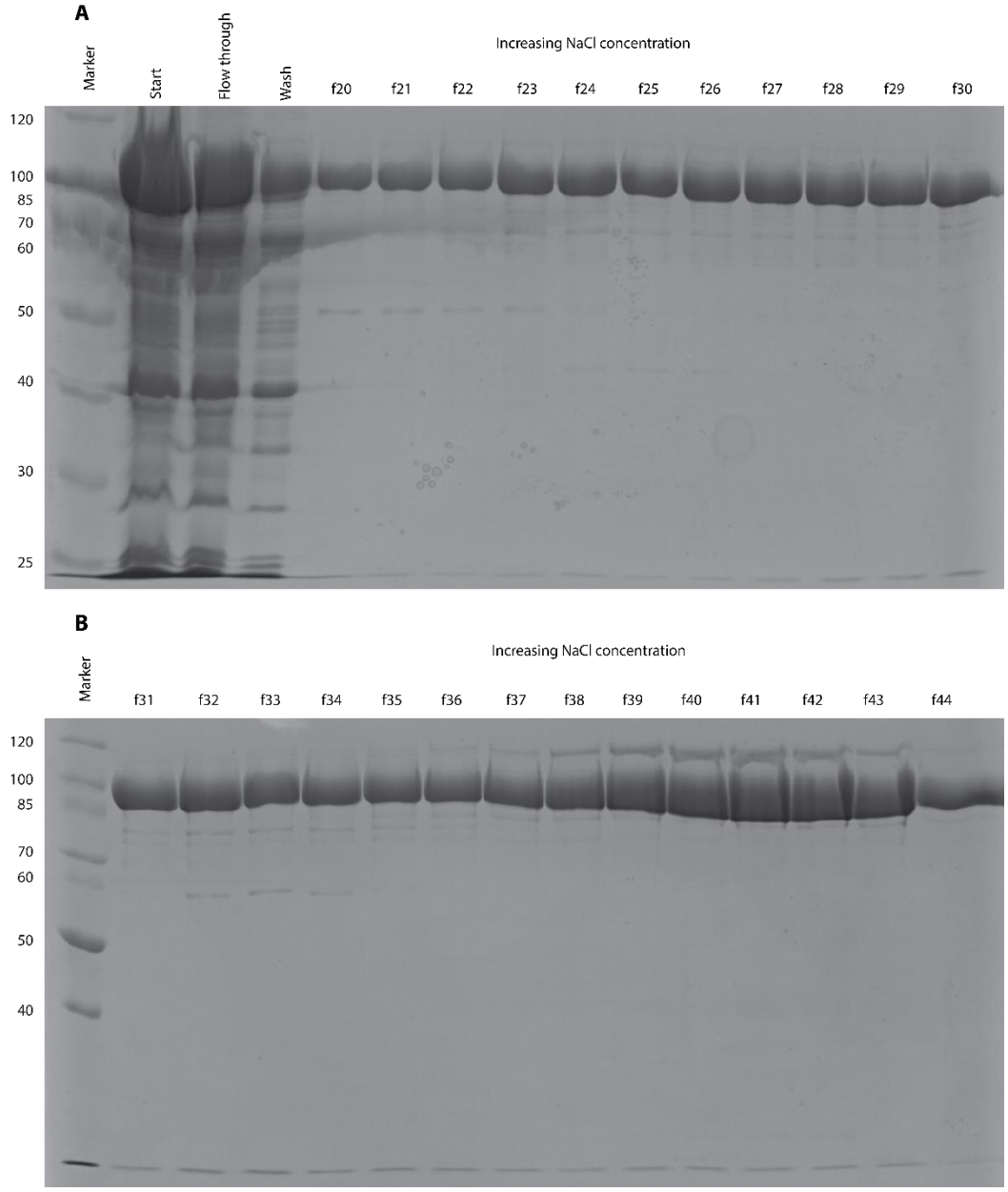

Figure 12: SDS-PAGE analysis on protein composition of SecA purification fractions. (a) Coomassie stained SDS-PAGE gel SecA purification process containing starting material, flow-through, wash and fractions 20 to 30. (b) Coomassie stained SDS-PAGE gel SecA purification process containing fractions 30 to 44. Both panels show the presence of SecA in the FPLC fraction at approximately 100 kDa.

Purifying additional components Sec-pathway: proOmpA

To determine the translocation activity of the labeled SecYEG proteoliposomes, fluorescein labeled proOmpA was used as a substrate for the SecYEG channel. This substrate was purified from fluorescein labeled inclusion bodies containing the overexpressed protein. This was achieved by disrupting the inclusion bodies and isolating the labeled substrate by an anion exchange column. Several samples from the purification process were analyzed using SDS-PAGE (fig 13a). Most of the proOmpA was eluted in one fraction, which was also confirmed by the fluorescent signal (fig 13b). The fluorescent substrate was aliquoted and used in the SecYEG translocation assays.

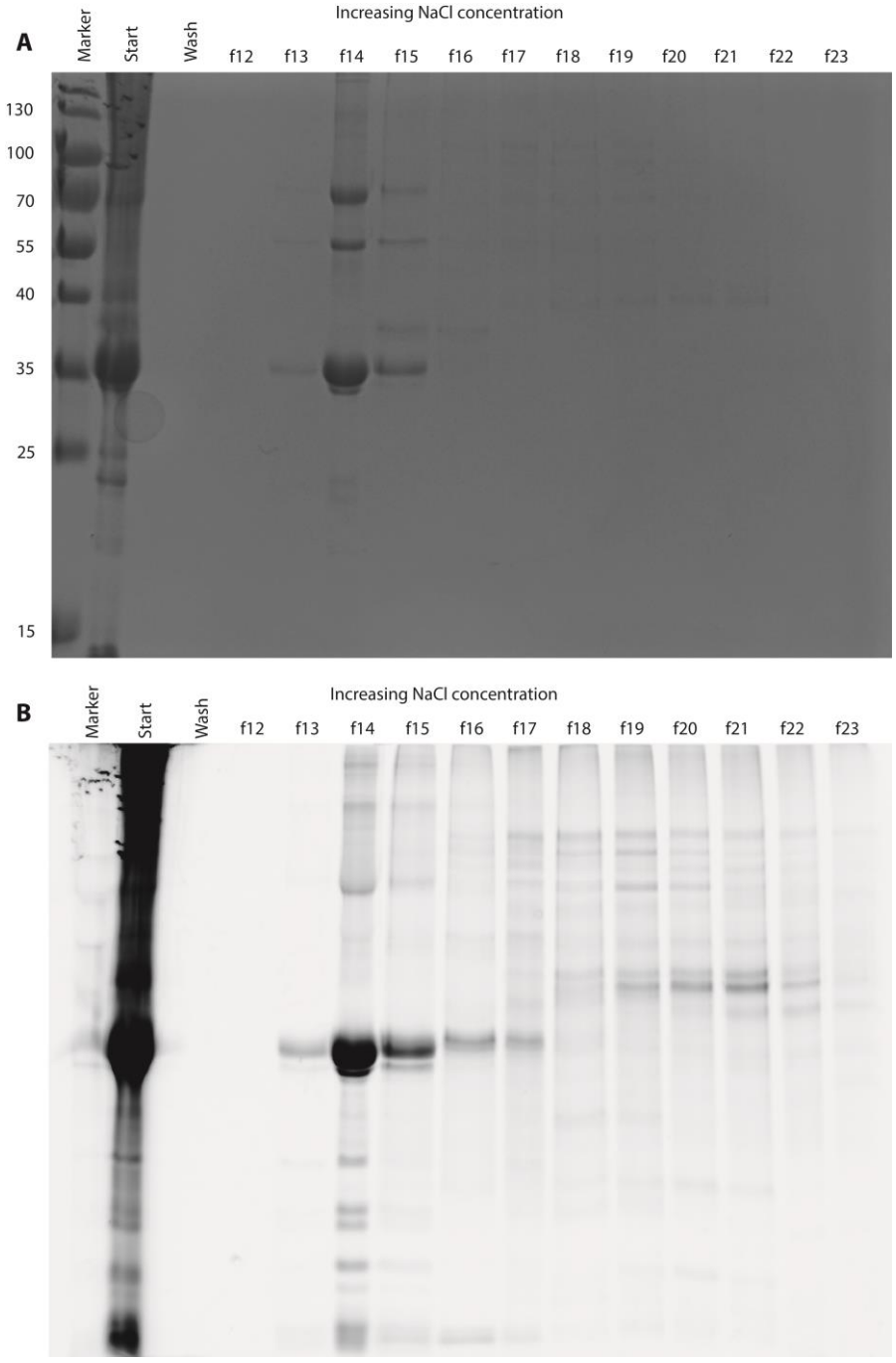

Figure 13: proOmpA anion exchange purification. (a) SDS-PAGE analysis Coomassie stained. Most proOmpA was mostly eluted infraction 14. Some impurities were present in the sample. (b) Fluorescence image of panel A. This image confirms the presence of fluorescein labeled proOmpA in fraction 14.

DISCUSSION

SecYEG translocates proteins via two routes across or into the cytoplasmic membrane. To incorporate newly synthesized proteins into the cytoplasmic membrane, corresponding proteins, associated with the translating ribosome are recruited to the SecYEG translocation channel through binding with the FtsY receptor. To translocate secretory proteins over the cytoplasmic membrane, the pre-protein is captured by SecB and transferred to the SecYEG-SecA complex. During this study, all components for the *in vitro* assays were overexpressed in *E. coli* strains and purified.

Even though, the labeled SecYEG proteoliposomes did not show identical translocation abilities compared to the unlabeled derivative. It could be suggested that some complexes conducted proOmpA translocation with a lower efficiency due to the attached Atto647N fluorophore. This could be caused by the characteristics of the fluorophore itself and/or be dependent on the labelling efficiency of the labelling process. It could be suggested that the higher labelling efficiency of Atto647N, 102% in respect to 37% for Cy5, caused the difference in translocation activity. If a 102% labelling efficiency was acquired with Cy5, the translocation efficiency could also drop, as observed for Atto647N SecYEG proteoliposomes. Repetition of the translocation assays and labelling with other fluorophores could be attempted to get more insight in the translocation abilities and/or increase the translocation activity of fluorescently labeled SecYEG proteoliposomes. This could also increase the trustworthiness of other experiments conducted with these complexes.

The overexpression of the RNCs in *E. coli* was tested in different strains combined with a variable induction time, ranging from 30 to 60 minutes to combat the low overexpression of the RNCs. This could be caused by different reasons. Either the nascent chain was never overexpressed in the first place or the stalled ribosomes were rescued, so that they release the nascent chain and it was lost during the isolation process. It has been shown that the BL21(DE3)Δtig strain, combined with 60 minutes of induction time, resulted in the optimal growth speed and RNC overexpression. These conditions may be incorporated in future RNC overexpression procedures to ensure a sufficient yield by the RNC purification.

After purifying RNCs with sufficient yields, they were tested for their binding affinity to SecYEG. This was done using Micro Scale Thermophoresis. Initial experiments were conducted with SecYEG proteoliposomes binding to wild type SecA. The resulting traces showed multiple peaks and inverted increasing fluorescence which indicated the presence of aggregates in the analyzed samples. To combat these events, SecYEG proteoliposomes were extruded in two steps (200 and 50 nM), L-arginine was added to the MST buffers and the traces were recorded at 37°C. These changes showed minor improvements towards the quality of the MST traces (fig 11a). These traces were transformed into a binding curve (fig 11b). However, due to the low quality of the traces, the resulting K_D of 1.4 µM is most likely inaccurate. Therefore, the setup was shifted towards SecYEG-Cy5 nanodiscs binding to RNCs. When using proteoliposomes, the complex formation between the purified SecYEG and the lipids is random, which results in a wide variety of SecYEG proteoliposomes: This ranges from proteoliposomes containing no SecYEG complex to proteoliposomes containing several SecYEG complexes. The fundamental difference between these complexes and nanodiscs is the presence of a size limiting scaffold protein, which is only present in nanodiscs. This is a polymer which limits the size of the lipid vesicles resulting in a controlled uptake of SecYEG complexes. This makes creates a more narrow size distribution across the nanodisc population, which ensures a constant amount of SecYEG per nanodiscs, hence a comparable fluorescent signal from each complex. Additionally, this prevents

the formation of aggregation between complexes. This resulted in smooth MST traces without any peaks or bumps, which indicates that there were little to no aggregates in the solution (fig 11c). This resulted in a reliable binding curve (fig 11d). Multiple experiment were done using this setup. All the experiments resulted in a K_D value in the nanomolar range (~0.5 nM). Previous binding study found values of 0.03 and 0.12 nM for ribosomes and RNCs respectively[30]. This is in the same range as the 0.5 nM from the acquired MST data. Even though the set-up is functional when SecYEG nanodiscs are used, this ignores the possibility that the RNCs have a different binding behavior to proteoliposomes compared to nanodiscs, although they are made out of the same lipid composition. Based on the fact that the proteoliposome set-up creates vesicles containing multiple SecYEG complexes, combined with the high affinity of SecYEG to RNCs, the binding between RNCs and SecYEG can be compromised by the presence of other SecYEG complexes. It is not clear if this poses a problem because, during microscope experiments, SecYEG proteoliposomes will fuse with a supported lipid bilayer where SecYEG can diffuse freely. Such an environment would have more resemblance with the nanodiscs set-up in terms of SecYEG per surface unit. If future microscopy experiments results in data that points towards binding inability due to aggregations or inactive complexes, the MST setup in combination with SecYEG proteoliposomes should be resolved for it to be a reliable tool to determine the activity of the RNCs in combination with these complexes before microscopy experiments are conducted.

Once current bottlenecks are resolved and sufficient data on the interaction between SecYEG and the RNCs is acquired, one could add other known components to reaction between SecYEG and its binding partners to visualize complex formation and acquire single molecule data on the versatile reaction within this system.

MATERIAL AND METHODS

Preparation of competent cells and transformation

Table 3: Strains and plasmids used in this study.

Strain	Characteristics	Source
E. coli DH5α	F- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K-m_K+), λ-	[31]
E. coli SF100	F-, Δ*lacX7*, *galE*, *galK*, *thi*, *rpsL*, *strA* 4, Δ*phoA*(pvuII), Δ*ompT*	[32]
E. coli BL21(DE3)Δtig	F-, *om_pT*, *lon*, *gal*, *dcm*, *hsdS*$_B$(r_B-m_B-)(DE3)Δ*tig*	[33]
E. coli BL21(DE3)	F-, *ompT*, *lon*, *gal*, *dcm*, *hsdS*$_B$(r_B-m_B-)	[34]
E. coli KC6(DE3)ΔSmpBΔSsrA	*Rna-19gdhA2his-95*^{a}*relA1spoT1metB1*, Δ*endAmet*, Δ*tonA*, Δ*speA*, Δ*tnaA*, Δ*sdaA*, Δ*sdaB*, Δ*gshA* (KC6) Δ*SmpB*, Δ*SsrA*	[35]
pEK20-148	SecY(L148C)EG	[36]
pUC19Strep$_3$FtsQSecM	RNC-FtsQ108	[28]
pTrc99A SecA	Wild type SecA	Lab stock

To prepare *E. coli* BL21Δtig competent cells 5 mL sterile LB supplemented with 0.5 % glucose was inoculated 1:100 with an overnight culture of the strain and grown at 37 °C until an OD_{600} ~ 0.6. 1 ml of the overday culture was spun down at 6000 *x g* for 1 min, resuspended in 1 ml 0.1 M ice-cold $CaCl_2$ and incubated on ice for 20 minutes. The cells were harvested by centrifugation (6000 *x g* for 1 min at room temperature (RT)) and resuspended in 100 µl 0.1 M ice-cold $CaCl_2$ and stored on ice.

Transformations were initiated by adding 0.1 – 1 µg of the desired plasmid to 100 µl of competent *E. coli* cells and incubated on ice for 10 minutes. The cells were heat shocked for 1 minute at 42°C, mixed with 900 µl LB and grown for 60 minutes at 37 °C. 50 µl of the cell suspension was plated on a LB plate containing 0.5% glucose and 100 µg/ml ampicillin. The remaining cells were harvested by centrifugation (1 min; 6000 *x g*), resuspended in 200 µl LB and plated. Both plates were grown overnight at 37°C and stored at 4°C the next day.

SecYEG overexpression and crude membrane isolation

SecYEG was overexpressed using pEK20 SecY_{C148}EG transformed into the *E. coli* SF100 expression strain[22]. In this SecYEG mutant the native amino acid on position 148 was replaced by a cysteine which enable specific fluorophore labelling by a maleimide reaction. 100 ml of LB containing 0.5% glucose and 100 µg/ml ampicillin was inoculated with a single colony from the transformation LB plate and grown overnight at 37°C. The next day this culture was 100 times diluted in sterile LB containing 100 µg/ml ampicillin and grown at 37°C until an OD_{600} of 0.6. Protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture was grown for an additional two hours. The cells were harvested by centrifugation (6000 *x g* for 15 min at 4°C) and resuspended in 1 ml 50 mM Tris/HCL, pH 8.0, 20% sucrose. To disrupt the cells, the culture was passed through a continuous cell disrupter at 13000 psi. A protease inhibitor tablet was dissolved in 10 ml 50 mM/Tris/HCl, pH 8.0.

0.5 ml of the protease inhibitor solution was added to the cell suspension after disruption and the disruption cycle was repeated. Whole cells and cell debris were removed by centrifugation (2100 *x g* for 10 min at 4°C and 7650 *x g* for 10 min at 4°C). Crude membranes were collected by centrifugation (185500 *x g* for 60 min at 4°C) and resuspended in 1 ml 50 mM Tris/HCl, pH 7.5, 20% glycerol.

SecYEG purification, labelling and reconstitution into proteoliposomes or nanodiscs

10 mg crude membranes, containing overexpressed SecYEG were solubilized in a buffer containing 50 mM Tris/HCl, pH 7, 100 mM KCl, 20% glycerol, 2% n-Dodecyl β-D-Maltopyranoside (DDM) and 200 µM Tris(2-carboxyethyl) phosphine (TCEP) and incubated for 30 minutes at 4°C. The solubilized protein was mixed with equilibrated 250 µl nickel-NTA beads agarose solution and incubated for 2 hours at 4°C. The protein-bead slurry was transferred to a biospin column and washed with washing buffer (50 mM Tris/HCl, pH 7, 100 mM KCl, 20% glycerol, 0.1% DDM and 10 mM imidazole). The mixture was then incubated overnight at 4°C in the presence of minimally a 5-fold excess of the desired fluorophore. Unbound fluorophores were removed by excessive washing. The labeled SecYEG was incubated with 500 µl elution buffer (50 mM Tris/HCl, pH 7, 100 mM KCl, 20% glycerol, 0.1% DDM and 300 mM imidazole) for 15 minutes at 4°C. The column was washed additional two times with 400µl elution buffer.

The purified labeled SecYEG was either reconstituted into liposomes or nanodiscs. For the liposome set-up the purified labeled SecYEG was mixed with a 4 mg/ml lipid solution containing 50 mM Tris/HCl, pH 8, 50 mM KCl and 0.5% Triton 100x and incubating the mixture for 30 minutes on ice. The detergent was removed sequentially using SM2 bio-beads. The first two portions consists of 20 mg and are each incubated for 90 minutes at 4°C. The final amount of detergent was removed out by adding 40 mg of bio-beads and an overnight incubation at 4°C. The SecYEG proteoliposomes were collected by centrifugation (250000 *x g* for 30 min at 4°C) and resuspended in 100 µl 50 mM Tris/HCl, pH 8, 50 mM KCl and 10% glycerol.

For the nanodisc set-up, a 10 mg/ml lipid solution was sonicated (15 sec on, 15 sec off for 15 cycles at power 7). After sonication, 0.5% Triton-X100 was added to solubilize the lipids. A protein buffer was prepared containing 50 mM HEPES/KOH pH 7.4, 100 mM KCl, 10% glycerol and 0.05% DDM. The purified labeled SecYEG was diluted in the protein buffer. The ApoE422k and the solubilized lipids were added to the protein mixture. This resulted in an internal ratio of 0.25 (SecYEG) : 10 (ApoE422k) : 1800 (lipids). This solution was incubated for 30 minutes at 4°C, while gently agitated. Subsequently, 200 mg bio-beads were added and the mixture was incubated overnight at 4°C, while gently agitated. Aggregates and other unwanted complexes were removed by centrifugation (250000 *x g* for 30 min at 4°C). The nanodiscs were purified from the supernatant using a Biorad FPLC system. The supernatant was concentrated before loading it on a superpose 6 10/300 GL column. The nanodisc solution was run through the column with a flowrate of 0.4 ml/min. A buffer containing 50 mM HEPES/KOH pH 7.4, 100 mM KCl and 5% glycerol was used to elute the labeled SecYEG nanodiscs in 0.4 ml fractions.

SecA overexpression, isolation and purification

SecA was overexpressed using pTrc99ASecA transformed into *E. coli* BL21. 20 ml of LB containing 0.5% glucose and 100 µg/ml ampicillin was inoculated with a single colony from the transformation LB plate and grown overnight at 37°C. The next day this culture was 100 times diluted in sterile LB containing 100 µg/ml ampicillin and grown at 37°C until an OD_{600} of 0.6. SecA overexpression was induced with

0.5 mM IPTG and the culture was grown for an additional two hours. The cells were harvested by centrifugation (6000 *x g* for 15 min at 4°C).

The cell pellet was resuspended in buffer A containing 20 mM HEPES and 10% glycerol. The cells were disrupted by sonication (10on/10off for 10 cycles) with and power amplitude of 8. Cellular debris was removed from the mixture by centrifugation (3000 *x g* for 10 min at 4°C), and membranes were removed by centrifugation (4440000 x *g* for 30 min at 4°C).

SecA was purified using a Biorad FPLC system. For this purification a 5 ml Hi Trap SP HP cation exchange column was used. The cell extract was loaded onto the column where SecA interacts with the available negative ions. SecA was eluted using a gradient of a buffer B, containing 20 mM HEPES, 10% glycerol and 1M NaCl. The purity of the purified SecA was analyzed by SDS-PAGE.

Translocation assay

Each reaction volume of the assay consisted of components: proteoliposomes in buffer and SecA in variable concentrations. Each reaction contained 25 mM Tris/HCL, pH 7.0, 25 mM KCl, 5 mM $MgCl_2$, 0.1 mg/ml BSA, 2 mM DDT, 24 mM creatine phosphate, 0.24 mg/ml creatine kinase, 0.04 mg/ml SecB, 29 µM proOmpA and excess of labeled SecYEG proteoliposomes. Subsequently, a series was made with an increasing concentration of SecA (0 nM, 50 nM, 100 nM, 150 nM, 200 nM and twice 250 nM). The final volume was adjusted to 50 µl with MilliQ. The translocation reaction was initiated by adding 5 mM ATP to the translocation mixture, except one of the two 250 nM SecA reaction, which will function as a negative control. All reactions were incubated at 37°C for 20 min. Meanwhile, new tubes containing a final concentration of 0.9 mg/ml Proteinase K (PK) were placed on ice. After incubation, 40 µl of all reactions was transferred into a PK tube, while the remaining reaction solution functioned as a control series as a reference for the total amount of proOmpA during the reaction. 40 µl of the translocation reaction was incubated for 30 minutes on ice in the presence of PK, after which 150 µl 10% trichloroacetic acid (TCA) was added. After a 30 minute incubation on ice, the proteoliposomes were separated from soluble debris by centrifugation (1000 *x g* for 15 min at 4°C). The supernatant was removed and 500 µl ice-cold acetone was added to the precipitated proteoliposomes. Proteoliposomes were harvested by centrifugation (17000 *x g* for 5 min at 4°C), dried at 37°C and dissolved in 10 µl 2x sample buffer. To verify the activity of the SecYEG proteoliposomes, both the control samples and the PK treated samples were loaded on a 12% SDS-PAGE gel for SDS-PAGE.

Ribosome Nascent chain Complex overexpression, isolation and purification

pUK19strep3FtsQSecM was transformed into *E. coli* BL21(DE3)Δtig. 50 ml sterile LB containing 100 µg/ml ampicillin was inoculated with a single transformant colony and grown overnight at 30°C. The next day, the culture was diluted 100 times in sterile LB containing 100 µg/ml ampicillin and grown at 30°C until an OD_{600} of 0.5 was reached. RNC overexpression was induced with 0.5 mM IPTG and the culture was grown at 30°C for at least 30 minutes. Subsequently, the culture was placed on ice and ice cubes, made from buffer R (50 mM Tris/HCl, pH 7.5, 150 mM KCl and 10 mM $MgCl_2$), were added. The cells were harvested by centrifugation (6000 *x g* for 20 min at 4°C) and the pellet was resuspended in 10 ml ice-cold buffer R. The cells were lysed by adding 1 ml of lysing solution to the cell suspension and one freeze-thaw cycle at -80°C. To decrease the viscosity of the lysed cell suspension, 20 µl DNase was added and the freeze-thaw cycle was repeated. Cellular debris was removed using 2 cycles of centrifugation (30000 *x g* for 30 min at 4°C). The ribosomes were harvested by laying the cleaned cellular content onto a sucrose cushion of minimally 80% during centrifugation (112000 *x g* for 17

hours at 4°C). The translucent ribosome pellet was dissolved in 500 µl cold buffer R by gentle shaking to avoid mechanical stress. The dissolved pellet was loaded onto equilibrated StrepTactin-beads and incubated for 60 minutes at 4°C. After incubation, the column was washed with 1 column volume (CV) ice-cold buffer R, 2 CV of ice-cold buffer R containing 0.5 M KCl and again ice-cold buffer R until an A_{260} lower than 0.01 was achieved. The RNCs were eluted by incubating the beads with 3 column volumes elution buffer (2.5 mM desthiobiotin in buffer R). The concentration was determined spectrophotometrically at 260 nm on 100 times diluted samples.

Semi dry western blot RNC strep-tag detection

A 15% SDS-PAGE gel, containing on average 50 ng total protein in each sample, was run to separate the RNCs by size and equilibrated in Tris-Buffered Saline (25 mM Tris, 192 mM Glycine and 20% methanol). The polyvinylidene fluoride (PVDF) membrane was activated by washing in methanol and Tris-Buffered Saline (TBS). The transfer complex was assembled by stacking 3 filter papers, soaked in TBS, the activated PVDF membrane, the equilibrated SDS-PAGE gel and 3 additional soaked filter papers. Before starting to blot from the gel to the membrane, air bubbles were removed from the complex to ensure proper transfer. The loaded samples were blotted onto the PVDF membrane for 45 minutes at 8 V. After the blotting, the membrane was rinsed in Tris-Buffered Saline containing 0.1% Tween 20 (TBST) before blocking it in blocking solution (TBS containing 5% skimmed milk powder) for 1 hour or overnight at 4°C. After the membrane was blocked, the membrane was washed in TBST. The membrane was incubated with a 1:4000 Strep-Tactin AP conjugate solution for one hour. Subsequently, the membrane was washed twice with TBST for 10 minutes and twice with assay buffer (50 mM Tris/HCl, pH 10, 1 mM $MgCl_2$) for 5 minutes. The membrane was incubated with 4.17 mM of chemiluminescent substrate CDP star for 5 minutes. After incubation the excess liquid was drained from the membrane and the nascent chain was visualized using chemo-luminescence.

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