Nearest Neighbor Analysis of the SecYEG Complex. 1. Identification of a SecY−SecG Interface†

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ABSTRACT: Integral membrane components SecY, SecE, and SecG of protein translocase form a complex in the *Escherichia coli* plasma membrane. To characterize subunit interactions of the SecYEG complex, a series of SecY variants having a single cysteine in its cytoplasmic (C1−C6) or periplasmic (P1−P5) domain were subjected to site-specific cross-linking experiments using bifunctional agents with thiol−amine reactivity. Experiments using inverted membrane vesicles revealed specific cross-linkings between a cysteine residue placed in the C2 or C3 domain of SecY and the cytosolic lysine (Lys26) near the first transmembrane segment of SecG. These SecY Cys residues also formed a disulfide bond with an engineered cytosolic cysteine at position 28 of SecG. Thus, the C2−C3 region of SecY is in the proximity of the N-terminal half of the SecG cytoplasmic loop. Experiments using spheroplasts revealed the physical proximity of P2 (SecY) and the C-terminal periplasmic region of SecG. In addition, mutations in *secG* were isolated as suppressors against a cold-sensitive mutation (*secY104*) affecting the TM4−C3 boundary of SecY. These results collectively suggest that a C2−TM3−P2−TM4−C3 region of SecY serves as an interface with SecG.

The protein translocation machinery (translocase) of *Escherichia coli* consists of three core integral membrane components, SecY, SecE, and SecG (*1*, *2*), as well as the translocation-driving ATPase, SecA. SecY has 10 transmembrane segments (TM1−TM10), six cytoplasmic domains (C1−C6), and five periplasmic regions (P1−P5) (ref *3*; see Figure 1). SecE and SecG have three and two transmembrane segments, respectively (*4*, *5*). These membrane components presumably constitute a channel through which preprotein is inserted and translocated in conjunction with the SecA actions. Probably, two or more units of the SecY−SecE−SecG heterotrimeric complex are involved in the formation of the active channel (*6*−*10*). SecA interacts specifically with the SecYEG complex and undergoes remarkable conformational changes that are termed the insertion−deinsertion cycles (*11*, *12*). It was proposed that SecG assists in the SecA cycle (*13*, *14*) and that SecG itself is mobile (*5*). Thus, SecG appears to assume different orientations relative to the membrane, and its topology inversion occurs under the conditions in which SecA would assume the "inserted" conformation (*5*).

To understand the nature of the translocation channel, basic characterization of subunit interactions in the SecYEG heterotrimeric complex would be important. A SecY−SecE-(−SecG) complex can be isolated from detergent extracts of membranes (*15*, *16*). Studies using His_6-tagged SecY or SecE, in combination with the appropriate mutations, indicate that SecY interacts independently with SecE and SecG (*16*). Although genetic and biochemical studies of the SecY−SecE interaction have yielded some detailed information (*17*−*19*), little is known about how SecY interacts with SecG. Although the apparently dynamic nature of this interaction (*5*) should be taken into consideration, we thought it worthwhile to carry out systematic cross-linking studies of the SecYEG complex. Thus, we engineered SecY to introduce a cysteine residue into its selected positions. Such SecY variants were modified with thiol-specific cross-linking agents for cross-linking experiments. To study interactions among soluble domains, we chose cytosolic and periplasmic domains as the targets of cysteine introduction. These studies as well as genetic suppression information provided evidence that SecG interacts with the C2−C3 segment of SecY.

EXPERIMENTAL PROCEDURES

E. coli Strains. Strains AD202 [an *ompT*::*kan* derivative of MC4100 (*20*)], GN31 (MC4100, *ompT*::*kan secY39*), and KN370 (C600, *recD1009 secG*::*kan*) were described previously (refs *21*−*23*, respectively). GN94 (MC4100, *secY104 rpsE*), GN96 (MC4100, *secY104 rpsE zha203*::Tn*10*), and SA101 (AD202, *secG*::*kan zha203*::Tn*10*) were constructed by appropriate P1 transduction experiments using *rpsE* [spectinomycin resistance (*24*)] or *zha203*::Tn*10* (*25*) as a selective marker.

Media. L medium contained 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1.7 mmol of NaOH per liter.

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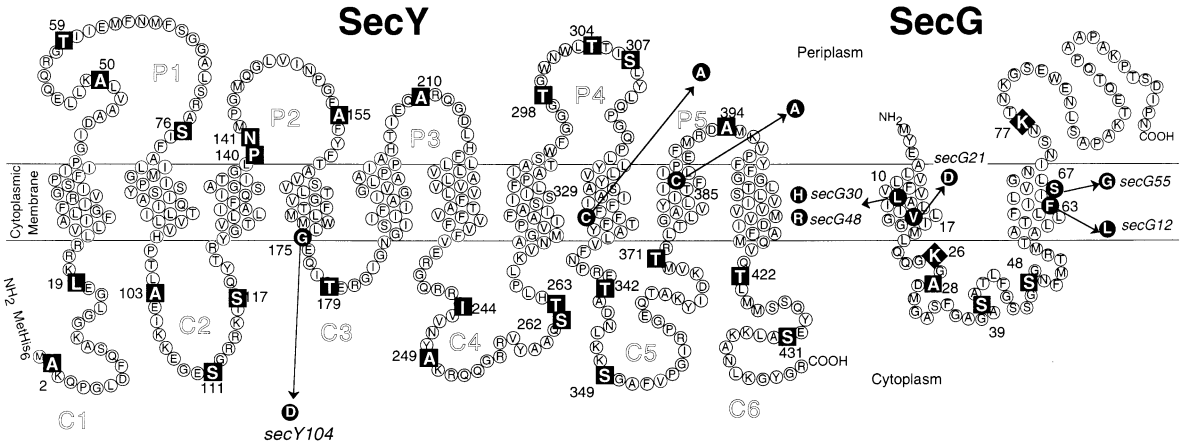

FIGURE 1: SecY and SecG variants used in this study. SecY and SecG amino acid sequences are shown according to their topology models (*3*, *5*). The two cysteine residues of SecY (shaded circles) were converted to alanine as indicated by arrows. Single-cysteine mutations were then introduced into residues shown as shaded squares. The *secY104* amino acid alteration is also shown. SecG residues that were site-directedly converted to Cys are also shown as shaded squares. Lys26 and Lys77 which were converted to arginine are highlighted as shaded diamonds. SecG amino acid changes identified as suppressors against *secY104* are denoted with arrows.

Minimal medium M9 was as described previously (*20*). Ampicillin (50 μg/mL) and/or chloramphenicol (20 μg/mL) was included for growing plasmid-bearing strains, as required. P1 transduction was carried out according to the standard procedures (*26*). Unselected markers were examined as described previously (*12*).

Isolation of secG Mutations as Suppressors against the secY104 Mutation. Cells (10^9) of strain GN96 were suspended in 10 mL of 0.1 M $MgSO_4$, irradiated with ultraviolet light (14 J/m^2 for 10 s), and divided into five portions, which were grown in L broth at 37 °C overnight. Cultures were then plated on L-tetracycline agar, after appropriate dilutions. Colonies that appeared after incubation at 20 °C for 3 days were pooled for preparation of P1*vir* lysates, which were then used to infect strain GN94 and to select Tet^R transductants at 20 °C. On the basis of growth phenotypes and the independence of their origin, a total of 21 mutants were chosen for further studies. They were shown to carry a *secY104*-suppressing mutation that was cotransducible with *zha203*::Tn*10*. The *secG* region of the chromosome was then amplified using primers 5′-CCTATTGGGCACTACCAC-CTCAA-3′ and 5′-TTGAAGCATTTGGTACGCGGC-3′ and sequenced using an ABI PRISM 310 genetic analyzer (ABI).

Plasmids. A pBR322-based plasmid pAJ51 encoded a cysteine-less variant of His_6-SecY under the control of the *tac* promoter. It carried *lacI*^{q} as well, and was constructed from pCM66 (*22*) by changing its Cys329 and Cys385 codons in *secY* to GCG (Ala) by site-directed mutagenesis using the QuickChange kit (Stratagene) and appropriate mutagenic primers. Plasmid pAJ57, a derivative of pCM4 (*27*), was similar to pAJ51 but without the N-terminal tag. pHM462, a derivative of pHMC5A (*22*), also encoded the cysteine-less variant of SecY with His_6 and Myc bipartite tags attached to the C-terminus. A series of site-directed mutations were introduced further into pAJ51, pAJ57, and pHM462 to construct plasmids encoding single-cysteine SecY variants, which are summarized in Table 1.

A pACYC184-based plasmid pTYE100 encoded SecE and SecG under the control of the *lac* promoter (*22*). Site-directed mutations were introduced into its *secG* region to construct plasmids encoding mutant SecGs as summarized also in Table 1. Plasmid pSA13 (encoding only SecE) was constructed by deleting the *Avr*II−*Sal*I *secG* region from pTYE100.

Table 1: Plasmids Encoding Site-Directed SecY and SecG Variants

SecY plasmid	amino acid substitution in SecY[a]	domain affected
pAJC2	Ala2Cys	C1
pAJC19	Leu19Cys	C1
pAJC750[b]	Ala50Cys	P1
pAJC759[b]	Thr59Cys	P1
pAJC776[b]	Ser76Cys	P1
pAJC103	Ala103Cys	C2
pAJC111	Ser111Cys	C2
pAJC117	Ser117Cys	C2
pHM473[c]	Pro140Cys	P2
pHM486[c]	Asn141Cys	P2
pAJC155	Ala155Cys	P2
pAJC179	Thr179Cys	C3
pAJC7210[b]	Ala210Cys	P3
pAJC244	Ile244Cys	C4
pAJC249	Ala249Cys	C4
pAJC262	Ser262Cys	C4
pAJC263	Thr263Cys	C4
pAJC7298[b]	Thr298Cys	P4
pAJC7304[b]	Thr304Cys	P4
pAJC7307[b]	Ser307Cys	P4
pAJC342	Thr342Cys	C5
pAJC349	Ser349Cys	C5
pAJC371	Thr371Cys	C5
pAJC7394[b]	Ala394Cys	P5
pAJC422	Thr422Cys	C6
pAJC431	Ser431Cys	C6

SecG−SecE plasmid	amino acid substitution in SecG[d]	domain affected
pSA11	Lys26Arg	C left
pSA12	Ala28Cys	C left
pSA22	Ser39Cys	C central
pSA23	Ser48Cys	C right
pSA24	Lys77Arg	P

[a] All the *secY* mutations were present in addition to the Cys329Ala and Cys385Ala mutations. Unless otherwise noted, plasmids were derived from pAJ51. [b] These plasmids were derived from pAJ57. [c] These plasmids were derived from pHM462. [d] These *secG* mutations were introduced into pTYE100 carrying *secG* and *secE*.

Preparation of Membrane Fractions. Preparations of either the total membrane fraction or inverted inner membrane vesicles were used for cross-linking experiments. Cells

carrying an appropriate combination of SecY and SecEG plasmids were grown at 37 °C in L medium containing appropriate antibiotics, and the expression of SecYEG complexes was induced with 1 mM isopropyl thio-β-D-thiogalactoside for 1 h. Cells were then harvested and converted to spheroplasts as described previously (*28*), which were finally resuspended in 10% (w/v) sucrose containing 3 mM EDTA (pH 7.0), 1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride. Spheroplasts were disrupted with a French press or sonication at 4 °C. Total membrane materials containing outer and inner membranes were precipitated by ultracentrifugation and resuspended in 50 mM HEPES-KOH (pH 7.2) and 20% glycerol. Inverted membrane vesicles were obtained by subjecting the total membrane fraction to centrifugation via a 33 to 48% step gradient of sucrose containing 3 mM EDTA (pH 7.0) and 1 mM dithiothreitol at 4 °C and at 100000*g* for 16 h; materials at the sucrose interface were taken, diluted, pelleted down, resuspended in 50 mM HEPES-KOH (pH 7.2) and 20% glycerol, and stored in small portions at −80 °C.

Cross-Linking Experiments. The total membrane or inverted membrane vesicle fraction in 50 mM HEPES-KOH (pH 7.2) and 20% glycerol was incubated with AMAS[1] or MBS (purchased from Pierce), after addition of $^1/_{10}$ volume of a 10 mM stock solution of one of the reagents in dimethyl sulfoxide (final concentration of 1 mM). The incubation temperature and time are specified in each experiment, and the reaction was terminated by mixing with a 2-fold concentrated SDS−PAGE sample buffer containing β-mercaptoethanol. To confirm the thiol specificity of the cross-linking, free thiol groups were alkylated by treatment of the membrane preparations with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (final concentration of 1 mM) for 2 h on ice. Subsequently, membranes were collected by centrifugation, resuspended in 50 mM HEPES-KOH (pH 7.2) and 20% glycerol, and subjected to the cross-linking reaction.

Formation of disulfide bonds was facilitated by addition of $K_3[Fe(CN)_6]$ ($^1/_{25}$ volume of 250 mM solution in water; final concentration of 10 mM) and incubation on ice for 30 min in 50 mM HEPES-KOH (pH 7.2) and 20% glycerol. Trichloroacetic acid (final concentration of 5%) was added to samples, and protein precipitates were collected by centrifugation, washed with acetone, and dissolved in 1.5% SDS, 100 mM Tris-HCl (pH 9.0), 5 mM EDTA, and 15 mM iodoacetamide by agitating at room temperature for 30 min, followed by incubation at 37 °C for 10 min. Iodoacetamide (purchased from Nacalai Tesque) was included to modify any free thiols and thereby to prevent artificial disulfide bond formation *in vitro* (*29*).

In the experiments described above, using a cross-linking or an oxidizing agent, samples were subjected to SDS−PAGE, under reducing or nonreducing conditions. Subsequently, SecY and SecG, as well as their covalent complexes, were visualized by immunoblotting using antibodies against SecY and those against SecG. Antibodies and immunoblotting conditions were described previously (*30*). Anti-SecG serum was raised in rabbit with a synthetic peptide as an antigen, having the amino acid sequence from the periplasmic carboxy-terminal tail of SecG (*5*). For antigen competition experiments, anti-SecG serum (10 μL) was mixed with 0.5 mL of phosphate-buffered saline containing 200 μg of the antigen peptide, before its use in immunoblotting.

In Vivo Protein Export Assay. Cells were grown at 37 °C in M9 medium supplemented with 0.4% glycerol, 0.4% maltose, and 18 amino acids (each 20 μg/mL, except Met and Cys) until an early log phase, and then cooled to 20 °C. After 30 min, they were pulse-labeled with [^{35}S]methionine for 1 min. Maltose-binding protein was immunoprecipitated as described previously (*12*), and radioactivities in its precursor and mature forms were determined using a BAS1800 phosphorimager (Fuji Film).

Other Methods. The *in vitro* translocation assay and the SecA translocation ATPase assay were carried out as described previously (*31*).

RESULTS

Variants of SecY Having Single Cysteines in Cytoplasmic Domains and Their Thiol−Amine Bifunctional Cross-Linking with SecG. To characterize subunit interactions of the SecYEG complex at the residue level, we designed site-specific cross-linking experiments using thiol-reactive cross-linking agents. SecY has two cysteine residues, in TM8 and TM9, whereas SecE and SecG lack this amino acid. A cysteine-less SecY was constructed by replacing Cys329 and Cys385 with alanine. A series of single-cysteine variants of SecY were then constructed by Cys substitution for some selected residues in each cytoplasmic domain; altogether, 15 mutants were constructed (Table 1 and Figure 1). All of them, when expressed from plasmids, complemented the cold-sensitive growth defect of the *secY39* mutant as potently as the wild-type SecY. Thus, none of the cysteine substitutions compromised the SecY function.

For cross-linking experiments, each SecY variant was co-overexpressed with SecE and SecG. Cellular accumulation levels of different SecY variants were similar and did not significantly differ from that of the wild-type SecY expressed in the same scheme (see Figure 2, lanes 2−11 vs lane 1). Inverted membrane vesicles were then prepared from the SecYEG-overexpressing cells; the mutant membrane vesicles exhibited significantly increased levels of activity for mediating SecA-dependent translocation of ^{35}S-labeled proOmpA as compared with membrane vesicles of the basal SecY content (data not shown). These inverted membrane vesicle preparations or total membrane preparations were subjected to cross-linking experiments using AMAS having reactivities to a thiol group and a primary amine with a spacer arm of 4.4 Å. Samples were separated by SDS−PAGE, and materials reacting with antibodies against each Sec component were detected by immunoblotting.

A lower-mobility band was produced when membranes from mutants having a single cysteine at the C2 domain (Ser111Cys and Ser117Cys) and at the C3 domain (Thr179Cys) were treated with AMAS. This product was found to react with antibodies against SecY (Figure 2, lanes 4−6) as well as those against SecG (Figure 2, lanes 15−17). It disappeared when the membranes were pretreated with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid to modify free and accessible thiol groups (Figure 3A). No cross-linked product could be detected with wild-type SecY

[1] Abbreviations: AMAS, *N*-(α-maleimidoacetoxy)succinimide ester; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; PAGE, polyacrylamide gel electrophoresis.

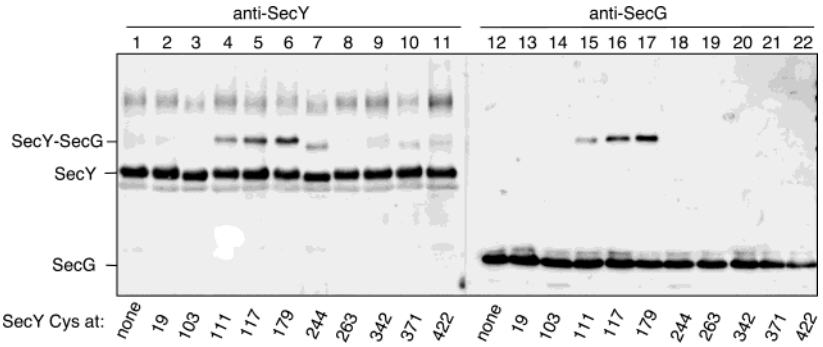

FIGURE 2: AMAS cross-linking of SecY and SecG. Strain AD202 carrying plasmid pTYE100 (encoding *secE* and *secG*) was transformed further with pAJ51 (Cys-less SecY) or one of its derivatives encoding a single-cysteine variant of SecY, as indicated by residue numbers at the bottom of each lane. The cloned gene products were induced, and total membrane fractions were prepared, which were subjected to cross-linking with AMAS (on ice for 2 h). Samples were analyzed by SDS−PAGE and anti-SecY (lanes 1−11) and anti-SecG (lanes 12−22) immunoblotting. Results were essentially unchanged when inverted membrane vesicles, instead of total membranes, were used. The SecY plasmids that were introduced were as follows: lanes 1 and 12, pAJ51; lanes 2 and 13, pAJC19 (Leu19Cys); lanes 3 and 14, pAJC103 (Ala103Cys); lanes 4 and 15, pAJC111 (Ser111Cys); lanes 5 and 16, pAJC117 (Ser117Cys); lanes 6 and 17, pAJC179 (Thr179Cys); lanes 7 and 18, pAJC244 (Ile244Cys); lanes 8 and 19, pAJC263 (Thr263Cys); lanes 9 and 20, pAJC342 (Thr342Cys); lanes 10 and 21, pAJC371 (Thr371Cys); and lanes 11 and 22, pAJC422 (Thr422Cys). In separate experiments, single-cysteine mutants at residues 2, 249, 349, and 429 gave negative results with respect to SecY−SecG cross-linking (data not shown).

(data not shown) or with the cysteine-less variant (Figure 2, lanes 1 and 12). Thus, the cross-linking was specifically generated through the introduced cysteine residues. The cross-linked product reacted with antibodies against neither SecE (except for the Ile244Cys mutant; see below) nor SecA (data not shown). Its detection with the SecG antibodies was abolished when an excess of antigen peptide was included in the immunodetection step (Figure 3B). Moreover, this cross-linked product was not produced when membranes were prepared from a strain lacking SecG (Figure 3C). Results of SecY immunoblotting revealed a product migrating slightly faster than the SecY−SecG complex for the SecY(Ile244Cys) variant (Figure 2, lane 7). This proved to represent a SecY−SecE cross-linkage (*32*).

The above results indicate that the cross-linking had occurred specifically between SecG and the three SecY cysteine residues that had been introduced into positions 111 and 117 in the C2 domain as well as position 179 in the C3 domain. Since the other reactive end of AMAS will react with a primary amine, the SecG residue that engaged in the cross-linking should have been either the amino terminus or one of its lysine moieties. In the "resting" conformation of SecG, the amino terminus and a few lysines near the carboxyl terminus face the periplasm (*5*), whereas Lys26 is the sole lysine in the cytoplasmic domain. We mutated Lys26 to arginine (Figure 1). The Lys26Arg variant of SecG was functional as it retained the complementation activity against a cold-sensitive Δ*secG* strain (data not shown). A single-cysteine SecY variant, either Ser111Cys or Thr179Cys, was coexpressed with SecE and SecG(Lys26Arg) in the Δ*secG* strain. AMAS cross-linking experiments using membranes prepared from either strain did not yield the SecY−SecG covalent complex (Figure 4). Thus, Lys26 in SecG in the cytoplasmic loop near the first membrane-spanning segment was responsible for the AMAS-mediated cross-linking observed with the cysteine residues in the C2−C3 region of SecY.

The Cys residues introduced into SecY were all in cytoplasmic regions, and many of them were presumably two to five residues away from the membrane surface, whereas Lys26 of SecG is three residues distant. Other mutations were farther from the nearby transmembrane segments, at least in the primary sequence. Taking these features of the mutation sites into consideration, we may conclude that the cysteine moieties at residues 111, 117, and 179 were specifically cross-linked with Lys26 of SecG by AMAS. It could still be argued that the cross-linkages had been due to some artificial elements in the experiments, in which SecY, SecE, and SecG proteins were overproduced. We thus carried out the same cross-linking experiments using membranes prepared from cells in which only a mutant SecY was expressed from a plasmid. Because cellular accumulation of SecY depends on SecE (*33*, *34*), the total amount of SecY (chromosomally encoded $SecY^+$ and the variant SecY from plasmid) should have been similar to the SecY content of the wild-type cells. Specific cross-linking between SecY and SecG was observed under these conditions, albeit at lower efficiencies, for the SecY single-cysteine mutants that gave positive cross-linking results in the experiments whose results are shown in Figure 2 (data not shown).

Disulfide Cross-Linking of SecY and SecG. The above results suggest that each of the SecY residues (positions 111, 117, and 179) can be in the proximity of Lys26 of SecG, probably as close as the spacer arm length (4.4 Å) of AMAS. To substantiate this conclusion, we introduced cysteine into position 28 of SecG, resulting in SecG(Ala28Cys) (Table 1 and Figure 1). One of the single-cysteine SecY variants, SecE, and SecG(Ala28Cys) were co-overexpressd in the Δ*secG* strain, for preparation of membranes, which were subjected to oxidation by $K_3[Fe(CN)_6]$. Samples were treated with trichloroacetic acid, and free SH groups in proteins were blocked with iodoacetamide modification, followed by SDS−PAGE under reducing (data not shown) and nonreducing (Figure 5) conditions. Under nonreducing conditions, a band with a mobility similar to that of the SecY−SecG cross-linked product was detected prominently for the SecY variants with cysteines at position 111, 117, or 179 (Figure 5, lanes 5−7). This band was undetectable or its magnitude greatly diminished for the other SecY variants that were examined. It reacted with both antibodies against SecY and SecG (data not shown for SecG immunoblotting). Its disappearance after reduction (data not shown) indicates that

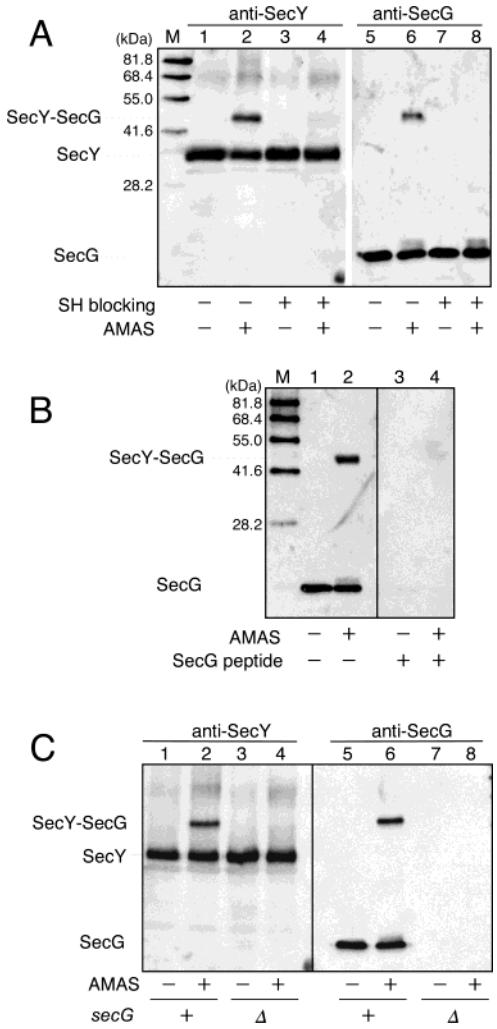

FIGURE 3: Specificity of cross-linking. (A) Thiol specificity. Inverted membrane vesicles were prepared from cells of strain AD202 carrying pTYE100 (SecE and SecG) and pAJC179 [SecY-(Thr179Cys)] and treated with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, and 6) 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid. Membranes were then subjected to cross-linking with AMAS. Lane M contains molecular mass markers. (B) Immunological specificity. Total membranes were prepared from cells of strain AD202 carrying pTYE100 and pAJC179 and subjected to cross-linking with AMAS. Anti-SecG immunoblotting was carried out using antiserum without (lanes 1 and 2) or with (lanes 3 and 4) added antigen peptide. (C) Genetic evidence. Total membrane fractions were prepared either from cells of strain AD202 carrying pTYE100 and pAJC179 (lanes 1, 2, 5, and 6) or from cells of strain SA101 (Δ*secG*) carrying pSA13 (SecE) and pAJC179 (lanes 3, 4, 7, and 8) and subjected to cross-linking with AMAS. SDS−PAGE patterns were visualized by anti-SecY (lanes 1−4) and anti-SecG (lanes 5−8) immunoblotting.

it represented a disulfide bond-linked product between SecY and SecG. These results, taken together, indicate that residues 26−28 of SecG are adjacent to the C2−C3 region of SecY. A Ser39Cys SecG variant very weakly produced the cross-linked product in combination with several SecY mutants (data not shown), but the significance of these weak cross-linkings as well as that of the faint SecY−SecG band observed in some combinations presented in Figure 5 requires further analyses. SecG(Ser48Cys) did not produce any detectable disulfide linkage with SecY (data not shown). These results show that the N-terminal part of the SecG cytoplasmic loop is close to the C2−C3 region of SecY.

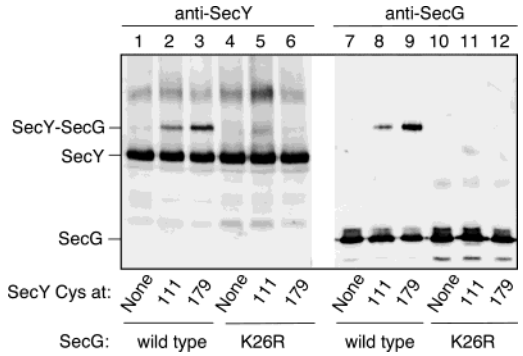

FIGURE 4: Lys26 of SecG in the cytoplasmic loop is the cross-linking partner. pTYE100 (SecG$^+$ and SecE$^+$) was introduced into strain AD202 (lanes 1−3 and 7−9), whereas pSA11 [SecG-(Lys26Arg) and SecE$^+$] was introduced into SA101 (AD202, Δ*secG*; lanes 4−6 and 10−12). The resulting plasmid-bearing cells were transformed further with pAJ51 (cysteine-less SecY; lanes 1, 4, 7, and 10), pAJC111 (Ser111Cys; lanes 2, 5, 8, and 11), or pAJC179 (Thr179Cys; lanes 3, 6, 9, and 12). Total membrane fractions were subjected to the cross-linking reaction with AMAS, followed by visualization with anti-SecY (lanes 1−6) and anti-SecG (lanes 7−12) as described in the legend of Figure 2.

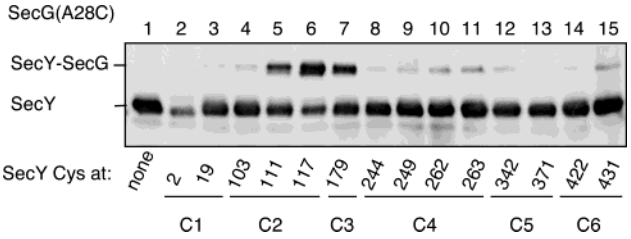

FIGURE 5: Disulfide cross-linking of SecY and SecG. Combinations of two plasmids, pSA12 [SecG(Ala28Cys) and SecE$^+$] and another plasmid encoding a SecY single-cysteine variant indicated by the position of Cys at the bottom of each lane, were introduced into strain SA101. These proteins were induced, and total membrane fractions were prepared, which were subjected to oxidation with $K_3[Fe(CN)_6]$ on ice for 1 h. Samples were then analyzed by SDS−PAGE followed by anti-SecY immunoblotting.

Cross-Linking on the Periplasmic Side. The proximity of the cytoplasmic loop of SecG to the C2−C3 region of SecY raises a question of whether the corresponding periplasmic region, P2, of SecY is adjacent to SecG. Thus, 11 single-cysteine mutants with the substitutions in different periplasmic regions of SecY were constructed (Table 1 and Figure 1). Cross-linking experiments were carried out using spheroplast samples. A cross-linked product was observed for a SecY(Ala155Cys) variant (Figure 6), but not for the other periplasmic variants (data not shown). Cross-linkers having the same reactivity but different arm lengths, AMAS (4.4 Å; Figure 6, lanes 2, 4, 6, 9, 11, and 13) and MBS (9.9 Å; Figure 6, lanes 3, 5, 7, 10, 12, and 14), were used. Cross-linking was evident at 37 °C with MBS (Figure 6, lane 5) but observed only weakly at 0 °C (Figure 6, lanes 1−3). Interestingly, when samples were incubated at 37 °C in the presence of NaN_3, significantly enhanced cross-linking was observed with AMAS (lane 6). The cross-linked products involving the cysteine residue that was introduced into position 155 were recognized by anti-SecY (Figure 6A) as well as by anti-SecG (Figure 6B) antibodies. This cross-linked product was not detected when the SecY variant was expressed in the Δ*secG* strain (Figure 7, lanes 12, 15, 18, and 21). Since it was detected even with the SecG(Lys26Arg) mutant (Figure 7, lanes 11, 14, 17, and 20), the reactive

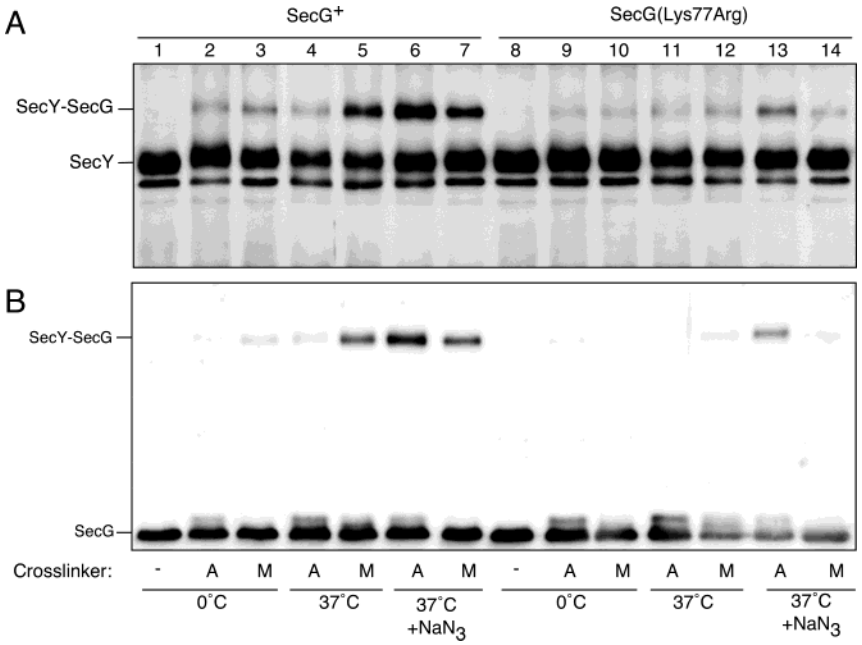

FIGURE 6: Cross-linking properties of a SecY variant having a single cysteine in the P2 domain. Strain SA101 (Δ*secG*) was transformed with two combinations of plasmids: lanes 1−7, pAJC155 [SecY(Ala155Cys)] and pTYE100 (SecG and SecE); and lanes 8−14, pAJC155 and pSA24 [SecG(Lys77Arg) and SecE]. Cells were grown at 37 °C, and spheroplasts were prepared, which were subjected to cross-linking with AMAS (lanes 2, 4, 6, 9, 11, and 13) or MBS (lanes 3, 5, 7, 10, 12, and 14). Samples for lanes 1 and 8 received only the solvent, dimethyl sulfoxide. Cross-linking reactions in 50 mM Hepes-KOH (pH 7.2) and 20% sucrose were carried out at either 0 °C for 1 h (lanes 1−3 and 8−10), 37 °C for 1 h (lanes 4, 5, 11, and 12), or 37 °C for 1 h with 30 mM NaN_3 added 5 min before the addition of a cross-linker (lanes 6, 7, 13, and 14). Samples were analyzed by SDS−PAGE and immunoblotting using either antibodies against SecY (A) or antibodies against SecG (B).

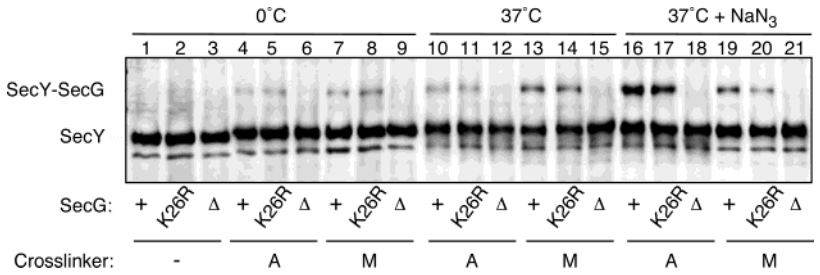

FIGURE 7: Periplasmic cross-linking depends on SecG but not on its cytoplasmic Lys26 residue. Strain SA101 (Δ*secG*) was transformed with three combinations of plasmids: lanes 1, 4, 7, 10, 13, 16, and 19, pTYE100 (SecG and SecE) and pAJC155 [SecY(Ala155Cys)]; lanes 2, 5, 8, 11, 14, 17 and 20, pSA11 [SecG(Lys26Arg)] and pAJC155; and lanes 3, 6, 9, 12, 15, 18 and 21, pSA13 (SecE) and pAJC155. Cells were grown at 37 °C, and spheroplasts were prepared, which were subjected to cross-linking with AMAS (lanes 4−6, 10−12, and 16−18) or MBS (lanes 7−9, 13−15, and 19−21). Samples for lanes 1−3 received only dimethyl sulfoxide. Cross-linking reactions in 50 mM Hepes-KOH (pH 7.2) and 20% sucrose were carried out at either 0 °C for 1 h (lanes 1−9), 37 °C for 1 h (lanes 10−15), or 37 °C for 1 h with 30 mM NaN_3 added 5 min before the addition of a cross-linker (lanes 16−21). Samples were subjected to SDS−PAGE and anti-SecY immunoblotting.

moiety in SecG should have been either the N-terminal amine or one of the four lysine residues in the periplasmic tail (Figure 1). Indeed, the Lys77Arg alteration in SecG (Figure 1) greatly reduced the cross-linking efficiency (Figure 6, lanes 11−14). These results suggest that the periplasmic tail of SecG is close to the P2 region of SecY on the periplasmic side of the membrane. The remaining weak cross-linking observed even with SecG(Lys77Arg) (Figure 6, lane 13) might have involved one or more of the three other lysine residues located in the carboxy-terminal region of this protein.

Genetic Suppression Studies. A cold-sensitive *secY* mutation, *secY104*, causes a Gly175Asp alteration in the boundary of the TM4 and C3 domains (*35*). We isolated *secG* mutations that suppressed the cold sensitivity of the *secY104* mutant (Experimental Procedures). We were thus able to identify five amino acid substitutions in the transmembrane domains of SecG (summarized in Table 2). Suppression by these *secG* mutations was specific against *secY104*; none of them suppressed other cold-sensitive *secY* mutants that were examined [*secY205*, *secY39*, *secY115*, *secY125*, and *secY129* (*35*)]. These suppressor *secG* mutations, when present in the *secY*$^+$ strain, did not confer any significant defect in growth or protein export (data not shown).

The suppression effects of the *secG* mutations were confirmed at the level of protein export. In the pulse labeling experiments (labeling for 1 min at 20 °C) whose results are given in Table 2, the *secY104* single mutant gave only a 24% mature form of maltose-binding protein. The values for the suppressed strains (*secY104-secG* double mutants) were close to the wild-type level (86% mature form). These results show that the five suppressor mutations in *secG* effectively compensate for the *secY104* protein export defect. Incidentally, the SecY104 alteration is within the region that our

Table 2: Mutations in *secG* that Suppress the *secY104* Mutational Defect

secG allele	nucleotide substitution[a]	SecG amino acid substitution	MBP export in the presence of *secY104* (% mature form)[b]
secG$^+$			24
secG12	T187C	Phe63Leu	70
secG21	T50A	Val17Asp	72
secG30	T29A	Leu10His	80
secG48	T29G	Leu10Arg	81
secG55	A209G	Ser67Gly	63

[a] The numbering starts from the first base of the *secG* initiation codon. [b] Mutations in *secG* that had been identified as suppressors against the *secY104* mutation were introduced into strain GN94 (*secY104*) by joint P1 transduction with *zha203*::Tn*10*. Resulting strains were grown at 37 °C, cooled to 20 °C, and pulse-labeled with [^{35}S]methionine for 1 min. After immunoprecipitation and SDS−PAGE, radioactivities associated with precursor and mature forms of maltose-binding protein (MBP) were quantified. Values indicate the proportions of the mature form. Wild-type (*secY*$^+$ *secG*$^+$) cells exhibited an 86% mature form under the conditions that were examined.

cross-linking analyses identified as a nearest neighbor of SecG.

DISCUSSION

SecY interacts independently with each of the SecE and SecG subunits of protein translocase. This was shown previously by His_6 tagging and pull-down experiments using detergent extracts of membranes (*16*). However, there had been no biochemical or genetic information about the SecY−SecG interaction domains, although van der Sluis et al. (*36*) reported on SecY−SecG cross-linking results after completion of this work. In this work, we employed site-specific cross-linking approaches to addressing this question. Specific cross-linkage was observed with AMAS between the thiol moiety of one of the cysteine residues of SecY variants (Ser111Cys, Ser117Cys, and Thr179Cys) and the ϵ-amino moiety of Lys26 in the cytoplasmic domain near the first transmembrane domain of SecG. These cysteine residues also formed disulfide bonds with a SecG variant, Ala28Cys. Thus, the α-carbon of residues 111, 117, and 179 of SecY can be as close as 5−6 Å to that of the residue 28 of SecG (*37*). Our results also suggest that, on the periplasmic side of the membrane, the P2 region of SecY is close to the C-terminal region of SecG. It is thus suggested that the C2−P2−C3 segment of SecY faces SecG.

Nishiyama et al. (*5*) showed that SecG has unusual mobility within the membrane such that its orientation relative to the membrane is reversed under the conditions of active translocation, in which the SecA ATPase undergoes insertion−deinsertion cycles. The topology inversion of SecG appears to coincide with the insertion phase of the cycle. Some of our cross-linking results are consistent with the mobile nature of SecG. We observed that cross-linking patterns of SecY and SecG on the periplasmic side were affected by NaN_3 that should act to stabilize the "inserted" state of SecA (*38*). The arm length-dependent change we observed in the cross-linking results could be related to the mobile nature of SecG. Our cross-linking experiments targeted to the central part of the SecG cytosolic loop detected very weak cross-linkings with a wide spectrum of SecY residues, especially those in C4−C6 regions (Figure 5 and data not shown). This might also have resulted from the mobile nature of SecG.

The physical proximity of the C2−TM3−P2−TM4−C3 region to SecG is consistent with the genetic evidence we obtained using a *secY104* mutation (Gly175Asp) affecting this region. This mutant shows a severe protein export defect at 20 °C (*35*). We have isolated several *secG* mutations that suppress the *secY104* cold sensitivity. Genetic suppression by these *secG* mutations exhibited allele specificity in that they did not suppress other *secY* mutational defects that were examined. Although the localizations of the suppressor *secG* alterations themselves were not unique, results of our suppression studies are consistent with the notion that the TM4−C3 region of SecY interacts functionally with the transmembrane domains of SecG. Although we have not sorted out the translocation step that is affected by the *secY104* mutation, the occurrence of the suppressor alterations within the SecG TM segments is intriguing in view of the apparent mobility of SecG (*5*).

It is also interesting to note that TM3 and TM4 of SecY are less hydrophobic than other TM regions in SecY (*3*). The nature of the TM3−TM4 regions may be suitable for supporting the conformational change of SecG. The SecG-interacting domain identified in this study may constitute a dynamic platform for the actions of the SecA−SecG components of protein translocase. Key questions are where this interface is located in the three-dimensional structure of SecYEG (*9*) and how it is transformed while the translocase is actually mediating translocation reactions.

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