Interaction between SecA and SecYEG in Micellar Solution and Formation of the Membrane-Inserted State †

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Interaction between SecA and SecYEG in Micellar Solution and Formation of the Membrane-Inserted State[†]

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ABSTRACT: Preprotein translocation in *Escherichia coli* is mediated by the *translocase* with SecA as peripheral ATPase and SecY, SecE, and SecG as membrane domain. To facilitate large-scale purification of the SecYEG heterotrimer, SecY was fused at its amino terminus with a hexahistidine tag and co-overexpressed with SecE and SecG. The presence of the His tag allowed purification of homogeneously pure SecYEG complex by a single anion-exchange chromatographic step starting from octyl glucoside-solubilized inner membranes. Endogenous levels of SecD and SecF copurified with the SecYEG protein. Purified SecYEG complex retained a nativelike, α-helical conformation in octyl glucoside and in micellar solution binds SecA with high affinity. In the presence of the nonhydrolyzable nucleotide analogue adenosine 5′-(*β*,*γ*-imidotriphosphate), octyl glucoside-solubilized SecYEG is nearly as effective as the reconstituted enzyme in inducing the formation of a proteinase K-protected 30 kDa fragment of ^{125}I-labeled SecA, while SecYEG is proteolyzed to fragments smaller than 6 kDa. These data demonstrate that the 30-kDa SecA fragment is not protected by the lipid phase nor by SecYEG but rather indicate that it represents a SecYEG- and nucleotide-induced stable conformational state of a SecA domain.

The last decade has seen a major advance in the study of bacterial protein translocation (for reviews see refs *1* and *2*). The components involved in the translocation reaction have been genetically identified and biochemically purified, and the translocation reaction as it proceeds across the inner membrane of *Escherichia coli* has been reconstituted in liposomes using purified components (*3, 4*). The general secretion pathway in *E. coli* consists of a cytoplasmic chaperone, SecB (*5*), a peripherally membrane-associated ATPase, SecA (*6*), and five inner membrane proteins, *i.e.*, SecY (*7*), SecE (*8*), SecG (*9*), SecD, and SecF (*10, 11*). The minimal requirements for preprotein translocation across the inner membrane are met by SecA, SecY, and SecE, which together with SecG form the translocase (*3*). SecG enhances the fidelity of the SecYE reconstituted protein translocation reaction at least 20-fold (*9*), while *secG* null strains are cold-sensitive for growth (*12*). SecD and SecF are integral membrane proteins that expose large hydrophilic domains to the periplasmic space (*10, 11, 13*). *SecD* and *secF* null strains are also cold-sensitive (*13*). SecY, SecE, and SecG can be immunoprecipitated, isolated, and purified as a stable heterotrimeric complex (*3, 14*). Genetic evidence suggests that SecY and SecE are dissociable subunits (*15*), but biochemical evidence suggests that they interact stably once assembled in the membrane (*16*). Together SecY and SecE suffice to constitute a high-affinity membrane binding site for SecA (*14, 17*). Recent evidence suggests that the proteins encoded by the *secD* operon, *i.e.*, SecD, SecF, and YajC, also form a heterotrimeric complex that associates with the SecYEG complex to form a large hexameric integral membrane protein domain (SecYEGDFYajC) that is stable in the mild detergent digitonin (*14*). Stabilization by overexpression (*18, 19*) experiments indicate that SecG and SecE interact with SecY, while SecF interacts with SecD and SecY. YajC is a dispensable protein that has no known function in protein export (*11*). When present on a high-copy plasmid, it suppresses the SecY^{-d} mutation and causes impaired growth of the SecY39 mutant at 42 °C (*20*). SecD and SecF have been implicated in the maintenance of protonmotive force (Δ*p*) across the cytoplasmic membrane (*21*), in the release of translocated proteins in the periplasmic space (*22*), and in the stabilization of membrane-inserted SecA (*14, 23*). Overexpression of SecD and SecF restores the translocation of preproteins with a defective signal peptide (*13*).

SecA is a dissociable subunit of the translocase and exists both in free cytosolic forms and as membrane-bound forms (*24*). SecA interacts in a nonsaturable manner with acidic phospholipids (*25*) and binds with high affinity to the SecY subunit of the SecYEG complex (*14, 17, 26, 27*). SecYEG-bound SecA exhibits a high affinity for the binary SecB−

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[1] Abbreviations: AMP-PNP, adenosine 5′-(*β*,*γ*-imidotriphosphate); BSA, bovine serum albumin; CBB, Coomassie brilliant blue; CD, circular dichroism; DTT, dithiothreitol; IMVs, inner membrane vesicles; IPTG, isopropyl *β*-D-thiogalactopyranoside; pAb, polyclonal antibody; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TID, 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine; TMS, transmembrane segment; Δ*p*, protonmotive force.

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preprotein complex and recognizes the preprotein by interacting with both the signal sequence and the mature domain of the preprotein (*25*, *28*). SecA is a preprotein-stimulated ATPase (*29*), which in the presence of acidic phospholipids and SecYEG is activated for ADP−ATP exchange (*3*, *29*). Binding of ATP elicits a conformational change (*30*) that releases SecB into the cytosol (*31*) and promotes insertion of a SecA carboxyl-terminal domain into the membrane (*23*, *32*, *33*). At the same time, limited translocation of SecA-bound preprotein polypeptide segments occurs (*34*). SecA releases the bound preprotein upon the hydrolysis of ATP (*34*), concomitantly with the deinsertion of the SecA domain from the membrane (*32*). Repeated cycles of ATP binding and hydrolysis coupled to the membrane insertion and deinsertion of SecA at the SecYEG complex ultimately allow the stepwise translocation of the preprotein across the membrane (*34*−*36*). Δp stimulates the rate of translocation (*37*).

The preprotein translocation reaction across the inner membrane of *E. coli* shares many characteristics with that of the eukaryotic endoplasmic reticulum (for reviews see refs *38* and *39*). The quaternary organization of the mammalian and yeast endoplasmic reticulum translocase largely resembles that of the SecYEG complex of *E. coli*. The mammalian Sec61p complex consists of three subunits, *i.e.*, Sec61α, Sec61β, and Sec61γ. Sec61α and Sec61γ are homologous to the bacterial SecY and SecE, respectively, while Sec61β has a similar organization as SecG but is not homologous. The mammalian Sec61p complex is found associated with the TRAM protein, which so far has no known homologues in bacteria. In yeast, also a posttranslational translocase has been identified, which in addition to Sec61 (Sec61α in mammals), Sbh1 (Sec61β in mammals), and Sss1 (Sec61γ in mammals), involves Sec62, Sec63, Sec71, and Sec72 (*40*). Yeast does not contain a SecA homologue, but lumenal BiP (Hsp70) couples the hydrolysis of ATP to the translocation of preproteins into the ER lumen. These complexes have been purified to homogeneity (*40*, *41*), and investigated by high-resolution electron microscopy, which shows ringlike structures, suggesting that the membrane domain consists of 3−4 Sec61p trimers with a central pore (*42*). The translocation pore of the mammalian Sec61p complex may have a diameter of 40−60 Å during protein translocation (*43*). A low-resolution structure of the bacterial SecYEG complex is not yet available.

The SecYEG complex can be purified from wild-type cells (*3*) or as separate, isolated subunits from overproducing strains (*44*). To facilitate studies on the structure and function of the bacterial translocase, we have developed a rapid purification protocol that permits isolation of milligram amounts of functional SecYEG protein using membranes derived from an *E. coli* strain that overexpresses the SecYEG complex with a His tag on SecY. The catalytic and functional properties of the SecYEG complex have been analyzed in micellar solution in the absence of compartmentalization. The solubilized complex binds stoichiometrical amounts of SecA with high affinity and, more strikingly, supports the nucleotide-induced formation of a 30 kDa proteinase-protected fragment of SecA that is thought to be membrane-integrated. Since the SecYEG complex is degraded under these conditions, it appears that this SecA fragment is not protected by the lipid bilayer nor by a transmembrane shell of SecYEG protein but rather represents a stable conformation of a SecA domain induced by its interactions with SecYEG and nucleotides.

EXPERIMENTAL PROCEDURES

Materials. *E. coli* SecA (*6*), SecB (*45*), and proOmpA (*46*) were purified as described. ^{35}S-Labeled proOmpA was synthesized by *in vitro* transcription/translation, affinity-purified (*47*), and stored frozen in 6 M urea and 50 mM Tris-HCl, pH 7.8. pAbs were raised against synthetic peptides that correspond to domains of SecG (H_2N-APAK-TEQTQP) (Research Genetics Inc., Huntsville, AL) (α-SecG IgG), YajC (H_2N-YRPQQKRTKEHKKLMDS), SecD (H_2N-KEELSNGRTVQQAIDEGYRG) (α-SecD IgG), and SecF (H_2N-MAQEYTVEQLNHGRKC) (α-SecF IgG) (Neosystem Laboratoire, Strassbourgh, France) as conjugates of bovine serum albumin (BSA). The pAb against leader peptidase (α-Lep IgG) was a generous gift of W. Wickner (Dartmouth College, Hanover, NH). *E. coli* lipids (Avanti Polar lipids, Inc., Brimingham, AL) were washed with acetone and ether (*48*) and suspended at 20 mg/mL in water containing 1 mM dithiothreitol (DTT).

Plasmid Construction. The vector pET340 (SecYEG$^+$) allows the overproduction of SecYEG under control of the isopropyl β-D-thiogalactopyranoside-(IPTG-) inducible *trc* promoter (*49*) and contains an ampicillin resistance gene and colE1 origin. The construction of plasmids overproducing the SecYEG complex with a hexahistidine tag at the amino terminus of SecY (pET349; SechYnEG$^+$) and SecE (pET320; SecYhEnG$^+$) and the carboxy-terminus of SecY (pET512; SechYcEG$^+$) has been described previously (*26*). The hexa-His tag followed (or preceded, in the case of the carboxy-terminal tag) by an enterokinase recognition site contains the amino acids sequence MH_6E_4KA. As a control plasmid, the linking sequence coding for the His tag and enterokinase site (*26*) was inserted in pET324 (*49*), resulting in pET302. All constructs were confirmed by sequence analysis on a Vistra DNA sequencer 725 using the automated Δtaq sequencing kit (Amersham, Buckinghamshire, U.K.). All other DNA techniques followed standard procedures (*51*).

Bacterial Strains and Growth Conditions. For all experiments, the OmpT and OmpP (A.K., unpublished results) protease-deficient strain SF100 (*52*) was used. Strains were grown aerobically at 37 °C on L-broth in a shaking incubator until the end of the logarithmic phase. Ampicillin was used at 50 μg/mL. For the induction of plasmid-encoded genes under control of an IPTG-inducible promoter, exponentially growing cultures were supplemented with 0.5 mM IPTG at an OD_{660} of 0.5 and grown for another 2 h. Large-scale production of cells was done in a 15-L fermenter (ADI 1065 Biobench, Applikon, The Netherlands) on L-broth supplemented with 1% glycerol. At an OD_{660} of about 4, cells were induced for 2 h, collected by centrifugation, resuspended into 20% sucrose and 50 mM Tris-HCl, pH 8.0, and frozen as nuggets in liquid nitrogen.

Isolation of Inner Membrane Vesicles. Cells were quickly thawn at 37 °C, and diluted with an equal volume of 20% glycerol and 50 mM Tris-HCl, pH 8.0 (buffer A) supplemented with 1 mg/mL DNase and RNase and 1 mM phenylmethanesulfonyl fluoride. The suspension was subjected to French press treatment (4 times at 8000 psi), diluted

with an equal volume of buffer A, and cleared from debris by centrifugation (10 min at 4000*g*). Membranes were isolated from the supernatant by centrifugation (90 min at 40000*g*), resuspended in buffer A, and applied onto a 30−60% sucrose gradient in 50 mM Tris-HCl, pH 8.0. After 18 h, IMVs were collected, diluted with 5 volumes of buffer A, and recollected by centrifugation (90 min at 40000*g*). Purified IMVs were resuspended in buffer A at 20 mg/mL and stored in liquid nitrogen.

Solubilization and Purification of SecYEG. SechYnEG$^+$ or SechYcEG$^+$ IMVs (60 mg of protein) were solubilized on ice for 20 min at 1 mg/mL in 1.25% *n*-octyl β-D-glucopyranoside (octyl glucoside), 10 mM Tris-HCl, pH 8.0, 20% glycerol, 0.5 mg/mL *E. coli* lipids, and 5 mM *p*-aminobenzamidine (buffer B). Nonsolubilized proteins and aggregates were removed by centrifugation (30 min at 40000*g*), and the cleared supernatant was loaded onto a DEAE column (volume 60 mL) (Whatman, DE-52) equilibrated with buffer B. The column was washed with 2 volumes of buffer B, and proteins were eluted with a linear KCl gradient (0−300 mM) in 3 volumes of buffer B. The ionic strength of the elution was monitored continuously with a conductivity monitor (Pharmacia Biotech, Uppsala, Sweden). Fractions of 6 mL were collected, immediately supplemented with 87% glycerol and 1.25% octyl glucoside to yield a final glycerol concentration of 40%, and stored at −18 °C. Fractions were analyzed by SDS−15% PAGE (sodium dodecyl sulfate−polyacylamide gel electrophoresis) (*53*) and stained with Coomassie brilliant blue (CBB). Immunoblotting was performed using a semidry blotter (Transblot-SD, Bio-Rad Laboratories, Hercules, CA), and blots were developed using several different pAbs and *p*-nitroblue tetrazolium chloride/sodium *5*-bromo-*4*-chloro-indolyl phosphate staining (Boehringer Mannheim, Mannheim, Germany) of alkaline phosphatase-conjugated secondary antibody (Boehringer Mannheim, Mannheim, Germany).

Preparation of Polyclonal Antibodies Directed against SecY and SecE. To obtain pAbs directed against purified SecY and SecE, membranes were isolated from *E. coli* strain SF100 harboring pET320 (SecYhEnG$^+$) or pET349 (SechYnEG$^+$), respectively. His-tagged proteins were purified from the octyl glucoside-solubilized IMVs by Ni^{2+}−NTA affinity chromatography using a HiTrap chelating column (Pharmacia Biotech) and a buffer containing 100 mM NaCl and 50 mM NaP_i, pH 8.0, and a gradient of 0−500 mM imidazole. Fractions containing SecY and SecE were separated on a preparative 15% polyacrylamide gel, and individual proteins were excised from the gel and collected by electroelution (Electro-Eluter, Bio-Rad Laboratories, Hercules, CA). Purified proteins (over 99% pure as checked by SDS−PAGE followed by CBB staining) were used as antigens to immunize rabbits to yield pAbs against the His-tagged SecY (α-SecY IgG) and SecE (α-SecE IgG).

Reconstitution of SecYEG into Liposomes. The SechYnEG complex was reconstituted into liposomes by a modification of the detergent dilution technique (*3*). Solubilized SechYnEG (100 μL; 0.25−0.5 mg/mL) was mixed with 20 μL of *E. coli* lipids (20 mg/mL) and incubated for 5 min on ice. The sample was diluted into 4 mL of buffer containing 50 mM Tris-HCl, pH 8.0, and 50 mM KCl, and after 5 min of incubation, proteoliposomes were collected by centrifugation (30 min, SW-28 rotor, 50 000 rpm, 4 °C) and resuspended in 100 μL of 50 mM Tris-HCl, pH 8.0, and 50 mM KCl. Reconstituted SechYnEG proteoliposomes were frozen and stored in liquid nitrogen. Before use, samples were thawed at 37 °C and sonicated 3 times for 10 s in a bath sonicator.

Translocation and SecA Translocation ATPase Assays. Translocation of ^{35}S-proOmpA into proteoliposomes was assayed by its accessibility to added proteinase K (*54*). Reaction mixtures (50 μL) contained buffer D [50 mM HEPES-KOH, pH 7.5, 30 mM KCl, 0.5 mg/mL BSA, 2 mM DTT, and 2 mM $Mg(OAc)_2$], 1.6 μg of SecB protein, 0.5 μg of SecA, 2 mM ATP, 10 mM creatine phosphate, and 0.5 μg of creatine kinase. Based on quantitative immunoblot analysis, reactions were supplemented with approximately equal amounts of SecY, *i.e.*, with SechYnEG proteoliposomes (20 μg/mL) or SechYnEG$^+$ IMVs (100 μg/mL). ^{35}S-proOmpA was diluted 50-fold from a solution containing 6 M urea and 50 mM Tris-HCl, pH 7.8. Translocation reactions were performed at 37 °C, and at various times, samples were taken, chilled on ice, and treated with proteinase K (0.1 mg/mL) for 15 min. Samples were analyzed by SDS−12% PAGE and quantified with the β-imager 2000 (Biospace Measures, Paris, France).

Translocation ATPase activity of urea-treated inner membranes or SecYEG proteoliposomes was measured with proOmpA as described (*29*).

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra of octyl glucoside-solubilized or reconstituted SechYnEG protein (95 μg/mL) were recorded in 10 mM NaP_i, pH 7.8, 10 mM KCl, and 1 mg/mL *E. coli* lipids. CD spectra of SecA (168 μg/mL) were determined in 10 mM NaP_i, pH 7.6, 10 mM KCl and 2 mM $MgCl_2$ in the absence and presence of 1.25% octyl glucoside. Measurements were performed with an Aviv CD spectrophotometer, Model 62a DS. The measuring cell was thermostated at 4 °C. Spectra were corrected for the lipid and detergent contributions and deconvoluted with the SELCON software package (*55*).

Enterokinase Digestion of His-Tagged SecY. The accessibility of the introduced enterokinase site at the amino terminus of SecY was determined as follows: solubilized or reconstituted SechYnEG complex (50 μg/mL) was incubated at 20 °C in a buffer (30 μL) containing 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 2 mM $CaCl_2$. To the samples, varying amounts of SecA or BSA were added, and after 10 min, the mixture was supplemented with 1 unit of enterokinase (New England Biolabs, Beverly, MA) and incubated for 1 h. Reactions were terminated by the addition of SDS sample buffer and analyzed by SDS−15% PAGE followed by CBB staining or immunoblotting using the pAbs directed against His-tagged SecY or SecE.

Formation of the ^{125}I-Labeled 30 kDa Fragment of SecA. Purified SecA was labeled with carrier-free ^{125}I as described (*32*, *56*). ^{125}I-SecA (2.5 ng/mL) was incubated on ice in buffer D containing either SechYnEG$^+$ IMVs (0.5 μg/mL) or purified SechYnEG (50 ng/mL) reconstituted into proteoliposomes or present in octyl glucoside solution. Incubations were performed with or without 1.25 mM adenosine 5′-(β,γ-imidotriphosphate) (AMP-PNP), and in the case of the solubilized SechYnEG, the buffer was supplemented with 1.25% octyl glucoside or 1% Triton X-100.. After 30 min, samples were treated with proteinase K (0.04−1 mg/mL) for 15 min on ice, precipitated with 7.5% (w/v) trichloroacetic acid, washed with acetone, and solubilized in SDS

Table 1: Plasmids Used in This Study

plasmid	relevant characteristics	source or reference
pET324	pTRC99A-derived vector containing *lacZ*α behind the *trc* promoter	*48*
pET302	pET324-derived vector containing a His tag and an enterokinase site	this work
pET340	pET324 with *secYEG* under control of the *trc* promoter	*48*
pET320	pET302 containing *secYEG* behind *trc* promoter with N-terminally His-tagged SecE	*26*
pET349	pET302 containing *secYEG* behind *trc* promoter with N-terminally His-tagged SecY	*26*
pET512	pET340 containing C-terminally His-tagged SecY	*26*

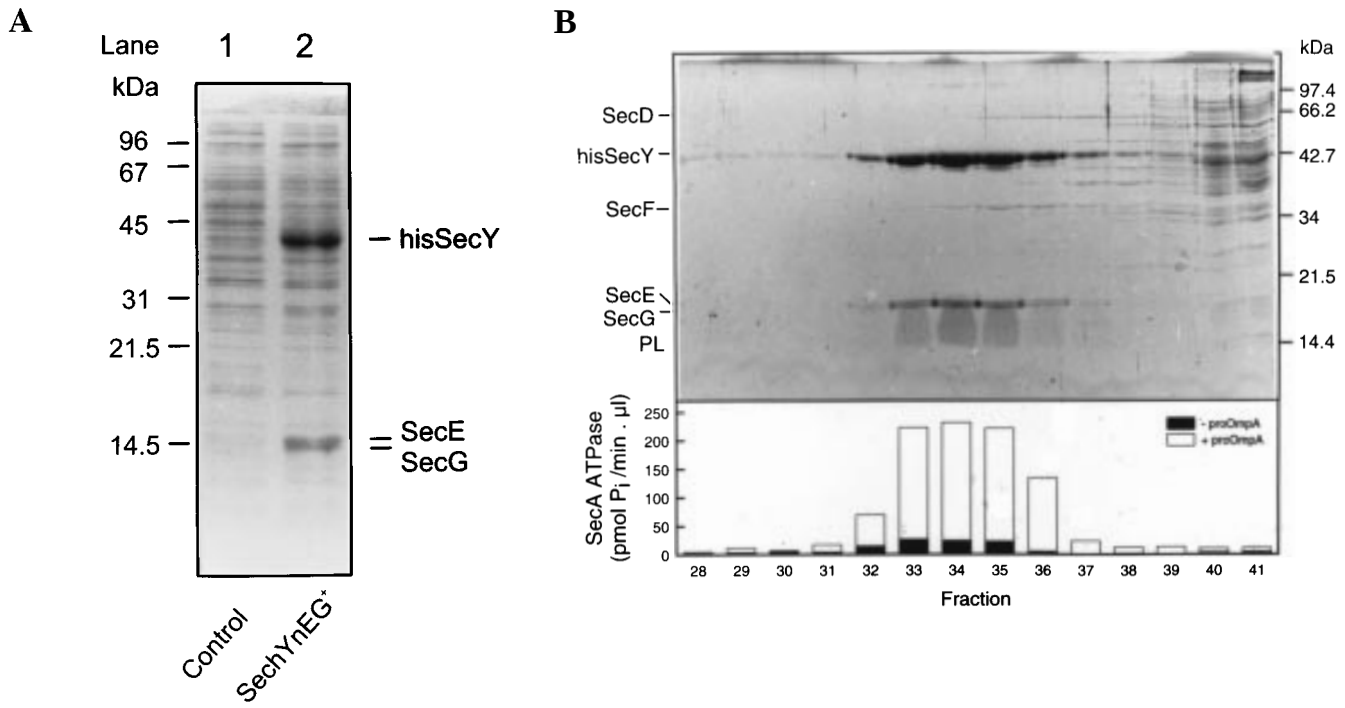

FIGURE 1: Co-overexpression of hexahistidine-tagged SecY with SecE, and SecG, and purification of the SecYEG complex. (A) CBB-stained SDS−PAGE of IMVs derived from SF100 cells harboring plasmids pET324 (control) (lane 1) and pET349 (SechYnEG$^+$) (lane 2). (B and C) Octyl glucoside-solubilized SechYnEG$^+$ IMVs were applied on a DEAE column and eluted with a linear gradient of 0−300 mM KCl. Column fractions were analyzed by SDS−PAGE and CBB staining (B), and after reconstitution into liposomes by rapid dilution, they were assayed for the SecA activity in the absence (solid bars) and presence (open bars) of proOmpA (C). The positions of the various Sec proteins and molecular mass markers are indicated.

sample buffer. Samples were separated by 12% PAGE and analyzed by autoradiography and β-imaging.

^{125}I-Labeling and Proteolysis of Purified SechYnEG Complex. Reconstituted SechYnEG protein (10 μg) in 50 mM KCl and 50 mM Tris-HCl, pH 8.0 (100 μL), was photoaffinity-labeled with 13.3 μCi of 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (^{125}I-TID) (Radiochemical centre, Amersham, U.K.) for 2 min at 0 °C with a 254 nm lamp (Model UVG-54, UVP Life Sciences Inc., Cambridge, U.K.) placed at a distance of 1 cm.

For proteolysis experiments, proteoliposomes bearing nonlabeled or ^{125}I-TID SechYnEG (0.5 μg), or solubilized SechYnEG complex, were incubated for 30 min at 4 °C in buffer D (50 μL) in the absence or presence of 1.25% octyl glucoside, 1.25 mM AMP-PNP, and/or 20 μg/mL of SecA protein. Subsequently, samples were incubated with proteinase K (1 mg/mL) for 15 min at 4 °C, solubilized in SDS sample buffer, separated on 16% Tricine gels (*57*), and analyzed by silver and CBB staining, autoradiography, or by western blotting using α-SecY IgG.

Other Analytical Techniques. Protein concentrations were determined by the method of Lowry (*58*) in the presence of SDS using BSA as a standard. The SecYEG concentration was estimated from quantitative total amino acid determination performed by Eurosequence (Groningen, The Netherlands). SecA protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient (ϵ) of 90.85 mM^{-1} cm^{-1} (T. den Blaauwen, personal communication).

RESULTS

Purification of Functional SecYEG Complex. To simplify the purification of the SecYEG complex from an overproducing strain (*49*), affinity purification of the complex with His-tagged SecY or SecE on Ni^{2+}−NTA columns was tried. For this purpose, vectors were used that express the *secYEG* genes in tandem with a hexahistidine tag and cleavable enterokinase site at either the amino- or carboxyl terminus of SecY (Table 1; *26*). The His-tagged SecY protein can be co-overexpressed to high levels together with SecE and SecG as shown by CBB staining of an SDS−polyacrylamide gel loaded with SechYnEG$^+$ IMVs (Figure 1A). We have previously shown that the His tag does not interfere with the level of SecYEG overexpression, the proOmpA-stimulated SecA translocation ATPase, and ^{35}S-proOmpA translocation into IMVs (*26*). Ni^{2+} affinity chromatography

Table 2: Purification of SecYEG Protein

sample		protein (mg)	SecA translocation ATPase: total activity[a] (mmol of P_i/min)	SecA translocation ATPase: specific activity [nmol of P_i/min^{-1} (mg of protein)$^{-1}$]
SecYEG$^+$	membranes	60 (100%)	7860 (100%)	131
	DEAE pool	15 (25%)	8118 (103%)	580

[a] The percentage of recovery of SecA translocation ATPase is indicated in parentheses.

with Triton X-100-, octyl glucoside-, or dodecyl maltoside-solubilized IMVs resulted only in the purification of His-tagged SecY or SecE protein with almost no detectable copurification of other components of the SecYEG complex. Moreover, *E. coli* phospholipids could not be included in the elution buffer as they interfered with the binding of the His-tagged proteins to the Ni^{2+}−NTA resin. Previously, it was shown that phospholipids are need to maintain the SecYEG complex in an active state during solubilization and reconstitution (*59*). Therefore, the conventional method for purification of the SecYEG complex was attempted, which is based on octyl glucoside solubilization of urea-treated IMVs followed by three consecutive chromatographic steps (*3*). Since the urea extraction reduced the efficiency of solubilization, this step was eliminated and octyl glucoside-extracted proteins were directly loaded on a DEAE column. In contrast to the native complex, the SecYEG heterotrimer with amino-terminally His-tagged SecY binds only weakly to the column and elutes at a KCl concentration of about 10 mM in an essentially pure form (Figure 1B). Similar results were obtained with carboxyl-terminally His-tagged SecY (data not shown). In contrast, wild-type SecYEG complex elutes at a KCl concentration of about 50−60 mM along with most of the other membrane protein (*3*). For convenience, the purified complex harboring the amino-terminally His-tagged SecY is termed SechYnEG in the following sections.

The identity of the protein bands in the purified fractions was verified by the use of pAbs directed against the His-tagged SecY and SecE proteins, a pAb raised against a synthetic peptide corresponding to a SecG domain, and by total amino acid analysis of the proteins excised from the gel. In addition, minor amounts of two other proteins were present that coelute with the SechYnEG protein. These proteins, with apparent molecular masses of 54 and 27 kDa, reacted with α-SecD and SecF antibodies, respectively. Immunoblot analysis further showed that the purified SechYnEG was devoid from YajC and leader peptidase (Lep) that were recovered in the flowthrough and bulk protein fractions, respectively (data not shown).

Column fractions were reconstituted by rapid dilution in liposomes composed of *E. coli* lipids and analyzed for the proOmpA-stimulated SecA translocation ATPase activity. Only the fractions containing SechYnEG protein were active in this assay (Figure 1C). Purification resulted in an almost 5-fold increase in the specific SecA ATPase activity, and apparently without loss of activity (Table 2). When analyzed at the same SecY content, proteoliposomes reconstituted with SechYnEG proteoliposomes were nearly as active in the ATP and SecA-dependent translocation of ^{35}S-proOmpA as non-urea-treated SechYnEG$^+$ IMVs (Figure 2). The use of a His-tagged SecY, therefore, allows for a rapid purification of

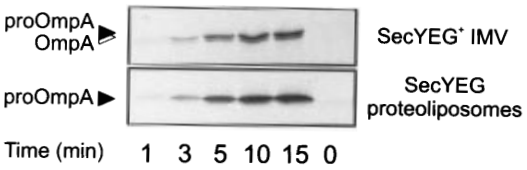

FIGURE 2: Purified SechYnEG is reconstituted in an active state. Time courses of the translocation of ^{35}S-proOmpA into SechYnEG$^+$ IMVs (A) and proteoliposomes reconstituted with purified SechYnEG$^+$ (B) in the presence of SecA and ATP are shown. Samples contained identical amounts of SecY as determined by quantitative immunoblotting. Positions of proOmpA and processed OmpA are indicated.

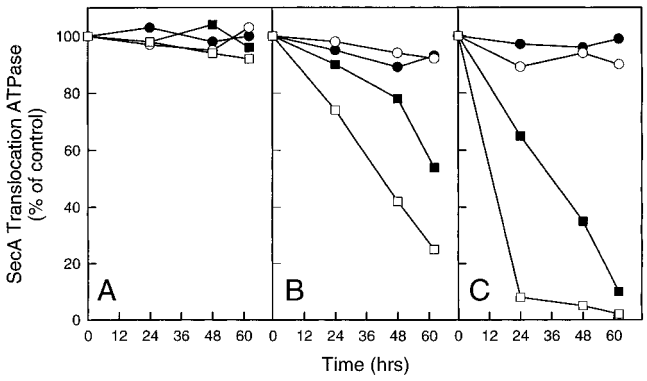

FIGURE 3: Temperature stability of the SechYnEG complex. The time courses of the remaining proOmpA-stimulated SecA ATPase activity of (A) SechYnEG$^+$ IMVs, (B) SechYnEG proteoliposomes, and (C) solubilized SechYnEG protein after incubation at −20 °C (●), 4 °C (○), 25 °C (■) and 37 °C (□) was measured as described in the Experimental Procedures section. The SecA translocation ATPase activity of SechYnEG$^+$ IMVs stored in liquid nitrogen was set to 100%.

large quantities of functional SechYnEG complex with only a minimal loss of activity.

Stability of the SecYEG Complex. To facilitate further biochemical and biophysical research, the stability of the detergent-solubilized SechYnEG was compared with the reconstituted enzyme and with SechYnEG$^+$ IMVs. For this purpose, the SecA translocation ATPase activity was measured after incubation under different sets of conditions. SechYnEG$^+$ IMVs appeared highly stable, and hardly any inactivation occurred after a 3 day incubation at 37 °C (Figure 3). Reconstituted SechYnEG complex was stable on ice and when stored at −20 °C or below but lost its activity when incubated for longer periods of time at 25 and 37 °C. Finally, the purified SechYnEG complex in octyl glucoside was stable only at 4 °C and below and was rapidly inactivated at 37 °C ($t_{1/2}$ = 15−20 min) (Figure 3). For stability, it was necessary that the glycerol concentration was kept at 40% (v/v) and that at least 0.2 mg/mL *E. coli* lipids are present. Under these conditions, it was possible to store the purified complex for at least 7 months at −20 °C without loss of activity.

Octyl Glucoside-Solubilized SecYEG Is in a Near-Native, α-Helical Conformation. The influence of octyl glucoside on the secondary structure of SechYnEG was analyzed by circular dichroism (CD) spectroscopy. The spectrum of reconstituted SechYnEG is typical for an α-helical protein (Figure 4A). Computational deconvolution of the CD spectrum revealed an α-helical content of at least 83%, indicating that the protein is largely in a nondenatured state.

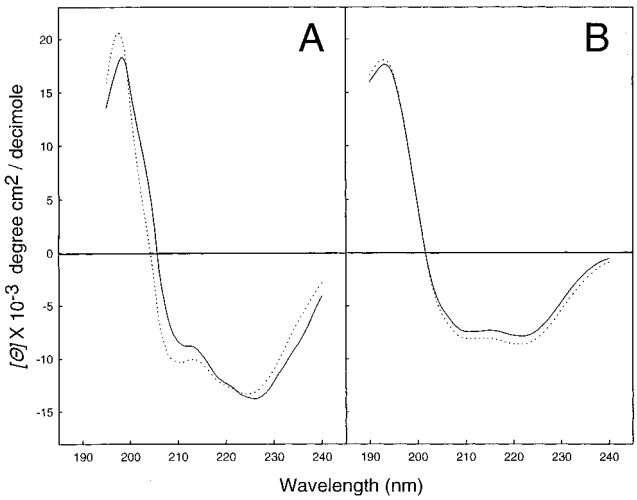

FIGURE 4: Circular dichroism spectra of SechYnEG (A) and SecA (B) in the absence (solid lines) and presence (dashed lines) of octyl glucoside. Shown spectra were corrected for the lipid and detergent contributions.

Although some loss of secondary structure seemed to occur, the SechYnEG complex remained mainly α-helical (72%) when solubilized in octyl glucoside. The CD spectrum of SecA (Figure 4B) points at a high amount of β-structure (55% β-sheet, 18% β-turn, and 18% α-helical). Octyl glucoside (1.25%) hardly altered the spectrum (48% β-sheet, 16% β-turn, and 21% α-helical). These data indicate that both SechYnEG and SecA maintain a nativelike secondary structure when present in detergent solution.

Octyl Glucoside-Solubilized SecYEG Binds SecA with High Affinity. To determine the orientation of reconstituted SechYnEG, the accessibility of the enterokinase recognition site at the amino terminus of SecY to externally added enterokinase was analyzed in the absence and presence of octyl glucoside. In the absence of octyl glucoside, enterokinase removes about 50% of the His tag from the reconstituted SecY protein (Figure 5, compare lanes 1 and 2), and the tag is removed completely upon solubilization by octylglucoside (lane 4). Immunoblot analysis of the samples using a mAb directed against the His tag confirms that enterokinase indeed removes the His tag from SecY (see Figure 6B). The data suggest that SechYnEG is reconstituted in a scrambled orientation.

Next, the ability of SecA to protect the His-tagged SecY of SechYnEG proteoliposomes against enterokinase cleavage was determined. Experiments were performed at 25 °C where the SechYnEG complex is reasonably stable in detergent solution when incubated for only short periods of time, *i.e.*, 30 min to 1 h (Figure 3C). When the amount of BSA in the buffer was gradually replaced by SecA, protection of the His-tagged SecY against proteolysis by enterokinase occurred (Figure 6A, B). Strikingly, the same protective effect of SecA was observed when the experiments were performed in the presence of octyl glucoside to solubilize the SechYnEG protein (Figure 6C, D). In the absence of SecA, enterokinase completely removed the His tag from SecY. On the other hand, full protection against proteolysis was obtained when SecA monomer (~0.95 μM) was added in a slight excess relative to SecY (~0.7 μM). The protective effect of SecA on reconstituted and detergent-solubilized His-tagged SecY was not influenced by nucleotides (ATP, AMP-PNP, or ADP) and/or proOmpA. In contrast to the reconstituted enzyme, the solubilized SechYnEG did not support SecA translocation ATPase activity (data not shown). These data demonstrate that SechYnEG maintains the ability to bind SecA with high affinity when present in detergent solution.

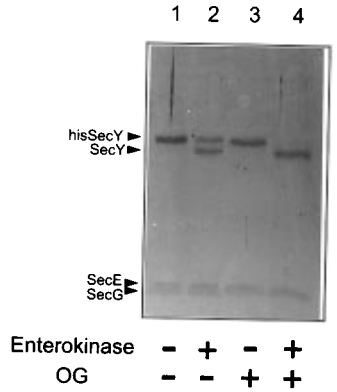

FIGURE 5: Orientation of SecY in reconstituted SechYnEG proteoliposomes. The accessibility of the introduced proteolytic enterokinase site at the amino-terminus of SecY toward purified enterokinase was used to probe the orientation of SecY in the reconstituted SechYnEG proteoliposomes. SechYnEG proteoliposomes were incubated in the absence (lanes 1 and 2) and presence of 1.25% octyl glucoside (OG) (lanes 3 and 4) and, as indicated, treated with enterokinase as described in the Experimental Procedures section. Incubations were at 25 °C in a buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 2 mM $CaCl_2$. After 1 h, samples were analyzed by SDS−12% PAGE and CBB staining. The positions of SecE, SecG, and his-tagged (hisSecY) and enterokinase-cleaved SecY (SecY) are indicated by an arrow.

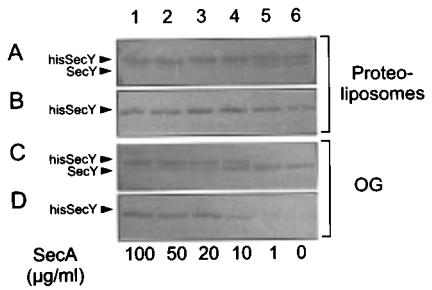

FIGURE 6: SechYnEG maintains its ability to interact with SecA when present in octyl glucoside micellar solution. Reconstituted SechYnEG proteoliposomes (50 μg/mL) (lanes A and B) or solubilized SechYnEG complex (lanes C and D) was incubated with 0−100 μg/mL SecA and treated with enterokinase for 1 h at 25 °C as described in the Experimental Procedures section. The amount of SecA was balanced by BSA at a constant level of 100 μg of protein/mL, while the samples containing solubilized SechYnEG complex were supplemented with 1.25% octyl glucoside. Samples were separated on SDS−15% PAGE and analyzed by Western blotting using α-SecY (lanes A and C) and α-SecE IgG (lanes B and D). The latter has been raised against His-tagged SecE and strongly cross-reacts with the His tag on the SecY protein but not with SecY.

SecYEG-Dependent and Nucleotide-Induced Formation of the SecA Membrane-Inserted State in Detergent Solution. At 37 °C, in the presence of ATP and preprotein, the SecYEG-bound SecA undergoes a conformational change that results in the formation of a membrane-protected, proteinase inaccessible 30 kDa carboxy-terminal domain (*23*, *32*, *33*). It has been suggested that this fragment corresponds to a membrane-inserted domain of SecA. The 30 kDa SecA fragment can also be formed without preprotein at 4 °C in the presence of nonhydrolyzable ATP, *i.e.*, AMP-PNP. To

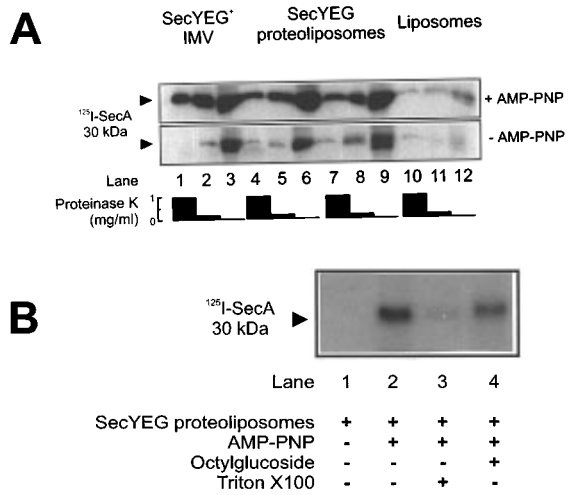

FIGURE 7: Octyl glucoside-solubilized SechYnEG supports the AMP-PNP-induced formation of the proteinase K-resistant 30 kDa ^{125}I-SecA fragment. (A) ^{125}I-SecA (2.5 ng/mL) was incubated at 4 °C with SechYnEG$^+$ IMVs (lanes 1−3), SechYnEG proteoliposomes (lanes 4−6), octyl glucoside-solubilized SechYnEG (lanes 7−9), or liposomes (lanes 10−12) with and without 1.25 mM AMP-PNP. Samples containing the solubilized SechYnEG complex were supplemented with 1.25% octyl glucoside. Subsequently, the amount of 30 kDa ^{125}I-SecA fragment was determined by incubation for 15 min with various concentrations of proteinase K (0.04, 0.2, and 1 mg/mL) as indicated by the black bars. Samples were precipitated with 7.5% (w/v) trichloroacetic acid, washed with acetone, solubilized in SDS sample buffer, and analyzed by 12% PAGE and β-imaging. (B) ^{125}I-SecA (2.5 ng/mL) was incubated at 4 °C with SechYnEG proteoliposomes (lanes 1 and 2), octyl glucoside- (lane 3) and Triton X-100- (lane 4) solubilized SechYnEG, with (lanes 2−4) or without (lane 1) AMP-PNP. Samples were further treated as described above.

determine whether the octyl glucoside-solubilized SechYnEG supports the formation of this membrane-inserted state, SecA was radiolabeled with ^{125}I, added to purified SechYnEG present either in octyl glucoside micellar solution or reconstituted in proteoliposomes, and treated with proteinase K (up to 1 mg/mL). In the presence of AMP-PNP, the solubilized SechYnEG supported the formation of the 30 kDa SecA fragment as effectively as proteoliposomes reconstituted with SechYnEG, and it was about half as active as urea-treated SechYnEG$^+$ IMVs (Figure 7A). In the absence of AMP-PNP or SechYnEG, no 30 kDa SecA fragment was formed. In contrast to octyl glucoside, Triton X-100 did not support the formation of the 30 kDa fragment (Figure 7B). Our data demonstrate that octyl glucoside-solubilized SechYnEG supports the formation of the ^{125}I-labeled 30 kDa fragment of SecA, which implies that the 30 kDa fragment is not protected by the lipid phase.

To establish whether the 30 kDa SecA domain is protected by SechYnEG, samples were also analyzed for remaining SechYnEG complex. In the absence of SecA and AMP-PNP, already at low proteinase K concentration (*i.e.*, 10 μg/mL) both the reconstituted (Figure 8A) and solubilized SechYnEG (data not shown) were rapidly degraded to fragments of 6 kDa and smaller that could be visualized by silver staining. Similar results were obtained by CBB staining (data not shown) and are in accordance with previous observations that SecY is highly susceptible to proteolysis (*17*). Since the staining assay could not be used for proteolysis experiments in which SecA was included, two other approaches were followed, *i.e.*, immunoblot analysis of the SecY using a pAb directed against the purified SecY protein (Figure 8B) and autoradiography of ^{125}I-labeled SechYnEG complex (Figure 8C). At the concentration of proteinase K (*i.e.*, 1 mg/mL) used to form the 30 kDa SecA fragment, immunoblotting with the α-SecY IgG revealed that SecA was unable to prevent proteolysis of SecY, in the absence or presence of AMP-PNP, either with the SechYnEG complex reconstituted into proteoliposomes (Figure 8B) or solubilized in octyl glucoside (data not shown). Although the pAb presumably recognizes multiple epitopes on SecY, these are likely confined to the exposed loops of SecY that are most readily proteolyzed. As an independent assay, reconstituted SechYnEG was labeled with the nonspecific lipophilic photoaffinity probe 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine (TID) that was added in ^{125}I-labeled form. ^{125}I-TID readily labeled the SecY, SecE, and SecG proteins (Figure 8C, lane 1). Since only radiochemical amounts of ^{125}I-TID were used for labeling, photo-cross-linking had little effect on the SecA translocation ATPase of the SechYnEG proteoliposomes (data not shown), indicating that the enzyme has retained most of its activity. Reconstituted or solubilized ^{125}I-labeled SechYnEG was completely digested by proteinase K (*i.e.*, 1 mg/mL) yielding only small fragments (Figure 8C, lanes 2−4 and 6−8). The fragments are poorly resolved in the lower molecular mass range due to the presence of ^{125}I-TID-labeled phospholipids in same region of the tricine gel. An excess of SecA, with or without AMP-PNP, did not protect the ^{125}I-SechYnEG, nor did it yield larger protected fragments of SecY (Figure 8C). These data demonstrate that under conditions that the 30 kDa SecA fragment can be formed, the SecYEG is proteolyzed to fragments of 6 kDa and smaller.

DISCUSSION

In this paper a simple method is described for the rapid isolation of large quantities (about 5 mg/L of cells) of homogeneously pure SecYEG protein using IMVs derived from *E. coli* cells that overexpress this complex with a hexahistidine-tagged SecY. The method is based on the weak anion-exchange column binding characteristics of the octyl glucoside-solubilized SechYnEG complex. The His tag on SecY causes a dramatic shift in the elution profile of the SecYEG complex, such that it elutes in a region which is essentially free from other contaminants. Wild-type SecYEG complex elutes from the DEAE column together with the bulk protein elution (*3*), and even when overproduced, more than one chromatographic step is needed to purify the complex (*60*). Due to the distribution of charges, the sequence bearing the hexahistidine tag and the enterokinase site will have a net negative charge at pH 8.0. Therefore, tighter instead of weaker binding to the anion-exchange resin is expected. The reason for this aberrant chromatographic behavior is unclear. On the other hand, protocols based on Ni^{2+} affinity column chromatography failed to purify the SechYnEG protein as a complex, and instead only the His-tagged protein is retained by the column (this paper and ref *26*). It thus appears that the subunit interaction cannot be maintained using Ni^{2+} affinity column chromatography, which is either due to the absence of phospholipids, extensive washing procedures, or both.

The presence of a His tag does not affect the activity of the SecYEG protein. Translocation and SecA translocation

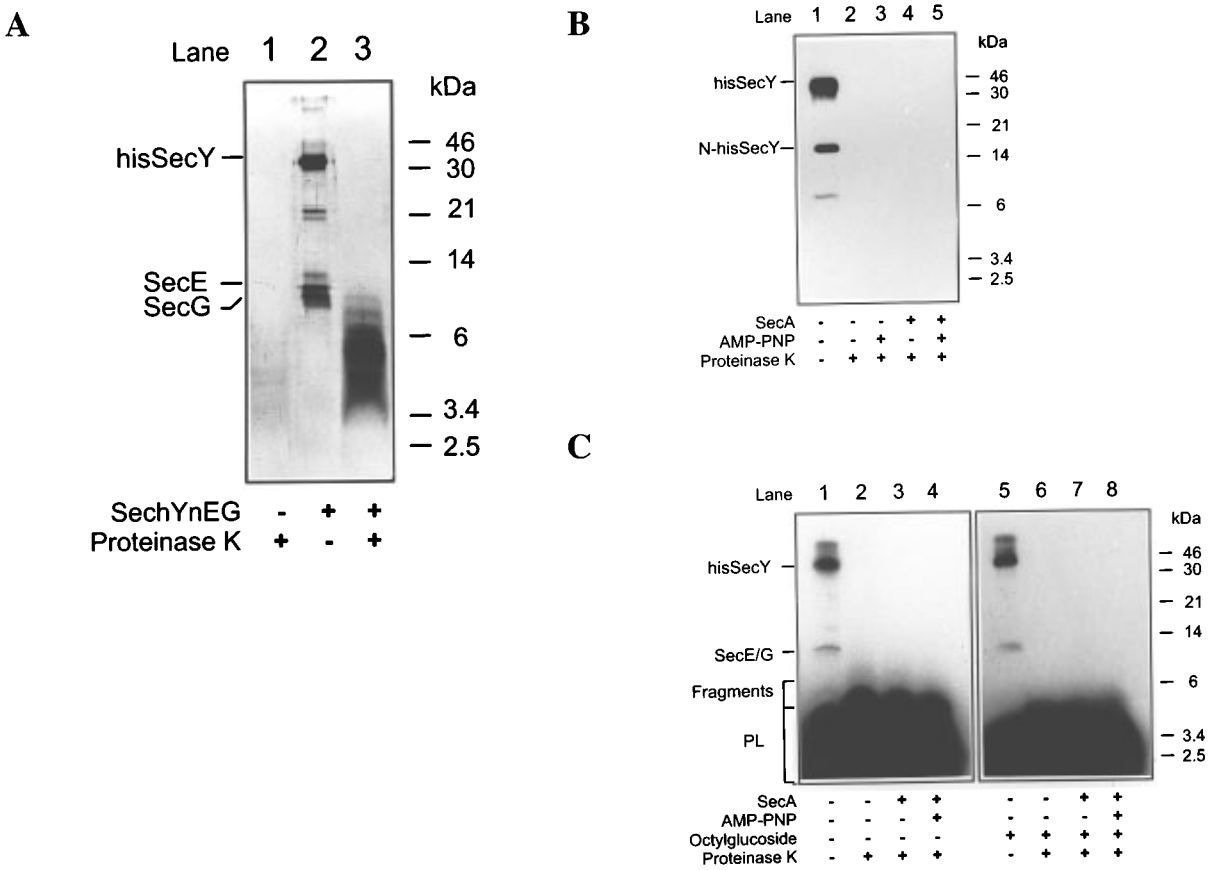

FIGURE 8: SecA and AMP-PNP do not protect SechYnEG against proteolysis by proteinase K. (A) SechYnEG proteoliposomes (100 μg/mL) in 50 mM KCl and 50 mM Tris-HCl, pH 8.0, were incubated for 15 min at 4 °C in the absence (lane 2) or presence (lane 3) of proteinase K (10 μg/mL). Samples were separated on a 16% Tricine gel and analyzed by silver staining. As a control, the amount of proteinase K present in the assay was silver-stained (lane 1). The position of hisSecY, SecE, and SecG are indicated. Additional polypeptide bands in lane 2 are proteolytic degradation products of His-tagged SecY. (B) SechYnEG proteoliposomes (10 μg/mL) (lane 1) were incubated for 30 min in the translocation buffer D without any additions (lane 2), with 1.25 mM AMP-PNP (lane 3), with 20 μg/mL SecA (lane 4), or with both SecA and AMP-PNP (lane 5). Samples were subsequently incubated with proteinase K (1 mg/mL) for 15 min at 4 °C, separated on a 16% Tricine gel, and analyzed by Western blotting using a pAb directed against the purified SecY protein (α-SecY IgG). The positions of hisSecY and an immunoreactive amino-terminal fragment of hisSecY are indicated. (C) ^{125}I-TID-labeled SechYnEG proteoliposomes (10 μg/mL) were incubated for 30 min at 4 °C in translocation buffer D in the absence (lanes 1−4) and presence (lanes 5−8) of 1.25% octyl glucoside. Samples (lanes 2 and 6) were supplemented with 20 μg/mL SecA (lanes 3 and 7) or with both SecA and 1.25 mM AMP-PNP (lanes 4 and 8). As indicated, samples were treated with proteinase K (1 mg/mL) for 15 min at 4 °C, solubilized in SDS sample buffer, separated on 16% Tricine gels, and analyzed by autoradiography. The positions of the His-tagged SecY(hisSecY), SecE/G, phospholipids (PL), and proteolytic fragments are indicated. Molecular mass standards are marked.

ATPase activities of SechYnEG$^+$ IMVs are identical to those of SecYEG$^+$ IMVs (*26*). Also, the specificity of the SecYEG complex appears not to be affected. Unlike proteoliposomes reconstituted with purified SechYnEG containing a *prlA* mutation, SechYnEG proteoliposomes and IMVs are unable to translocate a signal sequence mutant of proOmpA with a deletion of Ile at position 8 (A.K. and C.v.d.D., unpublished results).

As judged from the recovery of SecA translocation ATPase activity, slightly more than 100% of the total activity observed with SechYnEG$^+$ IMVs was recovered after purification. An increase in SecA translocation ATPase activity was noted before during the purification of the SecYEG complex from wild-type IMVs (*3*). When the mixed orientation of the reconstituted SechYnEG is taken into account, and assuming that the wrongly oriented molecules do not contribute to the SecA translocation ATPase activity, an almost 2-fold increase in total activity is predicted. These data are normalized to the activity observed with urea-treated IMVs. Urea treatment largely inactivates the SecA bound to the translocation sites (*29*), and in particular with SechYnEG$^+$ IMVs, a major part of the SecA protein remains bound to the membranes (*24*, *49*). It may well be that this SecA blocks translocation sites that are recovered when the SecA is dissociated from SecYEG during solubilization and purification. Alternatively, it may be that urea treatment results in a partial inactivation of the SecYEG complex. In this respect, we have noted that SecA readdition to urea-treated IMVs does not completely restore the translocation activity to the level observed with native membranes. Taken together, it appears that our purification methods allows for the isolation of pure SecYEG complex with a high recovery of activity.

Circular dichroism analysis of the secondary structure of the SechYnEG reconstituted into liposomes suggests that it is largely α-helical. The complex encompasses 15 transmembrane segments (TMS) that are connected by cytosolic and periplasmic loops. A high α-helical content is thus consistent with the predicted secondary structure of the SecYEG and readily accounts for the presence of 15 α-helical TMS. There is only a small loss of secondary structure when the protein is incubated with octyl glucoside or dodecyl

maltoside (C.v.d.D., unpublished data), suggesting that the complex retains a nearly to native secondary structure when present in detergent solution. The amino-terminal His tag on SecY can readily be removed by enterokinase, while SecA protects SechYn against cleavage when the SechYnEG is either reconstituted in proteoliposomes or present in detergent solution. The latter is remarkable as the translocation and lipid ATPase activity of SecA is (reversibly) inactivated by detergent (*25*). In detergent solution approximately stoichiometric amounts of the SecA monomer relative to SecY protein are needed for complete protection against enterokinase digestion. A one-to-one SecA−SecY interaction has been reported in wild-type IMVs (*17*). The SechYnEG complex (0.7 μM) was used at a concentration far above the binding affinity (30 nM) for the SecA interaction. Therefore, it is not possible to derive a K_d value from these proteolysis experiments. Since only a slight excess of SecA is needed relative to SechYnEG complex, it is evident that the interaction must be of high affinity. SecA also binds with high affinity to amino-terminal fragments of SecY on blotting membranes (*27*).

Octyl glucoside-solubilized SechYnEG not only binds SecA but also, in the presence of the nonhydrolyzable ATP analogue, AMP-PNP, supports the formation of the 30 kDa fragment of ^{125}I-labeled SecA. The 30 kDa fragment corresponds to a carboxyl-terminal domain of SecA (*33*) and is thought to insert into the membrane upon binding of AMP-PNP (*23*, *32*). We now show that this reaction can be carried out at 4 °C under conditions that the octyl glucoside-solubilized SechYnEG is stable. At 37 °C, the complex rapidly aggregates in detergent solution, and therefore it was not possible to analyze the formation of 30 kDa SecA fragment under "translocating" conditions, *i.e.*, in the presence of ATP and preprotein. Previously, solubilization of the membranes with Triton X-100 was used as a control to demonstrate that the 30 kDa proteolytic fragment is a membrane-integrated SecA domain (*32*), *i.e.*, in the presence of Triton X-100, the 30 kDa fragment is readily digested by proteinase K. Our studies show that, in contrast to octyl glucoside, Triton X-100 is unable to stabilize the 30 kDa fragment. Triton X-100 is a more potent detergent as compared to octyl glucoside due to its lower critical micellar concentration. It may well be destructive for the SecA−SecYEG interaction or disrupt the SecA conformation. Freezing and thawing of the solubilized SechYnEG complex in the presence of proteinase K did not result in a loss of the 30 kDa ^{125}I-SecA fragment (C.v.d.D., unpublished results). Recent studies suggest that the membrane-inserted form of SecA is not exposed to the lipid phase since it is inaccessible to lipid-embedded photoaffinity cross-linkers (*61*). This has led to the suggestion that the 30 kDa fragment entirely penetrates the membrane and exposes domains at the periplasmic membrane face or, alternatively, that it is shielded from lipid by the SecYEG complex (*61*, *62*). Our data with the SecYEG complex in octyl glucoside solution support the finding that the 30 kDa SecA fragment is not protected against proteinase digestion by the lipid membrane but also exclude the possibility that it is exposed to a different compartment, *i.e.*, the periplasmic membrane face of IMVs. The latter seems also unlikely on the basis of the observation that AMP-PNP or SecYEG-bound SecA is accessible from the cytosolic membrane face for binding toward a mAb that recognizes an epitope which is part of the 30 kDa SecA fragment (*56*). Most importantly, the conditions used to form the SecA fragment result in the complete digestion of the SecYEG complex, yielding degradation products that are smaller than 6 kDa. Therefore, it appears that the SecA fragment is also not protease-protected by a belt of SecYEG helices. Our data suggest that only small fragments of SecY suffice to maintain the SecA domain in a protease-resistant conformation or, alternatively, that the formation of the 30 kDa proteolytic fragment is irreversible. In this respect, SecA has been shown to bind to SecY fragments that cover only the 107 amino-terminal residues of the protein (*27*). It should be emphasized that the 30 kDa fragment is not unique for the SecYEG-bound form of SecA, and albeit less stable, it can also be formed in solution (*33*). This is an indication that the 30 kDa fragment might resemble a conformational state rather than being proteinase-inaccessible. Taken together, we conclude that the 30 kDa fragment represents a stable conformation of a SecA domain that is induced by its interaction with nucleotides and further stabilized by the SecYEG complex.

Our data do not argue with the hypothesis that SecA drives preprotein translocation across the membrane by the co-insertion of a SecA domain and bound preprotein (*32*) but demonstrate that any evidence for membrane insertion based on the formation of a protease-resistant conformation has to be taken with caution. Recently, it has been shown that SecA is also proteolyzed into a stable amino-terminal 65 kDa fragment under exactly the same conditions that result in the formation of the 30 kDa fragment, *i.e.*, in the presence of ATP and preprotein or AMP-PNP alone (*62*) or at low proteinase concentration in solution (*33*). Both fragments cover more than 90% of the molecular mass of SecA, which is a 102 kDa protein. The 65 kDa fragment bears both of the nucleotide binding sites of SecA and evidently this part must be accessible from the cytosol. Since the evidence that the 65 kDa fragment is membrane-inserted is also based on proteinase protection experiments, while it is also not photoaffinity-labeled from the lipid phase (*62*), its exact localization requires further investigation. In this respect, recent studies on the membrane topology of monocysteine SecA variants indicate that amino-terminal, central, and carboxy-terminal regions of SecA are periplasmically accessible to biotin maleimide (*63*). Biotin maleimide labeling can be blocked by prior treatment with a membrane-impermeable cysteine-reactive reagent, suggesting that the labeling does not occur within the membrane bilayer. These regions therefore either span the membrane or are located within a channel that is periplasmically accessible to molecules the size of biotin maleimide. These data confirm previous studies on the periplasmic accessibility of the carboxy-terminal (*49*) and other regions (*64*) of SecA to trypsin. The extreme carboxyl-terminal part of SecA contains the SecB binding domain (*31*) and dissociates the SecB upon the initiation of preprotein translocation by binding of ATP to SecA (*31*). Since other regions of the carboxyl-terminal part of SecA interact with SecY (*27*), it seems that the carboxyl terminus fullfils a critical role in anchoring of SecA to the SecYEG heterotrimer during a translocation cycle. Alternatively to the membrane insertion hypothesis, polypeptide segments may also be translocated across the membrane by a mechanism that involves only conformational

changes (and mechanical movements) of the SecA domains (*30*), while they are anchored at the SecYEG complex (*49*, *63*).

Summarizing, the SecYEG complex can now be purified in large quantities in a functional form and retains many of its catalytic and functional properties in micellar solution. This provides the unique opportunity to biochemists and biophysicists to study the mechanism of preprotein translocation in the absence of compartmentalization.

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