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Characterization of a Mutant Form of SecA That Alleviates a SecY Defect at Low Temperature and Shows a Synthetic Defect with SecY Alteration at High Temperature[1]

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The *secY205* mutant is cold-sensitive for protein export, with an *in vitro* defect in supporting ATP- and preprotein-dependent insertion of SecA into the membrane. We characterized SecA81 with a Gly516 to Asp substitution near the minor ATP-binding region, which suppresses the *secY205* defect at low temperature and exhibits an allele-specific synthetic defect with the same SecY alteration at 42°C. The overproduced SecA81 aggregated *in vivo* at temperatures above 37°C. Purified SecA81 exhibited markedly enhanced intrinsic and membrane ATPase activities at 30°C, while it was totally inactive at 42°C. The trypsin digestion patterns indicated that SecA81 has some disorder in the central region of SecA, which encompasses residues 421–575. This conformational abnormality may result in unregulated ATPase at low temperature as well as the thermosensitivity of the mutant protein. In the presence of both proOmpA and the wild-type membrane vesicles, however, the thermosensitivity was alleviated, and SecA81 was able to catalyze significant levels of proOmpA-stimulated ATP hydrolysis as well as proOmpA translocation at 42°C. While SecA81 was able to overcome the SecY205 defect at low temperature, the SecY205 membrane vesicles could not significantly support the translocation ATPase or the proOmpA translocation activity of SecA81 at 42°C. The inactivated SecA81 molecules seemed to jam the translocase since it interfered with translocase functions at 42°C. Based on these results, we propose that under preprotein-translocating conditions, the SecYEG channel can stabilize and activate SecA, and that this aspect is defective for the SecA81–SecY205 combination. The data also suggest that the conformation of the central region of SecA is important for the regulation of ATP hydrolysis and for the productive interaction of SecA with SecY.

Key words: ATPase, *Escherichia coli*, protein translocation, SecA, SecY.

Translocation of proteins across membranes is an obligatory process for cells. In *Escherichia coli*, newly synthesized preproteins are first recognized by the secretion-specific chaperone, SecB, which targets them to SecA, an ATPase residing both in the cytosol and on the plasma membrane (*1*, *2*). The binding of ATP to SecA drives the insertion of a 20–30 residue segment of the preprotein into the membrane, and ATP hydrolysis leads to the release of the bound precursor (*3*). In catalyzing this preprotein movement into the membrane, SecA itself undergoes striking conformational changes. In the presence of ATP, a preprotein, and functional membrane vesicles, substantial portions of SecA insert deeply into the membrane in such a way that they are protect them from proteases added to the cytosolic side and accessible from the periplasmic side (*4–6*). The inserted SecA segment deinserts upon the hydrolysis of ATP (*4*).

The membrane-embedded portion of protein translocase contains SecY, SecE, and SecG, which are thought to constitute a channel-like pathway for protein translocation (*1*, *2*, *7*). SecY, which has 10 transmembrane segments, serves as a central subunit with which SecE and SecG associate independently (*7*, *8*). A central question about the prokaryotic protein translocase functions is how the SecYEG channel complex interacts with the SecA motor protein to allow the insertion/deinsertion cycles of the latter. It has been reported that SecG undergoes topology inversion, which is apparently coupled with the SecA insertion event (*9*). We have been characterizing a series of cold-sensitive mutations in *secY* with respect to their interaction with SecA. It was found that several mutations in the two most C-terminal cytosolic domains of SecY affect SecA ATPase activities *in vitro* (*10*). More specifically, a mutant form of SecY (SecY205) was found to have a defect in the ability to allow the ATP- and preprotein-dependent productive insertion of SecA (*11*). We isolated *secA* mutations that suppressed the *secY205* translocation defect *in vivo*. The *in vitro* properties of one of the SecA variants agreed well with the *in vivo* genetic interactions, thus providing evidence that the SecA

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Abbreviation: IMV, inverted membrane vesicle.

insertion reaction is indeed important for protein translocation and that SecY plays a crucial role in this reaction. Another *secA* mutation, *secA81* for Gly516 to Asp alteration near the minor ATP-binding domain of SecA, showed a peculiar phenotype. While it suppressed the cold-sensitivity of the *secY205* mutant, the *secY205-secA81* double mutant was in turn unable to grow at 42°C (*11*). This synthetic temperature-sensitivity, observed for the specific combination of these *secY* and the *secA* alleles, may offer a unique opportunity to investigate the interactions between SecY and SecA. In the work reported in this paper, we have characterized the SecA81 mutant form of SecA.

EXPERIMENTAL PROCEDURES

Escherichia coli Strains and Media—The bacterial strains used in this study were all derived from MC4100 (*12*) and contained the *ompT::kan* marker (*13*). Additionally, they carried the following mutations as described previously (*11*). GN40 carried *leu-82*::Tn*10*. GN17 carried *secY205* and *leu-82*::Tn*10*. GN43 carried *secA81* and *leu-82*::Tn*10*. GN29 carried *secY205*, *secA81*, and *leu-82*::Tn*10*. TW156 carried Δ(*atpB-atpC*), *zhd-33*::Tn*10* and *rpsE*. GN5 carried *secY205*, Δ(*atpB-atpC*), and *zhd-33*::Tn*10*. GN45 and GN48 were F′ *lacI*^{q} *lacPL8* *lacZ*$^{+}$*Y*$^{+}$*A*$^{+}$ derivatives of GN40 and GN43, respectively. Unless otherwise stated, cells were grown in L-medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 1.7 mmol NaOH per liter).

Pulse-Chase Analysis of Protein Export—Cells were grown in minimal medium M9 (*12*) supplemented with 0.4% glycerol, 0.4% maltose, and 18 amino acids (20 μg/ml, other than Met and Cys). Pulse-labeling with [^{35}S]methionine, chase with unlabeled methionine, immunoprecipitation of maltose-binding proteins and SDS-PAGE were done essentially as described previously (*11*). Radioactivities in the precursor and mature forms of the maltose-binding protein were determined using a BAS1800 phosphor imager (Fuji Film). Values were corrected for the methionine residues present in the signal sequence.

Purification of SecA and Its Variants—Wild type SecA protein was overproduced in strain GN45/pKY173 and purified essentially as described previously (*11*). Overproduction and purification of SecA36 was also described previously (*11*). Similarly, a SecA81-overproducing plasmid (pGN102) was constructed and introduced into strain GN48 carrying the chromosomal *secA81* mutation. One of the resulting transformants was grown at 25°C in L-medium containing 50 μg/ml ampicillin and induced with 1 mM isopropyl-β-D-thiogalactoside for 2 hr. Cells were disrupted by a French pressure cell in 10 mM Tris-HCl (pH 8.0), 12 mM Mg-acetate, 60 mM KCl, 1 mM dithiothreitol, and 0.2 mM phenylmethyl sulfonylfluoride, and ultracentrifuged. The supernatant was then applied to a Red agarose column, and eluted with 0.06–2 M KCl in the same buffer. The 2 M KCl fractions were pooled and purified further by MonoQ HR 5/5 column chromatography.

Preparation of IMV—For the preparation of IMV with the wild-type set of SecYEG translocase components, strain TW156 was used, whereas IMV containing the SecY205 form of the SecY subunit was prepared from strain GN5. Cells were grown at 37°C in L-medium containing glucose (0.4%), harvested by centrifugation at 4°C, washed with 10 mM Tris-HCl (pH 8.1), and resuspended in buffer containing 30 mM Tris-HCl (pH 8.1), 20% (w/v) sucrose, and 1 mM dithiothreitol. To this suspension was added one-tenth volume of 1 mg/ml lysozyme dissolved in 0.1 M EDTA (pH 7.5). After incubation on ice for 30 min, the resulting spheroplasts were disrupted by repeated (2 times) passage through a French pressure cell at 7,500 psi at 4°C. Membranes were isolated by ultracentrifugation, resuspended in 50 mM Tris-HCl (pH 7.5) containing 3 mM EDTA and 1 mM dithiothreitol, and layered on a 33–48% step gradient of sucrose in 3 mM EDTA (pH 7.5)–1 mM dithiothreitol. The sample was then centrifuged at 4°C in a Beckmann 70 Ti rotor at 38,000 rpm for 1 h. Materials at the sucrose interphase were taken, diluted, pelleted, and resuspended in 50 mM HEPES-KOH (pH 7.5) containing 50 mM KCl, 5 mM Mg-acetate, and 10 mM 2-mercaptoethanol. After centrifugation, the membranes were suspended again in the same buffer containing 6 M urea and incubated on ice for 1 h. Urea-washed IMVs were pelleted and finally suspended in the same buffer without urea and stored in small portions at −80°C.

Measurement of ATPase Activities—The ATPase activities of SecA were assayed either by an enzyme coupling method (*14*) or by direct quantitation of inorganic phosphate liberated using malachite green (*15*). In the former assay, ADP generated by the ATPase action was recycled back to ATP using the coupled reactions of pyruvate kinase (phosphoenolpyruvate + ADP → pyruvate + ATP) and lactate dehydrogenase (pyruvate + NADH + H^{+} → lactate + NAD^{+}). The reaction was started by adding 10 μl of 100 mM ATP to 1 ml of mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM $MgSO_4$, 3 mM phosphoenolpyruvate, 0.25 mM NADH, 5 units of pyruvate kinase, 7.5 units of lactate dehydrogenase, and 30 μg of SecA. The rate of ATP hydrolysis was calculated from the linear phase of the decrease in A_{340} of NADH. The latter assay mixture consisted of 50 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 4 mM ATP, and SecA (40 μg/ml). Additionally, either IMV (60 μg protein/ml) or IMV plus urea-denatured proOmpA (60 μg/ml; Ref. *11*) was included to measure membrane-ATPase and translocation-ATPase activities, respectively. After incubation at 30 or 42°C for the indicated time, portions were subjected to malachite green color development.

In Vitro Translocation of proOmpA—The reaction mixture consisted of 50 mM Tris-HCl (pH 8.0), 5 mM $MgSO_4$, 500 μg bovine serum albumin/ml, 1 mM ATP, 16 μg SecB/ml, 10 μg SecA/ml, IMV (250 μg protein/ml), ATP regeneration system (5 mM creatine phosphate and 100 μg/ml of creatine kinase), and 1/20 volume of ^{35}S-labeled and 6 M urea-denatured proOmpA (*11*). Samples were incubated at the temperatures specified and removed for subsequent proteinase K (100 μg/ ml) treatment on ice for 20 min and SDS-PAGE (*11*).

Preparation of Site-Specific SecA Antibodies and Western Blotting—Synthetic polypeptides with amino acid sequences corresponding to residues 137–151, 345–362, 421–437, 560–575, and 704–719, of SecA were named peptide-1, 3, 4, 6, and 8, respectively. These peptides were conjugated with keyhole limpet hemocyanin and injected into rabbits. The antisera thus prepared (custom product service from Sawady Technology) were purified by affinity chromatography using HiTrap NHS-activated columns (Pharmacia) that were covalently conjugated with 0.5 mg of the respective SecA peptides. After washing with 25 mM Tris-HCl

(pH 7.5)–150 mM NaCl, the antibodies were eluted with 0.1 M glycine-HCl (pH 2.7) and neutralized immediately with 1 M Tris-HCl (pH 8.5). Western blotting was carried out as described previously (*8*).

RESULTS

Temperature-Sensitivity of the secA81-secY205 Double Mutant—The *secY205* mutant of *E. coli* cannot grow at 20°C and protein export in this mutant is retarded at this temperature. Even at 37°C, protein export is significantly defective (*16*), and the defect was reproduced *in vitro* using IMV (*10*). It was also shown that IMV from this mutant does not allow any significant ATP- and preprotein-dependent insertion of SecA at any temperature *in vitro* (*11*). We isolated several mutations in *secA* that suppress the cold-sensitive growth phenotype of the *secY205* mutant. Among them, *secA81* was peculiar in that it exhibited a severe temperature-sensitive phenotype when combined with the *secY205* mutation. The *secA81* mutation causes a single amino acid substitution (Gly516 to Asp) near the putative second ATP-binding site of SecA. Both its ability to suppress the cold-sensitivity and its inability to support growth at high temperature (42°C) were observed specifically in combination with *secY205*, but not with other *secY* mutations (*11*). The growth of *secA81-secY205* double mutant cells, as measured by turbidity increase, stopped about 60 min after a 30 to 42°C temperature shift (data not shown). The number of viable cells started to decline soon after the temperature shift (Fig. 1, solid triangles). The rapid expression of temperature sensitivity suggests that these two mutant proteins have a serious incompatibility at 42°C in their functions to support cell viability.

The protein export abilities of these strains were then examined by pulse-labeling the maltose-binding protein, a periplasmic protein, at various time points after temperature shift (30 to 42°C). As Fig. 2A shows, at 30°C (time zero), 80–90% of the maltose-binding protein is labeled as the mature (and presumably exported) form in both wild-type and *secA81* mutant cells (open circle and open triangle). This ratio was only about 30% or less for the *secY205* mutant (solid circle), consistent with its being a cold-sensitive *sec* mutant. Export of the maltose-binding protein was significantly recovered in the *secY205-secA81* double mutant cells (about 70% mature form; Fig. 2A, solid triangle at time 0), confirming that *secA81* is a suppressor of *secY205*. However, the export of maltose-binding protein in the double mutant cells was retarded (to about 25% mature form) within 2 min upon exposure to 42°C (Fig. 2A, solid triangles). Thus, the incompatibility of these two mutations at 42°C was expressed very rapidly at the level of protein export. The kinetics of export of the maltose-binding protein was examined by pulse-chase experiments at 4 min after a temperature shift from 37 to 42°C (Fig. 2B). In the double mutant cells, about 50% of the maltose-binding protein remained in the precursor form even after chase for 16

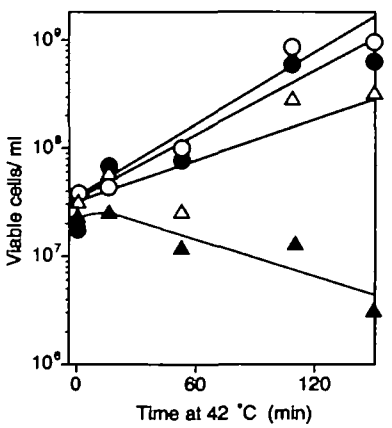

Fig. 1. **Temperature-sensitivity of the *secA81-secY205* double mutant.** Cells of GN40 (*sec*$^+$; open circles), GN17 (*secY205*; solid circles), GN43 (*secA81*; open triangles), and GN29 (*secA81 secY205*; solid triangles) were grown in L medium at 30°C. The temperature was then shifted to 42°C during early exponential phase. At each time point after the temperature shift, the cultures were diluted and 0.1 ml portions were spread on L-agar plates, which were then incubated at 30°C overnight. The numbers of viable cells was calculated from the numbers of colonies appeared.

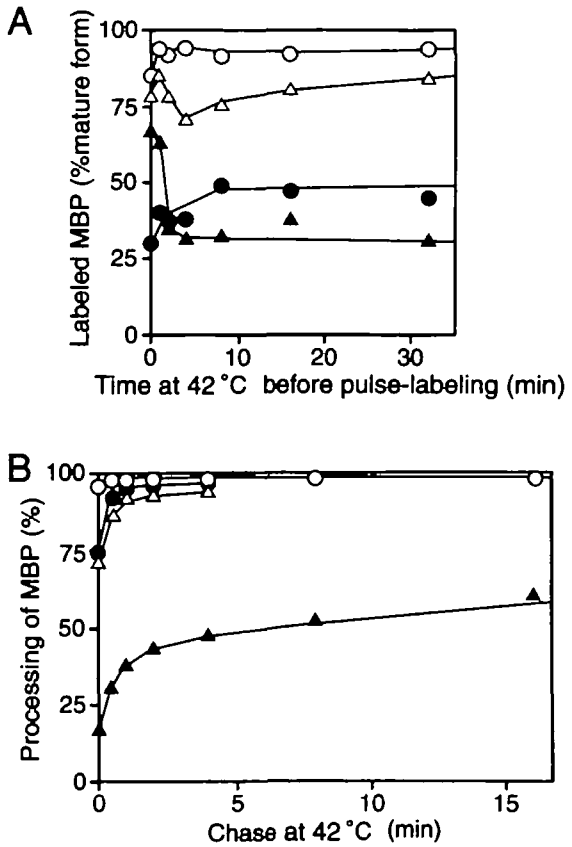

Fig. 2. **Export of maltose-binding protein.** (A) Cells of GN40 (*sec*$^+$; open circles), GN17 (*secY205*; solid circles), GN43 (*secA81*; open triangles), and GN 29 (*secA81 secY205*; solid triangles) were grown in amino-acid supplemented M9 medium, first at 30°C and then shifted to 42°C. At each time point after the temperature shift, cells were pulse-labeled with [^{35}S]methionine for 40 s. Maltose-binding protein was immunoprecipitated and separated by SDS-PAGE into the precursor and mature forms. The radioactivities associated with each species were determined using a BAS1800 phosphor imager (Fuji Film), and the proportions of the mature form in total maltose-binding protein are shown. (B) The same set of strains as shown in (A) were grown first at 37°C and then at 42°C for 4 min, after which the cells were pulse-labeled with [^{35}S]methionine for 40 s and chased with unlabeled excess methionine for the indicated periods. Samples were then processed as described in (A).

min (Fig. 2B, solid triangles). In contrast, the strains having only one of the mutations exhibited only a slight delay in export (Fig. 2B, open triangles and solid circles) as compared with the wild-type cells (open circles). Note that protein export in the *secY205* mutant was faster in the experiments shown in Fig. 2B, in which cells were first grown at 37°C, than in Fig. 2A, in which the cells were first grown at 30°C. Similar results were obtained when the export of OmpA was examined, although the defect was less severe for this protein than for maltose-binding protein (data not shown). These results suggest that the temperature sensitivity in the *secA81-secY205* double mutant strain is accom-

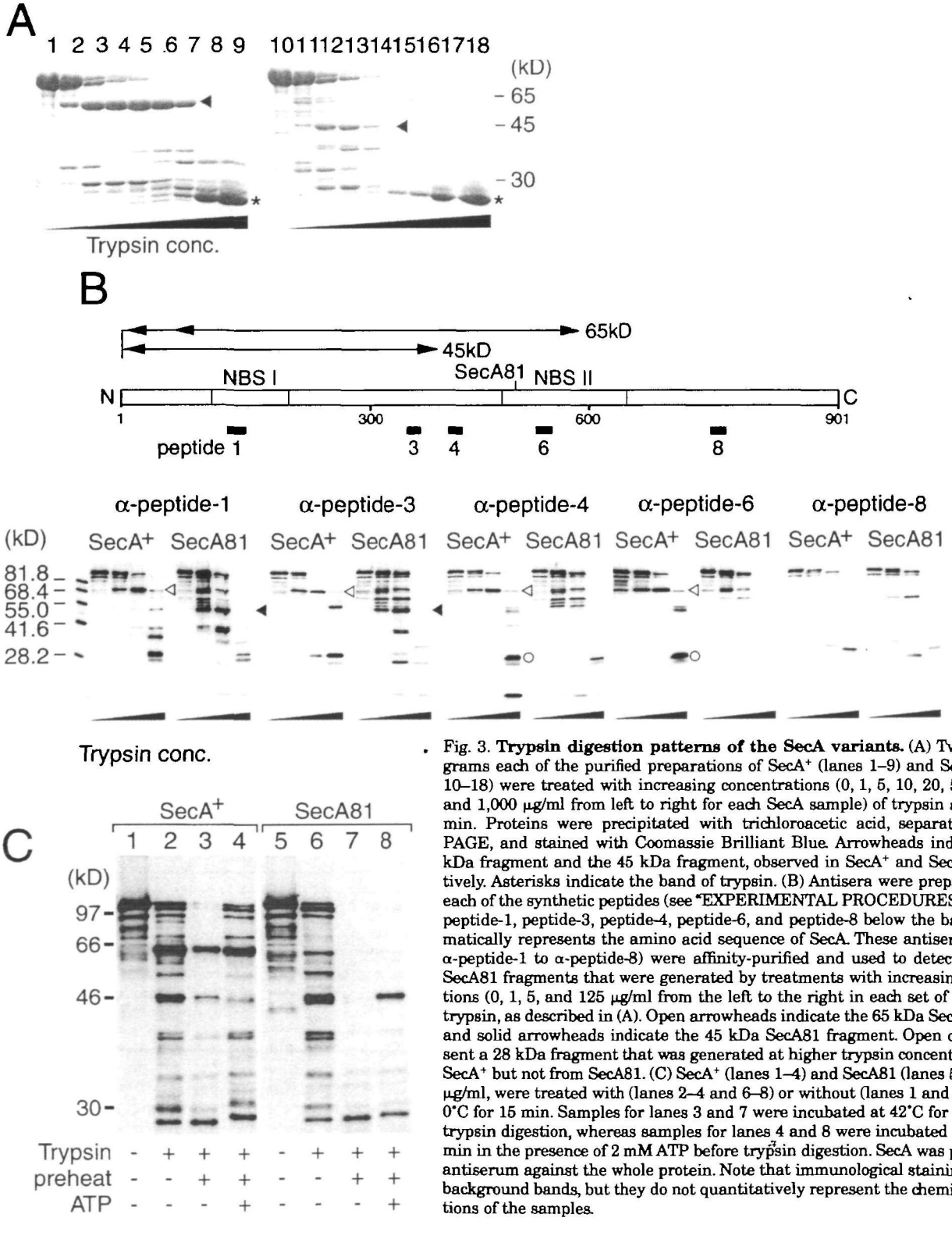

Fig. 3. **Trypsin digestion patterns of the SecA variants.** (A) Twenty micrograms each of the purified preparations of SecA$^+$ (lanes 1–9) and SecA81 (lanes 10–18) were treated with increasing concentrations (0, 1, 5, 10, 20, 50, 100, 500, and 1,000 μg/ml from left to right for each SecA sample) of trypsin at 0°C for 15 min. Proteins were precipitated with trichloroacetic acid, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue. Arrowheads indicate the 65 kDa fragment and the 45 kDa fragment, observed in SecA$^+$ and SecA81, respectively. Asterisks indicate the band of trypsin. (B) Antisera were prepared against each of the synthetic peptides (see "EXPERIMENTAL PROCEDURES"), shown as peptide-1, peptide-3, peptide-4, peptide-6, and peptide-8 below the bar that schematically represents the amino acid sequence of SecA. These antisera (shown as α-peptide-1 to α-peptide-8) were affinity-purified and used to detect SecA$^+$ and SecA81 fragments that were generated by treatments with increasing concentrations (0, 1, 5, and 125 μg/ml from the left to the right in each set of reactions) of trypsin, as described in (A). Open arrowheads indicate the 65 kDa SecA$^+$ fragment and solid arrowheads indicate the 45 kDa SecA81 fragment. Open circles represent a 28 kDa fragment that was generated at higher trypsin concentrations from SecA$^+$ but not from SecA81. (C) SecA$^+$ (lanes 1–4) and SecA81 (lanes 5–8), each 50 μg/ml, were treated with (lanes 2–4 and 6–8) or without (lanes 1 and 5) trypsin at 0°C for 15 min. Samples for lanes 3 and 7 were incubated at 42°C for 5 min before trypsin digestion, whereas samples for lanes 4 and 8 were incubated at 42°C for 5 min in the presence of 2 mM ATP before trypsin digestion. SecA was probed using antiserum against the whole protein. Note that immunological staining gave high background bands, but they do not quantitatively represent the chemical compositions of the samples.

panied by a strong defect in the protein export mechanism, which is probably the primary cause of the temperature sensitivity.

Properties of the SecA81 Mutant Protein—In order to purify the mutant form of SecA, SecA81 was overexpressed from a plasmid carrying the *secA* gene with this mutation. Although a high level of overproduction of the SecA81 protein was possible, a large fraction of it precipitated after low-speed and high-speed centrifugations. We found that the cultivation temperature affected the solubility of this protein. After growth at 37 or 42°C, most of the protein was recovered in the pellet fraction after high or low speed centrifugation, respectively, whereas recovery in the supernatant increased markedly when cells were grown at 25°C or lower (data not shown). Thus, SecA81 was induced at 25°C and purified by means of Red-Agarose and MonoQ column chromatographies. The wild-type and SecA36 form of SecA were also purified. Each of these preparations was at least about 90% pure as judged from the SDS-PAGE patterns (Fig. 3A, lanes 1 and 10; data not shown for SecA36). The results of N-terminal sequencing showed that $SecA^+$ and SecA81 retained the intact N-terminus, whereas SecA36, which was purified with a slightly different time schedule, comprised two species with the intact N-terminus and Val9 at the N-terminus.

To examine whether any gross conformational changes are present in SecA81, the trypsin digestion patterns of the SecA preparations were compared. $SecA^+$ produced a fragment of about 65 kDa at low trypsin concentrations (Fig. 3A, lanes 2–7; arrowheads), which was not produced significantly from the SecA81 protein. Instead, a new fragment of about 45 kDa was produced (Fig. 3A, lanes 12–14; arrowhead). The results of N-terminal sequencing indicated that the 65 kDa fragment of $SecA^+$ begins with Val78, whereas the 45 kDa fragment of SecA81 begins with Leu5. Thus, these fragments are derived from the N-terminal region of

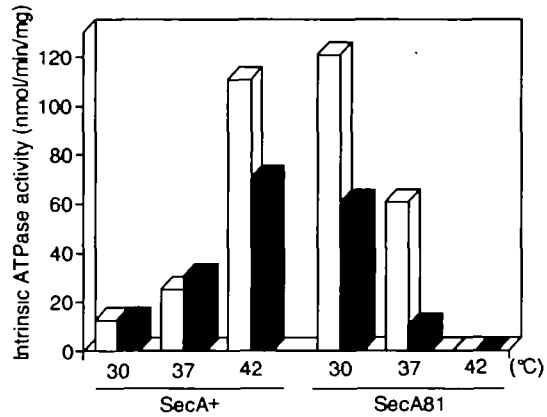

Fig. 4. **Intrinsic ATPase activities.** Intrinsic ATPase activities of wild-type SecA and SecA81, as indicated, were measured by the enzyme coupling assay ("EXPERIMENTAL PROCEDURES") at the temperatures indicated. In one series of assays (open columns), SecA, ATP and the other components were pre-mixed on ice and then the reaction mixtures were incubated at each temperature. In another series of reactions (hatched columns), the components other than ATP were preincubated at each temperature for 5 min followed by the addition of ATP. In both cases, the rate of ATP hydrolysis was calculated form the initial linear phase of the reaction.

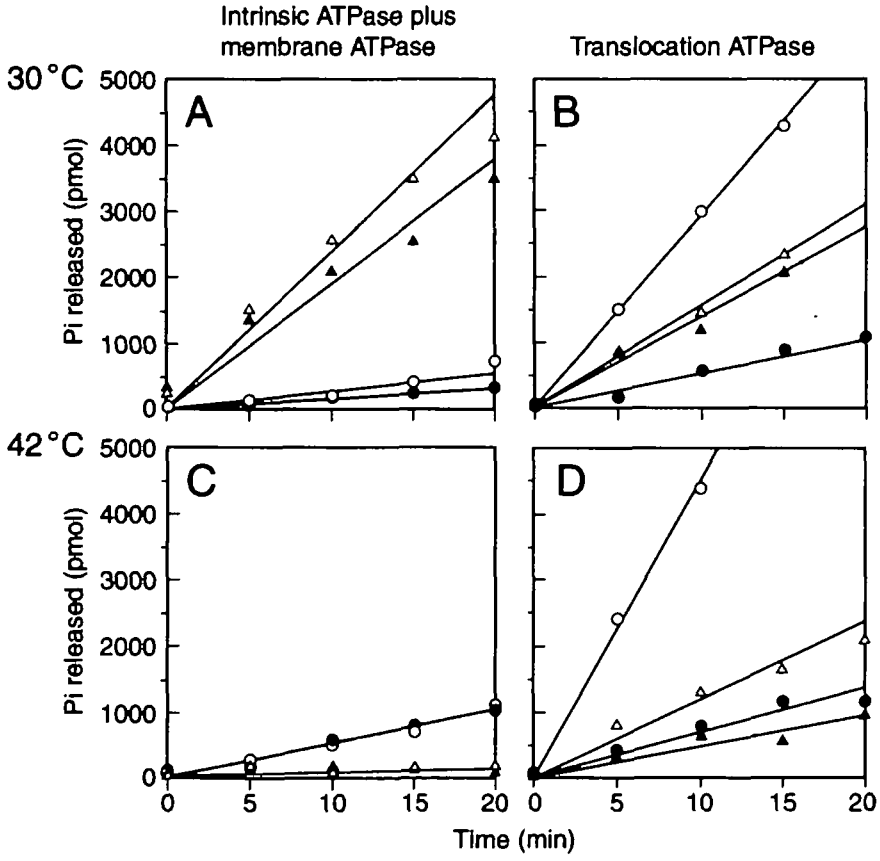

Fig. 5. **Membrane- and translocation-ATPase activities.** ATPase activities of $SecA^+$ (circles) and SecA81 (triangles) were assayed at 30°C (A and B) or at 42°C (C and D) by quantitating malachite green absorbance due to the liberation of inorganic phosphate. Reactions were carried out in the presence of the $SecY^+$ IMV (open symbols) or the SecY205 IMV (solid symbols). The membrane ATPase activities, after subtraction of the intrinsic ATPase activity (data not shown for the activities in the absence of IMV), were 41 and 299 nmol/min/mg for $SecA^+$ and SecA81, respectively, at 30°C. To measure the translocation ATPase activities, proOmpA was included in addition to IMV (B and D). In the latter cases, the values of the corresponding reactions in the absence of proOmpA were subtracted.

SecA, placing the C-terminal ends of the 65 kDa fragments within the NBS II region and that of the 45 kDa fragment in the region N-terminal to NBS II (Fig. 3B). Thus, the latter region of SecA is sensitized to trypsin by the SecA81 alteration.

We prepared a series of antibodies against several selected peptides derived from the SecA amino acid sequence (Fig. 3B), and used them to probe the trypsin digestion products. The 65 kDa fragment reacted with antibodies against peptide 1, peptide 3, peptide 4, and peptide 6 (Fig. 3B, open arrowheads), whereas the SecA81-specific 45 kDa fragment was recognized by antibodies against peptide 1 and peptide 3, but not by the other peptide antibodies used (Fig. 3B, solid arrowheads). These results confirm the identity of these fragments as shown in Fig. 3B. We also noticed that a prominent band of about 28 kDa was produced from $SecA^+$ at higher trypsin concentrations; it reacted with antibodies against peptide 4 and peptide 6 (Fig. 3B, open circles). This fragment was not produced from SecA81 (Fig. 3B). These results, taken together, indicate that the SecA81 mutant form of SecA has an altered conformation for the region (residues 420 to 580) corresponding to peptide 4 to peptide 6 of SecA. The N-terminal half of the NBS II region and its N-terminally adjacent segment are less tightly folded in the mutant protein.

To examine temperature-induced conformational changes, the $SecA^+$ and SecA81 preparations were incubated at 42°C for 5 min and subjected to trypsin digestion at 0°C. It was found that the trypsin-sensitivity of both proteins became exaggerated by preheating (Fig. 3C, lanes 3 and 7). Thus, they underwent irreversible conformational changes at 42°C, and this effect was more pronounced for SecA81 than for $SecA^+$. The inclusion of ATP during the heating period protected the wild-type protein almost completely and the mutant protein partially (Fig. 3C, lanes 4 and 8). These results indicate that SecA81 is more thermosensitive than the wild type SecA protein.

ATPase Activities—Intrinsic ATPase activities of $SecA^+$ and SecA81 were measured at 30, 37, and 42°C. At 30°C, SecA81 exhibited about 8 times as much activity as the wild type protein (Fig. 4, open columns). At 37°C, the activity of SecA81 was about two-fold higher than the wild-type value, whereas the mutant protein was almost inactive at 42°C. When these proteins were preincubated for 5 min in the absence of ATP at different temperatures before measurement of the ATPase activity, SecA81 showed reduced activities (Fig. 4, hatched columns). Although the wild-type protein was only slightly inactivated at 42°C, SecA81 activity dropped about 6-fold when a preincubation at 37°C was included. These results indicate that SecA81 is thermolabile. At the same time, SecA81 has higher than normal ATPase activity at lower temperatures in the absence of both membranes and preprotein.

ATPase activity in the presence of inverted membrane vesicles is referred to as "membrane ATPase." ATPase activity that is stimulated over the membrane ATPase activity by the presence of a preprotein is referred to as translocation ATPase activity (*15*). At 30°C, the membrane ATPase activities of SecA81, whether the IMVs used were from the *secY*$^+$ strain or *secY205* strain, were again about 10-fold higher than those of wild-type SecA (Fig. 5A, compare triangles with circles). While wild-type SecA, in combination with wild-type IMV, was activated more than 10-fold by proOmpA (Fig. 5B, open circles), SecA81 was activated less than two-fold, resulting in the translocation ATPase activity that was only about 50% of the wild-type activity (Fig. 5B, open triangles). Although the translocation ATPase activity of $SecA^+$ was markedly reduced when SecY205 IMV was used (Fig. 5B, solid circles), that of SecA81 was little affected by the IMV source (Fig. 5B, triangles). Thus, the translocation ATPase activity in combination with SecY205 IMV was more than 2-fold higher for SecA81 than for

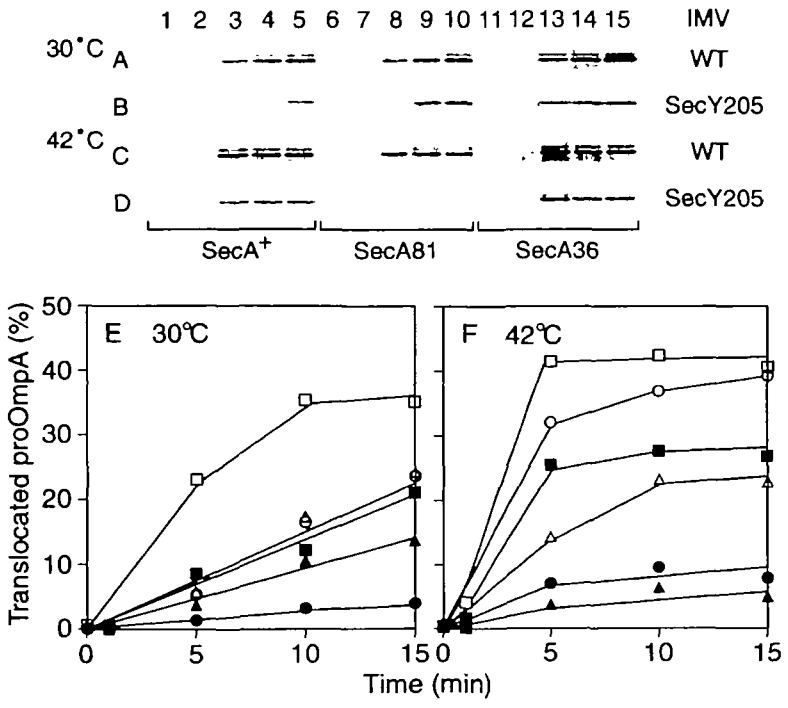

Fig. 6. ***In vitro* abilities of SecA to drive proOmpA translocation.** Translocation of ^{35}S-labeled proOmpA was assayed at 30°C (A and B) or at 42°C (C and D), using either the $SecY^+$ IMV (A and C) or the SecY205 IMV (B and D), in combination with either $SecA^+$ (lanes 1–5), SecA81 (lanes 6–10), or SecA36 (lanes 11–15). Reactions were followed for 0 (lanes 1, 6, and 11), 1 (lanes 2, 7, and 12), 5 (lanes 3, 8, and 13), 10 (lanes 4, 9, and 14), and 15 (lanes 5, 10, and 15) min in the presence of ATP. Samples were then treated with 0.1 mg/ml proteinase K and undigested OmpA was visualized after SDS-PAGE and phosphor imager exposure. The results in A and B (at 30°C) and results in C and D (at 42°C) are graphically depicted in E and F, respectively. Open symbols, $SecY^+$ IMV; solid symbols, SecY205 IMV; circles, $SecA^+$; squares, SecA36; triangles, SecA81.

$SecA^+$. This is consistent with the *in vivo* ability of SecA81 to suppress the *secY205* defect.

When assayed at 42°C, the wild-type SecA protein (in combination with wild-type IMV) showed membrane and translocation ATPase activities that were about 1.6-fold higher than at 30°C (Fig. 5C, and D, open circles). The translocation ATPase activity at this temperature again decreased when the SecY205 IMV was used (Fig. 5D, solid circles). SecA 81 was almost inactive with regard to membrane ATPase activities at 42°C (Fig. 5C, triangles), and was also inactive at 42°C when proOmpA alone was added (data not shown). Interestingly, however, SecA81 exhibited significant translocation ATPase activity even at 42°C in the presence of wild-type IMV and proOmpA (Fig. 5D, open triangles). In other words, its thermosensitivity is alleviated under the conditions of active translocation. In contrast, the same SecA protein showed a very low translocation ATPase activity at 42°C in combination with the SecY205 IMV (Fig. 5D, solid triangles). This result appears to be consistent with the synthetic temperature sensitivity of the *secY205* and *secA81* mutations observed *in vivo*.

Translocation Activity—SecA proteins were then examined for their ability to drive translocation of proOmpA into IMVs having wild-type SecY protein or the SecY205 mutant protein. Whereas $SecA^+$ had greatly decreased translocation activity in combination with SecY205 IMV at both 30°C (Fig. 6B, lanes 1–5; Fig. 6E, solid circles) and 42°C (Fig. 6D, lanes 1–5; Fig. 6F, solid circles), the activity at 42°C was slightly higher than that at 30°C. SecA36 was active even with the SecY205 IMV (Fig. 6, B and D, lanes 11–15; Fig. 6, E and F, solid squares). SecA81 was active in translocating proOmpA into wild-type IMV at both 30 and 42°C (Fig. 6, A and C, lanes 6–10; Fig. 6, E and F, open triangles). Its activity at 30°C was only slightly decreased when the SecY205 IMV was used (Fig. 6B, lanes 6–10; Fig. 6E, solid triangles), consistent with its being a suppressor of the SecY205 defect. SecA81 was barely active at 42°C in combination with the mutant IMV (Fig. 6D, lanes 6–10; Fig. 6F, solid triangles), an observation that is consistent with the *in vivo* temperature-sensitivity of this combination. The relative activities of various combinations and at different temperatures did not vary greatly when the assays were repeated in the presence of up to 100 ng of unlabeled proOmpA (data not shown). Thus, the activities measured quantitatively represent the catalytic abilities of the different *in vitro* reaction systems.

SecA81 Is Inactivated at 42°C on the SecYEG Channel Having the Altered SecY205 Subunit—The results shown above indicate that SecA81 does not function in combination with the mutationally altered SecY205 channel at 42°C. This inability, which was expressed specifically in this combination, may not be fully explained by the intrinsic thermal instability of SecA81 alone. Thus, it is conceivable that the defect is due to some abortive interaction between SecA81 and the SecY205 translocation channel. To address these questions, we examined whether SecA81 can block the translocation reaction in combination- and temperature-specific manners. We used the temperature-indepen-

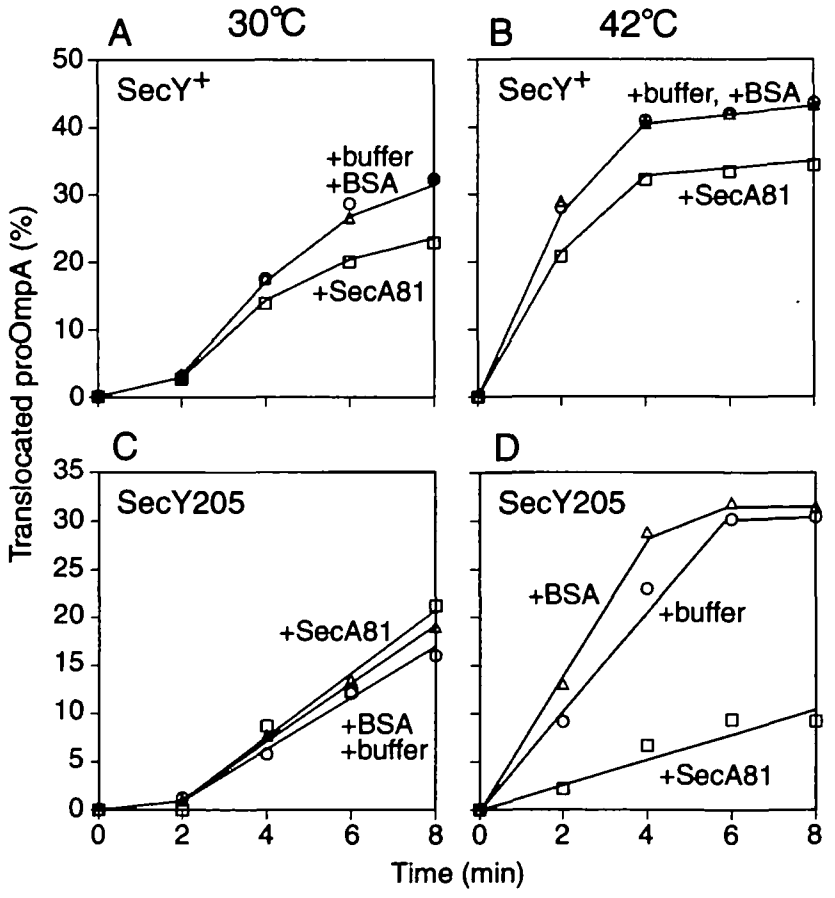

Fig. 7. **Interfering effects of SecA81 on proOmpA translocation.** Translocation of ^{35}S-labeled proOmpA into the $SecY^+$ IMV (A and B) or into the SecY205 IMV (C and D) was driven by SecA36. Concentrations of IMV and SecA36 were 250 μg proteins/ml and 10 μg/ml, respectively (circles). In addition, 500 μg/ml of either bovine serum albumin (triangles) or SecA81 (squares) was included in the reaction. Proteinase K–resistant OmpA was quantitated after SDS-PAGE and phosphor imager exposure.

dent suppressor variant, SecA36, to drive proOmpA translocation into both the SecY205 IMV and wild-type IMV (*11*). The combination of wild-type IMV and SecA36 (Fig. 7, A and B) as well as the combination of SecY205 IMV and SecA36 (Fig. 7, C and D) were subjected to proOmpA translocation assays in the presence of excess SecA81 or bovine serum albumin. The addition of SecA81 only slightly lowered the translocation reactions into the wild-type IMV (Fig. 7, A and B, squares). This lowering can be explained by the fact that SecA81, which was added in excess, was less active than SecA36 (Fig. 6). SecA81 did not inhibit the reaction carried out by the SecY205–SecA36 combination at 30°C (Fig. 7C, squares). However, SecA81 was significantly inhibitory at 42°C (Fig. 7D, squares), in comparison to bovine serum albumin (Fig. 7D, triangles), when added to the SecY205–SecA36 combination. Thus, at this temperature, SecA81 was not only inactive, but also was interfering with the functioning of the translocation machinery carrying the mutationally altered SecY205 subunit. These results suggest that the inactive form of SecA81 occupies the mutationally altered SecY205-EG channel, thus interfering with the action of the SecA36 protein at 42°C.

DISCUSSION

The SecYEG complex in the membrane presumably functions as a translocation channel in conjunction with the protein-driving SecA ATPase. Biophysical and morphological studies suggest that the diameter of the translocation channel might be as large as 40–60Å (*17*, *18*). Such a channel should be sealed while idling so that the topological segregation between the cytosol and periplasm is maintained. Even during the ongoing translocation reactions, leakage of non-specific molecules and ions through the channel should be minimized (*19*). To fully understand these important properties of the protein translocation system, it is crucial to address the question of how SecA interacts with the SecYEG channel components during the catalysis of preprotein movement into the membrane as well as during its own insertion into the membrane.

A number of *secY* mutations in the cytosolic domains 5 and 6 prevent the full execution of the SecA ATPase activities (*10*), suggesting the importance of these regions of SecY in the SecY–SecA interaction. We showed previously that the SecA insertion reaction is impaired by the *secY205* mutation affecting the most carboxy-terminal cytoplasmic domain of SecY (*11*). The IMV containing this mutant form of SecY does not allow any significant levels of insertion of the 30 kDa segment of SecA in response to a preprotein and ATP. However, the mutant IMV allows significant levels of futile (preprotein-independent) insertion that are observed in the presence of a non-hydrolyzable analog of ATP. Even this futile insertion depends on the intact SecYEG (as opposed to membrane lipids), since it is abolished by the combined effect of a SecY mutation and a specific SecY-interacting protein, Syd (*20*). These observations suggest that the SecY205 alteration transforms SecYEG to a state in which it cannot accept the productive SecA-preprotein complex. Consistent with this notion, we were able to isolate allele-specific mutations in *secA* that suppressed the *secY205* defect both *in vivo* and *in vitro* (*11*). More systematic isolation of suppressor mutations indicated that SecA can also mutate into "super active" forms having increased ATPase activities and overcoming a number of *sec* defects (G. Matsumoto, H. Nakatogawa, H. Mori, and K. Ito, manuscript in preparation). Many of these mutations have amino acid alterations in the central part of SecA around the minor ATPase domain (G. Matsumoto, H. Nakatogawa, H. Mori, and K. Ito, manuscript in preparation). We report here that the SecA81 alteration in this region activates the intrinsic and membrane ATPase activities at lower temperatures. The mutational alteration also destabilizes the conformation of a central region of SecA encompassing residues ~420 to ~580, resulting in the hypersensitivity of this region to trypsin. Thus, instead of the 65 kDa fragment, a 45 kDa fragment is produced from the N-terminal region by trypsin digestion. The latter fragment may correspond to the 48 kDa fragment observed with a membrane-bound state of SecA (*21*). Thus, the SecA81 conformation may mimic a partially activated state of SecA.

It has been proposed that an interaction between the N-terminal ATPase domain and the C-terminal domain is necessary to down-regulate the ATPase activity (*22*, *23*). Thus, trypsin cleavage between the N- and C-terminal domains (*22*), as well as low concentrations of guanidinium hydrochloride (*24*), stimulate the intrinsic ATPase activity of SecA. Probably, the SecA81 conformational change disrupts the inter-domain interaction of SecA, which is important for the regulation of ATPase activity. In this connection, it was also proposed that the functional state of SecA has some properties that are characteristic of unfolded proteins (*24*). Probably, the loosened conformation of the SecA81 mutant protein in its central region is advantageous not only for the elevation of ATP-hydrolysis but also for the protein to insert into the SecYEG channel that is altered by the SecY205 amino acid change. Thus, the SecY205 IMV restricts the entry of the wild-type SecA-proOmpA complex, but not the SecA81-proOmpA complex. We observed that, unlike wild-type SecA, SecA81 could indeed insert into the SecY205 IMV at 30°C (data not shown).

The SecA conformation/function seems to be subject to some intrinsic thermal modulation and the SecA actions include some endothermic component (*25*). Our results show that SecA81 has exaggerated temperature-sensitivity. Although it has markedly enhanced intrinsic and membrane ATPase activities at 30°C, these ATPase activities are totally lacking at 42°C. Strikingly, however, SecA81 has significant ATPase activity (translocation ATPase) even at 42°C when both wild-type IMV and proOmpA are included in the assay, although either IMV or proOmpA alone is ineffective. SecA81 was also active in translocating proOmpA into wild-type IMV at 42°C. *In vivo*, it is active at this temperature in combination with wild-type SecY or in combination with several mutations in SecY other than SecY205 (*11*). The aggregation of overproduced SecA81 at higher temperatures *in vivo* is also consistent with the thermosensitivity of SecA81 molecules that are not engaged in protein translocation. All of these results indicate that, despite its intrinsic thermosensitivity, SecA81 is somehow stabilized and activated under the preprotein-translocation conditions. It is possible that SecA is not released from the membranous environment during the catalysis of multiple rounds of translocation reactions (*26*, *27*). We assume that the SecY205 channel is defective in the ability to stabilize and activate SecA81 at 42°C. The

altered translocation channel may have some incompatibility with the SecA81 form of SecA, making the latter fit less well in the channel and subjecting it to thermal inactivation. Since SecA81 may be inactivated on site, it interferes with the action of other SecA molecules, as we observed in the mixing experiments.

The observations reported in this work provide additional evidence that a specific interaction between SecY and SecA is important for the ability of SecA to undergo insertion–deinsertion cycle and to drive preprotein translocation. In addition, our results, together with previous findings by others (*24*, *25*), suggest that SecA functions in the delicate balance between disordered and ordered structural states. The SecYEG integral membrane complex not only provides a channel-like space for the insertion of the SecA-preprotein complex, but also contributes to the fine tuning of the conformational transition of the SecA molecules. SecA81 may prove to be an interesting material for studies on the structural biology of this ATPase.

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