Supporting Information

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SI Materials and Methods

Purification of the Holo-Translocon. Freshly transformed *Escherichia coli* C43(DE3) containing pACEMBL:holo-translocon (HTL) were grown in 2×YT broth with antibiotics to an OD_{600} of 0.8, before 3 h of induction using 1 mM isopropyl β-D-1-thiogalactopyranoside and 0.2% (wt/vol) arabinose. Following centrifugation, cell pellets were broken at 25 kpsi using a cell disruptor (Constant Systems, Ltd.) in TSG_{130} buffer [20 mM Tris·Cl (pH 8.0), 130 mM NaCl, and 10% (vol/vol) glycerol]. Membranes were collected and solubilized by rotation in TSG_{130} buffer containing 2% (wt/vol) *n*-dodecyl-β-D-maltoside (DDM) for 1 h at 4 °C. The DDM-soluble fraction was clarified by further centrifugation and applied to a Chelating Ni^{2+}-Sepharose Fast Flow column (GE Healthcare) pre-equilibrated with TSG_{130} + 0.1% DDM. The resin bed was washed with 10 column volumes of buffer containing 30 mM imidazole before elution with the same buffer containing 500 mM imidazole. Peak fractions were loaded onto a Superdex 200, 26/60 gel filtration column (GE Healthcare) placed in-line with a Q-Sepharose ion exchange column equilibrated in TSG_{130} + 0.05% DDM. A well-defined A_{280} peak eluted at ∼190 mL and was concentrated in a 50-kDa-molecular-weight cutoff centrifugation filter (Amicon) to ∼10 mg/mL, using an experimentally determined molar extinction coefficient of ε_{HTL} = 497,000 $M^{-1}{\cdot}cm^{-1}$.

Coreconstitution of Bacteriorhodopsin with Translocation Machinery. Bacteriorhodopsin (BR) membranes were purified by standard methods (1) and solubilized with 2% Triton X-100 for 72 h at 21 °C. Proteoliposomes were made by combining BR (0.22 mg/mL = 8.9 mM) together with either SecYEG (0.22 mM, dimer) or HTL (0.22 mM) and *E. coli* total polar lipids: hen egg PC (3:1 ratio; 2.9 mg/mL) in the presence of 0.25% Triton X-100 in liposome buffer (20 mM Tris·Cl, 50 mM KCl, and 2 mM $MgCl_2$) at 23 °C in total darkness. The vesicles were then reconstituted by the removal of detergent via adsorption to polystyrene Bio-beads SM-2 Adsorbent (Bio-Rad), pelleted by ultracentrifugation, and reconstituted in liposome buffer together with purified BR (90 μM), SecYEG (2.3 μM dimers), or the HTL complex (2.3 μM).

Generation of Proton-Motive Force in Proteoliposomes. For in vitro generation of a proton-motive force (PMF) in proteoliposomes, BR from *Halobacterium halobium* purple membranes was coreconstituted together with the HTL or SecYEG. Purple membranes were purified by standard methods (1). Protein translocation was then established into the vesicles by standard methods (2) for 15 min at room temperature, either in the dark or 10 cm away from a saturating light source from a Kodak slide projector fitted with a yellow filter for the generation of a PMF. Carbonyl cyanide *m*-chlorophenyl hydrazine was used at a final concentration of 50 μM to collapse the PMF.

Negative-Stain EM and Image Processing. Detergent-solubilized, purified HTL complexes were subjected to GraFix (3) and subsequently analyzed by negative-stain EM. The complexes were absorbed onto carbon film for 30 s, followed by negative staining with 2% (wt/vol) uranyl acetate for 30 s. One hundred fifty micrographs of the complex were recorded under low-dose conditions with a bottom-mounted Orios SC600 camera (Gatan Inc.) in a Jeol 1200EX II transmission electron microscope running at 100 kV at a magnification of 40,000× (pixel size of 1.7 Å). A total of 8,000 individual HTL complexes were selected with Boxer (EMAN) (4) from the micrographs and processed with IMAGIC-5 (Image Science) (5). After a first "reference-free" alignment procedure, the particles were iteratively subjected to multivariate statistical analysis and classification. Selected 2D class averages were used as reference images for the subsequent rounds of alignment resulting in 300 2D class averages (6). Characteristic class averages are shown in Fig. S2.

SecA ATPase Stimulation. The data in the absence of cardiolipin (CL) were fitted to the one-site weak binding equation (Eq. **S1**):

$$v = \frac{B_{max} \cdot [L]}{K_d + [L]}, \quad \textbf{[S1]}$$

where $[L]$ is the total concentration of ligand (SecYEG or HTL), v is the enzyme velocity, B_{max} is the total capacity of SecA-ligand, and K_d is the dissociation constant. Data recorded in the presence of CL were fitted to a one-site tight binding equation with *Background* (basal activity in the absence of SecYEG or HTL) (Eq. **S2**):

$$v = B_{max} \cdot \frac{[L] + [E_0] + K_d - \sqrt{([L] + [E_0] + K_d)^2 - 4[E_0][L]}}{2[E_0]} + Background, \quad \textbf{[S2]}$$

where v is equal to enzyme velocity, B_{max} is the total capacity of SecA-ligand, $[L]$ is the total ligand (i.e., SecYEG or HTL) concentration, $[E_o]$ is the total SecA concentration, and K_d is the dissociation constant for SecA-ligand.

Affinity Measurements of SecA to Either SecYEG or HTL by Quenching of an Extrinsic Fluorescent Probe on SecA. SecYEG data measured in the absence of CL were fitted to the one-site weak binding equation (Eq. **S3**):

$$F = \frac{F_{max} \cdot [L]}{K_d + [L]}, \quad \textbf{[S3]}$$

where $[L]$ is the total concentration of ligand (SecYEG or HTL), F is the fluorescence change, F_{max} is the maximum fluorescence quench, and K_d is the dissociation constant. HTL data recorded in the absence of CL were fitted to the one-site weak binding equation with a linear component (m) (Eq. **S4**):

$$F = \frac{F_{max} \cdot [L]}{(K_d + [L]) + m \cdot [L]}. \quad \textbf{[S4]}$$

HTL and YEG data in the presence of CL were fitted to a one-site tight binding equation with linear phase (Eq. **S5**):

$$F = F_{max} \cdot \frac{[L] + [E_0] + K_d - \sqrt{([L] + [E_0] + K_d)^2 - 4 \cdot [E_0] \cdot [L]}}{2 \cdot [E_0]} + m \cdot [L], \quad \textbf{[S5]}$$

where F is the fluorescence change, $[L]$ is the concentration of ligand, $[E_o]$ is the total SecA concentration, K_d is the dissociation constant, m is the linear component, and F_{max} is the maximum signal change.

Cy3 Labeling of HTL and SecYEG. Membranes containing overexpressed cysteine mutants of SecYEG and HTL were incubated with 2 mM Tris(2-carboxyethyl)phosphine for 15 min on ice. Subsequently, Cy3 maleimide (Lumiprobe) was added at a concentration of 1 mM and incubated for 1 h at 4 °C in the dark. The reaction was quenched by adding 10 mM reduced glutathione. HTL and SecYEG were then purified as before.

Trypsin Proteolysis. SecYEG and HTL proteoliposomes were subjected to proteolysis using trypsin (15 μg/μL) at a ratio of 2:1. The reactions were incubated at room temperature for 20 min, solubilized in LDS sample buffer, and subsequently run on SDS/PAGE followed by Coomassie blue staining.

Ribosome Binding Assays. Binding of purified SecYEG, HTL, YidC, SecDF or DF–YajC–YidC to ribosome nascent chain complexes displaying the FtsQ transmembrane helix and to 70S nontranslating ribosomes was analyzed by cosedimentation experiments. The pellet fraction was dissolved in SDS loading dye and analyzed by SDS/PAGE followed by Coomassie staining of the gel. In all experiments, excess of translocon (sub)complexes compared with ribosomes was used (Fig. S6).

In Vitro Transcription/Translation/Insertion Assay. *E. coli* cytochrome bo_3 oxidase subunit CyoA was chosen as a substrate for monitoring cotranslational insertion into proteoliposomes. mRNA transcripts were generated using T7 RNA polymerase by in vitro transcription from PCR products containing *cyoA* downstream of a T7 promoter. These mRNAs were subsequently used in a coupled in vitro translation/insertion assay. Briefly, 8 μg of mRNA transcript was translated using an *E. coli* membrane-free cell extract (7) in the presence of *E. coli* single-chain signal recognition particle-SRP receptor fusion construct (8) and proteoliposomes containing SecYEG or the HTL complex. The nascent polypeptides were detected by incorporation of radiolabeled [^{35}S]methionine. To assay for membrane insertion, the in vitro translation reaction was allowed to proceed in the presence of the same proteoliposomes used for the in vitro translocation assays. Following a 90-min coupled translation/insertion at room temperature, the proteoliposomes were purified by sucrose flotation and treated with 5 M urea for 20 min on ice. The urea-washed proteoliposomes were then pelleted and resuspended in SDS/PAGE sample buffer and loaded onto a 4–12% Bis-Tris PAGE gel. Radioactive material was identified by phosphorimaging.

1. Oesterhelt D, Stoeckenius W (1974) Isolation of the cell membrane of Halobacterium halobium and its fractionation into red and purple membrane. *Methods Enzymol* 31: 667–678.
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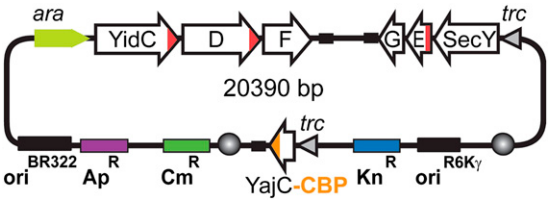

Fig. S1. Construction of the pACEMBL:HTL expression plasmid. The pACEMBL:HTL plasmid was constructed using the ACEMBL system (1). The plasmid consists of the pACE acceptor and the pDC and pDK donor vectors combined by Cre-loxP fusion (LoxP, gray circles). A polycistron encoding for YidC, SecD (D), SecF (F) with an arabinose promoter (ara, lime green) has been subcloned into pACE. A second polycistron encoding for SecY, SecE (E) and SecG (G) with a trc promoter (trc, gray triangles) has been cloned into pDC. Calmodulin-binding protein (CBP)-tagged YajC under the control of the trc promoter was cloned into pDK. Transcriptional terminators are shown as small black rectangles. The position of hexahistidine-tags in YidC, SecD, and SecE are indicated in red and the CBP-tag of YajC is indicated in orange. Origins of replication (BR322 and R6Kγ) are indicated by large black rectangles. Antibiotic resistance genes confer resistance to the following antibiotics: Ap (ampicillin, purple), Cm (chloramphenicol, green), and Kn (kanamycin, blue).

1. Bieniossek C, et al. (2009) Automated unrestricted multigene recombineering for multiprotein complex production. *Nat Methods* 6(6):447–450.

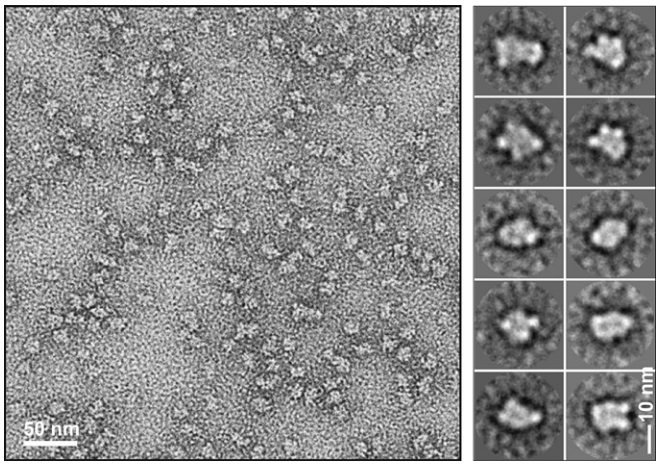

Fig. S2. Negative-stain EM of purified HTL complex. Micrograph of GraFix-treated HTL complexes reveals particles of a diameter of 13–15 nm (*Left*). The 2D class averages of the HTL reveal asymmetric particles composed of several domains (*Right*).

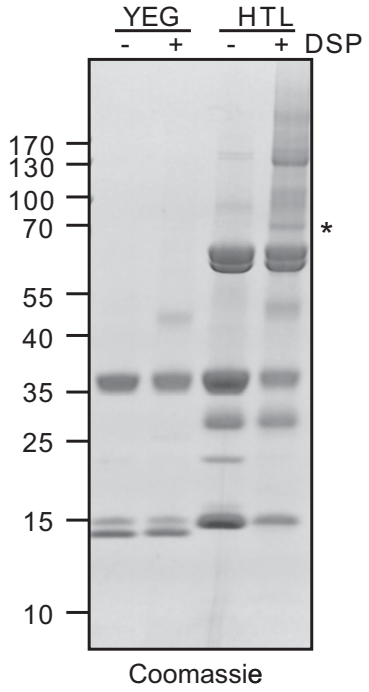

Fig. S3. Cross-linking of HTL subunits by DSP. Coomassie-stained SDS/PAGE gel of the same samples used for blotting in Fig. 2*B*. The HTL-specific cross-linked band excised from the Coomassie gel (asterisk) were found by mass spectrometry of tryptic fragments to contain both YidC and SecD, indicating the formation of higher-molecular weight products.

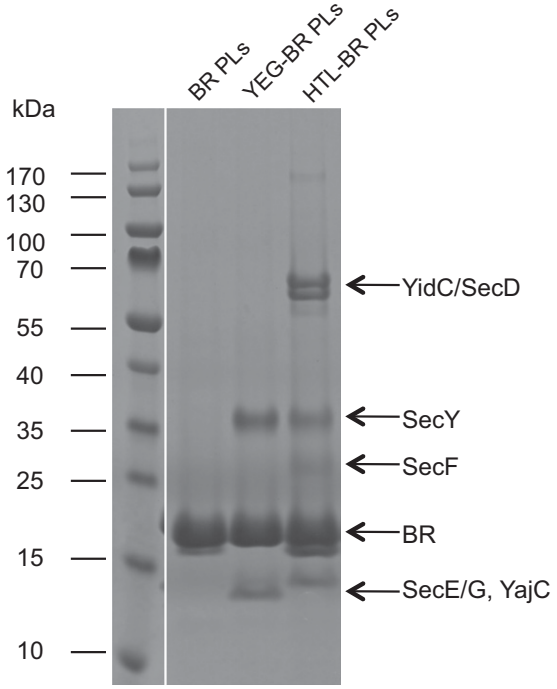

Fig. S4. Composition of proteoliposomes used for in vitro translocation experiments. Aliquots of proteoliposomes used for the translocation experiments were solubilized in LDS sample loading buffer and subjected to SDS/PAGE on 4–12% Bis-Tris gels. Proteins were visualized by Coomassie staining. Note that migration of SecE is slightly higher in the HTL construct compared with the SecYEG construct owing to slight differences in sequences of the affinity tags between the two.

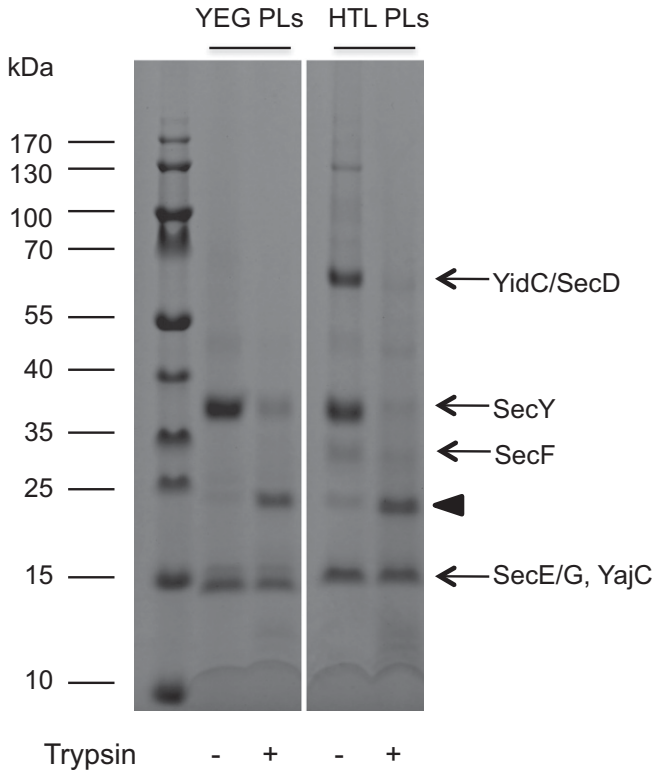

Fig. S5. Orientation of complexes in proteoliposomes. Aliquots of SecYEG- and HTL-containing proteoliposomes were treated with trypsin and subjected to SDS/PAGE followed by Coomassie staining. A black arrowhead indicates the generated proteolysis fragment.

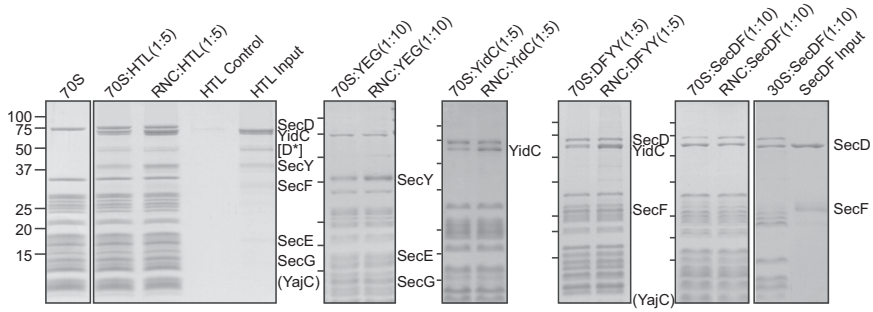

Fig. S6. Interaction of the HTL and its subcomplexes with ribosomes. Binding of purified HTL to translating (ribosome nascent chain complexes, RNCs) and nontranslating ribosomes (70S) analyzed by cosedimentation experiments. The pellet fractions were analyzed on a Coomassie-stained SDS gel. Input samples were not centrifuged. The HTL control shows that HTL is not found in the pellet fraction in absence of ribosomes. D* is a degradation product of SecD.

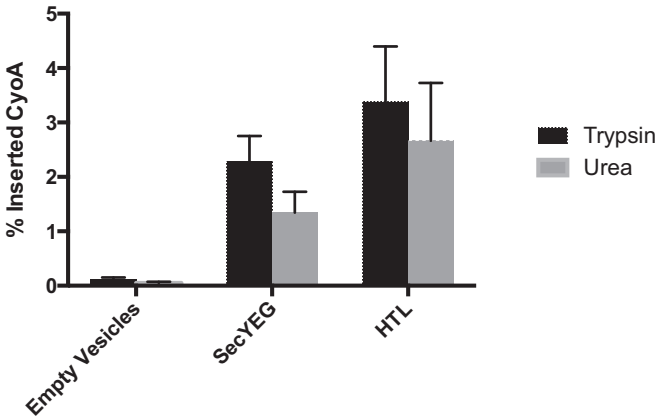

Fig. S7. In vitro insertion of CyoA into proteoliposomes. In vitro-synthesized CyoA was incubated in the presence of empty vesicles or SecYEG- or HTL-bearing proteoliposomes. Successfully incorporated protein was measured separately by resistance to urea extraction and proteolysis. Data represent four independent experiments and are displayed as an average percentage of CyoA inserted into the membrane ± SEM.