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# Protein Translocation Is Mediated by Oligomers of the SecY Complex with One SecY Copy Forming the Channel

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## SUMMARY

Many proteins are translocated across the bacterial plasma membrane by the interplay of the cytoplasmic ATPase SecA with a protein-conducting channel, formed from the evolutionarily conserved heterotrimeric SecY complex. Here, we have used purified *E. coli* components to address the mechanism of translocation. Disulfide bridge crosslinking demonstrates that SecA transfers both the signal sequence and the mature region of a secreted substrate into a single SecY molecule. However, protein translocation involves oligomers of the SecY complex, because a SecY molecule defective in translocation can be rescued by linking it covalently with a wild-type SecY copy. SecA interacts through one of its domains with a nontranslocating SecY copy and moves the polypeptide chain through a neighboring SecY copy. Oligomeric channels with only one active pore likely mediate protein translocation in all organisms.

## INTRODUCTION

Many proteins are transported across or are integrated into the endoplasmic reticulum (ER) membrane in eukaryotes or the cytoplasmic membrane in prokaryotes. These proteins are directed to the membrane by cleavable signal sequences or by transmembrane (TM) segments of membrane proteins. Translocation occurs through a hydrophilic channel that is formed from a conserved heterotrimeric membrane protein complex, called the Sec61p complex in eukaryotes and the SecY complex in bacteria and archaea (for review, see Osborne et al., 2005). The complex consists of a multispanning $\alpha$-subunit (Sec61p or SecY) and two smaller $\beta$- and $\gamma$-subunits, called SecG and SecE in bacteria. The channel can associate with different partners that provide the driving force for translocation. In cotranslational translocation, the Sec61p/SecY complex associates with the translating ribosome; in posttranslational translocation in eukaryotes, the Sec61p complex associates with the Sec62/63p complex, and the ER luminal ATPase BiP; in posttranslational translocation in bacteria, the SecY channel associates with the cytoplasmic ATPase SecA. How the Sec61p/SecY complex forms a channel and how it associates with its different partners to translocate polypeptide chains is unclear.

In the simplest model, the channel would be formed from a single copy of the Sec61p/SecY complex. This is supported by the crystal structure of an archaeal SecY complex (Van den Berg et al., 2004). The structure shows a monomer of SecY complex and is likely representative of the closed states of all Sec61p/SecY channels. SecY consists of two linked halves, TMs 1–5 and 6–10, which form a lateral gate at the front and are clamped together at the back by SecE. A cytoplasmic funnel leading into the channel is closed by a short helix, termed the "plug." During initiation of translocation, a signal sequence or TM sequence intercalates into the lateral gate of SecY and causes the plug to move toward the back of SecY (Tam et al., 2005). The open channel would have an hourglass shape, with a pore ring of hydrophobic amino acid residues at its constriction, through which translocating polypeptide chains move, as demonstrated by disulfide bridge crosslinking (Cannon et al., 2005). Hydrophobic segments of membrane proteins would move through the lateral gate into the lipid phase.

The idea that the active translocation pore is formed by a monomer of Sec61p/SecY complex is challenged by observations that the solubilized complex can form oligomers, containing between two and four copies (for discussion, see Eichler and Duong, 2004). Oligomers of the SecY complex have also been observed in intact membranes (Mori et al., 2003; Scheuring et al., 2005). The crystal structure excludes the formation of a hydrophilic channel by the simple association of several SecY copies, because these are entirely hydrophobic in their membrane-facing regions. However, it is conceivable that multiple complexes associate at their front surfaces and open their lateral gates to fuse their pores. This could explain fluorescence-quenching experiments, which indicated a pore diameter of at least 40 Å (Hamman et al., 1997), and it would be consistent with a near front-to-front orientation

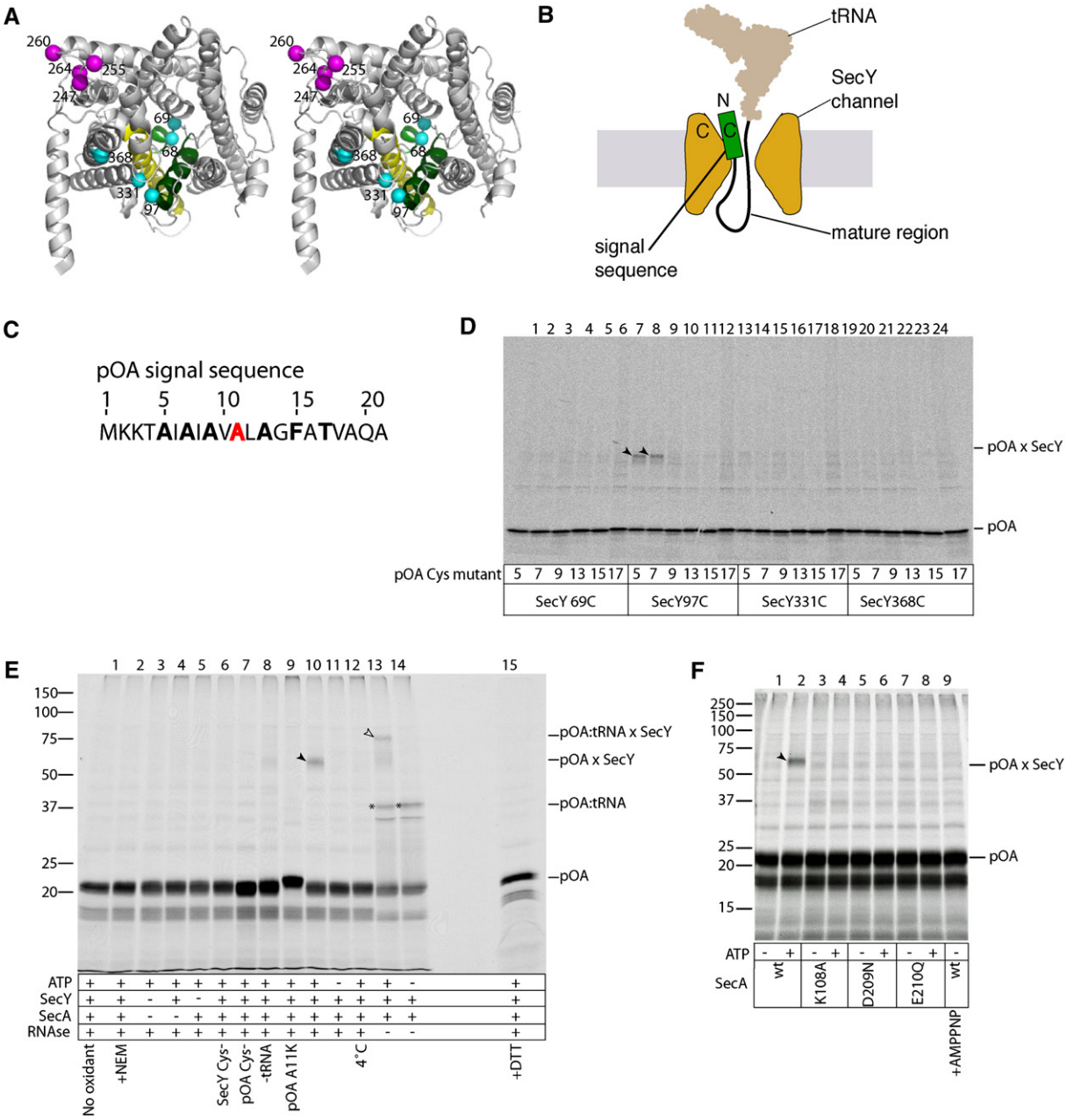


**Figure 1. Crosslinking of SecY to the Signal Sequence of a Translocation Substrate**

(A) Stereo view from the cytoplasm of the *M. jannashii* SecY complex. The plug (TM2a) is colored light green, and helices in the lateral gate (TM2b and TM7) are dark green and yellow, respectively. Cyan-colored spheres mark cysteines introduced into *E. coli* SecY for pOA-SecY crosslinking experiments. Magenta-colored spheres indicate cysteines placed into the 6/7 loop for SecA-Y crosslinking. Residues are numbered as in *E. coli* SecY. *E. coli* residue 255 is in an insertion; the closest position is labeled.

(B) Strategy of crosslinking. A translocation intermediate of proOmpA (pOA) was generated with a bulky tRNA at the C terminus (pOA:tRNA) to prevent its complete translocation through the SecY channel. Single cysteine residues (C) were placed into SecY and pOA to test their proximity by disulfide bond formation.

(C) Signal sequence of pOA. Residues mutated to cysteines are in bold and the inactivating A11K mutation in red.

(D) Proteoliposomes containing purified SecY mutants with cysteines at various positions were mixed with purified SecA, ATP, and $^{35}$S-methionine-labeled pOA:tRNA, containing 120 residues and cysteines at different positions of the signal sequence. After incubation at 37°C, the samples were treated on ice with an oxidant. NEM was added and the samples were digested with RNase prior to nonreducing SDS-PAGE and autoradiography. Filled arrows, crosslinked products.

(E) As in (D), with SecY containing a cysteine at position 97 (SecY-97C) and a pOA:tRNA substrate, containing 206 residues and a cysteine at position 5 (pOA-5C). The samples were incubated under different conditions, as indicated. +NEM, NEM added prior to the oxidant; SecY Cys- and pOA

proposed for a ribosome-bound dimer of SecY complexes (Mitra et al., 2005). However, oligomerization of the Sec61p/SecY complex may not necessarily indicate that the pore is formed from several copies; rather, it could serve another purpose, for example, to increase the number of interaction sites with channel partners.

In posttranslational translocation in bacteria, the SecY channel cooperates with the ATPase SecA, but the translocation mechanism is poorly understood. In one model, SecA would undergo nucleotide-dependent conformational changes to "push" successive segments of a polypeptide chain through the channel (Economou and Wickner, 1994). However, the originally proposed "plunging" model, according to which SecA reaches through the SecY channel (Economou and Wickner, 1994), is difficult to reconcile with the crystal structures. How SecA interacts with the SecY complex is also largely unknown, although several cytosolic loops of SecY appear to be involved (Mori and Ito, 2006).

Here, we address how the translocation channel is formed during SecA-mediated translocation, using purified SecY complexes reconstituted into proteoliposomes and purified SecA (Brundage et al., 1990). We show that the translocation pore is formed from a single copy of the SecY complex but that translocation is mediated by oligomers, with a nontranslocating SecY copy providing a static interaction site for SecA.

## RESULTS

### Signal Sequence Insertion into the Lateral Gate of the SecY Complex

To understand how the SecY complex forms a translocation pore, it is important to know where the translocating polypeptide chain is located. Previous crosslinking experiments suggested that the polypeptide chain inserts into the channel as a loop, with the signal sequence intercalated into the lateral gate between TM2b and TM7, and the following mature region in the actual pore (Cannon et al., 2005; Plath et al., 1998). We now asked whether the signal sequence and mature region of a substrate are both contained in the same SecY molecule. If so, this would demonstrate that a single copy of the SecY complex forms the pore. We incorporated single cysteines into *E. coli* SecY that would form specific disulfide bonds with cysteines in either the signal sequence or the mature region of a translocating substrate.

Using proOmpA (pOA) as a substrate, we first tested crosslinking of the signal sequence. Single cysteines were placed at the cytoplasmic side of the lateral gate of SecY, at positions 97 (in TM2b) and 331 (in TM8) (Figure 1A). Upon loop insertion of the substrate into the channel, these residues would be expected to be in proximity of the N terminus of the signal sequence (Figure 1B). Proteoliposomes generated with purified SecY mutant complexes supported the translocation of pOA when combined with purified SecA and ATP (Figure S1A, lanes 9–12, in the Supplemental Data available with this article online). Single cysteines were also introduced at several positions of the signal sequence of pOA (Figure 1C, residues in bold). All cysteine mutants of pOA were translocated by SecA into SecY complex-containing proteoliposomes (Figure S1B, lanes 6–17).

To increase the likelihood of disulfide bond formation, the polypeptide substrate was trapped in the channel by preventing its complete translocation with a bulky tRNA at the C terminus (see scheme in Figure 1B). $^{35}$S-methionine-labeled substrate was generated by translating in vitro a truncated mRNA coding for the first 120 residues of pOA. In the absence of a stop codon, the nascent polypeptide chain remains associated with the ribosome as peptidyl-tRNA and can be released from the ribosome by treatment with urea. Radiolabeled pOA120:tRNA was incubated at 37°C in the presence of purified SecA, ATP, and reconstituted proteoliposomes containing purified SecY complex. The trapped translocation intermediate was treated with the oxidant copper phenanthroline to induce disulfide bond formation. The products were digested with RNase to remove the tRNA and analyzed by nonreducing SDS-PAGE, followed by autoradiography.

Cysteines at positions 5 or 7 of pOA:tRNA substrates (pOA-5C or pOA-7C) formed a disulfide bridge with the cysteine at position 97 of the SecY mutant SecY-97C (Figure 1D, lanes 7 and 8). In contrast, cysteines at positions 9, 13, 15, or 17 of pOA did not crosslink (lanes 9–12). None of the substrates crosslinked to the SecY-331C mutant (lanes 13–18). Two other SecY mutants, one with a cysteine at position 69, which is located in TM2a of the plug, the other at position 368, which is located in the cytoplasmic loop between TM8 and TM9, did not crosslink to any of the cysteines in the signal sequence (lanes 1–6 and 19–24, respectively), although they were active in translocation (Figure S1A, lanes 7, 8, 13, and 14). We conclude that residues 5 and 7 in the signal sequence of pOA make a specific contact with residue 97 in SecY. Crosslinking between pOA-5C and SecY-97C was also observed with a significantly longer translocation intermediate of 206 residues (Figure 1E, lane 10). Thus, the signal sequence remains stationary while the C-terminal region of pOA is moving through the channel.

Several controls confirm that disulfide crosslinks are derived from a functionally relevant translocation intermediate. For example, no crosslink was observed in the absence of copper phenanthroline (Figure 1E, lane 1) or

Cys-, proteins lacking cysteines; -tRNA, substrate pretreated with RNase; pOA A11K, signal sequence mutant; +DTT, reduction of the sample prior to SDS-PAGE. Filled arrow, crosslink of SecY-97C with pOA-5C; open arrow, crosslink of SecY-97C with pOA-5C:tRNA; asterisk, noncrosslinked pOA-5C:tRNA.

(F) Crosslinking of SecY-97C with pOA:tRNA containing 206 residues and a cysteine at position 5 (pOA-5C) was tested with various ATPase mutants of SecA. Where indicated, AMPPNP was added instead of ATP. WT, SecA lacking cysteines.

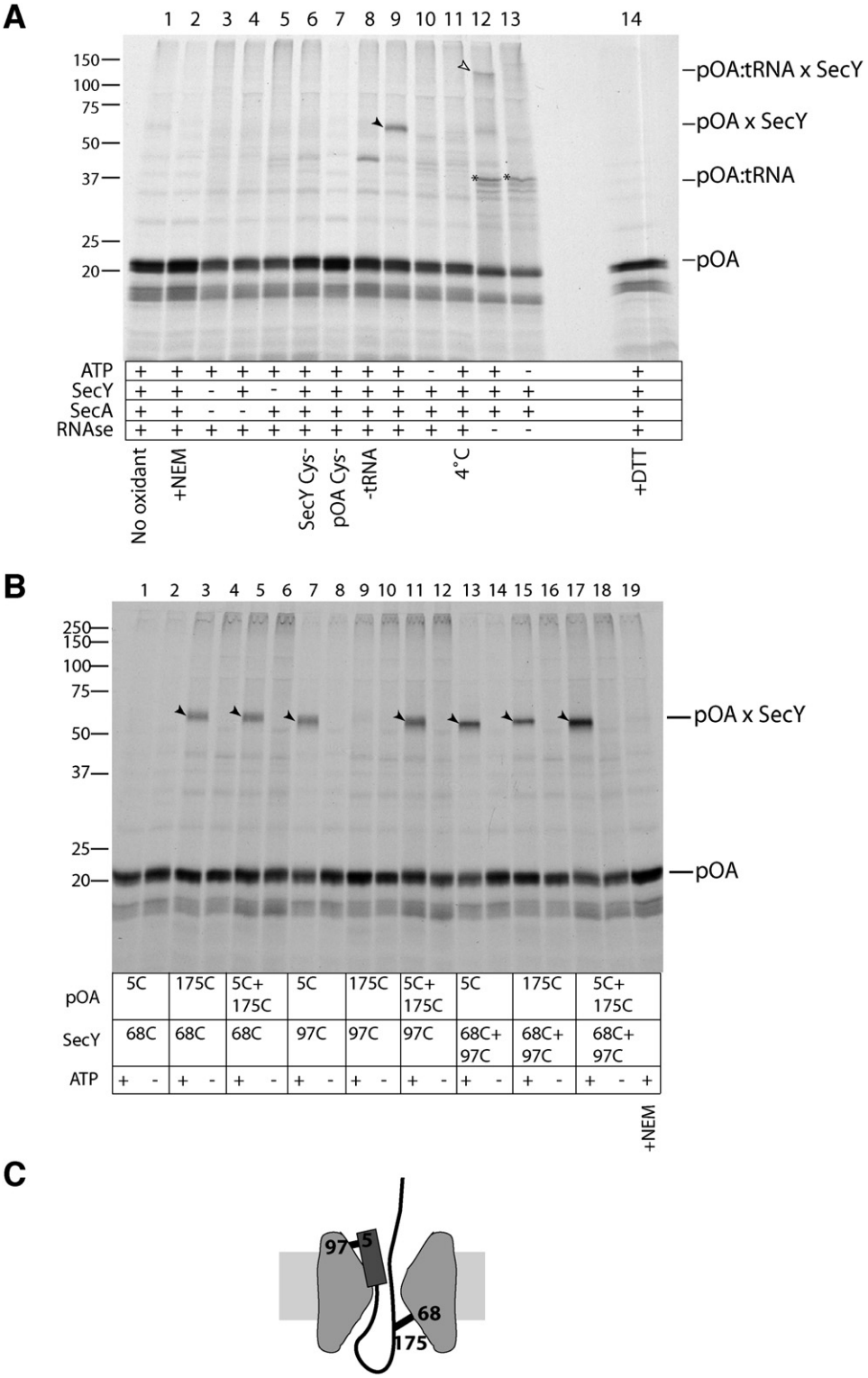


**Figure 2. The Signal Sequence and Mature Region of pOA Occupy Distinct Positions in the SecY Channel**

(A) Proteoliposomes containing SecY-68C were mixed with SecA, ATP, and $^{35}$S-methionine-labeled pOA:tRNA, containing 206 residues and a cysteine in the mature region at position 175 (pOA-175C). After incubation at 37°C, the samples were treated on ice with an oxidant. NEM was added and the samples were digested with RNase prior to nonreducing SDS-PAGE and autoradiography. Various controls were performed, as indicated (as in

if the cysteines were modified with N-ethylmaleimide (NEM) prior to addition of the oxidant (lane 2). The crosslink disappeared upon reduction with dithiothreitol (DTT) (lane 15). Disulfide bridge formation did not occur in the absence of SecA, SecY (lanes 3–5), or ATP (lane 11), if the samples were incubated at 4°C rather than at 37°C (lane 12), or if the appropriate cysteine residue was lacking in pOA or SecY (lanes 6 and 7). No crosslink was seen with a substrate that contained a mutation in the signal sequence (A11K) that renders it nonfunctional (Tam et al., 2005) (lane 9). Also, crosslinking was drastically reduced if the substrate was treated with RNase to remove the tRNA prior to translocation (lane 8), thus allowing the substrate to move all the way across the membrane. Finally, when the sample was not treated with RNase prior to SDS-PAGE, the crosslinked product was larger, as expected from the retention of the tRNA at the C terminus (lane 13).

Insertion of the signal sequence did not occur when ATP was depleted (Figure 1F, lane 1 versus 2). Also, no crosslinking was seen with a SecA mutant defective in ATP binding (K108A), with SecA mutants that bind ATP, but do not hydrolyze it (D209N and E210Q), or when ATP was replaced with the nonhydrolyzable analog AMPPNP (lanes 3–9). Thus, signal sequence insertion into the lateral gate of SecY requires a complete ATP hydrolysis cycle by SecA.

## Insertion of the Mature Region of a Substrate into the Center of SecY

Next, we tested crosslinking of the mature region of pOA. SecY with a cysteine at position 69 of the plug, close to the central constriction of the channel, can form a disulfide bridge with a cysteine in the mature region (Cannon et al., 2005). SecY-69C is a signal sequence suppressor mutation, and we therefore introduced a cysteine into the neighboring position 68 (SecY-68C), a mutation that is neutral (Tam et al., 2005). The substrate pOA contained 206 residues, representing an advanced stage of translocation, and had a single cysteine in its mature region at position 175 (pOA-175C), which is expected to be inside the channel. Oxidation of the trapped translocation intermediate indeed induced disulfide bridge formation between SecY-68C and pOA-175C (Figure 2A, lane 9). Crosslinking was not seen if the oxidant was omitted (lane 1), if the sample was pretreated with NEM (lane 2), if SecA, SecY-68C, or ATP were omitted (lanes 3–5; lane 10), or if the sample was incubated at 4°C (lane 11). Also, no crosslinking occurred if either cysteineless SecY or substrate were used (lanes 6 and 7) or if the substrate was allowed to move completely into the vesicles following RNase treatment (lane 8). The crosslinked product disappeared upon reduction with DTT (lane 14), and it increased in mass when the sample was not treated with RNase (lane 12), consistent with it being tRNA associated. These results show that during translocation, position 175 in the mature region of pOA comes close to position 68 of SecY.

Importantly, pOA-5C did not crosslink with SecY-68C (Figure 2B, lanes 1 and 2), and conversely, pOA-175C did not crosslink with SecY-97C (lanes 9 and 10). Thus, the signal sequence and mature region of the translocation substrate have specific, nonoverlapping contact points with the channel, the signal sequence with the lateral gate, and the mature region with the central constriction.

## The Signal Sequence and Mature Region of pOA Are Contained in One SecY Molecule

The specificity of disulfide crosslinking allowed us to determine whether the signal sequence and mature region of pOA reside within the same SecY molecule or are located in separate SecY molecules. We introduced cysteines into both the signal sequence and mature region of pOA, and two cysteines into SecY at the respective interaction sites, and asked whether the substrate forms disulfide bridges with one or two SecY molecules. As expected, pOA-5C+175C gave a crosslink to both SecY-68C and SecY-97C (Figure 2B, lanes 5, 6, 11, and 12). Likewise, pOA-5C and pOA-175C gave crosslinks with SecY-68C+97C (lanes 13–16). Importantly, when both the substrate and SecY carried two cysteines, i.e., when pOA-5C+175C was tested with SecY-68C+97C, a crosslinked product of the same size was observed and no higher molecular weight crosslinks were visible (lanes 17 and 18). Thus, a single pOA substrate was crosslinked to a single SecY (see scheme in Figure 2C).

To test directly whether the signal sequence and mature region of pOA are crosslinked to the same SecY molecule, the crosslinked products were treated with endoproteinase LysC, an enzyme that cleaves after lysine residues. Upon digestion with LysC, the two cysteines in SecY-68C+97C will remain in the same peptide, as there is no lysine between residues 68 and 97 of SecY (Figure 3A). The two cysteines in pOA, however, should end up in separate peptides, as there are multiple lysines in the segment between residues 5 and 175. Thus, digestion of a crosslinked species that contains two disulfide bridges should yield a SecY fragment that is disulfide bonded to two pOA peptide fragments. If only one of the disulfide bridges is formed, digestion of the crosslinked product should yield a smaller species, in which the SecY fragment is linked to only one of the two pOA fragments (Figure 3A).

Figure 1E). Filled arrow, crosslink of SecY-68C with pOA-175C; open arrow, crosslink of SecY-68C with pOA-175C:tRNA; asterisk, noncrosslinked pOA-175C:tRNA. The 40 kDa band in lane 8 appears to be background as seen in other lanes with lower intensity.

(B) Proteoliposomes containing purified SecY-68C, SecY-97C, or SecY-68C+97C were incubated at 37°C with SecA in the presence or absence of ATP with radiolabeled pOA:tRNA, containing 206 residues and cysteines at position 5, 175, or both (pOA-5C, pOA-175C, or pOA-5C+175C). The samples were analyzed as in (A).

(C) Scheme of SecY crosslinking to both the signal sequence and mature region of pOA.

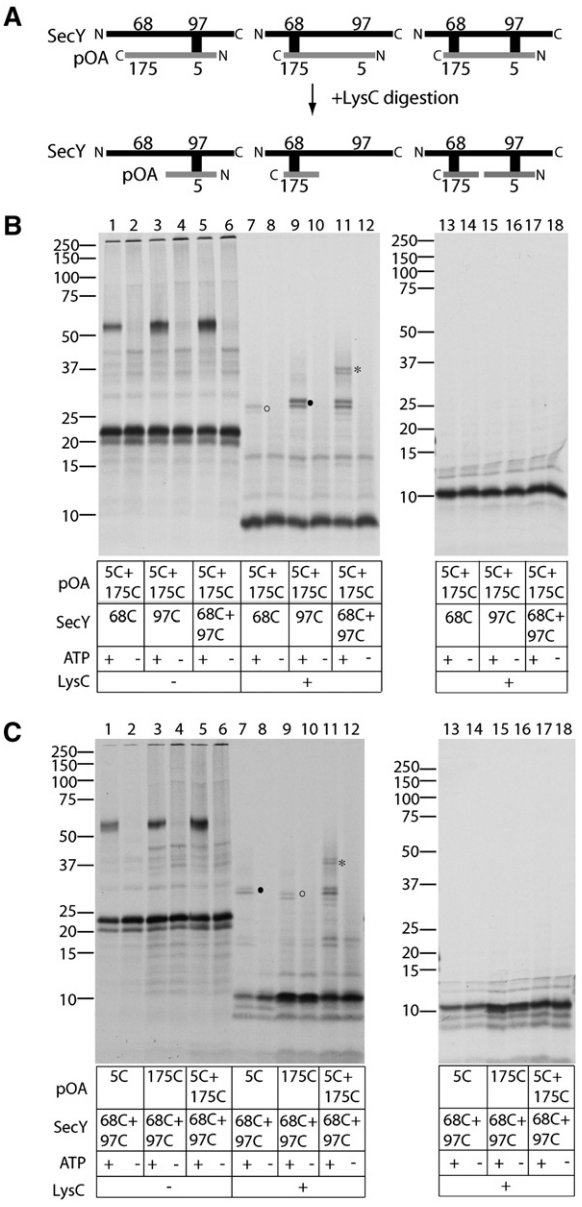


**Figure 3. The Signal Sequence and Mature Region of pOA Are Contained within a Single Copy of SecY**

(A) LysC digestion is used to determine whether one or two disulfide bridges are formed between SecY and pOA.

(B) Proteoliposomes containing SecY complexes with cysteines at different positions (SecY-68C, SecY-97C, or SecY-68C+97C) were incubated at 37°C with SecA and pOA:tRNA, containing 206 residues and cysteines in both the signal sequence and mature region (pOA-5C+175C). After incubation in the presence or absence of ATP, the samples were oxidized and analyzed by nonreducing SDS-PAGE (lanes 1–6). Equivalent samples were solubilized and digested with LysC prior to nonreducing SDS-PAGE (lanes 7–12). LysC-digested samples were also analyzed after reduction with DTT (lanes 13–18). Open circle, crosslink of the SecY fragment with the C-terminal pOA fragment; filled circle, crosslink of the SecY fragment with the N-terminal pOA fragment; asterisk, crosslinks of the SecY fragment with both pOA fragments.

(C) As in (B) but with proteoliposomes containing SecY with cysteines at positions 68 and 97 (SecY-68C+97C) and pOA substrates containing cysteines in the signal sequence, the mature region, or both (pOA-5C, pOA-175C, or pOA-5C+175C).

We first analyzed crosslinked species that contain a single disulfide bridge. The substrate itself was digested to a major band of ~10 kDa and several minor bands (Figures 3B and 3C, lanes 7–12 versus 1–6); the major band is likely a mixture of N- and C-terminal pOA fragments of similar mass. The crosslinked product of pOA-5C+175C and SecY-68C, which can only contain a single disulfide bridge, was cleaved by LysC to a doublet of bands of ~30 kDa (Figure 3B, lane 7, empty circle). Considering the size of the C-terminal fragment of pOA contained in these bands, the SecY fragment must be ~20 kDa, although the appearance of a doublet suggests that it is somewhat heterogeneous. The same fragment was generated when the crosslinking was performed with pOA-175C and SecY-68C+97C (Figure 3C, lane 9, empty circle). A slightly larger doublet of bands was obtained upon cleavage of the crosslinked products generated with either pOA-5C+175C and SecY-97C or pOA-5C and Sec68C+97C (Figure 3B, lane 9, filled circle and Figure 3C, lane 7, filled circle). These products contain the N-terminal fragment of pOA.

We then analyzed the crosslinked product generated with pOA-5C+175C and SecY-68C+97C. When treated with LysC, it gave rise to an additional species of ~37 kDa (Figure 3B, lane 11, Figure 3C, lane 11, asterisk), proving that indeed two disulfide bridges are formed. The size of the product is close to the expectation that the ~20 kDa SecY fragment is disulfide linked with the N- and C-terminal pOA fragments of ~10 kDa. All crosslinked products were only seen in the presence of ATP (Figures 3B and 3C, compare even and odd lanes), and they disappeared upon reduction with DTT (lanes 13–18). These results show that the signal sequence and mature region of pOA can simultaneously crosslink with the same SecY molecule. Thus, the pore is contained in a single SecY copy, even though the SecY complex forms oligomers in reconstituted proteoliposomes (Mori et al., 2003; Scheuring et al., 2005).

## Multiple Copies of SecY Form the Translocation Complex

To address the role of oligomers of SecY complex in translocation, we employed tandem SecY molecules, in which the C terminus of the first SecY copy is coupled covalently to the N terminus of the second SecY copy (Duong, 2003). Tandem SecY complexes generated by expressing fused SecY genes are active in translocation (Duong, 2003). We used purified tandem SecY complexes reconstituted into proteoliposomes to test whether a SecY copy with an inactivating mutation could be rescued for translocation by its association with a wild-type SecY copy, a result that would prove the involvement of SecY oligomers in translocation.

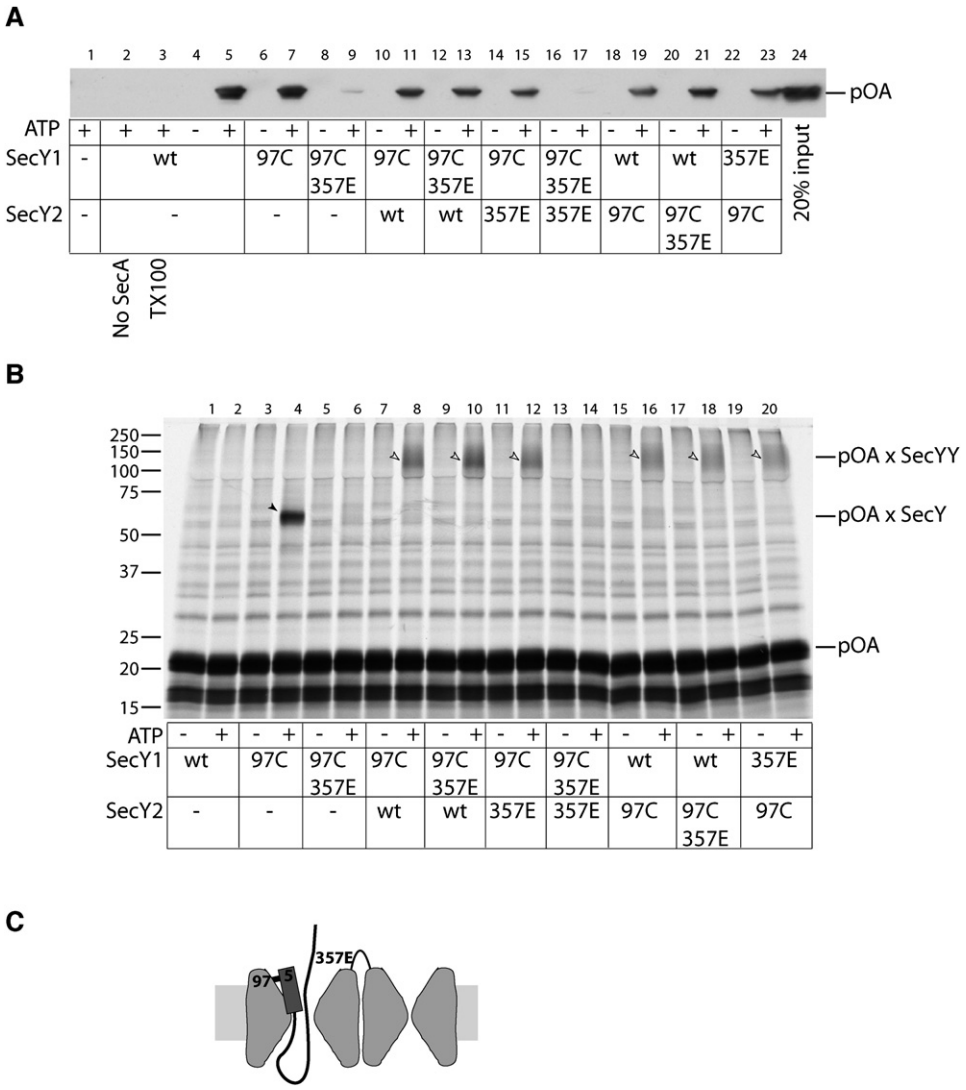


**Figure 4. Signal Sequence Interaction with Tandem SecY Molecules**

(A) Translocation of purified full-length pOA was tested with proteoliposomes containing purified SecY complex with either wild-type (WT) SecY, a SecY mutant containing a cysteine at position 97 (SecY-97C), or tandem SecY constructs (SecYY), in which the first SecY molecule (SecY1) is covalently linked with a second copy (SecY2). The SecY copies were either wild-type, contained a cysteine at position 97, or the R357E mutation. The proteoliposomes contained equivalent amounts of protein (Figure S2A). After incubation at 37°C with SecA in the presence or absence of ATP, proteinase K was added, and protease-protected material was analyzed by immunoblotting. The sample in lane 3 contained Triton X-100 during proteolysis. Lane 24 shows 20% of the input material.

(B) Proteoliposomes containing the indicated purified SecY complexes, were incubated with SecA and radiolabeled pOA:tRNA, containing 206 amino acids and a cysteine in the signal sequence at position 5 (pOA-5C). After oxidation, the samples were analyzed by nonreducing SDS-PAGE, followed by autoradiography. Filled arrow, crosslink to monomeric SecY complex; open arrows, crosslinks to tandem constructs.

(C) Scheme showing that a dead SecY copy, linked to a wild-type SecY copy, can crosslink to a signal sequence.

We first followed the insertion of the signal sequence of pOA into tandem SecY constructs that contained the 97C mutation in either the first or second SecY copy. Both proteins were able to translocate pOA (Figure 4A, lanes 11 and 19). They also inserted the signal sequence, as demonstrated by the ATP-dependent formation of a disulfide bond with pOA-5C (Figure 4B, lanes 8 and 16). Judged from the crosslinking efficiency, the activity of the SecY copies in the tandem is about the same. To inactivate one or both of the SecY copies, we introduced the R357E mutation into the cytoplasmic loop between TMs 8 and 9 of SecY. This mutation in monomeric SecY-97C drastically reduced protein translocation (Figure 4A, lane 9 versus 7), as expected (Mori and Ito, 2001), and it abolished crosslinking of the signal sequence (Figure 4B, lane 6 versus 4). As expected, when this mutation was present in both copies of a tandem construct, no translocation activity or signal sequence crosslinking was observed

(Figure 4A, lane 17; Figure 4B, lane 14). However, tandem constructs containing one wild-type and one R357E copy were active in translocation, regardless of whether the dead copy was at the N- or C terminus (Figure 4A, lanes 13, 15, 21, and 23). Remarkably, when linked to a wild-type SecY copy, the inactive SecY copy in these tandem constructs could insert the signal sequence; when the first SecY copy carried the R357E mutation and the second SecY copy was wild-type, the signal sequence gave a strong crosslink to the first copy (Figure 4B, lane 10; scheme in Figure 4C). With the wild-type copy at the N terminus and the R357E mutation in the C-terminal copy, the signal sequence could also insert into the inactive SecY copy (lane 18). These results strongly suggest that SecY functions as an oligomer during translocation. It should be noted that a SecY monomer with a R357E mutation could not be rescued by simply coreconstituting it with wild-type SecY monomer, probably because the desired dimer combination constituted only a small percentage and because the R357E mutation affects SecY dimerization (Tam et al., 2005).

Next, we followed the insertion of the mature region of pOA into tandem constructs, in which one SecY copy contained a cysteine at position 68, and the R357E mutation was present in one or both SecY copies. Proteoliposomes containing these purified mutant proteins were all able to translocate pOA, except if both SecY copies contained the R357E mutation (Figure 5A). Disulfide bond formation occurred between pOA-175C and monomeric SecY-68C (Figure 5B, lane 4), but was abolished when the R357E mutation was introduced (lane 6). Wild-type tandem SecY complexes containing 68C either in the first or second copy of SecY were able to form a disulfide bridge with pOA-175C (Figure 5B, lanes 8 and 16). When both SecY copies contained the R357E mutation, no crosslink was observed (lane 14). Most significantly, when the first SecY copy contained both the 68C and R357E mutations and the second copy was wild-type, disulfide bridge formation was observed (lane 10; scheme in Figure 5C). Surprisingly, the tandem construct in which the cysteine was in the first, otherwise wild-type copy, and the R357E mutation in the second, yielded weaker crosslinks (lane 12; by a factor of 2.7–5.7 in three experiments). Similarly, a wild-type SecY copy at the N terminus allowed significant crosslinking to a dead SecY copy at the C terminus (lane 18); by comparison, a dead copy at the N terminus showed weaker crosslinking by the associated wild-type copy (lane 20; by a factor of 2.7–5.9). These results again indicate that an oligomer of SecY mediates translocation. They also suggest that the translocating SecY copy is less sensitive to the R357E mutation than the nontranslocating copy.

### Oligomers Containing SecA-Interacting and Substrate-Translocating SecY Copies

Why is translocation mediated by oligomers of the SecY complex? One possibility is that one copy of SecY is primarily responsible for SecA binding, while another forms the pore. In a tandem construct, one may then expect that one SecY copy can be crosslinked to SecA, and simultaneously the other copy to the substrate.

To test this idea, we first screened for combinations of cysteines in SecY and SecA that would give efficient disulfide crosslinks. Because the N terminus of SecA interacts with SecY (Dapic and Oliver, 2000; Mori and Ito, 2006), we introduced single cysteines into the NBF1 domain. The cysteines in SecY were placed into the 6/7 loop, previously implicated in SecA binding (Mori and Ito, 2006). Purified SecA mutants were mixed with proteoliposomes containing purified SecY mutants, and treated with an oxidant to induce disulfide bridge formation. The products were separated by SDS-PAGE and visualized by staining with Coomassie blue. Cysteines at positions 48, 55, 163, and 202 of SecA formed a disulfide bond with cysteines introduced into the tip of the 6/7 loop of SecY (Figure 6A, lanes 7, 8, 12, 13, 27, 47, and 48). The identity of the crosslinked species was confirmed by immunoblotting with SecA and SecY antibodies (data not shown). Cysteines introduced at positions 148, 153, 180, 184, or 188 of SecA did not crosslink to the 6/7 loop. All SecA residues that gave crosslinks were localized to one face of NBF1 (Figure 6B; colored in red).

For subsequent experiments, we chose a cysteine at position 48 in the NBF1 domain of SecA (SecA-48C) and a cysteine at position 255 in the 6/7 loop of SecY (SecY-255C). This combination gave a prominent disulfide bridge in the presence or absence of ATP (Figure 6C, lane 9, and data not shown). A crosslink of the same intensity was seen if SecY contained an additional cysteine at position 97 (SecY-255C+97C) (lane 11). As expected, no crosslink was observed with SecY-97C (lane 10) or with SecA lacking cysteines (lanes 1, 2). Next, we introduced the cysteine at position 255 into either the first or second SecY copy of tandem constructs. All constructs gave disulfide crosslinks with SecA-48C, regardless of whether the cysteine was present in the N- or C-terminal SecY copy, or whether an additional cysteine at position 97 was present (lanes 12–15). No crosslinks were seen with a tandem construct lacking a cysteine at position 255 (lane 16). It should be noted that both SecY copies in a tandem construct are equally efficient in crosslinking to the NBF1 domain of SecA (Figure 6C, lanes 12 and 14) and in translocating polypeptides (Figures 4B and 5B), indicating that covalent linkage does not impair the function of either SecY copy.

We then used the tandem constructs to test whether the NBF1 domain of SecA interacts with a translocating or nontranslocating SecY copy. The SecY copies carried a cysteine at position 97 for crosslinking with the signal sequence in pOA-5C, a cysteine at position 255 for crosslinking with SecA-48C, or both cysteines. When the first SecY copy contained a cysteine at position 97 and the second SecY a cysteine at position 255, two ATP-dependent crosslinks of pOA-5C were observed: one containing the tandem SecY molecule alone, and the other both the tandem SecY and SecA (Figure 6D, lane 30). These data show that the first SecY copy contains the translocation

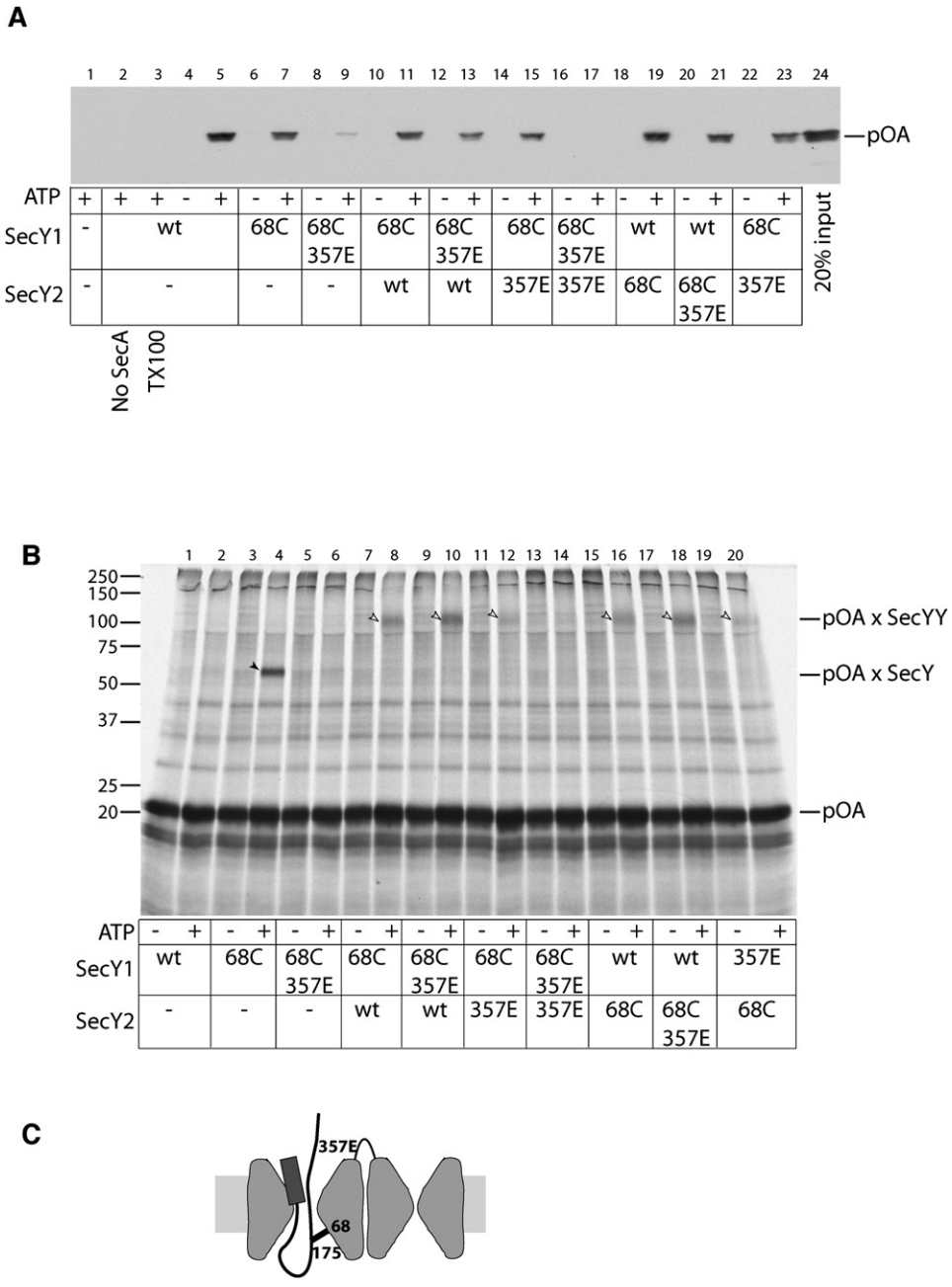


**Figure 5. Interaction of the Mature Region of a Substrate with Tandem SecY Molecules**

(A) Translocation of purified full-length pOA was tested with proteoliposomes containing purified SecY complex with either wild-type (WT) SecY, a SecY mutant containing a cysteine at position 68 (SecY-68C), or tandem SecY constructs (SecYY). The SecY copies were either wild-type, contained a cysteine at position 68, or the R357E mutation. The proteoliposomes contained equivalent amounts of protein (Figure S2B). After incubation at 37°C with SecA in the presence or absence of ATP, proteinase K was added, and translocated material was detected by immunoblotting. The sample in lane 3 contained Triton X-100 during proteolysis. Lane 24 shows 20% of the input material.

(B) Proteoliposomes containing the indicated purified SecY complexes were incubated with SecA and radiolabeled pOA:tRNA, containing 206 amino acids and a cysteine in the mature region at position 175 (pOA-175C). After oxidation, the samples were analyzed by nonreducing SDS-PAGE, followed by autoradiography. Filled arrow, crosslink to monomeric SecY complex; open arrows, crosslinks to tandem constructs.

(C) Scheme showing that a dead SecY copy, linked to a wild-type SecY copy, can crosslink to the mature region of a substrate.

substrate, and the second interacts with the NBF1 domain of SecA (scheme in Figure 6E). When both cysteines were present in the first SecY copy, individual crosslinks to pOA-5C and SecA-48C were seen, but double crosslinks were absent or very faint (lane 26). Thus, the translocation substrate does not insert into the SecY copy that interacts

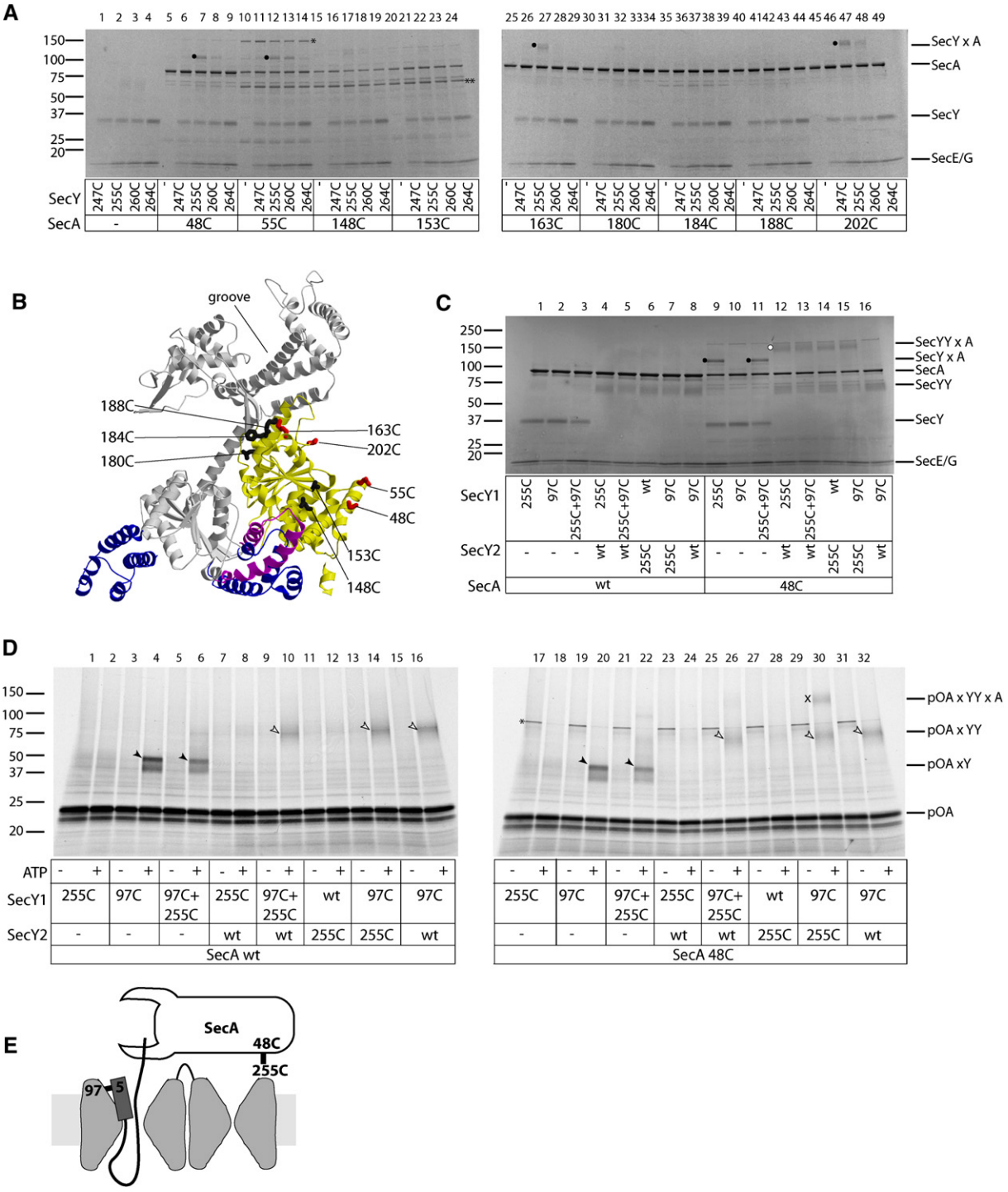


**Figure 6. Oligomers Containing SecA-Interacting and Substrate-Translocating SecY Copies**

(A) Proteoliposomes containing SecY complexes with single cysteines at the indicated positions in the 6/7 loop of SecY were incubated on ice with SecA mutants containing single cysteines at different positions in the NBF1 domain. The samples were oxidized, quenched with NEM, and analyzed by nonreducing SDS-PAGE, followed by staining with Coomassie blue. Filled circles, crosslinks between SecY and SecA; asterisk, crosslinks between two SecA molecules; double asterisk, likely a fragment of SecA.

(B) Ribbon diagram of *B. subtilis* SecA in an open conformation (Osborne et al., 2004). The NBF1 domain is highlighted in yellow. *E. coli* SecA residues that form disulfide bonds with residues in the 6/7 loop of SecY are in red. Residues that did not form disulfide bonds are black. Residues are numbered as in *E. coli* SecA. Nonconserved insertions in SecA from *M. tuberculosis* (Sharma et al., 2003) and *T. thermophilus* (Vassylyev et al., 2006) are superimposed in magenta and blue, respectively.

with the NBF1 domain of SecA. Similar results were obtained with SecA carrying a cysteine at position 55 (Figure S3). No or only faint double crosslinks were seen with monomeric SecY complexes (lanes 17–22), with tandem SecY complexes lacking one of the two cysteines (lanes 24, 28, and 32), or with SecA lacking cysteines (lanes 1–16). These results show that SecA interacts through its NBF1 domain with the nontranslocating SecY copy and inserts the polypeptide chain into the other copy. They also support the idea that only one SecY molecule forms the translocation pore.

## DISCUSSION

We show that one copy of the SecY complex forms the translocation pore but that oligomers mediate translocation, with a nontranslocating SecY copy providing an interaction site for the NBF1 domain of SecA. Based on these and other results, we propose a refined model of SecA-mediated protein translocation (Figure 7). At the beginning of translocation, SecA, anchored by its NBF1 domain to one SecY molecule, transfers the signal sequence of a translocation substrate into the neighboring SecY copy, a step requiring ATP hydrolysis. The substrate is inserted as a loop. Signal sequence insertion into the lateral gate causes the plug to move toward the back of the SecY molecule, and insertion of the mature region of the substrate into the pore fixes the channel in the open state. During further translocation, the signal sequence remains stationary, while the mature part of the translocation substrate passes through the central pore. Specifically, the peptide binding domain of SecA binds a segment of the substrate and moves toward the translocationally active SecY molecule. The peptide binding domain then releases the substrate segment into the channel, and moves back. These events are coupled to ATP hydrolysis cycles and are repeated until the polypeptide is all the way through the channel. The static interaction of NBF1 with the nontranslocating SecY copy prevents complete detachment of SecA when it moves away from the translocating SecY copy.

Our conclusion that a single SecY molecule forms the pore is based on the observation that both the signal sequence and mature region of proOmpA are contained in the same SecY molecule. Because the cysteines in the substrate were separated by a long polypeptide segment, this result is not caused by artificial constraint of the substrate to a single channel. The N terminus of the

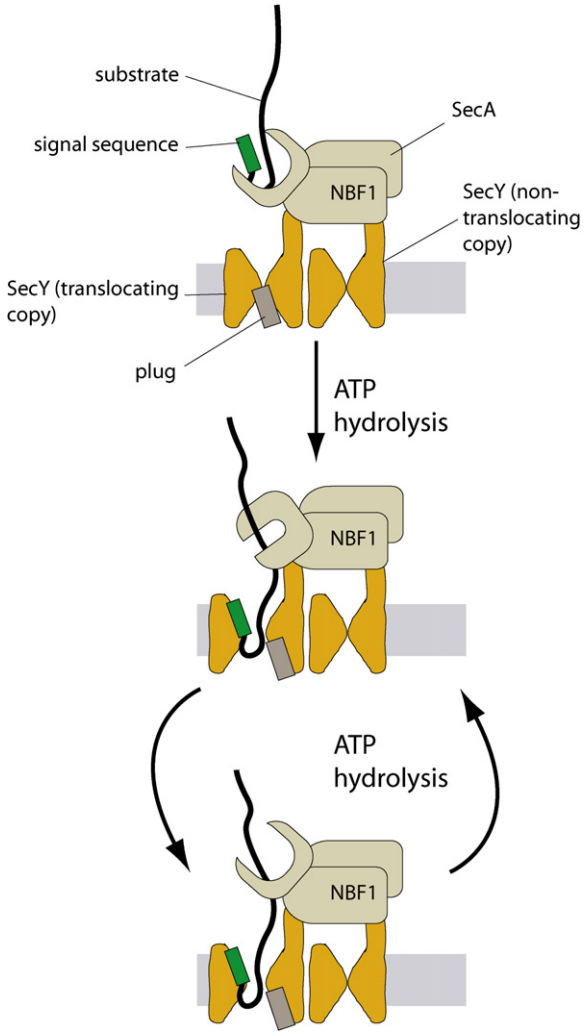


**Figure 7. A Model for SecA-Mediated Translocation through the SecY Channel**
See text.

(C) Proteoliposomes were generated that contained SecY complex with a cysteine at position 255 for crosslinking with SecA, a cysteine at position 97 for crosslinking with the signal sequence, or both cysteines. Other vesicles contained purified tandem constructs (SecYY) with the same cysteines in the first or second SecY copy (SecY1 and SecY2), as indicated. The samples were incubated at 37°C with either SecA lacking cysteines (WT) or SecA containing a cysteine at position 48 (SecA-48C). After oxidation on ice with copper phenanthroline, the samples were quenched with NEM, and analyzed by nonreducing SDS-PAGE, followed by Coomassie staining. Crosslinks of SecA-48C with monomeric and tandem SecY are indicated by filled and open circles, respectively.
(D) Proteoliposomes containing the indicated purified SecY proteins were incubated with radiolabeled pOA:tRNA, containing 206 residues and a cysteine in the signal sequence at position 5 (pOA-5C) in the presence of cysteineless SecA (WT) or SecA with a cysteine at position 48 of the NBF1 domain (SecA-48C). ATP was added as indicated. Crosslinks were induced with copper phenanthroline and analyzed by nonreducing SDS-PAGE and autoradiography. Filled arrows, crosslinks of pOA-5C with monomeric SecY; open arrows, crosslinks of pOA-5C with tandem SecYY; X, crosslink between SecA-48C, tandem Sec Y, and pOA.
(E) Scheme showing that one copy of SecY in a tandem construct crosslinks to the NBF1 domain of SecA, and the other SecY copy crosslinks to the signal sequence of pOA.

hydrophobic core of the signal sequence was in close proximity to the cytoplasmic end of TM2b, one of the helices forming the lateral gate. At the same time, a residue in the mature region of proOmpA could be crosslinked to the center of SecY. Although the lateral gate is only ~22 Å away from the central pore, these sites show exquisite specificity in their interactions with the signal sequence and mature region, respectively. The residue in the center of SecY is actually located in the plug and can also be crosslinked to a residue in the TM segment of SecE at the back of SecY (Tam et al., 2005), suggesting that the plug is mobile in the open channel.

The idea that the pore is formed from a single SecY molecule is supported by the observation that only one SecY molecule remains associated with a substrate molecule upon solubilization of the membrane (Duong, 2003); SecY oligomers dissociate in detergent, and therefore the nontranslocating copy is lost during isolation of the complex. Our data are also consistent with in vitro photo-crosslinking experiments, which showed that the hydrophobic core of a signal sequence forms a short helix that is contacted on opposite sides by TM2b and TM7 of yeast Sec61p (Plath et al., 1998). These two helices appear to belong to the same Sec61p molecule, because a signal sequence with two photoreactive probes could not be crosslinked to two Sec61p molecules (Plath et al., 2004).

The fact that TM2b and TM7, from the same SecY complex, sandwich the hydrophobic core of the signal sequence over its entire length (Plath et al., 1998) indicates that there is not a large opening in the lateral gate; rather, the gate appears to open just enough to allow the intercalation of the hydrophobic signal sequence, bringing it in contact with phospholipid molecules (Plath et al., 1998). Thus, with or without an intercalated hydrophobic sequence, the gate would prevent the lateral exit of a hydrophilic segment of a translocation substrate, effectively confining it to the central pore of a single Sec61p/SecY molecule. A hydrophobic segment, however, could partition through the lateral gate into the lipid phase.

Forming the channel from a single SecY molecule has implications for the estimated pore size. The crystal structure of a monomeric SecY complex indicates that moving the plug out of the way would create a pore with a diameter of ~5–8 Å (Van den Berg et al., 2004), which is probably too small to accommodate a translocating polypeptide chain. However, molecular dynamics simulations show that a helix, mimicking a translocation substrate, can be pulled through the SecY complex, causing the plug to move out of the way and the pore ring to widen (Gumbart and Schulten, 2006). When expanded, the pore may be large enough to explain the passage of bulky amino acid residues or of a disulfide-bonded loop of 13 residues (Tani et al., 1990). However, it is not large enough to allow tertiary structure formation in a polypeptide (Kowarik et al., 2002), and it can accommodate at most two hydrophobic segments of membrane proteins, one in the pore and one in the lateral gate (Ismail et al., 2006; Sadlish et al., 2005). A single SecY molecule could not form a channel with a diameter of >40 Å, a size deduced from fluorescence-quenching experiments for the Sec61p channel (Hamman et al., 1997). However, the same experiments overestimate the diameter of the ribosome tunnel (Voss et al., 2006).

Dimers of SecY complexes likely form the basic unit of active channels, in agreement with an electron microscopy structure of a ribosome-channel complex (Mitra et al., 2005). A near front-to-front arrangement of two SecY complexes was reported, based on fitting the crystal structure by normal mode analysis into an electron density map of 15 Å resolution. However, a higher resolution is required to unambiguously determine the orientation of the SecY monomers, particularly because crosslinking data and a 2D crystal structure suggest that they are associated back-to-back (Breyton et al., 2002; Kaufmann et al., 1999).

How exactly SecA binds to a SecY oligomer remains to be determined. The dimensions of SecA are consistent with one molecule interacting with two SecY complexes. With NBF1 bound to one SecY molecule, a large groove seen in the open conformation of SecA and postulated to be involved in peptide binding (Osborne et al., 2004) could be located above the cytoplasmic funnel of the other SecY molecule. A role for SecA monomers in translocation is supported by experimental data (Or et al., 2004), but the issue is controversial (Mitra et al., 2006). The interaction sites for SecA are likely different for the two SecY copies. The 6/7 loop of the nontranslocating SecY copy contacts one face of the NBF1 domain of SecA, a surface that does not contain amino acid insertions in other bacteria (Figure 6B), but the loop may be somewhat flexible as it crosslinks to several positions in SecA. The 8/9 loops of both the nontranslocating and translocating SecY copies may also contact SecA, and their interaction may contribute to SecY dimerization (Tam et al., 2005), but it appears that only one intact loop is essential, particularly the one in the nontranslocating SecY copy, as indicated by the observation that a mutation in it reduced translocation of a substrate through an associated wild-type SecY copy. Signal sequence insertion was less affected; the defect may be exacerbated at later stages of translocation, because multiple rounds of ATP hydrolysis by SecA are required.

Given the conservation of Sec61p/SecY complexes, it is likely that in all translocation modes and organisms the active translocation complex is an oligomer, with one Sec61p/SecY copy forming the pore. However, during cotranslational translocation, the nontranslocating copy could simply stabilize interactions of the translocating copy with the ribosome. Upon termination of translocation, dissociation of the Sec61p/SecY oligomer within the plane of the membrane could facilitate ribosome dissociation, allowing the next round of translocation to occur. The oligomeric state of the Sec61p complex indeed appears to change during cotranslational translocation (Schaletzky and Rapoport, 2006). Dissociable oligomers

may also allow the Sec61p/SecY complex to change channel partners.

Dimeric translocation channels, with only one active copy, have also been proposed for PapC, involved in the secretion of pili subunits across the outer membrane of *E. coli*, as well as for Tom40 and Tim22, involved in protein transport across the outer and inner mitochondrial membrane, respectively (for discussion, see Thanassi et al., 2005). PapC and Tom40 are β-barrel proteins, and thus dimers likely cannot form fused pores. Nonfused pores exist in many other channels, such as tetrameric aquaporins, trimeric porins, and dimeric chloride channels. Whereas in these cases all pores are active, it appears that in protein translocation systems, only one copy in an oligomeric assembly forms the actual channel.

## EXPERIMENTAL PROCEDURES

### Protein Expression

SecY mutants were expressed from a pBAD vector also encoding SecG and His-tagged SecE. SecY tandems were constructed by linking arginine 443 of the first SecY to alanine 2 of the second SecY. SecYEG complexes were purified by Ni-NTA agarose. SecY-97C tandems were further purified by gel filtration. 18 μg of SecY complex was mixed with 33 μl of *E.coli* polar lipid (20 mg/ml) and reconstituted into proteoliposomes using Biobeads to remove the detergent. Full-length proOmpA with a C-terminal Strep tag, was expressed in MM52 cells. The protein was solubilized from inclusion bodies with 6 M urea, purified on a Mono Q column, and dialyzed against 8 M urea in 50 mM HEPES (pH 7). His-tagged SecA mutants were purified with Ni-NTA agarose followed by MonoQ and Superdex200 columns. All SecA and SecY mutants used in this study lacked endogenous cysteine residues.

### Generating tRNA Associated pOA Substrates

Cysteines were introduced into a pOA derivative in which all endogenous cysteines were removed and residues 175 to 296 deleted. PCR was used to introduce an SP6 promoter and a Kozak sequence at the 5′ end. pOA coding fragments of different lengths, which lacked a stop codon, were used for in vitro transcription. RNA was translated in the presence of $^{35}$S-methionine in reticulocyte lysate for 25 min at 30°C. The proteins were precipitated by addition of three volumes of 80% saturated ammonium sulfate. After centrifugation at 22,000 × g for 20 min, the pellet was resuspended in 8 M urea, 50 mM HEPES (pH 7). The solubilized material was spun at 100,000 rpm in a Beckman TLA100 rotor, and the supernatant was used for experiments. To remove tRNA from the substrate, RNase A at 1 mg/ml was added on ice for 45 min prior to ammonium sulfate precipitation.

### Crosslinking

Crosslinking reactions were performed in a volume of 20 μl in buffer A (50 mM Tris [pH 7.9], 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$). SecA and ATP were added at 20 μg/ml and 1.25 mM, respectively. 2 μl of SecYEG-containing proteoliposomes were added to a 20 μl reaction. In some samples, ATP was depleted by addition of 3.5 μg/ml hexokinase, 10 mM glucose, and 1.25 mM ADP. Translocation intermediates were generated by adding 0.4 μl of urea-denatured pOA derivative to a 20 μl reaction. After incubation at 37°C for 10 min, the samples were placed on ice before addition of 50 μM copper phenathroline for 5 min (Kaufmann et al., 1999). The samples were incubated for 5 min with 10 mM NEM, followed by 20 min at room temperature with 100 μg/ml RNase A. The samples were analyzed by nonreducing SDS-PAGE and autoradiography. Crosslinking of SecA to SecY was performed similarly, except that oxidation was performed with 50 μM sodium tetrathionate.

### Protease Digestion of Crosslinked Products

The samples were crosslinked, treated with NEM and RNase as above, and then subjected to centrifugation for 25 min at 50,000 rpm in a Beckman TLA100 rotor. The pellets were resuspended in 10 μl of 1% SDS in 0.1 M Tris (pH 8.8), sonicated for 10 min, and heated to 65°C for 10 min. After dilution to a final volume of 100 μl with 0.1 M Tris (pH 8.8), remaining insoluble material was removed by centrifugation for 1 min at 15,000 rpm. Sequencing-grade endoproteinase LysC was added to half of the sample (final concentration of 10.3 μg/ml) and the other half remained untreated. All samples were incubated overnight at 37°C and subjected to electrophoresis using Tris-Tricine gels and autoradiography.

### Translocation Reactions

Translocation reactions were performed in 20 μl in buffer A containing 0.2 mg/ml BSA and 1 mM DTT. SecA, SecY-containing proteoliposomes, and ATP-depletion system were added as above. ATP was added at 1.25 mM in the presence of creatine phosphate and creatine kinase, and purified Strep-tagged pOA was added at 38 μg/ml. The samples were incubated at 37°C for 10 min. They were placed on ice and incubated with 0.1 mg/ml proteinase K for 30 min. The reaction was stopped with 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and the proteins precipitated with 10% TCA. The samples were analyzed by SDS-PAGE and immunoblotting with Strep tag antibodies. Translocation of signal sequence cysteine mutants of pOA were tested as above, but using proteins synthesized in a reticulocyte lysate in the presence of $^{35}$S-methionine. Protease-protected pOA was detected by SDS-PAGE and autoradiography.

### Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.cell.com/cgi/content/full/129/1/97/DC1/.

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