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Biochemical Characterization of a Mutationally Altered Protein Translocase: Proton Motive Force Stimulation of the Initiation Phase of Translocation

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Protein translocation across the *Escherichia coli* plasma membrane is facilitated by concerted actions of the SecYEG integral membrane complex and the SecA ATPase. A *secY* mutation (*secY39*) affects Arg357, an evolutionarily conserved and functionally important residue, and impairs the translocation function in vivo and in vitro. In this study, we used the "superactive" mutant forms of SecA, which suppress the SecY39 deficiency, to characterize the mutationally altered SecY39EG translocase. It was found that SecY39-mediated preprotein translocation exhibited absolute dependence on the proton motive force. The proton motive force-dependent step proved to lie before signal peptide cleavage. We suggest that the proton motive force assists in the initiation phase of protein translocation.

A significant fraction of *Escherichia coli* proteins are transported across the cytoplasmic membrane for their cell envelope localization. The major constituents of the Sec protein translocation system (translocase) include the SecA translocation ATPase and integral membrane components SecY, SecE, and SecG (18, 24). In the series of membrane translocation reactions, targeting of a preprotein molecule to the translocase site on the membrane is followed by initiation of translocation. The ATP- and preprotein-dependent conformational change of SecA is thought to be essential for the initiation reaction, in which the signal peptide region and its immediate C-terminal segment are inserted into the membrane, probably together with SecA itself (11, 30). In this event, which requires ATP but not its hydrolysis (30), the loop-like insertion of the N-terminal region (14) leads to periplasmic exposure of the signal peptide cleavage site. The initiation phase is followed by translocation of the main body of the preprotein, and this reaction step seems to be driven by repeated cycles of SecA-mediated ATP-binding and hydrolysis as well as by the proton motive force (PMF) across the membrane.

The postinitiation role of the PMF can be inferred from the in vitro observation that a preprotein with an internal disulfide-bonded loop near the carboxyl terminus can only complete translocation when the PMF has been imposed (31). It was also proposed that the PMF facilitates the SecA reaction cycles by stimulating its "deinsertion" from the membrane (28). The PMF appears to have a role in earlier steps of translocation as well. Mutations in the signal sequence or in the early mature region of a preprotein can affect the PMF dependence in the in vitro protein translocation system (13, 17). Some *prlA* mutations in *secY* not only suppress mutational defects of signal sequence but also alleviate the PMF dependence of protein translocation (10, 29). Such PrlA phenotypes are also accompanied by an apparent destabilization of the SecY-SecE interaction (10) as well as by an apparent increase in the SecY-SecA binding affinity (37). These results suggest that the PMF has multiple roles in protein translocation in *E. coli*. Obviously, full understanding of the PMF requirements in bacterial protein export requires additional cellular and molecular studies.

SecY and SecE are the minimum membrane components that are required for translocation (1, 6); they are thought to form a channel-like translocation pathway as well as a high-affinity SecA-binding site in the membrane. SecY is essential to activate SecA (12, 21). Our genetic studies suggest that among the six cytoplasmic domains of SecY, the two C-terminally located cytoplasmic regions (C5 and C6) are particularly important for the functions to support the SecA-driven translocation reactions (32, 33). Extensive mutational analyses revealed that Arg357 in the C5 domain is a critical residue in SecY (23). The mutation *secY39* results in an alteration of this residue to His, in cold sensitivity in growth, and in a severe defect in protein export (4).

The mutational inactivation of translocase has been demonstrated in vitro by using inverted membrane vesicles (IMVs) (32) as well as purified components after reconstitution into proteoliposomes (see reference 23 for another Arg357 mutation and this study for SecY39). In this study, we examined the in vitro translocation reaction that is mediated by translocase having the SecY39 defect. To enable such analyses, we took advantage of a class of functionally upregulated SecA variants that we had characterized earlier as "superactive" variants (20). The results obtained suggest that the PMF can facilitate the initiation step of translocation.

MATERIALS AND METHODS

***Escherichia coli* strains and plasmids.** Strains TW156 (*secY*$^+$), GN5 (*secY205*) (21), and GN4 (*secY39*) were derived from strain AD202 (2) and carried the Δ(*uncB-uncC*) marker (16) in addition to the *secY* mutation indicated. They were used for preparation of IMVs. Strain KI269/pKY173 (*secA*) was used to purify wild-type SecA (38). For overproduction of a mutant form of SecA, the *secA* mutation was first introduced into strain GN93 (AD202 *secY205 Tet*^{s}/ F′ *lacI*^{q}) (20) with *leu*::Tn*10* as a selective marker in P1 transduction, followed by introduction of a plasmid encoding the corresponding SecA variant.

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TABLE 1. Plasmids

Plasmid name	Cloned genes[a]	Promoter for expression	Reference
pHAsecEYG	HA-*secEYG*	*ara*	24
pHAsecEY205G	HA-*secEY205G*	*ara*	This study
pHAsecEY39G	HA-*secEY39G*	*ara*	This study
pKY173	*secA*$^+$	*lac*	23
pGN71	*secA71*	*lac*	This study
pGN117	*secA117*	*lac*	This study
pHM329	*secA (Ile502Ser)*	*lac*	This study
pHM348	*secA (Asp580Val)*	*lac*	27

[a] Mutations were introduced by using DNA fragments amplified from *secA* mutant strains, appropriate restriction enzymes, and fragment substitution procedures. HA denotes a hemagglutinin epitope sequence.

E. coli BL21(DE3) was used as a host for pHAsecEYG (8) for purification of the SecY$^+$EG complex. Strains HM501 (*secY39*) and GN19 (*secY205*) were *araD*$^+$ derivatives of GN31 and GN15 (21), respectively, and used as host cells for pHAsecEY39G and pHAsecEY205G for purification of the SecY39EG and the SecY205EG complexes, respectively. Plasmids used in this study are summarized in Table 1.

Purification of SecA and its superactive variants. SecA and its mutant derivatives were purified from their overproducing strains. Each mutation was transferred onto pKY173 (38). The mutant plasmids were expressed in a strain that carried the same mutation on the chromosome (20; H. Mori, K. Chiba, and K. Ito, unpublished data) to avoid any heterodimer formation. Cells were grown at 37°C (for wild-type SecA) or at 30°C (for mutant SecAs), induced for SecA expression with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h, and disrupted by French pressure cell (Aminco). Soluble fractions were applied to Red-agarose column (Amicon), and after washing the column with 0.5 M KCl, SecA protein was eluted with 1 M KCl. Peak SecA fractions were then applied to Phenyl Superose 5/5 (Amersham Biosciences), which was eluted with a linear gradient of 1 to 0 M $(NH_4)_2SO_4$. Peak fractions of SecA were dialyzed against 50 mM Tris-HCl (pH 7.5) and subjected to Hi-trap Q chromatography and linear elution with 0 to 0.4 M NaCl. SecA preparations thus obtained were at least 98% pure (data not shown). They were stored in 50 mM Tris-HCl (pH 7.5)–10% glycerol at −80°C.

In vitro translocation assay. Pro-OmpA was translated in vitro in the presence of [^{35}S]methionine (35), precipitated with 5% trichloroacetic acid, washed with 5% trichloroacetic acid and then with acetone, and solubilized in 6 M urea–50 mM Tris-HCl (pH 7.5)–1 mM dithiothreitol. Pro-OmpA with intramolecular disulfide bond was prepared after oxidation with potassium ferricyanide (final concentration 10 mM) (35). In vitro translocation assay conditions were described previously (23).

Purification of SecYEG and its mutant forms SecY39EG and SecY205EG. Plasmid pHAsecEYG (8) and its derivatives with the *secY39* and the *secY205* mutations were used for overproduction of SecYEG and mutant complexes. Cells carrying one of these plasmids were grown at 37°C on M9 medium containing 0.4% maltose, 0.4% glycerol, amino acids mixture (20 μg/ml), and ampicillin (50 μg/ml) until mid-log phase. SecYEG synthesis was induced with 0.4% arabinose for 1 h. IMVs prepared as described previously (22) were washed with 5 M urea–6% cholate and solubilized with 2.5% (wt/vol) *n*-octyl-β-D-thioglucoside (OG) (Dojindo) in 20 mM Tris-HCl (pH 8.0)–20% glycerol–7.5 mg of *E. coli* phospholipids per ml. Solubilization was for 10 min, and the final protein concentration was adjusted to 10 mg protein/ml.

After centrifugation at 100,000 × *g* for 30 min, the supernatant was applied to an EDAE2 column (Bio-Rad), equilibrated with 1.25% OG–20 mM Tris-HCl (pH 8.0)–20% glycerol–0.5 mg of *E. coli* phospholipids per ml. The column was developed with a linear gradient of KCl (0 to 0.2 M) in the same buffer. The SecYEG complex was eluted at KCl concentrations of 75 to 90 mM, away from many other membrane proteins; the purity was about 80% (Fig. 1B).

Protein concentration. Protein concentration was determined by the micro-BCA method (Pierce) with bovine serum albumin standards.

Reconstitution of SecYEG proteoliposomes. *E. coli* phospholipids (100 μg) were added to 25 μl of the purified preparation of SecYEG complex (0.2 to 0.5 mg/ml) in 1.25% OG. The mixture was diluted with 1 ml of 50 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, followed by incubation on ice for 5 min. The proteoliposomes were recovered by centrifugation at 100,000 × *g* for 30 min, suspended in 25 μl of 50 mM Tris-HCl (pH 7.5)–50 mM NaCl, and subjected to freezing and thawing as described by Tokuda et al. (34). To generate the PMF across the reconstituted membranes, valinomycin (2 nmol per mg of *E. coli* phospholipids) was added to the frozen and thawed proteoliposomes and incubated on ice for 10 min. The valinomycin-treated proteoliposomes were recovered by centrifugation and sonicated. The PMF was generated by addition of 50 mM potassium acetate (9), leading to selective import of K^+ ions (inside positive) and concomitant formation of hydrogen acetate outside the proteoliposomes (outside less acidic). As a control (no PMF), 50 mM NaCl was added.

Precursor binding assay. Binding of pro-OmpA to 4 M urea-treated IMVs was assayed as follows. SecB (6.4 μg/ml), SecA (50 μg/ml), IMVs (250 μg protein/ml), and a mixture of ^{35}S-labeled pro-OmpA and unlabeled pro-OmpA (0.6 ng/ml) were incubated on ice in 100 μl of buffer consisting of 50 mM Tris-HCl (pH 7.5) and bovine serum albumin (500 μg/ml). After 15 min, the mixture was centrifuged (for 30 min at 100,000 × *g*, 4°C) through a 100-μl sucrose cushion (0.2 M sucrose in the same buffer). Supernatant and precipitates were treated with 5% trichloroacetic acid and applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by phosphor image exposure for quantitation.

Measurement of SecA ATPase activity. Hydrolysis of ATP was assayed by ADP-coupled reactions involving pyruvate kinase and lactate dehydrogenase as described previously (25).

RESULTS

***secY39* Arg357 alteration of SecY impairs translocation function, especially in the absence of the PMF.** Residue Arg357 of SecY is functionally important (23). A mutation, *secY39*, causing an alteration of this residue to His renders the cell cold sensitive for growth, and this defect is accompanied by significant retardation of protein export to the periplasmic space and the outer membrane (4). Inverted membrane vesicles from cells this mutant exhibit impaired in vitro abilities to support SecA-dependent preprotein translocation and to activate the SecA ATPase (32). To study the nature of the *secY39* mutational defects in vitro, we used the superactive variants of SecA in place of the wild-type SecA protein in translocation reactions. We reasoned that if these mutant forms of SecA allow significant translocation even in combination with the SecY39 mutant IMV, it becomes possible to study the reaction parameters of the translocation reaction that is mediated by the mutationally altered SecY39 complex.

We purified two superactive forms of SecA, SecA71 (Ala507Val) and SecA117 (Ala488Thr). These mutations had been isolated as suppressors against the *secY205*(Cs) defect, but they proved to suppress a number of other mutations in *secY*, including *secY39*. Translocation of ^{35}S-labeled pro-OmpA was assayed with 4 M urea-washed IMVs prepared from wild-type cells, *secY205* mutant cells, or *secY39* mutant cells. These IMV preparations contained similar amounts of the wild-type and mutant SecY molecules, as demonstrated by immunoblotting experiments (32). The PMF was either imposed or dissipated before the assay. In the presence of the PMF, the wild-type IMV supported 50% or higher proportions of pro-OmpA translocation, irrespective of the SecA source (Fig. 1A, lanes 1 to 6). The translocated proportion decreased somewhat in the absence of the PMF (Fig. 1A, lanes 2, 4, and 6), but no more than twofold. Although this limited PMF dependence was a characteristic of the IMV-based in vitro reaction at 37°C; more pronounced PMF dependence was evident at 20°C (26).

The superactive SecA variants gave markedly higher levels of translocation (Fig. 1A, lanes 3 and 5), which was only marginally decreased upon PMF dissipation (Fig. 1A, lanes 4 and 6). The SecY205 IMV exhibited only low-level (13% translocation) activity in combination with SecA$^+$ (Fig. 1A, lane 13),

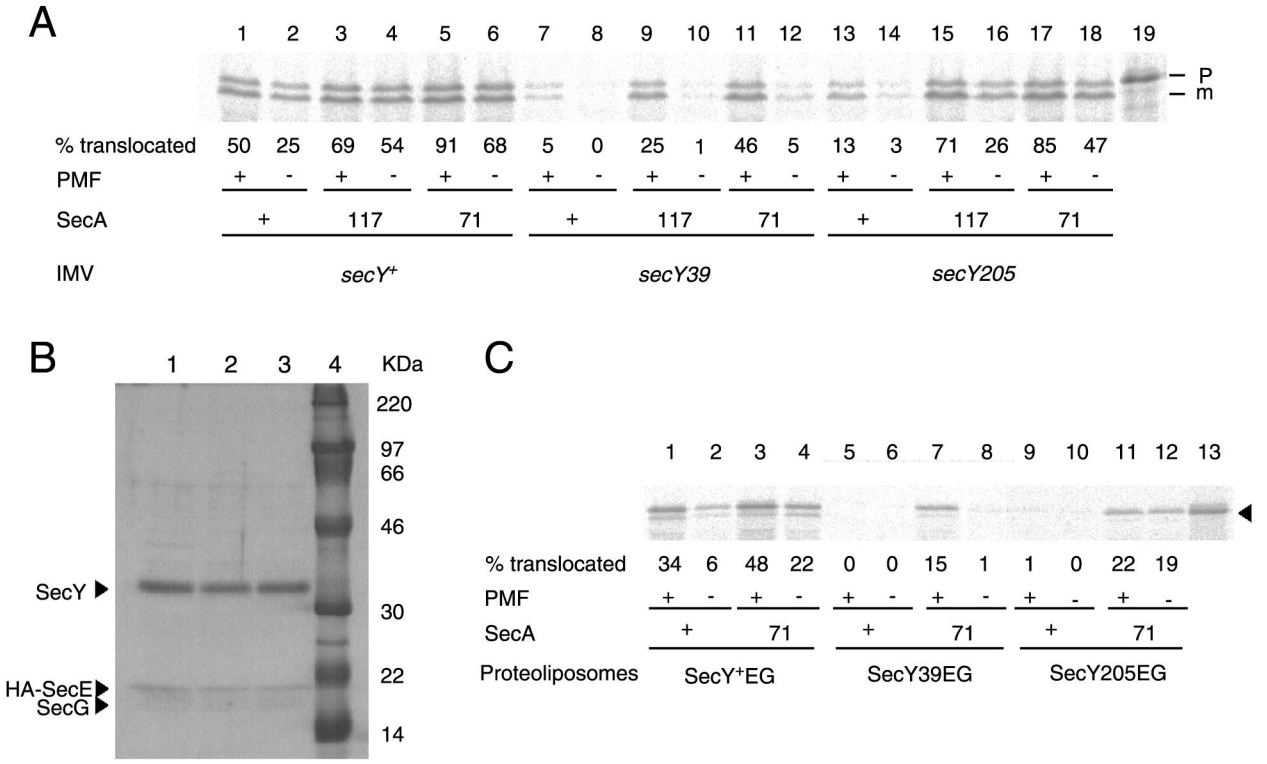

FIG. 1. SecY mutational effects on PMF dependence of pro-OmpA translocation. (A) Translocation into IMVs. We combined 4 M urea-washed IMV, prepared either from *secY*$^+$ strain TW156 (lanes 1 to 6), *secY39* strain GN4 (lanes 7 to 12), or *secY205* strain GN5 (lanes 13 to 18) with either wild-type SecA protein, the SecA117 mutant protein, or the SecA71 mutant protein for translocation of ^{35}S-labeled pro-OmpA. Translocation was allowed to proceed at 37°C for 10 min in the presence of 5 mM succinate (+ PMF) or in the presence of 10 μM carbonycyanide-*m*-chlorophenyl-hydrazone (−PMF). Phosphor images for ^{35}S radioactivity upon SDS-PAGE are shown for input (lane 19) as well as for samples after proteinase K treatment (other lanes). Proportions of the translocated molecules are indicated (% translocated). Positions of the precursor (p) and mature (m) species are shown on the right. (B) Purified SecYEG complexes used for reconstitution. About 3 μg each of purified SecYEG preparations containing either the wild-type SecY (lane 1), the SecY205 mutant form (lane 2), or the SecY39 mutant form (lane 3) was examined by SDS-PAGE and Coomassie brilliant blue staining. (C) Translocation into SecYEG proteoliposomes. Valinomycin-treated SecYEG proteoliposomes (with wild-type or mutant SecY subunit, as indicated) were used for in vitro translocation of ^{35}S-labeled pro-OmpA in combination with wild-type SecA or the SecA71 superactive form, as indicated. Reactions were allowed to proceed at 37°C for 10 min in the presence of 50 mM potassium acetate (+PMF) or 50 mM NaCl (−PMF). Proteinase K-resistant full-length pro-OmpA bands are shown.

and the activity was further lowered to an insignificant level (3% translocation) upon dissipation of the PMF (Fig. 1A, lane 14). However, the SecA117 and SecA71 mutant proteins effectively alleviated the defects in both the presence and absence of the PMF (Fig. 1A, lanes 15 to 18). The SecY39 IMV, the target of our present analysis, had only a very low level (5% translocation) of activity in combination with SecA$^+$; it was virtually inactive in the absence of the PMF (Fig. 1A, lane 8). The superactive forms of SecA, SecA117 and SecA71, allowed 25% and 46% translocation, respectively, when combined with the SecY39 IMV (Fig. 1A, lanes 9 and 11). Strikingly, such suppression effects of the SecA variants against the SecY39 defect were only observed in the presence of the PMF; in its absence, SecY39 IMV was inactive even in combination with either superactive variant of SecA (Fig. 1A, lanes 10 and 12).

The above results that the translocation defect of the SecY39 IMV could be circumvented by the superactive alterations in SecA but only in the presence of the PMF indicate that the SecY39 IMV has unusually strong dependence on the PMF. We were able to reproduce these characteristics of the mutational alterations with purified components. SecYEG complexes having either the wild-type, SecY205, or SecY39 form of the SecY subunit were purified with appropriate overproducing cells (Fig. 1B). They were reconstituted with *E. coli* phospholipids into proteoliposomes. To generate the PMF across the phospholipid bilayer of the proteoliposomes, they were made K$^+$ permeable by valinomycin treatment, followed by addition of potassium acetate. Their activities to translocate pro-OmpA were examined in combination with either SecA$^+$ or SecA71. Although proteoliposomes containing wild-type SecYEG complex were active in combination with both SecA$^+$ and SecA71 (Fig. 1C, lanes 1 and 3), those containing the SecY39EG complex or the SecY205EG complex were only active when the superactive SecA71 variant was used (Fig. 1C, lanes 7 and 11). In the absence of the PMF, the SecA71 form of SecA could not promote significant pro-OmpA translocation into the SecY39EG proteoliposomes (Fig. 1C, lane 8), while it was able to translocate the preprotein into the SecY205EG proteoliposomes (Fig. 1C, lane 12).

PMF is required for early posttargeting step of translocation. At which step does the SecY39-mediated translocation require the PMF? We first examined binding of a preprotein to

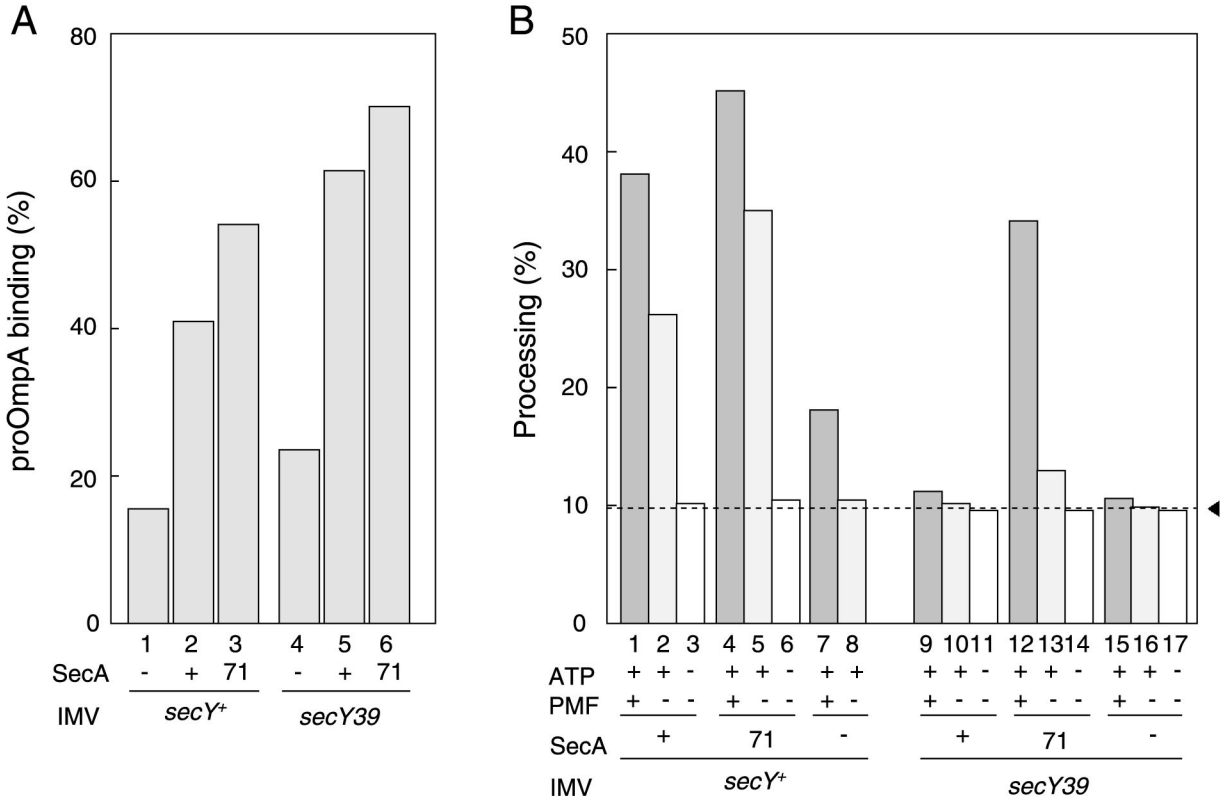

FIG. 2. Assays for precursor binding and signal peptide processing steps. (A) Pro-OmpA binding to IMVs. ^{35}S-labeled pro-OmpA (0.6 μg/ml) was incubated with wild-type and mutant IMVs on ice for 10 min. Samples were centrifuged, and supernatants and precipitates were examined by SDS-PAGE. The precipitated ^{35}S-labeled pro-OmpA proportions were quantified. (B) Processing. ^{35}S-labeled pro-OmpA was subjected to in vitro translocation reaction with combinations of IMVs and SecA$^+$ or SecA71, as indicated, under the PMF conditions indicated: +, PMF imposed; −, PMF dissipated. Reactions were allowed to proceed at 37°C for 10 min and terminated directly by trichloroacetic acid precipitation. The processed and nonprocessed species, separated by SDS-PAGE, were quantified. Dotted line shows the background processing level observed in the absence of ATP and SecA.

wild-type and SecY39 IMVs. A defined quantity of pro-OmpA mixed with ^{35}S-labeled pro-OmpA was incubated on ice with IMV, and IMV-bound pro-OmpA molecules were isolated by centrifugation. Although a certain fraction (about 20%) of pro-OmpA was recovered in the pellets even in the absence of SecA (Fig. 2A, column 1), a two- to threefold enhancement of recovery over the control was observed when SecA or SecA71 was included in the incubation (Fig. 2A, columns 2 and 3). We then compared the SecY39 IMV and the wild-type IMV for their abilities to support pro-OmpA binding in the presence of SecA. It was found that the mutant IMV was as able as the wild-type IMV to bind pro-OmpA, irrespective of the SecA source (Fig. 2A, columns 4 to 6). It should be noted that the binding assays were carried out without imposition of the PMF. These results suggest that the preprotein binding step is not compromised by the SecY39 alteration.

In the next step of translocation, signal peptide and the following N-terminal mature part are inserted into the membrane, leading to exposure of the leader peptide processing site to the periplasmic side of the membrane. This initiation step has some distinct properties compared to the "continuation" phase of translocation (3, 30). To examine the effects of mutation on the initiation process, we examined signal peptide processing upon incubation of pro-OmpA with IMV, SecA, and ATP at 37°C. Under the present reaction conditions, about 10% of pro-OmpA was cleaved for the signal peptide even in the absence of ATP (Fig. 2B, columns 3, 6, 11, 14, and 17). Presumably, this was due to the presence of some right-side-out membrane vesicles and/or membrane fragments with accessible leader peptidase in the IMV preparations.

The presence of SecA, ATP, and the PMF stimulated pro-OmpA processing by the SecY$^+$ IMV (Fig. 2B, columns 1 and 4). Lower but significant processing stimulation was observed even in the absence of the PMF for the wild-type IMV (Fig. 2B, columns 2 and 5). The SecY$^+$ IMV might have contained some residual SecA (Fig. 2B, column 7). The SecY39 mutant IMV was almost inactive in supporting SecA$^+$-stimulated processing reaction (Fig. 2B, columns 9 to 11). The mutated IMV supported significant levels of processing reaction only when combined with the SecA71 form of SecA in the presence of the PMF (Fig. 2B, column 12). In the absence of the PMF, pro-OmpA processing was almost completely blocked even in the presence of ATP. (Fig. 2B, lane 13). In the above processing experiments, only a chemically minute amount of in vitro-synthesized and ^{35}S-labeled pro-OmpA was used. It is thus unlikely that the low-level processing represented a secondary event caused

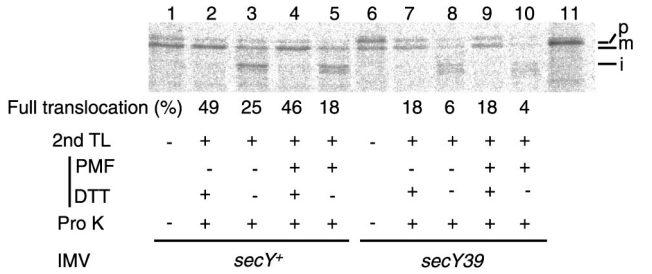

FIG. 3. Effects of PMF on a late step in pro-OmpA translocation. (A) ^{35}S-labeled pro-OmpA was oxidized to form a loop of 35 amino acid residues near the carboxyl terminus. It was subjected to the first in vitro translocation reaction at 37°C for 10 min with either SecY$^+$ IMV (lanes 1 to 5) or SecY39 IMV (lanes 6 to 10) in the presence of the PMF. Precursor-bound IMVs were recovered by centrifugation and subjected to the second translocation reaction (Materials and Methods) with or without PMF imposition and the dithiothreitol addition, as indicated. After an additional 10 min of incubation at 37°C, samples were treated with proteinase K (Pro K) where indicated. Proteinase K-resistant precursor and mature bands were quantified, and the sums of the values are shown as full translocation. Positions of the precursor (p), mature (m), and 26-kDa intermediate (i) forms are shown on the right. Input pro-OmpA is shown in lane 11.

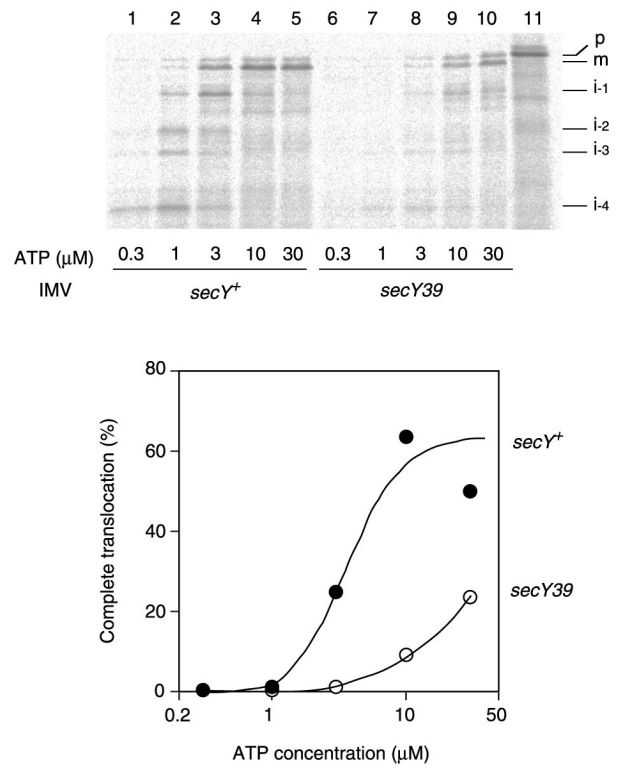

FIG. 4. Effects of ATP concentration on pro-OmpA translocation. In vitro translocation reaction of [^{35}S]pro-OmpA was assayed with either SecY$^+$ IMV (lanes 1 to 5) or SecY39 IMV (lanes 6 to 10) in combination with SecA71. Reactions at different ATP concentrations, as indicated, were allowed to proceed at 37°C for 10 min in the presence of succinate, which generated the PMF. Positions of translocation intermediates i-1, i-2, i-3, and i-4 are shown on the right. In the lower graph, the full-length translocated pro-OmpA band was quantitated, and the translocation yields are plotted against ATP concentrations.

by jamming of the translocase by untranslocated molecules. These results indicate that the SecY39 alteration and the PMF affect the initiation phase of the translocation reaction.

We then examined whether the PMF was essential for the continuation phase of translocation into the SecY39 IMV. For this purpose, we used a pro-OmpA derivative, pro-OmpA L35, which carried cysteines at residues 268 and 302. When a disulfide-bonded loop of 35 amino acid residues is formed, it acts as an obstacle in translocation of pro-OmpA L35, generating a translocation intermediate of a transmembrane configuration (35). Translocation can then be reinitiated to completion by addition of dithiothreitol, which breaks the disulfide loop. Translocation of oxidized pro-OmpA L35 was driven at 37°C by SecA71 into the SecY$^+$ and the SecY39 IMVs in the presence of the PMF.

The IMV–pro-OmpA L35 complexes were then reisolated by centrifugation and subjected to a second incubation in the presence and absence of dithiothreitol. Proteinase K treatment after the reaction without dithiothreitol generated intermediate bands of 26 kDa. This was true for both the SecY$^+$ IMV (Fig. 3, lanes 3 and 5) and the SecY39 IMV (Fig. 3, lanes 8 and 10). When dithiothreitol was included in the second reaction, the intermediate bands disappeared and the fully translocated product increased. This translocation continuation-completion occurred at significant levels for both the wild-type (Fig. 3, lanes 2 and 4) and mutant (Fig. 3, lanes 7 and 9) IMVs; the final translocation yield was about 50% for the former and about 20% for the latter. In both cases, the presence or absence of the PMF did not affect the reactions (Fig. 3, compare lanes 2 and 4 as well as lanes 7 and 9). Thus, in contrast to what was observed for the whole translocation reaction, the continuation phase of the reaction occurred, even with the SecY39 mutant IMV, without the aid of the PMF. These results together indicate that the PMF is essential for initiation of SecY39-mediated protein translocation.

Increased initiation barrier for translocation across SecY39 mutant channel. The higher PMF dependence in the initial phase of translocation across the SecY39 membrane might indicate that there is an increased energy barrier for initiation of translocation. We examined translocation reactions in the presence of low ATP concentrations. SecA71-driven pro-OmpA translocation at 37°C, in the presence of the PMF was examined in the presence of 0.3 to 30 μM ATP (Fig. 4). At low ATP concentrations (0.3 to 3 μM), several translocation intermediates were generated upon proteinase K digestion of the wild-type IMV reaction (Fig. 4, lanes 1 to 3, bands i-1, i-2, i-3, and i-4). The SecY39 IMV required higher ATP concentrations (10 μM and above) to achieve significant translocation of the full-length molecule (Fig. 4, lanes 9 and 10). Importantly, at lower ATP concentrations, the mutant IMV only slightly generated the lower-molecular-mass intermediates i-1, i-2, and i-3 (Fig. 4, lanes 6 to 8). In other words, translocation may not have been initiated under these conditions. Once initiated at higher ATP concentrations, the reaction continued beyond the i-4, i-3, and i-2 sites. Thus, mainly the i-1 intermediate and the full-length translocation were observed with the SecY39 mutant membrane vesicles (Fig. 4,

lanes 9 and 10). These results suggest that the *secY39* mutation increases the energy barrier required for the initial event.

Exaggerated PMF dependence is due primarily to a channel defect. The strong PMF dependence in translocation was observed with the SecY39-SecA71 and the SecY39-SecA117 combinations. To know to what extent the particular alterations in SecA contributed to this property, we isolated 20 new *secA* mutations that suppressed the cold-sensitive growth defect of the *secY39* mutation itself. They were all superactive in that they suppressed a number of different *secY* alleles. Most of the suppressor mutations were mapped within a segment of residues 451 to 580. Five alterations (to Ala, Asn, Glu, Gly, and Val) were identified at Asp580. We purified seven representative SecA variants with the alterations His484Arg, Ile502Ser, Trp541Arg, Leu571Trp, Asp580Ala, Asp580Gly, and Asp580Val. They were able to support pro-OmpA translocation into the *secY39* mutant IMV, but only when the PMF was imposed (data not shown). Thus, although a different set of SecA variants were isolated in our new mutant selection, they all behaved similarly in terms of the in vitro ability to drive pro-OmpA translocation into the SecY39 IMV. These results suggest that the exaggerated PMF dependence is caused primarily by the Arg357 alteration in the SecYEG channel.

ATPase activities of suppressor variants are not fully activated by the SecY39 IMV. We then studied functional interactions between different forms of SecA and SecY by measuring ATPase activities exhibited by SecA. We used the wild type and three variants of SecA, Ala507Val (SecA71), Ile502Ser, and Asp580Val. Assays were carried out under three conditions: (i) SecA alone was assayed to measure the intrinsic ATPase activity; (ii) assays were carried out in the presence of IMV to measure the membrane ATPase activity; and (iii) assays were carried out in the presence of IMV and pro-OmpA to measure the translocation ATPase activity. To highlight the SecYEG effects, we used IMVs in which these components were overproduced. The SecYEG contents of these IMVs were similar (data not shown). Results of assays i, ii, and iii are shown in Fig. 5 in that order from left to right for each SecA species. The meaningful values of membrane and translocation ATPase activities were obtained by subtracting the values of assay i and assay ii, respectively. Thus, filled, hatched, and open components of each column represent the intrinsic, membrane, and translocation ATPase activities.

It was noted that the mutant forms of SecA all possessed higher than normal intrinsic activities of ATPase (Fig. 5) (27). At 37°C, the activities of the SecA71, SecA(Ile502Ser), and SecA(Asp580Val) variants were 3.8-, 10.7-, and 12.0-fold higher than the wild-type activities (Fig. 5). These values were more striking at 20°C; these variants were 27.5-, 22.1-, and 86.5-fold more active than the wild-type protein. Similar features were also observed for all the other suppressor mutants of SecA examined (data not shown). Although the $SecA^+$ translocation ATPase activity was lowered by the *secY205* mutation, the suppressor forms of SecA were able to exhibit nearly normal levels of translocation ATPase activities even with the SecY205 IMV (Fig. 5B). In contrast, none on them was able to exhibit comparably high levels of the translocation ATPase activities in combination with the SecY39 IMV (Fig. 5C). The activities remained low even when the PMF was imposed (data not shown). These results suggest that the SecY39 IMV cannot fully activate even the superactive forms of SecA. The limited stimulation of the translocation ATPase activity may force the translocation reaction to depend absolutely on the PMF.

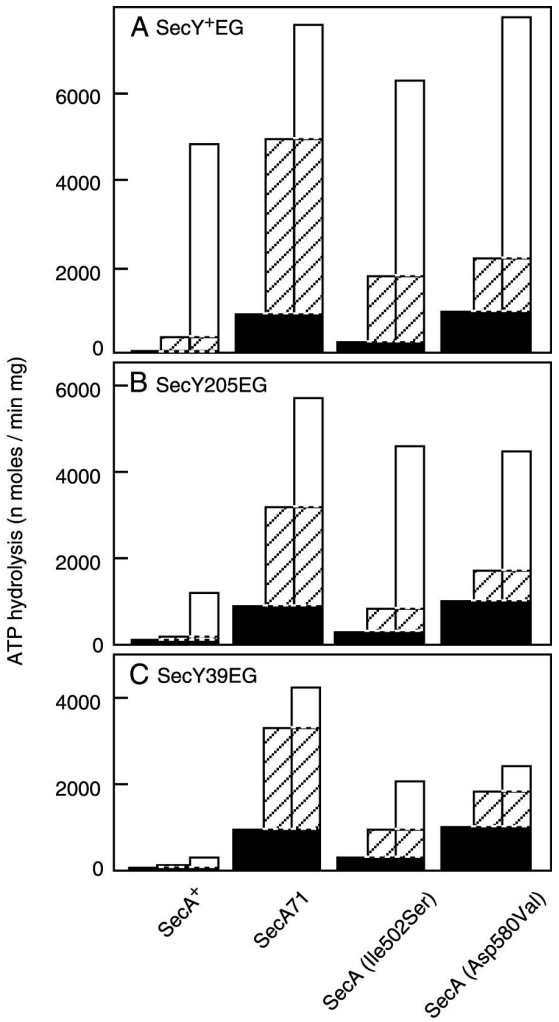

FIG. 5. ATPase activities of suppressor SecA variants. $SecA^+$, SecA71, SecA(Ile502Ser), and SecA(Asp580Val) were overproduced and purified. Their ATPase activities were measured at 37°C under three conditions: (i) SecA alone (left column in each group of three columns), (ii) in the presence of IMV (center), and (iii) in the presence of IMV and pro-OmpA (right). They are reported after subtraction of the background ATP hydrolysis in the absence of SecA. The IMVs used were prepared from cells overproducing SecYEG (A), SecY205EG (B), and SecY39EG (C). For each SecA preparation, the value shown by the left column represents the intrinsic ATPase activity, and this column and the corresponding portions of the center and right columns are colored black to indicate that these activities should have included this component. Similarly, the remaining part of the center column as well as the corresponding portion of the right column are hatched to indicate the membrane ATPase component. Thus, the open portion of the right column represents pro-OmpA-dependent translocation ATPase activity. Reaction mixtures (200 μl) contained SecA (1 μg), IMV (10 μg of protein), pro-OmpA (1 μg), SecB (15 μg/ml), 50 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 1 mM ATP, 5 mM KCN, and the ADP assay components of the coupled enzyme reaction (25). IMV and pro-OmpA were omitted for reaction i, whereas pro-OmpA was omitted for reaction ii.

DISCUSSION

We have characterized the in vitro protein translocation reaction that is mediated by the mutationally altered Sec machinery having the SecY39 subunit. Although the *secY39* mutant is viable at around 37°C, IMVs prepared from this mutant were virtually inactive in translocating pro-OmpA. As a means to investigate the mutationally altered reaction parameters in vitro, we used several SecA variants (superactive forms) that had been isolated as suppressors against this and other *secY* mutations. The altered forms of SecA indeed allowed the activity measurements. In every case, pro-OmpA translocation into the SecY39 IMV depended strongly on the PMF. We reason that the exaggerated PMF dependence is due primarily to the channel defect, as the SecA variants used were all produced by gain-of-function-type mutations in *secA*, which actually lowered the PMF dependence when combined with the wild-type SecYE.

The SecY39 mutant IMV can support the initial phase of translocation, in which signal sequence is processed, only when the PMF is imposed and when a superactive SecA is used to drive the reaction. In contrast, precursor binding to the IMV did not show this requirement. Thus, the PMF-dependent step should come after the binding of preprotein to the membrane but before translocation of the signal peptide cleavage site to the lumenal side of the IMV, where the leader peptidase active site resides. Strikingly, once translocation initiation had been allowed in the presence of the PMF, further forward movement of the polypeptide occurred even when the PMF was dissipated. These results indicate that the PMF is required for the initiation step of the translocation reaction that is mediated by the SecY39 translocase.

The ATPase activity of SecA is subject to regulation by interacting ligands, including a SecYEG channel component and preprotein. The SecA activation function of SecY is impaired by mutations such as *secY39* and *secY205* (21, 32). The superactive forms of SecA have markedly enhanced ATPase activity in the absence of the ligands. This enhancement was particularly evident at 20°C, the restrictive temperature for the *secY* mutations, and might therefore explain the suppression. This explanation may hold for the suppression against the *secY205* mutation, whose defect can be alleviated without imposition of the PMF and was accompanied by normal activation of the ATPase. In contrast, the SecY39 defect was not overcome by the ATPase alterations alone. It was only overcome by the combined effects of the upregulated ATPase and the PMF. We speculate that the Arg357 residue is so important that its replacement by His seriously compromised the SecA activation function of SecYEG. Thus, translocation mediated by the SecY39 translocase requires the PMF as well as elevated concentrations of ATP.

The SecY39 translocase was characterized by its requirement for higher than normal ATP concentrations to complete translocation of a model preprotein pro-OmpA. Although the wild-type reaction proceeds to certain distinct stages of pro-OmpA translocation at low concentrations (low micromolar) of ATP, SecY39 translocase was less competent in producing such intermediate species under the limited ATP availability. This observation is consistent with the notion that the mutant translocase requires a higher concentration of ATP for initiating the translocation reaction. Once initiated at the high ATP concentration, the reaction goes to completion. The SecY39 alteration might affect the initial steps of translocation so that it requires higher levels of energy supply in the forms of ATP and the PMF.

In the initiation phase of translocation, the signal peptide region of preprotein forms a loop, with its amino terminus remaining on the cytoplasmic surface of the membrane. Thus, a pair of antiparallel polypeptide chains must be inserted into the SecYEG channel. This initiation step should be contrasted with the continuation phase, in which only a single polypeptide chain may be moving through the channel. Thus, an initiation reaction that involves two strands may have a higher energy barrier than the continuation reaction. A segment of about 30 amino acids at the N-terminal region of the mature sequence was proposed to comprise, together with the signal sequence, a distinct translocation initiation domain; positively charged residues are to be avoided in this segment (3).

The PMF may facilitate the initiation reaction by assisting the Sec machinery recognition of the initiation domain or its subsequent insertion. In doing so, it might affect either the SecYEG channel or the preprotein. As model experiments show that the PMF can directly promote the penetration of signal peptide into the lipid bilayer (13, 36), a direct action of the PMF against a preprotein should not be excluded. Also, the PMF dependence differs among different preproteins (13). On the other hand, the observation that the PMF can stimulate translocation of a preprotein without any charged residues in the mature region points to a role of the PMF other than the electrophoretic action against the preprotein (15).

It is conceivable that the PMF also affects the conformation of the SecYE channel so that it becomes ready to accept the initiation loop of a preprotein. Such a role for the PMF might be related to its role in allowing translocation of a preprotein with a disulfide loop (35). Some mutational alterations of SecY are known to mimic the PMF-activated state of the translocase (10, 26, 29, 37), and we have shown that some such alterations in SecY can intragenically suppress the *secY39* defect (26). Recent studies suggest that the SecYEG complex undergoes higher-order assembly into an active oligomeric form (5, 19). The effects of the *secY39* alteration as well as those of the PMF should also be considered in the contexts of superassembly of the SecYEG complexes (7). The SecY39 mutant form of SecY may contribute to our understanding of the protein translocation channel at work through future study of the structural biology of this molecular machine.

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