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Characterization of the supporting role of SecE in protein translocation

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ABSTRACT

SecYEG functions as a membrane channel for protein export. SecY constitutes the protein-conducting pore, which is enwrapped by SecE in a V-shaped manner. In its minimal form SecE consists of a single transmembrane segment that is connected to a surface-exposed amphipathic α-helix via a flexible hinge. These two domains are the major sites of interaction between SecE and SecY. Specific cleavage of SecE at the hinge region, which destroys the interaction between the two SecE domains, reduced translocation. When SecE and SecY were disulfide bonded at the two sites of interaction, protein translocation was not affected. This suggests that the SecY and SecE interactions are static, while the hinge region provides flexibility to allow the SecY pore to open.

1. Introduction

In bacteria, periplasmic and inner membrane proteins that are synthesized in the cytosol need to be translocated across or inserted into the inner membrane, respectively. These processes are facilitated by the Sec-system that consists of an inner membrane complex termed SecYEG that functions as the translocation channel and a cytoplasmic motor protein SecA or the translating ribosome [1]. SecY resembles a clamshell that encompasses a central hourglass shaped pore [2]. Secretory proteins are vectorially translocated, while transmembrane proteins are laterally inserted in the membrane via the so-called lateral gate of SecYEG. The SecY subunit is enwrapped by the SecE subunit, which consists of a highly tilted transmembrane helix associated with one half of the SecY clamshell, while the cytosolic surface exposed amphipathic helix of SecE interacts with the other SecY half. Both SecY and SecE constitute essential subunits of the SecYEG complex, whereas the third subunit SecG that is peripherally associated with the channel seems functionally redundant. Although most bacterial SecE proteins harbor only one transmembrane segment (TMS), the *Escherichia coli* SecE contains two additional non-essential TMSs that are N-terminally associated with the amphipathic helix. The amphipathic helix and the tilted TMS are connected by a hinge region that harbors several conserved residues. While the deletion of these amino acid residues abolished the SecE function, amino acid substitutions had little effect on the activity implying that the conservation is not of functional importance [3,4]. The hinge region together with the tilted helixwere shown to be essential for the stability of the SecYEG complex, whereas a large part of the amphipathic helix seems redundant [5]. In this respect, SecY that is not stably associated with SecE is readily degraded by the membrane protease FtsH as part of a quality control process [6]. It is likely that the supporting role of SecE is sustained during protein translocation, where the opening of the central SecY constriction allows space for the translocating preprotein. This is accompanied by the opening of the clamshell, partly exposing the interior of the channel to the lipid bilayer via the lateral gate. How the expansion of the channel diameter influences interactions between SecY and SecE is unknown.

We have investigated the dynamics of the interaction between SecY and SecE by a cysteine-based crosslinking approach. Herein, the two main sites of SecY–E interaction were stabilized by means of disulfide bonding and the effect of this immobilization on the protein translocation activity was examined. Furthermore, we examined the importance of the hinge region by a targeted proteolytic analysis.

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2. Materials and methods

2.1. Chemicals and biochemicals

The isolation of inner membrane vesicles (IMVs) containing levels of overexpressed SecYEG and the purification of SecA, SecB and proOmpA were performed as described [7]. ProOmpA(245C) was labeled with fluorescein-maleimide (Invitrogen™) according to a previously developed protocol [8]. OmpT was expressed and outer membranes were isolatedas described [9]. DNA modification enzymes were obtained from Fermentas, all other chemicals were from Sigma.

2.2. Bacterial strains and plasmids

All strains and plasmids used are shown in Table 1. All cloning procedures were performed with DH5α cells. The template vector used for further cloning was constructed by ligation of the *NcoI-BamHI* fragment of pEK20 into pET401. Site directed mutagenesis according the Stratagene QuickChange® kit was used to introduce cysteine mutations in the template pET401-YE. In case of the multiple cysteine constructs multiple rounds of site directed mutagenesis with a cysteine-containing construct as template was performed. After cysteine introduction the*NcoI-BamHI* SecY-E fragments were swapped for the ones in the expression vector pEK20. Specific amino acids in SecE were substituted for Factor Xa recognition sites (IEGR) by overlap PCR. DNA sequences on both sides of the target substitution site were amplified with primers carrying a 5′-ATTGAAGGTCGT-3′ overlapping complementary sequence. The resulting PCR fragments were used in a second PCR reaction. The final fragment was digested with *NcoI* and *XbaI* and swapped for the SecYEG fragment in the expression vector pEK20 or pZW1 [10,11]. All introduced mutations were checked by sequencing. *E. coli* strain SF100 was used for the overproduction of the different SecYEG complexes.

2.3. Factor Xa and OmpT cleavage

IMVs overexpressing the various SecYEG mutants were diluted to at least 1 mg/ml and further normalized for SecY content based on the intensities of the SecY band using SDS–PAGE and CBB staining. Stock solutions of Factor Xa (NEB) were diluted to 200 μg/ml with 20 mM TRIS/HCl pH 6.8; 50 mM NaCl; 1 mM $CaCl_2$. Of this solution, 2 μl was added to a volume of 30 μl of IMVs (~1 mg/ml). Reactions were typically incubated for 2 h at 30 °C.

For chemical crosslinking IMVs were than incubated for 30 min with 100 μM Cu^{2+}(phenanthroline)$_3$ in a volume of 30 μl at 37 °C. An OmpT protease assay was used to determine the crosslinking efficiency in the quadruple cysteine mutants [9]. Outer membranes containing overexpressed OmpT were diluted to 1 mg/ml in 50 mM TRIS/HCl pH 8.0 and 0.3% Triton X-100. The OmpT solution (5 μl) was mixed with the crosslinked IMVs (10 μl) and incubated for 30 min at 37 °C. Samples were loaded on 15% non-reducing SDS–PAGE and stained with CBB R250 or blotted on PVDF membranes and immunostained with anti-SecE IgG [18].

2.4. Other techniques

In vitro translocation of proOmpA was performed as described [8] with 5 μg of IMVs. Translocated protease resistant proOmpA was visualized in-gel with a Fujifilm LAS-4000 image analyzer using a 460 nm excitation and an emission filter of 510DF20 for fluorescein. Protein concentrations were determined with the Bio-Rad RC DC protein assay kit using BSA as a standard.

3. Results

3.1. SecE supports the activity of a split SecY protein

Previous deletion studies showed that a major part of the *E. coli* SecE protein is not essential for protein translocation [4,12]. To investigate more precisely which regions of SecE arenecessary for protein translocation we introduced Factor Xa recognition sites (IEGR) at various points in and around the hinge region, to subsequently cleave SecE and analyze its effect on protein translocation. Using the crystal structure of the *Methanocaldococcus jannaschii* SecYEβ [2] and an alignment to the *E. coli* SecYEG, two sites were chosen within the hinge region (Xa2 and Xa3) and one N-terminally located of the hinge region (Xa1) (Fig. 1). SecYEG proteins with these Factor Xa sites were expressed and inner membrane vesicles (IMVs) were isolated. The in vitro proOmpA translocation activity of the different IMVs were similar (Fig. 2A). When the mutant SecYEG complexes containing the IEGR motif in SecE weretreated with Factor Xa, the SecE band disappeared almost completely as compared to the cysteine less SecYEG (Fig. 2B, compare lanes 4, 6, and 8 with lane 2). An unforeseen side effect was the cleavage of

Table 1
Strains and plasmids used in this study.

Strains/plasmids	Characteristics	Source
E. coli DH5α	*supE44, ΔlacU169 (Δ80lacZ_M15) hsdR17, recA1, endA1, gyrA96 thi-1, relA1*	[14]
E. coli SF100	*F⁻, ΔlacX74, galE, galK, thi, rpsL, strA, ΔphoA(pvuII), ΔompT*	[15]
E. coli NN100	SF100, *unc⁻*	[16]
pET36	proOmpA(245C)	[17]
pEK1	Cysteine-less SecY	[10]
pEK20	Cysteine-less SecYEG	[10]
pEK20-Xa1	As pEK20 with SecE: ^{71}FARE substituted for IEGR	This study
pEK20-Xa2	As pEK20 with SecE: ^{80}RKVI substituted for IEGR	This study
pEK20-Xa3	As pEK20 with SecE: ^{82}VIWP substituted for IEGR	This study
pZW1	Cysteine-less SecY(R255E,R256E)EG	[11]
pZW1-Xa1	As pZW1 with SecE: ^{71}FARE substituted for IEGR	This study
pZW1-Xa2	As pZW1 with SecE: ^{80}RKVI substituted for IEGR	This study
pZW1-Xa3	As pZW1 with SecE: ^{82}VIWP substituted for IEGR	This study
pET401	Derivative of pBluescript SK with *NcoI* site	[10]
pET401-YE	As pET401 with SecY-E	This study
pEK20-2C1	SecY(A197C)E(S105C)G	This study
pEK20-2C2	SecY(R372C)E(A75C)G	This study
pEK20-2C3	SecY(I413C)E(V100C)G	This study
pEK20-4C1	SecY(A197C,R372C)E(A75C,S105C)G	This study
pEK20-4C2	SecY(R372C,I413C)E(A75C,V100C)G	This study

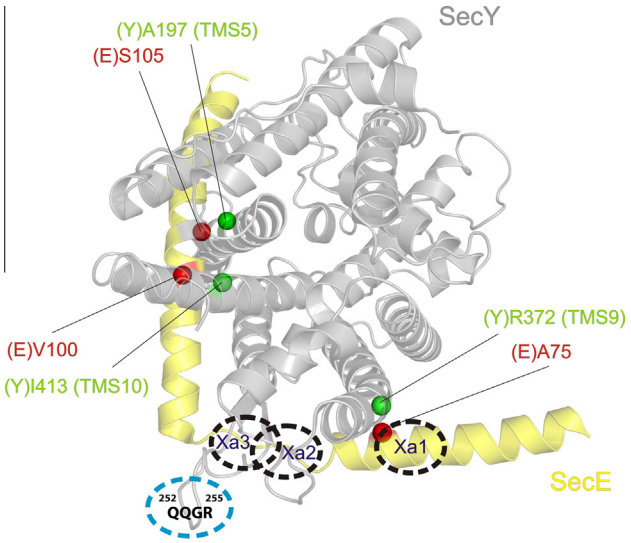

Fig. 1. (A) Positions of the cysteine mutations introduced in SecE (red spheres) and SecY (green spheres) introduced into the *E. coli* SecYE and mapped on the structure of the *Methanocaldococcus jannaschii* SecYE. The Factor Xa recognition sites are visualized as bracketed circles. The non-canonical Factor Xa cleavage site in SecY is shown as a blue bracketed circle. SecE is shown in yellow and SecY in gray.

SecY in two fragments with apparent molecular masses of 25 and 18 kDa, respectively. Surprisingly, the translocation activity of cysteine less SecYEG was only marginally affected when SecY was cleaved by Factor Xa (Fig. 2C, lane 4). However, when also SecE was cleaved at the different Factor Xa recognition sites, this resulted in an almost complete inhibition of protein translocation (Fig. 2C, lane 6, 8 and 10). These data suggest that SecE supports the translocation activity of cleaved SecY most likely by keeping the two fragments together.

3.2. The hinge region of SecE is essential for protein translocation

We hypothesized that the SecY was cleaved at the imperfect Factor Xa site ^{252}QQGR^{255} in the hinge region of SecY (Fig. 1). To test this hypothesis we examined a mutant in which the amino acids R255 and R256 are substituted for glutamic acid. The altered charge causes a different migration behavior of SecY on SDS–PAGE migrates at a higher molecular mass slightly above 40 kDa as reported previously [11]. Treatment of IMVs bearing overexpressed levels of SecY(R255E,R256E)EG with Factor Xa did not result in cleavage of SecY (Fig. 3A). This implies that these mutations effectively disrupt the non-canonical Factor Xa recognition site. Next, we combined the R255E/R256E mutation of SecY with the different SecE subunits containing the Xa1, Xa2 and Xa3 cleavage sites. Treatment of the SecYEG complexes with Factor Xa resulted in

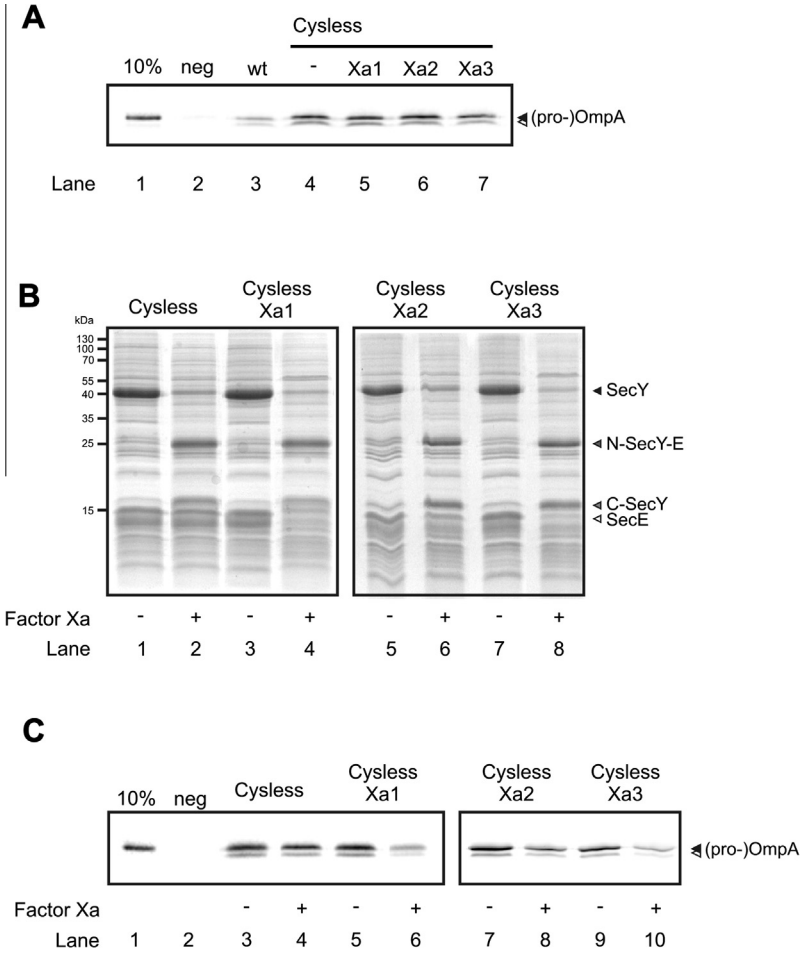

Fig. 2. (A) Translocation of fluorescein-proOmpA into inner membrane vesicles containing wild-type (lane 3) or overexpressed levels of cysteine-less SecYEG with the indicated factor Xa recognition sites introduced in SecE (lane 4–7). Translocation of proOmpA was assayed by its protection against proteinase K, followed by SDS–PAGE and fluorescence imaging. Lane 1 shows 10% of the total fluorescein-proOmpA used in one reaction. Lane 2 is the negative control where no ATP was added to the translocation assay. (B) IMVs containing the different cysteine-less SecYEG mutants were treated with Factor Xa (13 μg/ml) and the cleavage was checked on SDS–PAGE followed by coomassie staining. (C) ProOmpA translocation by the indicated IMVs not treated (lanes 3, 5, 7 and 9) and treated with Factor Xa (lane 4, 6, 8 and 10. In lane 2, ATP was omitted from the reaction.

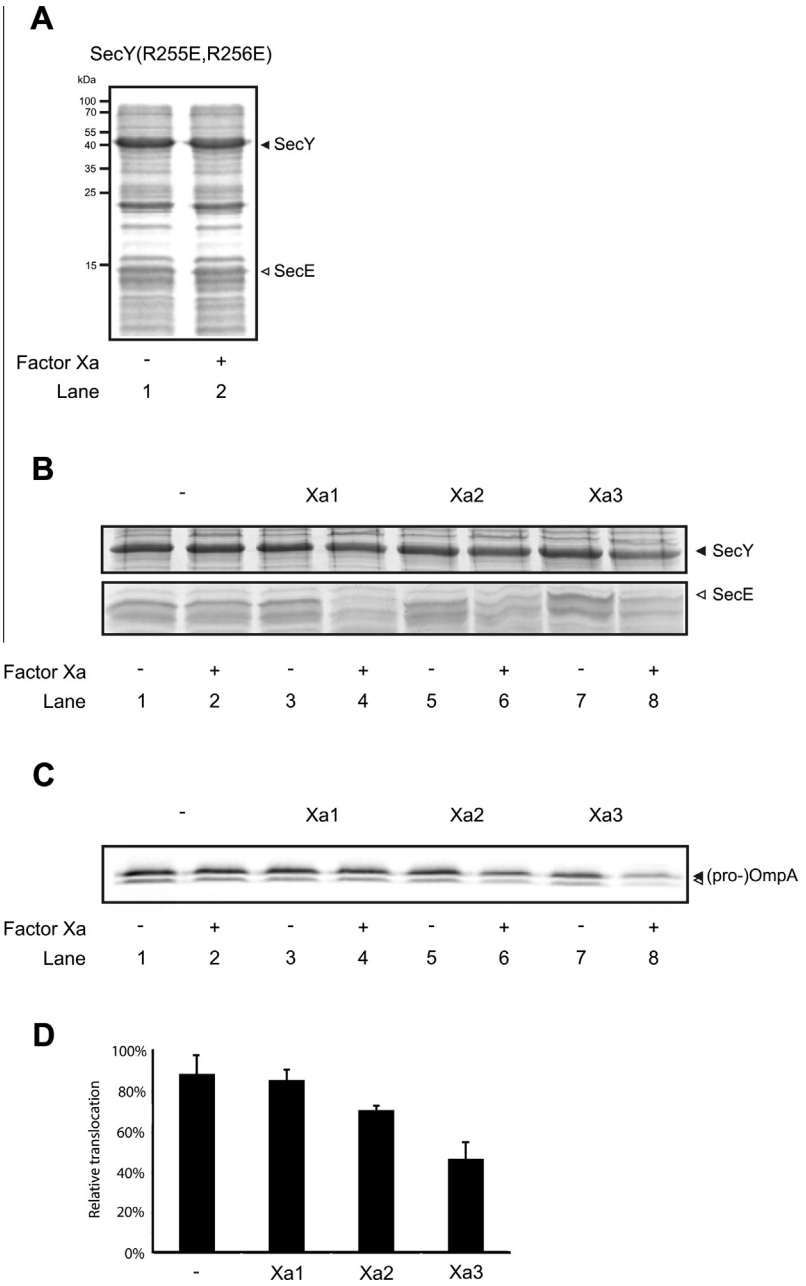

Fig. 3. (A) IMVs containing the SecY(R255E,R256E)EG mutant (B) or the different SecY(R255E,R256E)E(IEGR)G mutants were treated with Factor Xa (13 μg/ml) and the cleavage was checked on SDS–PAGE followed by coomassie staining. (C) Fluorescence image of ProOmpA translocation by the indicated IMVs not treated (lanes 1, 3, 5 and 7) and treated with Factor Xa (lane 2, 4, 6 and 8). (D) Quantification of the translocation reactions by IMVs treated with Factor Xa. Reactions were related to untreated IMVs.

different degrees of cleavage of the SecE protein, while the control SecY(R255E,R256E)EG complex was not cleaved (Fig. 3B). Importantly, IMVs containing the SecY(R255E,R256E) mutant showed normal translocation activity irrespective of the treatment with Factor Xa (Fig. 3C and D). Cleavage of SecE at position Xa1, which is N-terminally located of the hinge region also had no effect on the proOmpA translocation activity (Fig. 3C, lane 4). However, when SecE was cleaved in the hinge region at position Xa2, but more severely at the Xa3 site, the translocation activity was inhibited. These results indicate that the hinge region of SecE is needed for efficient protein translocation.

3.3. SecY and SecE function through a flexible interaction

To investigate the flexibility needed at the SecY–SecE interface we introduced cysteines on SecE and SecY at three potential points of interaction that are predicted to be in close vicinity according to the *M. jannaschii* SecY structure (Fig. 1). Two of the points of contact were between the tilted helix of SecE and SecY, i.e., at the positions SecE(S105) and SecY(A197) in TMS5, and at SecE(V100) and SecY(I413) in TMS10. The other point of contact was chosen between the amphipathic helix of SecE(A75) and SecY(R372) at TMS9. At these positions, cysteine substitutions were introduced by site-directed mutagenesis. The various cysteinemutations were expressed as pairs of SecY and SecE. To be able to immobilize SecE to SecY at two points of interaction, the cysteine pairs at the tilted helix and those at the amphipathic helix of SecE where combined yielding two quadruple cysteine mutants. These double and quadruple cysteine SecYEG mutants were overexpressed in *E. coli* strain SF100 and IMVs were isolated. SDS–PAGE analysis showed similar levels of overexpression as compared to the cysteine-less SecYEG (See Fig. 4A). High crosslinking efficiencies were observed

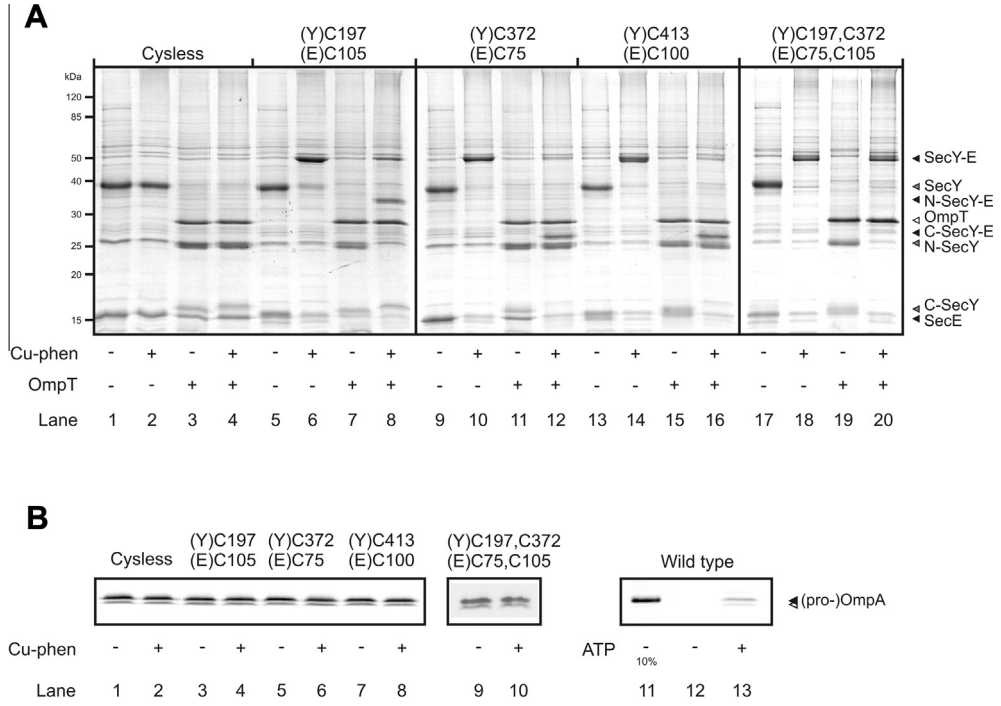

Fig. 4. (A) IMVs containing the different SecYEG mutants were treated with Cu-phen (100 μM) and the crosslinking efficiency was checked on SDS–PAGE. The crosslinking efficiency of the quadruple cysteine mutant was checked by OmpT treatment. Double cysteine mutants were used as a control. (B) ProOmpA translocation by the indicated IMVs not treated (lanes 1, 3, 5, 7 and 9), oxidized with Cu-phen (lanes 2, 4, 6, 8 and 10). Lane 11 shows 10% of the total fluorescein-ProOmpA in one reaction. In lane 12, ATP was omitted from the reaction, while in lane 13, wild type vesicles without overexpression of the SecYEG complex were used.

for all sites when the hydrophobic oxidizer copper phenanthroline (Cu-phen) was used. The three double cysteine mutants showed a complete shift of SecY to a high molecular mass species indicating that full oxidation of the available cysteines was obtained consistent with their predicted close proximity (Fig.4A, lanes 6, 10 and 14). To assess the degree of crosslinking in the quadruple cysteine mutants we used a previously developed OmpT assay [9]. OmpT protease cleaves between arginine 255 and 256 in the cytoplasmic loop between TMS 6 and TMS 7. Cleaved SecY yields an N- and C-terminal SecY fragment with calculated molecular masses of 28 and 20 kDa, respectively. On SDS–PAGE, these fragments migrate at apparent molecular masses of 25 and 18 kDa, respectively (Fig. 4A, lane 3, 7, 11, 15 and 19). In case of a single disulfide bond between SecE and SecY, the SecY fragment crosslinked to the SecE subunit should shift to a higher apparent molecular mass. When oxidized, the SecY(C197)E(C105)G complex is crosslinked between SecE and TMS5 in the N-terminal part of SecY. Upon subsequent OmpT treatment, indeed a shift of the N-terminal SecYfragment is observed from 25 to 35 kDa (Fig. 4A, lane 8). SecY(C372)E(C75)G and SecY(C413)E(C100)G both form disulfide bonds between SecE and the C-terminal SecY fragment, TMS 9 and 10, respectively. When these complexes are treated with Cu-phen and OmpT, the expected shift of the C-terminal SecY fragment from 18 to 27 kDa is observed (Fig. 4A, lane 12 and 16). The quadruple cysteine mutant SecY(C197,C372)E(C75,C105)G combines cysteine pairs at sites of interaction between SecE and both the N-terminal and the C-terminal SecY halves. Similar to the double cysteine mutants, oxidation of SecY(C197,C372)E(C75,C105)G with Cu-phen resulted in a complete shift of SecY and SecE to the higher molecular mass species (Fig. 4A, lanes 8). The efficiency of crosslinking was also evident from immunostainedgels using an antibody directed against SecE (Supplementary Fig. 1). When this mutant is oxidized and treated with OmpT, none of the previously mentioned fragments appear and instead the entire SecYE complex stays together as a crosslinked species (Fig. 4A, lane 20, Supplemental Fig. 1). Taken together, these data demonstrate that the introduced cysteine mutations allow for an efficient crosslinking of SecY with SecE at single and at specific double positions.

The effect of crosslinking SecE to SecY on translocation was determined by a proOmpA translocation assay. Cu-phen treatment of the cysteine-less SecYEG complex had no effect on proOmpA translocation (Fig. 4B, lane 2). Interestingly, the oxidation of SecY (C197)E(C105)G, SecY(C372)E(C75)G and SecY(C413)E(C100)G and thus yielding singular points of immobilization of SecY and SecE also had little effect on the translocation activity for proOmpA (Fig. 4B, lanes 4, 6 and 8). Additionally, when the SecY(C197, C372)E(C75,C105)G complex was oxidized thus immobilizing the SecY–SecE interaction at the two interacting regions, no inhibition of translocation was observed (Fig. 4B, lane 10). This suggests that the SecE–SecY contact points are maintained during translocation activity.

4. Discussion

Here we have investigated the needed flexibility in the interaction between SecE and SecY. To examine the functional dynamics of SecE, we first addressed the role of the hinge region of SecE. This region in particularcontains several amino acid residues that are highly conserved among eubacterial SecE proteins. Introduction of the Factor Xa sites resulted in a substitution of some of these conserved residues for example F71 and K81/V82. However, substitution of the sequences ^{71}FARE^{74} (Xa1) and ^{80}RKVI^{83} (Xa2) for the IEGR recognition site had no influence on the ability of the SecYEG complex to translocate proOmpA (Fig. 2A). The W84/P85 residues are even more conserved and when substituted in the ^{82}VIWP^{85} (Xa3) sequence for IEGR, this resulted in a slight loss of translocation activity. Likely these amino acids are important for structural integrity of the hinge region and thus for the SecE

function. Previous studies showed that the deletion of residues W84 and P85 abolishes the SecE function, but that substitutions are tolerated [3] yielding only minor growth defects in vivo [5].

Cleavage of SecY by Factor Xa was unsuspected but closer examination revealed that ^{252}QQGR^{255} may be a possible cleavage site (Fig. 1). When SecY was cleaved while SecE was still intact, translocation activity was retained (Fig. 2C), consistent with earlier observations upon OmpT cleavage [13]. Likely, SecE keeps the two SecY fragments together thereby maintaining the protein translocation activity. Upon combination of the R255 and R256 mutations in SecY and the factor Xa site in SecE, SecE could be exclusively cleaved by Factor Xa. Translocation activity assays showed that cleavage of SecE at position Xa1 does not result in any reduction of translocation, indicating that the tilted helix, the hinge region and only a small part of the amphipathic helix is sufficient for the SecYEG translocation function (Fig. 3C and D). Other in vivo and in vitro deletion studies show a similar effect [3,4]. Cleavage at positions Xa2 and Xa3 markedly reduces translocation activity. Both positions are located in the hinge region and the observation that cleavage at position Xa1 does not affect translocation indicates that at least some part between Xa2 and Xa1 is essential for protein translocation. Most likely the first part of the amphipathic helix acts as an anchor to ensure that the hinge region is properly located to support the SecY subunit.

The anchoring capability of SecE is a consequence of stable contact points between SecE and SecY. During opening of the SecY-translocation channel, likely a certain degree of flexibility in these contacts might be required. To test this hypothesis we fixed three points of contact between SecY and SecE using a cysteine based crosslinking approach. Crosslinking of the tilted helix and the amphipathic helix of SecE to the SecY subunit as well as a combination of the two did not result in a visible decrease in proOmpA translocation (Fig. 4B). This indicates that the interaction points between SecE and SecY are maintained even during active translocation. We hypothesize that the flexibility needed in SecE during channel opening arises from the unstructured hinge region.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.07.046.

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