J Biochem. 128, 129–137 (2000)

Two SecG Molecules Present in a Single Protein Translocation Machinery Are Functional Even after Crosslinking[1]

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Received April 6, 2000; accepted May 8, 2000

SecG, a membrane component of the protein translocation apparatus of *Escherichia coli*, undergoes membrane topology inversion, which is coupled to the membrane insertion and deinsertion cycle of SecA. Eighteen SecG derivatives possessing a single cysteine residue at various positions were constructed and expressed in a *secG* null mutant. All the SecG-Cys derivatives retained the SecG function, and stimulated protein translocation both *in vivo* and *in vitro*. Inverted membrane vesicles containing a SecG-Cys derivative were labeled with a membrane-permeable or -impermeable sulfhydryl reagent before or after solubilization with a detergent. The accessibility of these reagents to the cysteine residue of each derivative determined the topological arrangement of SecG in the membrane. Derivatives having the cysteine residue in the periplasmic region each existed as a homodimer crosslinked through disulfide bonds, indicating that two SecG molecules closely co-exist in a single translocation machinery. The crosslinking did not abolish the SecG function and the crosslinked SecG dimer underwent topology inversion upon protein translocation.

Key words: cysteine mutagenesis, *E. coli*, protein translocation, SecG, sulfhydryl reagent.

The translocation of presecretory proteins across the *Escherichia coli* cytoplasmic membrane is mediated by a SecA/D/E/F/G/Y complex with the assistance of a molecular chaperone, SecB (*1–3*). This protein translocation is thought to be driven by the membrane insertion-deinsertion cycle of SecA, which is coupled to ATP binding and hydrolysis (*4*, *5*). A proton motive force was recently found to stimulate SecA deinsertion, thereby accelerating the SecA cycle (*6*). SecG, a small membrane protein, undergoes a cycle of topology inversion that is coupled to and facilitates the SecA cycle (*7*, *8*). The efficiency of protein translocation therefore becomes maximum in the presence of both SecG and the proton motive force (*9–11*). A *secG* null mutant exhibits a cold-sensitive growth defect in a strain-specific manner (*12*), whereas proOmpA translocation at low temperature was defective in all *secG* null mutants examined (*13*).

Based on the alkaline phosphatase (PhoA) activities of a series of SecG-PhoA fusion proteins, it has been proposed that the C-terminal hydrophilic region of SecG is exposed to the periplasm whereas the central weakly hydrophobic region, which is flanked by two highly hydrophobic sequences, faces the cytoplasm (*7*). Furthermore, the cytoplasmic region, but not the C-terminal region, was shown to be accessible to external proteinase K in inverted membrane vesicles (IMVs). Although no direct evidence is available for the periplasmic exposure of the N-terminus, the introduction of a positive charge at the N-terminus rendered the C-terminus accessible to external proteinase K in IMVs, indicating that the topology of SecG is inverted in accordance with the positive inside rule (*14*). The membrane topology of SecG was further confirmed by identifying proteinase K-sensitive sites in spheroplasts (*7*). Taken together, these results indicate that SecG spans the membrane twice, and that both its N- and C-termini are exposed to the periplasm, leaving the weakly hydrophobic region on the cytoplasmic side of the membrane (*7*). Strikingly, however, the membrane topology of SecG observed in the absence of protein translocation was found to be inverted upon the initiation and subsequent blockage of protein translocation (*7*). Since membrane proteins are generally believed to exist in a fixed topology, the detailed properties of SecG are of great interest.

Cysteine scanning mutagenesis has been applied to several membrane proteins to reveal their topological arrangements in membranes in detail (*15–17*). The substitution of a pre-existing residue with Cys in most cases does not abolish functions, which enables detailed analysis of active proteins. The introduction of Cys also enables SH-specific labeling of proteins. Moreover, the disulfide bonds formed between pairs of Cys residues indicate that these residues are close to each other. Cysteine scanning mutagenesis was recently used to reveal the site of contact between SecY and SecE (*18*, *19*). It was also found that a SecE derivative possessing a cysteine residue in the third transmembrane region forms a homodimer with crosslinking through disul-

[1] This work was supported by grants to H.T. from CREST of the Japan Science and Technology Corporation, and the Ministry of Education, Science, Sports and Culture of Japan, and a JSPS Research Fellowship for Young Scientists to S.N

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Abbreviations: IMVs, inverted membrane vesicles; AMP-PNP, β,γ-imido adenosine 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide; octylglucoside, β-D-octylglucopyranoside; DTT, dithiothreitol; AMS, 4-acetamido-4′-maleimidylstilbene-2-2′-disulfonic acid; TCA, trichloroacetic acid.

fide bonds (*19*).

Electron microscopic data indicate that the protein-conducting channels of the prokaryotic and eukaryotic protein translocation machineries comprise multiunits of a Sec complex (*20*, *21*). Therefore, a single translocation machinery is expected to contain multicopies of each Sec component.

To clarify the membrane structure of SecG in detail, eighteen SecG-Cys derivatives were constructed. We report here that a single translocation machinery contains at least two molecules of SecG that are close to each other.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]NEM (2,220 GBq/mmol) and non-radioactive NEM were purchased from Dupont NEN and Sigma, respectively. Tran^{35}S-label (37 TBq/mmol as [^{35}S] Met) was obtained from ICN. Octylglucoside was from Dojindo Laboratories. Digitonin was from Nacalai Tesque AMS was from Molecular Probes. ATP and AMP-PNP were from Boehringer Mannheim. Proteinase K was from Merck. Restriction enzymes were obtained from Takara Shuzo and Toyobo. The site-directed mutagenesis system was from Amersham. Oligonucleotides were synthesized using a Beckman Oligo 1000 DNA synthesizer. Anti-SecG antibodies were raised in rabbits against a synthetic peptide corresponding to the C-terminal 16 amino acid residues of SecG (*9*). Anti-SecE (*22*), -SecD (*23*), and -OmpA (*24*) antibodies were prepared as described.

Bacterial Strains—*E. coli* K003 (HfrH *pnp-13 tyr met* RNaseI$^-$ Lpp$^-$ *ΔuncB-C::Tn10*) (*25*), FS1576 (C600 *recD-1009*) (*26*, *27*), KN370 (FS1576 *ΔsecG::kan*) (*28*), and KN553 (K003 *ΔsecG::kan*) (*11*) were used. The latter strain was used to prepare IMVs according to the reported method (*29*). IMVs were kept frozen in 50 mM potassium phosphate (pH 7.5), 10% glycerol, and 8.5% sucrose with or without 10 mM DTT.

Construction of SecG-Cys Derivatives—A numeral followed by C in the names of plasmids and phage clones encoding SecG-Cys derivatives represents the position of the Cys residue in each derivative, except in the case of 3.5C, in which the Cys is between positions 3 and 4. To construct pCG111C, a synthetic linker comprising a pair of primers, 111C+ and 111C– (Table I), which encodes the 108–110 region, Cys at 111 and the termination codon, was inserted into the *Eco*RV–*Kpn*I sites of pCG6 (*7*). The *Eco*RV and *Kpn*I sites are located in the 107th codon and immediately downstream of *secG*, respectively. Other SecG-Cys derivatives were constructed by means of site-directed mutagenesis using a Sculptor *in vitro* mutagenesis system (Amersham) and the primers listed in Table I. To construct pMG3.5C and pMG4C, the specified primers were used to mutagenize pMG5E, a derivative of M13mp18, encoding Glu3Arg-SecG and possessing a unique *Sac*I site around the N-terminus of SecG (*7*). The mutagenesis deleted both the Glu3Arg mutation and the *Sac*I site. To construct pMG8C, pMG18C, pMG33C, pMG39C, pMG45C, pMG48C, pMG57C, pMG60C, pMG67C, pMG71C, pMG75C, and pMG88C, the wild type *secG* gene on pMG5 (*7*) was mutagenized with the respective primers. Each mutation created the new restriction site indicated in Table I. Construction of pMG13C from pMG5 resulted in loss of the *Mun*I site. To construct pMG28C and pMG82C, pMG7, and pMG9 encoding truncated SecG derivatives (*7*) were mutagenized with the specified primers, respectively. Upon the mutation, pMG28C and pMG82C lost the *Eco*RV–*Nae*I and *Ssp*I sites, respectively. A ~350 bp *Bam*HI–*Eco*RV fragment of pMG3.5C through pMG88C was cloned into the same sites of pCG6 to construct pCG3.5C through pCG88C, respectively. The mutations in these constructs were confirmed by sequencing.

TABLE I. **Oligonucleotides used to construct 18 SecG-Cys derivatives.**

SecG-Cys derivative	Sequence* (5'–>3')
3.5C	TTTATGTATGAATGCGCTCTTTTAG
4C	TATGTATGAATGCCTTTTAGTAG
8C	GTATG**AAGCTT**TGTTAGTATGCTTCCTTATTG (*Hin*dIII)
13C	CCTTATTGTGTGCATTGGCCTTG
18C	ATTGTGG**CCA**TTGGCT**TGG**TTTGTCTGATC (*Bst*XI)
28C	CTGCAGCAAGGTAAAGGCTGCGATATGGGAGCC
33C	ATATGGGA**GCATGC**TTCGGAGCA (*Sph*I)
39C	GAGCAGGC**GCATGC**GCTACGCTG (*Sph*I)
45C	CGCTGTTTGG**CTGCAG**TGGTTCTG (*Pst*I)
48C	GTTTGGTTCA**TCCGGA**TGCGGTAACTTCA (*Acc*III)
57C	CTTCATG**ACGCGT**ATGACGTGCCTGGCAAC (*Mlu*I)
60C	CGCATGAC**AGCGCT**GCTGTGCACGTTATTCTTC (*Aor*51HI)
67C	TCATCATCTGTCT**GGTCC**TGGGTAAC (*Eco*47I)
71C	CTGGTGCTGTGC**AATATT**AATAGCAAC (*Ssp*I)
75C	TGGGTAAC**ATTAAT**TGCAACAAAAC (*Vsp*I)
82C	CAAAACCAATAAAGGTTGCGAATGGGA
88C	GGGAAAATCTG**TGCGCA**CCGGCGAAAACC (*Fsp*I)
111C (+)	ATCCCGAACTGCTAAAAAGGTAC
(-)	CTTTTTAGCAGTTCGGGAT

*The codons for Cys are indicated by bold letters. New restriction sites created for the specified restriction enzymes, indicated in parenthesis, are underlined.

A ~380 bp *Bam*HI–*Bgl*II fragment of pCG3.5C through pCG111C carrying the gene for a SecG-Cys derivative was cloned into the *Bgl*II site located downstream of the *ara* regulon of pKQ2 (*28*) to construct pAG3.5C through pAG111C.

Translocation of proOmpF-Lpp—[^{35}S]proOmpF-Lpp, a chimeric model secretory protein containing the signal peptide and the N-terminal 11 amino acid residues of OmpF, and the C-terminal 51 amino acid residues of the major outer membrane lipoprotein (Lpp), was synthesized *in vitro* as described (*25*). The reaction mixture (25 μl), comprising 0.2 mg/ml IMVs, that had been kept in the absence of DTT, 1 mM ATP, 1 mM $MgSO_4$, [^{35}S]proOmpF-Lpp (4.5–9 × 10^7 cpm/ml), and 50 mM potassium phosphate (pH 7.5), was incubated at 37°C for 10 min and then treated on ice with 0.8 mg/ml proteinase K to terminate the reaction. After 30 min digestion, OmpF-Lpp was precipitated with 10% TCA, successively washed with acetone and ether, and then analyzed by SDS-PAGE and fluorography. The translocation activity was determined by densitometric quantification of OmpF-Lpp with an ATTO densitograph and expressed as a percentage, taking the activity of wild type SecG as 100%.

Labeling of SecG-Cys Derivatives with [^{3}H] NEM—IMVs (20 μg protein, kept in the absence of DTT) in 15 μl of potassium phosphate (pH 7.5) were preincubated at 30°C for 5 min. After the addition of 5 μl of 20 μM [^{3}H]NEM, the

mixture was incubated for 10 min. To terminate the labeling, 5 μl of 50 mM non-radioactive NEM was added and the incubation was continued for 5 min. IMVs were then solubilized by the addition of 1 ml of a solution comprising 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2% Triton X-100, 1 mM EDTA, and 1 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride. Supernatants obtained on centrifugation at 18,000 ×*g* for 30 min were mixed with 1 μl of the anti-SecG antibody, incubated for 2 h at room temperature, and then treated with 40 μl of 10% IgG sorb for 20 min on ice. Pellets were obtained by brief centrifugation and washed twice with 50 mM Tris-HCl (pH 7 5) containing 500 mM NaCl and 0.05% SDS. Each washed pellet was dissolved in Laemmli sample buffer and then analyzed by SDS-PAGE and fluorography.

Labeling of SecG-Cys Derivatives with AMS—AMS labeling was examined with IMVs, which had been kept frozen at 10 mg/ml in the presence of 10 mM DTT. The reaction mixture containing 0.1 mg/ml IMVs and 5 mM AMS was incubated on ice for 20 min. Where indicated, the labeling was carried out in the presence of 2.5% octylglucoside on ice or at 37°C. The labeling was terminated by the addition of the Laemmli sample buffer (*30*). Aliquots of the reaction mixture containing 0.5 μg protein, or 1 μg protein for 111C, were analyzed by SDS-PAGE and immunoblotting with the anti-SecG antibody. The amounts of SecG on the immunoblots were determined with an ATTO densitograph. The amounts of SecG-Cys derivatives labeled with AMS were expressed as percentages, taking the total amounts of derivatives as 100%.

Detection of SecG-Cys Oligomers Formed In Vivo—SecG-Cys was expressed in KN370 by the addition of arabinose. When the OD at 660 nm reached 1.2, cells were harvested and treated with 10 mM NEM in 50 mM potassium phosphate (pH 7.5) for 30 min on ice. Cellular proteins were precipitated with TCA and then analyzed by SDS-PAGE and immunoblotting with the anti-SecG antibody.

Membrane Topology Inversion of the 111C Derivative—The topology inversion assay was carried out as described previously (*7*). Briefly, the reaction mixture (370 μl) comprised 0.1 mg/ml IMVs containing 111C, 60 μg/ml SecA (*31*), 50 μg/ml SecB (*32*), 25 μg/ml proOmpA (*24*), 1 mM ATP, an ATP generating system comprising 2.5 mM creatine phosphate and 5 μg/ml creatine kinase, and 1 mM $MgSO_4$ in 50 mM potassium phosphate (pH 7.5). The mixture was incubated at 37°C for 10 min. Where specified, AMP-PNP and $MgSO_4$, each at 20 mM, were added at 5 min. Reactions were terminated by chilling the mixtures on ice for 2 min, and then aliquots (25 μl) of the mixtures were treated with 25 μl of proteinase K at the indicated concentration on ice for 30 min. To prevent the disulfide bond formation of 111C or its fragments, the proteinase K–treatment was performed in the presence of 1 mM NEM. After TCA treatment, the precipitate containing 1 μg IMVs was analyzed by SDS-PAGE and immunoblotting with the anti-SecG antibody. IMVs (1 μg protein) not treated with proteinase K were also analyzed.

SDS-PAGE—Two gel systems were used; one (L-gel) was that of Laemmli (*30*) with a gel composed of 15% acrylamide and 0.4% *N,N'*-methylenebisacrylamide, and the other (HI-gel) that of Hussain *et al.* (*33*) with a gel composed of 12.5% acrylamide and 0.27% *N,N'*-methylenebisacrylamide.

RESULTS

Construction, Expression and Functional Analysis of SecG-Cys Mutants—SecG comprises 110 amino acid residues and has no Cys. When analyzed by the reported method (*34*, www.enzim.hu/hmmtop/index.html), SecG was predicted to possess two membrane spanning regions, Ala4–Leu22 and Phe51–Gly71. The prediction accuracy of this method was reported to be higher than that of other prediction methods. This method also predicted that both the N- and C-termini of SecG are oriented toward the cytoplasm, causing periplasmic exposure of the weakly hydrophobic region flanked by two membrane spanning segments. In contrast, biochemical data revealed that the C-terminus and weakly hydrophobic region are exposed to the periplasm and cytoplasm, respectively (*7*). The incorrect prediction of the SecG topology seems to indicate that the topology determinant of SecG is not strong. This property may be important for the topology inversion of SecG.

To examine the membrane topology of SecG in detail with special regard to its function, eighteen SecG derivatives possessing a single Cys at various positions were constructed by either substitution with or addition of Cys (Fig. 1). SecG-3.5C has an additional Cys between positions 3 and 4, and SecG-111C was constructed by the addition of Cys to the C-terminus Residues at various positions were replaced by Cys to construct SecG-4C to SecG-88C. The genes encoding SecG-Cys derivatives were placed under the control of the arabinose promoter on a plasmid and then expressed in the *ΔsecG::kan* mutant by the addition of 0.2% arabinose. The cold-sensitive growth defect of KN370 (*ΔsecG::kan*) was suppressed by the expression of all the SecG-Cys derivatives (data not shown), indicating that these derivatives retain the SecG function. Suppression of the cold sensitive phenotype by 111C was weaker than that by the other derivatives. Immunoblotting with anti-SecG antibodies revealed that the levels of all derivatives except

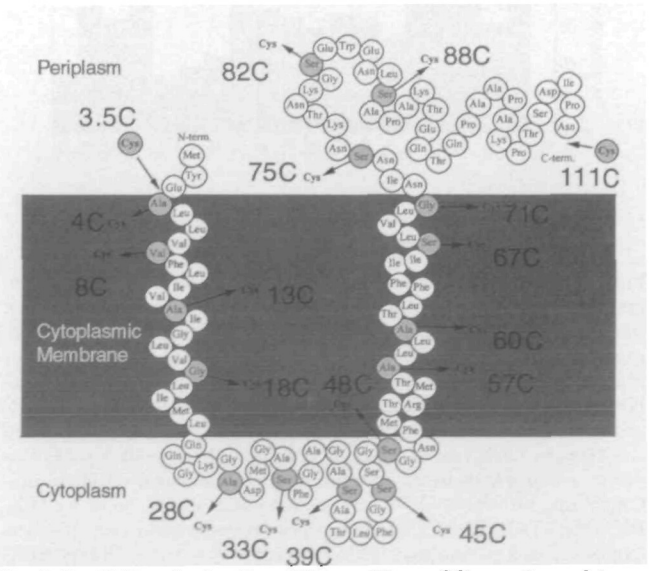

Fig 1 **SecG-Cys derivatives.** The positions of Cys in the eighteen SecG-Cys derivatives are indicated. Based on the predicted transmembrane sequences and biochemically determined membrane sidedness of the central weakly hydrophobic region and C-terminal region, SecG is topologically arranged in the membrane.

111C were similar to that of the wild type SecG encoded by pAG5 (Fig. 2A). The 9 kDa band migrating faster than each intact molecule (12 kDa) represents the C-terminal proteolyzed fragment (*7*).

Translocation of proOmpF-Lpp in the absence of the proton motive force absolutely requires SecG (Fig. 2B) and can be used for evaluation of the SecG function *in vitro*, although the reason for this SecG requirement is not fully understood. The translocation activity of IMVs prepared from KN553 (*Δunc::*Tn*10 ΔsecG::kan*) expressing one of the SecG-Cys derivatives varied to some extent depending on the species of derivative. However, proOmpF-Lpp was translocated in the absence of the proton motive force when SecG or its Cys derivatives were expressed (Fig. 2B). Taken together, these results indicate that all SecG-Cys derivatives are functional both *in vivo* and *in vitro*.

Labeling of SecG-Cys Derivatives with NEM—Based on the transmembrane sequence prediction and biochemically determined membrane topology (*7*), the Cys residues in 28C to 48C and 75C to 111C were predicted to be exposed to the outside and inside of IMVs, respectively. A membrane-permeable SH reagent, NEM, was therefore expected to label Cys residues in these derivatives. The Cys residues of 28C to 48C were intensely labeled with [^{3}H]NEM (Fig. 2C). In contrast, among the derivatives possessing Cys in the putative periplasmic region (75C to 88C), only 75C was labeled with NEM. The derivatives possessing Cys in the proposed transmembrane region (4C to 18C and 57C to 71C) or near the membrane surface (3.5C) were not labeled with NEM.

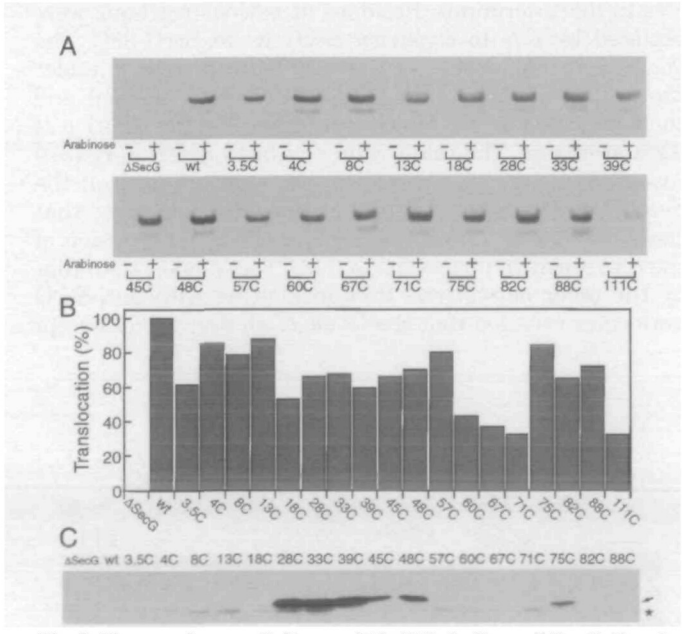

Fig. 2. **Expression, activity, and NEM labeling of SecG-Cys derivatives.** (A) *E. coli* KN370 (*ΔsecG::kan*) harboring pKQ2, a vector, pAG5 carrying the wild type *secG*, or one of the 18 plasmids encoding the specified SecG-Cys derivative was grown in the presence or absence of 0.2% arabinose. Total cellular proteins (5 μg) were analyzed by SDS-PAGE (HI-gel) and immunoblotting with anti-SecG antibodies. (B) IMVs were prepared from KN553 (*Δunc::*Tn*10 ΔsecG.kan*) harboring a plasmid encoding the specified SecG-Cys derivative and kept in the absence of DTT. The translocation of proOmpF-Lpp into these IMVs was carried out as described under "EXPERIMENTAL PROCEDURES." The translocation activity was expressed as a percentage, taking the activity of the wild type SecG as 100%. (C) The IMVs used in B were labeled with [^{3}H]NEM, immunoprecipitated with the anti-SecG antibody, and then analyzed by SDS-PAGE (L-gel) and fluorography as described under "EXPERIMENTAL PROCEDURES." The migration position of SecG is indicated by an arrow. The asterisk indicates a non-specific band.

The IMVs used for the NEM labeling were not pretreated with reducing agents. When IMVs were reduced and then labeled, 82C and 88C were modified with NEM (data not shown). However, 3.5C to 18C and 57C to 71C remained unlabeled on such treatment, suggesting that these derivatives have Cys in the transmembrane regions.

Labeling of SecG-Cys Derivatives with a Membrane-Impermeable SH Reagent—A membrane-impermeable SH reagent, AMS, has been used to determine the membrane sidedness of membrane proteins (*15*). The labeling of SecG-Cys derivatives with AMS was examined on ice before and after the solubilization of IMVs, which had been kept frozen in the presence of 10 mM DTT (Fig. 3, A and B). SDS-PAGE and immunoblotting with anti-SecG antibodies revealed that the migration positions of AMS-labeled derivatives on SDS-PAGE were slightly different from those of the unlabeled ones (Fig. 3A). The amount of each derivative labeled with AMS was then determined (Fig. 3B). Among the derivatives, only 28C to 48C possessing Cys in the cytoplasmic region were substantially labeled with AMS before solubilization. The labeling of these derivatives was little affected by solubilization. About half of 28C to 48C remained unlabeled even after solubilization, indicating that the Cys residues in these derivatives are heterogenous as to ASM labeling. This heterogeneity was not caused by overexpression of these derivatives but was presumably caused by interaction with other Sec factors since they were near completely labeled in the presence of Triton X-100 at 37°C (data not shown).

The AMS labeling of 3.5C and 75C to 111C having Cys in the periplasmic region was only marginal before solubilization but markedly increased upon solubilization. The incomplete labeling of 75C to 111C after solubilization most likely resulted from incomplete reduction of disulfide bonds (see below). Contamination by right side-out membrane vesicles or partial leakiness of membranes may account for the labeling of small amounts of 75C to 111C before solubilization. Solubilization was essential for the AMS labeling of 57C to 71C having Cys in the second transmembrane region. In contrast, 4C to 18C possessing Cys in the first transmembrane region were not labeled on ice even after solubilization (Fig. 3, A and B). These derivatives were efficiently labeled when incubated at 37°C in the presence of octylglucoside (Fig. 3C). The AMS labeling caused the faster migration of 3.5C and 4C to 18C, whereas the migration of other derivatives became slower upon the labeling (Fig. 3, A and C), suggesting that the faster migration is peculiar to the labeling of the first transmembrane region.

The helical arrangement of the first and second transmembrane regions revealed that the properties of these two transmembrane regions are considerably different (Fig. 3D). More polar residues are present in the second than the first transmembrane region. The polar residues in the second transmembrane region are localized on one side of the helix. The highly hydrophobic property of the first transmembrane region may be the reason why the introduction of hydrophilic AMS to this region was temperature-dependent and caused faster migration on SDS-PAGE. Tempera-

ture-dependent dissociation of a solubilized SecY/E/G complex (*35*) may also increase the efficiency of AMS labeling.

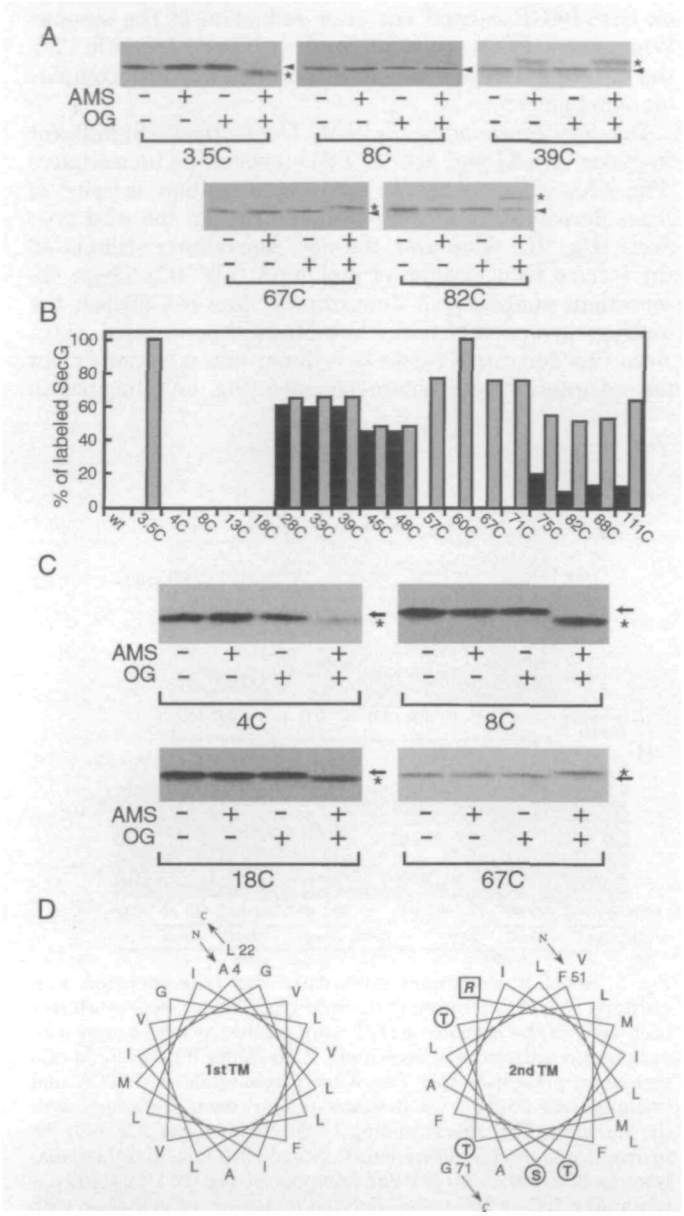

Fig. 3 **Labeling of SecG-Cys derivatives with AMS.** IMVs kept frozen at 10 mg/ml in the presence of 10 mM DTT were used for AMS labeling after 100-fold dilution with 50 mM potassium phosphate (pH 7.5). Aliquots of the reaction mixture containing 0.5 μg protein were then analyzed by SDS-PAGE (L-gel) and immunoblotting with the anti-SecG antibody. (A) IMVs containing the specified SecG-Cys derivatives were incubated at 0 1 mg/ml with or without 5 mM AMS on ice for 20 min. AMS labeling was also examined after the solubilization of IMVs with 2.5% octylglucoside (OG) on ice for 20 min. Arrowheads and asterisks indicate unlabeled and labeled derivatives, respectively (B) The labeling of the eighteen SecG-Cys derivatives and wild type SecG (wt) with AMS was examined before (filled bars) and after (dotted bars) solubilization with octylglucoside as described in A. The amounts of AMS-labeled derivatives were determined as described under "EXPERIMENTAL PROCEDURES." (C) IMVs containing the specified derivatives were subjected to AMS labeling as described in A at 37°C, but not on ice. (D) Helical arrangement of the first and second transmembrane (TM) regions. Polar residues are indicated by circled bold letters. The bold letter in a square represents a charged residue.

SecG homologs of *Salmonella typhi, Yersinia pestis, Haemophilus influenzae, Pseudomonas aeruginosa*, and *Neisseria meningitidis* found in the database provided by the National Center for Biotechnology Information are predicted to possess two membrane spanning regions. The more hydrophobic property of the first membrane spanning region seems to be conserved in the SecG homologs of these Gram-negative bacteria.

Taking these results together, we conclude that the proposed membrane topology of SecG was established.

SecG Homodimer Formed through a Disulfide Bond—When IMVs kept in the absence of DTT were analyzed by SDS-PAGE under non-reducing conditions, bands corresponding to molecular masses of higher than that of the SecG monomer were detected on immunoblotting with anti-SecG antibodies (Fig. 4A). These bands were prominent for 3.5C, 82C, and 88C, but not detected for the wild type SecG, and completely disappeared if the SDS-PAGE was carried out after reduction of the samples, indicating that these bands represent SecG oligomers formed through disulfide bonds. Oligomers of 82C and 88C were also present in IMVs kept frozen in the presence of 10 mM DTT, albeit in greatly reduced amounts (data not shown).

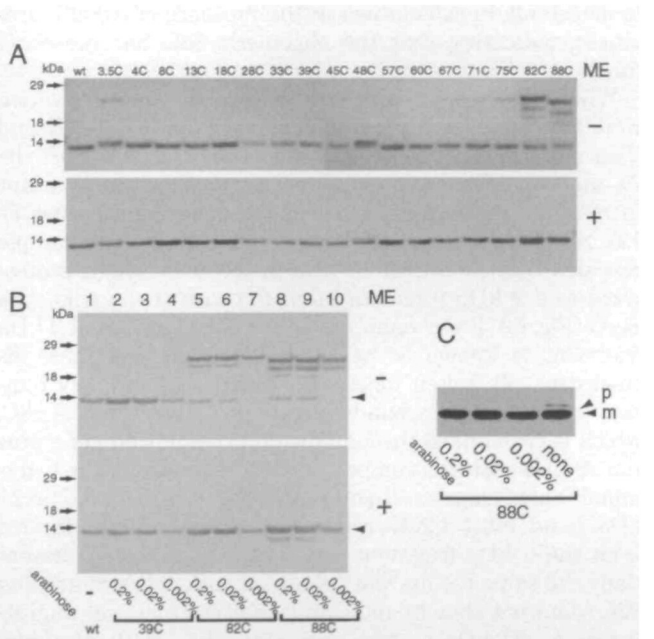

Fig. 4 **Formation of SecG oligomers through disulfide bonds.** (A) IMVs (0.5 μg protein) containing the specified SecG-Cys derivatives or wild type SecG (wt) which had been kept in the absence of DTT were solubilized with the sample buffer containing (lower panel) or not containing (upper panel) 2% mercaptoethanol (ME), and then analyzed by SDS-PAGE (HI-gel) and immunoblotting. (B) KN370 harboring a plasmid, which encodes the specified SecG-Cys or wild type SecG, was grown in the presence of the indicated concentrations of arabinose, harvested and then treated with 10 mM NEM on ice for 30 min. As a control, FS1576 grown in the absence of arabinose was also treated with NEM (lane 1). The cellular proteins (5 μg) were solubilized and analyzed by SDS-PAGE as in A. The position of the monomer is indicated by an arrowhead. (C) Expression of 88C was induced in KN370 cells by the addition of specified concentrations of arabinose at 37°C. At OD_{600} = 1.0, the cells were transferred to 20°C and then incubated for 3 h. Cellular proteins (5 μg) were precipitated with TCA, and then analyzed by SDS-PAGE and immunoblotting with anti-OmpA antibodies. The positions of proOmpA (p) and mature OmpA (m) are indicated.

To determine whether the oligomers are formed *in vivo* or during the preparation of IMVs, *E. coli* cells were harvested and immediately treated with NEM. Cellular proteins were then precipitated with TCA, and analyzed by non-reducing SDS-PAGE and immunoblotting (Fig. 4B, upper panel). Essentially the same species of 82C and 88C oligomers as those observed with IMVs were detected in the cells. Furthermore, the decreases in the levels of derivatives caused by a decrease in the amount of arabinose had no effect on the profile of the oligomers, indicating that the oligomerization is not caused by their overproduction. Bands corresponding to molecular masses of higher than 24 kDa were not detected. No oligomer was found for the wild type SecG. Only the monomeric form of 82C and 88C was detected on SDS-PAGE carried out after reduction of the sample (Fig. 4B, lower panel). The amount of 88C in cells grown in the presence of 0.002% arabinose was similar to that of the chromosomally-encoded SecG (compare lanes 1 and 10 in the lower panel of Fig. 4B). Moreover, essentially all 88C molecules in these cells existed as oligomers (lane 10 in the upper panel). Cells grown in the absence of arabinose accumulated proOmpA at low temperature (Fig. 4C) due to the cold-sensitive translocation defect of the *secG* null mutant (*28*). In contrast, proOmpA was undetectable in cells grown in the presence of 0 002% arabinose, indicating that the oligomeric 88C has the SecG function.

When IMVs containing the oligomeric 88C derivative were treated with increasing amounts of proteinase K and then analyzed by SDS-PAGE without reduction, both the 24 and 21 kDa bands disappeared with a concomitant increase in the 18 kDa band (Fig. 5A, upper panel, lanes 7–12). SDS-PAGE performed after reduction of the sample revealed that the intact 12 kDa molecule of 88C is proteolyzed to a 9 kDa fragment as in the case of the wild type SecG (Fig. 5A, lower panel), from which a C-terminal 9 kDa fragment is known to be generated upon proteinase K-treatment (*7*). Taken together, these results strongly indicate that the 24 kDa band represents a homodimer of 88C, which is crosslinked through disulfide bonds and generates the 18 kDa material comprising two molecules of the C-terminal 9 kDa fragment upon proteinase K digestion. The 21 kDa band most likely represents a heterodimer formed from the 9 kDa fragment and intact 12 kDa 88C. Essentially the same results were obtained with IMVs containing 82C (data not shown), indicating that the material migrating as a ~24 kDa protein represents the 82C homodimer. The mobility of the 82C dimer on SDS-PAGE was slightly lower than that of the 88C dimer, whereas their monomers migrated to the same position (Fig. 5A), suggesting that the structure of the 82C dimer is bulkier than that of the 88C dimer. These results also indicate that a single translocation machinery contains at least two molecules of each derivative existing close to each other, whereby spontaneous crosslinking occurs through disulfide bonds. A SecE derivative possessing Cys in the third transmembrane region was also reported to form a homodimer crosslinked through disulfide bonds (*19*).

IMVs containing the 88C dimer were solubilized with digitonin (*36*), precipitated with anti-SecE or anti-SecD antibodies, and then analyzed by SDS-PAGE without reduction or after reduction (Fig. 5B). The 88C homodimer was co-precipitated with SecE and SecD, as the monomer of 39C derivative was, indicating that dimerization does not perturb the interaction with other components of the translocation machinery. Only the 88C monomer was detected on SDS-PAGE carried out after reduction of the sample. When the IMVs were solubilized with octylglucoside (*37*), the 88C dimer was precipitated as a SecY/E/G complex (data not shown).

Topology Inversion of the SecG Homodimer—Significant fractions of 82C and 88C in IMVs existed as homodimers (Fig. 4A), whereas the *in vitro* translocation activity of these derivatives was comparable to that of the wild type SecG (Fig. 2B). Moreover, the 88C homodimer stimulated the *in vivo* translocation of proOmpA (Fig. 4C). These observations suggest that dimerization does not abolish the topology inversion of SecG. To address this, we used 111C, since this derivative exists as a dimer and a monomer but not an intermediate hetero-oligomer (Fig. 6A). Incubation

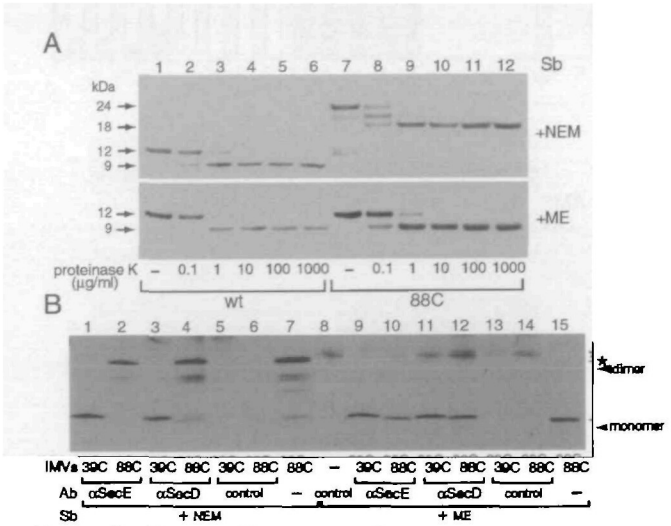

Fig. 5 **SecG homodimers constitute the translocation machinery.** (A) IMVs containing the wild type SecG or 88C which had been kept in the absence of DTT were treated at 0 05 mg/ml with various concentrations of proteinase K on ice for 30 min in 50 mM potassium phosphate (pH 7 5) After precipitation with TCA and washing with acetone, the proteins (0.5 μg) were solubilized with the sample buffer (Sb) containing 1 mM NEM (upper panel) or 2% mercaptoethanol (ME, lower panel), boiled for 5 min, and then analyzed by SDS-PAGE (HI-gel) and immunoblotting. (B) IMVs (100 μg) containing 39C or 88C were subjected to immunoprecipitation with the specified antibodies after solubilization. IMVs kept in the absence of DTT were solubilized at 0 1 mg/ml in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% digitonin and 40% (w/v) glycerol on ice. Supernatants obtained on centrifugation at 40, 000 rpm for 30 min at 4°C with a Beckman TLA-45 were treated with 1 μl of the anti-SecE, anti-SecD, or non-immune (control) antibody (Ab) for 3 h on ice. The mixture was then treated with 40 μl of protein A Sepharose (Pharmacia) on ice for 30 min with occasional mixing. Pellets were collected by brief centrifugation, washed twice with 500 μl of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% digitonin, and 20% (w/v) glycerol, and then resuspended in 15 μl of the Laemmli sample buffer (Sb) containing 2% mercaptoethanol (ME) or 1 mM NEM (NEM). Aliquots of the supernatants obtained on brief centrifugation were analyzed by SDS-PAGE (L-gel) and immunoblotting with the anti-SecG antibody. The blots were visualized with ECL (Amersham Pharmacia Biotech). The migration positions of the dimer and monomer of SecG are indicated by arrowheads, and the bands indicated by an asterisk were due to antibodies. Only the antibody was applied to lane 8 IMVs (1 μg) containing 88C were also analyzed without immunoprecipitation in lanes 7 and 15.

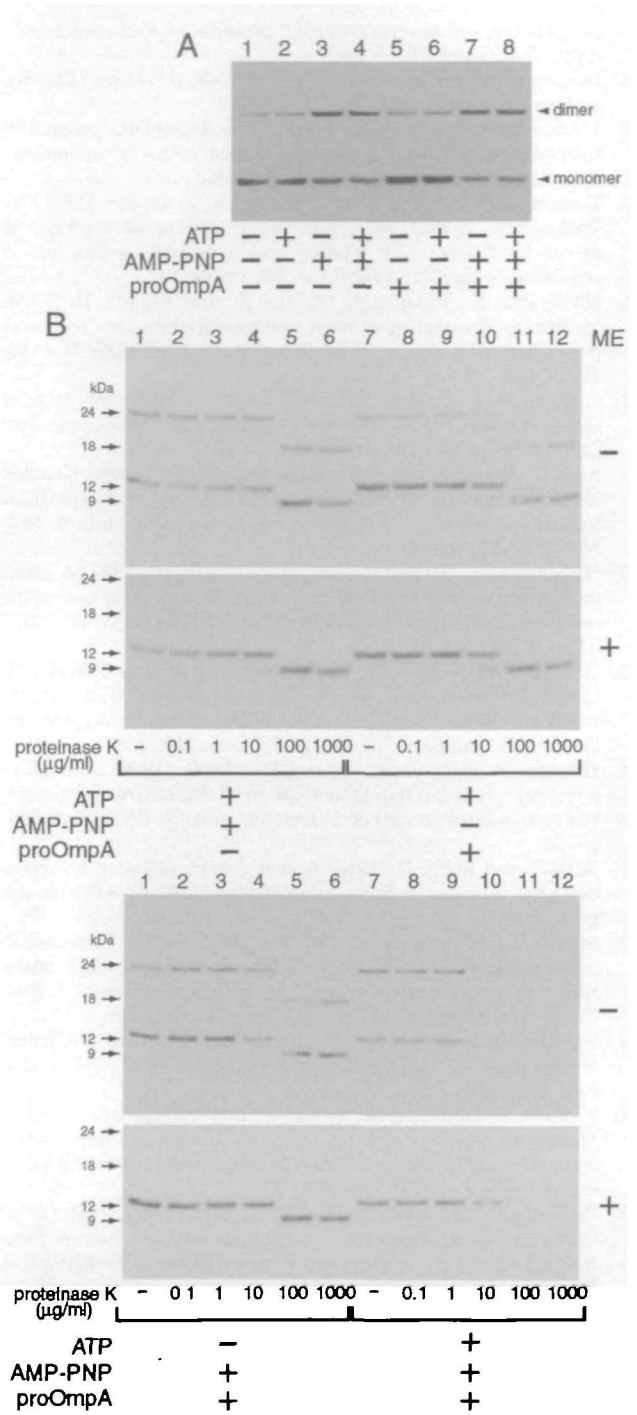

Fig. 6 **The SecG homodimer undergoes membrane topology inversion on proOmpA translocation.** IMVs containing 111C were subjected to topology inversion assaying as described under "EXPERIMENTAL PROCEDURES." (A) IMVs were incubated under the specified conditions and then analyzed by non-reducing SDS-PAGE (HI-gel) without proteinase K treatment. The sample buffer contained 1 mM NEM instead of mercaptoethanol The positions of the monomer and dimer are indicated. (B) The topology inversion assay was carried out under the specified conditions. IMVs were treated with various amounts of proteinase K in the presence of NEM, solubilized with the sample buffer containing or not containing 2% mercaptoethanol (ME), and then analyzed by SDS-PAGE (HI-gel).

of IMVs with AMP-PNP was found to increase the amount of the 111C dimer for an unknown reason. This increase was dependent on AMP-PNP but not proOmpA (Fig. 6A). This seems to suggest that the C-termini of two 111C molecules become closer to each other in the presence of AMP-PNP. It has been reported that AMP-PNP also stimulates the crosslinking of the SecE-Cys homodimer in a proOmpA-dependent manner (*19*).

To examine the topology inversion of 111C, IMVs were subjected to protein translocation assaying at 37°C and then treated with increasing amounts of proteinase K on ice for 30 min. The proteolyzed fragments of 111C were analyzed by reducing and non-reducing SDS-PAGE and immunoblotting with anti-SecG antibodies (Fig. 6B). When protein translocation was not initiated due to the omission of proOmpA (upper two panels, lanes 1–6) or ATP (lower two panels, lanes 1–6), or initiated but not blocked by AMP-PNP (upper two panels, lanes 7–12), the 111C dimer and monomer were completely converted to the 18 kDa and 9 kDa fragments, respectively. On reducing SDS-PAGE, only the 9 kDa fragment was detected with higher concentrations of proteinase K, indicating that the 111C monomer and dimer exist in the original topologies under these conditions, and that the C-terminal fragment is generated upon proteinase K digestion (*7*). When protein translocation was initiated and then blocked by AMP-PNP (lower two panels, lanes 7–12), both the 111C dimer and monomer disappeared without generation of the 18 kDa and 9 kDa fragments upon proteinase K treatment. Reducing SDS-PAGE revealed that neither the intact 12 kDa molecule nor the 9 kDa fragment was detectable with higher concentrations of proteinase K, indicating that the crosslinked SecG dimer undergoes topology inversion. On the other hand, the SecE-Cys homodimer crosslinked through disulfide bonds was reported to be non-functional (*19*). The 88C dimer (24 kDa) also underwent topology inversion although the amount of 18 kDa fragment of this derivative remained unchanged (data not shown).

DISCUSSION

The membrane topology of SecG was precisely determined on the basis of the accessibility of SH-specific reagents to Cys residues of SecG-Cys derivatives. The N-terminus of SecG has been speculated to be exposed to the periplasm based on indirect evidence obtained with a non-functional SecG derivative (*7*). This was proved by the octylglucoside-dependent labeling of functional 3.5C with AMS (Fig. 3). Moreover, that the 3.5C dimer was formed through disulfide bonds also indicates that the Cys residue in 3.5C is exposed to the periplasmic oxidative environment. AMS labeling revealed a difference in not only membrane sidedness between the weakly hydrophobic region and the C-terminal region, but also the difference in properties between the first and second transmembrane regions (Fig. 3). It is not clear whether or not the different properties of the two transmembrane regions are of any physiological significance, although they seem to be conserved in SecG homologs of Gram-negative bacteria.

SecG-Cys derivatives possessing Cys in the periplasmic region were found to each form a homodimer through disulfide bonds. In order for an intermolecular disulfide bond to form, two Cys residues must exist very close to each other

(*38*, *39*). Major fractions of 82C and 88C, and certain fractions of 3.5C and 75C in IMVs existed as homodimers formed through disulfide bonds (Fig. 4A). When the level of expression was low, both 82C and 88C only existed as homodimers *in vivo* (Fig. 4B). Moreover, the 88C dimer was co-immunoprecipitated with SecE and SecD, as the SecG monomer was (Fig. 5B). These results strongly indicate that at least two SecG molecules co-exist in a single translocation machinery. Since the Cys residues in 3.5C and 75C are each located at a membrane proximal site, it is highly unlikely that the two Cys residues crosslinked together are localized in different machineries. It has been proposed, based on electron microscopic observations, that a single protein translocation machinery in bacteria is composed of multiple copies of a SecY/E complex (*21*), as the eukaryotic machinery comprising the Sec61p complex is (*20*). The bacterial protein-conducting channel formed by SecY/E of *Bacillus subtilis* was reported to have an outer diameter of ~85 Å and a pore size of ~15–20 Å (*21*). On the other hand, the distance found for natural disulfide bonds in proteins is 4–8 Å (*38*, *39*). These observations also support that two SecG-Cys molecules in a single translocation machinery are crosslinked together.

The SecY/E/G complex has been reported to contain equal numbers of SecY and SecE molecules (*40*), but the number of SecG molecules in the complex is unknown. Our data do not reveal the copy number of the SecY/E complex in a single translocation machinery. However, since SecE-Cys derivatives also form a crosslinked homodimer (*19*), it seems likely that a single translocation machinery of *E. coli* comprises multicopies of the SecY/E/G complex, as indicated by electron microscopic data (*21*). It was recently reported that protein translocation induces the formation of a tetrameric SecY/E/G complex (*41*).

The crosslinked SecG dimer supported the *in vivo* translocation of proOmpA (Fig. 4, B and C) and exhibited topology inversion on proOmpA translocation (Fig. 6). The topology inversion of SecG is dependent on the membrane insertion of SecA (*7*). However, no further details of the topology inversion are known at present. If one molecule of the crosslinked dimer undergoes topology inversion while the other one remains in the native topology, intermediate fragments with molecular masses of 9–24 kDa may be generated on proteinase K digestion. However, the 24 kDa 111C dimer disappeared without the generation of such fragments, suggesting that the two SecG molecules underwent topology inversion almost simultaneously. In order to clarify the molecular events involved in the SecG topology inversion in detail and to control the inversion, SecG-Cys derivatives seem to be useful tools. It is noteworthy that the crosslinking of the SecE homodimer through disulfide bonds at a specific site in the third transmembrane region inhibits protein translocation by preventing the functional SecY-SecE interaction (*19*).

We thank R. Ishihara for the secretarial support.

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