The Plug Domain of the SecY Protein Stabilizes the Closed State of the Translocation Channel and Maintains a Membrane Seal

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SUMMARY

Proteins are translocated across membranes through a channel that is formed by the prokaryotic SecY or eukaryotic Sec61 complex. The crystal structure of the SecY channel from Methanococcus jannaschii revealed a plug domain that appears to seal the channel in its closed state. However, the role of the plug remains unclear, particularly because plug deletion mutants in S. cerevisiae are functional. Here, we demonstrate that plug deletion mutants in E. coli SecY are also functional and even efficiently translocate proteins with defective or missing signal sequences. The crystal structures of equivalent plug deletions in SecY of M. jannaschii show that, although the overall structures are maintained, new plugs are formed. These lack many interactions that normally stabilize the closed channel, explaining why the channels can open for proteins with signal-sequence mutations. Our data show that the plug domain is required to maintain a closed state of the channel and suggest a mechanism for channel gating.

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 conserved protein-conducting channel that is formed by a heterotrimERIC membrane protein complex, called the Sec61 complex in eukaryotes and the SecY complex in prokaryotes (for review, see Osborne et al. [2005]). The channel binds the ribosome during cotranslational translocation, collaborates with the Sec62/63p membrane protein complex and the ATPase BiP during posttranslational translocation in eukaryotes, and associates with the cytoplasmic ATPase SecA during posttranslational translocation in bacteria.

The crystal structure of the SecY complex from Methanococcus jannaschii is likely representative of all Sec61 and SecY complexes (Van den Berg et al., 2004). The structure shows that 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 the SecE subunit. Viewed from the side, the channel has an hourglass shape with a hydrophilic conduit through its center. The intracellular and extracellular funnels meet at a central constriction, created by a ring of six amino acids, “the pore ring.” A short helix, TM2a or “the plug,” is located in the center of the extracellular funnel abutting the pore ring. The intercalation of a signal sequence between TM2b and TM7 at the cytoplasmic side of the lateral gate (Plath et al., 1998) would cause displacement of the plug toward the back of SecY and thus open the channel (Tam et al., 2005). During translocation, the polypeptide chain would then move from the cytoplasmic funnel through the pore ring into the extracellular funnel. Crosslinking experiments confirm that the translocation pore is located at the center of a single SecY molecule (Cannon et al., 2005; Osborne and Rapoport, 2007).

The role of the plug in channel function is unclear. One possibility is that the plug seals the channel in its closed state, preventing the permeation of ions or other small molecules. However, molecular dynamics simulations predict that the pore ring alone can significantly slow the flux of ions and water (Gumbart and Schulten, 2006; Haider et al., 2006). In addition, deletion of sequences corresponding to TM2a in S. cerevisiae Sec61p do not inactivate the channel (Junne et al., 2006); the mutant cells are viable and have only mild translocation defects. These results are surprising because fixing the E. coli channel in a permanently open conformation by disulfide crosslinking of the plug with SecE is lethal (Harris and Silhavy, 1999). One possible explanation is that the plug plays...
a more important role as a seal in bacteria, because they need to maintain an electrochemical potential and gradients for many small molecules across the plasma membrane, whereas the eukaryotic ER is somewhat permeable to small molecules (Le Gail et al., 2004) and may thus better tolerate a leaky channel. Alternatively, it is possible that plug deletion and plug relocation are not equivalent. For example, the deleted sequences might be compensated for by structural changes in Sec61p that enable the seal to be maintained. The plug has also been proposed to stabilize the interactions of the three subunits of the Sec61 complex during translocation (Junne et al., 2006) or to affect oligomerization of the complex (Tam et al., 2005). Although it is clear that the plug needs to relocate during translocation, it is unknown how plug movement is triggered and the channel is opened.

In this study, we use functional and structural approaches to address the role of the plug. We demonstrate that E. coli SecY mutants lacking TM2a are functional in vivo and in vitro. In addition, they allow the efficient translocation of proteins with defective or missing signal sequences (suppression of signal-sequence mutations). The crystal structures of equivalent TM2a deletion mutants in M. jannaschii reveal that new helical plugs seal the resting channel. The new plugs lack numerous interactions that are present in the wild-type protein, particularly contacts with the lateral gate and the pore ring, providing a structural basis for destabilization of the closed channel state and explaining the signal-sequence-suppressor phenotype. Our data also explain why the plug deletion mutants are viable. Taken together, these results show that the plug is required to maintain a closed state of the channel and suggest a mechanism for channel gating.

RESULTS

Plug Deletion Mutants of E. coli SecY Are Viable

To clarify the role of the plug in SecY function, we deleted residues 65–70 or 60–74 from the E. coli protein, which correspond to half or all of the TM2a helix from M. jannaschii (Van den Berg et al., 2004). The mutant proteins were expressed from a plasmid in an E. coli strain that harbors a temperature-sensitive (ts) SecY allele (Shiba et al., 1984). RecA was removed from the strain to reduce the probability of recombination between the SecY genes on the plasmid and the chromosome. Although the ts strain did not grow at the nonpermissive temperature of 42°C, the presence of the plasmids coding for the mutant SecYs restored viability (Figure 1A). The half-plug deletion supported growth almost as well as the wild-type SecY protein, whereas the full-plug deletion was less active. These results show that the plug deletions in E. coli SecY are functional, similar to the mutants made in S. cerevisiae (Junne et al., 2006).

To directly determine whether the deletion mutants have protein translocation activity, we used a genetic assay in which the localization of a translocated domain of the membrane protein MalF is tested (Boyd et al., 1987; Froshauer et al., 1988). The translocated domain is fused to β-galactosidase encoded by the lacZ gene such that when properly localized to the periplasm, the enzyme is inactive; when translocation is impaired, the enzyme is retained in the cytosol and becomes active (D.B. and J.B., unpublished data). The MalF-lacZ fusion was expressed from the chromosome in the SecY ts strain. When grown in the presence of an empty plasmid at 37°C, a significant level of β-galactosidase activity was detected (Figure 1B), indicating that the ts allele of SecY is not fully functional even at the permissive growth temperature. When the plasmids coding for wild-type or plug deletion SecYs were introduced, the translocation defect disappeared or was greatly reduced. These results confirm that the deletion mutants are functional.

To exclude that the observed activity of the mutant SecYs is dependent upon cooperation with another gene product, we tested the translocation activity of the deletion mutants in vitro. The mutant SecY complexes were purified and reconstituted into proteoliposomes. These proteoliposomes contained about the same amount of protein as control vesicles generated with the wild-type protein (Figure S1 in the Supplemental Data available with this article online). The proteoliposomes were tested for translocation of 35S-labeled proOmpA in the presence of SecA and ATP. Translocated material was detected by its protection from proteinase K digestion. The mutant SecY complexes showed at least as much translocation activity as the wild-type protein (Figure 1C, lanes 5 and 8 versus lane 2). As expected, no protease-protected material was seen in the absence of ATP (lanes 4, 7, and 10) or if Triton X-100 was added prior to protease treatment (lanes 3, 6, and 9). Thus, the half- and full-plug deletion mutants are capable of supporting translocation in a reconstituted system.

Plug Deletion Mutants Suppress Defective Signal Sequences

A number of SecY mutants allow the translocation of proteins with defective or missing signal sequences (prlA mutants), and many of the mutations are proposed to destabilize the closed state of the channel (Derman et al., 1993; Emr et al., 1981; Smith et al., 2005). We suspected that the deletion of residues from helix TM2a might have the same effect. We therefore tested the plug deletion mutants for their ability to suppress signal-sequence mutations in alkaline phosphatase. Plasmids encoding SecY complex were introduced into cells expressing chromosomal copies of alkaline phosphatase with defective signal sequences (Michaelis et al., 1983). We first tested the suppression of a mild signal-sequence mutation in which a Leu residue is replaced by a Gln residue (Figure 2A). Both plug deletion mutants allowed much more secretion of mutant alkaline phosphatase than the wild-type. The efficiency of signal-sequence mutant suppression was as high as with the prlA4 mutant, one of the strongest prl alleles. The plug deletion mutants also suppressed a more severe point mutation in the signal sequence F (Figure 2B). This suppression was as high as with the prlA4 mutant and fully consistent with the idea that the plug deletion mutants are able to support signal translocation. The loss of secretion in the wild-type strain is consistent with the channel being closed in the absence of ATP (lanes 4 and 7), whereas in the presence of ATP (lanes 5 and 8), the signal sequence is suppressed by the mutant SecY complexes (Figure 2C).
sequence of alkaline phosphatase in which a Leu residue is replaced by an Arg residue (Figure 2B). Finally, we tested whether the plug deletion mutants would allow the secretion of alkaline phosphatase lacking a signal sequence altogether. In this case, the alkaline phosphatase gene was placed on a plasmid, resulting in higher enzyme activity. Again, the plug deletion mutants, like the prlA4 mutant, allowed efficient secretion of alkaline phosphatase (Figure 2C). Together, these experiments show that the plug deletion mutants are very strong suppressors of defective or missing signal sequences.

**Structure Determination of Plug Deletion Mutants**
To understand why the plug deletion mutants are functional and display a signal-sequence suppressor phenotype, we decided to determine the crystal structures of plug deletion mutants in *Methanococcus jannaschii* SecY. These mutants (Δ60–65 and Δ57–67, respectively) are equivalent to the *E. coli* half- and full-plug deletion mutants (Van den Berg et al., 2004). The mutant *M. jannaschii* SecY complexes were purified and crystallized under the same conditions as the wild-type complex (Van den Berg et al., 2004). The crystals belonged to the space group P2_12_12 and diffracted to a resolution of 3.5 Å. The unit cell dimensions were close to those seen in one of the two previously observed crystal forms. The structures of the half- and full-plug deletions were solved by molecular replacement using the Phaser program (McCoy et al., 2005). In order to reduce model bias, the search model lacked the entire plug region (PDB file 1RH5 excluding residues 53–67) and was only refined as a rigid body.

The electron density maps of the half-plug deletion revealed strong density corresponding to a newly formed...
plug domain (above 1.5 σ in the 2Fo − Fc map and above 3.5 σ in the Fo − Fc map). In the Fo − Fc map, this was the only strong density (>3.5 σ) in the entire unit cell. The new plug displayed connectivity of the electron density, and the side chains for Arg, Phe, and Trp were clearly visible after solvent flattening using data corrected for anisotropy (Figure 3A). We built an α helix into the density but did not attempt to model the side chains of small amino acids. When the search model lacked a larger portion of the plug (Δ47–74), the density map looked similar (data not shown). The final model was refined to Rwork and Rfree of 30% and 32%, respectively (Table 1).

For the full-plug deletion mutant, we also observed a large and strong density at the plug region in the 2Fo − Fc map, indicating that a new plug was again formed. However, a discontinuity in the density map was observed. To improve the density map and reduce model bias, we used the prime-and-switch method, which maximizes a probability function of the electron density (Terwilliger, 2004). The resulting map showed continuous density between TM2b and the new plug (Figure 3B), although the density for the loop between the new plug and TM1 was fragmented, likely because of the solvent flattening employed. A similar density map was obtained when the prime-and-switch method was used with a model that lacked a larger region of the plug domain (Δ47–74). We built the new plug as an α helix, using the large side chain of Phe56 as a landmark. The model was refined to Rwork and Rfree of 30% and 33%, respectively (Table 1).

Except for the plug regions, the structures of both deletion mutants were almost identical to that of the wild-type protein, as demonstrated by the calculation of composite-omit maps (data not shown). In addition, deletion of individual helices in the model resulted in the reappearance of density for these helices in the calculated 2Fo − Fc and Fo − Fc maps, supporting the conclusion that the overall structures are maintained.

**Description of the New Plugs in the Deletion Mutants**

In the half-plug deletion mutant (Δ60–65), the new α-helical plug is comprised of residues Ile55–Trp59 (Figures 3A and 4A). Compared to the location of TM2a in the native structure, the new plug is moved away from the pore ring and toward the periplasmic side of the channel (Figures 4B and 4C). The residues of the new helix are recruited from residues that in the wild-type protein belong to the N-terminal part of helix TM2a and to the loop preceding TM2a (Figures 3A and 4B). The remaining part of this loop remains essentially unchanged. One of the two strands of the β-hairpin loop that connects TM2a with TM2b in the wild-type protein also maintains its conformation. The new plug has lost all interactions with TM5 and TM10 and most interactions with TM2b and TM7. The new helix is held in place by its association with the loop between TM7 and TM8, as well as by interactions of the segment preceding the helix with the loop between TM3 and TM4, and by the insertion of the segment following the helix between TM1 and TM4.

In the full-plug deletion mutant (Δ57–67), the new α helix is comprised of residues Ile55–Thr56 and Gly68–Ile71
The recruitment of the segment Gly68–Ile71 to the helix completely disrupts the β-hairpin loop formed by these residues in the wild-type structure (Figures 3B and 4A). The new plug becomes more aligned with TM2b by rotating away from the pore ring (Figure 4B). The newly formed helix only interacts with TM5 and TM10. All interactions with TM1 and TM4 are lost.

In the wild-type protein, the helices TM2b and TM7, which form the lateral gate, make few contacts with one another and their association is largely mediated by the plug (Figures 5A–5C). In the plug deletion mutants, the new plugs have lost most interactions with the helices TM2b and TM7 (Figures 5D and 5E) and thus can no longer stabilize the lateral gate.
### Table 1. Crystallographic Data and Refinement Statistics for the Crystals from Half- and Full-Plug Deletion Mutants

<table>
<thead>
<tr>
<th>SecY Deletion Mutants</th>
<th>Half Plug (Δ60–65)</th>
<th>Full Plug (Δ57–67)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2₁2₁2</td>
<td>P2₁2₁2</td>
</tr>
<tr>
<td>Solvent content</td>
<td>72.6%</td>
<td>72.9%</td>
</tr>
<tr>
<td>Unit cell dimension</td>
<td>92 x 148 x 81</td>
<td>92 x 150 x 79</td>
</tr>
</tbody>
</table>

**Native Data**

- Resolution: 3.5 Å, 3.6 Å
- R_{sym} (overall/last shell): 0.067/0.787, 0.087/0.739
- R_{free} (overall/last shell): 21.9/2.3, 16.8/3.3
- Completeness: 98.6%, 99.6%
- Redundancy: 9–12x, 7–8x

**Refinement Statistics**

- Resolution range: 50–3.5 Å, 56–3.6 Å
- Number of Atoms: 4055, 4002
- Rmsd bond length: 0.010, 0.010
- Rmsd bond angle: 1.31, 1.18
- Rwork: 30.1%, 29.8%
- Rfree: 31.7%, 33.5%

### DISCUSSION

Our results show that *E. coli* SecY mutants lacking TM2a are functional in protein translocation both in vivo and in vitro. Consequently, *E. coli* cells containing these mutant SecYs are viable, similar to *S. cerevisiae* cells with plug deletions in Sec61p [Junne et al., 2006]. While the present work was in its final stages, Maillard et al. (2007) also reported that plug deletions in *E. coli* SecY are functional. These results are surprising in light of the crystal structure of a wild-type archaeal SecY complex, which predicted that the plug has an important role in sealing the channel in its closed state. Recent electrophysiology data show that the wild-type resting channel formed by the *E. coli* SecY complex is always closed, whereas the channels formed by the half- and full-plug deletion mutants continuously switch between closed and open states (Saparov et al., 2007). Thus, the new plugs are not as efficient as the wild-type plugs in keeping the channel in its closed state. The structures of the closed channels indicate that the pore ring alone leaves a relatively small hole (Figure 6A; the program HOLE [Smart et al., 1993] predicts the passage of spheres up to a diameter of 2.2 Å). The ion conductance seen in the electrophysiology experiments therefore suggests that the pore ring can widen spontaneously once it no longer interacts with the plug. Thus, in addition to providing a physical barrier to the free flow of ions, the plug also restricts the movement of pore residues to ensure their contribution to the seal. The relative importance of the pore and plug in maintaining a membrane barrier remains to be established.

The transient opening of the channel in the plug deletion mutants, as seen in the electrophysiology experiments (Saparov et al., 2007), would allow the occasional passage of small molecules. Such a situation is obviously compatible with cell viability, perhaps because the breach of the permeability barrier can be compensated for by pumps. In contrast, locking the plug in a constitutively open position by crosslinking it to the TM segment of SecE results in massive ion and water flux in electrophysiology experiments (Saparov et al., 2007) and causes lethality of *E. coli* cells (Harris and Silhavy, 1999).

The SecY complex shows an amazing ability to restore an essential structural feature even after drastic alterations. There appears to be a strong energetic pressure that forces plug deletion mutants to sacrifice residues that are normally part of the loops connecting TM2a with TM1 and TM2b to form a new plug. Most of the driving force might come from interactions of hydrophobic residues in the plug domain with hydrophobic residues in the extra-cytoplasmic funnel. In support of this idea, some *prl* mutations affect the hydrophobicity of plug residues (e.g., *prlA3*, F67C). Helix formation in the plug would maximize hydrogen bonding of the backbone amide and carbonyl groups and present amino acid side chains in all directions, thus generating a stable structure that can seal the channel.

Although we have structural information for only two plug deletion mutants, it seems likely that the essential conclusions are not dependent on the specific deletions. However, with other mutants it is conceivable that the new plugs could adopt several different conformations within the extra-cytoplasmic funnel and yet still seal the channel. Surprisingly, even mutants carrying much larger deletions than made in the present study are viable, for example, the deletion of residues 42–74 in *E. coli* SecY or of residues 52–74 in *S. cerevisiae* Sec61p (corresponding...
Figure 4. Comparison of the Structures of Wild-Type SecY with Those of the Plug Deletion Mutants

(A) Top views of the channels. The original and new plugs are shown in red, the β-hairpin connecting TM2a and TM2b in brown, TM2b in gold, and TM5 and TM10 in green.

(B) Side views of the channels.

(C) An enlarged view of the plug region, with the wild-type plug in red, the new plug of the half-plug deletion mutant in dark pink, and the new plug of the full-plug deletion mutant in purple, all superimposed onto the rest of the structures. The helices surrounding the plug that contain pore ring residues are shown in the same colors as in (A). The pore residues are shown in ball-and-stick representation.
to 46–67 and 49–70 in *M. jannaschii* (Junne et al., 2006; Maillard et al., 2007). In these cases, the loop following TM1 and perhaps even some C-terminal residues of TM1 might be recruited to form a new plug. The large deletions show partial dissociation of the small subunits both from *E. coli* SecY and *S. cerevisiae* Sec61p (Junne et al., 2006; Maillard et al., 2007); we have made similar observations for *M. jannaschii* SecY, precluding structural determination (data not shown). Dissociation may be caused by perturbation of TM1, which is a major interaction site for both small subunits (Van den Berg et al., 2004). Thus, although these large deletions maintain a surprising level of activity, they are significantly compromised. In fact, we have found that the deletion of residues 42–77 in *E. coli* SecY results in a protein that does not complement a ts SecY mutant at elevated temperatures but can still suppress signal sequence mutations at lower temperatures (data not shown).

The mutagenesis results indicate that many different sequences can form a plug. This is consistent with the observation that the plug domain is only poorly conserved in sequence among different species (Van den Berg et al., 2004). The only sequence requirements may be some hydrophobic and α-helix-forming residues, as well as flexible linkers on either side of the plug to allow its movement during translocation. In the wild-type protein and in our deletion mutants, the linkers appear to be provided by the sequences PFXG at the N terminus of the plug and GFXP at the C terminus (Φ stands for a hydrophobic residue) (Van den Berg et al., 2004).

In agreement with results by Maillard et al. (2007), we found that the plug deletion mutants allow proteins with defective or missing signal sequences to be translocated (signal sequence mutant suppressor or prl mutants). These deletion mutants are in fact as strong or stronger than the known prl mutants. The mutant channels are expected to open more easily than the wild-type channel, and our structures support this prediction. Although not all amino acid side chains could be visualized at the relatively low resolution of our structures, it is clear that the

![Figure 5. Interactions of the Plugs with the Lateral Gate](image)

(A) The helices TM2b and TM7 that form the lateral gate interact only weakly.
(B and C) In the wild-type, the interaction between TM2b and TM7 is stabilized by multiple interactions with the plug (TM2a) in red and the following β-hairpin in brown.
(D and E) The new plugs of the half- and full-plug deletion mutants interact only weakly with TM2b and TM7.
new plugs have lost many interactions with helices TM2b and TM7, which need to separate for signal-sequence intercalation. Movement of TM2b and TM7 may require the separation of the two halves of the SecY molecule (TM1–5 and TM6–10), which should be facilitated in the mutants because, in contrast to the wild-type, the new plugs interact with only one of the two halves of SecY (in the half-plug deletion mutant with N-terminal half, and in the full-plug deletion mutant predominantly with the C-terminal half). Finally, the new plugs no longer interact with the six hydrophobic residues that form the pore ring. These changes would be expected to destabilize the closed state of the mutant channels and facilitate signal-sequence insertion and pore ring widening, explaining the prl phenotype.

How then would the channel open in the wild-type situation? The polypeptide chain would insert as a loop, with the hydrophobic part of the signal sequence intercalated between TM2b and TM7, gaining access to the surrounding lipid phase (Figure 7). Intercalation requires that the helices TM2b and TM7 undergo spontaneous separation and reassociation. This may be induced by the dimerization of the SecY complex in the plane of the membrane, as the lateral gate was observed to be partially open in an electron microscopy structure of the dimeric E. coli SecY (Bostina et al., 2005). The binding of a channel partner, such as the ribosome or SecA, may also favor “breathing” of the lateral gate. As a result of signal-sequence intercalation, TM2b would move, pulling on the terminal residues of the adjacent plug domain (arrows in Figure 7B). This would reduce the number of plug residues available to interact within the extra-cytoplasmic funnel, resulting in a destabilizing effect similar to that seen in our deletion mutants. The major consequence of plug movement would be that the plug no longer interacts with residues of the lateral gate, the pore ring, and the

Figure 6. Space-Filling Models of the Wild-Type Channel and Plug Deletion Mutants
(A) The wild-type channel with the plug domain removed. Pore ring residues are shown in ball-and-stick representation. (B–D) The wild-type channel, the half-plug mutant, and the full-plug mutant, all with the plug domain in red.
helices to which the pore ring residues are attached (TM2b, 5, 7, and 10). The pore ring would thus be free to widen, a state that would be maintained after the insertion of the mature region of the polypeptide chain (Figure 7B). Whether these events would occur in a strictly sequential order or in a more concerted fashion remains to be established. In this model, the plug would serve not only as a seal but also as a lock that stabilizes the channel in its closed state.

**EXPERIMENTAL PROCEDURES**

**Alkaline Phosphatase Assays**

Wild-type and mutant SecY complex or control plasmid were transformed into various strains (Table S1) and expressed without induction from a pBAD22 vector. Alkaline phosphatase was endogenously expressed from MPh1061 (ploA61, L14R) and MPh1068 cells (ploA68, L8Q). APu2-22 was induced with 1 mM isoproryl β-D-1-thiogalactopyranoside (PTG) from the pACYC135 plasmid in C43 cells. The cells were grown to midlog phase in rich media from an overnight culture, and alkaline phosphatase activity was quantified as previously described (Derman et al., 1993). Activity units are defined as (OD420 nm – 1.75 OD550 nm) × 1000/(OD600 nm × volume of cell suspension × min). All experiments were done in quadruplicate (two independent colonies in duplicate).

**Complementation Testing and β-Galactosidase Assays**

Wild-type and mutant SecY complex or control plasmid were transformed into DH7302 cells and expressed without induction from a pBAD22 vector (Table S1). Transformants were reisolated twice on LB/ampicillin media. To test for growth complementation, DH7302 cells containing the indicated plasmid or the DH7301 parent strain were streaked onto LB or M6 minimal media and grown at the restrictive temperature of 42°C. For the plate assay, cells were then transferred to LB/ampicillin containing 0.2% maltose and grown to midlog phase in rich media from an overnight culture, and β-galactosidase activity was measured in Miller units (Miller, 1972).

**Preparation of 35S-Methionine-Labeled Substrates**

Full-length preOmpA was amplified via PCR using primers inserting a 5’ SP6 promoter. The PCR product was purified, transcribed in vitro, and translated at 30°C for 20 min in a reticulocyte lysate supplemented with 35S-methionine. Protein was precipitated with saturated ammonium sulfate, pelleted, and resuspended in 8 M urea, 50 mM HEPES (pH 7).

**Translocation Assays**

Reactions were performed in 50 mM Tris (pH 7.9), 50 mM NaCl, 50 mM KCl, and 5 mM MgCl2 buffer supplemented with 1 mM DTT and 0.2 mg/ml BSA. Where indicated, 1.25 mM ATP, 1 μg SecA, and 1 μl of SecY complex reconstituted into proteoliposomes were added. To remove ATP, 3.5 μg/ml hexokinase and 10 mM glucose were added. Reactions were initiated by adding 1 μl 35S-methionine-labeled substrate. In all cases, the final reaction volume was 50 μl. Translocation proceeded for 30 min at 30°C. Nontranslocated protein was then digested on ice with 0.1 mg/ml protease K for 30 min; some samples received 5.5 μl 20% Triton X-100 prior to proteolysis. 4-(2-aminoethyl)benzenesulfonlfy fluoride (PMSF) was added to 1 mM to stop proteolysis before addition of 10% TCA. Total protein was pelleted, resuspended in sample buffer, and analyzed by SDS-PAGE, followed by analysis with a PhosphorImager.

**Protein Purification and Crystallization**

Wild-type and mutant E. coli SecY complexes were expressed from a pBAD22 vector encoding His-tagged SecE, SecY, and SecG (Table S1). The proteins were purified in DDM by Ni-NTA agarose chromatography as previously described (Cannon et al., 2005). They were reconstituted into proteoliposomes by adding 18 μl SecY complex to 33 μl of E. coli polar lipids (20 mg/ml). The detergent was subsequently removed by the addition of Biobeads.

The full-plug deletion (Δ57–67) and the half-plug deletion (Δ60–65) mutants of SecYEi complex from Methanococcus jannaschii were expressed and purified as described (Van den Berg et al., 2004). After purification, the proteins were exchanged from MPh1061 (ploA61, L14R) and then combined with the 1RHZ model lacking the plug region (Van den Berg et al., 2004) to form a glycine-HCl buffer pH 8.5–10, similar to the conditions reported before (Van den Berg et al., 2004). The crystals were flash-frozen by direct transfer from mother liquor to liquid nitrogen.

**Structure Determination**

The 1RH5 model (Van den Berg et al., 2004) lacking the plug region (residues 53–67) of SecY was used as a search model in the molecular replacement program Phaser (McCoy et al., 2005). For both deletion mutants, the model was refined only as a rigid body using REFMAC (Winn et al., 2001). The 1RH5 model was then used as a model in the molecular replacement program Phaser (McCoy et al., 2005). For both deletion mutants, the model bias was further reduced by the replacement program Phaser (McCoy et al., 2005). For both deletion mutants, the model bias was further reduced by the replacement program Phaser (McCoy et al., 2005). For both deletion mutants, the model bias was further reduced by the replacement program Phaser (McCoy et al., 2005). For both deletion mutants, the model bias was further reduced by the replacement program Phaser (McCoy et al., 2005). For both deletion mutants, the model bias was further reduced by the replacement program Phaser (McCoy et al., 2005). For both deletion mutants, the model bias was further reduced by the replacement program Phaser (McCoy et al., 2005). For both deletion mutants, the model bias was further reduced by the replacement program Phaser (McCoy et al., 2005). For both deletion mutants, the model bias was further reduced by the replacement program Phaser (McCoy et al., 2005).
The Function of the SecY Plug Domain

Molecular Cell

Supplemental Data
Supplemental Data include Supplemental References, one figure, and one table and can be found with this article online at http://www.molecule.org/cgi/content/full/26/4/511/DC1/.

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REFERENCES


Accession Numbers
The coordinates for the half plug (2YXQ) and full plug (2YXR) deletion mutants were deposited in the RCSB protein data bank.