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A Single Amino Acid Substitution in SecY Stabilizes the Interaction with SecA*

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The components of the bacterial protein secretion pathway have originally been identified in *Escherichia coli* through both genetic (1, 2) and biochemical studies (3). The translocation reaction across the cytoplasmic membrane is mediated by an enzyme complex termed the translocase. The SecYEG complex constitutes a protein conducting channel across the bacterial cytoplasmic membrane. It binds the peripheral ATPase SecA to form the translocon. When isoleucine 278 in transmembrane segment 7 of the SecY subunit was replaced by a unique cysteine, SecYEG supported an increased preprotein translocation and SecA translocation ATPase activity, and allowed translocation of a preprotein with a defective signal sequence. SecY(I278C)EG binds SecA with a higher affinity than normal SecYEG, in particular in the presence of ATP. The increased translocation activity of SecY(I278C)EG was confirmed in a purified system consisting of SecYEG proteoliposomes, while immunoprecipitation in detergent solution reveals that translocase-preprotein complexes are more stable with SecY(I278C) than with normal SecY. These data imply an important role for SecY transmembrane segment 7 in SecA binding. As improved SecA binding to SecY was also observed with the prlA4 suppressor mutation, it may be a general mechanism underlying signal sequence suppression.

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† The abbreviations used are: TMS, transmembrane segment; IMVs, inner membrane vesicles; octyl glucoside, n-octyl-b-D-glucopyranoside.
A synthetic secYEG operon behind the isopropyl-β-D-thiogalactoside-inducible tcr promoter was used for the plasmid-derived overexpression of the SecYEG complex. All plasmids encoding single cysteine SecYEG were constructed via polymerase chain reaction mutagenesis, resulting in the indicated mutations.

### Table I

| Plasmid | Relevant characteristics | Mutations | Source |
|---------|--------------------------|-----------|--------|
| pET340  | SecYEG tandem behind tcr promoter | L60L (CTG->CTC) | Ref. 20 |
| pET349  | His-tagged SecYEG in pET340 | V274C (GTA->TGT) | This study |
| pET602  | Cysteine-less YEG in pET605 | I275C (ATC->TGC) | This study |
| pET605  | pET340 with ΔFinelII in secE | F276C (CGC->GGC) | This study |
| pET610  | pET349 with ΔFinelII in secE | A277C (GCA->GGA) | This study |
| pET611  | SecY TMS7 mutant 1 in pET602 | L60L (CTG->CTC) | Ref. 38 |
| pET612  | SecY TMS7 mutant 2 in pET602 | I275C (ATC->TGC) | This study |
| pET613  | SecY TMS7 mutant 3 in pET602 | F276C (CGC->GGC) | This study |
| pET614  | SecY TMS7 mutant 4 in pET602 | A277C (GCA->GGA) | This study |
| pET615  | SecY TMS7 mutant 5 in pET602 | I275C (ATC->TGC) | This study |
| pET616  | SecY TMS7 mutant 6 in pET602 | F276C (CGC->GGC) | This study |
| pET617  | SecY TMS7 mutant 7 in pET602 | A280C (GCT->GGA) | This study |
| pET618  | SecY TMS7 mutant 8 in pET602 | S281C (TCC->GGA) | This study |

### EXPERIMENTAL PROCEDURES

**Materials**—Monoclonal antibodies against OmpA were raised and selected by Prof. Dr. L. de Leij, Academic Hospital Groningen. Polyclonal antiserum against purified SecY or SecA were obtained as described (20). Western blots were developed as films using chemiluminescence (Tropix, Bedford, MA). For densitometry a Dextra DF-2400T scanner (Dextra Technology Corp., Taipei, Taiwan) and ImageScan/ Image Software (Jandel Corp., San Rafael, CA) were used. DNA sequencing was performed on a Vistra DNA sequencing 725 using the automated Δtaq sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Protein A-Sepharose was from Amersham Pharmacia Biotech (Uppsala, Sweden), n-octyl-β-D-glucopyranoside (octyl glucoside) from Sigma, and E. coli phospholipids from Avanti Polar Lipids Inc. (Alabaster, AL).

**Plasmids**—All plasmids used for this study are described in Table I. The construction of plasmids that allow the overexpression of SecYEG, (His)_6-tagged SecYEG (20), or cysteine-less SecYEG (38) has been described previously. Cysteines were introduced in SecY TMS 7 by a one-step polymerase chain reaction mutagenesis. To facilitate the screening for correct mutants, cysteine mutagenesis was accompanied by the GGT→GGG (G350C) mutation, leading to the insertion of an BspEII restriction site. An amino-terminal (His)_6-tag on SecYEG was obtained by cloning the Ncol/BamHI fragment from pET615 (Table I) into Ncol/BamHI-digested pET302 (20). All constructs were confirmed by sequence analysis.

**Translocation Reactions**—(His)_6-tagged SecYEG was purified and reconstituted into proteoliposomes as described (20), and other components of the translocation reaction were obtained as in Ref. 32. Concentrations of the different components are mentioned in the text or figure legends. Reactions were incubated at 37 °C in a total volume of 100 μl of translocation buffer (50 mM Hepes-KOH, pH 7.6, 50 mM KCl, 5 mM MgCl₂, 0.5 mg/ml bovine serum albumin, and 10 mM dithiothreitol) and stopped by chilling on ice and protease K treatment (10).

**Immunoprecipitation**—Proteoliposomes from two translocation reactions were collected by centrifugation (20 min, 120,000 × g), and solubilized in buffer C (1.2% (w/v) octyl glucoside, 0.3 mg/ml E. coli phospholipids, 20% (v/v) glycerol, 50 mM KCl and 50 mM Tris-HCl, pH 8.0) for 1 h on ice. Protein A-Sepharose slurry (10 μl) was incubated with 20 μl of antisera diluted in 200 μl of buffer A for 1 h at 4 °C, washed, and mixed with the solubilized proteoliposomes. After 90 min of constant shaking at 4 °C, Sepharose beads were collected (3 min, 12,000 × g) and washed five times with 0.3 ml of buffer C. Bound proteins were eluted by incubation with 60 μl of SDS sample buffer for 10 min at 60 °C and separated from the Sepharose beads by centrifugation.

**RESULTS**

**Identification of a Mutation in SecY That Supports Increased Translocation**—As part of a larger cysteine-scanning mutagenesis study (38), unique cysteine residues were introduced in TMS 7 of SecY. To cover at least two turns of the putative α-helical structure, 8 residues in TMS 7 (Val(274)Sec(281)) were mutated to cysteine residues (Fig. 1). Substitutions of two of these residues that face the same side of the helix, Val(274) and Ile(278), have been reported to give rise to suppressor phenotypes (37). The mutant secY genes were cloned in pET602, a vector that allows overexpression of cysteine-less SecYEG, which is functionally indistinguishable from normal SecYEG (38). Although the expression of SecYEG was similar with all TMS 7 mutants (Fig. 2A), there was a pronounced increase in SecA ATPase activity with SecYEG IMVs (Fig. 2B). Moreover, this resulted in increased translocation of the preprotein proOmpA (Fig. 2C). To test whether the increased activity of the SecYEG complex affected its specificity, we examined the translocation of ΔsproOmpA, a variant precursor carrying a defective signal sequence due to the deletion of Ile(6) (41) that is efficiently translocated by PrlA IMVs (32). This precursor was transported only into the SecYEG complex affected its specificity. Apparently, the introduction of cysteines at the other positions of SecY TMS 7, including Val(274), did not alter the activity or specificity of translocase.

**Increased SecA Binding to SecY(278C)EG**—With the PrlA suppressor, which contains the F286Y substitution in TMS 7 and I408N in TMS 10, an increased affinity for SecA was observed as compared with normal PrlA (SecY). The difference in SecA binding is even larger upon the addition of ATP, which...
lowers the affinity, but to a much lesser extent with PrlA4 (32). Since SecY(I278C) allows translocation of D8proOmpA, we compared the binding of SecA to IMVs containing overproduced SecYEG, cysteine-less SecYEG or SecY(I278C)EG (Fig. 3). As expected from their similar activity (Fig. 2), the binding of SecA to SecYEG or cysteine-less SecYEG was nearly identical (Fig. 3A, closed bars) and was reduced to the same level in the presence of ATP (open bars). In contrast, the binding of SecA to SecY(I278C)EG IMVs was significantly higher and was only slightly reduced in the presence of ATP. Using a concentration range of SecA, we determined the affinity of SecA binding to the IMVs containing cysteine-less SecYEG (Fig. 3B) or SecY(I278C)EG (Fig. 3C) by Scatchard analysis (42). IMVs contained 2.1–2.4 μM/mg high affinity SecA-binding sites, a 25–30-fold increase as compared with IMVs harboring endogenous levels of SecYEG (80 nM/mg; Ref. 32). SecA binds to overproduced cysteine-less SecYEG with a $K_d$ of 4 nM in the absence and a $K_d$ of 16 nM in the presence of ATP. These affinities are somewhat higher, but comparable, to those observed with endogenous SecYEG, i.e. 7 nM without and 24 nM with ATP (32), and confirm that SecYEG is functionally overexpressed. Compared with cysteine-less SecYEG, the affinity of SecA binding to SecY(I278C)EG was 2.5-fold higher in the absence of ATP ($K_d$ = 1.6 nM) and 5.7-fold higher in the presence of ATP ($K_d$ = 2.8 nM). These data demonstrate that the SecY(I278C) mutation results in an increased affinity of the SecYEG complex for SecA, especially in the presence of ATP.

With PrlA4, SecA binding occurs with a $K_d$ of 1.4 nM in the absence and a $K_d$ of 3.6 in the presence of ATP. The increased affinity leads to a decreased rejection of SecA-precursor complexes, and less dissociation of SecA during translocation (32). We propose that the same phenomenon is responsible for the increased translocation activity and lowered specificity of SecY(I278C).

Translocation Activity of Purified SecY(I278C)EG—To study the translocation activity of the purified SecY(I278C)EG complex, a (His)$_6$-tag was positioned at the amino terminus of SecY. The complex was then overexpressed, purified, and reconstituted as described previously (20). (His)$_6$SecY(I278C)EG proteoliposomes were compared with those reconstituted with the same amount of normal (His)$_6$SecYEG. As observed with IMVs, the proOmpA-stimulated ATPase activity was highest with SecY(I278C)EG proteoliposomes (Fig. 4A, closed bars). However, when D8proOmpA was used in the translocation reaction, hardly any stimulation of the SecA translocation ATPase activity was observed (Fig. 4A). Therefore, the amounts of translocated precursor were visualized by Western blotting using monoclonal antibodies against OmpA. Quantitative analysis of these blots demonstrated that after 20 min only a minor fraction (<0.5%) of the D8proOmpA was translocated in SecY(I278C)EG proteoliposomes, as compared with normal proOmpA (about 25%) (Fig. 4, B and C, closed symbols). To demonstrate that the translocated protein was truly D8proOmpA, and not the result of an impurity with endoge-
nous proOmpA from the host strain used for purification, we repeated the experiment with in vitro synthesized and purified [35S]methionine-labeled D8proOmpA (Fig. 4D). This clearly showed that purified SecY(I278C)EG allows the translocation of this defective precursor. The sensitivity of the autoradiograms (Fig. 4D) was somewhat higher than that obtained by Western blots (Fig. 4C) and revealed a minimal level of D8proOmpA translocation with normal SecYEG, confirming in vivo data (41).

In the absence of reducing agents, proOmpA is blocked for further translocation at the position of a disulfide-bond between two unique cysteine residues (Cys290 and Cys302) in its carboxyl terminus (9, 43). In proteoliposomes, this results in the accumulation of a 31-kDa translocation intermediate (I31) (Fig. 4E). This intermediate occupies the translocation sites and blocks them for a second round of translocation (Ref. 22 and data not shown). With the normal proteoliposomes, maximal I31 translocation was reached after 10 min, whereas proteoliposomes with SecY(I278C) accumulated maximal amounts within the first 5 min of the translocation reaction (Fig. 4E). SecY(I278C) did not allow the full-length translocation of oxidized proOmpA and thus differs in this respect from PrlA4 (33). The fast kinetics of the translocation reaction with SecY(I278C), as compared with normal SecY, is apparent from the initial rate of translocation (Fig. 4B) and the shorter time required to saturate the translocation sites with I31 (Fig. 4E). The experiments with proteoliposomes demonstrate that the SecY(I278C) mutation stimulates SecA- and ATP-driven translocation, and that this effect does not require proteinaceous factors other than the SecYEG complex.
SecY(I278C) enforces the translocation of Δ8proOmpA with purified translocase.

SecY(I278C) Stabilizes Translocase-Precursor Complexes—Co-immunoprecipitation was used to assay the stability of translocase-precursor complexes formed during ongoing translocation or at halted stages of the translocation reaction. Proteoliposomes were incubated with SecA and proOmpA in the absence of ATP (targeting of SecA and the precursor), the presence of ATP (ongoing translocation), or with ATP under oxidizing conditions (blocked translocation, yielding I31). The proteoliposomes were then harvested by centrifugation and solubilized in the detergent octyl glucoside. Samples were immunoprecipitated with polyclonal antiserum against SecA or SecY, and co-precipitation of proOmpA was visualized by immunoblotting using a monoclonal antibody against OmpA (Fig. 5A). No, or only very little, interaction between proOmpA and either SecY or SecA was observed with normal translocase after incubation in the absence of ATP (lanes I and 7). After translocation under reducing conditions, a fraction of proOmpA was associated with SecY but not with SecA (lanes 2 and 8). Apparently, SecA has dissociated from these SecYEG-precursor complexes. Only after translocation of I31, fully stable translocase-precursor complexes were formed (lanes 3 and 9). In contrast, SecY(I278C) translocase-precursor complexes were precipitated independent of the preincubation (lanes 4–6 and 10–12). Only immunoprecipitation with anti-SecYE serum yielded a significantly lowered amount of proOmpA after incubation in the absence of ATP (lane 4). Antibodies against SecY interfere with SecA binding (44, 45) and therefore may destabilize translocase. In conclusion, SecY(I278C) translocase-precursor complexes are more stable than their counterparts containing normal SecY. Wild-type translocase is, however, stabilized by the I31 translocation intermediate. This is consistent with experiments in IMVs that suggest a stable association of this intermediate with SecA at translocation sites (9).
DISCUSSION

The *E. coli* translocase is composed of the SecA ATPase bound to a transmembrane protein conducting channel with SecY and SecE as core components (19), and with SecG as an additional subunit (14, 21). The identification and reconstruction of its minimal constituents (19–21) have made translocase an intriguing model to study subunit dynamics in a membrane protein complex. To allow site-directed labeling of functionally important regions in translocase and to detect specific intermolecular contacts, we have employed cysteine mutagenesis of regions in SecY and SecE that contain clusters of prlA or prlG suppressor mutations, respectively (31, 37, 38). From the single cysteine mutants at positions 274–281 of SecY TMS 7, the I278C substitution resulted in an increased translocation activity and gave rise to *in vitro* detectable signal sequence suppression, as measured by the translocation of Δ8proOmpA. Previously, prlA suppressor mutations have been identified that lead to substitutions of I278 for Ser (prlA202, 203, 204, and 207), Asn (prlA208), or Thr (prlA303) residues (37). It thus seems that Ile<sup>278</sup> is a hot spot for such suppressor mutations. SecY<sup>V274C</sup> did not alter the activity and specificity of translocase, although prlA suppressor mutations have been identified that result in a V274G substitution (prlA1, 2, 5 and 201; Ref. 37). Apparently, the amino acid substitutions that give rise to prl suppression depend not only on the position but also on the nature of the substituted amino acid. We have also constructed plasmids that allowed co-overexpression of the SecY mutants with cysteines at positions 105–109 of SecE TMS 3. Although synthetic lethality was observed between prlA208 (I278N) and prlG1 (L108R) (31), none of the combined mutants yielded cross-links between SecY and SecE upon oxidation (data not shown). This implies that synthetic lethality does not necessarily result from a direct interaction between two amino acids.

One of the earliest identified prlA suppressor mutations is prlA4 (24). Its suppressor phenotype is caused by the I408N substitution in SecY TMS 10, but this mutation is generally accompanied by the F286Y substitution in TMS 7 or, with prlA6, S188L in TMS 5 (37, 46, 47). The apparently unavoidable occurrence of secondary mutations may reflect a detrimental effect of the I408N substitution on the *E. coli* cell. SecA binds to PrlA4 with an increased affinity, and this results in a decreased rejection of SecA and the preprotein at the onset of translocation (32). We now report on a similar phenomenon with SecY carrying the I278C amino acid substitution in TMS 7 in a cysteine-less background. This mutant supports an increased translocase activity and translocates Δ8proOmpA, carrying a defective signal sequence. As the activity of cysteine-less SecYEG is indistinguishable from normal SecYEG (this study and Ref. 38), the I278C mutation appears solely responsible for the observed phenotype (see Fig. 2D). Using affinity blotting, the amino-terminal half of SecY was detected as a binding site for SecA (48). The increased affinity for SecA caused by this I278C substitution, however, indicates that SecY TMS 7 also serves as a site of interaction with SecA. Alternatively, this mutation affects binding of SecA to the amino-terminal half of SecY. Our data support a model in which prlA suppression is the result of improved binding between SecA and SecY. This will optimize the translocation of normal preproteins due to a better targeting of SecA to SecYEG and less dissociation of the translocase components during ATP-driven translocation. At the same time, it lowers the proofreading activity of translocase as SecA carrying a defective preprotein is less easily rejected from translocation sites at the onset of translocation, likely upon the binding of ATP. Since proton-motive force-driven translocation is prevented by the presence of SecA (9), an increased affinity for SecA may explain why PrlA suppressors render the translocation reaction proton-motive force-independent (33).

The stabilization of translocase-preprotein complexes by SecY<sup>V278C</sup> during translocation was directly demonstrated by co-immunoprecipitation. With normal SecY, a soluble translocase-preprotein complex required the presence of a stable translocation intermediate I<sub>31</sub>. No complexes between SecA or SecY with the precursor were observed after incubation in the absence of ATP, and SecA readily dissociated from the complex during an ongoing translocation reaction. With SecY<sup>V278C</sup>, translocase-preprotein complexes were completely stable after incubation under translocating conditions, as compared with complexes with trapped I<sub>31</sub>. Incubation in the absence of ATP yielded complexes that were susceptible for dissociation by an antibody against SecY. This suggests that the SecYEG channel alters its conformation during translocation, rendering the interaction with SecA and the preprotein more stable. This conformational change may involve subunit rearrangements, or channel “opening,” as has been observed with the Sec61p complex during translocation (49, 50).

Whereas increased SecA binding is a clear phenotype of both PrlA4 and SecY<sup>V278C</sup>, they are functionally different in two aspects. First, PrlA4 allows translocation of a disulfide bonded loop of 10 amino acids in the mature region of proOmpA (33), whereas SecY<sup>V278C</sup> does not (Fig. 4E). Second, PrlA4 supports increased translocation with a lowered SecA ATPase activity (32), whereas with SecY<sup>V278C</sup>, the increased translocation is accompanied by a concomitant increase in the rate of ATP hydrolysis by SecA. The affinity of PrlA4 and SecY<sup>V278C</sup> for SecA is hardly different and we therefore hypothesize that PrlA4 and SecY<sup>V278C</sup> differ mechanistically. Suppressor mutations in SecY TMS 10 appear to strongly affect the interaction with SecE TMS 3 (31, 37). As conformational changes in SecE TMS 3 and SecA membrane cycling are interrelated (38), the I408N mutation in PrlA4 may slow the SecA reaction cycle due to an altered interaction with SecE. A slowed ATPase activity has been proposed as a mechanism for prlD (secA) suppression by increasing the lifetime of SecA-preprotein complexes during translocation (27). With PrlA4, improved SecA binding to the SecYEG complex at the same time makes translocation highly efficient. We propose that SecY<sup>V278C</sup> is a milder suppressor than PrlA4 because it does not affect the SecA reaction cycle. Extensive biochemical analysis of other Prl suppressors will unravel more of the mechanistic aspects underlying signal sequence recognition and the activity of translocase.

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