

Supplementary Materials and methods

Genetic constructs:

Construction of proPhoA-His, proPhoA(L8Q)-His, proPhoA(L14R)-His, PhoA(Δ 2-22)-His and proPhoA(1-62)-His for purification purposes:

proPhoA-His₆: The wild type *proPhoA* gene (Exposy access code: P00634) was isolated by PCR from the chromosome of *E.coli* JM109(DE3) using X560 (5' GGG AAT TCC ATA TGA AAC AAA GCA CTA TTG CA 3') that inserts an *Nde* I site as forward primer and X561 (5' GAC CCG CTC GAG TTT CAG CCC CAG AGC GGC 3') that inserts a *Xho* I site as reverse primer. The 1,4kb *Nde* I-*Xho* I PCR fragment was cloned into the corresponding sites of pET22b resulting in pIMBB882.

proPhoA(L8Q)-His₆: the *proPhoA(L8Q)* gene was isolated by PCR from the chromosome of *E.coli* MPh1068(phoA68) strain¹, using X560 (5' GGG AAT TCC ATA TGA AAC AAA GCA CTA TTG CA 3') that inserts an *Nde* I site as forward primer and X561 (5' GAC CCG CTC GAG TTT CAG CCC CAG AGC GGC 3') that inserts a *Xho* I site as reverse primer. The 1,4kb *Nde* I-*Xho* I PCR fragment was cloned into the corresponding sites of pET22b resulting in pIMBB883.

proPhoA(L14R)-His₆: the *proPhoA(L14R)* gene was isolated by PCR from the chromosome of *E.coli* Mph1061(phoA61) strain¹, using X560 (5' GGG AAT TCC ATA TGA AAC AAA GCA CTA TTG CA 3') that inserts an *Nde* I site as forward primer and X561 (5' GAC CCG CTC GAG TTT CAG CCC CAG AGC GGC 3') that inserts a *Xho* I site as reverse primer. The 1,4kb *Nde* I-*Xho* I PCR fragment was cloned into the corresponding sites of pET22b resulting in pIMBB884.

phoA(Δ 2-22)-His₆: the gene encoding the mature domain of PhoA (*phoA* Δ 2-22) was isolated by PCR from pIMBB882 (proPhoAHis₆), using X646 (5' GGG AAT TCC ATA TGA CCC CAG AAA TGC CTG TT 3') that inserts an *Nde* I site as forward primer and X561 (5' GAC CCG CTC GAG TTT CAG CCC CAG AGC GGC 3') that inserts a *Xho* I site as reverse primer. The 1,3kb *Nde* I-*Xho* I PCR fragment was cloned into the corresponding sites of pET22b resulting in pIMBB953.

proPhoA(1-62)-His₆: the gene encoding the N1-62aa of proPhoA (removal of 409 residues from the C-terminus of proPhoA) was generated by PCR from pIMBB882 (proPhoAHis₆), using X560 (5' GGG AAT TCC ATA TGA AAC AAA GCA CTA TTG CA 3') as forward primer that introduces an *Nde* I site and X728 (5' GAC CCG CTC GAG ATA TTT ATC GCT AAG AGA ATC ACG 3') as reverse primer that introduces a *Xho* I site. The 0,186kb *Nde* I-*Xho* I PCR fragment was cloned into the corresponding sites of pET22b resulting in pIMBB1001.

Construction of proPhoA-His, proPhoA(L8Q)-His, proPhoA(L14R)-His, PhoA(Δ2-22)-His and proPhoA(1-62)-His for in vivo secretion assays:

For *in vivo* secretion assays proPhoA and derivatives were placed under the arabinose promoter of the pBAD33² vector, a pACYC derivative.

proPhoA-His₆: Using pIMBB882 as template and primers X629 (5' CGG GGT ACC GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG 3') that introduces a *Kpn* I site and X630 (5' AAA CCC AAG CTT TCA GTG GTG GTG GTG GTG GTG 3') that introduces a *Hind* III site, the *Kpn* I- *Hind* III PCR product was cloned in the corresponding sites of pBAD33 resulting in pIMBB932.

proPhoA(L8Q)-His₆: Using as template pIMBB883 and primers X629 (5'CGG GGT ACC GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG3') that introduces a *Kpn* I site and X630 (5'AAA CCC AAG CTT TCA GTG GTG GTG GTG GTG GTG 3') that inserts a *Hind* III site, the *Kpn* I- *Hind* III PCR product was cloned in the corresponding sites of pBAD33 resulting in pIMBB933.

PhoA(Δ2-22)-His: Using as template pIMBB932 and primers X646 (5' GGG AAT TCC ATA TGA CCC CAG AAA TGC CTG TT 3') that introduces a *Nde* I site and X561 (5'GAC CCG CTC GAG TTT CAG CCC CAG AGC GGC 3') that introduces a *Xho* I site, the generated *Nde* I-*Xho* I PCR fragment replaced the *Nde* I- *Xho* I fragment of pIMBB932 vector resulting in pIMBB954.

Construction of SecY prlA3 and prlA4 mutants:

His₆SecYprlA3/SecE/SecG was constructed by the Quick-Change PCR site-directed Mutagenesis method (Stratagene) using *PfuUltra*TM High Fidelity DNA polymerase, plasmid pET610³ as

template, X522 (5'GAG ATG TTT AAC ATG **TGC** TCT GGT GGT GCT CTC 3') as forward mutagenic primer and X523 (5' GAG AGC ACC ACC AGA **GCA** CAT GTT AAA CAT CTC 3') as the reverse. The resulting construct is pIMBB843.

His₆SecYprlA4-EG was constructed by the Quick-Change PCR site-directed Mutagenesis method (Stratagene) using *PfuUltra*TM High Fidelity DNA polymerase at two steps. prlA4 (I408N) mutation was introduced in the first round using plasmid pET610 as template, X524 (5'GGG ACC TCA CTG CTT **AAC** GTT GTT GTC GTG ATT 3') as forward mutagenic primer and X525 (5'AAT CAC GAC AAC AAC **GTT** AAG CAG TGA GGT CCC 3') as the reverse. In the second round (F286Y) mutation was introduced using plasmid pET610(I408N) as template, X526 (5'TCC AGT ATT ATT CTG **TAC** CCG GCG ACC ATC GCG 3') as the forward mutagenic primer and X527 (5' CGC GAT GGT CGC CGG **GTA** CAG AAT AAT ACT GGA 3') as the reverse. The resulting construct is pIMBB842.

Construction of SecA I304A/L306A mutant:

His₆SecA I304A/L306A was constructed by “megaprimer” PCR using pIMBB7 as template, X413 (5' CT CCG GCC AAC **GCC** ATG **GCG** ATG CAC CAC GT 3') as forward mutagenic primer and X182 (5'GGCCTTTCGCAGTACGTTTC3') as reverse. The 0.45 kb PCR product was then used as reverse primer together with X395 (5' CTA ACA ACA ATA AAC CTT TAC TTC 3') as forward primer. The 1.42 kb *Nsi* I/*Bgl* II PCR product was cloned into the corresponding sites of pIMBB7 resulting in pIMBB691.

Cloning of other secretory proteins

proBglX-His₆: the *proBglX* gene (access code: P33363) was isolated by PCR from the chromosome of *E. coli* DH5 α using X732 (5' GGG AAT TCC ATA TGA AAT GGC TAT GTT CAG TAG GAA TCG CG 3') that inserts an *Nde* I site as forward primer and X733 (5' GAC CCG CTC GAG CAG CAA CTC AAA CTC GCC TTT CTT AAC G 3') that inserts a *Xho* I site as reverse primer. The 2.3kb *Nde* I-*Xho* I PCR fragment was cloned in the corresponding sites of pET22b resulting in pIMBB1036.

BglX-His₆ : the gene encoding the mature domain of BglX was isolated by PCR from the chromosome of *E. coli* DH5 α using X734 (5' GGG AAT TCC ATA TGG ATG ATT TAT TCG GCA ACC ATC CAT TAA CGC 3') that inserts an *Nde* I site as forward primer and X733 (5' GAC CCG CTC GAG CAG CAA CTC AAA CTC GCC TTT CTT AAC G 3') that inserts a *Xho* I site as reverse primer. The 2.3kb *Nde* I-*Xho* I PCR fragment was cloned in the corresponding sites of pET22b resulting in pIMBB1037.

proPpiA-His₆ : the *proPpiA* gene (access code: P0AFL3) was isolated by PCR from the chromosome of *E. coli* DH5 α using X741 (5' GGG AAT TCC ATA TGT TCA AAT CGA CCC TGG CGG CG 3') that inserts an *Nde* I site as forward primer and X742 (5' GAC CCG CTC GAG CGG CAG GAC TTT AGC GGA AAG GAT AA 3') that inserts a *Xho* I site as reverse primer. The 0.6kb *Nde* I-*Xho* I PCR fragment was cloned in the corresponding sites of pET22b resulting in pIMBB1042.

PpiA-His₆ : the gene encoding the mature domain of PpiA was isolated by PCR from the chromosome of *E. coli* DH5 α using X743 (5' GGG AAT TCC ATA TGG CAG CGA AAG GGG ACC CG 3') that inserts an *Nde* I site as forward primer and X742 (5' GACCCG CTC GAG CGG CAG GAC TTT AGC GGA AAG GAT AA 3') that inserts a *Xho* I site as reverse primer. The 0.5kb *Nde* I-*Xho* I PCR fragment was cloned in the corresponding sites of pET22b resulting in pIMBB1043.

proAmy1-His₆ : the *proAmy1* gene (access code: P25718) was isolated by PCR from the chromosome of *E. coli* DH5 α using X744 (5' GGG AAT TCC ATA TGA AAC TCG CCG CCT GTT TTC TGA CA 3') that inserts an *Nde* I site as forward primer and X745 (5' GAC CCG CTC GAG CTG TTG CCC TGC CCA GAC GAC 3') that inserts a *Xho* I site as reverse primer. The 2.0kb *Nde* I-*Xho* I PCR fragment was cloned in the corresponding sites of pET22b resulting in pIMBB1044.

Amy1-His₆ : the gene encoding the mature domain of Amy1 was isolated by PCR from the chromosome of *E. coli* DH5 α using X746 (5' GGG AAT TCC ATA TGG CCA GCT GGA CTT CTC CGG G 3') that inserts an *Nde* I site as forward primer and X745 (5' GAC CCG CTC GAG CTG TTG CCC TGC CCA GAC GAC 3') that inserts a *Xho* I site as reverse primer. The 1.9kb

Nde I-*Xho* I PCR fragment was cloned in the corresponding sites of pET22b resulting in pIMBB1045.

proYdeN-His₆ : the *proYdeN* gene (access code: P77318) was isolated by PCR from the chromosome of *E.coli* DH5 α using X747 (5' GGG AAT TCC ATA TGA AGT CTG CAT TAA AGA AAA GTG TCG TAA GTA C 3') that inserts an *Nde* I site as forward primer and X748 (5' GAC CCG CTC GAG TTT CGC TTC GCT TAG TGC TTT CTT GAT ATT GTT AAA 3') that inserts a *Xho* I site as reverse primer. The 1.7kb *Nde* I-*Xho* I PCR fragment was cloned in the corresponding sites of pET22b resulting in pIMBB1046.

YdeN-His₆ : the gene encoding the mature domain of YdeN was isolated by PCR from the chromosome of *E.coli* DH5 α using X749 (5' GGG AAT TCC ATA TGG CAG ATG ATG TAA AGC TGA AAG CAA CCA AAA C 3') that inserts an *Nde* I site as forward primer and X748 (5' GAC CCG CTC GAG TTT CGC TTC GCT TAG TGC TTT CTT GAT ATT GTT AAA 3') that inserts a *Xho* I site as reverse primer. The 1.6kb *Nde* I-*Xho* I PCR fragment was cloned to the corresponding sites of pET22b resulting in pIMBB1047.

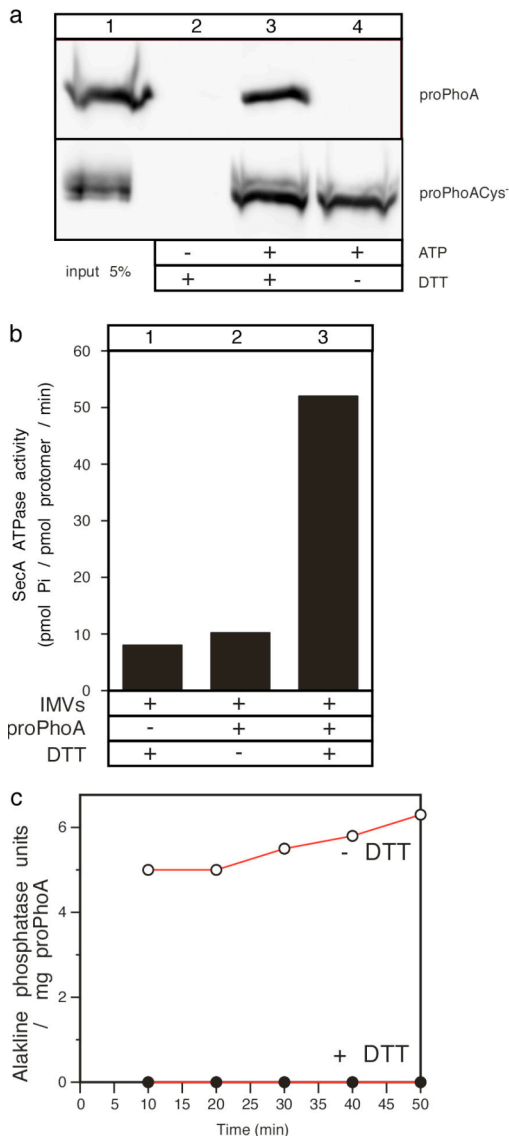
Purification of secretory proteins

Secreted protein derivatives were purified by Ni⁺² affinity chromatography in Buffer C (50 mM Tris-HCl pH 8.0; 50 mM KCl; 6M Urea; 10 % glycerol v/v) and dialyzed in buffer D (50 mM Tris-HCl pH 8.0; 50 mM KCl; 6M Urea; 1 mM EDTA; 10 % glycerol v/v).

Antibodies

Polyclonal rabbit antibodies were raised against purified proPhoA (Davids biotechnologie, Germany). His-tagged proteins were immunostained with α -His antibodies (Serotech) following analysis by SDS-PAGE.

Supplementary Figures

Fig. S1 Translocation competence versus phosphatase activity of proPhoA in non-native and native forms

a. *In vitro* ATP-driven translocation of proPhoA or proPhoACys⁻ (8.5 μ M) into IMVs (1.0 μ M SecY) catalyzed by SecA (0.4 μ M). proPhoA, purified in the absence of a reducing agent, was treated with 2 mM DTT for 8 h prior to the reaction (only where indicated). Reactions [100 μ l; buffer B, 0.5 mg/ml BSA, 2.5 mM ATP, 1 mM DTT where indicated] were incubated at 37°C for 12 min. Translocation was terminated by addition of proteinase K (1 mg/ml; 20 min; 4°C). Proteins were precipitated with trichloroacetic acid (TCA; 15% w/v), analyzed by SDS-PAGE and immuno-stained with α -PhoA antibody. Translocation *per se* does not require a reducing environment since a cysteineless proPhoA variant gets translocated equally well with or without DTT (bottom panel). However, only the reduced proPhoA is translocation competent.

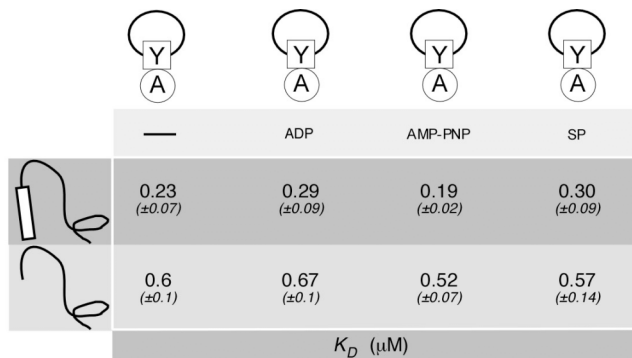
b. SecA ATPase activity is stimulated by proPhoA. The K_{cat} values (pmoles Pi/pmol SecA protomer/min) of membrane (IMVs; 0.4 μ M SecY) and translocation (IMVs plus 8.5 μ M proPhoA prepared as in "a") ATPase activities of SecA were determined (10 min; 37°C) in the presence or absence of 1mM DTT (as indicated).


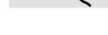
Released phosphate was measured as described^{4,5}. Only the reduced form of proPhoA stimulates SecA ATPase.

c. proPhoA phosphatase activity was determined spectroscopically at O.D._{425nm}^{6,7,8} (in 1 M Tris pH:8.0, 5 mM MgCl₂, 5 mM ZnCl₂) in the presence or absence of 1mM DTT. One unit of PhoA hydrolyzes 1.0 μ mole of p-nitrophenyl phosphate/min at 37 °C. proPhoA was diluted into the

reaction from 6 M urea stock to 0.1 M Urea. Only the oxidized form of proPhoA (as for PhoA, data not shown) is active as phosphatase.

Fig. S2 Effect of nucleotide or SP addition on the K_D of proPhoA or PhoA for the translocase.



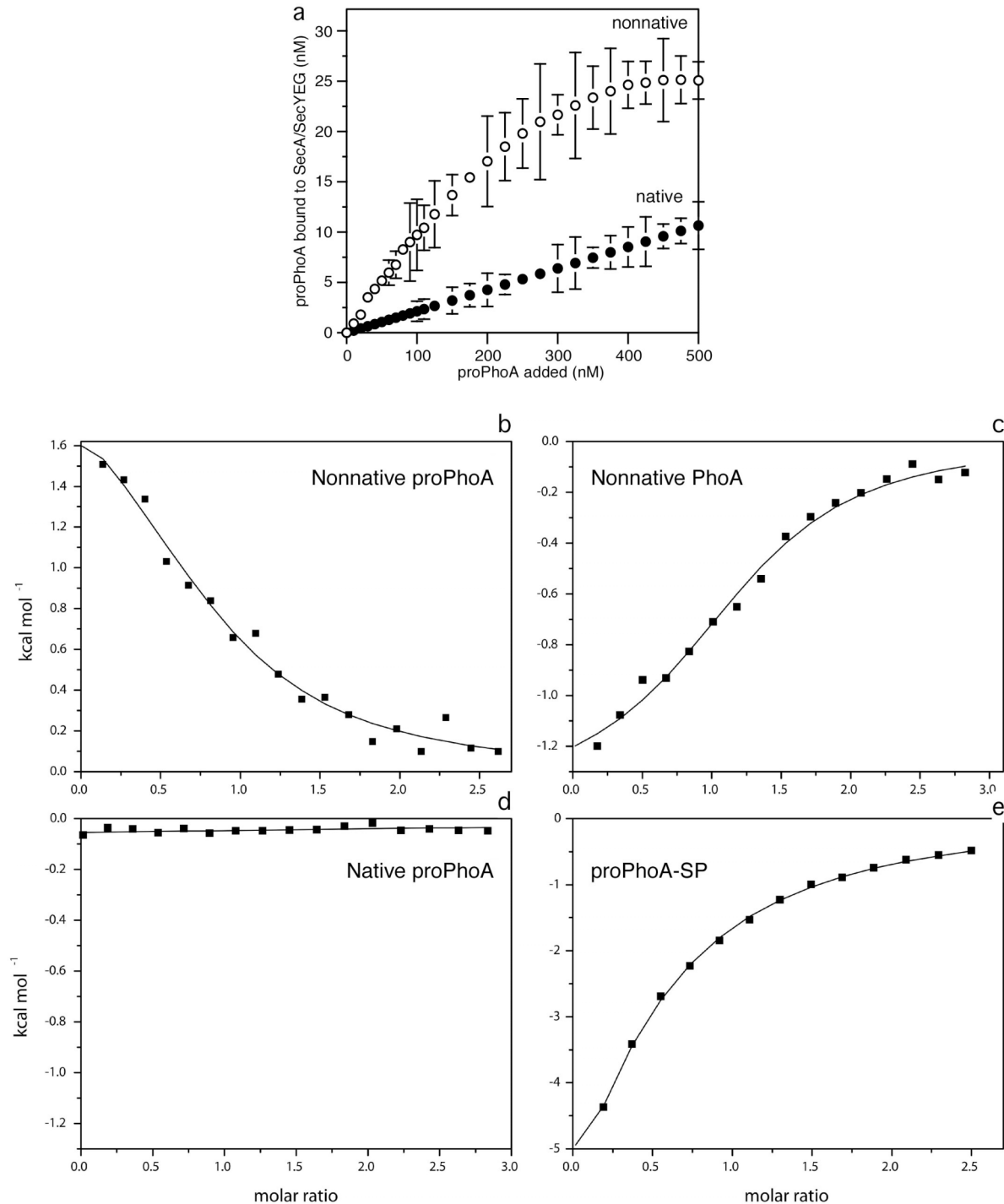
	—	ADP	AMP-PNP	SP
	0.23 (±0.07)	0.29 (±0.09)	0.19 (±0.02)	0.30 (±0.09)
	0.6 (±0.1)	0.67 (±0.1)	0.52 (±0.07)	0.57 (±0.14)
	K_D (μM)			

a. Equilibrium dissociation constants (K_D ; as in Fig. 1a) of proPhoA and PhoA for SecYEG-bound SecA were measured in the presence or absence of nucleotides (2mM) or synthetic proPhoA signal peptide (20 μM), as indicated.

Fig. S3 Affinity of native and non-native proPhoA for free and SecYEG-bound SecA.

a. Equilibrium dissociation constants (K_D) of oxidized (native) versus reduced (nonnative) proPhoA onto SecYEG-bound SecA, [^{35}S]-proPhoA (added in 1-500 nM range) that remained associated to SecYEG-IMVs with bound SecA was immobilized on nitrocellulose (using a vacuum manifold) and then quantitated (by phosphorimaging). Data were analyzed by non-linear regression as described⁹. Data represent average values ($n=3-7$) and error bars the standard deviation. Only the "Non-native" proPhoA (with DTT) can associate to SecYEG-bound SecA, in complete agreement with its translocation competence state (Fig. S1a and b).

b-e. Binding isotherms of the calorimetric titration of proPhoA and derivatives with SecA in solution were performed on a VP-Isothermal Titration Calorimeter (ITC; Microcal), in 20 mM Tris-HCl pH 8.0, 20 mM KCl, supplemented with 2 mM Tris (2-carboxyethyl) phosphine (TCEP) as a reducing agent (where indicated). proPhoA or its derivatives were in the cell, at 80 μM . SecA (at 1 mM in the syringe) was titrated (20 μl injections) to purified "non-native" proPhoA (b), mature PhoA (c), "native" proPhoA (d) and the proPhoA signal peptide (e). Binding isotherms were generated by plotting heats of reaction normalized by the modes of injectant versus the ratio of total injectant to total protein per injection. Data were analyzed as described¹⁰.



“Non-native” PhoA or proPhoA, as well as the isolated signal peptide, bind to SecA with a 1:1 stoichiometry. In contrast, “native” proPhoA does not bind to SecA as judged by the flat binding isotherm (Panel d). Interestingly, the energetics of non-native proPhoA binding to SecA is not the simple sum of the binding energetics of its individual domains (signal peptide and mature). Non-native proPhoA binding to SecA is enthalpically opposed (ΔH positive) and

entropically favored whereas binding of non-native PhoA and the isolated signal peptide is enthalpically favored (ΔH negative). This observation suggests that the signal sequence and the mature domain act synergistically in the context of the preprotein.

Fig. S4 Activation energy of the translocase - stimulation of SecA ATPase by proPhoA and derivatives as a function of temperature.

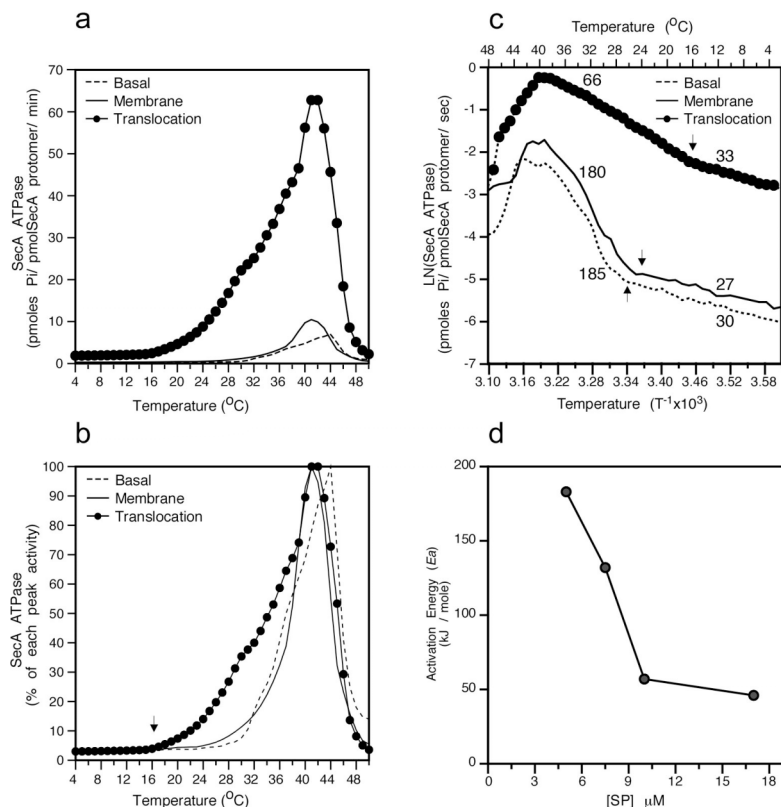
a and **b**. The K_{cat} values (pmoles Pi/pmol SecA protomer/min) of basal, membrane (urea-treated IMVs; 0.4 μM SecY) and

translocation (IMVs plus 8.5 μM proPhoA) ATPase activities of SecA (0.4 μM) were determined, as a function of temperature (4–50°C), as described (Fig. S3a)¹¹. The actual data for the wild type translocase are shown in **a**.

In **b**, the peak K_{cat} value of each ATPase activity, presented in **a**, was considered as 100%; all other values are expressed as a percentage of that. Experimental data were fitted in Matlab using spline interpolation (Mathworks, Matlab spline toolbox). For each

condition a number of 4–15 experimental data sets were used, the values were compared and averaged.

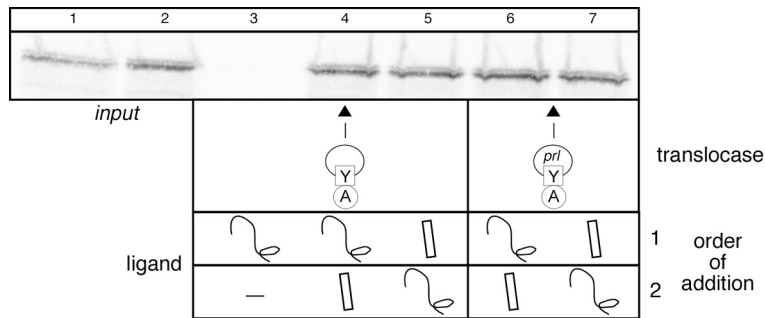
c. Arrhenius plots of the data presented in **a**. in. The Y axis represents the natural logarithm of the K_{cat} values [$\ln(K_{cat})$] and the X axis the inversed temperature values ($1/T$) expressed in Kelvin. The Arrhenius plots obtained with the three SecA ATPase activities are discontinuous and concave upwards^{12,13} till 41°C. Above that temperature ATP turnovers start a rapid reduction trend and the system most probably collapses (thermal denaturation of translocase components, complexes dissociate, etc.). Therefore data above 41°C should not be taken into account. The



curves in the remaining temperature range are concave for all three SecA ATPase activities including "basal ATPase". This demonstrates that the characteristic non-linear Arrhenius plot reflects an inherent property of SecA catalysis and does not relate to the presence of other ligands. The concave curves in all three cases comprise two distinct linear components. A minimum of 150 points was used to determine the slope of the lines ($R^2 > 0.980$). The activation energies (E_a) of the SecA ATPase under different conditions was calculated (in kJ/mole) from the slopes of the two linear parts. The breakpoints [also known as transitions temperature (T_m)] in the linearity of an Arrhenius plot¹³ are indicated on the plot (filled arrows). The first linear component (seen at low temperature) represents a low activation energy value (~30 kJ/mole) that remains unaltered in the three SecA ATPase states examined. Actual translocation is not observed in this temperature range. The second linear component (seen from 16-40°C in "translocation ATPase") displays a dramatic alteration both in terms of activation energy as well as in terms of turnovers when preproteins are present and is representative of the translocation-active enzyme. As observed, free or membrane-bound SecA have similar T_m (26 and 24°C respectively) and the activation energies of their ATPase are 180-185 kJ/mole. Addition of the preprotein substrate (proPhoA) significantly lowers the T_m of SecA bound to SecYEG (16°C) and lowers its activation energy to 66 kJ/mole. The different positioning of Arrhenius plots relative to the Y axis represents differences in ATP turnover under basal, membrane and translocation conditions.

d. The K_{cat} values (pmoles Pi/pmol SecA protomer/min) of membrane bound SecA in the presence of various concentrations of synthetic proPhoA signal peptide (as indicated) were determined (as in a and b). The calculated activation energies (as in c) were then plotted as a function of SP concentration (μM).

Fig. S5 Signal peptides added *in trans* promote PhoA translocation in wild type or *prl* translocases.



a. *In vitro* translocation of [³⁵S]-PhoA in wild type (lanes 4,5) or *prlA4* (lanes 6,7) SecYEG IMVs, by adding *in trans* the synthetic signal peptide of proPhoA. The order of ligand addition is indicated.

Translocated material was quantified

(*n* =3) relative to lane 5 (100%) as follows: lane 4, 97 (±3.1) %; lane 6, 108 (±7.4) %, lane 7, 120 (±9.4) %. Lanes 1 and 2 contain 30% and 60% respectively of the "input" [³⁵S]-PhoA, not treated with protease.

Fig. S6 Kinetics and concentration-dependence of mature PhoA translocation driven by signal peptide *in trans*

a. *In vitro* translocation of [³⁵S]-PhoA (300 fmoles) by addition “*in trans*” of the proPhoA-signal peptide in a concentration dependent manner. Reactions run for 12 minutes.

b. *In vitro* translocation of reconstituted [³⁵S]-PhoA + SP (50 μM), in the presence or absence of DTT (2 mM) is compared to proPhoA translocation.

In both **a.** and **b.** wild type SecA and SecYEG-IMVs were used. On the left part of panels values from repeat experiments ($n=3$) were quantitated; on the right a representative gel is shown. In all gels lane 1 represents 60% of the input protein, not treated with protease.

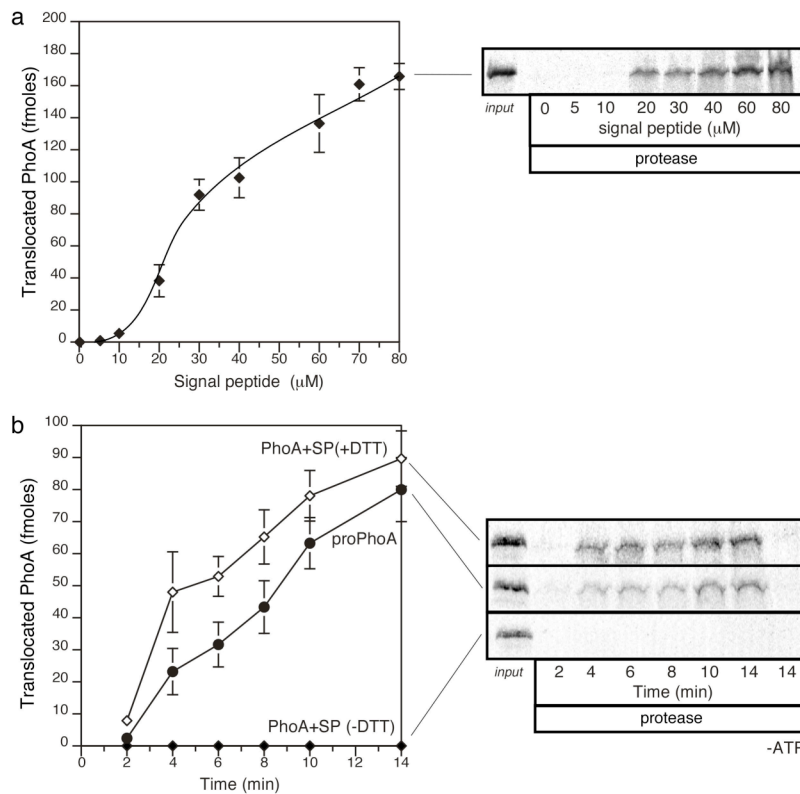
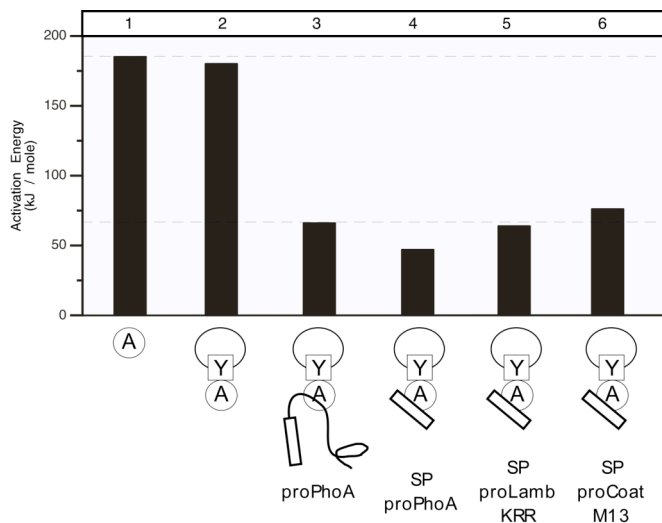


Fig. S7 Translocase "triggering" by synthetic signal peptides.

Activation energies (E_a ; kJ/mole) were calculated via Arrhenius plots (as in Fig. S3c). All reactions were with wild type SecA or/and SecYEG. Added substrates were proPhoA (lane 3). Synthetic signal peptides (added at 15 μ M) were: SP of proPhoA (lane 4), SP of proLamb KRR (lane 5) and the SP of proCoat M13 (lane 6).

References

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