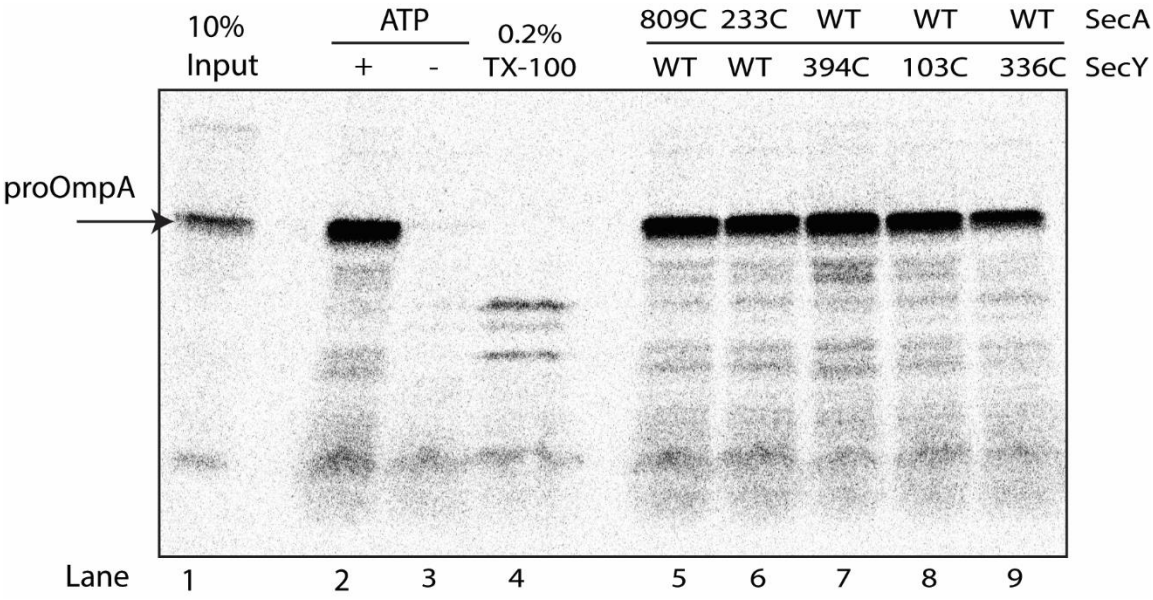
Appendix

Appendix

Table S1. Sample Sizes in Analysis

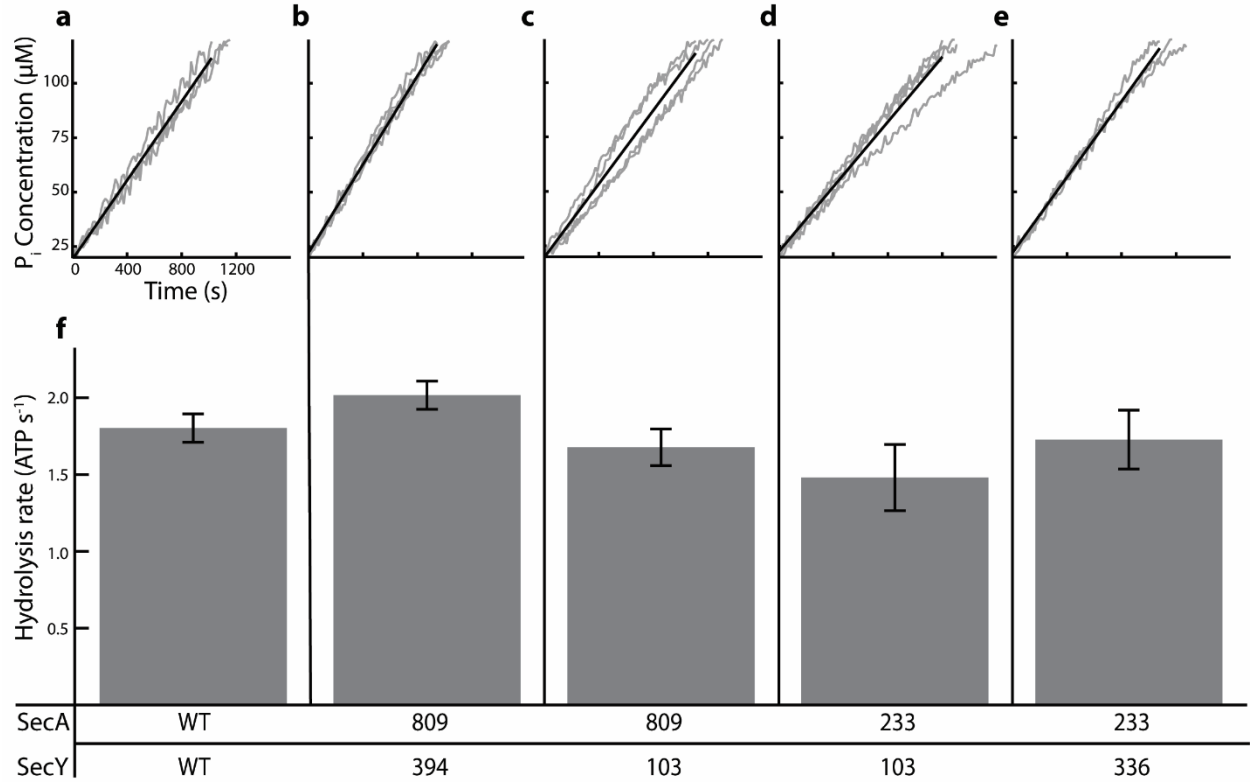
SecA Mutant	SecY Mutant	Nucleotide	# Videos	# Colocalized Spots	# FRET spots
809	**394**	**ADP•BeF_x**	43	473	97
809	**394**	**ATP**	113	1469	257
809	**394**	**ATPγS**	107	1106	168
809	**394**	**ADP•P_i**	96	1184	227
809	**394**	**ADP•V_i**	89	326	202
809	**103**	**ADP•BeF_x**	64	299	106
809	**103**	**ATP**	101	1448	200
233	**103**	**ADP•BeF_x**	72	648	197
233	**103**	**ATP**	98	1568	228
233	**103**	**ATPγS**	91	1153	315
233	**103**	**ADP•P_i**	92	940	274
233	**103**	**ADP•V_i**	66	506	157
233	**336**	**ADP•BeF_x**	57	760	163
233	**336**	**ATP**	90	1260	178

Figure S1. Translocation activity of fluorescently labeled SecA and SecY.



ProOmpA was synthesized in reticulocyte lysate in the presence of ^{35}S-methionine. Translocation was tested with either wild type SecA or SecA in which single cysteines were introduced and labeled with Cy5. Proteoliposomes contained either SecYEG with wild type SecY or SecY labeled at the indicated positions with Cy3. After incubation in the presence of ATP, the samples were treated with proteinase K to degrade any non-translocated material. Where indicated, the reaction was performed with wild type components in the absence of ATP and proteolysis in the presence of Triton X-100 (TX-100). All samples were analyzed by SDS PAGE followed by autoradiography. Lane 1 shows 10 % of the input sample.

Figure S2. ATPase activity measured with fluorescently labeled SecA and SecY.

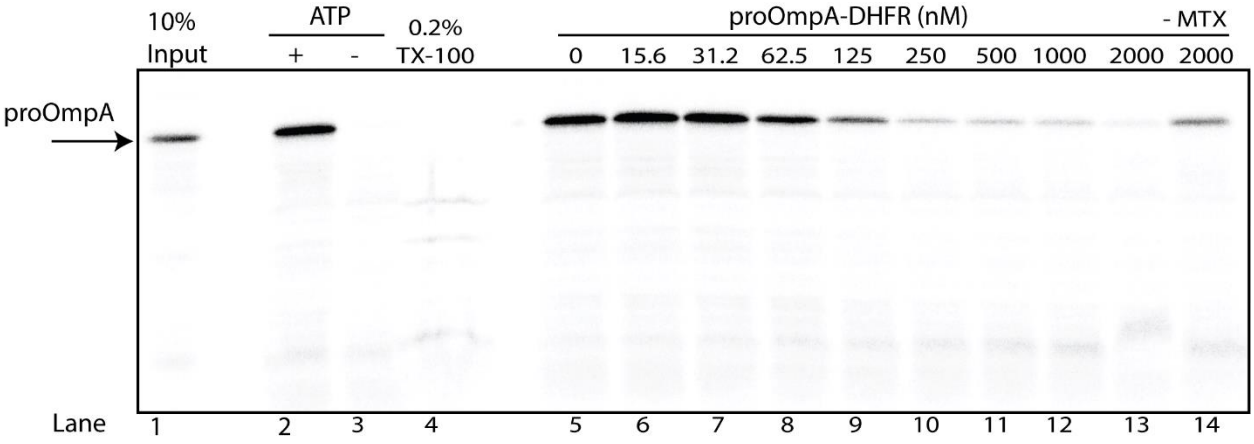


(A) Wild type SecA and SecYEG complex reconstituted into proteoliposomes was incubated at 24 ^{o}C with a fusion of proOmpA with DHFR and ATP. The release of inorganic phosphate (P_i) over time was measured with an enzymatic assay. The grey curves show the actual data of four experiments and the black curve the average.

(B-E) As in (A), but with single-cysteine mutants of SecA and SecY labeled with fluorophores at the indicated positions.

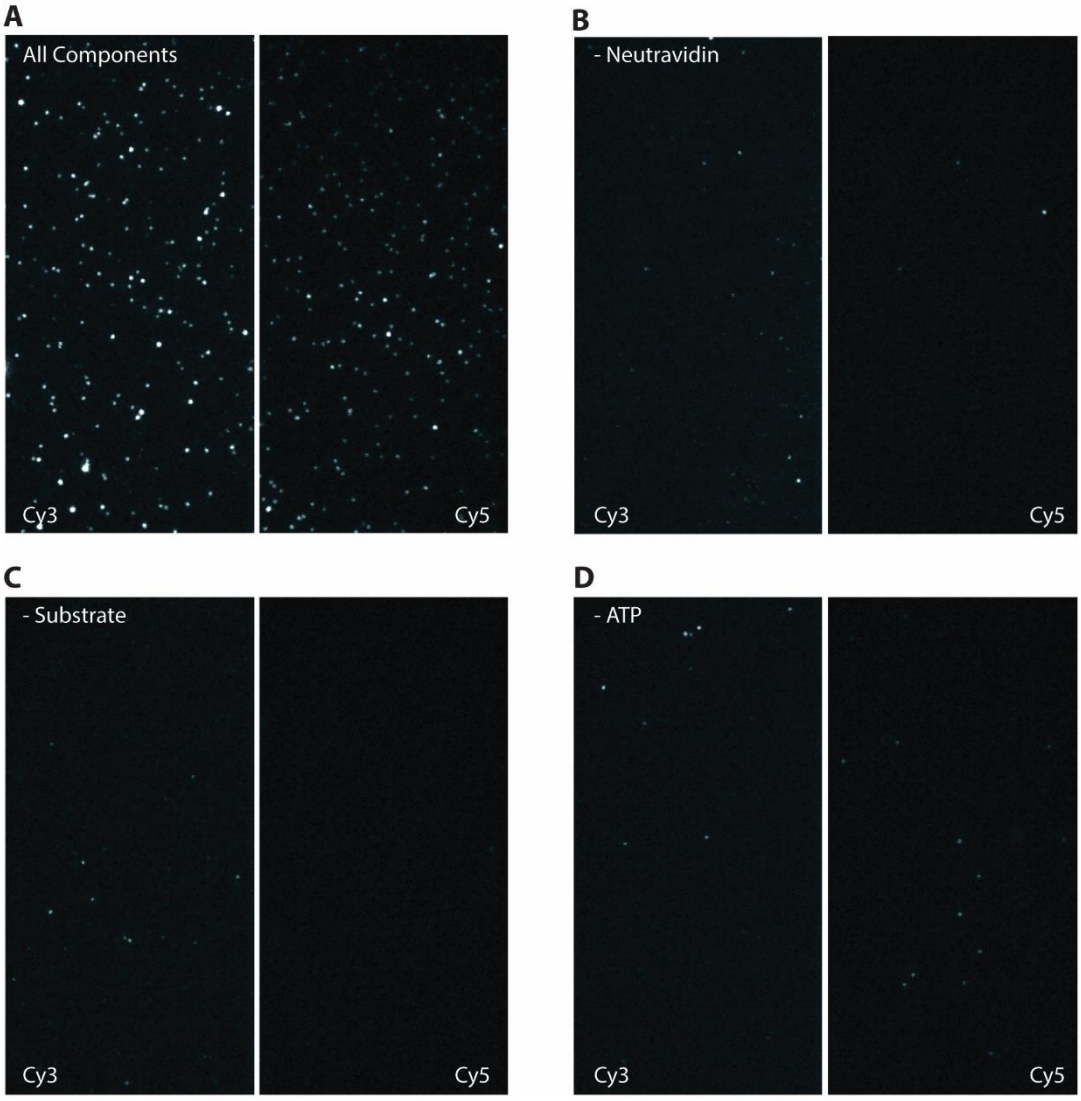
(F) The slopes in (A-E) and the dependence of ATPase activity on SecA concentration were used to calculate the hydrolysis rates per SecA molecule. The error bars show standard deviations of four replicates.

Figure S3. Saturation of translocation sites with proOmpA-DHFR.



ProOmpA was synthesized in reticulocyte lysate in the presence of ^{35}S-methionine and incubated with proteoliposomes containing SecYEG in the presence of increasing concentrations of unlabeled proOmpA-DHFR and methotrexate (MTX), a substrate analog of DHFR. The fusion protein generates a stalled translocation intermediate with the MTX-stabilized DHFR domain remaining outside the vesicles. After incubation, proteinase K was added to digest all non-translocated material, and the samples were analyzed by SDS PAGE and autoradiography. As a control, MTX was omitted (lane 14), so that proOmpA-DHFR was completely translocated into the liposomes and did not plug the translocation sites. Lanes 2-4 show samples in the absence of proOmpA-DHFR. In lane 4, proteolysis was performed in the presence of Triton X-100 (TX-100). Lane 1 shows 10% of the input material. Note that proOmpA-DHFR was used for the single-molecule experiments at 1 µM concentration, which is more than sufficient to saturate all translocation sites.

Figure S4. Assembly of translocation complexes for single molecule imaging.



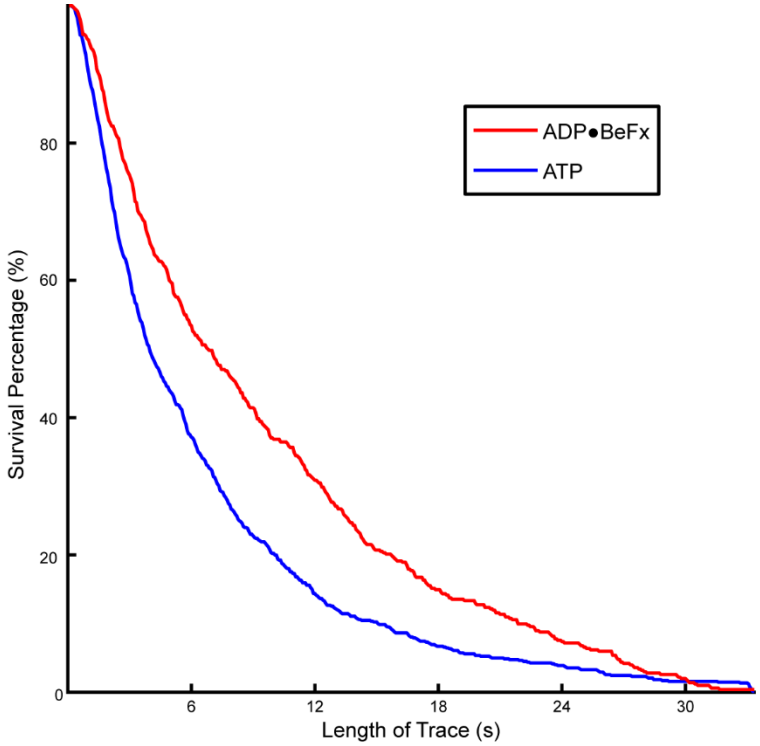
(A) Translocation intermediates were assembled with proOmpA-DHFR, Cy5-labeled SecA, and proteoliposomes containing SecYEG labeled with Cy3 on SecY. The complexes were immobilized via a biotin-tag at the C-terminus of the substrate to a glass surface containing biotinylated PEG, which was pretreated with neutravidin. Individual SecA and SecY molecules were visualized by TIRF via direct excitation of their fluorophores (left and right halves of the image).

(B) As in (A), but without neutravidin preincubation.

(C) As in (A), but without substrate.

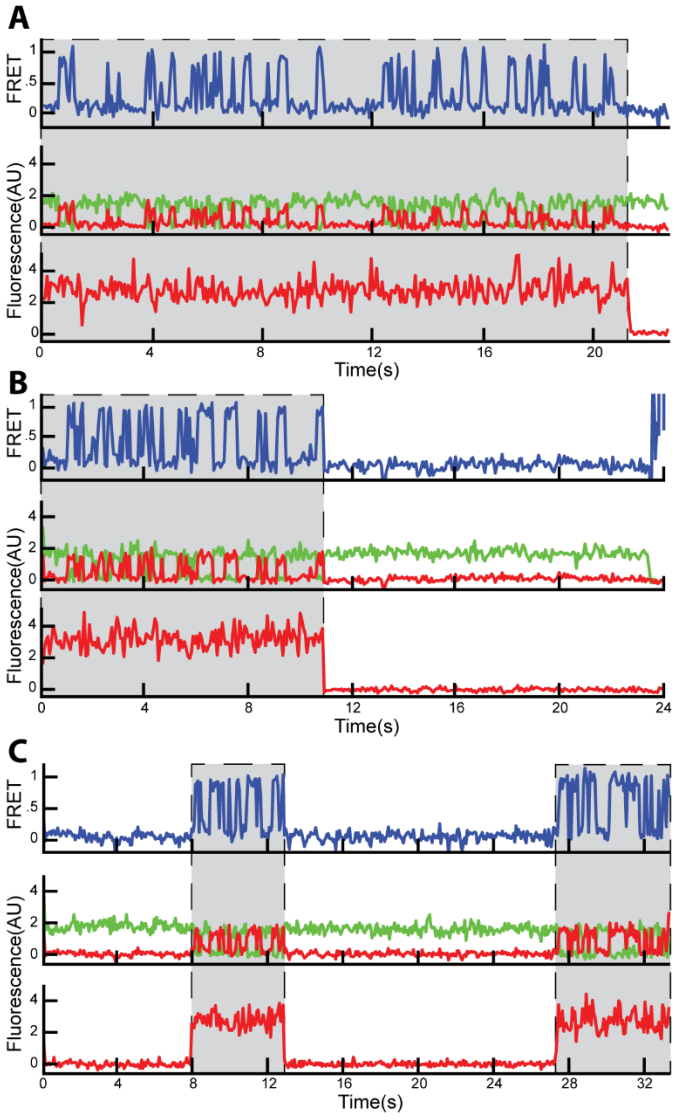
(D) As in (A), but without ATP, so that no translocation intermediate is generated.

Figure S5. Binding lifetime of SecA in ATP and ADP•BeF_x



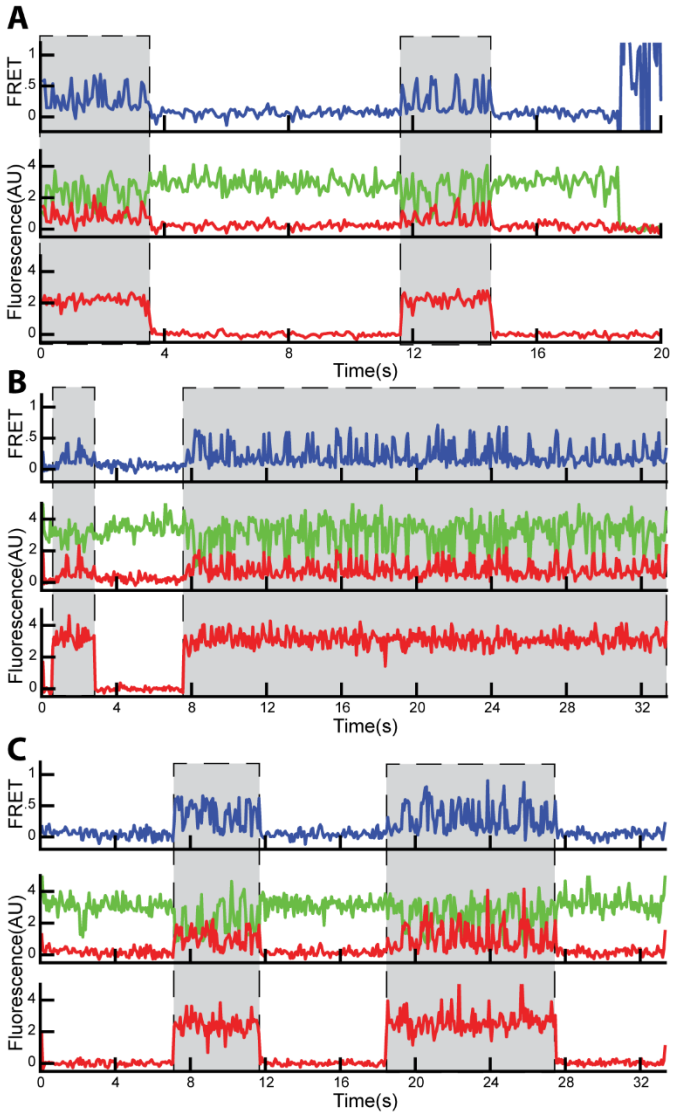
Survival plot of all SecA binding events delimited by SecA dissociation or bleaching. Binding lifetimes in the presence of ADP•BeF_x are in plotted red and lifetimes in the presence of ATP in blue.

Figure S6. Representative FRET traces for two-helix finger movements in the presence of ATP.



(A-C) Representative traces obtained with ATP. The upper FRET trace was calculated from the middle traces obtained by exciting the donor fluorophore and measuring both donor (green) and acceptor (red) fluorescence. The lowest trace was obtained by exciting the acceptor fluorophore directly. Periods in which a fluorescently labeled SecA molecule is bound are indicated by grey shading.

Figure S7. Representative FRET traces for clamp movements in the presence of ATP.



(A-C) Representative traces obtained with ATP. The upper FRET trace was calculated from the middle traces obtained by exciting the donor fluorophore and measuring both donor (green) and acceptor (red) fluorescence. The lowest trace was obtained by exciting the acceptor fluorophore directly. Periods in which a fluorescently labeled SecA molecule is bound are indicated by grey shading.