Disulfide bridge formation between SecY and a translocating polypeptide localizes the translocation pore to the center of SecY

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During their biosynthesis, many proteins pass through the membrane via a hydrophilic channel formed by the heterotrimeric Sec61/SecY complex. Whether this channel forms at the interface of multiple copies of Sec61/SecY or is intrinsic to a monomeric complex, as suggested by the recently solved X-ray structure of the *Methanococcus jannaschii* SecY complex, is a matter of contention. By introducing a single cysteine at various positions in *Escherichia coli* SecY and testing its ability to form a disulfide bond with a single cysteine in a translocating chain, we provide evidence that translocating polypeptides pass through the center of the SecY complex. The strongest cross-links were observed with residues that would form a constriction in an hourglass-shaped pore. This suggests that the channel makes only limited contact with a translocating polypeptide, thus minimizing the energy required for translocation.

Introduction

Many proteins that are exported from the cytosol pass through a membrane channel into the ER in eukaryotes or the extracellular space in prokaryotes (for reviews see Rapoport et al., 1996; Pohlschroder et al., 1997; Matlack et al., 1998; Johnson and van Waes, 1999). The channel is formed by a heterotrimeric complex of proteins called the Sec61 complex in eukaryotes and the SecY complex in bacteria and archaea. The channel has a hydrophilic interior, as shown by electrophysiology and fluorescence lifetime measurements (Simon and Blobel, 1991; Crowley et al., 1994). Previous models assumed that the channel is formed at the interface between three or four copies of the Sec61/SecY complex (Hanein et al., 1996; Beckmann et al., 1997; Hamman et al., 1997; Manting et al., 2000; Menetret et al., 2000). However, the recently solved X-ray structure of the SecY complex from *M. jannaschii* is of a monomer with no exterior hydrophilic surfaces in the membrane (van den Berg et al., 2004); thus, the channel pore could not be formed by simple association of several Sec61/SecY complexes. The channel, visualized in a closed state in the X-ray structure, features a cytoplasmic funnel that is lined by a number of evolutionarily conserved hydrophilic residues. The funnel narrows to a close at a plug formed by a short helix (helix 2a) near the center of the membrane. It was postulated that when the channel opens, helix 2a swings outward, revealing an extracellular funnel which, combined with the cytoplasmic funnel, results in an hourglass-shaped pore (van den Berg et al., 2004). Translocating polypeptides would be threaded through a ring of hydrophobic residues at the neck of the hourglass before reaching the extracellular space. Although this is an attractive hypothesis, there is as yet no conclusive evidence that a translocating polypeptide passes through the center of the SecY complex.

Cross-linking is the method of choice to identify residues in Sec61p/SecY that line the path of a translocating polypeptide chain through the membrane. So far, cross-linking has been performed at a rather crude level. Photo-activatible probes incorporated at different positions in a translocating polypeptide allowed the identification of Sec61p/SecY as the main component of the channel (Görlich et al., 1992; Musch et al., 1992; Sanders et al., 1992; High et al., 1993; Joly and Wickner, 1993; Mothes et al., 1994). In a more refined set of cross-linking experiments, probes were positioned in the signal sequence of prepro-α-factor. The site of cross-linking to Sec61p was mapped to individual transmembrane (TM) segments by the use of Sec61p mutants, each with a single protease cleavage site in a cytosolic or luminal loop (Plath et al., 1998). These experiments showed that the signal sequence binds specifically to TM segments 2 and 7. To identify TM segments that line the pore, a similar approach was tried with probes in the mature region of prepro-α-factor (Plath et al., 2004). Simultaneous

cross-linking to several different TM segments of Sec61p was observed, making it difficult to derive any firm conclusions about the location of the pore.

To define the precise location of the pore, we introduced single cysteines at 30 positions in *E. coli* SecY, selected on the basis of the 3-D structure of the *M. jannaschii* SecY complex. We then tested which positions support formation of a disulfide bond with a cysteine on a translocating polypeptide in the channel. Our results show that the mature region of a translocating chain mainly contacts residues in the narrowest part of the hourglass-shaped pore, and support the idea that the translocation pore is located in the center of SecY, rather than at the interface of multiple SecY molecules.

Results and discussion

Generating a translocation intermediate

Our strategy was to introduce a single cysteine at select positions of SecY and test whether it could form a disulfide bridge with a cysteine in a translocating polypeptide chain. To increase the likelihood of bond formation, the polypeptide substrate was trapped in the channel by preventing its complete translocation with a bulky t-RNA/ribosome at the COOH terminus. This was accomplished by in vitro translation of a truncated mRNA coding for the first 220 aa of proOmpA (Fig. 1 A). The lack of a stop codon results in a nascent polypeptide chain associated with the ribosome as a peptidyl-tRNA (Gilmore and Blobel, 1985). The substrate (pOA220:tRNA) contained a single cysteine at position 175, which is expected to be inside the channel upon formation of the translocation intermediate (the COOH-terminal ∼36 residues of pOA220 are inside the ribosome).

We first tested the ability of pOA220:tRNA to generate a translocation intermediate. For a control, pOA220:tRNA was treated with RNase to remove the tRNA, yielding a "standard" bacterial translocation substrate, pOA220 (Fig. 1 B, lane 1). In both cases the driving force for translocation was provided by

the bacterial ATPase SecA and a protease-protection assay was used to confirm translocation. In the presence of SecA, ATP, and proteoliposomes containing purified *E. coli* SecY complex, the translocated free pOA220 was protected from proteolysis (Fig. 1 B, lane 4). In the absence of ATP or if Triton X-100 was added after translocation to solubilize the membranes, most of the labeled material was digested by the protease (Fig. 1 B, lanes 2 and 5, respectively). In the absence of RNase pretreatment, two major bands were visible (Fig. 1 B, lane 6); the top band is the peptidyl-tRNA (pOA220:tRNA), the bottom one (pOA220) lacks the tRNA moiety and is presumably freed by hydrolysis of the peptidyl-tRNA bond during translation or sample preparation. When tRNA-associated pOA was first translocated and then digested with protease, some full-length pOA220:tRNA was protected (Fig. 1 B, lane 9, topmost asterisk). These may be chains in which the bulky tRNA/ribosome closely abuts the channel and prevents the protease from cleaving. In addition, a number of fragments appeared that were smaller than pOA220 (Fig. 1 B, lane 9, asterisks), suggesting that the nascent chain may slide back into the cytosol, so that the protease can cleave at internal positions. It should be stressed that in this situation the ribosome functions solely as an obstacle; it does not move the polypeptide chain as in cotranslational translocation.

Generation of a disulfide bridge to specific sites in SecY

To introduce single cysteines into *E. coli* SecY, we first mutated its two endogenous cysteines to serines (Fig. 3 A, underlined). Using the crystal structure of *M. jannaschii* and a sequence alignment of all known Sec61/SecY molecules as a guide, we next introduced single cysteine substitutions at 30 selected positions (Fig. 3 A). We then tested the translocation activity of the purified mutant SecY complexes after their reconstitution into proteoliposomes, using full-length proOmpA as a substrate (unpublished data). Five of the mutants (I82C, I86C, I189C, S323C, and F387C) had <40% activity compared with the wild-type SecY complex and were discarded. Proteoliposomes generated

Figure 1. **Generating a translocation intermediate.** (A) A tRNA-associated fragment of ^{35}S-proOmpA (pOA) containing a single cysteine is trapped in the translocation channel. Another single cysteine is placed in SecY at a position that may line the channel. If the two cysteines are close to one another they can form an intermolecular disulfide bond upon exposure to oxidizing conditions. (B) A fragment of pOA containing 220 aa (pOA220) was synthesized in vitro by translation of truncated mRNA in the presence of [^{35}S]methionine. Where indicated the samples were treated with RNase to remove the tRNA moiety. The translation products were then incubated with SecY complex–containing proteoliposomes and SecA in the presence or absence of ATP. Translocation was tested by treatment with protease in the absence or presence of Triton X-100. Asterisks highlight protected fragments of pOA220. Arrows point to positions of full-length pOA220 and full-length pOA220:tRNA.
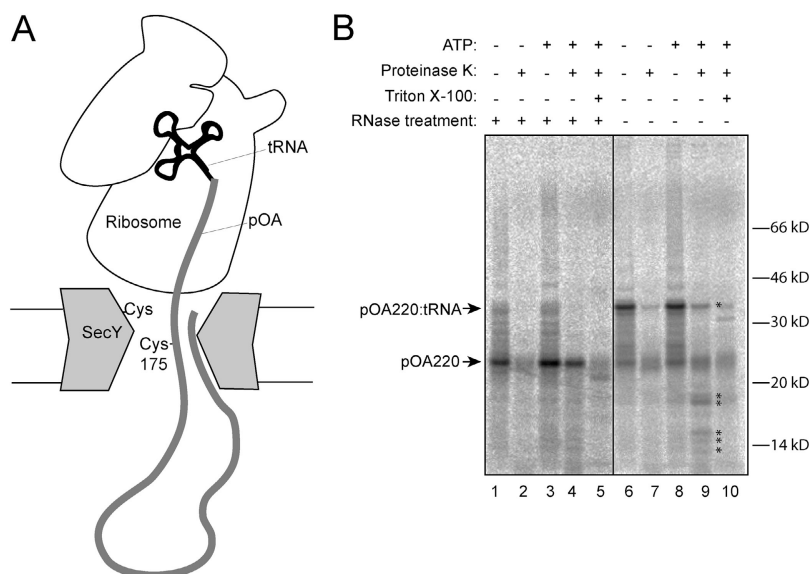
with the active complexes were then incubated with pOA220:tRNA in the presence of a cysteine-free mutant of SecA. The samples were pelleted by centrifugation, resuspended in the presence of an oxidizing agent to facilitate disulfide bridge formation, and analyzed by nonreducing SDS-PAGE.

Initial experiments revealed two SecY mutants, G69C and S282C, that gave efficient cross-links to pOA220:tRNA. We characterized these cross-links with a number of controls. A cross-linked band of the expected size was seen in the complete system (Fig. 2 A, lanes 1 and 6, arrowheads), but was missing in samples that lacked ATP (lanes 2 and 7), SecA (lanes 3 and 8), or the oxidizing agent (lanes 5 and 10). The cross-links were absent in samples that contained cysteine-free proOmpA (Fig. 2 A, lanes 4 and 9) or cysteine-free SecY (Fig. 2 B, lane 2), or that were generated with translocation substrate that lacked a signal

sequence (Fig. 2 B, lane 10). The presence of SecY in the cross-linked product was confirmed by immunoprecipitation under denaturing conditions with purified anti-SecY antibodies (Fig. 2 C). When the samples were treated with RNase after cross-linking, the cross-linked product displayed an increased mobility, of ~15 kD, as expected from loss of the tRNA moiety (Fig. 2 A, lane 12, labeled "pOA220xSecY"). If the translation mix was treated with RNase before translocation and cross-linking, this band was absent (lane 13), indicating that a fully translocated substrate does not form cross-links to SecY. Together, these results confirm that disulfide bond formation with an arrested translocation intermediate can be used to identify specific amino acids in SecY that surround the nascent chain.

When wild-type SecA, which contains four endogenous cysteines, was used to generate the translocation intermediate,
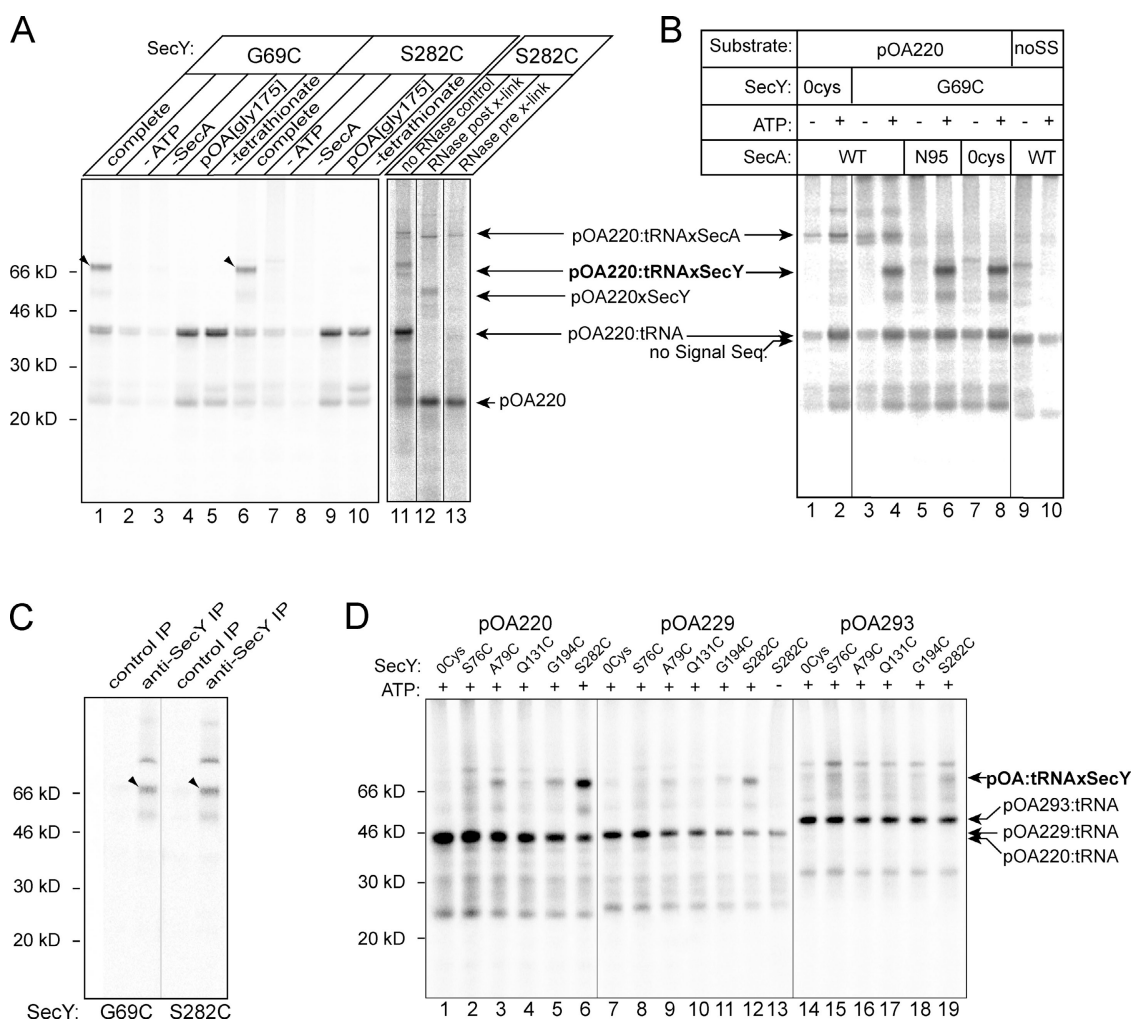

Figure 2. **Cysteines at positions 69 and 282 of SecY form a disulfide bond with pOA220:tRNA.** (A) SecY mutants G69C and S282C were purified, reconstituted into proteoliposomes, incubated with cysteine-free SecA and ^{35}S-pOA220:tRNA in the presence of ATP, and treated with 100 μM tetrathionate to facilitate disulfide bond formation. "Complete" contains all components; "−" indicates the omitted component. "pOA[Gly175]" is pOA220 with a glycine at position 175 instead of cysteine. Where indicated, samples were treated with RNase before or after cross-linking (pre- and post-X-link). Arrows point to the positions of the substrates and to cross-links with SecY and SecA. The cross-links to SecY are also highlighted by arrowheads. (B) Cross-linking was performed with pOA220 or substrate lacking the signal sequence (deletion of the NH2-terminal 21 aa of pOA220; noSS). SecY mutants lacked cysteines (0Cys) or contained a single cysteine at position 69 (G69C). SecA was either wild type (WT), or lacked three (N95) or all four (0Cys) cysteines. (C) Cross-linked samples were denatured and immunoprecipitated (IP) with affinity-purified polyclonal anti-SecY or control antibodies. (D) ^{35}S-pOA:tRNA fragments of 220, 229, or 293 aa were translocated into proteoliposomes with SecY that contains no cysteines (0cys), or a single cysteine at positions 76, 79, 131, 194, or 282.
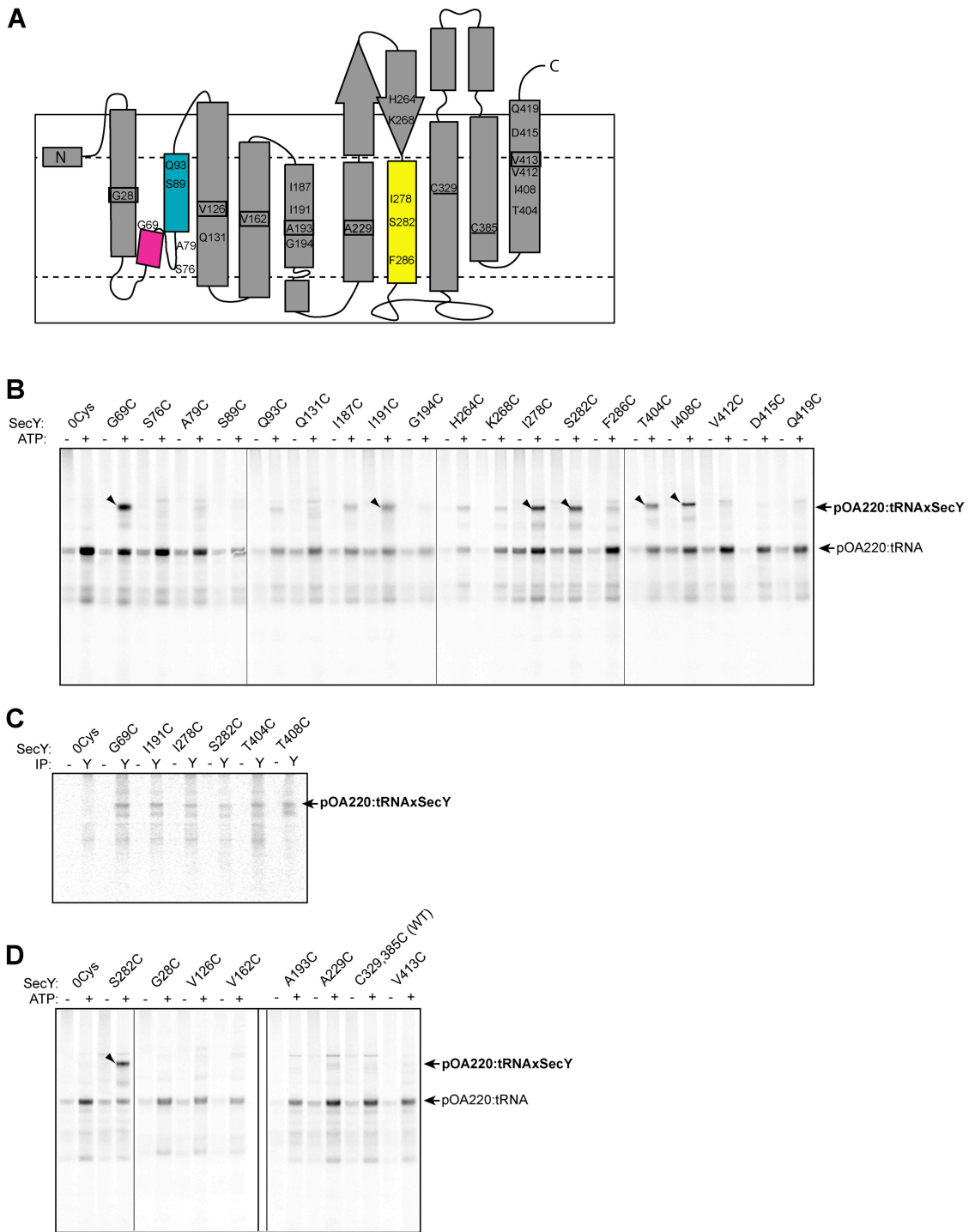
Figure 3. **Identification of amino acids in SecY that contact the translocating polypeptide.** (A) Cartoon showing the topology of SecY and positions where a cysteine was substituted for the endogenous residue. The two endogenous cysteines are underlined. Boxed numbers indicate positions that are predicted to face the exterior of the molecule. TM helices TM2a (magenta), TM2b (blue), and TM7 (yellow) are highlighted. (B) Purified SecY complexes with a single-cysteine substitution at a position predicted to face the interior of molecule were tested for their ability to form disulfide bonds with pOA220:tRNA in the presence of ATP as in Fig. 2; 0Cys, SecY lacking cysteines. Arrows indicate the substrate and its cross-link to SecY. Arrowheads highlight the strongest cross-links (>30% of total pOA220:tRNA linked to SecY). The gel shown is representative of at least three independent experiments. (C) Cross-linked samples were denatured and precipitated with affinity-purified polyclonal anti-SecY (Y) or control (−) antibodies. (D) SecY molecules with a single-cysteine substitution at positions on the exterior were screened for their ability to form disulfide bridges to pOA220. S282C is included as a positive control for cross-linking. 0Cys, SecY lacking cysteines; WT, wild-type SecY (cysteines at amino acids 329 and 385). An arrowhead highlights the cross-link to SecY S282C. The gel shown is representative of at least three independent experiments.
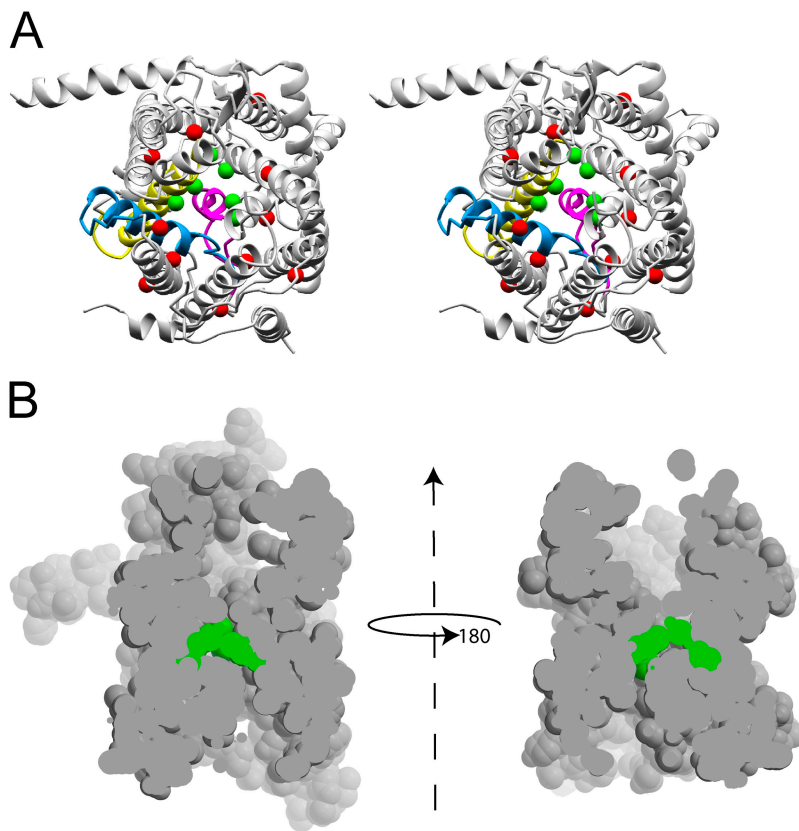
A

B

Figure 4. **Positions in the X-ray structure of SecY that contact a translocating polypeptide.** (A) Stereo view from the cytosol of the *M. jannaschii* SecY complex. Green spheres highlight the position of residues that in *E. coli* SecY give strong cross-links to a translocating chain (G69, I191, I278, S282, T404, I408). Red spheres indicate residues that did not give cross-links (G28, S76, S89, V126, Q131, V162, A193, A229, C329, C385, V413). TM helices TM2a (magenta), TM2b (blue), and TM7 (yellow) are highlighted. (B) Space-filling model of the SecY complex, viewed from the side. The complex has been sliced through the middle and opened to show the interior. Residues that form the strongest disulfide bonds with a translocation substrate are colored in green.

additional bands were seen above the cross-link to SecY (Fig. 2 B, lanes 1–4). These represent cross-links to SecA as they became fainter and decreased slightly in size if a truncated form of SecA, which lacks three of the four endogenous cysteines (N95), was used (Fig. 2 B, lanes 5 and 6). The SecA cross-links disappeared completely if the remaining cysteine was mutated (Fig. 2 B, lanes 7 and 8). We used this cysteine-free mutant in the remainder of our experiments.

We next generated translocation intermediates of varying lengths, keeping the cysteine at position 175, and compared their ability to form cross-links with a cysteine at several positions in SecY. Cross-links to S282C were observed with a chain length of 211 aa, but not with chains of 183 or 200 residues (unpublished data), in which Cys175 is expected to be inside the ribosome. With a chain of 229 residues (pOA229), positions 76, 79, 131, and 194 gave similar cross-links as with pOA220, but the intensity was lower (Fig. 2 D, 1–13). Most cross-links disappeared at a chain length of 293 aa (Fig. 2 D, lanes 14–18). Weak cross-links were still detectable with position S282C (Fig. 2 D, lane 19), presumably because random back and forth movements of the translocating substrate occasionally position Cys175 inside the channel. Because pOA220 gave the most efficient cross-links, it was used in more extensive screens for positions in SecY that interact with the nascent chain.

Screening SecY for interaction sites with the translocating polypeptide

We first tested positions in the interior of SecY, choosing residues that would form the neck of the postulated hourglass-shaped channel, as well as residues above and below the constriction. Six of the chosen positions—G69 in TM2a, I191 in TM5, I278 and S282 in TM7, as well as T404 and I408 in TM10—gave the strongest cross-links to SecY (Fig. 3 B). Immunoprecipitation with anti-SecY antibodies confirmed the identity of these cross-links (Fig. 3 C). Weaker cross-links to several other positions were also seen, particularly to Q93, I187, H264, K268, and F286 (Fig. 3 B). As expected, SecY complex lacking cysteines (0Cys) failed to give cross-links. We next tested positions on the exterior of the molecule, choosing residues that would be expected to be near the middle of the membrane, facing the outside of the SecY complex and encircling the perimeter (G28, V126, V162, A193, A229, and V413). None of these positions gave significant cross-links (Fig. 3 D). Likewise, no cross-links were observed with wild-type SecY (WT), which contains cysteines at positions 329 and 385 (Fig. 3 D). These positions are located on the exterior of SecY near the middle of the membrane, but do not point directly outwards. Together, these results indicate that the translocation pore is located in the interior of the SecY complex.

We used a ribosome as a bulky object to halt posttranslational translocation of a polypeptide because it minimized the aggregation of the substrate. However, similar cross-linking results were obtained in experiments using a purified proOmpA fragment (227 aa), which contained a 13–amino acid long disulfide-bonded loop at its COOH terminus to stop translocation (Tani et al., 1990), and a free cysteine at position 175 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200412019/DC1).

Conclusions

Our data support the idea that an internal cavity in SecY forms the pore of the protein-conducting channel. The six strongest cross-linked positions cluster in the center of the SecY molecule (Fig. 4, A and B). Three of the six residues–I191C, I278C, and I408C–are part of an isoleucine ring that is at the neck of the hourglass-shaped pore in the postulated open state of the channel (van den Berg et al., 2004). Weaker cross-links to a fourth member of the ring, I187C were also seen. Links to these residues and to S282C and T404C, which are directly below the ring on the periplasmic side of the channel, provide evidence that the isoleucine ring surrounds the translocating nascent chain. This finding is consistent with the proposal that the hydrophobic ring fits like a gasket around the translocating chain, providing a simple means of preventing ions and other small molecules from flooding through the channel (van den Berg et al., 2004). We consider it significant that the strongest cross-links of the translocating chain were seen with residues located at the narrowest point of the postulated hourglass-shaped channel. Restricting the interactions between a substrate and the channel to a narrow zone may minimize the energy required for a protein to cross the membrane.

Note that five of the strong cross-links (G69, I191, I278, S282, I408) come from positions at which signal sequence suppressor (prlA) mutations have been observed. These are thought to facilitate the opening of the channel and thus allow the translocation of proteins with altered signal sequences (Emr et al., 1981; Bankaitis et al., 1984). Our data suggest that residues that contact a translocating chain play an important role in channel gating.

In the closed channel visualized by the crystal structure, the residue in *M. jannaschii* that corresponds to *E. coli* G69 is located at the upper end of helix 2a (the plug), pointing away from the center of the molecule. However, in the open state of the channel, modeled on the basis of experiments that showed disulfide bridge formation between *E. coli* SecY residue 67 and a position in SecE near the luminal face of the membrane (Harris and Silhavy, 1999), the side chain of residue 69 would point toward the interior of the channel (van den Berg et al., 2004). The strong cross-linking seen with G69 thus supports the idea that the plug moves to provide a path for the polypeptide chain through the channel.

We cannot rule out the possibility that multiple SecY molecules open and combine their hydrophilic inner surfaces to form a channel. However, upon solubilization of a translocation intermediate in detergent, only one SecY was found to be associated with one copy each of SecA and proOmpA (Duong, 2003). Thus, a far simpler interpretation of our data is that a single SecY molecule forms the pore (van den Berg et al., 2004).

Materials and methods

Single-cysteine derivatives of SecY

A version of SecY lacking cysteine was generated and single cysteine codons were subsequently introduced at various positions through PCR-based mutagenesis (QuikChange; Stratagene) of pBAD-E$_{his}$YG (Collinson et al., 2001). The expression of these constructs in C43 (DE3) cells was induced with arabinose for 3 h at 30°C. Complexes were purified after solubilization of the membranes in 1.25% *n*-dodecyl-β-D-maltopyranoside (Anatrace) by binding to a Ni^{2+}-chelating column, followed by cation exchange chromatography (MonoS; van den Berg et al., 2004). Protein concentrations were determined with the Bradford reagent (Bio-Rad Laboratories). Purified SecY derivatives in TNG buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10% glycerol, 10 mM DTT, 0.03% *n*-dodecyl-β-D-maltopyranoside) were stored at −80°C.

Preparation of proteoliposomes, cross-linking experiments and translocation assays

Purified SecY mutants (16.8 μg) in 16.8 μl TNG buffer were reconstituted into phospholipid vesicles as described previously (Collinson et al., 2001).

Shortened DNA templates coding for proOmpA (pOA) with a glycine to cysteine substitution at amino acid 175 and lacking a stop codon were generated by PCR with appropriate primers and used to make mRNA by in vitro transcription with SP6 polymerase. Truncated pOA fragments (pOA#:tRNA, where # represents the last amino acid present in the truncated protein) were synthesized in the presence of [^{35}S]methionine by in vitro translation (Rabbit Reticulocyte Lysate; Promega) for 20 min at 30°C. Where indicated, translation mixtures were depleted of ATP by incubation with 20 mM glucose and 0.2 U/μl hexokinase (15 min at 30°C). Except where samples were treated with RNase, all buffers contained 1 U/μl SUPERase-In (Ambion). When tRNA was removed before cross-linking, samples were treated with 1/50 volume of RNase Cocktail (Ambion) for 5 min at 30°C. When RNase digestions were performed after cross-linking, samples were treated for 30 min at 37°C with one-third volume RNase Cocktail supplemented with 0.01 U/μl RNase V$_1$ (Ambion) and 3% Triton X-100.

To generate an arrested translocation intermediate, 1.8 μl proteoliposomes containing SecY complex were mixed with 1 μl in vitro–translated ^{35}S-pOA220:tRNA and 1 μg of cysteine-free (except where otherwise indicated) SecA (Osborne et al., 2004) in 7.2 μl of buffer. Final concentrations were 50 mM KCl, 50 mM Hepes, pH 7.5, 1.8% glycerol, 5 mM MgCl$_2$, 0.5 mg/ml acetylated BSA (B-2518; Sigma-Aldrich), and 4 mM ATP. After 15 min of incubation at 37°C, samples were mixed with 90 μl SM buffer (150 mM NaCl, 5 mM MgCl$_2$, 10 mM Hepes, pH 7.5) containing 2 mg/ml acetylated BSA and pelleted at 14,000 rpm for 15 min at 4°C. Pellets were washed with 100 μl ST buffer (150 mM NaCl, 5 mM MgCl$_2$, 10 mM Tris, pH 8), resuspended in 10 μl ST buffer containing 0.1 mM sodium tetrathionate (Sigma-Aldrich) and incubated for 10 min at 37°C. *N*-ethylmaleimide (Sigma-Aldrich) was added to 20 mM for 5 min on ice. Samples were then solubilized for 3 min at 40°C with 3 μl of 5× sample buffer (250 mM MES, pH 6.5, 25% glycerol, 10% SDS, 0.25 mg/ml bromophenol blue). Translocation assays using samples resuspended in 10 μl ST buffer without tetrathionate were performed as described previously (Or et al., 2002). Samples were separated on NuPage 10% Bis-Tris mini-gels (Invitrogen) with MES running buffer, visualized, and quantified by phosphorimaging (Fujix BAS 2000). Linear background subtraction and cropping of images was performed with Adobe Photoshop.

Analysis of cross-linked products

For immunoprecipitations, pellets were dissolved in 10 μl 8 M urea plus 1% SDS in SM buffer, heated to 40°C for 10 min, diluted 20-fold with 1% Triton X-100 in SM buffer then mixed with bead-bound purified antibodies directed against a COOH-terminal SecY peptide or an unrelated control peptide. After 1 h of incubation at 4°C the beads were washed three times with 1% Triton X-100 in SM buffer and bound proteins were eluted with sample buffer.

Online supplemental material

Fig. S1 demonstrates that similar cross-linking results are seen when a translocation intermediate is trapped in the channel by a 13–amino acid long disulfide-bonded loop at its COOH terminus instead of by attachment to a ribosome. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200412019/DC1.

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