Investigation of the polypeptide movement during SecA-mediated protein translocation

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ABSTRACT

The Sec-pathway of protein translocation is a decisive step in the biogenesis of secreted or integral membrane proteins. Its central component is a passive protein conducting channel, called SecY in bacteria and Sec61p in eukaryotes. Translocation of a protein occurs during or after its synthesis at the ribosome. In bacteria, the cytosolic ATPase SecA associates with SecY to translocate proteins after their translation. The molecular mechanism of SecA-mediated translocation is still unclear, but a recent structure of SecA in complex with SecY suggests how the ATPase could move a polypeptide chain through SecY. In this work, a disulfide bridge crosslinking approach was applied to test the interactions of a translocating polypeptide with SecA. The results show that SecA passes the polypeptide from its clamp down to the two-helix finger and into the SecY-pore. This observation is in agreement with a model of SecA-mediated translocation in which SecA´s clamp positions the polypeptide chain and the two-helix finger moves it into the channel. An attempt was made to distinguish between an active and a passive role of SecA´s clamp during translocation by looking at the backsliding of different polypeptide segments after ATP depletion. However, the results are inconclusive and do not confirm any of the proposed roles.

Contents

1 Introduction

1.1 Protein translocation

Cellular processes are orchestrated in a spatial and temporal manner, often separated by membranes that act as barriers to maintain different biological conditions. Proteins as the catalysts of these processes perform their task in the cytosol, the lumen of organelles, they are integrated into the plasmamembrane or organelle membranes, or they are secreted into the extracellular space. Information about the localization of a protein is encoded in a cleavable signal sequence of hydrophobic amino acids, which is recognized by specific receptors. Unless the final site of activity is the cytosol itself, newly synthesized proteins need to cross at least one membrane to reach their destination. The transport of proteins across or their integration into membranes is referred to as protein translocation.

The main pathway responsible for protein translocation is located in the plasma membrane of bacteria or the endoplasmatic reticulum membrane of eukaryotic cells and is called the Sec-pathway (see Rapoport, 2007). It´s central component is a passive protein conducting channel, called SecY in gram-negative bacteria and Sec61p in eukaryotes. Translocation occurs during the synthesis of a protein at the ribosome (cotranslational translocation) or after it´s synthesis (posttranslational translocation).

During cotranslational translocation, the signal sequence is recognized by the signal recognition particle (SRP) as soon as it emerges from the ribosome. The binding of the SRP to its cognate receptor targets the ribosome to the SecY/61p channel complex. Segments of the nascent chain are then either transferred through the pore or, in the case of a membrane embedded protein, they exit through a lateral gate in SecY/61p into the lipid environment. The driving force for cotranslational translocation is provided by the elongation of the polypeptide inside the ribosome.

In posttranslational translocation, synthesis of the protein is completed prior to translocation and the protein is kept in an unfolded state by chaperones. In this case, the driving force of transport is provided by the cytosolic ATPase SecA (in bacteria) or BiP (in eukaryotes), which is located in the lumen of the ER. During the ATP hydrolysis cycle, SecA is thought to push segments of the polypeptide chain through SecY (Economou & Wickner, 1994). BiP, on the other hand, acts as a molecular ratchet by grabbing segments of the polypeptide as soon they emerge from the luminal side of the channel (Matlack et al., 1999).

In spite of extensive research performed on both pathways of translocation, the exact molecular mechanism of protein transport across membranes remains unclear. However, substantial progress has been made in the recent years through the efforts of structural biology. Especially in the case of bacterial posttranslational translocation, X-ray structures of the channel in detergent and structures of SecA in different conformations have provided new insights into the process. The highlight of these studies is a structure that shows SecA in direct interaction with SecY.

1.2 The structure of SecYEG

The bacterial SecY-complex is composed of a large α-subunit, called SecY, and two smaller β- and γ-subunits, called SecE and SecG, respectively. X-ray structures of the archaeal analogues of SecYEG show that the complex roughly has the shape of an hourglass, with a cytoplasmatic and a periplasmatic funnel (Tsukazaki et al., 2008; van den Berg et al., 2004; Zimmer at al., 2008). The pore itself is formed by the ten transmembrane helices (TMs) of SecY. Viewed from the cytosol, it can be divided into two halves, TMs 1 to 5 and TMs 6 to 10, clamped together by SecG. The pore ring, the central constriction of the hourglass, consists of hydrophobic amino acids that project their side chains radially into the lumen of the channel and form a tight seal for small molecules around the polypeptide during translocation (figure 1).

In the inactive state, the periplasmatic funnel is fully plugged by the interaction of a short helix (TM 2a) with the pore ring. During translocation the polypeptide chain inserts as a loop into the SecY-channel, with the signal sequence intercalating between transmembrane helices 2b and 7. The binding of the signal sequence triggers the opening of the channel by the deposition of the plug and the following segments of the polypeptide chain are moved through the pore (figure 1A). Transmembrane helices of a newly synthesized membrane protein can exit the channel laterally through the interface between TM 2b and TM 7, referred to as the lateral gate (figure 1B). At some point during or after translocation, the signal sequence is cleaved off.

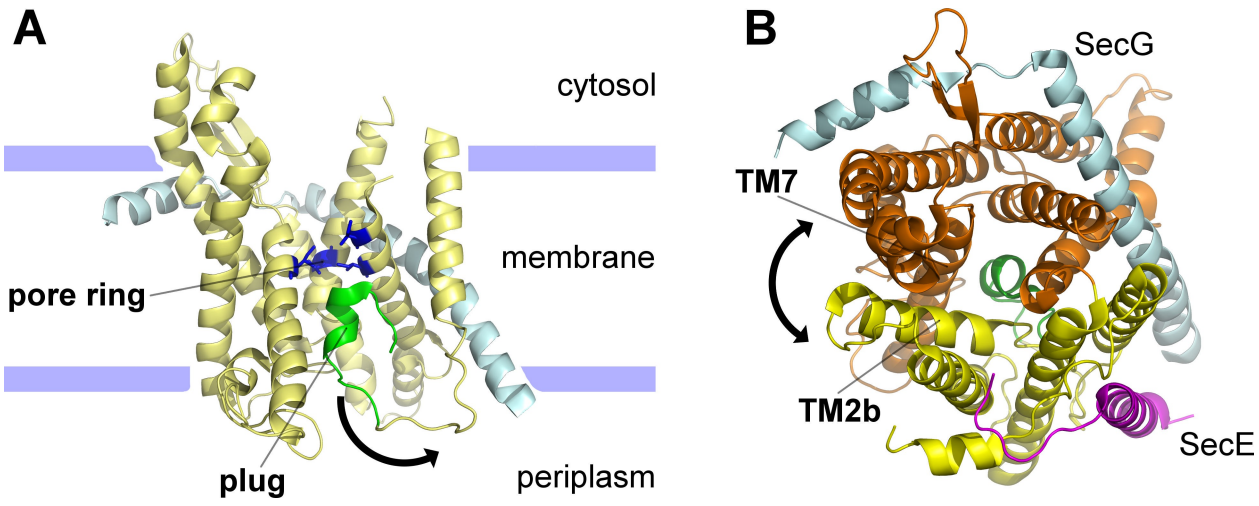


Figure 1. Struture of the SecYEG complex from *Methanococcus Jannaschii* (PDB accession: 1RH5). (A) Side view of a section through the center of the SecY-complex. SecY is displayed in yellow, SecG in cyan. Residues of the pore ring are shown in blue as stick models. The short plug helix (transmembrane helix 2a) is coloured green. The movement of the plug helix during translocation is indicated by the arrow. **(B)** Cytosolic view of the SecY-complex. The two halves of the Y-subunit are shown in yellow (TM1-5) and brown (TM6-10), respectively. SecG is shown in cyan and SecE in magenta. During translocation, the signal sequence intercalates between TM7 and TM2b. The movement of the two halves of SecY during the lateral release of transmembrane helices into the lipid bilayer is indicated by the arrow.

1.3 The structure of SecA

The ATPase SecA mediates posttranslational translocation of a polypeptide chain by a pushing mechanism that is coupled to the hydrolysis of ATP (Economou & Wickner, 1994). SecA consists of a polypeptide crosslinking domain (PPXD), two nucleotide binding domains (NBD1 and NBD2), a helical wing domain (HWD), and a helical scaffold domain (HSD) (Hunt et al., 2002). The latter consists of a long helix and two shorter ones that form a two-helix finger (Zimmer et al., 2008). NBD1 and NBD2 bind ATP at their interface and move relative to one another during the ATP-hydrolysis cycle (Hunt et al., 2002). X-ray structures of *Bacillus subtilis* SecA (Hunt et al., 2002; Osbourne et al., 2004) show the protein in different states of nucleotide binding: the "closed", nucleotide-free and the "open", ADP-bound conformation, described by a large body rotation of the PPXD from the HWD towards the NBD2 (figure 2A and B, respectively). In a recent structure of *Thermotoga maritima* SecA bound to SecY and ADP/BeF_x (Zimmer et al, 2008), a transition state analogue of ATP, this movement is even more pronounced, with the PPXD directly interacting with

NBD2 (figure 2C). Most importantly, the SecY bound structure shows that a clamp formed by this movement is perfectly aligned with the channel and that the two-helix finger inserts into the entrance of the pore (figure 2D).

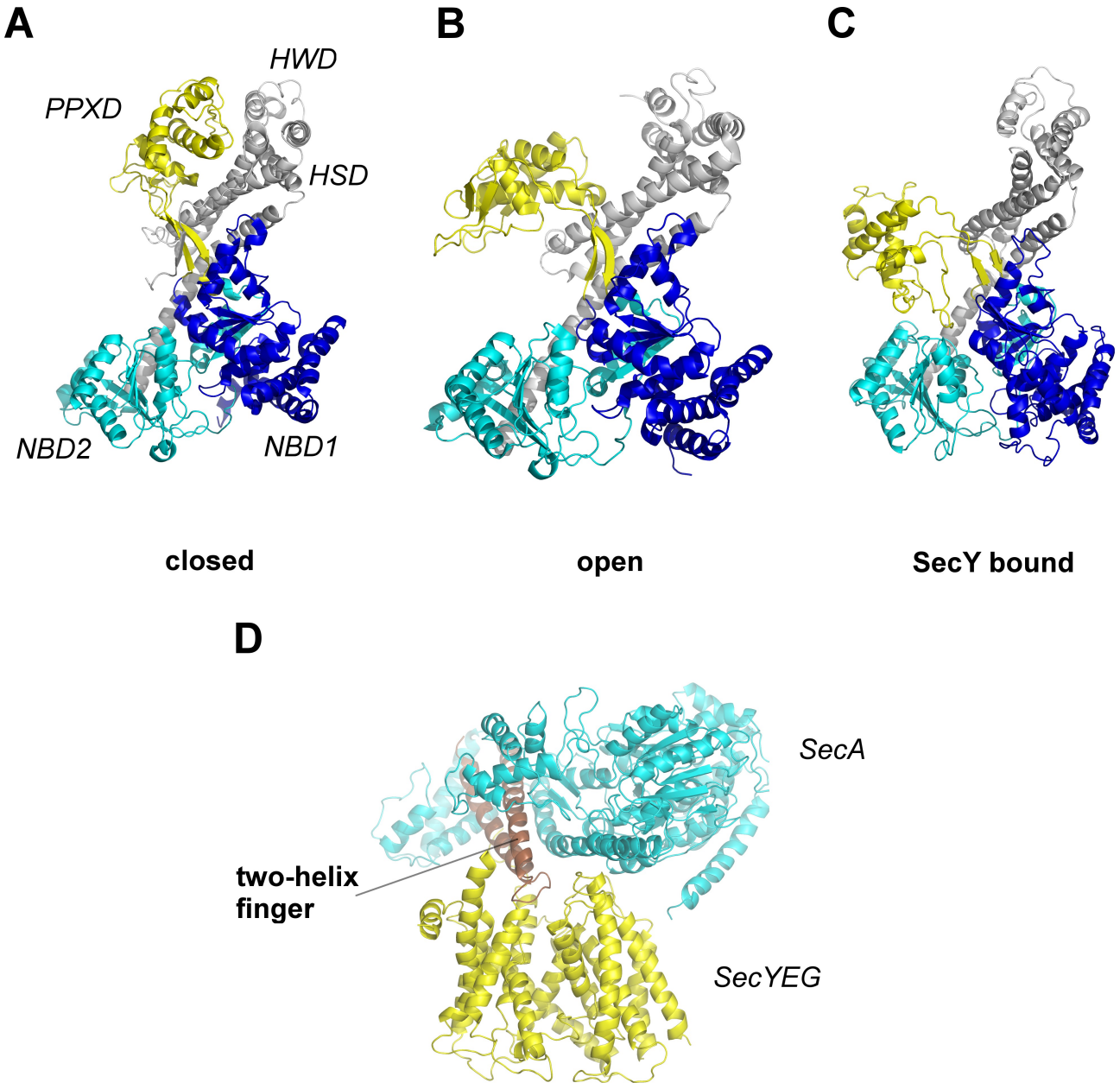


Figure 2. Different conformations of SecA. (A) "Closed" conformation, represented by the structure of *Bacillus subtilis* SecA in a nucleotide free state (PDB accession: 1M6N). The domains of SecA are shown in different colours: the polypeptide binding domain (PPXD) in yellow, the helical wing domain (HWD) and helical scaffold domain (HSD) in grey, and the nucleotide binding domains 1 and 2 (NBD1 and NBD2) in blue and cyan, respectively. **(B)** "Open" conformation of *Bacillus subtilis* SecA in the ADP-bound conformation (PBD accession: 1TF2). Colour coding is the same as in A. **(C)** Conformation of *Thermotoga maritima* SecA bound to SecY and ADP/BeF_x (PBD accession: 3DIN). Colour coding is the same as in A. **(D)** Structure of the SecY/SecA complex in a side view. SecA (cyan) lies flat on top of SecYEG (yellow), parallel to the membrane. In this conformation, the two-helix finger (brown) is inserted into the entrance of the pore.

1.4 Model of SecA-mediated translocation

The conformation of the complex lead to the proposal of a new model of SecA-mediated protein translocation (Zimmer et al., 2008). According to this model, the clamp formed by parts of the PPXD, HSD and NBD2 would capture the polypeptide and the described movement of the PPXD towards the NBD2 would close the clamp, positioning the polypepitde chain above the entrance of the channel. Conformational changes at the interface of NBD1 and NBD2 due to the hydrolysis of ATP would then be translated into an up-and-down movement of the two-helix finger. The loop that connects the helices of the two-helix finger would form intermediate contacts with the translocating polypeptide chain, providing enough friction to drag segments of the chain into the channel. To avoid reversal of the pushing movement, the polypeptide chain needs to be held in position while the two-helix finger is resetting for another powerstroke. Besides aligning the translocation substrate to the entrance of the SecY pore, the role of SecA´s clamp would be to prevent this reversal by tightening around the polypeptide in the ADP bound state, when the two-helix finger is supposedly resetting, and widening in the ATP bound state to allow free movement of the polypeptide (figure 3). Tightening and widening of the clamp would result from an increase and decrease of contacts of the PPXD with NBD2 in response to ATP-hydrolysis.

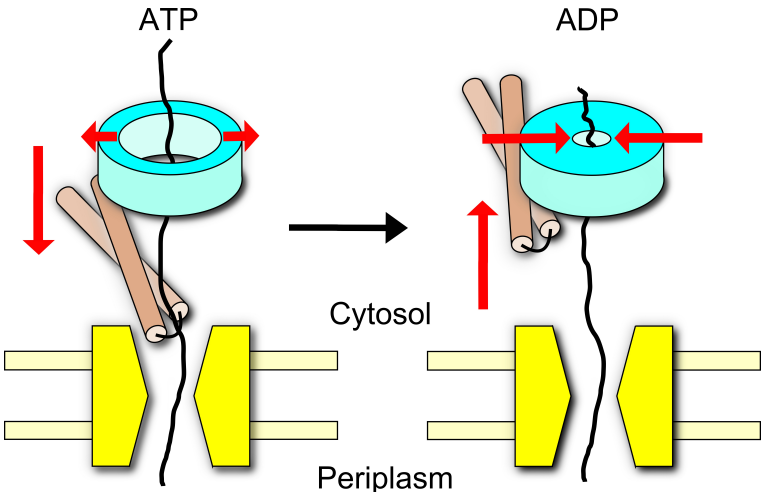


Figure 3. Proposed model of translocation by SecA. Two states of ATP hydrolysis are depicted. In the ATP-bound conformation (left), SecA´s two-helix finger moves into the channel dragging the polypeptide chain with it. In this state, the clamp is widened to allow the passage of the polypeptide. In the ADP-bound conformation (right), the two-helix finger resets for another power stroke while the clamp tightens around the polypeptide thus holding it in position.

Crosslinking experiments have shown that the loop indeed contacts a translocating polypeptide chain and that a conserved tyrosine at the tip of the loop is essential for translocation (Erlandson et al., 2008b). However in the structure, the mouth of the clamp is almost fully closed, leaving little space for a translocating protein chain. This emphasizes the need for experimental testing of the proposed model.

Recent spin-label perturbation experiments have shown that residues inside the clamp are in contact with the polypeptide chain, but several positions outside the clamp also showed interactions (Cooper et al., 2008). Furthermore, a cysteine positioned inside a translocation substrate can be crosslinked to cysteines placed at virtually all positions on the surface of SecA, representing a rather promiscuous interaction between SecA and its substrate in solution (unpublished results, Rapoport lab). These experiments were performed with non-translocating SecA.

In the current study, a doublecrosslinking approach was applied to determine the pathway of a polypeptide chain during posttranslational translocation. The data shows that the polypeptide passes in a stretched conformation from SecA´s clamp down to the tip of the two-helix finger and into the pore of the SecY-channel. The results are in accordance with the model of translocation in which

the clamp positions the polypeptide chain while movements of the two-helix finger push it into the channel.

In addition, an attempt was made to distinguish between an active and a passive role of SecA´s clamp during translocation by looking at the backsliding of different segments of the substrate. The data show that the whole polypeptide chain slides back when ATP is depleted, as seen by the modification of two substrate cysteines initially positioned in SecY´s pore and SecA´s clamp. Unfortunately, these results are inconclusive and neither confirm nor falsify any of the proposed roles.

2 Results

2.1 Experimental strategy

The pathway of a translocating polypeptide chain in SecA was determined using a disulfide bridge crosslinking approach. Disulfide bridge formation is often assessed to study protein interactions because two cysteines need to be in close contact to form a disulfide bond. Since SecA interacts unspecifically with polypeptide chains in solution, a crosslinking strategy with two cysteines in the translocation substrate had to be applied (figure 4). Crosslinking of a first cysteine to a cysteine placed in the pore ring of SecY would ensure that the substrate is engaged and only accessible to domains of SecA that really participate in translocation. A second cysteine could then be used to scan for interactions with cysteines placed at various positions in SecA. The resulting pattern of doublecrosslinks should describe the pathway of the polypeptide chain in SecA.

To increase the probability of disulfide bond formation, the translocation substrate needs to be arrested in an intermediate state of translocation, during which a bulky group at the C-terminus of the substrate prevents its complete translocation. Translocation intermediates have been generated using a C-terminal disulfide bonded peptide loop (Erlandson et al., 2008a) or a t-RNA (Erlandson, 2008b). While the disulfide loop couldn´t be used for a crosslinking assay due to the additional number of cysteines, the t-RNA also was found to be unsuitable for the current study. A t-RNA covalently attached to the C-terminus of a polypeptide chain is generated by the expression of a truncated mRNA (lacking a stop codon) and release of the ribosome from the resulting ribosome-nascent chain complex by urea treatment. Crosslinking experiments performed with this construct found that the loop at the tip of the two-helix finger was in close contact with residues inside the substrate ultimately preceding the t-RNA. This indicates that the t-RNA had moved all the way through SecA (Erlandson, 2008b), probably because of denaturation by the urea treatment, and provided a block for translocation only at the entrance of SecY.

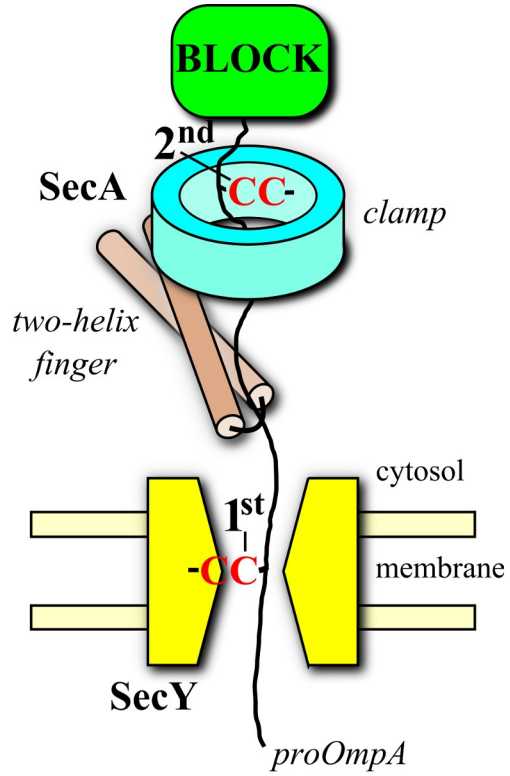


Figure 4. Crosslinking strategy employing a translocation intermediate. Two cysteines were introduced into the translocation substrate proOmpA (shown as a black chain), one for crosslinking to a cysteine placed into the pore ring of SecY ("1st") and the other for crosslinking to a cysteine placed at various positions inside of SecA ("2nd"; shown is a cysteine that has been placed into the clamp). A bulky domain at the C-terminus of proOmpA ("BLOCK", shown in green), prevents the substrate from moving all the way through the channel. SecA ´s two-helix finger and clamp are shown in brown and cyan, respectively. The SecY-complex is shown in yellow.

2.2 Generation of a stable translocation intermediate

Investigation of the pathway of the polypeptide chain would require the formation of a translocation intermediate, in which the C-terminus of the substrate has not moved all the way through SecA. Therefore, new fusion proteins were designed that introduced potentially blocking groups to the C-terminus of a fragment of proOmpA (pOA), a substrate of posttranslational translocation (figure 5). To test if a translocation intermediate could be established with the new constructs, the fusion proteins were synthesized *in vitro* in the presence of ^{35}S-methionine and incubated with SecA, ATP,

and proteoliposomes containing the purified SecY complex. After the translocation reaction, proteinase K was added to digest all proteins that were not in the protective environment of the lumen of the proteoliposomes, i.e. that had not been translocated. While a fully protease protected substrate would be expected of complete translocation, partial protection of the substrate would indicate that the fused domain was exposed to proteinase K and thus had not passed through the SecY channel. The pattern of proteolysis was analyzed by SDS-PAGE and autoradiography (figure 6).

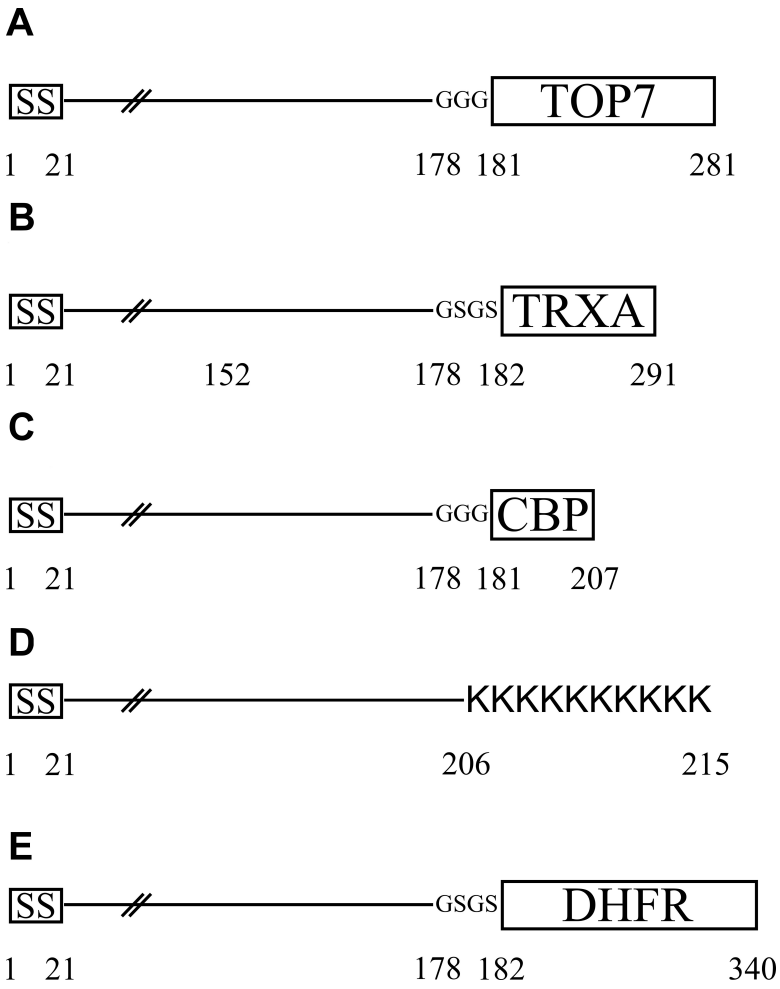


Figure 5. Schemes of the different pOA fusion constructs. (A) pOA-TOP7. The TOP7-protein is fused C-terminally to the first 177 amino acids of pOA. Amino acid positions in the construct are indicated. SS represents pOA´s signal sequence (amino acids 1-21), GGG is a linker sequence. **(B)** pOA-TRXA. Same as in (A) but with *Escherichia coli* thioredoxin (TRXA) fused to the C-terminus of the pOA-fragment. GSGS is a linker sequence. **(C)** pOA-CBP. Same as in (A) but with a calmodulin binding peptide (CBP) fused to the C-terminus of the pOA-fragment. **(D)** pOA-10K. A chain of ten lysine residues is fused to the C-terminus of the first 205 amino acids of pOA. **(E)** pOA-DHFR. Same as in (A) but with *Escherichia coli* dehydrofolate reductase (DHFR) fused to the first 177 amino acids of pOA.

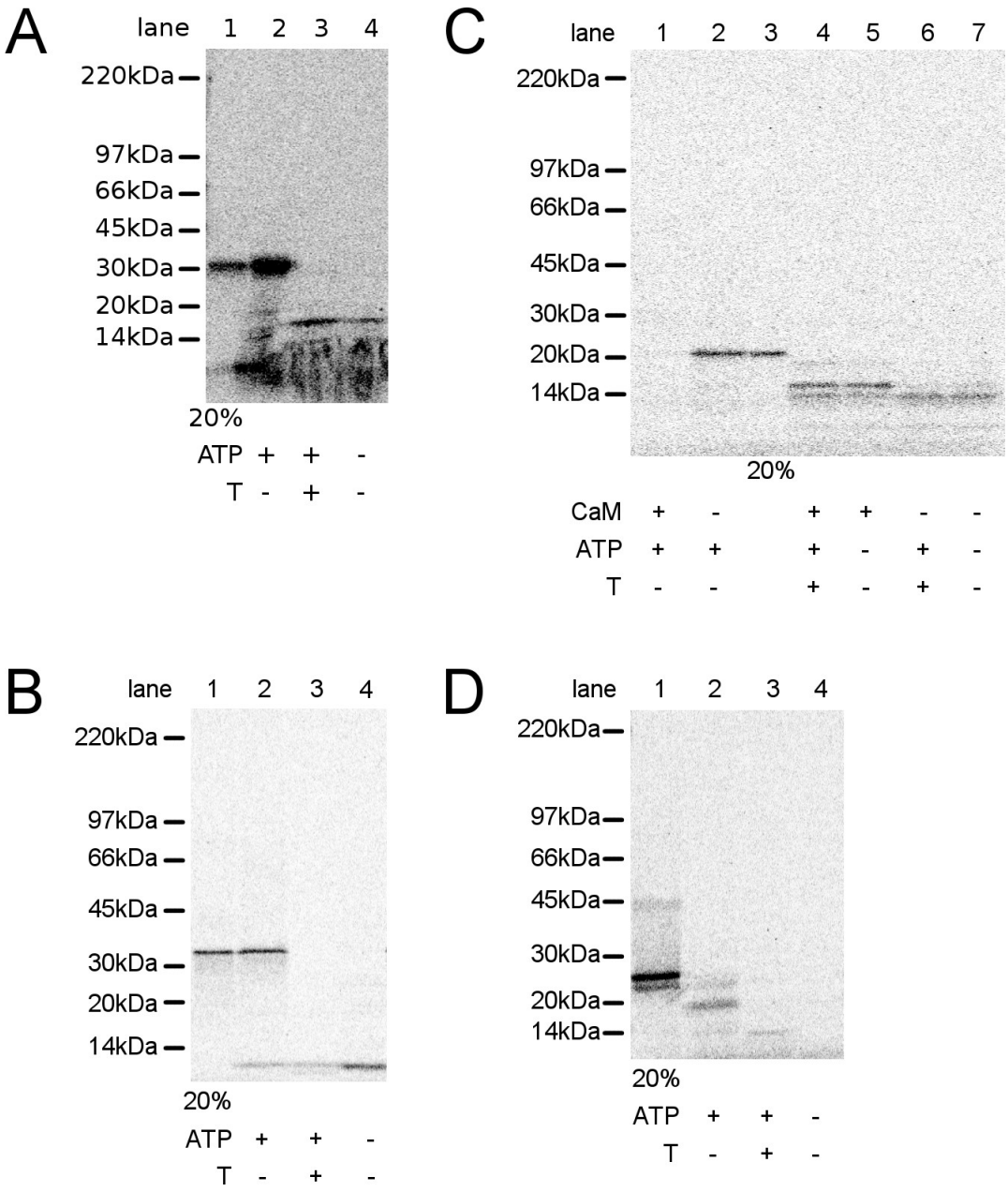


Figure 6. Stability of different translocation intermediates. (A) Protease protection pattern pOA-TOP7. pOA-TOP7 was synthesized *in vitro* and incubated with SecA and proteoliposomes containing purified SecYEG complex in the presence or absence of ATP. After translocation, samples were treated with proteinase K in the absence or presence of Triton X-100 ("T") and analyzed by SDS-PAGE and autoradiography. 20% of the input material was loaded onto lane 1. Approximate molecular weigths are indicated. **(B)** Protease protection pattern of pOA-TRXA. As in (A) but with the pOA-TRXA fusion. **(C)** Protease protection pattern of pOA-CBP. The assay was performed with the pOA-CBP as in (A) but in the presence of Ca^{2+} and in the presence or absence of calmodulin ("CaM"). 20% of the input material was loaded onto lane 3. **(D)** Protease protection pattern of pOA-10K. As in (A) but with the pOA-10K fusion.

pOA-Top7, the fusion construct of the first 177 amino acids of pOA and TOP7, a *de novo* designed globular protein fold, reported to be extremely stable to heat- and urea denaturation (Kuhlman et al., 2003) was completely translocated in the presence of ATP as indicated by the same molecular weigths of the translocated substrate and the undigested input material (figure 6A, lane 2 vs. 1). No protection could be observed when the proteoliposomes had been disruped by the detergent Triton X-100 or in the absence of ATP (figure 6A, lanes 3 and 4). The observed bands below 20kDa in presence of detergent and absence of ATP are likely due to protease resistance of the TOP7-domain.
A similar pattern was obtained with a fusion of pOA and *Escherichia coli* thioredoxin, pOA-TRXA (figure 6B). Thioredoxin, fused to a signal sequence has been shown to inhibit phage display due to a block of translocation (Steiner et al., 2006). Nevertheless it could not prevent the new construct from being completely translocated into the vesicles (figure 6B, lane 2 vs. 1).
Another strategy employed the interaction of calmodulin to a calmodulin binding peptide (CBP), fused to the C-terminus of pOA (pOA-CBP, figure 6C). The high specificity and affinity of calmodulin to CBP in the presence of Ca^{2+} can be used for the purification of recombinant proteins (Stofko-Hahn et al., 1992). However, upon addition of calmodulin to the reaction, translocation was completely inhibited (figure 6B, lane 2 vs. 1), probably due to an interaction of calmodulin with pOA´s signal sequence.
Two fusion proteins, pOA with a chain of ten lysines at the C-terminus (pOA-10K) and pOA with a C-terminal dehydrofolate reductase (pOA-DHFR), were found to successfully stall translocation (figure 6D and figure 7).
It has been reported previously that a chain of positively or negatively charged amino acids at the C-terminus prevents a polypeptide chain from being completely transported through the channel during SecA-mediated translocation (Nouwen et al., 2009), although the reason for this is unclear. The finding could be reproduced as seen in figure 6D by a shift of the substrate band towards a lower molecular weight as compared to the full length fusion protein (lane 2 vs. 1).
Dehydrofolate reductase (DHFR) is stabilized when bound to its substrate analogue methotrexate (Arkowitz et al., 1993). For this reason, DHFR fusions have been used to investigate import of mitochondrial proteins (e.g. Mukhopadhyay et al., 2004). The protease protection as described above was complicated by the fact that methotrexate-stabilized DHFR was resistant to proteolysis even in the presence of Triton X-100 and that the pOA-segment had approximately the same molecular size as the DHFR domain (figure 7, lane 2 vs. 3). To test if DHFR could block translocation, methotrexate was removed by sedimentation and resuspension of the proteoliposomes

after the intermediate had been formed and translocation was assessed by proteinase K digestion followed by SDS-PAGE and autoradiography. Removal of methotrexate resulted in the protection of the full length pOA-DHFR in the absence but not in the presence of detergent (figure 7, lane 4 vs. 5). On the other hand, when methotrexate was added back to the reaction, the protected fragment reappeared in the presence and absence of Triton X-100 (figure 7, lane 6 vs. 7). This shows that the DHFR-domain is stable enough to generate a translocation intermediate in the presence but not in the absence of methotrexate.

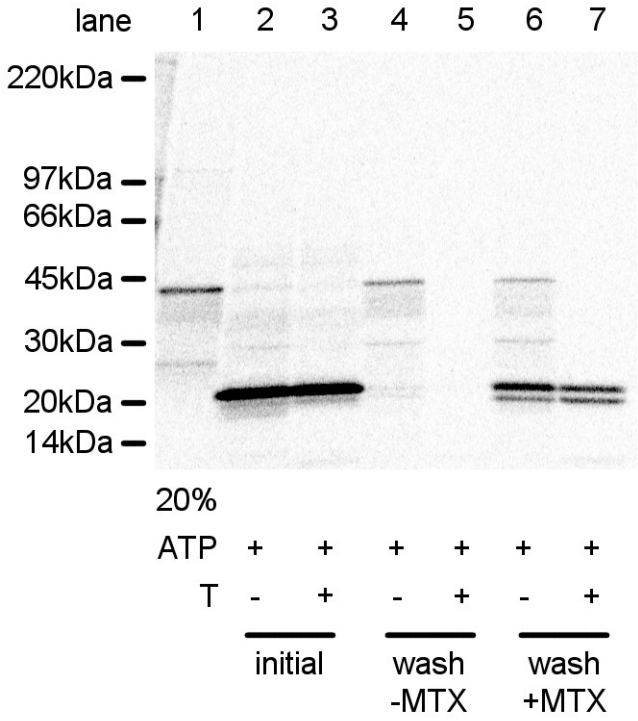


Figure 7. Generation of a translocation intermediate with the pOA-DHFR fusion. *In vitro* synthesized pOA-DHFR was incubated with SecA, ATP, proteoliposomes containing purified SecYEG and methotrexate ("MTX"). After the translocation reaction, two samples were taken and digested with proteinase K in the absence or presence of Triton X-100 ("T") ("initial"). The remainder of the reaction was subjected to sedimentation and resuspension in reaction buffer with or without methotrexate ("wash +MTX" and "wash -MTX"). After resuspension, these samples were digested with proteinase K in the absence or presence of Triton X-100. Results were analyzed by SDS-PAGE and autoradiography. Lane 1 was loaded with 20% of the input material. Approximate molecular weights are indicated.

2.3 Interactions of pOA-10K with Secy and SecA

Preliminary insights into the interaction of a polypeptide chain with the translocation components could be achieved by investigating the crosslinking behavior of the stalled pOA-10K substrate. To show that the substrate is captured inside the SecY channel, single cysteines were introduced at various positions into pOA-10K and the substrates were synthesized *in vitro* in the presence of ^{35}S-methionine. The translation products were then incubated with cysteine free SecA, ATP and proteoliposomes containing purified SecY complex with a single cysteine in the pore ring of SecY (position 282). After intermediate formation, disulfide crosslinking was induced by addition of the oxidant copper phenanthroline. The crosslinking products were separated by non-reducing SDS-PAGE and autoradiography (figure 8). The results show that the substrate is indeed translocated through the pore and that it can be crosslinked over a long range of residues (tested positions 184 to 199). Disulfide bridge formation was only seen in the presence of ATP (figure 8, odd vs. even numbered lanes), when the ATPase SecA is in its active state. Interestingly, crosslinking is almost completely absent beginning with the tested position 202, indicating that at least some segment of the polypeptide chain (with a total of 215 residues) is located far enough on the outside of the pore to be enganged with SecA. For the following experiments, pOA-10K with a cysteine at position 194 was used, which gave prominent crosslinking (figure 8A, quantification in figure 8B).

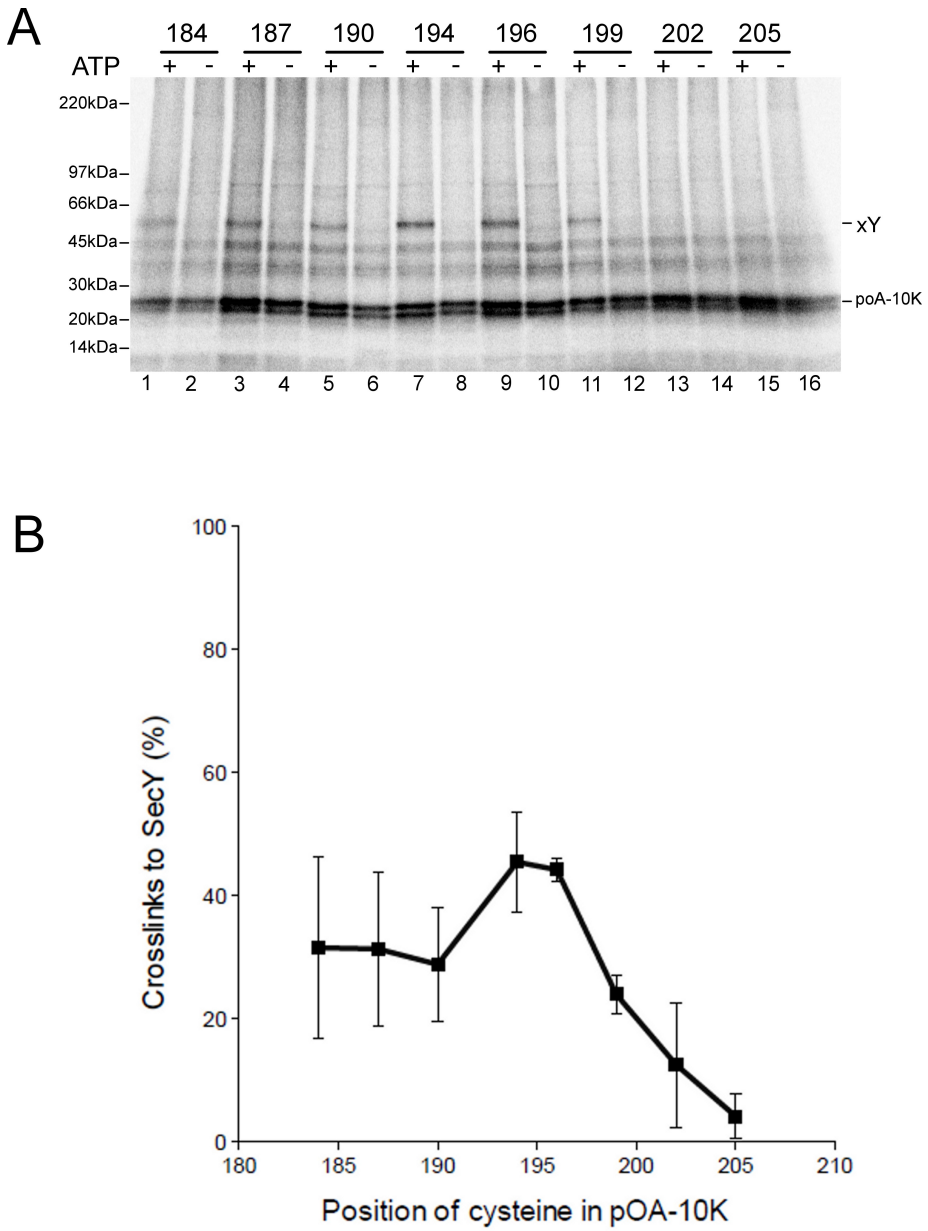


Figure 8. Interactions of pOA-10K with SecY. (A) *In vitro* synthesized and ^{35}S-labeled pOA-10K with a single cysteine at the indicated positons was incubated in the presence or absence of ATP with cysteine free SecA and proteoliposomes containing SecY-complex with a single cysteine at position 282. After intermediate formation, disulfide bridge formation was induced by oxidation and the crosslinked products were subjected to non-reducing SDS-PAGE and autoradiography. Uncrosslinked substrate and single crosslinked bands are indicated ("pOA-10K" and "xY", respectively). **(B)** Quantification of three experiments as performed in (A) (mean and standard deviation). Crosslinking efficiency is expressed as the ratio of counts of single crosslinked bands to the sum of counts of single crosslinked bands and uncrosslinked bands (xY/(xY+pOA-10K)).

To probe for interactions with SecA, a second cysteine was introduced at various positions into pOA-10K and tested for crosslinking with a single cysteine placed at a position inside the clamp of SecA (position 353). The substrates were synthesized *in vitro* and incubated with the described SecA mutant, ATP and proteoliposomes containing SecY complex with a cysteine in the pore ring (position 282). Crosslinking was induced by oxidation and the reaction products were subjected to non-reducing SDS-PAGE and autoradiography (figure 9A). Double crosslinks to SecA and SecY were observed from positions 210 to 214 in the presence but not in the absence of ATP. Single crosslinks to SecY and SecA were yielded at all positions. However, the single crosslinks to SecA were not ATP dependent, representing the unspecific interaction of SecA and the translocation substrate in solution.

Crosslinking of pOA-10K with SecA´s two-helix finger was also tested (figure 9B). The most efficient doublecrosslinks were observed from position 201 to 204. These data indicate that during translocation, the polypeptide chain is indeed in contact with the clamp and the two-helix finger of SecA. Unfortunately, crosslinking efficiencies for the pOA-10K construct were very low and the intensities of crosslinked bands could often hardly be discerned from the background noise. This might be due to the fact that the polylysine chain (from position 206 to 215 in the pOA-10K construct) had to be interrupted in order to introduce the second cysteines and that this interruption decreased the blocking efficiency (data not shown).

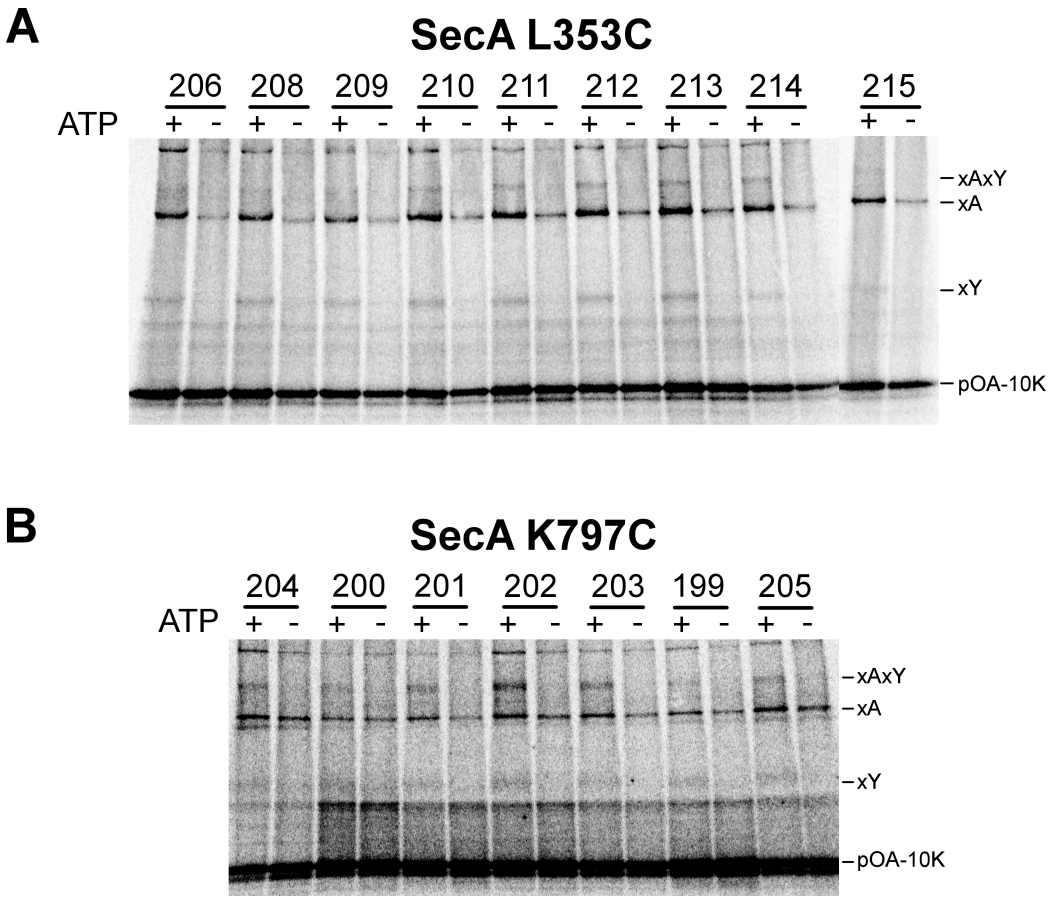


Figure 9. Interactions of pOA-10K with both SecY and SecA. (A) Interactions of pOA-10K with SecA´s clamp. pOA-10K with the first cysteine at position 194 and a second cysteine at the indicated positions was synthesized *in vitro* in the presence of ^{35}S-methionine and incubated with SecA bearing a single cysteine at position 353 and proteoliposomes containing SecY-complex with a single cysteine at position 282 in the presence or absence of ATP. Disulfide bridge formation was induced by oxidation and the crosslinked products were analyzed by non-reducing SDS-PAGE and autoradiography. Uncrosslinked pOA-10K, single crosslinks to SecY and SecA and double crosslinks to SecA and SecY are indicated ("pOA-10K", "xY", "xA" and "xAxY", respectively). **(B)** Interactions of pOA-10K with SecA´s two-helix finger. As in (A) but with SecA containing a single cysteine at position 797.

2.4 Interactions of pOA-DHFR with SecY and SecA

The crosslinking strategy described above was also applied for the pOA-DHFR construct. First, a position inside the new construct was determined that would yield the highest crosslinking efficiencies to the pore ring. Single cysteines were introduced into pOA-DHFR from position 140 to 161 and the *in vitro* synthesized substrates were incubated with cysteine free SecA, proteoliposomes containing SecY complex with a single cysteine in the pore ring (position 282C), ATP and methotrexate. After formation of a translocation intermediate, crosslinking was induced by copper phenanthroline and the products of the reaction were analyzed by non-reducing SDS-PAGE and autoradiography (figure 10A). Again, disulfide bridges to the channel could only be observed in the presence of ATP. Crosslinks occured over a wide range of amino acids (from 140 to 161), although the most prominent crosslinks were seen at positions 149 to 155 (figure 10A, quantification in figure 10B). For the following experiments, pOA-DHFR substrates with the first cysteine at position 152 were used.

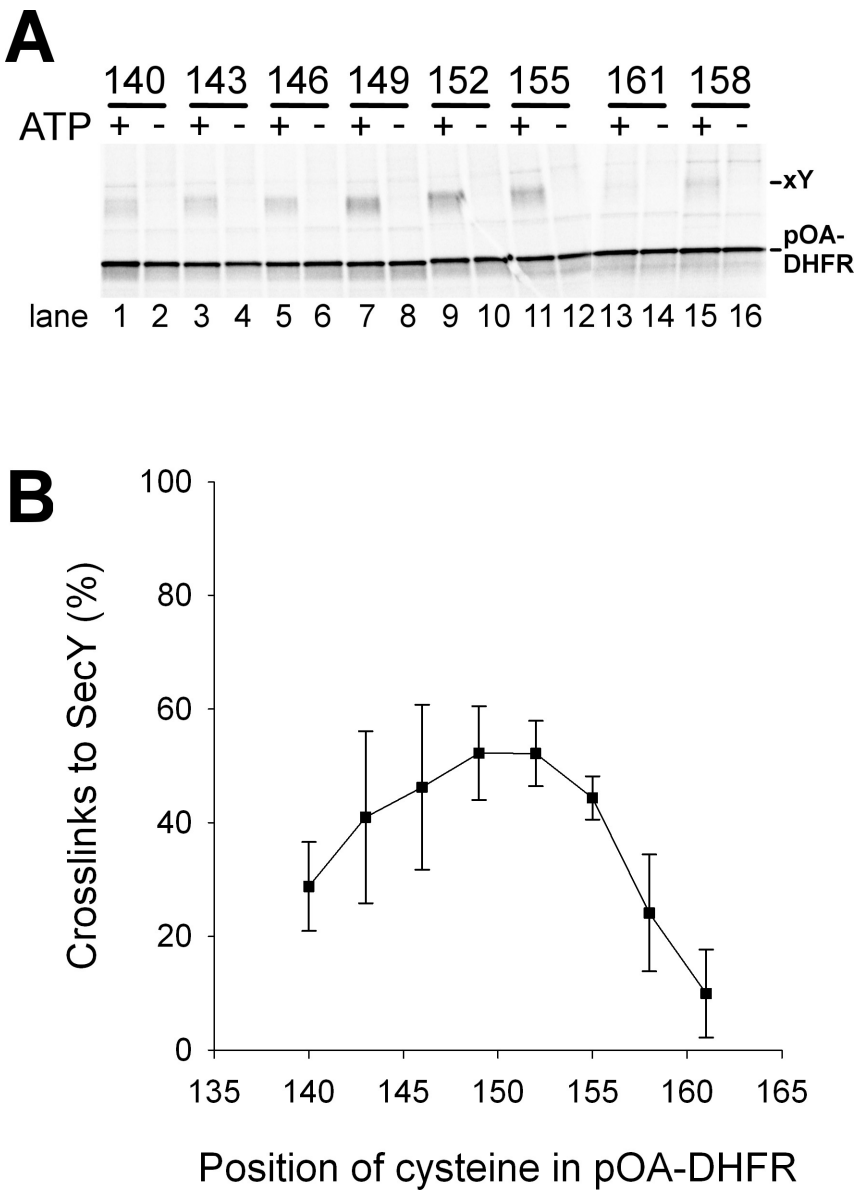


Figure 10. Interactions of pOA-DHFR with SecY. (A) *In vitro* synthesized and ^{35}S-labeled pOA-DHFR with a single cysteine at the indicated positons was incubated in the presence or absence of ATP with cysteine free SecA, proteoliposomes containing SecY-complex with a single cysteine at position 282 and methotrexate. After intermediate formation, disulfide bridge formation was induced by oxidation and the crosslinked products were subjected to non-reducing SDS-PAGE and autoradiography. Uncrosslinked pOA-DHFR and single crosslinked bands are indicated ("pOA-DHFR" and "xY", respectively). **(B)** Quantification of three experiments as performed in (A) (mean and standard deviation). Crosslinking efficiency is expressed as the ratio of counts of single crosslinked bands to the sum of counts of single crosslinked bands and uncrosslinked bands (xY/(xY+pOA-DHFR)).

Next, second cysteines were introduced into the substrate from position 157 to 184 (for orientation, see figure 5E) and tested systematically for crosslinking to a cysteine placed into SecA. The *in vitro* synthesized and ^{35}S-labeled substrates were incubated with the SecA mutants, proteoliposomes containing SecYEG with a single cysteine at position 282, ATP and methotrexate. After intermediate formation, cysteines were oxidized and crosslinking was assessed by non-reducing SDS-PAGE and autoradiography.

A first experiment showed that efficient doublecrosslinks could be yielded with a SecA mutant containing a cysteine in the membrane proximal part of the PPXD at position 269, which is part of SecA´s clamp (figure 11A). The most prominent doublecrosslinks with this residue were seen from position 165 to 171 in the substrate. At these positions more than 75% of the pOA-DHFR molecules that had been crosslinked to the channel also formed disulfide bonds to SecA (figure 11B). Again single crosslinks to the pore ring of SecY and to SecA were also seen, but only the single crosslinks to SecY were dependent on ATP. This indicates that SecA residue 269 is in the path of the translocating polypeptide chain.

The identity of the single crosslinked and doublecrosslinked bands was confirmed by immunoprecipitation. *In vitro* synthesized and ^{35}S-labeled pOA-DHFR with the second cysteine at position 165 was incubated with SecA(269C), proteoliposomes containing SecYEG with a cysteine in the pore ring (position 282), ATP and methotrexate. The translocation intermediate was formed and crosslinking induced by oxidation. The crosslinked products were then loaded onto protein G beads bound to antibodies directed against SecA or SecY. Bound material was eluded and subjected to non-reducing SDS-PAGE and autoradiography (figure 11C). As expected, the doublecrosslinked band, containing SecA as well as SecY, was efficiently captured by both antibodies, while the single crosslinked band, containing only SecY, could only be captured by the SecY antibody (figure 11C, lane 5 vs. 7). The single crosslink to SecA was efficiently precipitated by the SecA antibody, but could also be captured by the antibody directed against SecY (figure 11C, lane 5 vs. 7). This is probably due to unspecific binding of pOA-DHFR with the antibody that was also seen with the uncrossslinked band. No bands were precipitated with protein G beads alone (figure 11C, lane 3).

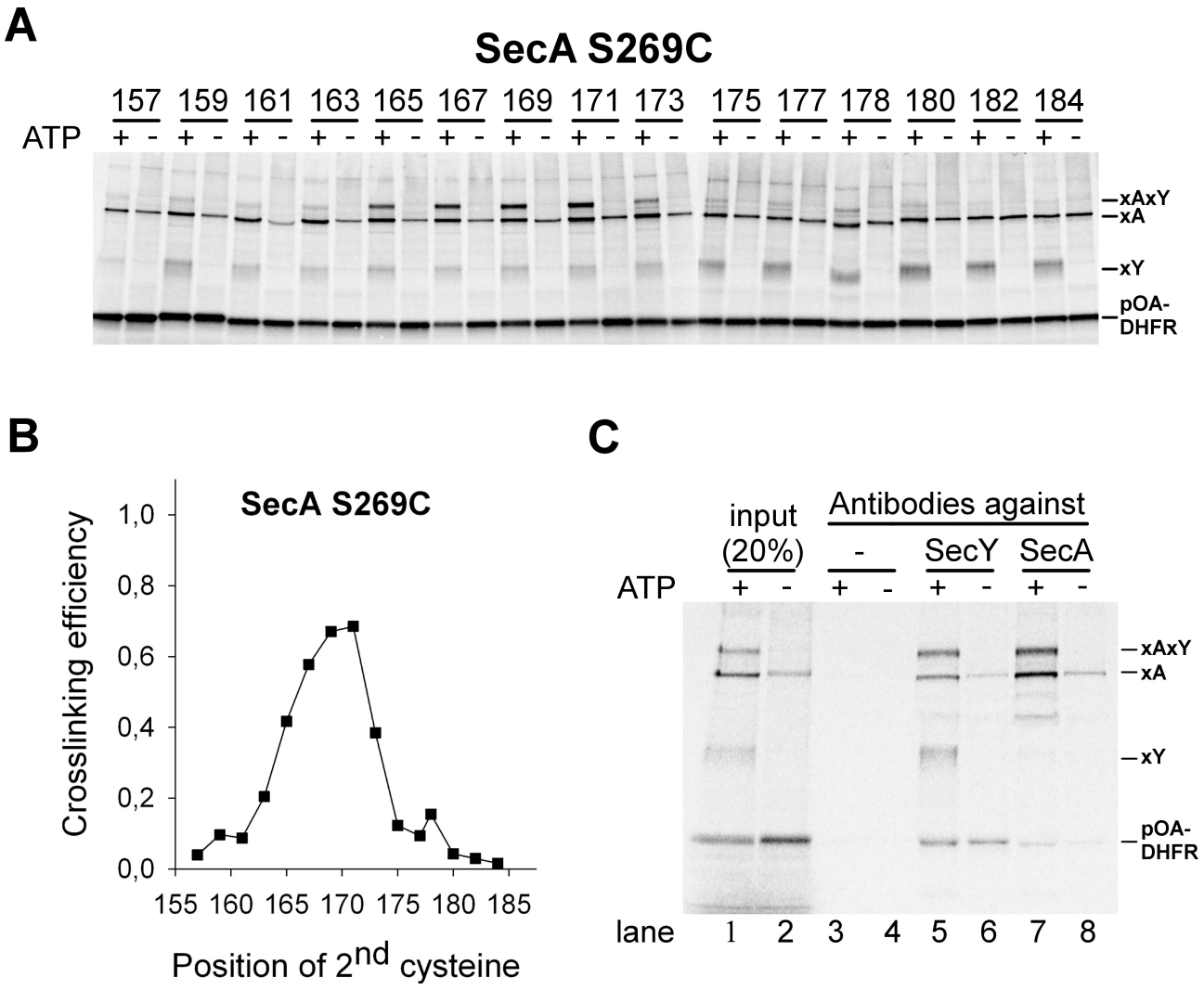
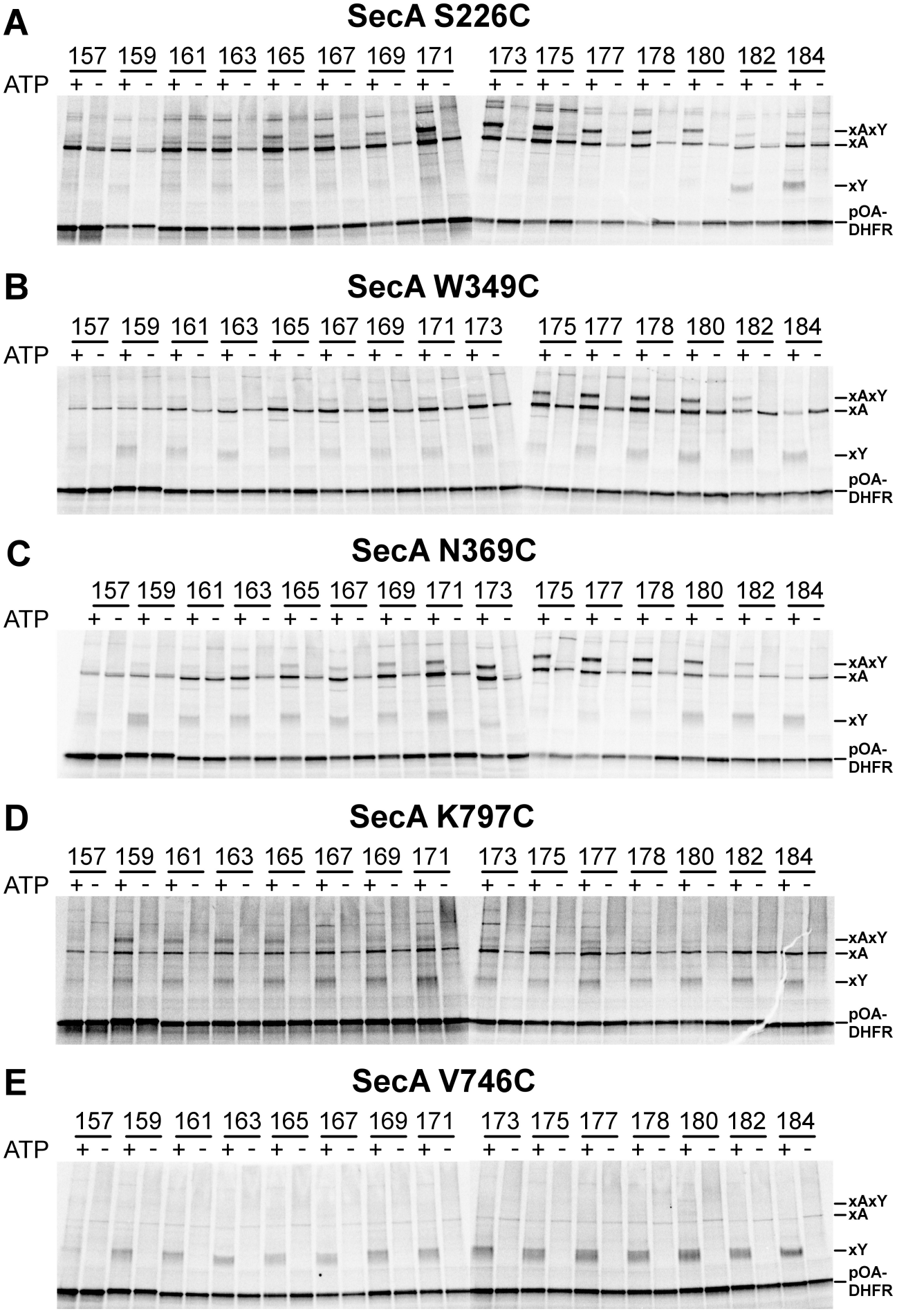


Figure 11. Interactions of pOA-DHFR with SecA´s clamp. (A) pOA-DHFR with a first cysteine at position 152 and a second cysteine at indicated positions was synthesized *in vitro* in the presence of ^{35}S-methionine. The substrate was incubated in the presence or absence of ATP with SecA containing a single cysteine at position 269, proteoliposomes containing SecY-complex with a single cysteine at position 282 and methotrexate. After intermediate formation, cysteines were oxidized and crosslinked products analyzed by non-reducing SDS-PAGE and autoradiography. Uncrosslinked pOA-DHFR, single crosslinks to SecY or SecA and double crosslinks to SecA and SecY are indicated ("pOA-DHFR", "xY", "xA" and "xAxY", respectively). **(B)** Quantification of the experiment performed in (A). Crosslinking efficiency is expressed as the ratio of counts of double crosslinked bands to the sum of counts of single crosslinked and doublecrosslinked bands (xAxY/(xY+xAxY)). **(C)** Immunoprecipitation of the crosslinked bands. Translocation was performed as in (A) but with pOA-DHFR conatining cysteines at positions 152 and 165. After oxidation, two samples were taken as input material and the remainder was precipitated with antibodies directed against either SecY or SecA. Samples were subjected to non-reducing SDS-PAGE and autoradiography.

Other cysteines were introduced at various positions inside of SecA and systematically probed for interactions with cysteines placed at positions 157 to 184 in pOA-DHFR. Efficient double doublecrosslinking was observed with all cysteines located inside or close to SecA´s clamp (positions 226, 349, and 369 are shown in figure 12 A,B and C, respectively; other positions tested are depicted in supplemental figure 1, A-D). Quantification shows that these positions yielded crosslinking efficiencies as high as 90% (figure 12, A-C; supplemental figure 1, A-D; quantification in figure 13, A-C, and supplemental figure 3, A-D). Especially residues 226, 369 (figure 12A and C) and 221 (supplemental figure 1A), which are located near the two β-sheets that connect the PPXD with NBD1 and NBD2, showed tight interactions with the translocating polypeptide chain.

In all cases, the intensities of the doublecrosslinked bands were dependent on the position of the second cysteine placed in pOA-DHFR and displayed a rather symmetrical peak at positions with the highest crosslinking efficiencies (see quantifications in figure 13A-D and supplemental figure 3A-D). This indicates that the observed crosslinking pattern represents the actual distance that pOA-DHFR spans between the pore ring of SecY and the tested positions in SecA. Furthermore, crosslinking of SecA cysteines was observed to a range of positions in pOA-DHFR, rather than single residues, so polypeptide chains might have acquired different conformations inside of SecA during translocation. In accordance with previous findings and the crosslinking experiments with the pOA-10K construct (see above), doublecrosslinks were also found with a cysteine placed at the tip of SecA´s two-helix finger (figure 12 D).

On the other hand, cysteines placed at random positions inside of SecA either didn´t give doublecrosslinks at all or they crosslinked only very weakly (position 746 is shown in figure 12E, other positions are shown in supplemental figure 2). While SecA contacts polypeptide chains rather unspecifically in solution, represented by the single crosslinks to SecA in the presence and absence of ATP, these findings demonstrate that only certain domains of SecA are engaged with the polypeptide chain during translocation. Some weak doublecrosslinking was observed with cysteines placed on the "back" of the PPXD (positons 306, 304 and 237; supplemental figure 2F, G, and H, respectively) and on the opposite site of the clamp in the HSD (position 665, supplemental figure 2E). The addition of copper phenanthroline might have caused denaturation of a small population of SecA molecules allowing non-specific interactions of pOA-DHFR with these positions. Denaturation of proteins as a result of treatment with copper phenanthroline or other oxidants has been reported in other studies (e.g. du Plessis et al., 2009). In any case, the data shows that the majority of pOA-DHFR molecules interacts specifically with SecA´s clamp and two-helix finger.


A
SecA S226C
157 159 161 163 165 167 169 171 173 175 177 178 180 182 184
ATP
+ - + - + - + - + - + - + - + - + - + - + - + - + - + - + -
xAxY
xA
xY
pOA-
DHFR
B
SecA W349C
157 159 161 163 165 167 169 171 173 175 177 178 180 182 184
ATP
+ - + - + - + - + - + - + - + - + - + - + - + - + - + - + -
xAxY
xA
xY
pOA-
DHFR
C
SecA N369C
157 159 161 163 165 167 169 171 173 175 177 178 180 182 184
ATP
+ - + - + - + - + - + - + - + - + - + - + - + - + - + - + -
xAxY
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pOA-
DHFR
D
SecA K797C
157 159 161 163 165 167 169 171 173 175 177 178 180 182 184
ATP
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xY
pOA-
DHFR
E
SecA V746C
157 159 161 163 165 167 169 171 173 175 177 178 180 182 184
ATP
+ - + - + - + - + - + - + - + - + - + - + - + - + - + - + -
xAxY
xA
xY
pOA-
DHFR

Figure 12. Probing interactions between pOA-DHFR and SecA. (A) Interaction of pOA-DHFR with SecA´s clamp. pOA-DHFR with a first cysteine at position 152 and second cysteines at indicated positions was synthesized *in vitro* in the presence of ^{35}S-methionine. The substrates were incubated with SecA containing a single cysteine at position 226, proteoliposomes containing SecY-complex with a cysteine at position 282, ATP and methotrexate. After intermediate formation, crosslinking was induced by oxidation. Crosslinked products were analyzed by non-reducing SDS-PAGE and autoradiography. Uncrosslinked pOA-DHFR, single crosslinks to SecY or SecA and double crosslinks to SecA and SecY are indicated ("pOA-DHFR", "xY", "xA" and "xAxY", respectively) **(B)** As in (A), but with a cysteine in the SecA clamp at position 349. **(C)** As in (A), but with a cysteine in the SecA clamp at position 369. **(D)** Interactions of pOA-DHFR with SecA´s two-helix finger. As in (A), but with a cysteine at position 797. **(E)** A cysteine placed outside of the clamp does not interact with the translocation substrate. As in (A) but with a cysteine at position 746 of SecA.

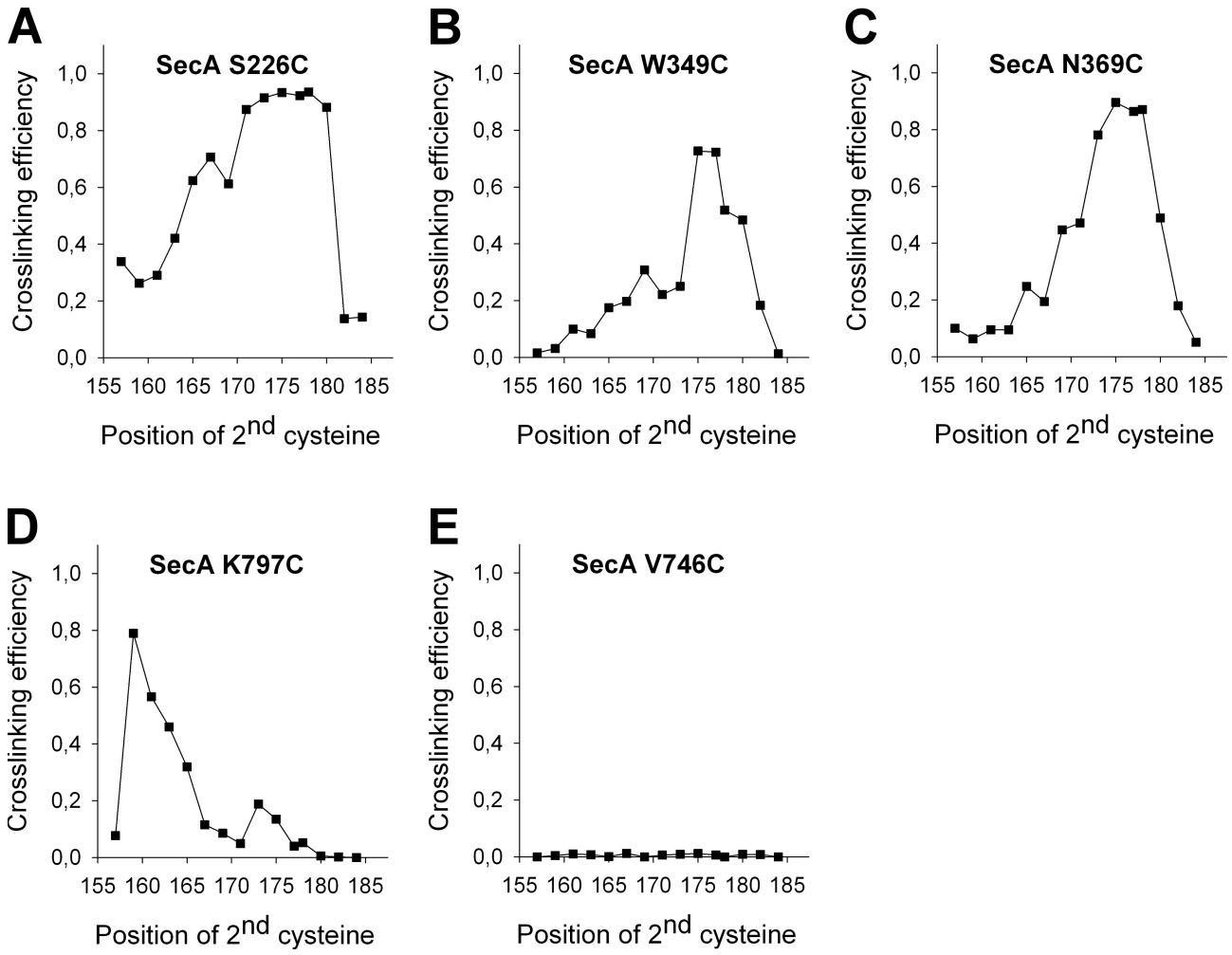

Figure 13. Quantification of the interactions of pOA-DHFR with SecA. (A)-(E) The experiments shown in figure 12 were quantified. Crosslinking efficiency is expressed as the ratio of counts of double crosslinked bands to the sum of counts of single crosslinked and doublecrosslinked bands (xAxY/(xY+xAxY)).

2.5 The pathway of a translocating polypeptide chain

Mapped into the *Thermotoga maritima* structure of the SecA-SecY complex, the crosslinking residues delineate the pathway of pOA-DHFR during translocation (figure 14). In a top view of SecA, tested positions inside the clamp describe the outlines of a tight conduit through which the polypeptide chain passes on its way to the SecY pore (figures 14A and B). The conduit is aligned with the entrance of the pore and at its end, the polypeptide contacts the tip of the two-helix finger. In a side view (figure 14C), positions in this passage that are most distant from the pore ring of

SecY (position 226) contact residues in pOA-DHFR that ultimately precede the DHFR domain (around position 177). Closer to SecY, positions 369 and 349 are in contact with residues 173 to 175 and residue 269 interacts with position 170 in pOA-DHFR. Finally, residues around 159 are in contact with position 797 at the tip of the two-helix finger, which is inserted into the SecY-pore.
Measuring direct distances in the structure reveals, that the peaks of crosslinking in pOA-DHFR to given residues in SecA seem to represent populations of polypeptide chains that are completely extended or close to an extended conformation. For example, pOA-DHFR requires seven amino acids to span the distance of 25Å between the cysteine in SecY´s pore ring and the cysteine in the two-helix finger, corresponding to 3.6Å per amino acid. Similarly with position 226 inside the clamp, the polypeptide chain requires a stretch of 25 amino acids (from position 152 to 177) to cover a distance of 66Å (2.6Å per amino acid).

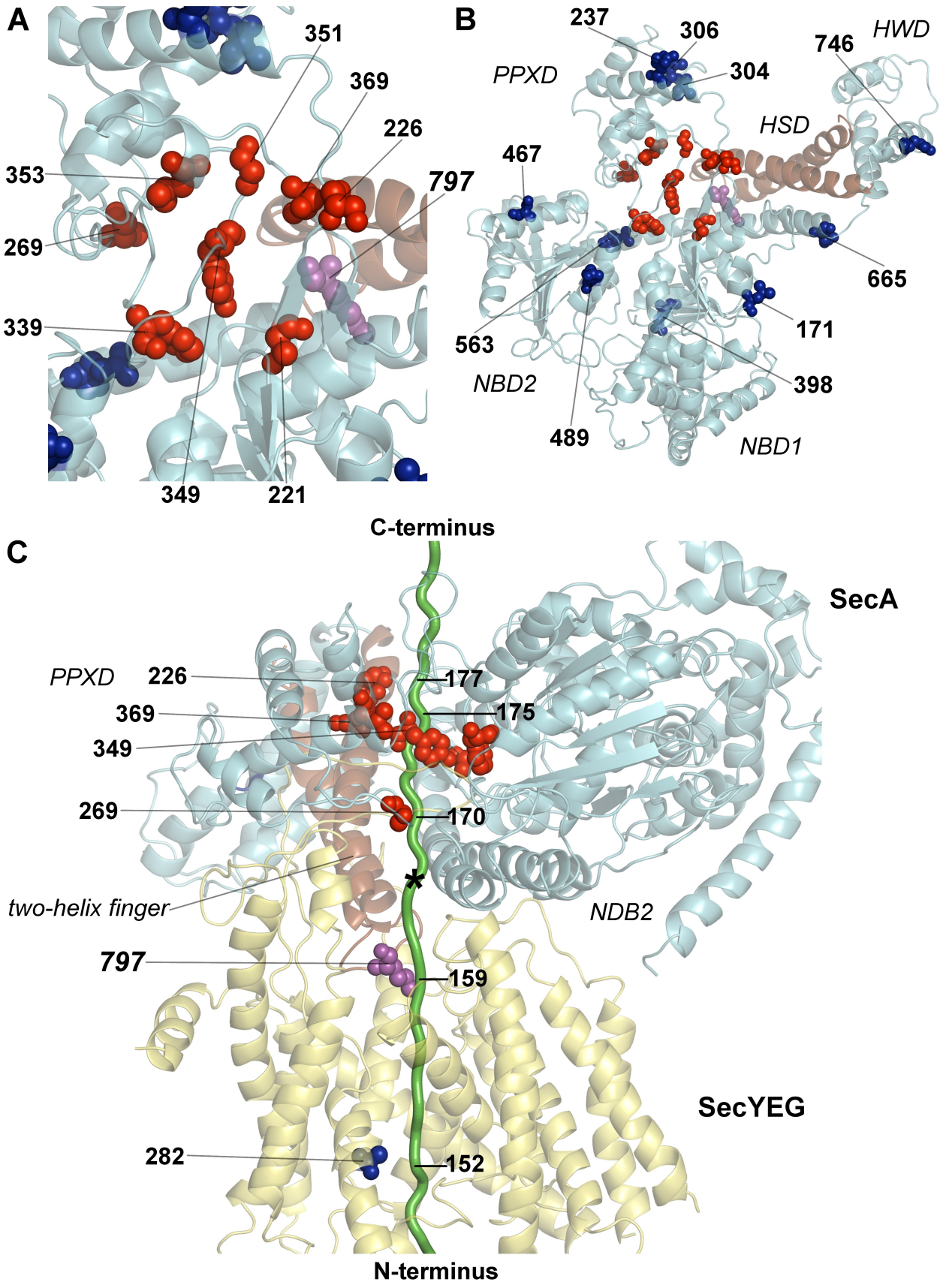
A
351
369
226
353
797
269
339
349
221
B
237
306
304
746
HWD
PPXD
HSD
467
665
171
563
NBD2
398
489
NBD1
C
C-terminus
SecA
PPXD
226
177
369
175
349
269
170
two-helix finger
NDB2
797
159
SecYEG
282
152
N-terminus

Figure 14. Interaction sites with pOA-DHFR mapped onto the *Thermotoga maritima* SecA and SecY structures. (A) View from the cytosol on SecA. Crosslinking residues in SecA´s clamp and two-helix finger are shown as red and magenta blalls, respectively. Residues that did not crosslink are shown as blue balls. The two-helix finger is shown in brown. **(B)** As in (A), but in a zoomed-out view. **(C)** Side view of the SecA-SecY structure with a modelled translocating polypeptide chain (shown in green). Positions inside SecA that were found to crosslink to pOA-DHFR are shown as red balls. The residue at the tip of the two-helix finger (position 797) is shown as magenta balls. The residue in the pore ring of SecY is shown as blue balls. The gap at the interface of SecA and SecY (see text) is marked by the asterisc.

2.6 Conformations of the polypeptide chain during backsliding

The model of translocation inferred from the SecA-SecY structure proposes that the conformational changes resulting from the hydrolysis of ATP at the interface of NBD1 and NBD2 would be translated into coordinated movements of SecA´s clamp and the two-helix finger. The states of the clamp would alternate between a widened conformation during ATP hydrolysis, allowing the free passage of the polypeptide chain, and a tightened conformation preventing its backsliding when SecA is bound to ADP (see introduction).

It has been observed that rendering SecA into the ADP-bound conformation by the depletion of ATP results in the backsliding of a stalled substrate out of the SecY pore, as seen by the disappearance of a protease-protected fragment with time (Erlandson et al., 2008a).

The model gives a prediction on the observed backsliding behavior of the polypeptide chain during translocation (figure 15). With the SecA clamp tightening in the ADP-bound conformation, the model would predict that backsliding had to occur through the interface of SecA and SecY. Indeed the translocating polypeptide chain has to pass an open cavity on its way to the pore (figure 14C). The cavity has a diameter of about 12Å and would be large enough to encompass two extended polypeptide chains. On the other hand, if the clamp´s role would be the mere positioning of the polypeptide during translocation, the backsliding had to occur through the clamp.

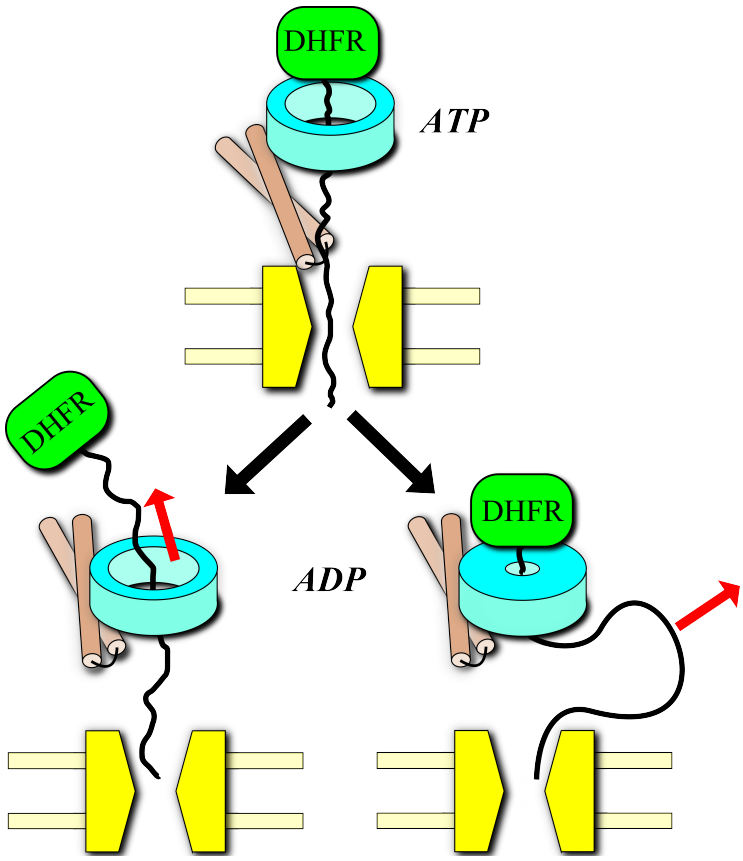

Figure 15. Two possibilities of backsliding of a polypeptide chain when SecA is in its ADP-bound state. On the left, the polypeptide would move back through the clamp. On the right, it would loop out sideways between SecA and SecY.

An assay was established to test these predictions. Single cysteines were placed into the substrate pOA-DHFR either at a position predicted to be inside the SecY pore (position 152) or inside the clamp (position 171) in the pOA-DHFR construct. In the translocation indermediate, these positions would be protected from modification by the 5kDA cysteine capping agent methyl-polyethyleneglycol-maleimide 5000 (malPEG). With the clamp holding the polypeptide in the ADP bound conformation, depletion of ATP by hexokinase and glucose would result in the backsliding of only the cysteine positioned in the SecY pore and its concomittant modification by malPEG, while the cysteine positioned in the clamp would be protected even in the absence of ATP. On the other hand, both cysteines would be subjected to backsliding after ATP-depletion if SecA´s role would be the passive positioning of the translocation substrate.

First, it was tested if the backsliding could also be observed with the pOA-DHFR construct. pOA-

DHFR with a single cysteine at position 152 was synthesized *in vitro* in the presence of ^{35}S-methionine. The substrate was then incubated with cysteine free SecA, proteoliposomes containing SecYEG with a cysteine at position 282, ATP and methotrexate. After intermediate formation, ATP was depleted by the addition of hexokinase and glucose. At different time points after ATP depletion, samples were taken and oxidized by the addition of copper phenanthroline. Crosslinked products were then analyzed by non-reducing SDS-PAGE and autoradiography (figure 16).

Backsliding could be reproduced with the pOA-DHFR construct. Within five minutes after ATP-depletion, crosslinking of pOA-DHFR to the pore ring was completely abolished (figure 16, lanes 2-6). On the other hand, when ATP hadn´t been depleted, the translocation intermediate was stable over the entire time course of the experiment (figure 16, lanes 7-11).

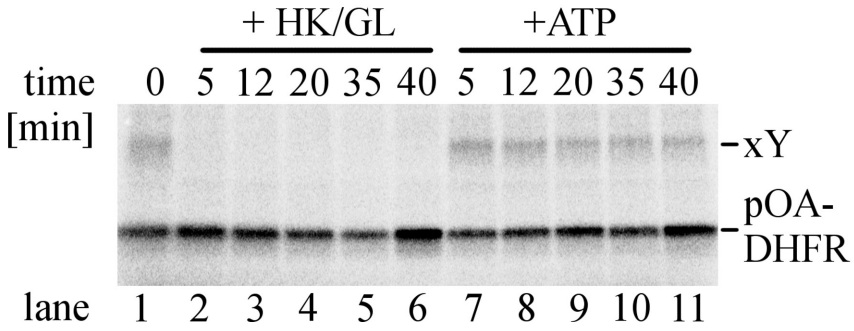

Figure 16. Backsliding of pOA-DHFR out of the SecY-pore. *In vitro* synthesized and ^{35}S-labeled pOA-DHFR with a cysteine at position 152 was used to form a translocation intermediate in the presence of ATP, cysteine-free SecA, proteoliposomes containing SecY-complex with a cysteine at position 282 and methotrexate. An aliquot was immediately treated with an oxidant (lane 1) and the remainder was either incubated in the presence of ATP (lanes 7-11) or hexokinase and glucose ("+HK/GL") to deplete ATP (lanes 2-6). Aliquots were taken at different time points, treated with oxidant, and analyzed by non-reducing SDS-PAGE and autoradiography.

Next, the movement of the two cysteines upon ATP-depletion was assessed by malPEG-modification. Translocation intermediates were formed as described above either with pOA-DHFR containing a single cysteine at position 152 or at position 171. ATP was depleted and at different timepoints thereafter, malPEG was added to the reaction. Modification of exposed cysteines was analyzed by SDS-PAGE and autoradiography (figure 17).

A cysteine at position 152 in pOA-DHFR was completely protected from modification, as seen by

the absence of a modified band in the presence of ATP during the whole course of the experiment (figure 17, lanes 2, 5 and 6). No translocation occured if ATP was lacking from the beginning of the experiment, so the cysteine could not be protected in this case (figure 17, lane 1). When ATP was depleted by hexokinase and glucose, backsliding occured and modification was seen within five minutes (figure 17, lanes 3 and 4).

The cysteine at position 171 of pOA-DHFR wasn´t completely protected by SecA´s clamp, so modification by malPEG was also seen in the presence of ATP (figure 17, lane 8). Inspite of the background modification, differences between protection and exposure to malPEG could be observed when comparing the ratio of both bands between the experimental conditions. In the presence of ATP, the fraction of protected cysteines was more pronounced than the fraction of modified ones (figure 17, lane 11 and 12). Upon depletion of ATP, this relation was reversed, with most of the cysteines becoming modified within the first five minutes of the experiment (figure 17, lane 9 and 10).

Together, these results demonstrate that both cysteines, altough initially located at different positions inside of the translocation intermediate, become exposed to modification once ATP is depleted.

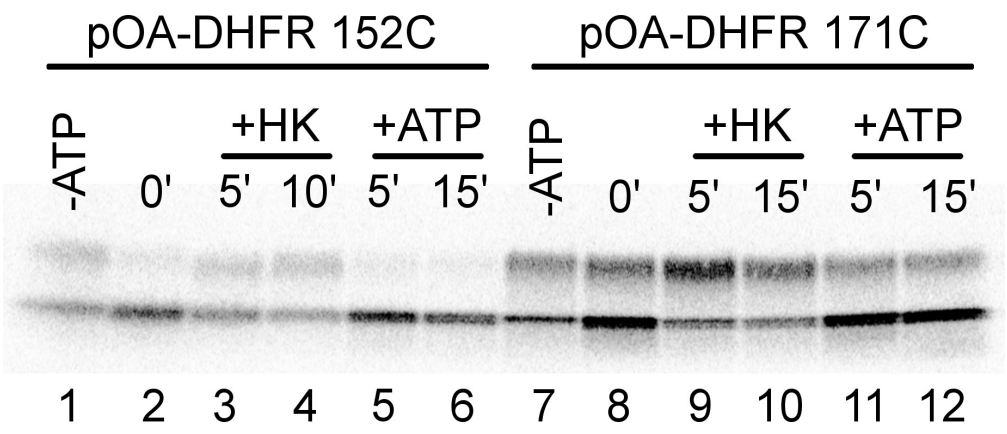

Figure 17. Backsliding of different segments of pOA-DHFR in the translocation intermediate. *In vitro* synthesized and ^{35}S-labeled pOA-DHFR with a single cystein at position 152 or 171 was incubated in the presence or absence of ATP with SecA, proteoliposomes containing purified SecY-complex and methotrexate. After intermediate formation, an aliquot was immediately treated with mPEG-maleimide (lanes 2 and 8, respectively). The remainder was either incubated with hexokinase and glucose ("HK") to deplete ATP (lanes 3, 4 and 9,10, respectively) or replenished with ATP (lanes 5,6 and 11,12, respectively). At indicated times thereafter, samples were taken and treated with mPEG-maleimide. Modifaction patterns were analyzed by SDS-PAGE and autoradiography.

3 Discussion

The Sec-mediated pathway of translocation is an early step in the biogenesis of secreted or integral membrane proteins. The central component of this pathway is a passive channel, called SecY/61p, that conducts proteins during or after their synthesis across the endoplasmatic reticulum membrane in eukaryotes or the plasma membrane in bacteria. Translocation of a polypeptide is either driven by its elongation inside the ribosome or it is fueled by ATP hydrolsis in the bacterial ATPase SecA or the eukaryotic ATPase BiP.

The current study focussed on the bacterial pathway of posttranslational translocation, mediated by SecA. How SecA transports proteins through the SecY-pore is still unknown. However, recent progress in the understanding of the process has been made in the field of structural biology. Especially a structure showing SecA in interaction with SecY lead to the proposal of a new model of translocation (Zimmer et al. 2008). According to the model, translocation of a polypeptide chain through SecY is mediated by the coordinated movements of two domains in SecA, a two-helix finger that pushes the polypeptide and a clamp that holds it and prevents its backsliding. The importance of the two-helix finger has been demonstrated in a previous study (Erlandson et al. 2008b). Here, the model´s predictions on the role of SecA´s clamp were tested.

Using a disulfide bridge crosslinking assay, it could be shown that a polypeptide chain specifically contacts residues inside the clamp and the two-helix finger of SecA. The design of the crosslinking experiments, using two cysteines placed into the substrate to test for disulfide bridge formation with cysteines in SecA as well as SecY, ensured that the observed crosslinking patterns represented true states of translocation. This approach was taken due to the fact that SecA and its substrate interact rather promiscuously in solution.

Disulfide bridge formation between cysteines in the substrate, SecA and SecY could only be observed in a stalled state of translocation, during which a bulky group at the C-terminus of the substrate prevents its complete translocation. For the question at hand, it was important that translocation was stalled at the level of SecA. Two groups were found to be suitable in this sense - a C-terminal chain of ten lysine residues and a C-terminally fused dehydrofolate reductase (DHFR).
The reason why a polylysine chain is capable of blocking translocation is not well understood (Nouwen et al. 2009). It might be that the positive charge of ten lysines in a row or the bulkiness of the long amino-alkylchains somehow presents an obstacle to the channel or SecA. Considering that

the pOA-10K substrate could be crosslinked to a cysteine placed inside the clamp makes it likely that translocation was stalled at the level of SecA.

The experiments performed with the translocation intermediate stalled by the lysine chain displayed only low crosslinking efficiencies. For this reason, the final crosslinking experiments were performed with the fusion of pOA and DHFR (Arkowitz, 1993). It could be shown that in the presence of its substrate analogue methotrexate, DHFR becomes resistant to proteolyis by proteinase K and stable enough to withstand the unfoldase activity of SecA. In addition, this state is reversible by the removal of methotrexate.

The positions found to crosslink to pOA-DHFR delineate its pathway through SecA and SecY: pOA-DHFR moves into the cavity of the clamp, passes the two-helix finger and enters the pore of the SecY. In the structure of SecY-SecA-complex, the mouth of the clamp is partially occluded by a loop of the PPXD (Zimmer et al., 2008). Accomodation of the translocating polypeptide chain in this region of the clamp might be faciliated by the fact that it acquires a rather extended conformation and that the described loop might be flexible enough to be displaced by the polypeptide.

The described pathway is strongly supported by the fact that positions found outside of the clamp and the two-helix finger didn´t crosslink at all or crosslinked only weakly. Some of these positions, namely positions 304, 306 and 237, are found in groove on the outer surface of the PPXD that NMR-experiments have reported to be involved in SecA´s binding of the signal sequence (Gelis et al., 2007). In the light of the current findings, it is unclear how a polypeptide chain could be moved from the outside of the PPXD into the clamp and finally into the SecY pore, especially since the structure of SecA and SecY in complex shows that a direct passage is blocked by interactions of the PPXD with loops of SecY. Signal sequence binding and transfer of the signal sequence into SecY might precede an interaction of SecY and SecA or there might be an additional binding site for the signal sequence inside the hydrophobic interior of the clamp.

The model of SecA´s molecular function proposes that the clamp tightens around the translocating polypeptide chain while SecA is in its ADP-bound conformation (Zimmer et al. 2008). It is known that depletion of ATP by the addition of hexokinase and glucose to the translocation reaction leads to the backsliding of a stalled polypeptide chain (Erlandson et al., 2008a). Hexokinase/glucose treatment generates ADP by the phosphorylation of glucose and should render SecA in the ADP-bound state. With the clamp tightening around the substrate in this conformation, the observed backsliding should occur through the interface of SecA and SecY, as opposed to a backsliding

through the clamp. This hypothesis was tested by looking at the protection of substrate cysteines from modification by mPEG-maleimide. The cysteines were initially positioned inside the protected environment of either the clamp or the SecY-pore. Depletion of ATP by hexokinase/glucose lead to the modification of both cysteines, indicating that all segments of the substrate were subject to backsliding.

While being opposite to the expectation that only the cysteine inside the pore should have been modified upon backsliding if the clamp had an active role during translocation, the data do not exclude this possibility. An alternative explanation for the backsliding would be the dissociation of SecA from SecY due to hexokinase/glucose treatment. For this reason, the results do not confirm a passive role of SecA´s clamp either. The backsliding paradigm is based on the assumption that SecA stays associated to SecY upon ATP depletion. Unfortunately, an experimental control to test this assumption has not been established yet and will be a task of future studies investigating the molecular mechanism of SecA-mediated translocation.

4 Methods and Materials

4.1 Molecular cloning and mutagenesis

The first 177 or 205 amino acids of *Escherichia coli (E.coli)* proOmpA (pOA) were cloned into pMAL p4E (NEB) using the plasmid´s *NdeI* and *SalI* restriction sites. Different domains were then introduced C-terminally using the plasmid´s *SalI* and *HindIII* restriction sites. The domains were either amplified from existing plasmids (TOP7) or from *E. coli* K12 genomic DNA (ER2508).
The C-terminal polylysine (10K) and calmodulin binding peptides (CBP) were generated by PCR. Forward and reverse primers were designed, each containing half of the sequence to be introduced and 5´ or 3´ extensions containing the sequences flanking the site of introduction. PCR resulted in the linearization of the plasmid. The methylated template DNA was digested with *DpnI* for 2hrs and the PCR products purified by gelextraction (Quiagen). 5´ and 3´ ends of the linear PCR-products were phosphorylated with calf intestinal alkaline phosphatase (NEB), ligated with T4 DNA ligase (NEB) and transformed into 5-alpha competent *E.coli* cells (NEB) .
Point mutations introducing cysteines at various positions into *E. coli* SecA, *E.coli* SecY and pOA were generated following the Quickchange method (Stratagene). After PCR, methylated template DNA was *DpnI*-digested for 2 hours and the PCR products directly used to transform 5-alpha competent *E.coli* cells (NEB). Success of mutagenesis and cloning was confirmed by sequencing.

4.2 Overexpression and purification of *E.coli* SecA

E.coli SecA lacking the C-terminal 70 residues with a C-terminal hexahistidine tag was overexpressed from the Isopropyl-β-D-thiogalactopyranosid (IPTG)-inducible pET30b vector (Novagen). BL21(DE3) competent cells (Stratagene) were transformed with the plasmid by electroporation and plated out on LB-agar plates containing 50µg/ml kanamycin. After incubation overnight at 37°C, the cells were resuspended in 2xYT medium and the suspension used to inoculate 1L of the same medium supplemented with 50µg/ml kanamycin. The cells were grown with vigorous shaking at 37°C to an OD_{600} of 0.6 and overexpression was induced by the addition of 1mM IPTG for another 3 hours at 37°C. Cells were harvested by centrifugation (7500 rpm, 15 min, 4°C, Beckman JLA 8.100) and the pellet stored at -80°C.

For purification, the cells were resuspended in 25ml buffer A (20mM Tris pH 7.5, 500mM NaCl, 3mM ß-mercaptoethanol) and supplemented with 1mM phenylmethylsulfonylfluorid (PMSF) and 0.2mg/ml benzamidine. The cells were lysed by passing them twice through a microfluidizer at 20000psi. Insoluble components of the lysate were removed by ultracentrifugation (42000 rpm, 45 min, 4°C, Beckman Ti45) and the supernatant was incubated with 1.5ml of Nickel-NTA beads (Quiagen) for 1 hour on a rocking table. The beads had been washed with water and equilibrated with buffer A before. After binding, the beads were sedimented by centrifugation (5000 rpm, 10 min, 4°C, Eppendorf 5804R (A 4-44)) and the supernatant was discarded. The beads were then washed twice with 50ml buffer B (20mM Tris pH 7.5, 500mM NaCl, 3mM ß-ME, 20mM imidazole) and bound protein was eluded in 5ml buffer C (20mM Tris pH 7.5, 100mM NaCl, 3mM ß-ME, 200mM imidazole). To remove imidazole from the protein solution, the samples were overconcentrated using 100kDa molecular weight cut-off (MWCO) amicon concentrators (Millipore) and diluted in the final storage buffer (20mM Tris pH 7.5, 100mM NaCl, 5mM DTT). Aggregates were removed by centrifugation (14000rpm, 15min, 4°C, Eppendorf 5417C). The final concentration of the sample was determined by its absorbance at 280nm and the molar extinction coefficient of the SecA construct (ε= 94980$M^{-1}cm^{-1}$).

4.3 Overexpression and purification of *E.coli* SecYEG

E.coli SecEYG with a N-terminal hexahistidine tag on SecE was overexpressed from the arabinose-inducible pBAD22 vector (Invitrogen). The plasmid was transformed into BL21(DE3) competent *E.coli* cells (Stratagene) by electroporation and the cells were plated out on LB-agar plates supplemented with 100µg/ml carbenicillin. After incubation at 37°C overnight, transformed cells were resuspended in 2xYT and the suspension used to inoculate 8l of the same medium supplemented with 100µg/ml ampicillin. The cells were grown with rapid shaking to an OD600 of 0.6. Overexpression of SecYEG was then induced by the addition of 2g/l arabinose. The cells were grown at 37°C for another 5 hours and then harvested by centrifugation (7500 rpm, 15 min, 4°C, Beckman JLA 8.100) and the pellet stored at -80°C.

For purification, the cells were resuspended in 150ml of buffer A (300mM NaCl, 10% glycerol, 20mM Tris pH 7.5) and supplemented with one tablet of complete EDTA free protease inhibitor cocktail (Sigma Aldrich), 1mM PMSF and 0.2mg/ml benzamidine. Cells were subjected to two rounds of lysis in a microfluidizer at 20000 psi. Membranes were then isolated by

ultracentrifugation (42000rpm, 1hr, 4°C, Beckmann Ti45) and solubilized with 60ml buffer B (20mM Tris pH 7.5, 300mM NaCl, 10% glycerol, 20mM n-dodecyl-ß-D-maltopyranoside (DDM)) for 1 hour at 4°C. At this point all protease inhibitors were replenished. Nonsoluble components were finally removed by ultracentrifugation (42000rpm, 30min, 4°C, Beckmann Ti45) and the supernatant was loaded onto 8ml Nickel-NTA beads (Quiagen). The beads had been washed with water and equilibrated with buffer B before incubation. Proteins were allowed to bind for one hour at 4°C with constant rocking. The suspension was then transferred to a plastic column and the flowthrough was discarded. After washing with 160ml buffer C (20mM Tris pH7.5, 300mM NaCl, 10% glycerol, 0.6mM DDM, 20mM imidazole), bound protein was eluded in 25ml buffer D(20mM Tris pH7.5, 100mM NaCl, 10% glycerol, 0.6mM DDM, 200mM imidazole). Buffers were exchanged by overconcentration of the sample with 100kDa MWCO amicon concentrators (Millipore) and dilution in the final storage buffer (20mM Tris pH 7.5, 100mM NaCl, 10% glycerol, 0.6mM DDM). The final protein concentration was determined using the sample´s absorbance at 280nm and the molar extinction coefficient of SecYEG (ε=68560$M^{-1}cm^{-1}$).

4.4 Reconstitution of SecYEG into liposomes

100µl of a 100mg/ml stock of *E.coli* polar lipids in chloroform (Avanti) was purged with a stream of nitrogen to remove chloroform. A mild vacuum was applied for 1 hour to remove the remaining solvent. The resulting lipid film was resuspended in 1ml of a 60mM DDM solution in water, giving a 10mg/ml lipid/detergent stock. The lipids were solubilized overnight at room temperature with vigorous shaking.

Proteoliposomes were formed by the removal of detergent from the lipid solution by biobeads (Biorad). 500µl of the beads were washed twice with 1ml water, three times with 1ml of buffer A (50mM HEPES 7.5, 200mM K-acetate, 12.5% glycerol) and finally resuspended in 500µl buffer A. 70µl of the 10mg/ml lipid stock solution in DDM and 40µg SecYEG in DDM were added to the beads and the mixture was vigorously shaken overnight at 4°C. After the beads had been removed, the proteoliposomes were isolated from the solution by ultracentrifugation (50000rpm, 30min, 4°C, Beckman TLA 100.3) washed twice with 100µl of buffer B (50mM HEPES pH 7.0, 10% glycerol) and resuspended in the same buffer. The solution of proteoliposomes was aliquoted and snap frozen in liquid nitrogen for storage at -80°C.

4.5 *In vitro* transcription and translation of translocation substrates

Linear fragments of the translocation substrates containing a 5´ SP6 promoter and a 3´ Stop codon were generated and amplified by PCR using phusion polymerase (Finnzymes). The DNA fragment served as a template for *in vitro* transcription by SP6-polymerase (Promega). After 4 hours of *in vitro* trancription at 37°C, mRNA was extracted by PCI (phenol:chloroform:isoamylalcohol in a 25:24:1ratio) and precipitated by ethanol and high salt. The mRNA pellet was washed with 70% ethanol, dried on air and resuspended in 15µl RNAse free water.

In vitro translation was performed in rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-labeled methionine (Perkin Elmer) according to the manufacturer´s instructions for 1.5 hours at 30°C. After *in vitro* translation, proteins were precipitated with a buffered solution of saturated ammonium sulfate (in 50mM Hepes pH 7.5) for 40min on ice and pelleted (14000rpm, 15min, 4°C, Eppendorf 5417C). The pellet was washed with saturated ammonium sulfate and resuspended in 35µl of a 8M urea, 50mM Hepes pH 7.0 buffer. In the case of the proOmpA-DHFR, proOmpA-TrxA, and proOmpA-Top7 constructs, the crude lysate was used for the translocation reactions.

4.6 Translocation assays

Translocation reactions were performed in 50µl volumes containing 50mM Tris pH 7.9, 50mM NaCl, 50mM KCl, 5mM $MgCl_2$, 2.5mM ATP, 0.1mg/ml bovine serum albumin (BSA), 32µg/ml SecY-complex in proteoliposomes (PL), 1mM dithiothreitol (DTT) and 1µl *in vitro* synthesized and ^{35}S-labelled tranlocation substrate. In the case of the pOA-DHFR construct, 10µM methotrexate was also added. After 15min at 37°C, the samples were digested with 0.2mg/ml proteinase K in the presence or absence of Triton X-100 for 30min on ice. After proteinase K digestion, samples were treated with 2mM PMSF for 10min on ice and precipitated with 10% trichloroacetic acid for 40min on ice. After sedimentation (14000rpm, 15min, 4°C, Eppendorf 5417C), the pellets were washed with acetone, resuspended in SDS-buffer (50mM Tris pH 7.5, 8M urea, 5% SDS, 10mM EDTA, 0.25mg/ml bromophenol blue, 100mM DDT) and subjected to SDS-PAGE on 4-20% Tris-HCl gels (Biorad) and autoradiography.

4.7 Crosslinking assays

Crosslinking was performed in 20µl reactions containing 50mM Hepes/KOH pH 7.5, 50mM KCl, 5mM MgCl2, 2.5mM ATP, 0.5mM BSA, 40µg/ml SecA, 20µg/ml SecY complex in PL, and 1µl of *in vitro* sythesized and ^{35}S-labelled pOA-DHFR or pOA-10K. After intermediate formation for 15min at 37°C, crosslinking was induced by the addition of 50µM copper phenanthroline for 10min at 37°C. The reaction was quenched with 10mM N-ethylmaleimide (NEM) for 5min on ice and then subjected to non-reducing SDS-PAGE on 4-20% Tris-HCl gels (Biorad). Bands were visualized by phosphorimaging. QuantityOne (Biorad) was used for local background substraction and quantification. Crosslinking efficiencies were expressed as the ratio of counts of doublecrosslinked bands (xAxY) to the sum of counts of doublecrosslinked and single SecY-crosslinked bands (xYxA/(xYxY+xY)).

4.8 Immunoprecipitation

Intermediates were formed with the poA-DHFR construct in a volume of 160µl for 15min at 37°C. After crosslinking (see above), two 20µl samples were taken as input and the remainder was sedimented (14000rpm, 15min, 4°C, Eppendorf 5417C) and resuspended in 30µl of 50mM Hepes/KOH pH 7.5, 50mM KCl, 50mM NaCl, 8M urea, 1% SDS. After solubilization for 10min at 65°C, SDS was diluted in 510µl 50mM Hepes/KOH pH 7.5, 50mM KCl, 50mM NaCl, 1% TritonX-100. Insoluble aggregates were removed by ultracentrifugation (70000rpm, 15min, 4°C, Beckmann TLA100) and the supernatant was incubated with 1mg/ml BSA for 10min at room temperature. The reactions were loaded onto protein G beads (NEB) prebound to antibodies directed against either SecY or SecA (Cannon et al., 2005). After 30min at room temperature, the beads were washed three times with 50mM Hepes/KOH pH 7.5, 50mM KCl, 50mM NaCl, 1% Triton X-100, and bound material was eluded in SDS buffer for 10min at 65°C. The samples were subjected to non-reducing SDS-PAGE and autoradiography.

4.9 Backsliding assay

A translocation intermediate was generated for 15min at 37°C in 50mM Hepes/KOH pH 7.5,

50mM KCl, 5mM MgCl2, 0.5mM ATP, 0.5mM BSA, 40µg/ml cysteine free SecA, 20µg/ml SecY(282C)EG in PL, and 1µl lysate containing *in vitro* synthesized pOA-DHFR with a single cysteine at position 152. A sample was taken (0´ of backsliding), and oxidized with 50µM copper phenanthroline for 10min at 37°C, followed by treatment with 10mM NEM for 5min on ice. The remainder was either depleted of ATP by addition of 0.5 units of hexokinase (Sigma Aldrich) and 10mM glucose, or replenished with 2.5mM ATP. Aliquots were taken from both reactions at different time points, oxidized and treated with NEM. The reactions were subjected to nonreducing SDS-PAGE and autoradiography.

4.10 mPEG-maleimide protection assay

Translocation intermediates were formed for 15min at 37°C in 50mM Hepes/KOH pH 7.5, 50mM KCl, 5mM MgCl2, 2.5mM ATP, 0.5mM BSA, 40µg/ml cysteine free SecA, 20µg/ml SecY(282C)EG in PL, and 1µl lysate containing *in vitro* synthesized pOA-DHFR with a single cysteine at position 152 or 171. ATP was then depleted either by the addition of 0.5units of hexokinase and 10mM glucose. Samples were taken at different time points after ATP-depletion and treated with 1.5mM mPEG-maleimide for 30min on ice. The reaction was quenched by 10mM DTT on ice and samples were subjected to SDS-PAGE and autoradiography.

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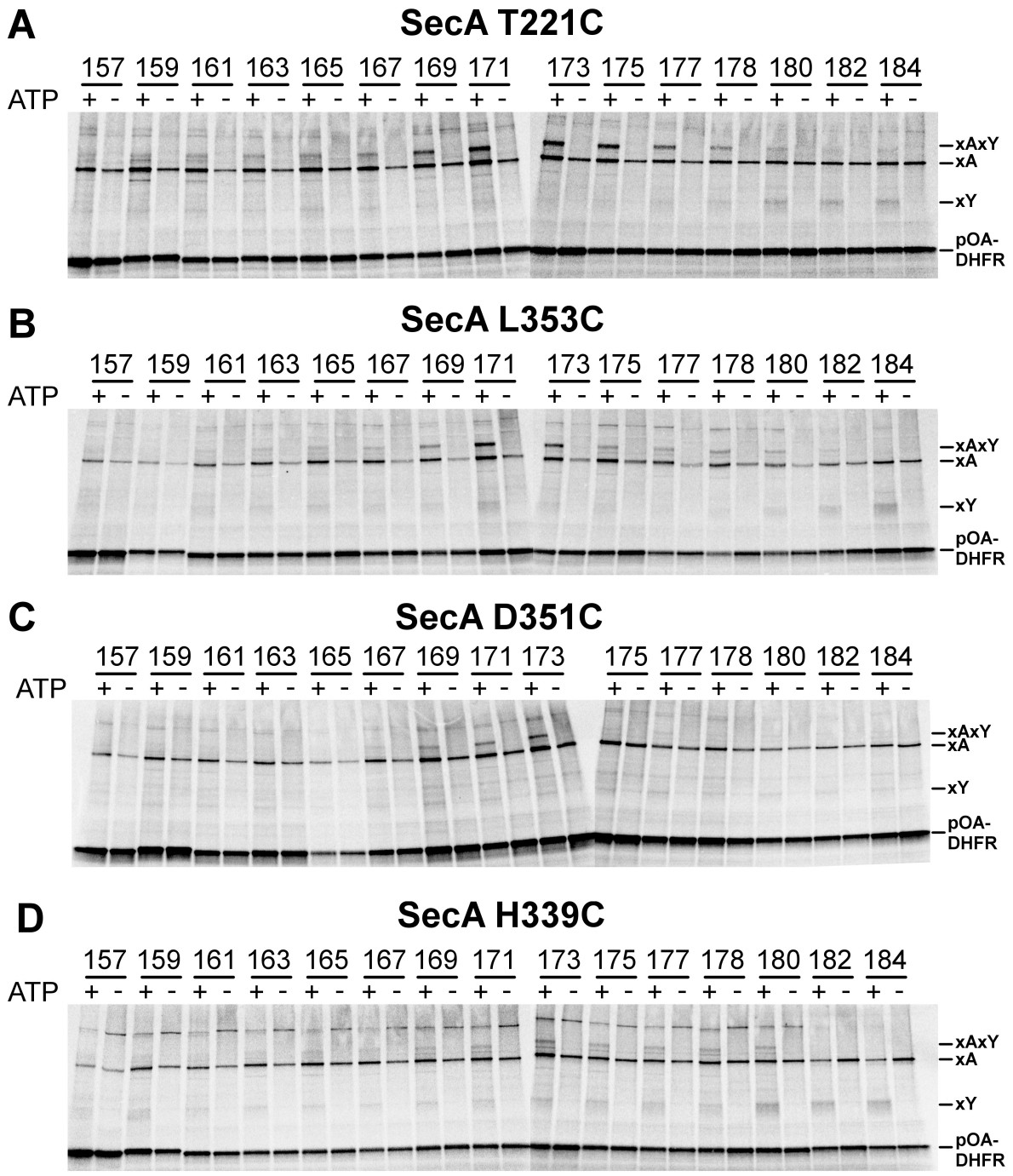
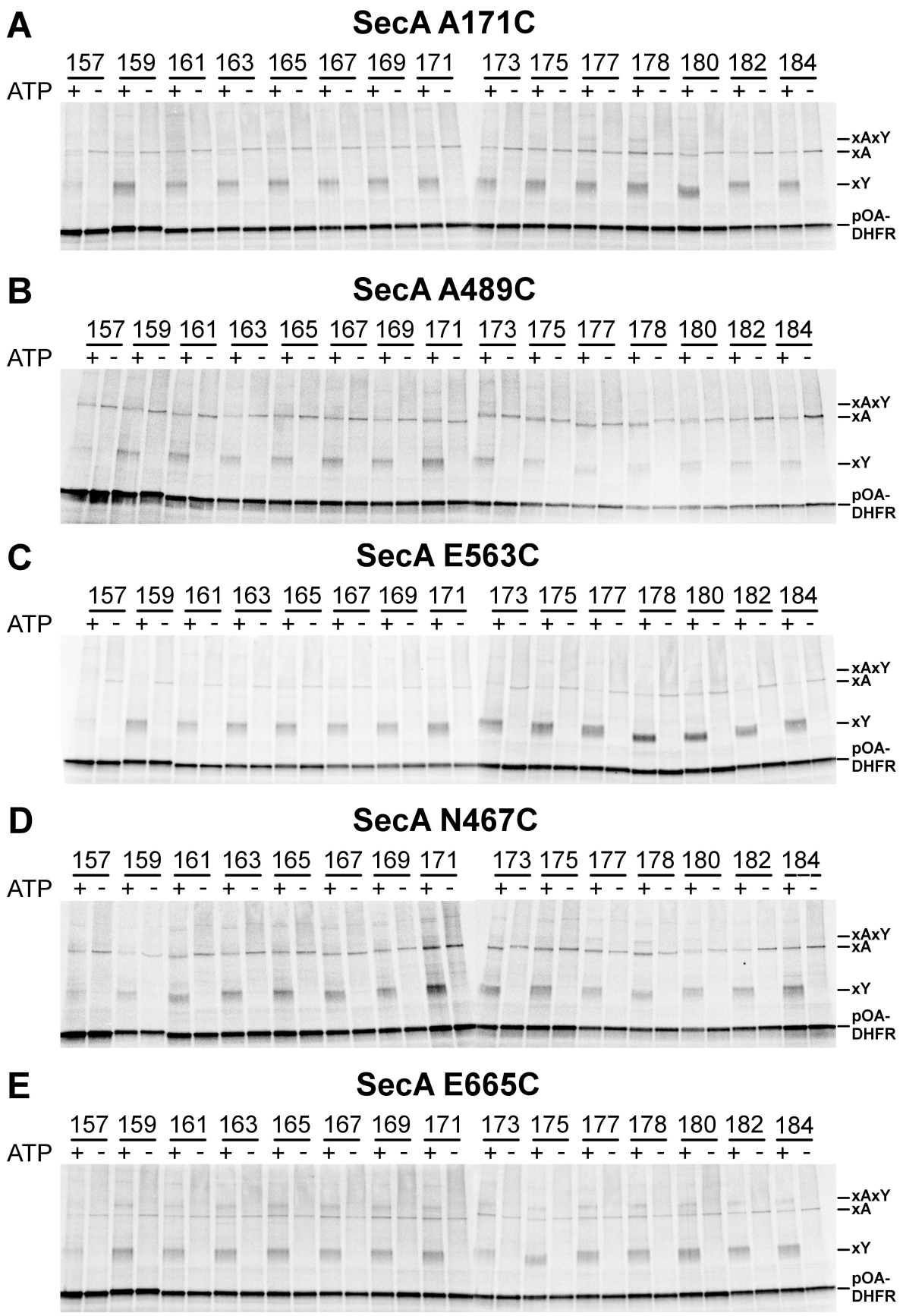
Supplemental figures



Supplemental figure 1. Interactions of a translocation intermediate with the clamp of SecA. (A) pOA-DHFR containing a cysteine at positions 152 and a second cysteine at the indicated positions was synthesized *in vitro* in the presence of ^{35}S-methionine. The substrate was incubated with ATP, SecA containing a single cysteine in the clamp at position 221 and with proteoliposomes containing SecY with a cysteine at position 282. The samples were treated with an oxidant and analyzed by SDS-PAGE and autoradiography. The positions of uncrosslinked pOA-DHFR, SecA-, SecY- and double-crosslinked substrate (pOA-DHFR, xA, xY, and xYxA, respectively) are indicated. **(B)** As in (A), but with a cysteine in the SecA clamp at position 353. **(C)** As in (A), but with a cysteine in the SecA clamp at position 351 **(D)** As in (A), but with a cysteine in the SecA clamp at position 339.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xA
xY
pOA-DHFR
B
SecA A489C
157 159 161 163 165 167 169 171 173 175 177 178 180 182 184
ATP + - + - + - + - + - + - + - + - + - + - + - + - + - + - + -
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xA
xY
pOA-DHFR
C
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157 159 161 163 165 167 169 171 173 175 177 178 180 182 184
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xA
xY
pOA-DHFR
D
SecA N467C
157 159 161 163 165 167 169 171 173 175 177 178 180 182 184
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xA
xY
pOA-DHFR
E
SecA E665C
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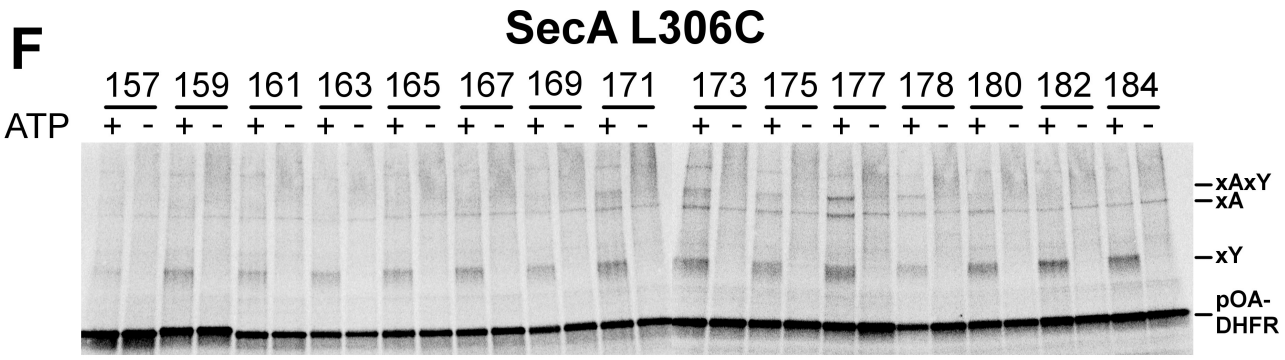
F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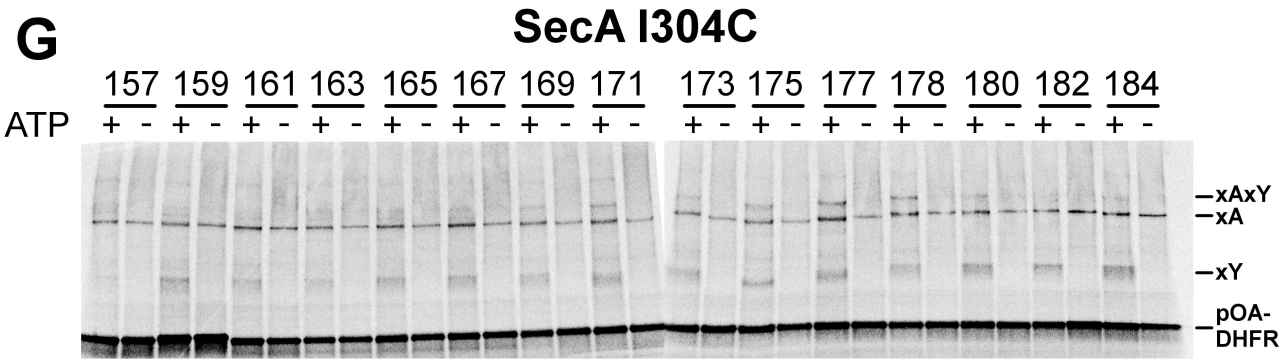
G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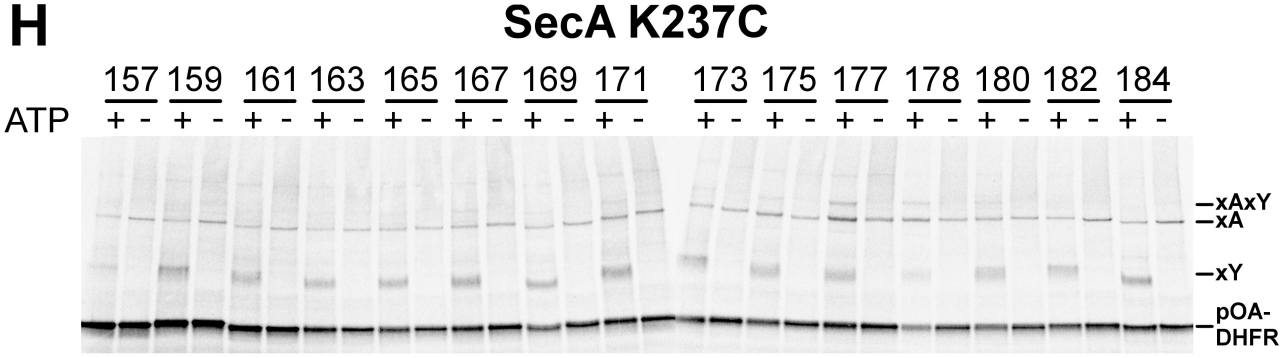
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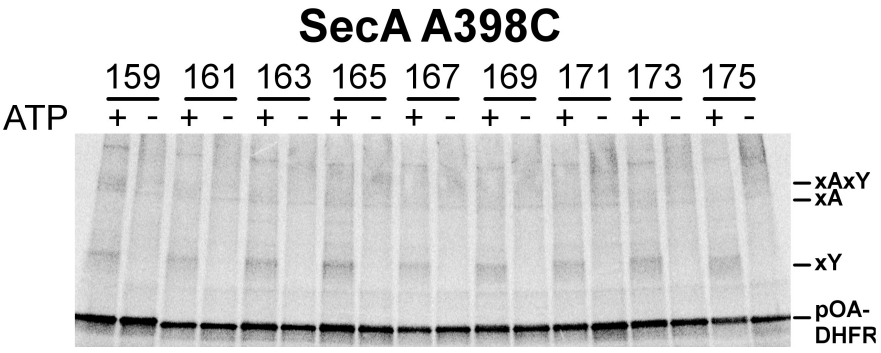
I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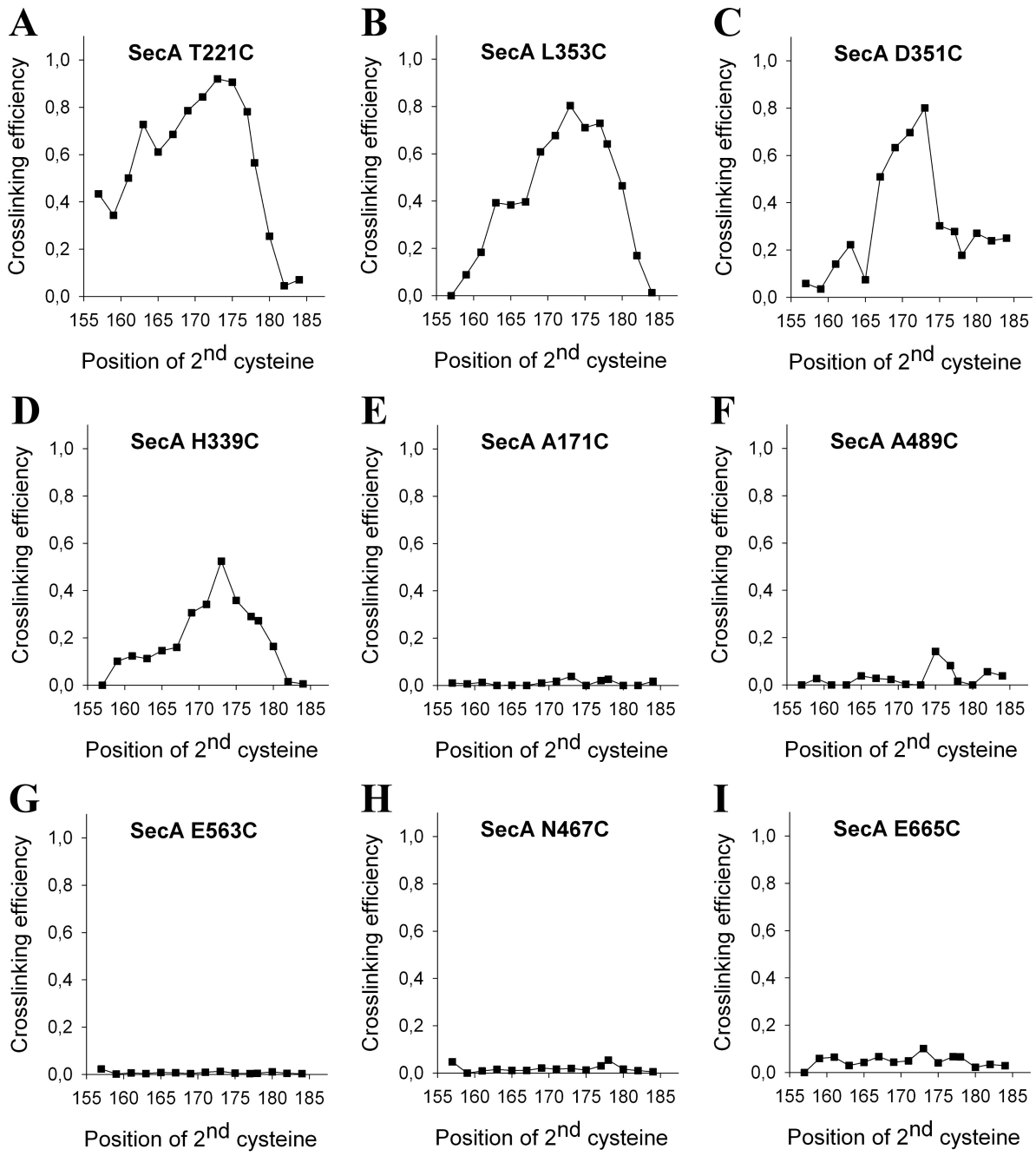
xAxY
xA
xY
pOA-
DHFR

Supplemental figure 2. pOA-DHFR does not interact with positions placed outside the postulated path. (A) pOA-DHFR containing a cysteine at positions 152 and a second cysteine at the indicated positions was synthesized *in vitro* in the presence of ^{35}S-methionine. The substrate was incubated with ATP, SecA containing a single cysteine at position 171 and with proteoliposomes containing SecY with a cysteine at position 282. The samples were treated with an oxidant and analyzed by SDS-PAGE and autoradiography. The positions of uncrosslinked pOA-DHFR, SecA-, SecY- and double-crosslinked substrate (pOA-DHFR, xA, xY, and xYxA) are indicated. **(B)** As in (A), but with a cysteine at position 489. **(C)** As in (A), but with a cysteine at position 563 **(D)** As in (A), but with a cysteine at position 467. **(E)** As in (A), but with a cysteine at position 665. **(F)** As in (A), but with a cysteine at position 306. **(G)** As in (A), but with a cysteine at position 304. **(H)** As in (A), but with a cysteine at position 237. **(I)** As in (A), but with a cysteine at position 398.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B
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C
SecA D351C
D
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E
SecA A171C
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G
SecA E563C
H
SecA N467C
I
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0,4
0,6
0,8
1,0
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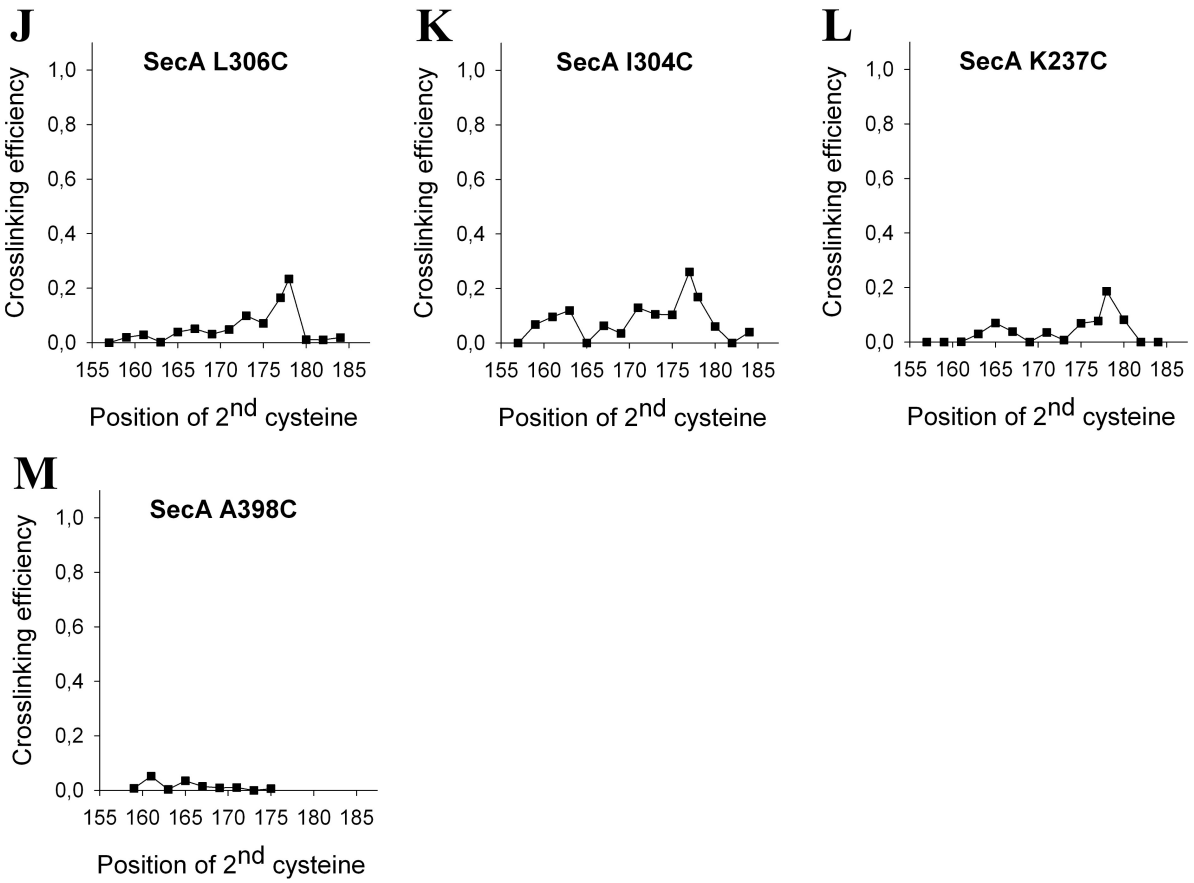
Position of 2nd cysteine



Supplemental figure 3. Quantification of the interactions of pOA-DHFR with SecA. (A)-(M) The experiments shown in Figures S1A-D and S2A-I were quantified. The crosslinking efficiency is expressed as the ratio of double-crosslinks over the total SecY crosslinks (xYxA/(xYxA + xY)).