Structure of the SecY channel during initiation of protein translocation

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Many secretory proteins are targeted by signal sequences to a protein-conducting channel, formed by prokaryotic SecY or eukaryotic Sec61 complexes, and are translocated across the membrane during their synthesis2–4. Crystal structures of the inactive channel show that the SecY subunit of the heterotrimeric complex consists of two halves that form an hourglass-shaped pore with a constriction in the middle of the membrane and a lateral gate that faces the lipid phase7,8. The closed channel has an empty cytoplasmic funnel and an extracellular funnel that is filled with a small helical domain, called the plug. During initiation of translocation, a ribosome–nascent chain complex binds to the SecY (or Sec61) complex, resulting in insertion of the nascent chain. However, the mechanism of channel opening during translocation is unclear. Here we have addressed this question by determining structures of inactive and active ribosome–channel complexes with cryo-electron microscopy. Non-translating ribosome–SecY channel complexes derived from Methanocaldococcus jannaschii or Escherichia coli show the channel in its closed state, and indicate that ribosome binding per se causes only minor changes. The structure of an active E. coli ribosome–channel complex demonstrates that the nascent chain opens the channel, causing mostly rigid body movements of the amino- and carboxy-terminal halves of SecY. In this early translocation intermediate, the polypeptide inserts as a loop into the SecY channel with the hydrophobic signal sequence intercalated into the open lateral gate. The nascent chain also forms a loop on the cytoplasmic surface of SecY rather than entering the channel directly.

Opening of the SecY channel during initiation of translocation involves two events: binding of the ribosome and insertion of the nascent chain. To analyse how ribosome binding per se affects the structure of a translocation channel, we first determined the structure of complexes lacking a nascent chain. Initial experiments were performed with complexes from M. jannaschii, because this allows a direct comparison with a crystal structure of SecY1. Purified M. jannaschii ribosomes were incubated with an excess of SecY complex, and complexes were imaged by cryo-electron microscopy. A total of ~37,000 particles were analysed, resulting in an electron density map with a resolution of 9.0 Å for the ribosome and ~12.7 Å for the channel (Supplementary Table 1).

A ribosome model from Pyrococcus furiosus6, a species related to M. jannaschii, was fit into the density map, allowing the identification of essentially all RNA helices and many helical features of ribosomal proteins (Fig. 1a and Supplementary Fig. 1). A crystal structure of the M. jannaschii SecY complex could be docked into density for the SecY channel (Fig. 1b and Supplementary Fig. 2), and molecular dynamics flexible fitting (MDFF)3 resulted in only small changes (Fig. 1c). All transmembrane segments (TMs), including the 10 TMs of SecY, and the single TMs of the SecE and SecM subunits, could be accounted for in the map. Several TM helices and the extracellular loop between TMs 5 and 6 were partially resolved (Supplementary Fig. 3). A comparison with the crystal structure shows that, with the exception of some adjustments in the cytoplasmic helix of SecE, membrane-embedded domains remained essentially unaltered (Fig. 1c). As observed previously with other species3–5, loops between TMs 6 and 7 (6/7 loop) and TMs 8 and 9 (8/9 loop) of SecY, as well as the cytoplasmic helix of SecE (Fig. 1b), interact with components of the large ribosomal subunit at the tunnel exit (Supplementary Fig. 4a–c). These interactions do not induce major structural changes in the SecY channel and leave the lateral gate closed.

Next we determined the structure of a non-translating ribosome–channel complex from E. coli, with a larger data set than used previously4. A total of ~39,000 particles were analysed, resulting in a density map with a resolution of ~9.5 Å for the ribosome and ~14 Å for the channel (Supplementary Table 1). Models for ribosomal subunits4,13 were docked into the density map (Fig. 1d) and all RNA helices were visible, as well as some partially resolved helices of ribosomal proteins (Supplementary Fig. 5). Because there is no crystal structure of the E. coli SecY complex, we generated a homology model on the basis of crystal structures of Thermus thermophilus and Thermotoga maritima complexes6,7 (Supplementary Figs 6 and 7). This model was subjected to MDFF using the entire density map of the ribosomal large subunit and channel as a restraint. This resulted in movements of cytoplasmic loops, whereas membrane-embedded domains remained essentially unchanged (Supplementary Fig. 8). Many features of the channel are clearly visible in a segmented map (Fig. 1e and Supplementary Figs 9 and 10), including cytoplasmic loops of SecY, two helices of SecE, two TMs of SecG (the bacterial equivalent of archaeal SecB) and some partially resolved TMs of SecY. Connections between the channel and ribosome were similar to those in the M. jannaschii complex, with the exception of the longer 6/7 loop of SecY, which is repositioned between RNA helices 6 and 7 (Supplementary Fig. 4d–f). Importantly, the ribosome alone does not induce major changes in the channel structure, so the lateral gate remains closed (Fig. 1f).

To determine the structure of an active E. coli ribosome–channel complex, we used a new strategy. Previous attempts to obtain a structure of an active translocation channel showed that a translating ribosome was bound to the channel, but there was little biochemical evidence that a nascent chain was inserted in the channel and no clear electron density was visible for the polypeptide10,11. These studies used small amounts of ribosome–nascent chain complexes (RNCs) that were formed in vitro and subsequently added to purified channels. To obtain a more physiological sample, we generated an early translocation intermediate of a secretory protein in living E. coli cells by expressing a polypeptide with 100 amino acids from an inducible promoter14,15. The polypeptide has an N-terminal signal sequence derived from DsbA, which targets the protein to the co-translational translocation pathway16, and a C-terminal SecM-stalling sequence, which arrests translation of the ribosome17 (Fig. 2a). We also expressed the endoribonuclease MazF from an inducible promoter to cleave messenger RNA between ribosomes, which results in the depletion of nascent chains associated with non-stalled ribosomes18. To generate a stable complex between the SecM-stalled ribosome–channel complex, we used a new strategy. Previous attempts to obtain a structure of an active translocation channel showed that a translating ribosome was bound to the channel, but there was little biochemical evidence that a nascent chain was inserted in the channel and no clear electron density was visible for the polypeptide10,11. These studies used small amounts of ribosome–nascent chain complexes (RNCs) that were formed in vitro and subsequently added to purified channels. To obtain a more physiological sample, we generated an early translocation intermediate of a secretory protein in living E. coli cells by expressing a polypeptide with 100 amino acids from an inducible promoter14,15. The polypeptide has an N-terminal signal sequence derived from DsbA, which targets the protein to the co-translational translocation pathway16, and a C-terminal SecM-stalling sequence, which arrests translation of the ribosome17 (Fig. 2a). We also expressed the endoribonuclease MazF from an inducible promoter to cleave messenger RNA between ribosomes, which results in the depletion of nascent chains associated with non-stalled ribosomes18. To generate a stable complex between the SecM-stalled
RNC and the channel, we used disulphide crosslinking. The nascent chain contained a cysteine at position 19 of the signal sequence, which can be crosslinked to a cysteine at position 68 in the SecY plug. Disulphide bond formation was achieved by adding an oxidant to the E. coli culture, resulting in 70% of nascent chains being linked to SecY.

To purify the RNC–channel complex, we replaced the endogenous ribosomal protein L12 with a Strep-tagged version, allowing the enrichment of ribosomes on a Strep-Tactin column. This purification step was performed at high salt concentration to remove SecY complexes lacking a nascent chain (Supplementary Fig. 11a). A second purification step exploited a His-tag inserted into a fusion between SecE and SecG, and allowed the enrichment of channel-containing complexes by Co2+-affinity chromatography. Finally, the sample was subjected to gel filtration. The purified RNC–channel complex eluted as a homogeneous peak at the position of monosomes (Supplementary Fig. 11b).

On a Coomassie-stained SDS gel, the SecY–nascent chain–transfer RNA species was the only major band besides those from ribosomal proteins (Fig. 2b, lane 1). As expected, the band disappeared when the sample was treated with a reducing agent to remove the disulphide bridge or with RNase A to degrade the tRNA (Fig. 2b, lanes 2 and 3). We found that the previous protocol of adding purified RNCs to SecY complex, either in detergent or in nanodiscs, resulted in inefficient insertion of the nascent chain into the channel (Supplementary Fig. 12). Also, when RNC–channel complexes were generated in vivo and crosslinked after purification, crosslinks between different nascent chain molecules and between the nascent chain and unidentified proteins were observed (Supplementary Fig. 13). Hence, crosslinking in vivo is required to maintain the nascent chain in the channel.

Purified RNC–channel complexes were frozen over holes on electron microscopy grids, as the channel was lost when complexes were placed on a carbon film. A total of ∼167,000 individual particles were used, of which ∼50% contained the channel. Additional sorting for the best signal-to-noise ratio identified ∼53,000 particles for structure determination and resulted in a density map at ∼10 Å resolution for the ribosome and ∼11 Å for the channel (Fig. 3a and Supplementary Table 1).

Ribosomal RNAs and proteins were clearly visible in the density map (Supplementary Fig. 14), along with aminoacyl (A-site) and peptidyl (P-site) tRNAs, as expected for a SecM-stalled ribosome (Supplementary Fig. 15a). Moreover, there was density for mRNA underneath the anticodon regions of tRNAs (Supplementary Fig. 15b). We

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**Figure 1 | Structures of non-translating ribosome–channel complexes.**

a. Density map for the M. jannaschii complex. Models for ribosomal RNA and proteins of the small and large ribosomal subunits (ssu and lsu; in gold and blue, respectively) and of the SecY complex (in red) were docked into the map. b. Fit of the M. jannaschii SecY complex into the segmented density map, as viewed from the cytoplasm (top view) and from the side. The N- and C-terminal halves of SecY are in light blue and red, respectively. SecE is in dark blue and Secβ in brown. c. Comparison between the crystal structure of an M. jannaschii SecY complex (grey) and the electron microscopy structure (in colour), as viewed facing the lateral gate (front view). d, e. As in a and b, but for the E. coli complex. SecG, the bacterial equivalent of Secβ, is in brown. f. A model for the E. coli channel in a front view.

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**Figure 2 | Purification of a RNC–channel complex.** a. The complex was generated in living E. coli cells by expressing a nascent chain (NC) of 100 amino acids with a signal sequence and SecM-stalling sequence. The nascent chain also contains a Myc-tag. A cysteine at position 19 of the nascent chain (19C) was disulphide-crosslinked to a cysteine in the plug of SecY (68C). Disulphide bond formation was achieved by adding an oxidant to the sample. The red arrow indicates the crosslinked product of SecY and the NC–tRNA adduct. This band disappears after treatment with β-mercaptoethanol (β-ME) or RNaseA (lanes 2 and 3). Ribosomal proteins (including S1) and the fusion between SecE and SecG are indicated.
also observed density for ribosomal protein S1 that was more extensive than seen before20 (Fig. 3a and Supplementary Fig. 15c–e).

To generate a model for the active channel, we created an *E. coli* homology model on the basis of a crystal structure of the SecY complex from *P. furiosus*5, which has the most open lateral gate among known crystal structures (Supplementary Fig. 16), and used MDFF to adjust the model to the experimental density map. The 6/7 loop and TM9 of SecY were well resolved (Fig. 3b), and ribosomal components interacting with the channel were the same as with the non-translating complex. The cytoplasmic helix of SecE and TM10 of SecY were clearly visible, and there was good density for SecG (Supplementary Figs 17 and 18). In addition, many TMs were partially resolved, with only occasional density breaks in the helices. Density for the nascent chain was clearly identifiable without segmentation of the density map. Specifically, additional density for a helix was visible in the cytoplasmic part of the lateral gate (see below), explaining why a channel with a fully open lateral gate could be fit into the density map. In fact, the lateral gate is more open than in the *P. furiosus* crystal structure1 (Supplementary Table 2). Calculated cross correlation coefficients showed that the model for the open SecY channel is a better fit in the density map than the model for the closed channel (Supplementary Table 1).

The modelled conformational change of the *E. coli* channel is supported by the fact that the conversion from a closed to an open channel involves mostly rigid body movements of the N- and C-terminal halves of SecY (Supplementary Fig. 19). To open the lateral gate, the N-terminal half of SecY undergoes a large rotation and tilt, whereas the C-terminal half moves less in the opposite direction (Fig. 3c; see also Supplementary Video 1). SecE undergoes a tilting motion to accommodate movements of SecY, and SecG moves with the N-terminal half of SecY. These conformational changes would maintain the hydrophobic belt of the SecY complex within the lipid environment. In addition to rigid body movements, there are changes in the 5/6 loop that connect the two halves of SecY to accommodate the large opening motion. There are also movements in TM8 and the lower part of TM7. One particularly large change occurs in the upper part of TM8 (helix 8b), which is displaced towards the membrane surface (Fig. 3d). The 6/7 loop and TM9, as well as preceding loop residues, including a conserved arginine (Arg 357), do not move appreciably (Fig. 3d), consistent with their role in tethering the channel to the ribosome. The plug domain moves only a small distance, probably because it is restrained by the disulphide bridge to the signal sequence. However, the plug does not have to move much to allow translocation21. When viewed from the cytoplasmic side, these conformational changes open a pore adjacent to the lateral gate (Fig. 3e; see also Supplementary Video 2). Overall, the changes are more pronounced than seen previously10,11.

Density for the nascent chain was seen inside the ribosomal tunnel, on the cytoplasmic surface of the SecY complex, inside the channel, and on its periplasmic side (Fig. 4a and Supplementary Fig. 20). On the

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**Figure 3 | Structure of the active SecY channel.** a, Structure of the *E. coli* RNC–SecY channel complex, with large and small ribosomal subunits in blue and gold, respectively, the SecY complex in red, and ribosomal protein S1 in tan. b, Front (left) and side (right) views of the channel fit into the segmented density map (grey). The nascent chain was omitted for clarity. The N-terminal half of SecY is in light blue, the C-terminal half in red, SecE in dark blue and SecG in brown. c, Comparison of front views of the closed (left) and open (right) *E. coli* SecY channels, with the approximate position of the membrane indicated by solid horizontal lines. The N-terminal half of SecY is in light blue, the C-terminal half in red, SecE in dark blue, SecG in brown, and the plug in yellow. Some movements during channel opening are indicated, such as the rotation and tilting of the N-terminal half of SecY, the tilting of SecE, and the movement of helix 8b. Labels for helices 2b and 7 are placed at the same position in the closed and open channel. Pore residues forming the constriction in the closed channel are indicated with grey balls and sticks. d, Connections of the ribosome with the 8/9 loop of SecY and the cytoplasmic helix of SecE in the closed and open channels (top and bottom panels, respectively). Note the large movement of helix 8b towards the membrane. e, As in c, but viewed from the top.
basis of biochemical data, an approximate model for the nascent chain in the RNC–channel complex was built into the density. The last ~40 amino acids are located inside the ribosome, as cysteines introduced into this segment are inaccessible to a bulky modification reagent. In addition, cysteines at positions 19–34 are most favourably to form a disulphide bridge with a cysteine in the plug. Finally, the position of the end of the signal sequence in our structure is constrained by the disulphide crosslink between position 19 of the nascent chain and position 68 of the plug.

The resulting model shows that the hydrophobic core of the signal sequence forms a helix in the lateral gate (residues 1–15) (Fig. 4b–d and Supplementary Fig. 21), consistent with crosslinking data obtained with the yeast Sec61 complex. The signal sequence helix is contacted by TM2b, helix 8b and TM7 of SecY (Fig. 4b). In a lipid bilayer, much of the signal sequence, including parts that follow the hydrophobic region, would be exposed to the hydrocarbon chains of phospholipids, again in agreement with crosslinking experiments. Additional density below and adjacent to the signal sequence helix can account for the other side of the nascent chain loop. The pore through which the mature region of the nascent chain would move into the extracellular funnel is not exactly in the centre of the channel, but the translocating polypeptide may still be surrounded by pore ring residues that form a constriction in the closed channel (Supplementary Video 2). Crosslinking to the nascent chain may restrain the plug, keeping it in the centre of the channel. However, there is still room for the nascent chain to form a loop in the pore.

We modelled density on the cytoplasmic surface of the channel as a loop that extends parallel to the surface and towards the back of the channel (residues ~45–63) (Fig. 4a, e). This part of the nascent chain lies in a V-shaped groove, which is framed by the base of the 6/7 loop and TM10 of SecY (Supplementary Fig. 22 and Supplementary Video 3). However, the nascent chain may adopt an alternative orientation with a loop that extends above the lateral gate (marked with an asterisk in Fig. 4a, d). The nascent chain may also slide up and down the axis of the channel to some extent, as there is density on the periplasmic side that is not fully accounted for in our model.

In summary, our structures show that ribosome binding alone does not induce major changes in the SecY channel, although it may cause transient opening. Rather, stable opening of the channel requires loop insertion of the nascent chain. As predicted, the hydrophobic part of the signal sequence forms a helix that occupies the open lateral gate. The signal sequence would thus become part of the channel wall, thereby increasing the size of the pore through which the polypeptide moves across the membrane. At later stages of translocation, the signal sequence is cleaved from the nascent chain and released from the lateral gate, which may result in a narrower pore. It is also possible that the signal sequence leaves the lateral gate before cleavage. This hypothesis would be consistent with a two-dimensional crystal structure of the SecY complex that showed a synthetic signal peptide bound to the outside of an essentially closed channel.

Our results also indicate that most nascent chains form a loop on the cytoplasmic surface of SecY, rather than adopting a fully extended conformation between the ribosome and channel. Although the observed looping of the nascent chain at the cytoplasmic surface of the channel needs to be confirmed with other substrates, it seems possible that a pulling force or ratcheting mechanism may be required to achieve efficient translocation. SecDF could use a proton gradient across the membrane together with movements of a periplasmic domain to pull on the nascent chain. In addition, polypeptide chain folding or the binding of periplasmic chaperones may help to move the polypeptide chain across the membrane.

**METHODS SUMMARY**

The purification of E. coli 70S ribosomes and M. jannaschii E. coli SecY complexes were each described previously. M. jannaschii 70S ribosomes were purified by sucrose gradient centrifugation, dissociated into 50S and 30S subunits, and re-associated. Non-translocating ribosome–SecY complexes were reconstituted by mixing ribosomes with a five- to eightfold molar excess of the SecY channels in n-dodecyl-β-D-maltoside (DDM). An RNC–SecY complex was generated in E. coli cells by expressing a SecM-stalled nascent chain under the arabinose promoter. In addition, MazF endoribonuclease was expressed from a Tetr promoter. After forming a disulphide bridge between the nascent chain and SecY by addition of 5,5′-dithiobis-(2-nitrobenzoic acid), RNC–SecY complexes were solubilized in DDM, and purified by tandem affinity chromatography using a Strep-tag on the ribosomal protein L12 and a His-tag on a fusion of SecE and SecG. Complexes were further purified by size-exclusion chromatography on Superose 6. Samples for cryo-electron microscopy were applied to holey grids or to grids with a continuous carbon film and vitrified. Images were collected at 160 and 200 kV on a Tecnai FEG 20 (FEI) with nominal magnifications of ×40,000 and ×52,000, using a TVIPS 4096 × 4096 charge-coupled device or film. Image processing and single-particle analysis were done with EMAN software. Molecular docking was carried out with Chimera and MDFF.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Information Electron density maps have been submitted to the Electron Microscopy Data Bank (http://www.emdbdatabank.org/) under accession numbers EMD-5691, EMD-5692 and EMD-5693, and modelled structures to the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) under accession numbers 3J44, 3J45, 3J46 and 1VVK. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to T.A.R. (Tom_Rapoport@hms.harvard.edu) or C.W.A. (cakey@bu.edu).
Purification of disulphide-crosslinked E. coli RNC–SecY complexes. EP72 (Aromf DompT rpl-rpl-strep::aadA SecY::hph PACYC-EgH/68c) cells harbouring pBAD(MazF)-NC100 were grown to logarithmic phase in a medium containing 5 g l−1 tryptophan, 2.5 g l−1 yeast extract, 10 g l−1 casamino acids and 5 g l−1 NaCl. The expression of the nascent chain was induced by addition of 0.06% arabinose for 2 h at 37 °C, followed by E. coli MazF induction with 10 ng ml−1 anhydrotracrine for 30 min at 30 °C. Disulphide crosslinking between NC100(19C) and SecY(68c) was then induced by addition of 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to the culture medium for 20 min. DTNB facilitates disulphide-bond formation between SecY and the nascent chain as efficiently as Cu-phenanthroline (Coomassie Brilliant Blue G250; CBB; CuPh). The cells were pelleted, washed once with buffer containing 50 mM Tris–HCl, pH 7.2, 5 mM Mg(OAc)2, 150 mM KCl, and frozen. RNC–SecY complexes were purified as follows. The cells were re-suspended in buffer containing 50 mM Tris-acetate, pH 7.2, 25 mM Mg(OAc)2, 0.3 M NH4Cl and homogenized with a French press. One per cent n-dodecyl-β-D-maltoside (DDM) was added to the cell lysate for 1 h to solubilize membranes. After centrifugation (SS-34 rotor, 13,000 r.p.m., 30 min), ribosomes containing Strep-tagged L12 were purified by applying the lysate to a Strep-Tactin Sepharose column (IBA). The column was washed with 8 column volumes (CV) of buffer containing 50 mM Tris-acetate, pH 7.2, 25 mM Mg(OAc)2, 0.4 M NH4Cl, 0.03% DDM, and then with 2 CV of buffer (TMP750) containing 50 mM Tris-acetate, pH 7.2, 25 mM Mg(OAc)2, 0.2 M KOAc, 0.03% DDM. Ribosomes were eluted from the column with 4 CV of buffer containing 4 mM desthiobiotin. To enrich for channel-bound RNCs containing His-tagged SecE–SecG fusion protein, the eluate was incubated with Dynal-Talon beads (Invitrogen) for 30 min. The beads were washed three times with TMP200 buffer, and bound complexes were eluted with TMP200 buffer containing 120 mM imidazole. The complexes were further purified by gel filtration on a Superose 6 column (GE Healthcare) equilibrated with buffer containing 50 mM Tris–acetate, pH 7.2, 10 mM Mg(OAc)2, 80 mM KOAc, 0.03% DDM. Monomeric ribosome fractions were collected and concentrated to 8–9 mg ml−1.

Test for in vitro reconstitution of the RNC–SecY complex. For the experiments shown in Supplementary Fig. 12, RNCs containing the DsbA108His- or NC100 nascent chain were isolated as follows. pBAD-DsbA108His(19C) or pBAD-NC100(19C) was transformed into Aromf DompT cells (EP51) harbouring the pBAD2plasmid. Cells were grown to log phase in 2 × YT medium (16 g l−1 tryptophan, 10 g l−1 yeast extract and 5 g l−1 NaCl) supplemented with 100 μg ml−1 ampicillin and 40 μg ml−1 chloramphenicol. Nascent chain expression was induced by addition of 0.4% arabinose for 3 h. The cells were re-suspended in buffer (TMA750) containing 50 mM Tris-acetate, pH 7.2, 25 mM Mg(OAc)2, 0.75 M NH4Cl and 1.5 mM DTT and homogenized in a French press. To solubilize the membranes, 1% DDM was added to the cell extract. The extract was cleared by centrifugation at 13,000 r.p.m. for 1 h. The ribosomes were sedimented through a sucrose cushion (TMA750, 30% sucrose, 0.03% DDM) and re-suspended in TMA750. The buffer was exchanged on a TMA750 Superose 6 column (GE Healthcare) to buffer Tris–acetate, pH 7.2, 25 mM Mg(OAc)2, 0.1 M KOAc. To purify RNCs containing monosomes, the ribosomes (OD260nm = 500–1,000) in TMA750 were briefly incubated with 20 μg ml−1 RNase A at room temperature (23 °C) and immediately injected into a Superose 6 gel-filtration column (GE Healthcare) equilibrated with TMP100 containing 50 mM Tris–acetate, pH 7.2, 25 mM Mg(OAc)2, and 100 mM KOAc. Fractions containing monomeric ribosomes were collected. DsbA108His- or NC100-containing RNCs (0.27 μM total ribosomes) were mixed with a 15-fold excess (4.1 μM) of the SecY(68c) complex in TMP100 containing 0.03% DDM. When SecY–nanodiscs were used instead of SecY–detergent complexes, 0.138 μM of RNCs were mixed with a fivefold (0.7 μM) excess of SecY–nanodisc. The three buffer lacking detergent. After incubation at 4 °C for 1 h or at 37 °C for 30 min, the ribosome–detergent complexes were induced by addition of 0.1 mM CuPh2, for 20 min at room temperature. The reaction was stopped by addition of 20 mM N-ethyl maleimide for 30 min at 4 °C. The samples were subjected to non-reducing SDS–PAGE and analysed by immunoblotting with Myc and SecY antibodies.

Nanodiscs containing SecY(68c) complex were generated as previously described12 using the scaffold protein MSP1D1 (ref. 33). In brief, SecY(68c) complexes, MSP1D1 and deoxyBigChap-solubilized E. coli polar lipid (Avanti Polar Lipids) were mixed in a molar ratio of 1:4:100 in 50 mM Tris-acetate, pH 7.2, 150 mM KOAc. After removal of the detergent with Biobeads (Bio-Rad), the sample was injected into a Superdex 200 column equilibrated with buffer TMP100. Fractions containing the SecY–nanodisc complex were pooled and concentrated with an Amicon Ultra device (100-kDa cut-off).

SDS–PAGE and immunoblotting. SDS–PAGE was performed using 4–12% Bis-Tris gels (Bio-Rad) with either MES-SDS or MOPS-SDS running buffer (Invitrogen). Images of immunoblots were recorded with a charge-coupled device (CCD)-based device (Fujifilm LAS-3000) and a standard ECL reagent. Antibodies against the
were aligned in Chimera and averaged to give the final three-dimensional map. (two from each structure path calculated with different refinement parameters) (EMDB code, 5036) scaled to 2.12 Å per pixel. After convergence, the four best maps, electron microscopy and imaged at 3
refinements carried out with different parameters and estimated resolutions of 9.2–9.5 Å (based on half-data set comparisons) were aligned in Chimera and averaged to obtain a final three-dimensional density map.

Non-programmed \textit{E. coli} ribosome–channel complexes were prepared for cryo-electron microscopy and imaged at \(\times 50,000\) with a Gatan (626-DH) cold holder at 200 kV, as described previously. After identifying and removing complexes without channels, \(\sim 90,000\) particles were processed with EMAN1 (ref. 35) at a pixel size of 2.73 Å (for details see ref. 8). Aliquots of \textit{E. coli} RNCs with SecYEG (OD\(_{\text{abs}}\) = 120–160 in \(-0.06–0.1\%\) DDM) were thawed and kept on ice. Samples were applied to 300 mesh Cu grids with a holey support film (Quantafold 2/1) for imaging at \(\times 42,000\) and 400 mesh grids (Quantafold 1.2/1.3) for imaging at \(\times 50,000\). The holey grids had a very thin layer of carbon freshly applied by evaporation and were air-dried discharged before use. A Vitrobot or a manual plunger was used to plunge-freeze grids after blotting into liquid ethane, with the chamber at room temperature and a relative humidity of \(-95\%\). Samples were loaded onto an Oxford cold holder and images obtained at 160 kV on a 4096 \times 4096 CCD (TVIPS) with a semi-automated, single-particle collection program in EMTools (TVIPS) on a TF-20. Particle images were selected using e2boxer and further processed with EMAN2 (ref. 29).

The CTF correction was based on all particles from each CCD frame (\(-450,000\) from \(-3500\) frames), including RNC–channel complexes that formed aggregates, after scaling data collected at \(\times 50,000\) to 2.12 Å per pixel. Subsequently, multiple cycles of reference free classification in EMAN2 were used to extract \(-167,000\) single particles without close nearest neighbours for final processing. A ribosome at 25 Å resolution, with and without the channel, was used as a starting model. The program e2freemulti.py was used to separate the data set into two groups, which were refined separately to a resolution of \(-11–12\) Å. A final supervised classification with e2freemulti.py at an angular step size appropriate for 14 Å resolution was then carried out with the full data set, using three-dimensional references with and without the channel filtered to 14 Å. This step used the Fourier ring correlation comparator and provided an improved separation of the data set. At this stage \(-83,000\) particles with channels from the supervised classification were sorted further with e2ligandclassify.py, on the basis of their signal-to-noise ratio, to give a final data set of \(-53,000\) particles. Two separate structure refinements were then done, starting with either the best three-dimensional reference from the original low-resolution ribosome model or using a 6.8 Å resolution \textit{E. coli} ribosome map (EMDB code, 5036) scaled to 2.12 Å per pixel. After convergence, the four best maps (two from each structure path calculated with different refinement parameters) were aligned in Chimera and averaged to give the final three-dimensional map.

**Molecular modelling and docking.** Maps from \textit{M. jannaschii} and active \textit{E. coli} ribosome–channel complexes were subjected to a local normalization in EMAN2 to allow densities for ribosomal proteins, RNA, channel and miscelle to be displayed and analysed using a single density cut-off. Maps were segmented with Chimera using Zone and difference map options (\textit{vop subtract})\(^8\). Small and large ribosomal subunit models were fit into the ribosome–channel density maps using Chimera fit in map option\(^30\) and MDFF\(^1\) with runs of 500,000 steps (0.5 ns). Because no model was available for the \textit{M. jannaschii} ribosome, we used a model of the related complex from \textit{P. furiosus} (ref. 6, PDB ID, 3J01; and 3J21). Extra copies of ribosomal proteins and ribosomal RNA loops from the \textit{P. furiosus} model that are absent in \textit{M. jannaschii} were omitted. For \textit{E. coli} ribosome–channel complexes, a nearly complete model of the large ribosomal subunit based on electron microscopy modelling and a crystal structure (ref. 11, PDB ID: 3J01; ref. 12, PDB ID: 212T) were used, along with a crystal structure of the small subunit (ref. 12, PDB ID: 212P). Models for tRNAs and mRNA were obtained from a crystal structure of a programmed \textit{T. thermophilus} ribosome (ref. 36, PDB ID: 318G).

The global resolution in experimental density maps was determined separately for the ribosome and channel in each structure using Fourier shell correlation (FSC) in EMAN2, with reference maps calculated from Protein Data Bank files of docked models. Reference maps were calculated with pdb2mrc in EMAN at 7 Å resolution and aligned in Chimera to the appropriate experimental map, then saved with vop resample onGrid. Experimental maps of ribosomes, as part of their cognate ribosome–channel complex, had a soft mask applied after calculation in EMAN2. Density maps for channels were created by segmentation in Chimera which also effectively created a mask. However, no masks were created for reference maps to prevent spurious correlations between similar masks in the FSC calculations between the two volumes being compared. The 0.5 criterion was used in all cases to identify the resolution.

Models for closed and open \textit{E. coli} SecYEG channels were constructed as follows. SecY in the closed channel was based on individual structural elements (helices and turns) from the crystal structure of \textit{T. thermophilus} SecY\(^1\). These segments were docked onto the closed crystal structure of SecY from \textit{M. jannaschii} in Chimera, on the basis of sequence alignments between the three organisms. Loops were then regularized and additional residues added as needed in Coot\(^7\), SecE and SecG subunits were taken from the crystal structure of \textit{T. maritima} SecYEG\(^1\). The structural model was then mutated to \textit{E. coli} sequences, energy minimized with NAMD\(^3\) and fit into the map with Chimera\(^3\) and MDFF.\(^1\) A model for the open \textit{E. coli} channel was constructed in a similar way, on the basis of a crystal structure of a partially open SecYEG channel from \textit{P. furiosus}. SecYEG models were positioned initially in the maps by docking the 6/7 and 8/9 loops into their density with Rosetta\(^2\). All MDFF runs with these components were done with segmented maps that contained the large ribosomal subunit and complete density for the channel and miscelle. Models for the large subunit and SecY channel were minimized together. Importantly, the model of a partially open channel moved into correct density, to reveal the signal sequence helix and associated density for the nascent chain. Finally, no density was observed for the first two TMs of \textit{E. coli} SecY, which are connected by an extended linker to the surface helix of this subunit, and thus may be flexible.

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