

## Indecisive M13 Procoat Protein Mutants Bind to SecA but Do Not Activate the Translocation ATPase\*

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**The M13 procoat protein serves as the paradigm for the Sec-independent membrane insertion pathway. This protein is inserted into the inner membrane of *Escherichia coli* with two hydrophobic regions and a central periplasmic loop region of 20 amino acid residues. Extension of the periplasmic loop region renders M13 procoat membrane insertion Sec-dependent. Loop regions with 118 or more residues required SecA and SecYEG and were efficiently translocated *in vivo*. Two mutants having loop regions of 80 and 100 residues, respectively, interacted with SecA but failed to activate the membrane translocation ATPase of SecA *in vitro*. Similarly, a procoat mutant with two additional glutamyl residues in the loop region showed binding to SecA but did not stimulate the ATPase. The three mutants were also defective for precursor-stimulated binding of SecA to the membrane surface. Remarkably, the mutant proteins act as competitive inhibitors of the Sec translocase. This suggests that the region to be translocated is sensed by SecA but the activation of the SecA translocation ATPase is only successful for substrates with a minimum length of the translocated region.**

Bacterial preproteins are translocated across the inner membrane enzymatically involving chaperones (1) and targeting factors such as the signal recognition particle (2), and an integral translocase complex (3, 4). The energy-utilizing subunit SecA binds the precursor protein in the cytoplasm or at the membrane surface and hands over sequential portions of the precursor protein chain to the SecYEG complex (5). One question is how SecA recognizes the precursor protein as a protein to translocate. Previous studies have shown that SecA recognizes parts of the leader and the mature sequence of the precursor proOmpA (6). A region to which preproteins were cross-linked has been localized to the central part of SecA (7). However, during the translocation reaction, the moving preprotein might be bound to additional binding sites within SecA and SecY along the interior of the translocase complex (5).

Small membrane proteins, such as the M13 procoat protein (hereafter "procoat") of 73 amino acid residues and the Pf3 coat protein of 44 residues, can bypass the preprotein translocase

and insert directly into the membrane bilayer (8, 9). Although procoat has a cleavable signal sequence, it neither contacts the signal recognition particle nor SecB in the cytoplasm (10). Therefore, the mere presence of a signal sequence is not sufficient to control the interaction with the components of the translocation pathway. In addition, membrane insertion and cleavage of procoat is not affected at the nonpermissive temperature in a *secA<sup>ts51</sup>* strain (11). Procoat insertion also occurs in a temperature-sensitive *secY<sup>ts</sup>* strain and in a SecE depletion strain (10, 11). How do the small coat proteins then insert into the membrane without contacting the translocase? It has been suggested that the small membrane proteins insert into the cytoplasmic membrane spontaneously, because they do this readily *in vitro* with artificial liposomes (9, 12). However, recent results show that procoat requires the YidC protein for efficient membrane insertion (13). YidC might therefore operate independently of the Sec translocase to insert small membrane proteins as is the case for Oxa-1, the mitochondrial homologue of YidC (14).

There have been M13 procoat mutants isolated that use the preprotein translocase pathway. They have an extension in the periplasmic region, the region that is actually translocated (15). One additional *sec*-dependent mutant was found in which the periplasmic region was extended by only two glutamic acid residues after position +2 in the mature region (16). Exchange of the hydrophobic anchor region of the M13 procoat region with the more hydrophobic region of leader peptidase also provoked the requirement of translocase (10). In conclusion, features of the mature region of M13 procoat determine its mode of insertion. How is it then decided whether the preprotein contacts the translocase components and activates the enzymatic function?

To address this question we investigated whether the *sec*-independent mutant proteins actually do not contact SecA or whether they just cannot stimulate the translocation ATPase. Three different phenotypes were observed. First were procoat mutants that bind to SecA and activate the translocation ATPase, representing a Sec-dependent phenotype. Second were mutants that interact with SecA but do not activate the ATPase; these mutants appeared as indecisive between the two translocation modes and were slowly translocated. Third were the Sec-independent proteins that do not bind to SecA at all. We conclude that SecA binds substrates in different modes and that only a subset of the substrates can efficiently start the translocation motor.

### EXPERIMENTAL PROCEDURES

*Strains and Plasmids*—*Escherichia coli* BL21(DE3) *ompT* *r<sub>B</sub>m<sub>B</sub>* *lon* *lac* UV5, F' (T7 gene 1); HB101 *supE44* *hsdS20* (*r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>*) *rec* A13 *ara-14 pro* A2 *lac* Y1 *gal* K2 *rps* L20 *xyl-5 mtl-1*; HJM114 ( $\Delta$  *lac pro*) F'

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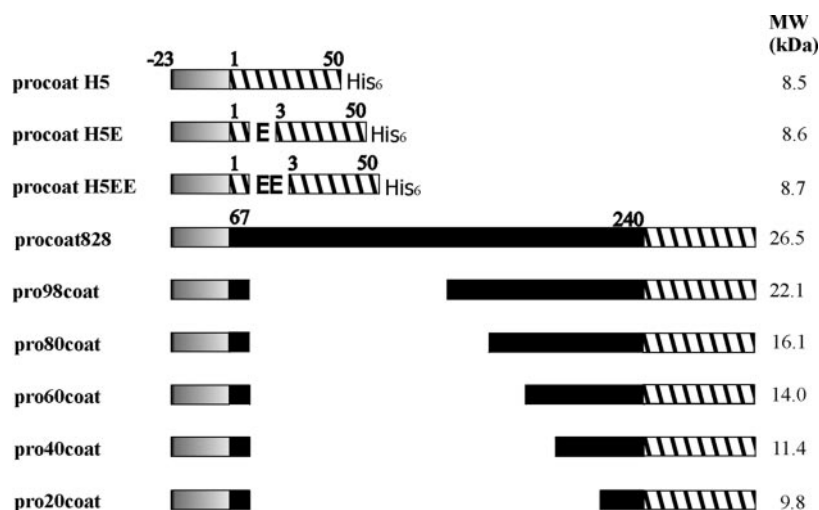


FIG. 1. Schematic representation of M13 procoat mutant and M13 procoat-OmpA hybrid proteins. The black bars represent the OmpA-derived sequence, and the gray and hatched bars refer to the M13 procoat leader and mature sequence, respectively.

(*lac*, *pro*); MRE600 (DSM3901); KM9 *unc*<sup>-</sup>::Tn10 *rel* A1 *spo*T1 *met* B1; MC4100 *ara* D139  $\Delta$ (*argF-lac*) U169 *rps* L150 *rel* A1 *flb* B5301 *deo* C1 *pts* F25 *rbs* R; and MM66 *secA*<sup>am</sup> Tn10 *su3*<sup>ts</sup> *trp*<sup>am</sup> were from our collection. CK1953 *secB*::Tn5 was obtained from C. Kumamoto (Tufts University, Boston).

The plasmids pQN8, pQN828, pQN8A1 (15), and pJQ8 with the procoat gene VIII cloned into pJF119HE (32), pTrcOmpA (33) were described. Plasmids pJQ8-H5E and pJQ8-H5EE were constructed from pQN8-4PC-lep and pQN8-5PC-lep (16), respectively, by ligation of a *Pst*I restriction fragment. The pQN828 derivatives coding for the various procoat-OmpA hybrid proteins were constructed by site-directed mutagenesis (16).

**In Vivo Translocation**—Cultures of MC4100 with the respective plasmid were grown at 37 °C to mid-log phase in M9 minimal medium supplemented with 0.5% fructose and 20  $\mu$ g/ml each amino acid except methionine. The cells were induced with 0.5% arabinose for 5 min, pulse-labeled with [<sup>35</sup>S]methionine for the indicated times, and chased with excess L-methionine. For translocation experiments, the cells were converted to spheroplasts and treated with 1 mg/ml protease as described elsewhere (16).

**In Vitro Expression and Co-immunoprecipitation**—The precursor proteins were expressed in a coupled transcription-translation system as described by Müller and Blobel (37) with modifications. The cell free extracts were prepared from strain MM66 (*secA*<sup>ts</sup>) shifted to the non-permissive temperature for 2 h. Residual SecA was removed from the extract by immunodepletion with SecA antibody coupled to *Staphylococcus aureus* protein A (Zymed Laboratories Inc.). Immunodepletion of the cell extract was performed for 2 h at 4 °C. For *in vitro* binding studies, purified SecA was added back to the depleted extracts with a final concentration in the translation mixture of 0.4  $\mu$ g/ml. Controls received translocation buffer only. Co-immunoprecipitation was performed by incubating the *in vitro* samples with SecA antibody coupled to Pierce UltraLink protein A in phosphate-buffered saline (PBS),<sup>1</sup> 0.1% Tween for 15 min at room temperature after translation had been completed. The coupling matrix was washed twice with PBS-Tween and prepared for SDS-PAGE by boiling in SDS sample buffer for 5 min. Translation products were separated on 22% acrylamide-SDS gels containing urea and visualized on a Fuji phosphorimaging device. Quantitation of the data was performed using AIDA software (Raytest). As a control for nonspecific background, samples were treated with Pierce UltraLink not coupled to an antibody. The respective values were subtracted from the values for SecA antibody-containing samples. Co-immunoprecipitation was evaluated by calculating the percentage of precipitable translation product with respect to the amount of totally synthesized precursor protein.

**Protein Purification**—SecA was purified from the overproducing strain *E. coli* HB101/pMAN (34) as described previously (35) by P11 phosphocellulose chromatography and Sephacryl S200 gel filtration. Purified SecA was stored at -80 °C in translocation buffer (50 mM KCl,

5 mM MgCl<sub>2</sub>, 50 mM Tris-HCl, pH 8.0) containing 10% glycerol and 1 mM dithiothreitol.

C-terminal His-tagged M13 procoat H5, procoat H5E, and procoat H5EE were purified on nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany). The overproducing *E. coli* HB101 with the respective pJQ plasmid was grown in LB medium induced with 0.5 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside at A<sub>600</sub> = 0.6, and the culture was continued for 3 h. The cells were collected and resuspended in 50 mM Tris-HCl, pH 7.6, 10% sucrose (1 ml/g cells). For lysis 0.4 mg/ml lysozyme was added for 1 h on ice, and 0.2 mg/ml DNase was added with 5 mM MgCl<sub>2</sub> for another 30 min on ice. Cell membranes were collected (40 000  $\times$  g for 30 min at 4 °C) and resuspended in 10 mM triethanolamine, 10% glycerol, pH 7.5. After centrifugation (40 000  $\times$  g for 30 min at 4 °C) the pellets were solubilized in 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 1% sodium *N*-lauroyl sarcosine (United States Biochemicals, Cleveland, OH), 10% glycerol and homogenized in a Dounce homogenizer. The supernatant was incubated with nickel-nitrilotriacetic acid-agarose (0.25 ml bed volume/liter of culture) for 2 h at 4 °C. After preparing the column for chromatography, the agarose was washed with 6 column volumes of buffer I (0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 1% sodium *N*-lauroyl sarcosine (1 ml/min) and equilibrated with 5 column volumes of buffer I containing 5 mM imidazole (Sigma). The proteins were eluted by steps of 2 column volumes of buffer I each containing 100, 200, 300, and 500 mM imidazole, respectively, and 2-ml fractions were collected. The procoat proteins were dialyzed overnight against 8 M urea, 20 mM Tris-HCl, pH 7.9.

Procoat-OmpA hybrid proteins were isolated from inclusion bodies. The overproducing *E. coli* HB101 with the respective pQN plasmid was induced with 1% arabinose at A<sub>600</sub> = 0.8 for 3 h. The cells were pelleted and resuspended in 20 mM Tris-HCl, pH 8.0, 10% sucrose (1 ml/g cells) and were broken by 0.4 mg/ml lysozyme and sonication. The pH was increased by the addition of 50 mM Tris-HCl, pH 9.0, and 0.4 mg/ml DNase was added with 5 mM MgCl<sub>2</sub> and 1  $\mu$ M phenylmethylsulfonyl fluoride (Sigma). After centrifugation (40,000  $\times$  g for 30 min at 4 °C) the pellet was solubilized in 4 ml/g cells of buffer S (1% Triton X-100, 100 mM EDTA, 20 mM Tris-HCl, pH 9.0) and homogenized in a Dounce homogenizer. The inclusion bodies were collected by centrifugation (30,000  $\times$  g for 30 min at 4 °C) and homogenized in 4 ml/g cells of buffer S until the supernatant was clear. The pellet was resuspended in ~10 volumes of 50 mM Tris-HCl, pH 8.0, and centrifuged as before. The inclusion bodies were resuspended in 6 M urea, 2% sodium *N*-lauroyl sarcosine, 20 mM Tris-HCl, pH 8.0, and stored at -20 °C. For further purification of the preproteins, the proteins were trichloroacetic acid (15%)-precipitated, washed with acetone, and resuspended in SDS-PAGE sample buffer. The proteins were applied on a preparative SDS-PAGE (Bio-Rad, 5-cm 15% separating gel). After elution of the dye, 4 ml of fractions were collected and analyzed by 19% SDS-PAGE containing 5 M urea and Western blotting with antibodies to M13 and to OmpA. The procoat-OmpA hybrid proteins were concentrated by trichloroacetic acid precipitation, washed by acetone, and resolved in 8 M urea, 20 mM Tris-HCl, pH 8.0.

ProOmpA was isolated from inclusion bodies as described (36) and further purified by MonoQ (Amersham Pharmacia Biotech) chromatography (19). Protein concentration was determined using Bradford reagent.

<sup>1</sup> The abbreviations used are: PBS, phosphate-buffered saline; BSA, bovine serum albumin; INV, inverted membrane vesicles; U-INV, urea-treated inner membrane vesicles; PAGE, polyacrylamide gel electrophoresis; SRP, signal recognition particle; invLep, inverted leader peptidase.

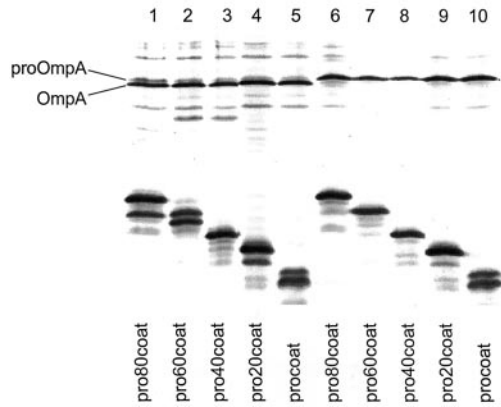


FIG. 2. *In vivo* translocation and processing of M13 procoat-OmpA hybrid proteins in the absence (lanes 1–5) and presence (lanes 6–10) of 2 mM azide. Pro80coat (lanes 1 and 6), pro60coat (lanes 2 and 7), pro40coat (lanes 3 and 8), pro20coat (lanes 4 and 9), and M13 procoat (lanes 5 and 10) were expressed in *E. coli* MC 4100 bearing the respective plasmid at 37 °C. [<sup>35</sup>S]Methionine was added for 1 min. The proteins were immunoprecipitated with antibodies to M13 coat protein and OmpA and analyzed by SDS-PAGE and fluorography.

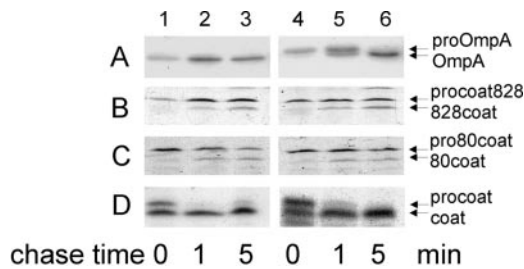


FIG. 3. *In vivo* translocation and processing in the presence and absence of SecB. ProOmpA (A), procoat 828 (B), pro80coat (C), and wild-type procoat (D) expressed in *E. coli* MC 4100 cells (left panels) or in CK1953 cells (right panels) bearing the respective plasmid were grown to mid-logarithmic phase at 30 °C. [<sup>35</sup>S]Methionine was added for 30 s (lanes 1) and chased with nonradioactive methionine for 1 min (lanes 2) and 5 min (lanes 3). The proteins were immunoprecipitated with antibodies to M13 coat protein and OmpA and analyzed by SDS-PAGE and fluorography.

gent (Bio-Rad) with BSA as a standard. The purity of the proteins was analyzed by SDS-PAGE and stained with Coomassie blue R250.

**SecA Translocation ATPase**—ATPase activity reactions (50  $\mu$ l) contained 1 mM ATP (Sigma), 0.1 mg/ml BSA (Roth, Karlsruhe, Germany), 20  $\mu$ g/ml SecA, 40  $\mu$ g/ml preprotein, and 100  $\mu$ g/ml urea-treated inner membrane vesicles (U-INV) in translocation buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl<sub>2</sub>). INVs were prepared as described elsewhere (37) from *E. coli* strains KM9, HB101/pHasecYEG, or MRE600, respectively. U-INV's were prepared from MRE600 vesicles and treated with 6 M urea (30 min on ice) to remove membrane-bound SecA as described (35). After the components were mixed, the reactions were incubated at 37 °C for 30 min. The release of phosphate was measured colorimetrically by the malachite green reaction at A<sub>640 nm</sub> as described (6).

**Resonant Mirror Spectroscopy**—Resonant mirror spectroscopy was carried out using the IAsys system (Affinity Sensors, Cambridge, UK) at 25 °C as described previously (29). Cuvettes (hydroxy surface) were coated with lipids in the presence of 1.25% *n*-octyl- $\beta$ -glucoside (Anatrace, Maumee, OH) in PBS, pH 7.4. The buffer was removed, and 50  $\mu$ l of dimyristoylphosphatidylethanolamine (Sigma, 2 mg/ml) in *n*-octyl- $\beta$ -glucoside/PBS were applied to the cuvette. After saturation of lipid binding, PBS (3  $\times$  50  $\mu$ l) was added directly. Binding of the proteins was measured in 100  $\mu$ l of translocation buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol) containing 0.1 mg/ml BSA to saturation, and the cuvette was regenerated (by the addition of 5 M urea for 1 min and washing several times with translocation buffer). The binding rate was calculated from the response [arc s] during the first 100 s.

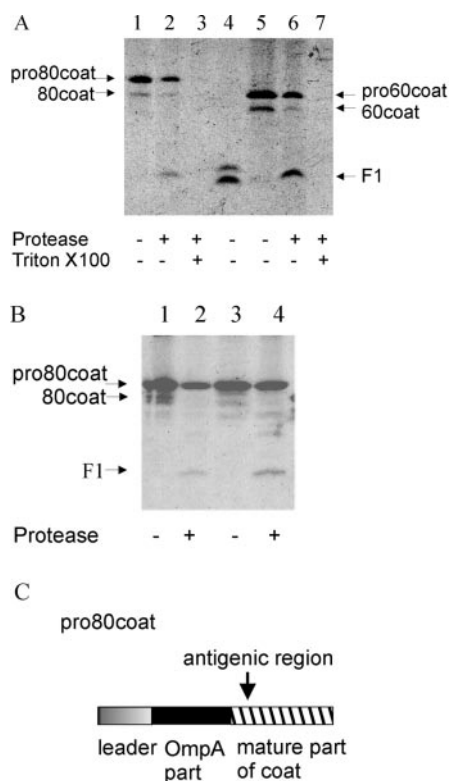
## RESULTS

**Construction of M13 Procoat Mutants with Different Loop Regions**—To investigate the features of a preprotein that are necessary to interact functionally with the translocase, hybrid proteins between the sec-dependent proOmpA protein and the sec-independent M13 procoat protein were constructed. Between the leader sequence and the mature part of procoat, OmpA sequences of different lengths were inserted (Fig. 1). These hybrid proteins were termed pro20coat, pro40coat, pro60coat, pro80coat, and pro98coat, where the number refers to the additional amino acid residues inserted. The previously studied procoat828, which has a 174-residue insertion (15), and procoat H5E and H5EE, which have 1 or 2 additional glutamic acid residues in the early mature region, respectively, were also included in this study. Similar mutants to H5E and H5EE, procoat-lep(-4) and (-5), two procoat leader peptidase fusion proteins, have previously been studied (16).

**Requirements for SecA and SecB of the Procoat-OmpA Hybrid Proteins**—The participation of SecA in the membrane insertion of the procoat mutant proteins was first investigated in a pulse-chase experiment (Fig. 2). Prior to pulse labeling (90 s), SecA was inactivated by 2 mM sodium azide for one set of experiments (lanes 6–10). [<sup>35</sup>S]Methionine was added for 1 min, and the samples were analyzed by immunoprecipitation to M13 coat and OmpA followed by SDS-PAGE. Pro60coat (lane 2) was cleaved efficiently by leader peptidase during the pulse period, which indicates a rapid membrane insertion of the protein. If the cells were pretreated with sodium azide, the cleavage to the mature form was strongly inhibited (lane 7). Similar results were observed for the proOmpA protein in each case and also for the mutants procoat828 and pro98coat (data not shown). Pro80coat was cleaved to the mature form inefficiently in the absence of azide (lane 1). In the presence of azide, however, cleavage to the mature form was completely inhibited (lane 6). The mutant pro40coat was membrane-inserted very inefficiently in the absence or presence of azide (lanes 3 and 8). Finally, the pro20coat and the wild type were membrane-inserted and processed by leader peptidase regardless of whether the cells had been treated by sodium azide (lanes 4, 5, 9, and 10).

To study whether the membrane insertion of the procoat mutants depends on SecB the translocation kinetics were analyzed in the *secB*<sup>null</sup> strain, CK1953 (Fig. 3). The cells were pulse-labeled with [<sup>35</sup>S]methionine for 30 s and chased for 1 and 5 min by the addition of 200  $\mu$ g/ml cold L-methionine. Whereas proOmpA showed a remarkable retardation of processing (Fig. 3A), M13 procoat (Fig. 3D) and the procoat-OmpA mutants (Fig. 3, B and C) were not affected by the absence of the SecB protein. In conclusion, membrane targeting of the procoat-proOmpA hybrid proteins does not require SecB.

**Characterization of a Translocation Intermediate**—Because some of the hybrid proteins were translocated inefficiently, we suspected that they might be trapped within the translocase. To address this possibility, we investigated the translocation of pro60coat and pro80coat in more detail. *E. coli* cells bearing the respective plasmid were induced to express the mutant procoat protein and pulse-labeled with [<sup>35</sup>S]methionine for 30 s. At that time point, most of either preprotein is still unprocessed. The cells were converted to spheroplasts, and proteinase K was added to detect translocation (Fig. 4A). Strikingly, a major portion of the precursor protein was digested by the external protease suggesting that it had been translocated across the inner membrane but was not yet cleaved by leader peptidase. A 6.5-kDa proteolytic peptide, termed F1, was recognized by antibodies specific to the M13 coat periplasmic region. The F1 fragment only appeared after a short pulse; at a later chase

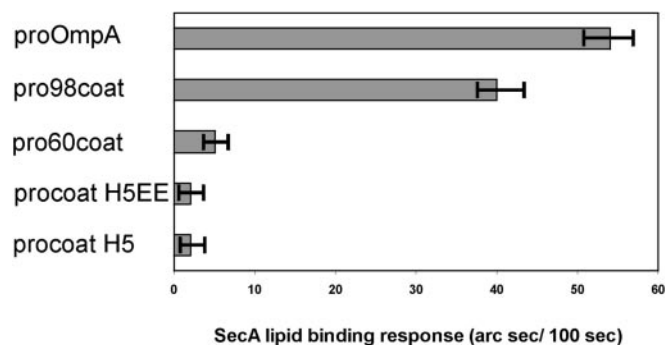


**FIG. 4. Partial translocation of pro60coat and pro80coat.** *E. coli* MC4100 bearing the respective plasmid were pulse-labeled with [<sup>35</sup>S]methionine for 30 s. Excess L-methionine was added, and the cells were converted to spheroplasts. **A**, 1 mg/ml proteinase K was added for 30 min in the absence (lanes 2 and 6) or presence of 2% Triton X-100 (lanes 3 and 7). For size comparisons, M13 procoat and coat are shown (lane 4). **B**, 90 s prior to pulse labeling, 2 mM azide was added (lanes 3 and 4). Cells were converted to spheroplasts and digested with 1 mg/ml proteinase K (lanes 2 and 4). F1 indicates the proteolytic fragment generated by the proteinase K treatment. The samples were immunoprecipitated and analyzed by SDS-PAGE and phosphorimaging. **C**, schematic representation of pro80coat and its antigenic region to illustrate the protease-protected parts of the protein.

time, the F1 fragment was not detectable (data not shown). We conclude that the procoat mutants pro60coat and pro80coat are partially translocated and proteolytically digested in the OmpA-derived portion of the proteins. However, the adjacent hydrophilic portion of the protein, including the region recognized by the M13 antibody, is protected from the protease. In addition, the leader peptidase has no access to the partially translocated intermediate.

The F1 peptide of pro80coat was also generated when the cells had been treated with azide prior to labeling (Fig. 4B, lanes 3 and 4). In the azide-treated cells, cleavage by leader peptidase was totally inhibited, whereas the formation of the F1 peptide was unaffected. Based on these results, we conclude that the N-terminal part of the precursor protein may be trapped in the translocase and is not released for processing by the leader peptidase, whereas a region C-terminal to the signal sequence is translocated in a Sec-independent manner.

**Stimulation of Precursor-triggered Lipid Binding of SecA**—To determine the translocation defect of the mutant proteins in more detail, the precursor proteins were purified and their membrane insertion was analyzed *in vitro*. Binding of SecA to a lipid monolayer is modulated by nucleotides (18). We used this feature of SecA to investigate the interaction of the mutant precursor proteins with SecA. Binding of SecA to a dimyristoylphosphatidylethanolamine surface was analyzed by resonant mirror spectroscopy. Increased binding of SecA to the lipid surface was observed in the presence of proOmpA and



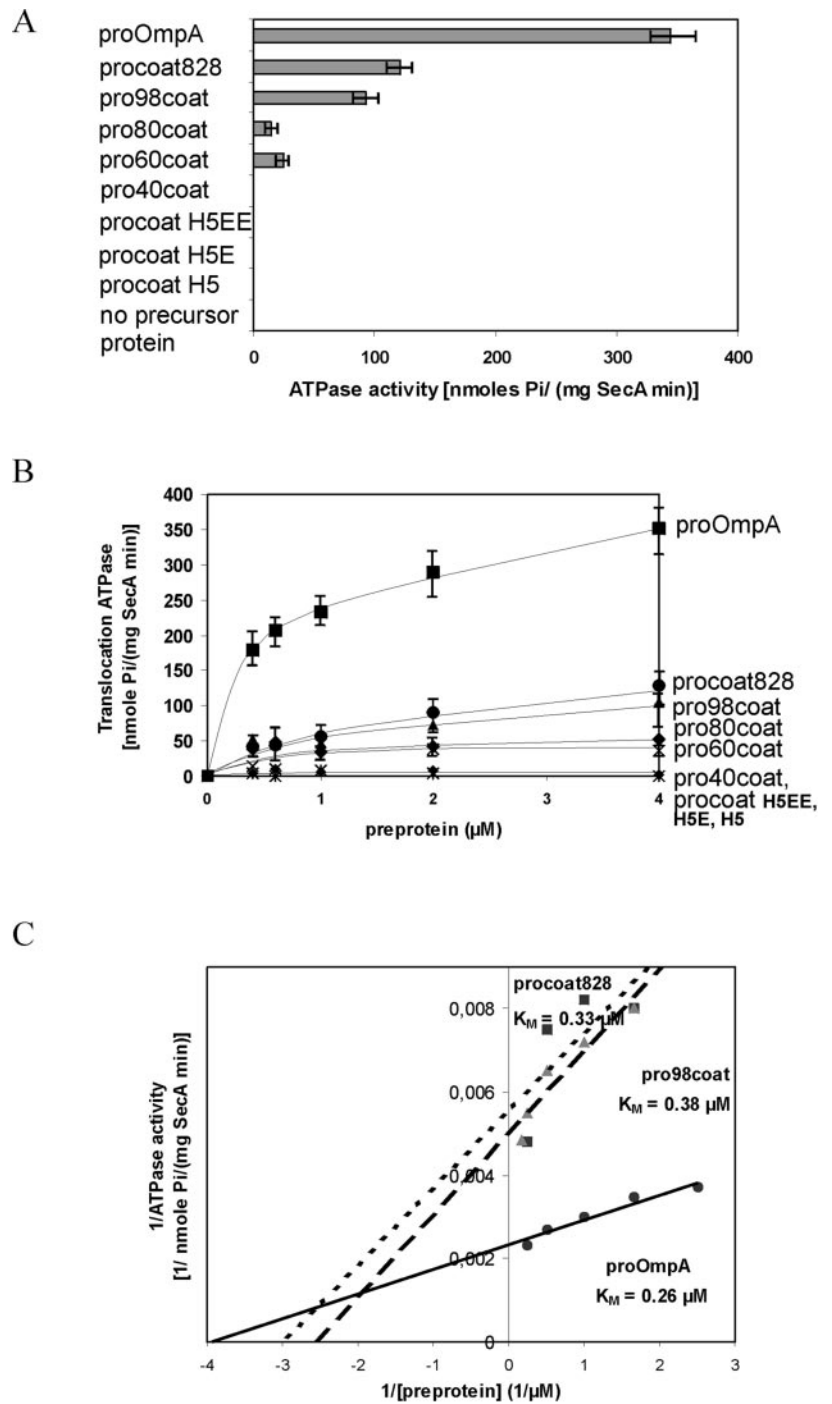
**FIG. 5. Stimulation of the SecA binding to a lipid surface by preproteins.** 1  $\mu$ g of SecA was incubated with 2  $\mu$ g of preprotein in 100  $\mu$ l of 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 3 mM ADP for 5 min at 25 °C. The binding to a dimyristoylphosphatidylethanolamine-coated surface was measured at 25 °C by resonant mirror spectroscopy.

ADP (Fig. 5). Similarly, the purified sec-dependent pro98coat protein stimulated SecA binding, whereas the sec-independent procoat H5 and the mutant proteins pro60coat and H5EE did not stimulate the binding of SecA to the lipid surface. Procoat mutants H5, H5E, and H5EE have a mutation at -3, Ser to Phe, that prevents cleavage by leader peptidase (17) and therefore allows the isolation of the precursor proteins. H5E and H5EE have a further mutation in the mature region at +2 that codes for one or two additional Glu residues, respectively.

**Mutant Procoat Proteins That Fail to Stimulate the SecA ATPase**—The ATPase activity of SecA stimulated by binding of the precursor protein SecYEG and negatively charged lipids is referred to as the translocation ATPase (19). The various M13 procoat mutants were tested for stimulation of the translocation ATPase activity. Purified SecA protein was preincubated with U-INV<sub>s</sub>, and purified precursor protein was added. The release of inorganic phosphate was measured photometrically at A<sub>640 nm</sub> after the addition of the malachite green reagent. ProOmpA, procoat 828, and pro98coat efficiently stimulated the ATPase activity (Fig. 6A). Pro80coat and pro60coat showed little stimulation, whereas pro40coat, H5, H5E, and H5EE showed no stimulation. Quantitative evaluation revealed that the translocation ATPase stimulation by proOmpA was considerably higher than for the procoat 828 and pro98coat, with an apparent K<sub>m</sub> of 0.26  $\mu$ M (Fig. 6C).

**Binding of Procoat Mutants to SecA**—SecA binding was first examined in a co-immunoprecipitation experiment (Fig. 7). The precursor proteins were expressed *in vitro* in a SecA-depleted extract in the absence or presence of purified SecA protein. The amount of precursor bound to SecA was calculated by co-immunoprecipitation with antiserum to SecA. When proOmpA, pro80coat, and pro60coat were expressed, between 25 and 30% of the precursor was found bound to the SecA protein. Procoat H5 did not bind (about 1.5%), but procoat H5EE showed measurable binding (7%) in this assay.

To test whether the precursors interact with SecA in a functional way we used a competition assay. Increasing amounts of purified precursor of procoat H5, H5E, H5EE, pro40coat, pro60coat, and pro80coat were added to SecA and proOmpA and assayed for ATPase activity (Fig. 8). The addition of procoat H5, H5E, and pro40coat had no inhibitory effect and, therefore, did not compete with proOmpA for the translocation ATPase. However, H5EE, pro60coat, and pro80coat clearly inhibited the translocation ATPase competitively at higher concentrations, suggesting that the precursor proteins bind to SecA at the same site as proOmpA.



**FIG. 6. Stimulation of the translocation ATPase by various preproteins.** A, purified SecA was added to U-INVes in the presence of the purified 40  $\mu\text{g/ml}$  preproteins and incubated for 30 min at 37  $^{\circ}\text{C}$ . B, stimulation of the translocation ATPase by preproteins at the indicated concentrations was measured after incubation for 10 min at 37  $^{\circ}\text{C}$ . C, the ATPase activity was analyzed by Lineweaver-Burk plots of proOmpA ( $\bullet$ ), procoat 828 ( $\blacksquare$ ), and pro98coat ( $\blacktriangle$ ) to determine the apparent  $K_m$ . The ATPase activity was determined photometrically with malachite green color reagent at  $A_{640\text{ nm}}$  (6). The lipid-stimulated basal ATPase activity (without preprotein) was subtracted from all values.

DISCUSSION

Newly synthesized membrane and secretory proteins can participate in a number of possible interactions with other cellular components. To date, the molecular basis of these interactions is still elusive. In the cytoplasm, molecular chaperones, *e.g.* SecB, have been shown to bind to precursor proteins and maintain their structural competence for the subsequent translocation event (20). Alternatively, bacterial membrane proteins use the signal recognition particle complex, SRP, that targets the proteins to SecYEG (2). At the membrane, SecA interacts with precursor proteins to sequentially feed them into the SecYEG translocase (5). To date, it remains unknown as to which features of a precursor protein are recognized by the targeting and translocation components. Early experiments with proOmpA deletion mutants of 68 and 72 amino acids showed an accumulation in the

cytoplasm when studied *in vivo* (21). Because longer precursor proteins of 123 residues were transported into the periplasm, it was suggested that the precursor protein must have a certain length.

To investigate the molecular interactions that direct a preprotein to a Sec-independent or -dependent pathway, we constructed a number of M13 procoat protein mutants that have alterations in the periplasmic region. For mutants that had larger periplasmic regions resulting in loop sizes of more than 80 residues, membrane insertion was inhibited in the presence of azide, a SecA inhibitor (22). A similar result has been described for inverted leader peptidase (*invLep*), a protein with a 25-residue periplasmic domain (23). Extension of the periplasmic loop in this protein to more than 80 residues also clearly led to Sec dependence (24). However, translocation of *invLep* in a

SecE-depleted strain was retarded, suggesting that an interaction with translocase is occurring (10). Therefore, a minimal amount of Sec translocase might be sufficient for *invLep* membrane insertion, and Sec-translocase may only accelerate the translocation process, but it is not really required for *invLep*.

The requirement of the Sec translocase for protein translocation is analyzed for the most part in deficient mutants or by addition of inhibitors. These systems leading to a loss of function cannot always prove an essential role for a given component. *In vitro* studies provide a more direct way to explore the interaction of a precursor protein with components of the translocation pathway. Earlier studies have shown that a 74-amino acid-long proOmpA fragment did not efficiently stimulate the SecA translocation ATPase activity, suggesting that the

smaller precursors are no true substrates for SecA (25). We therefore purified the procoat-OmpA hybrid precursor proteins of different lengths and tested whether they can bind to SecA and activate the translocation ATPase. The M13 procoat mutants that had periplasmic regions extended by 171 or 98 residues, respectively, activated the translocation ATPase *in vitro* and therefore exhibit a Sec-dependent phenotype. When compared with proOmpA, the apparent  $K_m$  values of the ATPase were higher, suggesting that the affinity for the translocase increases with the length of the precursor.

No activation of the SecA translocase was observed when the periplasmic extension was less than 40 residues. This finding is consistent with the *in vivo* results that membrane insertion of the mutants pro40coat and pro20coat was not affected by azide. Notably, the efficiency of membrane insertion within this class of mutants was decreased with the increasing length of the periplasmic region. These mutants can be classified as clearly Sec-independent.

Surprisingly, we found precursor protein mutants pro60coat, pro80coat, and H5EE that were sensitive to azide for membrane insertion but were incapable of efficiently activating the SecA ATPase. Co-immunoprecipitation experiments and a competition assay with proOmpA showed that these proteins did actually bind to SecA (Fig. 8). Although effective in binding, these mutants did not induce the enzymatic activity of SecA and were therefore most likely delayed in moving across the membrane. This might also explain their delay in translocation in the presence of azide. Azide has been proposed to inhibit the deinsertion step of SecA (26). As a result, the translocating protein would remain trapped within the translocase. Analysis of intermediates of pro60coat and pro80coat suggests that these proteins are partially translocated. Whereas a part of the hydrophilic domain was exposed to the periplasm and cleaved by externally added proteinase K, the signal peptide region

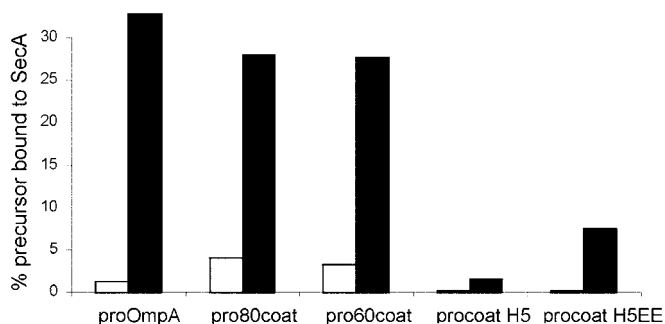
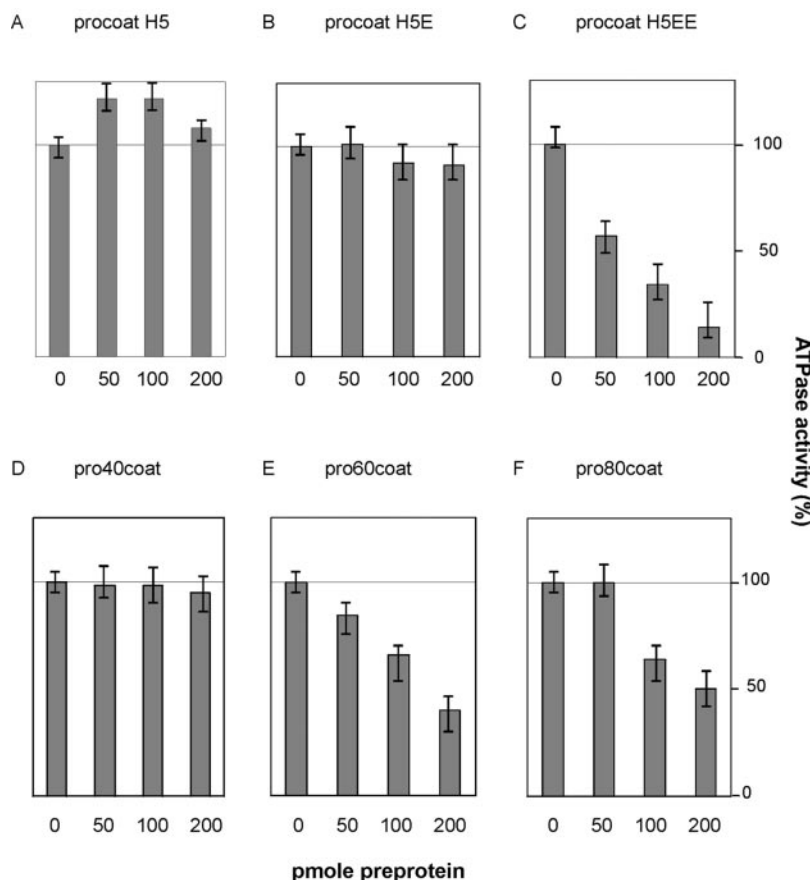


FIG. 7. Co-immunoprecipitation of *in vitro* synthesized precursor proteins by antibodies against SecA. The precursor proteins were expressed *in vitro* in a SecA-depleted cell-free extract and labeled by [ $^{35}$ S]methionine. Translation products were separated by SDS-PAGE, co-immunoprecipitated by SecA-antibodies coupled to Pierce UltraLink protein A, and visualized and quantified on a phosphorimaging device. Filled bars, the translation system was supplemented with purified SecA; open bars, control samples were supplemented with translocation buffer only.

FIG. 8. Competitive inhibition of the SecA ATPase activity by M13 procoat mutants and hybrid proteins. The ATPase activity of SecA was measured in 50  $\mu$ l of translocation buffer containing 5  $\mu$ g of BSA, 4  $\mu$ g of U-INV, and 40 pmol of proOmpA in the presence of increasing amounts of M13 procoat H5 (A), procoat H5E (B), procoat H5EE (C), pro40coat (D), pro60coat (E), and pro80coat (F). The values obtained with proOmpA alone were set to 100%.



remained protected from leader peptidase. Also, the part of the hydrophilic region close to the mature anchor was protected from the protease as judged from the size of the proteolytic fragment. Notably, this partial translocation was also observed when the cells had been treated with azide. We conclude that pro80coat, pro60coat, and H5EE bind to SecA but their membrane translocation is retarded because they do not stimulate the translocation ATPase.

We anticipate that precursor recognition by SecA is regulated by more than just one recognition site. Additional sites might ensure the coupling of the translocation process with ATP hydrolysis (25). Previous experiments with synthetic signal peptides have shown that they bind efficiently to SecA and activate the SecA-ATPase (27, 28). Because these substrates have no protein region to translocate, we suspect that for these cases the ATPase activity is uncoupled from protein movement. SecA activation by our substrates with small translocating domains is different. Pro60coat, pro80coat, and also H5EE bind to SecA but do not efficiently activate the ATPase. In contrast to the synthetic signal peptides, they might bind with their mature region, which couples translocation to ATP hydrolysis. Within SecA, a molecular switch regulating the translocation-dependent ATP hydrolysis has been recently localized (IRA1 domain). IRA1 communicates between the N- and C-terminal domains of SecA. Interestingly, mutants in the IRA1 domain uncouple ATP hydrolysis from membrane translocation (29). We suspect that the mutants H5EE, pro60coat, and pro80coat lack sufficient size to activate the IRA1 domain.

Our results also show that the size of the precursor for functional binding to SecA is not critical. The 75-amino acid-long H5EE protein was a very efficient competitive inhibitor of SecA. Previous data had shown that regions for SecA binding are localized in the signal sequence and in the mature region of precursor proteins (19). In the signal sequence, the hydrophobic character seems important (10, 28). Subtle changes in the region close to the leader peptidase cleavage site also affect the interaction with SecA as shown with the procoat mutants H5E and H5EE (Fig. 7). Obviously, specific features of the substrate protein are required for functional interaction with each component. Shortly after synthesis, membrane and secretory proteins interact with SRP (30), GroE (31), SecB (20), and/or SecA to reach the membrane surface. Further studies are necessary to understand the molecular details of recognition. M13 procoat mutants have helped to identify some of these features that allow interaction with SRP (10) and as shown in this work with SecA. Even small changes in procoat, such as two additional glutamate residues, enhance the binding to SecA. Intriguingly, the simple binding to a component may not be sufficient for optimal use of the respective pathway efficiently. A functional

activation is often also necessary. These events impose a multistep quality control to select specific substrates for each pathway.

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