The SecY complex forms a channel capable of ionic discrimination

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Protein translocation across the bacterial membrane occurs at the SecY complex or channel. The resting SecY channel is impermeable to small molecules owing to a plug domain that creates a seal. Here, we report that a channel loosely sealed, or with a plug locked open, does not, however, lead to general membrane permeability. Instead, strong selectivity towards small monovalent anions, especially chloride, is observed. Mutations in the pore ring-structure increase both the translocation activity of the channel and its ionic conductance, however the selectivity is maintained. The same ionic specificity also occurs at the onset of protein translocation and across the archaeal SecY complex. Thus, the ion-conducting characteristic of the channel seems to be conserved as a normal consequence of protein translocation. We propose that the pore ring-structure forms a selectivity filter, allowing cells to tolerate channels with imperfect plugs.

Keywords: channel; membrane; SecY; translocon; translocation
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INTRODUCTION

The Sec channel or translocon is a ubiquitous heterotrimeric protein complex found in the plasma membrane of bacteria and archaea (SecY), and the membrane of the endoplasmic reticulum (Sec61; Rapoport, 2007). The channel ensures the selective transport of large polypeptide substrates, without apparently compromising the permeability barrier of the membrane. X-ray crystallographic analysis of the archaeal SecY complex has provided the structural basis for understanding this astonishing transporter (van den Berg *et al*, 2004). The SecY subunit forms an hourglass-shaped transmembrane protein-conductive channel with, at its narrowest point, a pore structure lined by a ring of hydrophobic amino acids. On the *trans* side, the channel is obstructed by a short SecY α -helix domain, known as the plug. In the presence of a preprotein substrate, the plug is dislocated away

from the centre of the channel (Tam *et al*, 2005) and the pore ring expands to accommodate the polypeptide chain (Gumbart & Schulten, 2006; Tian & Andricioaei, 2006; Erlandson *et al*, 2008).

Recent *in vitro* electrophysiology experiments have shown that the Sec channel is impermeable to ions and small molecules, whereas a channel locked open creates a pore of strong ionic conductance (Saparov *et al*, 2007). Surprisingly, *in vivo*, the toxicity induced by the locked-open channel does not originate from immediate membrane depolarization or cell rupture, as would be expected from a permanently open membrane channel (Harris & Silhavy, 1999). It is also unexpected that a SecY complex carrying destabilizing *prl* mutations or truncations in the plug domain, then creating a loosely sealed channel, can be overproduced in the *Escherichia coli* membrane (Li *et al*, 2007; Maillard *et al*, 2007). In yeast, similar alterations in the Sec61 complex do not produce any obvious growth defect either (Junne *et al*, 2006, 2007), but the membrane of the endoplasmic reticulum is naturally leaky for small molecules (Le Gall *et al*, 2004; Lizak *et al*, 2008; Wonderlin, 2009). In bacteria, however, tightly regulated ion permeability is crucial to maintaining a correct electro-chemical gradient between the cytosol and the cellular environment. A loosely sealed or open SecY channel might be tolerated if, for example, strong counter-acting pumps compensate the ion leakage. Alternatively, the tolerance might be explained if the open channel only conducts certain ions. In some circumstances, chloride and proton leakage has been reported during protein transport (Schiebel & Wickner, 1992; Kawasaki *et al*, 1993), suggesting that the functioning translocon might indeed have some particular ionic channel characteristics.

Here, we analysed the ionic specificity of the translocon in both resting and active states. We show that the open channel is selective for small monovalent anions, with a strong preference for Cl^- ; however, K^+ , Na^+ , SO_4^{2-} , PO_4^{3-} or even H^+ cannot easily permeate through it. Mutation of the pore ring residues increases the ionic conductance, but the specificity is maintained. The ionic selectivity is also conserved in the archaeal SecY complex and at the onset of the protein translocation reaction. We propose that the channel forms a dual-stage conductance regulator, with the plug domain acting as a seal and the pore ring acting as a selective filter. This unique characteristic might explain why imperfectly sealed SecY channels can be tolerated by the cell.

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RESULTS AND DISCUSSION

Ionic specificity of the open SecY channel

Conductance experiments were carried out using inner membrane vesicles (IMVs) prepared from *E. coli* KM9. An electrochemical membrane potential—inside positive and acidic—was established with NADH and monitored using the voltage-sensitive dye oxonol VI (Clarke & Apell, 1989). The stability of the electrical potential ($\Delta\psi$) was then challenged in the presence of various salts. The IMVs prepared from *E. coli* wild type or from *E. coli* overproducing the SecY complex (termed Y^{WT}) were largely impermeable to small or organic ions, as only marginal dissipation of the membrane potential occurred on addition of these salts (Fig 1A and supplementary Fig S1 online). Halide anions such as Br[−] and especially I[−] have the ability to permeate through phospholipid bilayers and, accordingly, to dissipate the electrical gradient depending on their concentration (supplementary Figs S1 and S2 online; Paula *et al*, 1998).

The conductance experiments were then carried out using IMVs enriched with the SecY *prlA4* mutant complex (mutation I408N in the pore domain). This *prl* mutation is thought to stabilize the open state of the channel (van den Berg *et al*, 2004; Smith *et al*, 2005). We also used a SecY mutant carrying a deletion in the plug domain (SecY^{A33}; Maillard *et al*, 2007). This deletion creates an imperfect plug (Li *et al*, 2007) and the channel is thus poorly sealed (Saparov *et al*, 2007). The conductance measurements were also performed using a mutant carrying a pair of cysteine residues at position 67 of SecY (also known as mutation *prlA3*) and at position 120 of SecE. In this mutant (termed SecY^{67CC}), the plug domain is locked away from the centre of the channel on cysteine crosslinking (Tam *et al*, 2005). IMVs with these mutants were all able to generate a stable proton gradient on the addition of NADH (for example, see Fig 1B right panel). However, in sharp contrast to the wild-type IMVs, monovalent anions, including chloride, formate, bicarbonate and to a lesser extent acetate, could significantly dissipate the membrane potential (Fig 1B). Strikingly, the monovalent phosphate (H₂PO₄[−]) was permeable, whereas the divalent form (HPO₄^{2−}) was not. The greatest conductance was for chloride, and titration experiments showed that the $\Delta\psi$ could be collapsed with as little as 1 mM of KCl (supplementary Figs S1 and S2 online). Accordingly, $\Delta\psi$ could not be generated in these membranes if chloride was present in the buffer (supplementary Fig S3 online). By contrast, the membrane potential remained stable in the presence of K⁺, Na⁺, Mg²⁺, SO₄^{2−} or glutamate (Fig 1B). The impermeability to K⁺ was tested further using potassium acetate in the presence of the ionophore valinomycin. A K⁺-specific electrical potential was generated with a magnitude and stability identical to those of the wild type and mutants (supplementary Fig S3 online). Taken together, these results show that the various mutant SecY channels tested are highly selective for monovalent anions, with a strong preference for chloride.

Contribution of the pore ring residues

The atomic structure of the archaeal SecY channel suggests that the pore is formed by a ring of six hydrophobic amino acids, generally isoleucine (van den Berg *et al*, 2004). Our results show that the substitution I408N—that is, mutation *prlA4*—in *E. coli* leads to a specific leakage of ions. Thus, we tested the contribution of the other five hydrophobic side chains—that is, Ile 82, Ile 86, Ile 187, Ile 191 and Ile 278—after substituting them with asparagine.

The IMVs with the mutations I82N and I187N showed ionic conductance as low as that observed for the wild type (Fig 2A). By contrast, the other mutants—I86N, I191N and I278N—acquired a strong permeability for chloride, formate, H₂PO₄[−] and bicarbonate ions (Fig 2A). These last three mutants also showed an enhanced *in vivo* signal-sequence suppression activity and *in vitro* translocation activity (Fig 2B,C). Thus, the mutations in the pore ring that lead to increased translocation activity also lead to an increased ionic permeability, but the selectivity remains unchanged.

Ionic selectivity is a conserved characteristic

The hydrophobicity of the residues that form the pore ring is a conserved characteristic, but amino-acyl variations exist. The specificity for small molecules was thus tested using IMVs prepared from *E. coli* KM9 enriched with the evolutionarily distant SecY complex from *Methanococcus jannaschii*. The archaeal SecY complex does not support *in vitro* protein translocation (F.D., unpublished data) and membranes enriched for this complex do not significantly conduct any ions (Fig 3). However, these membranes become leaky for chloride, formate, H₂PO₄[−] and bicarbonate when a *prlA4*-like mutation is introduced into the pore ring of the archaeal SecY complex (mutation L406N). Thus, the ionic selectivity of the Sec complex is a conserved characteristic of the SecY channel.

Proton permeability of the open channel

As an additional means to probe the specificity of the SecY channel, we used the Δ pH-dependent dye 9-amino-6-chloro-2-methoxyacridine (ACMA). The magnitude of the proton gradient created in wild-type and mutant membranes was similar and the proton gradient was not dissipated with a solution of KCl (Fig 4A). Instead, greater acidification was observed with the mutant membranes, probably because a flow of Cl[−] ions in the lumen of the vesicles dissipates the membrane potential and allows for further pumping of protons. Finally, we used limiting amounts of NADH to allow the detection of a minor proton outflow (Fig 4B). In these conditions, proton leakage could be observed, but it was similar for both the wild-type and all SecY mutant complexes tested, including the archaeal ones (Fig 4B,C). Thus, destabilization of the closed state of the channel (with either a *prl* mutation or partial deletion of the plug) or the stabilization of its open state (through covalent linkage of the plug) does not render membranes leaky for protons.

Ionic conductance of the active channel

Schiebel & Wickner (1992) reported that a preprotein jammed across the membrane creates halide anion permeability, whereas Kawasaki *et al* (1993) reported that a counter-movement of protons occurs at the onset of protein translocation. Here, we revisited these pioneering observations using IMVs enriched for the SecYEG or SecYE complex (Fig 5). When IMVs were incubated in Tris-Cl buffer with the SecA ATPase and the preprotein proOmpA, a striking and immediate collapse of the membrane potential was triggered with ATP (Fig 5A). Translocation also caused a limited dissipation of $\Delta\psi$ in the presence of formate and H₂PO₄[−] (Fig 5B). The large conductance for chloride was, however, not observed on addition of the non-hydrolysable ATP analogue ATP γ S, or when the protein substrate had an altered

Fig 1 | Ionic conductance of the SecY complex. (A) Inner membrane vesicles (IMVs) with the wild-type (SecY^{WT}) complex were diluted in buffer A (50 mM Tris-SO₄ pH 7.9, 25 mM K₂SO₄, 10 mM MgSO₄, 0.2 mg/ml BSA) in the presence of oxonol VI (2 μM). The membrane potential $\Delta\psi$, inside positive, was generated with NADH (5 mM). The stability of $\Delta\psi$ was then challenged using various potassium salts (each 5 mM) for approximately 1 min and then dissipated with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; 1 μM). The graph (left panel) represents the percentage of $\Delta\psi$ that has been dissipated 10 s after the addition of salt. The traces (right panel) are examples of fluorescence recordings. (B,C) IMVs with the indicated SecY mutant complex were analysed as described above. The SecY mutants that were tested are (B) SecY^{PrI44}—mutations I408N and F286Y in SecY (recordings shown in the right panel); (C, left panel) SecY^{Δ33}—deletion of 33 amino-acid residues in the SecY plug domain; and (C, right panel) SecY^{67CC} + bismaleimidoethane (BMOE)—SecY complex with cysteine residues at position 67 of SecY and position 120 of SecE in a crosslinked state. Error bars indicate the standard deviation between three experiments.

(LpK) or deleted (OmpA) leader peptide (Fig 5C, and, for example of the recordings provided in supplementary Fig S4 online). Thus, SecA, nucleotides and preprotein do not have the capacity on their own to induce chloride permeability and to trigger opening of the channel (this study and Tam *et al.*, 2005). The chloride permeability was partial or null when using membranes with the translocation-inactivating mutation R357E or RPG357EDP introduced into the large cytosolic loop of SecY (Fig 5C; Tam *et al.*, 2005). Furthermore, when the translocation was initiated at lower temperatures to restrict molecular motions, a corresponding reduction in chloride conductance was recorded (Fig 5C and

traces in supplementary Fig S5 online). Clearly, the initiation of protein transport, and not only a stalled translocation intermediate (Schiebel & Wickner, 1992), creates chloride permeability across the SecY channel. In the same conditions, we could not detect a counter-movement of protons (Fig 5D,E). Thus, we conclude that protein translocation dissipates the $\Delta\psi$ component but not the ΔpH component of the bacterial membrane proton motive force.

Conclusions

This study shows that the SecY channel, despite having a strong ionic conductance when stabilized in the open state with

Fig 2 | The SecY pore mutants that are the most active in protein translocation are also the most leaky, but for only monovalent anions. (A) The ionic conductance of inner membrane vesicles with the indicated SecY mutant complex was analysed as described in Fig 1A. (B) The *in vivo* protein translocation activity of the mutants was assayed using the alkaline phosphatase 14R as a reporter. (C) The *in vitro* protein translocation activity of the mutants was assayed using ¹²⁵I-labelled proOmpA (pOmpA). The quantification was carried out by densitometry scanning using Image J (build 1.40 g). WT, wild type.

prl mutations or a locked-open plug (Saparov *et al*, 2007), is nonetheless able to maintain astonishing ionic specificity. The channel can discriminate between anions and cations, and between the monovalent and divalent forms of the same anion—that is, H₂PO₄⁻ versus HPO₄²⁻. Furthermore, assays with formate, acetate and glutamate indicate that the selectivity also operates based on the physical size of the anion. The amino-acyl side chains in and around the pore ring are probably the structures

that create the selectivity filter because specificity is maintained when the plug is locked away from the centre of the channel and the permeability of the channel is modulated by the nature of pore-ring residues. It is possible that, as is also proposed for other ion channels (Beckstein & Sansom, 2004), the exclusion of water from the SecY hydrophobic pore environment, together with a discreet distribution of positive and negative charges along the channel walls, would account for the observed selectivity. It is

Fig 3 | The ionic specificity of the SecY complex is a conserved characteristic. A membrane potential $\Delta\psi$ was generated and measured as described in Fig 1A, but using inner membrane vesicles with the *Methanococcus jannaschii* SecY complex, either (A) wild-type or (B) carrying a *prlA4*-like mutation L406N. The graphs represent the percentage of $\Delta\psi$ that has been dissipated 10 s after the addition of salt.

Fig 4 | The Cl^- -conductive SecY mutant channels are impermeable to protons. (A) The inner membrane vesicles (IMVs) with the indicated SecY mutant were incubated in buffer A in the presence of 9-amino-6-chloro-2-methoxyacridine (ACMA; 4 μM). A gradient of protons, inside acidic, was generated with 1 mM NADH and abolished with 1 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). The injection of 5 mM KCl causes a Cl^- influx in the mutant allowing for further acidification inside the vesicles. (B) IMVs bearing the SecY $\Delta 33$ complex were prepared as described in (A), but mixed with limiting amounts of NADH (final concentration as indicated). (C) The experiment in (B) was repeated using IMVs with the indicated SecY mutants. For each mutant listed, 100% corresponds to the ΔpH that was generated with 1 mM NADH.

also possible that the opening of the lateral gate would lead to localized re-organization of phospholipids, which, in turn, might enhance the conductance of anions, and especially halide ions, across the lipid bilayer. Site-directed crosslinking studies have indicated that the leader peptide and the lateral groove that forms the leader peptide-binding site are indeed in direct contact with phospholipids (Plath *et al*, 1998).

The ionic specificity of the SecY channel is intriguing and the precise mechanism needs to be elucidated, especially as, in eukaryotes, the Sec61 homologue seems to be naturally leaky for small molecules (Le Gall *et al*, 2004; Wonderlin, 2009). In bacteria, the observed anion conductance is clearly not required for the translocation efficiency of proteins (Schiebel & Wickner, 1992). Perhaps, the selectivity of the SecY channel

Fig 5 | Ionic conductance during protein translocation. (A) 40 μ g of inner membrane vesicles (IMVs) with the SecY^{WT} complex was incubated in 150 μ l of buffer B (containing 25 mM KCl) in the presence of bovine serum albumin (30 μ g), SecA (3 μ g) and the protein substrate proOmpA (pOmpA; 3 μ g). $\Delta\psi$ was generated with 5 mM NADH and protein translocation was initiated with 1 mM ATP. (B) IMVs with the wild-type SecY complex (40 μ g) were tested as described above, but in buffer A in which K₂SO₄ had been replaced by 25 mM of the indicated salt. The graph represents the percentage of $\Delta\psi$ that was dissipated 10 s after the addition of ATP. (C) SecY^{WT} IMVs (40 μ g) were tested as described in (A), but the translocation reaction was carried out under the following conditions: 1 mM ATP γ S; 3 μ g of the translocation-incompetent LpK or OmpA; and 40 μ g of IMVs with the indicated SecY mutant, at 4–25 °C. The graph was generated as described in (B). (D,E) IMVs enriched for the SecY^{EG} or SecY^E complexes were tested as in (A) but using 4 μ M 9-amino-6-chloro-2-methoxyacridine (ACMA) to follow the variations of $\Delta\psi$. CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; WT, wild type.

and, most importantly, the exclusion of protons might simply represent a selective advantage in bacteria because most of the metabolic energy is derived from the proton gradient across the inner membrane. This selectivity would allow the cell, for example, to tolerate leaky channels because of a *prl* mutation leading to an imperfect seal. The limited conductance would also explain why the expression of channels with their plug locked open, although lethal, does not lead to an immediate membrane depolarization and cell rupture (Harris & Silhavy, 1999).

METHODS

Biological and chemical agents. Strain KM9 (*unc::Tn10*, *rna10*, *relA1*, *spoT1* and *metB1*) is a derivative of DK8 (Klionsky *et al*, 1984). Plasmid pBAD22 encoding for the wild-type, mutant and

archeal SecY complexes has been described previously (Collinson *et al*, 2001; van den Berg *et al*, 2004; Maillard *et al*, 2007). Plasmids and methods for preparation of IMVs, SecA, OmpA, proOmpA and LpK (mutation A11K in the leader peptide of proOmpA) were as described previously (Tam *et al*, 2005; Alami *et al*, 2007). Overproduction of wild-type and mutant SecY complex was confirmed by Western blot analysis (supplementary Fig S6 online). Nucleotides, ionophores and bismaleimidoethane were purchased from Sigma (www.sigmaaldrich.com). Oxonol VI (bis-3-propyl-5-oxoisoxazol-4-yl pentamethine oxonol) and ACMA (9-amino-6-chloro-2-methoxyacridine) were obtained from Molecular Probes (www.invitrogen.com). Unless stated otherwise, all ions used in $\Delta\psi$ dissipation experiments were potassium salts.

Measurement of $\Delta\psi$ and ΔpH . Measurements were taken using a Varian Cary Eclipse spectrofluorometer. A typical experiment that consisted of mixing 40 μg of IMVs and 150 μl of buffer A (50 mM Tris- SO_4 , pH 7.9, 25 mM K_2SO_4 , 10 mM MgSO_4 and 0.2 mg/ml BSA) or buffer B (50 mM Tris-HCl, pH 7.9, 25 mM KCl, 10 mM MgSO_4 and 0.2 mg/ml BSA) in the quartz cuvette was carried out. All experiments were carried out at 25 °C and we applied the Henderson-Hasselbalch equation to determine the distribution anions. For example, at pH 8.2, the concentration of divalent $\text{K}_2\text{HPO}_4^{2-}$ was 4.65 mM, whereas that of monovalent KH_2PO_4^- was 0.35 mM; by contrast, at pH 7.4, the distribution was 1.93 mM for monovalent KH_2PO_4^- and 3.07 mM for divalent $\text{K}_2\text{HPO}_4^{2-}$. Unless stated otherwise, the proton gradient was generated using the electron donor NADH. The membrane potential $\Delta\psi$ was followed with oxonol VI (2 μM) at excitation and emission wavelengths of 610 and 640 nm (each of 10 nm slit width), respectively. Calibration of oxonol VI fluorescence with known membrane potentials is described in supplementary Fig S7 online. The proton gradient ΔpH was followed with ACMA (4 μM) at excitation and emission wavelengths of 409 nm (10 nm slit width) and 474 nm (20 nm slit width), respectively. The $\Delta\psi$ and ΔpH components were collapsed by the addition of 1 μM carbonyl cyanide *m*-chlorophenyl hydrazone. Fluorescence experiments with oxonol VI were repeated three times and each bar on the graph reports the mean and standard deviation of the values.

Protein translocation and alkaline phosphatase assays. *E. coli* strain MPH1061 (*phoA61*, L14R, a generous gift from Dr J. Beckwith) expresses *phoA* with the mutation L14R (Derman *et al*, 1993). The strain was transformed with plasmid pBAD22 encoding for the SecY complex and mutant forms. Cells were grown to mid-log phase in rich media and induced for 1 h with 0.2% arabinose. Alkaline phosphatase activity and *in vitro* translocation assays were carried out as described previously (Derman *et al*, 1993; Maillard *et al*, 2007). One unit of alkaline phosphatase is defined as $(\text{OD}_{420} - 1.75 \text{ OD}_{550}) / (\text{OD}_{600} \times \text{volume} \times \text{time}) \times 1,000$.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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