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Determining the Conductance of the SecY Protein Translocation Channel for Small Molecules

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SUMMARY

The channel formed by the SecY complex must maintain the membrane barrier for ions and other small molecules during the translocation of membrane or secretory proteins. We have tested the permeability of the channel by using planar bilayers containing reconstituted purified *E. coli* SecY complex. Wild-type SecY complex did not show any conductance for ions or water. Deletion of the "plug," a short helix normally located in the center of the SecY complex, or modification of a cysteine introduced into the plug resulted in transient channel openings; a similar effect was seen with a mutation in the pore ring, a constriction in the center of the channel. Permanent channel opening occurred when the plug was moved out of the way by disulfide-bridge formation. These data show that the resting channel on its own forms a barrier for small molecules, with both the pore ring and the plug required for the seal; channel opening requires movement of the plug.

INTRODUCTION

Many proteins are transported during or after their synthesis across the eukaryotic endoplasmic reticulum (ER) membrane or across the prokaryotic plasma membrane (for review, see Johnson and van Waes [1999], Osborne et al. [2005], and Veenendaal et al. [2004]). Transport occurs through a protein-conducting channel that is formed by an evolutionarily conserved heterotrimeric membrane protein complex, called the Sec61p complex in eukaryotes and the SecY complex in bacteria and archaea. The complex consists of a multispanning large subunit (Sec61p/SecY) and two smaller β- and γ subunits (Sec61β and Sec61γ in eukaryotes, SecG or Secβ and SecE in prokaryotes). While the channel allows protein translocation, it must prevent the free movement of small molecules, such as ions or metabolites. Maintaining the membrane barrier for protons is particularly important for the viability of prokaryotes, as the proton gradient across the membrane is the main source of their energy. The ER membrane may be somewhat leaky for small molecules (Le Gall et al., 2004), but it must also prevent the free flow of ions, so that Ca^{2+} ions can be accumulated in the ER lumen. How the membrane barrier is maintained during protein translocation is unclear, but two models have been proposed.

In one model, supported by fluorescence-quenching experiments with ER membranes (Crowley et al., 1994; Hamman et al., 1997, 1998; Liao et al., 1997), the channel itself would not provide a barrier: the seal would be formed by either a ribosome, binding to the cytosolic side of the channel, or the ER chaperone BiP, binding to the lumenal end of the channel. The experiments indicated that a ribosome with a translocating polypeptide chain covers a pore of at least 40 Å in diameter (Hamman et al., 1997). Even the resting channel had a pore size of 9 Å, closed on the lumenal side by BiP. This model does not explain how the membrane barrier is maintained in posttranslational translocation, when a polypeptide chain is transported after its release from the ribosome, or in prokaryotes, where BiP does not exist. In addition, a tight seal of the ribosome-channel junction is at odds with electron-microscopy structures that reveal a gap of 12–15 Å between the ribosome and channel (Beckmann et al., 2001; Menetret et al., 2000, 2005).

The alternative model, based on the crystal structure of an archaeal SecY complex (Van den Berg et al., 2004), posits that the membrane barrier is formed by the channel itself. The structure of the closed state of the channel suggested that the translocation pore may be formed by one copy of the SecY complex and may have a significantly smaller size than indicated by the fluorescence-quenching experiments. The structure shows a cytoplasmic funnel that tapers to a close in the middle of the membrane (see scheme in Figure 1A). The resting channel would thus be impermeable to ions or other small molecules, even in the

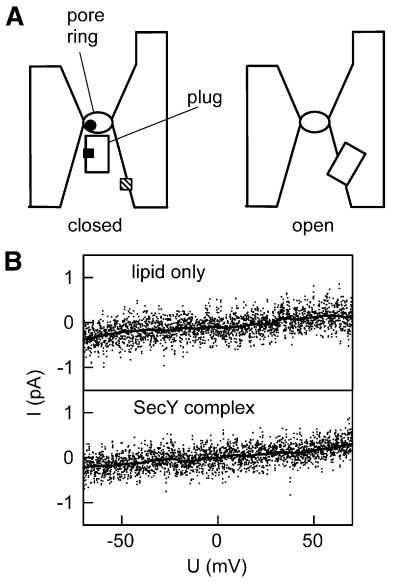

Figure 1. Permeability of the SecY Channel

(A) Scheme of the SecY channel in its closed state, based on the crystal structure, and in its postulated open state. The black dot indicates the position of I408, which is mutated in the *prlA4* mutant. The black and striped squares show positions 67 of SecY and 120 of SecE, respectively, where cysteines were introduced and can form a disulfide bridge in the open state.

(B) The ion conductivity of pure lipid bilayers (upper panel) and of bilayers reconstituted with wild-type SecY complex (lower panel) was measured. A voltage ramp was applied, and the measured current was filtered at 5 Hz (dots) and 0.05 Hz (spline lines). The slope of about 5 nS/cm^2 was the same with and without protein present in the bilayer. No channel openings were observed.

absence of interacting partners. When engaged in protein translocation, the channel would open by the movement of a short helix, the plug, adopting an hourglass shape with a pore ring at its constriction (Van den Berg et al., 2004) (Figure 1A). The pore ring would form a gasket-like seal around the translocating polypeptide. Although the seal is not perfect, as shown by the partial loss of the electrochemical gradient upon accumulation of an arrested translocation intermediate in *E. coli* membranes (Schiebel and Wickner, 1992), it would prevent the free flow of ions. In the absence of a translocating chain, the open channel would be significantly more permeable to small molecules. The model explains how the membrane barrier can be maintained in both co- and posttranslational translocation and why a gap between the ribosome and channel may not compromise the barrier.

The second model is consistent with the original interpretation of experiments in which the ion conductance of ER membranes was measured after their fusion into planar lipid bilayers (Simon and Blobel, 1991); conductance was seen only upon release of the nascent chains from the ribosomes by puromycin, suggesting that the channels, formed by the Sec61p complex (Wirth et al., 2003), were previously plugged by the nascent polypeptide chains. However, fluorescence-quenching experiments raised the possibility that the initially observed seal was not provided by the nascent chain inside the channel but rather by the ribosome-channel junction and that the release of the nascent chain by puromycin allowed small molecules to pass from the peptidyl transferase site in the ribosome all the way into the ER lumen (Hamman et al., 1997).

In this paper, we demonstrate that the channel itself provides a barrier for small molecules. In addition, we identify both the pore ring and the plug as features contributing to the tightness of the resting channel. Our results also support the proposal that the channel opens by plug movement.

RESULTS

We used a method, originally developed by Schindler (1989), to generate planar bilayers containing the protein-conducting channel. SecY complex was purified from *E. coli* and reconstituted with *E. coli* polar phospholipids into proteoliposomes. A suspension of these proteoliposomes was added to two compartments, separated by a diaphragm with a small hole. In each compartment, the liposomes are in equilibrium with a surface monolayer of phospholipids that have their hydrocarbon chains sticking out into the air (Schindler, 1979). A bilayer can be reconstituted from two monolayers by raising the liquid level in the compartments to cover the hole (Montal et al., 1981). Although not well understood, some protein molecules originally present in the proteoliposomes end up in the reconstituted bilayer. This procedure avoids the use of organic solvents or detergents as in common black-lipid experiments, and the bilayer is significantly thinner, as in physiological membranes. We then used standard electrophysiological methods to measure conductance for ions across the planar membrane.

When wild-type SecY complex was present in the bilayer, the conductivity of the membrane was as low as in the absence of protein (Figure 1B). Assuming that all added protein was reconstituted into proteoliposomes and that the ratio of protein to lipid in bulk phase is identical to that in the planar bilayer, we estimate that the tested membrane area (1.5×10^{-4} cm^2) contains 2.6×10^6 channels (calculated from the protein to lipid mass ratio of 1:200 and the molecular mass of the protein [~60 kDa] and of lipid [0.7 kDa]) and yet not a single channel opening event was detected. Thus, the resting wild-type channel appears to be impermeable to ions.

Next, we tested whether the plug in the SecY complex has the proposed role in preventing the permeation of small molecules through the channel (Figure 1A). We tested two plug-deletion mutants in which amino acid residues 65–70 or 60–74 were missing. Both mutants showed transient ion channel openings (Figures 2A and 2B); the conductance was voltage independent (Figure 2C). With the deletion 65–70, the single-channel amplitude was estimated to be 202 pS, a number close to that reported for the open channel in mammalian ER membranes (Simon

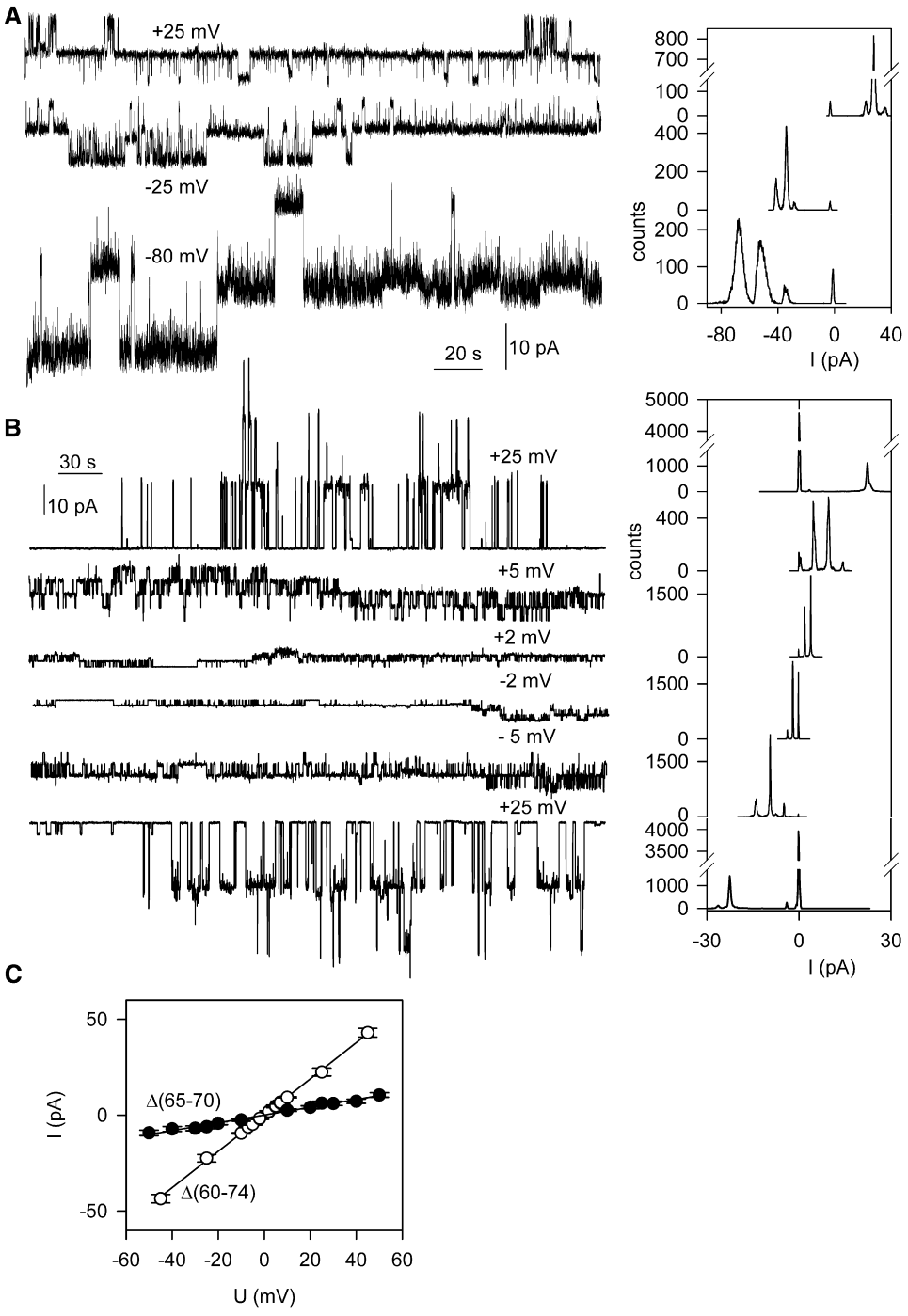

Figure 2. Single-Channel Recordings of Plug-Deletion Mutants of SecY

(A) SecY complex lacking amino acids 65–70 was reconstituted into a planar bilayer and single-channel activity was recorded at different voltages (left panels). The distribution of the channel amplitudes is shown in the histograms on the right.

(B) The same experiment as in (A) was performed with a deletion mutant lacking amino acids 60–74.

(C) Shown are the current-voltage characteristics of both plug-deletion mutants. Mean values and standard deviations were obtained from histograms of at least five independent runs of the experiments demonstrated in (A) and (B). Their single-channel permeabilities were voltage independent and estimated to be 202 and 944 pS.

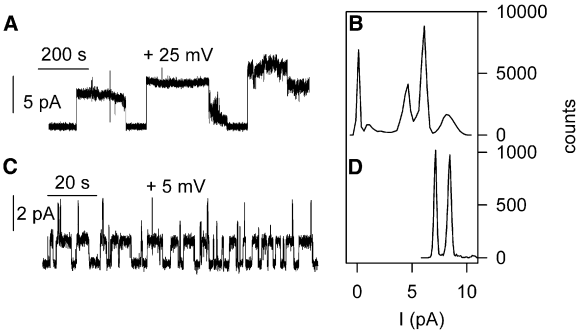

Figure 3. Single-Channel Recordings with a Signal-Suppressor Mutant, *prlA4*

(A) SecY complex containing a mutation in the pore ring was reconstituted into a planar bilayer, and single-channel activity was recorded. In the particular experiment shown, the characteristic open time was in the range of minutes.

(B) Histogram of the experiment shown in (A), demonstrating that the amplitude increased from 0.2 to 0.3 nS.

(C) Continuation of the experiment shown in (A). A further increase of conductivity to 0.4 nS was observed. The open time was now in the range of seconds.

(D) Histogram of the experiment in (C). In contrast to (A), channel openings occurred on top of already opened channels.

and Blobel, 1991). However, the previous experiments were performed in solutions with a 3-fold-lower ionic strength, and thus, the ion conductance of the channel in our experiments is actually smaller. With the other deletion mutant (missing amino acids 60–74), the conductance was significantly larger (~940 pS). These data indicate that the plug residues are indeed required to prevent the movement of ions through the channel.

A rough estimate of the pore size can be obtained from Equation 1,

$$\frac{1}{g} = \left(l + \frac{\pi r}{2}\right)\frac{1}{\sigma \pi r^2}, \quad (1)$$

in which g, r, l, and σ are the single channel amplitude, the channel radius, the channel length, and the conductivity of the solution, respectively. With σ = 2 S/m and l = 3 nm, we calculated r = 3.7 and 7.3 Å for the 65–70 and 60–74 deletion mutants, respectively. Equation 1 has to be used with caution, because channel conductance, calculated with Ohm's law, is often a poor measure of pore size (Rostovtseva et al., 2002; Smart et al., 1997). Nevertheless, both numbers are in reasonable agreement with the diameter of the pore ring seen in the crystal structure (Van den Berg et al., 2004). They indicate that the pore size can widen, as has been postulated to occur during protein translocation (Van den Berg et al., 2004).

To test the role of the pore ring in preventing ion flow through the channel, we used the *prlA4* mutant. This mutant SecY complex carries two mutations, one of which is a change of the pore-ring residue Ile408 to Asn (Figure 1A, black dot). The mutant belongs to a class of signal-suppressor mutants (*prl* mutants) that allow secretory proteins with defective or missing signal sequences to be translocated, likely because the closed state of the channel is destabilized (Bieker et al., 1990; Derman et al., 1993; Smith et al., 2005). Upon reconstitution into a planar lipid bilayer, significant ion channel activity was again observed (Figure 3). In contrast to the plug-deletion mutants, the amplitude and time of channel opening varied during and among different experiments. For example, in Figure 3A, the pore conductivity increased from 0.2 to 0.3 nS (see also histogram in Figure 3B). In Figure 3C, a constant conductivity of 0.4 nS was measured (see also Figure 3D), while the time of channel opening was much shorter. The variability may be caused by time-dependent structural changes of the open channel. These data show that the pore ring contributes to stabilizing the closed state of the channel and support the idea that the signal-suppressor mutant channel is more easily opened.

Next, we used a more defined method to lock the SecY channel into an open state. We used a double-cysteine mutant that contains a cysteine in the plug of SecY at position 67 and a cysteine at position 120 of the transmembrane segment of SecE (Harris and Silhavy, 1999) (Figure 1A, squares). In the closed state of the channel, the two cysteines are too far apart to form a disulfide bridge, but in the open state, they come into close proximity (Van den Berg et al., 2004). Although plug movement is normally facilitated by the binding of a translocation substrate, disulfide-bridge formation can be induced with oxidants even in the absence of substrate (Tam et al., 2005). The reduced double-cysteine mutant was incorporated into a planar bilayer and ion conductance was measured as before (Figures 4A and 4B). Similarly to the wild-type protein, the channel was impermeable to ions (data not

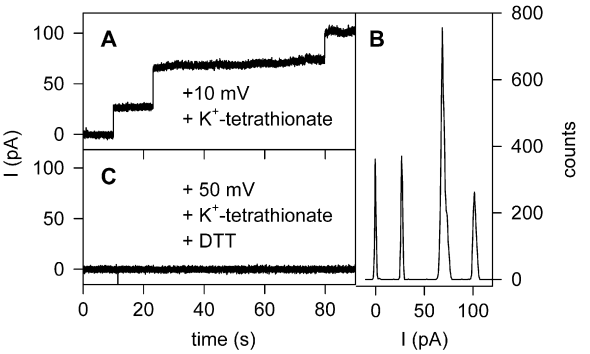

Figure 4. Ion Conductivity of a SecY Channel Locked into the Open State

(A) SecY complex containing cysteines at position 67 of SecY and position 120 of SecE was reconstituted into planar bilayers. One millimolar tetrathionate was added to induce disulfide-bridge formation between the plug and the transmembrane segment of SecE. The conductivity was measured at 10 mV.

(B) Histogram of the experiment in (A), revealing conductivities of 2.7, 4.0, and 2.8 nS for the three subsequent channel openings.

(C) After 90 min, 1 mM DTT was added to reduce the disulfide bridge and the current was measured at 50 mV.

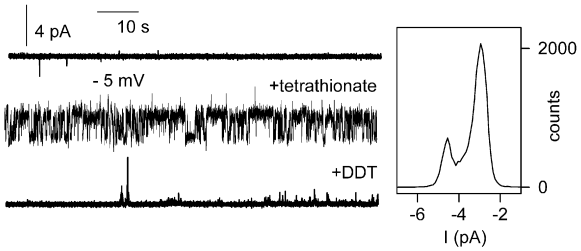

Figure 5. Channel Activity of SecY Complex after Chemical Modification of a Plug Residue

SecY complex containing a single cysteine in the plug at position 67 was reconstituted into a planar bilayer. Channel activity was recorded at −5 mV without additives (upper trace), after addition of 5 mM tetrathionate (middle trace), and after subsequent addition of 5 mM DTT (lower trace).

shown). However, when tetrathionate was added to induce disulfide-bridge formation, massive ion flux was observed. We rarely saw channel-closing events, as expected from the irreversible nature of disulfide-bridge formation. When dithiothreitol (DTT) was added to reduce the disulfide bridge, the channel reverted to its closed state (Figure 4C). These data support the idea that plug movement is linked to channel opening. In different experiments, we observed open-channel amplitudes from 0.3 to 5 nS. This large range suggests that the pore size of the open channel can vary, with a maximum diameter estimated from Equation 1 to be 24 Å.

Interestingly, a mutant SecY complex that contained a single cysteine in the plug at position 67 (Figure 1A, black square) also showed channel-opening events with an amplitude of 0.32 nS in response to the addition of tetrathionate (Figure 5). In contrast to the results with the double-cysteine mutant, both channel-opening and -closing events were observed. Tetrathionate is known to modify single cysteines (Parker and Allison, 1969), and modification of the cysteine in the plug may have destabilized the closed state of the channel. Consistent with this assumption, channel opening was abolished by the addition of DTT.

Finally, we tested water permeation through the SecY channel. Water permeation was measured by adding urea to one compartment, so that water flows from one compartment to the other by osmotic pressure. In the vicinity of the membrane, water flux generates a potassium ion gradient; in the compartment lacking urea, the potassium ion concentration will be high close to the membrane and will gradually decrease with distance from the membrane to reach the bulk concentration. The stationary gradient can be measured with an ion-sensitive microelectrode that is moved perpendicular to the membrane by a hydraulic microdrive, and the profiles can be used to calculate water flux and permeability (Pohl et al., 1997). Planar bilayers containing wild-type SecY complex or the reduced double-cysteine mutant (Figure 1A, squares) had the same water permeability as a pure lipid bilayer, indicating that

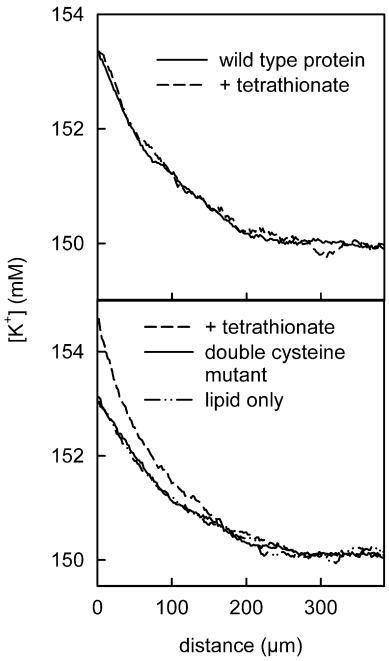

Figure 6. Water Permeability of the SecY Complex

Wild-type SecY complex (upper panel) or a double-cysteine mutant (cysteines at positions 67 of SecY and 120 of SecE; lower panel) was reconstituted into a planar lipid bilayer. One molar of urea was added to one compartment, and the potassium ion gradient perpendicular to the membrane was measured in the other compartment by using an ion-sensitive electrode. Where indicated, 10 mM tetrathionate was added. P_f of bilayers lacking protein or containing the wild-type channel or the reduced double mutant complex were calculated to be 22 μm/s. Upon oxidization with tetrathionate, ion conductivity of the double mutant increased from 5 nS/cm^2 to 70 mS/cm^2. Water conductivity increased as well, as indicated by the larger concentration of K^+ ions adjacent to the membrane. Because the SecY channel is permeable to K^+ ions, P_f cannot be calculated.

the channels were entirely impermeable to water (Figure 6). However, upon addition of tetrathionate to form a disulfide bridge in the double-cysteine mutant, the potassium ion concentration close to the membrane was significantly increased in the compartment lacking urea, indicating an increase in water permeability (lower panel). As expected, the addition of tetrathionate to the wild-type protein had no effect (upper panel). For the mutant, we measured an increase of overall membrane ion conductance from 5 nS/cm^2 to 70 mS/cm^2. However, because the pores are so large that both the osmolyte and ions can pass, we cannot calculate the water permeability of the channel molecules. Assuming an average single-channel ion permeability of 3.5 nS, the increase in bilayer conductance suggested that about 3000 channels were open in the tested membrane patch (it should be noted that these experiments were performed with a significantly higher protein concentration than the electrophysiological experiments). These results show that the resting SecY channel is tight not only for ions but also for water. Taken together with the other results, we conclude that the SecY

channel itself provides a barrier to the passage of small molecules and that both the plug and the pore ring contribute to the formation of a tight seal.

DISCUSSION

Our results provide evidence that the resting protein-conducting channel itself is impermeable to small molecules. Previous experiments were done with native ER membranes in which it was possible that the channels were sealed by bound ribosome-nascent chain complexes or by lumenal BiP (Hamman et al., 1997; Simon and Blobel, 1991). Experiments with purified Sec61p complex did show channel activity for the precursor polypeptide-activated channel (Wirth et al., 2003), but the assay did not allow the incorporation of resting channels into the planar bilayer. The lack of permeability of the channel itself is entirely consistent with the crystal structure of the archaeal SecY complex, which indicated that not even small molecules can pass. The results are also in agreement with molecular-dynamics simulations that show that neither ion nor water molecules can permeate the SecY channel (Gumbart and Schulten, 2006; Tian and Andricioaei, 2006). We thus conclude that the binding of a channel partner is not required for closing the SecY channel. Given the sequence conservation between SecY and Sec61 complexes, it seems likely that this conclusion is applicable to eukaryotic channels as well. The role for BiP in sealing the resting Sec61 channel, deduced from fluorescence-quenching experiments (Hamman et al., 1998), might be explained by BiP acting as a chaperone to refold Sec61p after the alkali treatment used to deplete BiP from microsomes, rather than by it acting as a plug. However, fluorescence measurements also give different answers than other methods for the diameter of the membrane and ribosome channels and for the tightness of the ribosome-channel junction, and the reasons are currently unclear.

As predicted from the crystal structure (Van den Berg et al., 2004), both the plug and the pore ring play a role in keeping the SecY channel closed for small molecules. Of course, the two features do not act independently; a pore-ring mutation may affect interactions that keep the plug in the center of the SecY molecule, and plug dislocation may result in widening of the pore ring. Molecular-dynamics simulations show that the pore ring alone can provide an efficient barrier for small molecules in the nanosecond time range (Gumbart and Schulten, 2006; Tian and Andricioaei, 2006). The pore ring may resemble hydrophobic gates seen in the acetylcholine receptor or the mechanosensitive channel of small conductance (Beckstein and Sansom, 2006; Miyazawa et al., 2003; Spronk et al., 2006). These gates are actually wide enough to let ions pass, but conductance is prevented by the exclusion of water. A reduced water density inside membrane pores has recently been confirmed experimentally (Saparov et al., 2006). However, the pore ring alone cannot prevent the passage of ions over periods of seconds or minutes, as shown by our electrophysiology experiments, explaining why a plug domain is required to seal the resting channel.

Our data indicate significant variation of the pore size, with an estimated pore diameter equal to or larger than that seen in the crystal structure. Variability of the pore size is required because the channel must translocate polypeptide segments of different amino acid sequence. The diameter of the pore ring seen in the crystal structure might be sufficient to accommodate an extended polypeptide chain but would have to expand to allow the passage of an α helix. A variable pore width would also be required to explain how even bulky residues attached to the side chains of amino acids in vitro (Kurzchalia et al., 1988), or a disulfide-bonded polypeptide loop of 13 residues in a secretory protein (Tani et al., 1990), can be transferred through the channel. Widening of the pore as seen in our electrophysiology experiments may be explained by lateral shifts of the helices to which the pore residues are attached (Van den Berg et al., 2004). Variable conductivities in the same range as we observed have been reported for the Sec61p channel (Wirth et al., 2003). The maximum pore diameters calculated from the electrophysiology experiments appear to be somewhat larger than expected from the crystal structure, but these estimates are inaccurate because bulk properties for water and electrolyte molecules are assumed. In reality, both the local ion and water diffusion coefficients inside the pore may be lower or higher than in bulk solution (Dani and Levitt, 1981; Saparov and Pohl, 2004), and the magnitude of water dipoles inside the channel can be up to 2 D, giving rise to large local fields (Tieleman and Berendsen, 1998).

The plug appears to play a major role in keeping the channel closed for small molecules. It is therefore surprising that plug deletion does not drastically affect the viability of *E. coli* or *S. cerevsiaie* (Maillard et al., 2007; Junne et al., 2006; our unpublished data). This is explained by the recently determined crystal structures of plug-deletion mutants in *M. jannaschii* SecY, equivalent to those studied in the present paper (Li et al., 2007). These structures show that a new plug is formed from residues that were previously not part of this domain. Space-filling models show that the resting mutant channels would still not be expected to be permeable to small molecules. However, the new plugs have lost numerous interactions that normally keep the plug in the center of the SecY molecule, explaining why the mutant channels can transiently open in the electrophysiological experiments. In vivo, the occasional opening and closing of the channel might not be detrimental if the passage of small molecules is compensated for by powerful pumps. Only the permanent opening of the channel might be catastrophic, consistent with the reported lethality of the double-cysteine mutant, in which disulfide-bridge formation is induced (Harris and Silhavy, 1999).

Our results support the postulated mechanism of channel opening by movement of the plug (Van den Berg et al., 2004). In our experiments, plug movement and corresponding channel opening occurred in the absence of a

translocation substrate. This could be due to the fact that the F67C mutation is a signal-suppressor mutation (Tam et al., 2005), which would be expected to destabilize the closed state of the channel as in the *prlA4* mutant. However, the destabilization by the F67C mutation must be less pronounced, because we did not observe channel activity unless the cysteine was modified with tetrathionate. Consistent with this assumption, whereas both the F67C and *prlA4* mutations suppressed a weakly defective signal sequence of alkaline phosphatase (Maillard et al., 2007; our unpublished data), only the *prlA4* mutation significantly suppressed a severely defective signal sequence (Figure S1 in the Supplemental Data available with this article online). The correlation between signal-sequence suppression in vivo and ion conductivity in vitro supports the idea that signal-sequence-suppressor mutations in the SecY complex facilitate channel opening. In the wild-type protein, binding of a translocation substrate is required to stimulate plug movement (Tam et al., 2005). At the same time, insertion of the translocating polypeptide chain into the pore would be expected to hinder the free flow of ions. The establishment of a system in which ion and water flux can be measured with purified components now paves the way to test the mechanism by which the membrane barrier is maintained during protein translocation.

EXPERIMENTAL PROCEDURES

Protein Purification

Mutants were generated by PCR mutagenesis, adding a GSGS linker to the 60–74 mutant, and verified by sequencing. SecY complex was essentially purified as described (Cannon et al., 2005). The expression of SecY complex in C43 (DE3) cells was induced with arabinose for 4 hr at 37°C. The membranes were solubilized in 1% dodecyl-β-D-maltopyranoside (Anatrace) and the extract passed over a Ni^{2+}-chelating column. Protein eluted with imidazole was further purified by chromatography on a MonoS column (Van den Berg et al., 2004). Protein concentrations were determined with the Bradford reagent (Bio-Rad Laboratories). Purified SecY complexes were stored at −80°C in TNG buffer (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 10% glycerol, 10 mM DTT, and 0.03% dodecyl-β-D-maltopyranoside).

Protein Reconstitution

Purified SecY complex was reconstituted into proteoliposomes by dialysis. In brief, the reconstitution mixture was prepared at room temperature by sequentially adding 50 mM K-HEPES, 1 mM DTT, 6% (w/v) deoxyBig-CHAP, purified protein (about 100 μg in detergent), and 10 mg of preformed *Escherichia coli* polar phospholipids (Avanti Polar Lipids, Alabaster, AL) vesicles. The mixture was placed into SPECTRA/POR 2.1 dialysis tubing, molecular mass cut-off 15,000 (Spectrum Laboratories, Laguna Hills, CA), and dialyzed against 100 volumes of assay buffer for 72 hr at 4°C. The assay buffer contained 50 mM K-HEPES (pH 7.5), 200 mM K-acetate, 1 mM DTT, 10% glycerol, and protease inhibitor. The proteoliposomes were harvested by ultracentrifugation (60 min at 100,000 × g) and resuspended into assay buffer at a concentration of 5–10 mg/ml. Water transport studies and experiments with the wild-type SecY complex were carried out at a protein to lipid ratio of 1:200. For single-channel experiments, the ratio was reduced to <1:1300 by mixing proteoliposomes and pure lipid vesicles at the appropriate ratio. The suspensions were added into the two aqueous compartments (1.5 ml each) of a Teflon chamber that was divided by a diaphragm. The final lipid concentration was between 1 and 2 mg/ml. Two monolayers formed at the water-air interfaces (Schindler, 1979) were combined to a planar bilayer in a 150 μm-diameter aperture of a 25 μm-thick polytetrafluoroethylene septum (Montal et al., 1981), separating the two aqueous phases of the chambers. The septum was pretreated with a hexadecane-hexane mixture (volume ratio of 1:200).

Water-Flux Measurements

Water passing a membrane dilutes the solution it enters and concentrates the solution it leaves. The water flux can be calculated from the concentration changes of a membrane-impermeable solute close to the membrane surface (for review, see Pohl [2004]). In brief, the solute concentration at the interface, C_s, is a function of the distance, x, to the membrane: $C(x) = C_s \exp(-vx / D + bx^3 / 3D)$, where v and b are the linear drift velocity of the osmotic volume flow and the stirring parameter, respectively (Pohl et al., 1997). In the steady state, v was obtained by fitting the K^+ ion concentration obtained for $x < 50$ μm to this equation. v is related to P_f by $P_f = v / (C_{osm} V_w)$ (Finkelstein, 1987). The water-flux J_w is the product of water concentration and v. K^+ concentrations were measured by microelectrodes made of glass capillaries, the tips (1–2 μm in diameter) of which were filled with a viscous cocktail of potassium ionophore (Fluka, Dreisenhofen, Germany). Movement of the electrodes relative to the membrane was performed with a hydraulic stepdrive (Narishige, Japan).

Single-Ion Channel Measurements

Ag/AgCl reference electrodes were immersed into the buffer solutions on both sides of the planar bilayers. Under voltage clamp conditions, the transmembrane current was measured by a patch clamp amplifier (model EPC9, HEKA electronics, Germany). The recording filter was a 4 pole Bessel with 3 dB corner frequency of 0.1 kHz. The acquired raw data were analyzed with the help of the TAC software package (Bruxton Corporation, Seattle, WA). Gaussian filters of 12 Hz were applied to reduce noise.

To ensure the absence of lipid channels, the first step in all experiments was the formation of a protein-free planar bilayer. After ~20 min of control current recordings, the membrane was ruptured by lowering the level of the aqueous phases. A tiny amount of proteoliposmes was added to both compartments, allowing some of the added material to enter the lipid monolayer at the air-water interface. Then the membrane was formed again by raising the level of the aqueous phases above the aperture in the diaphragm. The procedure of membrane rupture, proteoliposome addition, and de novo formation of the bilayer was repeated until single-ion channel activity was observed. Thus, the protein:lipid mass ratio varied from experiment to experiment, but it was usually well below 1: 1,300.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.molecule.org/cgi/content/full/26/4/501/DC1/.

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