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The Purified E. coli Integral Membrane Protein SecY/E Is Sufficient for Reconstitution of SecA-Dependent Precursor Protein Translocation

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Summary

We have previously reconstituted the soluble phase of precursor protein translocation in vitro using purified proteins (the precursor proOmpA, the chaperone SecB, and the ATPase SecA) in addition to isolated inner membrane vesicles. We now report the isolation of the SecY/E protein, the integral membrane protein component of the E. coli preprotein translocase. The SecY/E protein, reconstituted into proteoliposomes, acts together with SecA protein to support translocation of proOmpA, the precursor form of outer membrane protein A. This translocation requires ATP and is strongly stimulated by the protonmotive force. The initial rates and the extents of translocation into either native membrane vesicles or proteoliposomes with pure SecY/E are comparable. The SecY/E protein consists of SecY, SecE, and an additional polypeptide. Antiserum against SecY immunoprecipitates all three components of the SecY/E protein.

Introduction

There are many common features of precursor protein translocation across the membranes of mitochondria, endoplasmic reticulum, chloroplasts, peroxisomes, and bacteria (Wickner and Lodish, 1985; Verner and Schatz, 1988). Regions of preproteins, termed leader or signal sequences, are of particular importance for translocation. Many precursors are stabilized for translocation by interaction with cytosolic factors, such as signal recognition particle and chaperone proteins. Binding to the target organelle is mediated by receptor proteins. Translocation requires energy in the forms of ATP or GTP and, in some cases, a protonmotive force. An important approach to this work has been to synthesize specific preproteins in cell-free extracts supplemented with the isolated organelle. The chemical complexity of these extracts and organelles, however, has been a major limitation.

Bacterial protein export has been studied with a unique combination of genetics, physiology, and biochemistry. The leader regions of exported bacterial proteins are similar to their counterparts for the endoplasmic reticulum of eukaryotes (von Heijne, 1984). After translocation, the leaders are cleaved by one of two endoproteases, lipoprotein signal peptidase (Yu et al., 1984) or leader peptidase (Wolfe et al., 1982). Translocation can occur at any time after a preprotein reaches a certain critical molecular weight (Randall, 1983), but is not coupled to ongoing polypeptide chain growth (Randall, 1983; Zimmermann and Wickner, 1983). Two forms of metabolic energy, the protonmotive force (Date et al., 1980) and ATP (Chen and Tai, 1985), are required for membrane transit. While small proteins such as M13 coat protein can assemble into the plasma membrane without the aid of other proteins (Ohno-Iwashita and Wickner, 1983; Wolfe et al., 1985), most protein export requires the enzymes encoded by *sec* genes A, B, D, E, F, and Y (reviewed in Bieker et al., 1990). SecA is a peripheral membrane protein (Oliver and Beckwith, 1982), while SecY (Emr et al., 1981; Shiba et al., 1984) and SecE (Schatz et al., 1989) are integral to the membrane. SecY spans the membrane ten times (Akiyama and Ito, 1987) and SecE spans the membrane three times (Schatz et al., 1989). Many of these genes have also been discovered through *prl* mutations (Bieker et al., 1990), which suppress the export defect of a preprotein with an altered leader region.

The enzymology of bacterial export is well advanced. Precursor proteins and the two leader peptidases have been purified. Three chaperone proteins, SecB (Kumamoto et al., 1989; Weiss et al., 1988), trigger factor (Crooke et al., 1988), and GroEL (Fayet et al., 1986), have been identified and isolated. These proteins increase the efficiency of export by preventing the premature folding, misfolding, or aggregation (Lecker et al., 1990) of precursor proteins. Each, in vitro, can form a 1:1 stoichiometric complex with a precursor protein such as proOmpA (Lecker et al., 1989). SecB, the least chemically abundant of the three chaperones, appears to have the most general role in export (B. Guthrie and W. Wickner, submitted; Kusukawa et al., 1989). SecB has an affinity for the SecA protein, and this specific recognition may contribute to the role of SecA as the membrane receptor for precursor proteins (F.-U. Hartl, S. Lecker, E. Schiebel, J. Hendrick, and W. Wickner, submitted). SecA, a large peripheral membrane protein, is an ATPase (Lill et al., 1989). Maximal hydrolysis of ATP by SecA requires functional SecY protein, acidic lipids of the membrane, and precursor proteins (Lill et al., 1989, 1990). SecA recognition involves features of both the leader and mature domains of the preprotein. In vitro translocation has been reconstituted with pure precursor proteins, chaperone proteins, SecA protein, ATP, and isolated inner membrane vesicles (Cunningham et al., 1989; Lill et al., 1989).

We recently reported conditions for the solubilization of bacterial membranes and reconstitution of proteoliposomes that support the translocation reaction (Driessen and Wickner, 1990). From this membrane extract, we have now isolated an oligomeric integral membrane protein that is necessary and sufficient to function with SecA to support precursor protein translocation. This reconstituted translocation requires ATP and is stimulated by the protonmotive force.

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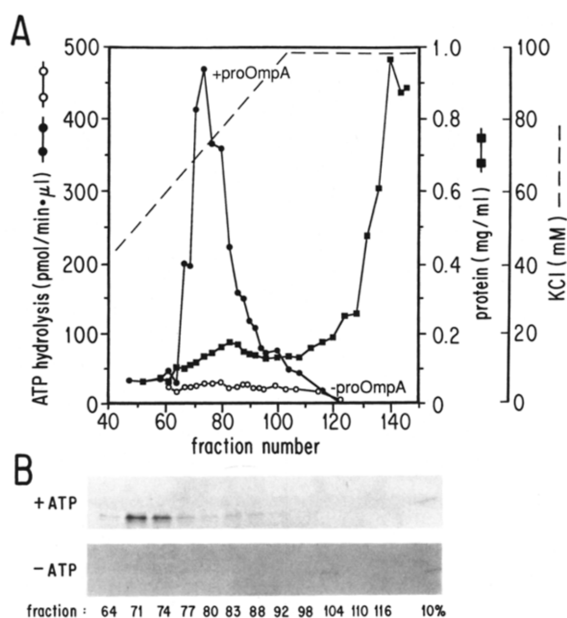

Figure 1. Translocation and Translocation ATPase Activities Copurify on DEAE Cellulose

(A) E. coli membranes (2 g) were solubilized as described in Experimental Procedures, and the extract was applied to a 7 cm × 10.4 cm column of DE52 resin (flow rate 8 ml/cm^2·hr; fraction size 10 ml). The resin was eluted with 400 ml of buffer A followed by a 1400 ml linear gradient of 0 to 100 mM KCl in buffer A followed by 300 ml of 100 mM KCl in buffer A. Proteoliposomes were formed from 100 μl of each fraction. Assays of translocation ATPase were performed using liposomes from each fraction in place of inner membrane vesicles. Closed circles, ATP hydrolysis for 15 min in the presence of 80 μg/ml proOmpA; open circles, ATP hydrolysis in the absence of proOmpA. Filled squares, protein concentration in mg/ml.

(B) Translocation of ^{35}S-labeled proOmpA into proteoliposomes was assayed in the presence or absence (as indicated) of ATP. Translocation reactions were assayed by digestion with 0.2 mg/ml proteinase K for 20 min at 0°C. The lane labeled 10% represents one-tenth of the proOmpA added to the other incubations, but without protease treatment.

Table 1. Purification of SecY/E Complex

Sample	Protein (mg)	Total Activity (nmol/min)[a]	Specific Activity (nmol/min·mg)	Recovery (%)
Extract	240	5,920	25	–
DE-52	12.5	14,760	1,183	100
Q Sepharose	0.44	4,203	9,531	28
Hydroxyapatite	0.15	1,340	8,766	9

See Experimental Procedures for details.
[a] Translocation ATPase activity.

Results

Isolation of the SecY/E Protein

Escherichia coli membranes were extracted with a mixture of octylglucoside, E. coli phospholipids, and glycerol (Driessen and Wickner, 1990), and the extracted proteins were chromatographed on a DEAE cellulose column. Aliquots of individual fractions were mixed with additional lipid, then diluted to reconstitute proteins into proteoliposomes. Proteoliposomes prepared from each fraction were mixed with purified SecA protein, and ATP hydrolysis was assayed in the presence or absence of pure proOmpA. The strong enhancement of ATP hydrolysis by SecA upon interaction with inner membrane vesicles and precursor protein is the "translocation ATPase" (Lill et al., 1989). Translocation ATPase activity was recovered in proteoliposomes reconstituted from a distinct group of fractions from the DEAE cellulose chromatography (Figure 1A). When these same proteoliposomes were mixed with ^{35}S-labeled proOmpA and assayed for translocation, there was a peak of translocation activity (Figure 1B), which required ATP. Immunoblot analysis indicates that the SecY protein, which is essential for protein translocation in vivo and in vitro and for the translocation ATPase reaction, eluted in a broad peak, which was centered at the position of the translocation and translocation ATPase activities (data not shown). The peaks of SecY polypeptide, translocation ATPase, and translocation activity were not perfectly coincident, and there clearly was some SecY that was not associated with translocation activity. This may be due to either a variable loss of other associated polypeptides (SecE or band 1; see below) or the inhibitors of translocation and translocation ATPase present in the crude extract, seen as a yield of greater than 100% in this DEAE purification step (Table 1). These inhibitors and inactive SecY have not been characterized further. The material from the DEAE cellulose chromatography was applied to a Q Sepharose anion exchange resin at pH 9.4, eluted with a salt gradient, and concentrated by adsorption to a column of hydroxyapatite and salt elution. This purification is summarized in Table 1. There was a 3-fold increase in total units of translocation ATPase activity between the starting detergent extract and the DEAE-purified fraction. We estimate that the DEAE cellulose chromatography yielded an approximately 15-fold purification, although the presence of inhibitors in the crude extract makes precise quantitation difficult. Since assays of the initial extract have proven variable, the purification data are normalized to the activities recovered from the DEAE chromatography. This procedure yielded a 9-fold purification with a 9% yield from the DEAE step. Overall, the activity has been purified approximately 130-fold from crude membranes.

Samples from each stage of the purification were analyzed by silver-stained SDS–PAGE (Figure 2A). The hydroxyapatite fraction contained four major polypeptide species. These were analyzed by immunoblots with antisera to SecY and SecE and by N-terminal sequence analysis. The largest polypeptide of the purified preparation, which reacted with antibodies to the SecY N-terminus, migrates on SDS gels with an apparent molecular weight of 21,000. It has the sequence AKQPGL . . ., which is the sequence of the SecY protein minus its amino-terminal methionine (Cerretti et al., 1983). The next smaller polypeptide has the sequence RVYAAQSTHL . . ., which is identical to a sequence that starts at residue 256 in the SecY protein. Thus the SecY protein has undergone proteolysis during isolation. The next protein, labeled "Band 1" in Figure 2,

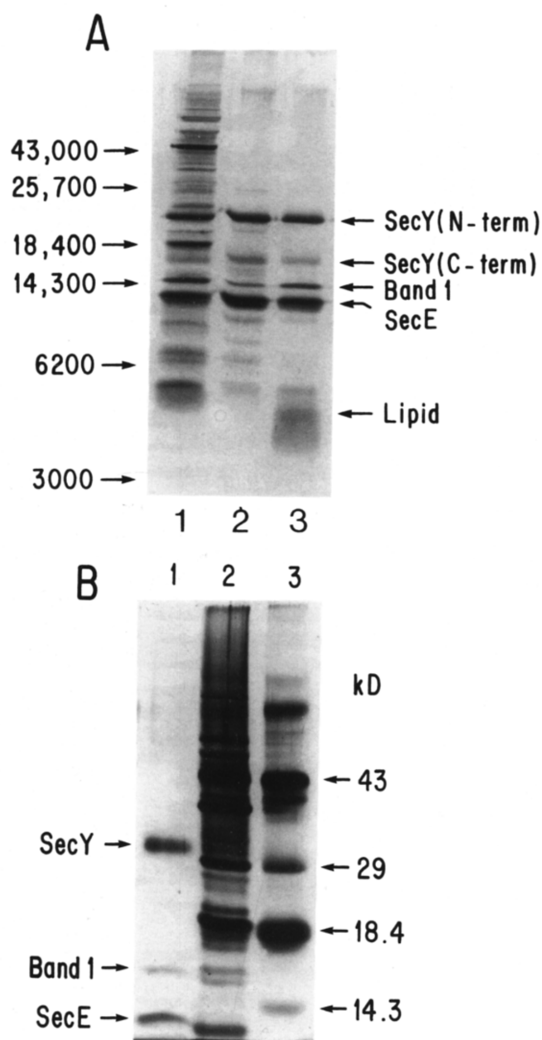

Figure 2. Structure of Purified SecY/E Protein

(A) SecY/E from E. coli D10. The fractions from Table 1 (10 U of translocation ATPase activity of each fraction) were analyzed on a silver-stained high Tris SDS–PAGE. Lane 1, DEAE cellulose; lane 2, Q Sepharose; lane 3, hydroxyapatite. Identification of bands was by N-terminal sequence analysis.

(B) Uncleaved SecY/E from E. coli UT5600. Samples (3.5 U of translocation ATPase) from the Fast-Flow Q step (lane 1) or the DEAE step (lane 2) were analyzed on a 15% polyacrylamide gel by silver staining. Lane 3 contains molecular weight markers.

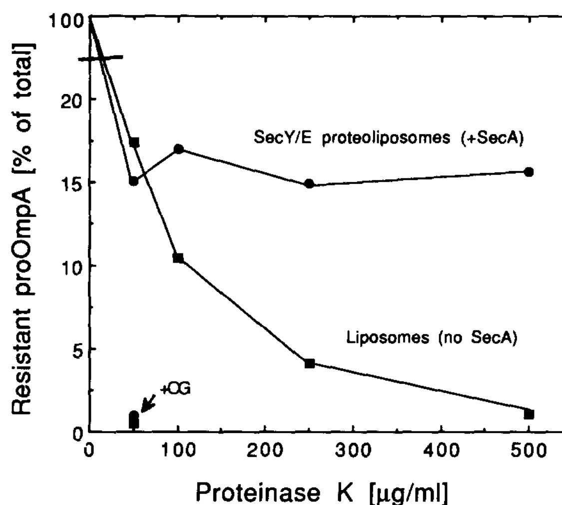

Figure 3. Experimental Distinction between Adsorption and Translocation

Urea-denatured ^{35}S-labeled proOmpA (~150,000 cpm) was diluted 50-fold into translocation buffer containing SecY/E proteoliposomes (200 µg/ml phospholipid, 400 ng/ml SecY/E) or protein-free liposomes. SecA (50 µg/ml) and 2 mM ATP were added to the former reaction to allow translocation of proOmpA into the proteoliposomes. After incubation for 15 min at 37°C, each reaction was divided into five aliquots, which received 0–500 µg/ml proteinase K for 15 min at 0°C. TCA precipitates were analyzed by SDS–PAGE, fluorography, and densitometry. Full-length proOmpA and pseudomature OmpA (the band immediately below proOmpA) are expressed as the percentage of total proOmpA added to the reactions. The arrow indicates the amount of proOmpA resistant to 50 µg/ml proteinase K, detected in reactions containing 2% octylglucoside (+OG).

has not been identified with respect to its DNA or to its amino acid sequence. Quantitative amino acid analysis indicated that it was present in substoichiometric amounts with respect to either the SecY or SecE subunits. The next smaller peptide was recognized by antibodies to SecE and has the sequence SANTE . . ., which is identical to the N-terminal sequence of the SecE protein minus its initiating methionine (Schatz et al., 1989). Following the recent observation of Akiyama and Ito (1990) that SecY is sensitive to cleavage by OmpT, we have been able to purify the SecY/E protein without cleavage from a strain lacking the OmpT protease. We find that it has a translocation ATPase specific activity at the Fast-Flow Q stage of purification of 3450 nmol/min·mg. A silver-stained gel of this material (Figure 2B, lane 1) shows three prominent bands; full-length SecY, band 1, and SecE.

To ensure that the process we have reconstituted with SecA protein and SecY/E proteoliposomes is truly translocation, we compared the ability of protease to digest proOmpA incubated either with these proteoliposomes or with liposomes prepared without proteins (Figure 3). ProOmpA, upon dilution from urea in the absence of lipid, was readily digested by concentrations of proteinase K as low as 5–10 µg/ml at 0°C (data not shown). When liposomes bearing purified SecA and SecY/E proteins were incubated with proOmpA and ATP prior to digestion with proteinase K, a "plateau" amount of undigested proOmpA was seen (Figure 3, circles), indicating that true translocation had occurred. Although proOmpA adsorbed to protein-free liposomes and became resistant to proteolysis (Figure 3, squares), there was no range of protease concentration over which a constant protease resistance could be seen. Essentially all the proOmpA was degraded at high protease concentrations. It is noteworthy that the resistance to low concentrations of protease conferred by either adsorption or translocation is relieved by detergent (Figure 3, arrow). This commonly employed criterion for translocation is not as rigorous as establishing a plateau of protease resistance. As an additional test of the authenticity of the translocation reaction, we examined its depen-

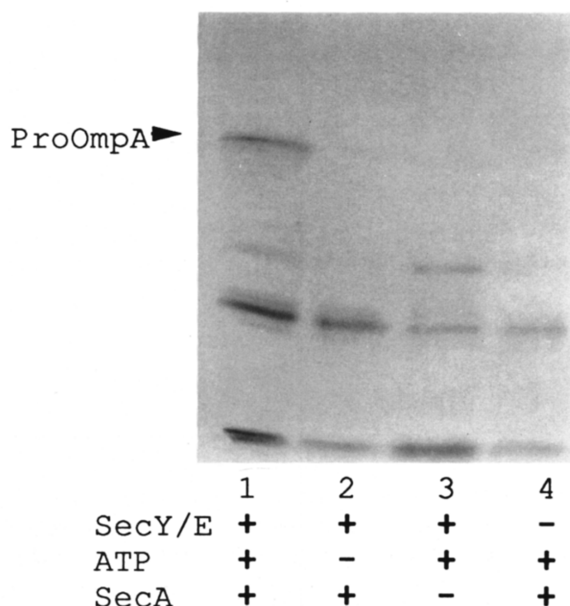

Figure 4. Requirements for Translocation

^{35}S-labeled proOmpA (~250,000 cpm) was incubated (40°C, 10 min) in translocation buffer containing 7 μg/ml SecB and, as indicated, SecY/E proteoliposomes (350 μg/ml phopholipid, 550 ng/ml SecY/E) or liposomes (350 μg/ml phopholipid), 4 mM ATP, and 100 μg/ml SecA. Translocation was assayed by digesting samples with 500 μg/ml proteinase K for 20 min at 0°C.

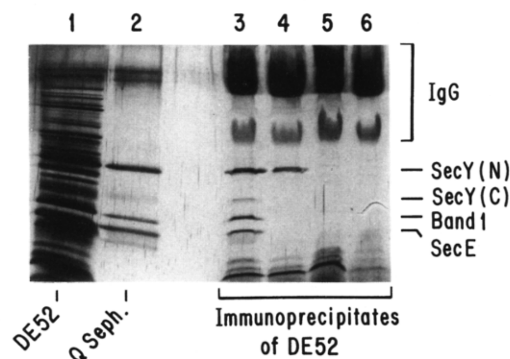

Figure 5. Immunoprecipitation of the SecY/E Protein

Anti-SecY IgG and control IgG were bound to protein A–Sepharose by saturating the antisera with phenylmethylsulfonyl fluoride, adding EDTA to 10 mM, and incubating with one-fourth volume of protein A–Sepharose 6MB (2 hr, continuous mixing). The resin with bound antibody was freed of unbound material by suspending twice in 10 vol each of 50 mM Tris–HCl (pH 8.0), 50 mM KCl, and 1 mM dithiothreitol and once in 5 vol of immunoprecipitation (IP) buffer (50 mM Tris–HCl [pH 8.0], 150 mM KCl, 1.25% octylglucoside, 40% [v/v] glycerol, 0.4 mM dithiothreitol). SecY/E protein was then immunoprecipitated from a DE52 fraction as follows: Samples of the indicated antibody resin (200 μl of a 50% slurry in IP buffer) were supplemented with E. coli phospholipids (450 μg) and incubated (2 hr, 4°C, continuous mixing) with 200 μl (23 μg) of DE52-purified SecY/E. The resin was sedimented (15 s, Brinkman microfuge), mixed four times with IP buffer (900 μl) containing 1.5 mg/ml E. coli phospholipids, and mixed once with 50 mM Tris–HCl (pH 8.0), 0.75% octylglucoside, 0.1% SDS, 20% (v/v) glycerol, 0.5 mg/ml E. coli phospholipids, and 0.4 mM dithiothreitol. The resin was sedimented as before, bound proteins were eluted with 100 μl of SDS sample buffer (5 min, 37°C), and 60 μl portions of the eluents were analyzed by SDS–PAGE and silver staining. Lane 1, 2.3 μg of DE52-purified SecY/E. Lane 2, 1 μg of highly purified (Q Sepharose) SecY/E. (Lanes 3–5, immunoprecipitation of DE52-purified SecY/E with anti-SecY resin.) Lane 3, immunopreciptiation was performed as described. Lane 4, SDS was added to DE52-purified SecY/E to 0.9%. The mixture was incubated at 37°C for 5 min to dissociate the peptides of SecY/E, then diluted with 1 ml of IP buffer containing 1.5 mg/ml E. coli phospholipids prior to immunoprecipitation. Lane 5, Anti-SecY resin was incubated with 100 μg of SecY N-terminal peptide (20 min, 0°C), prior to immunoprecipitation. Lane 6, immunoprecipitation with control IgG resin.

dence on ATP and the genetically and biochemically defined catalysts (SecA and SecY/E). ProOmpA translocation was seen in the complete reaction (Figure 4, lane 1) but was not observed upon omission of either ATP (lane 2), SecA (lane 3), or SecY/E (lane 4). The protonmotive force, which is required for normal rates of translocation in vivo, also strongly stimulates the reconstituted reaction (see below and Driessen and Wickner, 1990).

Since the SecY polypeptide and the translocation and translocation ATPase activities were not always coincident during the early steps of purification, immunoprecipitation was used to provide an independent means to establish that the SecY, SecE, and band 1 polypeptides are bound to each other as a single multisubunit protein. Antibodies that are specific for an amino-terminal SecY peptide (Lill et al., 1989) were used to immunoprecipitate the complex from the DE52 fraction. Despite the crude nature of the DE52 fraction (Figure 5, lane 1), the SecE subunit, band 1, and both halves of the SecY subunit were immunoprecipitated (lane 3). The precipitated bands comigrated with the subunits of highly purified SecY/E protein (lane 2). When the DE52 fraction was heated in SDS prior to immunoprecipitation, only the N-terminal fragment of SecY was recovered (Figure 5, lane 4). None of the subunits of the SecY/E protein were immunoprecipitated by either control IgG (lane 6) or by anti-SecY IgG that had been preincubated with the peptide to which it was raised (lane 5). The relative proportion of the components when immunoprecipitated (lane 3) and when copurified (lane 2) were similar. These data indicate that SecY, SecE, and band 1 form a stable complex, termed the SecY/E protein, which supports precursor protein translocation. We note that our current data do not yet establish whether polypeptide 1 is a functionally essential subunit of the SecY/E protein.

The Preprotein Translocase of E. coli

Is the SecY/E protein the major integral membrane protein needed for efficient translocation of proOmpA and translocation ATPase, or is another important component lost during the fractionation? To determine whether we might have lost a factor that helps to couple translocation and ATP hydrolysis, proteoliposomes reconstituted from fractions at each step of the purification were assayed for their ability to support translocation and translocation ATPase. The ratio of these activities, measured for proteoliposomes from each stage of purification, was very similar to the ratio measured for inner membrane vesicles (Table 2). This result suggests that there are no additional factors

Table 2. Copurification of the Translocation and Translocation ATPase Activities

Sample	Translocation (pg/min·µl)[a]	Translocation ATPase (nmol/min·µl)[b]	Translocation: Translocation ATPase
Membranes[c]	22.5	0.212	100
DE-52	20.0	0.305	60
Q Sepharose	1.5	0.020	75
Hydroxyapatite	16.5	0.276	60

[a] Translocation was assayed in 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 0.5 mg/ml fatty acid-free bovine serum albumin, 15 µg/ml SecA, 80 µg/ml SecB, and 4 mM ATP. Translocation of ^{35}S-labeled proOmpA was quantitated by densitometry. Assay time was 5 min.

[b] Translocation ATPase was assayed as described for translocation except that 20 µg/ml unlabeled proOmpA replaced ^{35}S-labeled proOmpA. Assay time was 15 min.

[c] Inner membrane vesicles purified as by Chang et al. (1979).

that are required for translocation beyond those that support translocation ATPase.

To determine whether factors that catalyze translocation were lost during the purification, we compared the SecY content of purified inner membrane vesicles and proteoliposomes reconstituted with purified SecY/E protein. Quantitative immunoblot analysis was performed with samples with similar units of translocation activity (Figure 6). Approximately twice the SecY/E protein was required in proteoliposomes to achieve the same rate of translocation seen with inner membrane vesicles. Several factors might account for this difference. Among these are some inactivation of SecY/E during its isolation or loss of asymmetry during its reconstitution. We conclude that the purified SecY/E protein has all the components that are essential for in vitro proOmpA translocation. Had another protein of the starting membranes been involved in the translocation reaction, its loss during the fractionation would have led to diminished translocation activity per SecY/E protein molecule. We define the "preprotein translocase" of E. coli as the complex of the peripherally bound SecA protein and the integral SecY/E protein.

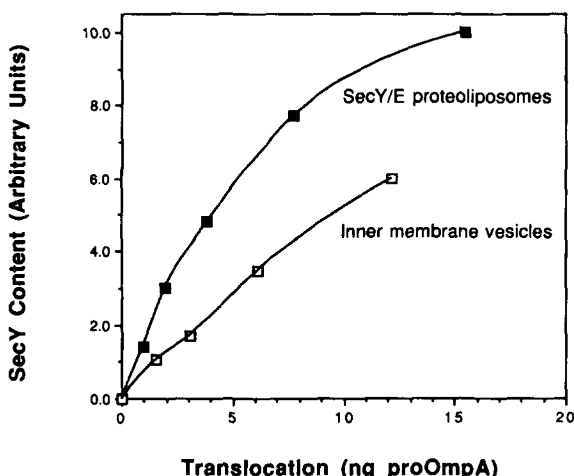

Figure 6. SecY Content of Inner Membrane Vesicles and SecY/E Proteoliposomes As a Function of Translocation Activity

Translocation activities of SecY/E proteoliposomes and urea-treated inner membrane vesicles were determined under conditions in which they were limiting. Translocation assays (7 min, 40°C) contained 50 mM HEPES-KOH (pH 7.5), 30 mM potassium acetate, 30 mM NH_4Cl, 1 mM dithiothreitol, 2.5 mM $MgCL_2$, 4 mM ATP, 0.5 mg/ml bovine serum albumin, 50 µg/ml SecA, 5 µg/ml SecB, and 280,000 cpm of ^{35}S-labeled proOmpA. Translocation was quatified by densitometry relative to a known standard of ^{35}S-labeled proOmpA. The SecY content of proteoliposomes and membranes was determined by immunoblotting a dilution series of each with antiserum to SecY. Quantitation of the immunoblot, normalized for translocation activity, is shown.

Protonmotive Force

The protonmotive force is required for proOmpA translocation in vivo (Zimmermann and Wickner, 1983) and stimulates in vitro translocation into inner membrane vesicles 5- to 10-fold (Geller et al., 1986). The purified SecY/E protein was co-reconstituted with bacteriorhodopsin, a light-driven proton pump. Upon illumination, a protonmotive force is generated, inside positive and acidic, which stimulates the rate of translocation (Figure 7). This stimulatory effect of light on the rate of translocation was prevented by the ionophores valinomycin plus nigericin (data not shown). Since the effect of a protonmotive force is seen both in vivo and, in the current experiments, in a reaction with chemically defined components (proOmpA, SecA, SecY/E protein, bacteriorhodopsin, E. coli lipids, ATP, and buffer), it is likely to reflect a direct part of the translocation mechanism rather than an indirect effect of the protonmotive force on cellular physiology.

Discussion

Biochemical reactions have classically been dissected by the purification of individual components and their recon-

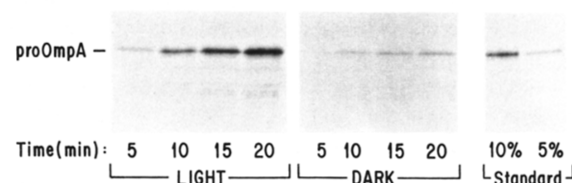

Figure 7. The Rate of Translocation of proOmpA into SecY/E Proteoliposomes Is Stimulated by a Protonmotive Force

Purified SecY/E protein was coreconstituted into proteoliposomes with the light-driven proton pump bacteriorhodopsin. Bacteriorhodopsin proteoliposomes (330 µg of E. coli phospholipid, 1.3 nmol bacteriorhodopsin [Sigma]), prepared by sonication (Driessen and Wickner, 1990), were mixed with 0.7 µg of SecY/E protein dissolved in 200 µl of 20 mM Tris-HCl (pH 7.9), 500 mM KCl, 50% (v/v) glycerol, 1.25% (w/v) octylglucoside, 0.5 mg/ml E. coli phospholipid, and 1 mM dithiothreitol. Proteoliposomes were formed by detergent dilution as described in Experimental Procedures. Translocation of ^{35}S-labeled proOmpA was in an assay (200 µl) containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 20 µg of fatty acid-free bovine serum albumin, 1 mM dithiothreitol, 5 mM ATP, 8 µg of SecA protein, 1 µg of SecB protein, and proteoliposomes (40 µl). Where indicated, vials were illuminated with saturating levels of actinic light from a 150 W slide projector lamp via a glass fiber optic bundle. At indicated times, samples of 45 µl were removed, chilled on ice, and assayed for translocation as described in Experimental Procedures. The lanes labeled "Standard" have the indicated percentage of the total proOmpA added to the other lanes, but without protease treatment.

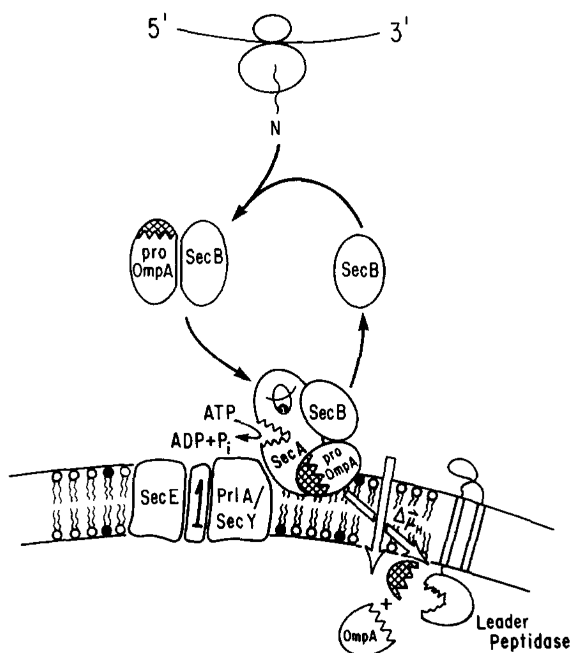

Figure 8. A Current Model of proOmpA Translocation across the Plasma Membrane

We do not know the spatial relationship or stoichiometry of the subunits of the translocase. The hatched region of proOmpA symbolizes its leader peptide.

stitution into a functional, chemically defined reaction. This strategy was previously applied to the membrane insertion of M13 procoat (Ohno-Iwashita and Wickner, 1983). Like other exported proteins, this precursor protein is made with a typical N-terminal leader sequence, requires the protonmotive force for insertion in vivo, and is cleaved by leader peptidase (Wickner, 1988). However, it spontaneously assembles into liposomes in vitro and its membrane insertion does not require catalysis by the Sec proteins. To understand the roles of these Sec proteins in catalysis of translocation, we therefore turned to an analysis of the translocation of proOmpA across the plasma membrane. The focus of these studies has been to isolate each component and study its role in the reconstituted translocation reaction. With the isolation of the SecY/E protein, we have reconstituted membrane translocation with a strikingly simple set of purified components. The reconstituted reaction that employs these purified components shows similar translocation rates as the starting in vitro reaction with the intact organelle.

The Secretion Pathway

Our current model of this process is shown in Figure 8. Several important features of the mechanisms of protein translocation have been discovered by isolation of the proteins of this pathway. The soluble component of the secretion pathway is a 1:1 stoichiometric complex between the precursor protein (such as proOmpA) and the chaperone protein SecB. The membrane-bound "translocase" consists of a peripheral membrane protein, SecA, and the integral membrane protein SecY/E. ProOmpA itself was isolated in urea solution (Crooke and Wickner, 1987; Crooke et al., 1988) and shown to fold upon dilution from urea into a structure that is competent for membrane assembly. However, this form of proOmpA tends to aggregate (Lecker et al., 1990), which prevents its membrane insertion. Formation of a stoichiometric complex with either SecB, trigger factor, or GroEL prevents aggregation and renders proOmpA stable for membrane insertion. While each of these chaperones functions well in vitro, genetic analysis (Kusukawa et al., 1989; B. Guthrie and W. Wickner, submitted) suggests that SecB has the major role in vivo.

SecA protein is the sole peripheral membrane protein with a direct role in the translocation reaction (Cunningham et al., 1989). It is an essential ATPase, and its ATP hydrolysis is coupled to its interactions with each of the other components of the translocation reaction (Lill et al., 1989). Thus, optimal activity and stability of SecA require its interaction with SecY, acidic lipids of the membrane bilayer, and the leader and mature domains of the precursor protein (Lill et al., 1990). The SecA protein binds both proOmpA and SecB and functions as the membrane receptor for the proOmpA/SecB complex (F.-U. Hartl, S. Lecker, E. Schiebel, J. Hendrick, and W. Wickner, submitted). SecA is needed for the stability of membrane-bound proOmpA for subsequent membrane translocation (Cunningham et al., 1989). ATP hydrolysis may be required for the release of proOmpA from its association with SecA protein (F.-U. Hartl, S. Lecker, E. Schiebel, J. Hendrick, and W. Wickner, submitted). In this manner, the SecB and SecA proteins constitute a two-stage chaperone system, the first stage (SecB/proOmpA) functioning in the cytoplasm while the second (SecA/proOmpA) is membrane bound. Hydrolysis of ATP causes release of proOmpA into the membrane and drives the overall chaperone and membrane association reactions.

The availability of pure SecY/E protein may now allow its functions to be more fully defined. It has been suggested that translocation may be reconstituted without SecY protein (Watanabe et al., 1990). However, as established through prior in vivo studies (Emr et al., 1981; Shiba et al., 1984; Schatz et al., 1989), we find a strict requirement for the SecY/E protein in the reconstituted proOmpA translocation reaction with all-purified components. We have thus far established two biochemical functions of SecY/E. It serves as the high affinity SecA receptor (F.-U. Hartl, S. Lecker, E. Schiebel, J. Hendrick, and W. Wickner, submitted) and is essential to activate and stabilize the ATP hydrolytic capacity of SecA (Lill et al., 1989). Thus, both binding and catalytic data establish that SecA and the SecY/E protein function together as a translocase enzyme, in a manner reminiscent of the F_1 and F_0 domains of ATP synthase. Further functions of the SecY/E protein are now open to experimentation. Current studies will test four possible additional functions of this complex protein: first, it might function with SecA as part of the proOmpA/SecB receptor; second, as suggested by the *prlA* mutants that originally defined the *prlA/secY* gene (Emr et al., 1981) and the *prlG* mutants of the *prlG/secE* gene (Bieker et al., 1990), the SecY/E protein might directly bind precursor proteins

after their release from SecA; third, the SecY/E protein might conduct protons and couple the proton flux to either the action of SecA itself or to work performed on the pro-OmpA molecule; and fourth, a major question will be whether the SecY/E protein serves as a pore to conduct proOmpA and other precursor proteins across the bilayer, or whether these proteins cross through the lipid phase per se.

While our current experiments suggest that we have faithfully reconstituted each of the proteins that function in crude in vitro translocation reactions, it is still quite possible that other proteins or conditions remain to be discovered that will dramatically improve the rate and extent of the translocation reaction. Since E. coli grows with a 20 min doubling time, exports 10% of its protein to the envelope layers, and has approximately 5% of its protein as plasma membrane, each milligram of this membrane must translocate approximately 0.1 mg of protein per min. Individual precursor proteins are translocated within 2–20 s at nearly 100% efficiency. It will be necessary to approach these kinetics with a reconstituted system to be completely satisfied that all components of this translocation reaction have been identified.

Experimental Procedures

Total E. coli Membranes

E. coli D10 (*rna*10, *relA*1, *spoT*1, *metB*1) was grown and stored as frozen nuggets as described by Wickner et al. (1972). Nuggets (80 g) of frozen cell suspension were added to 200 ml of rapidly stirred, room-temperature buffer E (20% [v/v] glycerol, 0.05 M HEPES–KOH [pH 7.0], 0.05 M KCl, 1 mM dithiothreitol) and broken with ultrasound (20 min, 0°C, power 3, 50% duty cycle with a Branson sonifier). Lysates were centrifuged (10 min, 10,000 × g, 0°C) to remove unbroken cells, then centrifuged (60 min, 50,000 rpm, Beckman Ti60 rotor, 4°C) to collect the membranes. Pellets were suspended in 200 ml of buffer E containing 6 M urea at 0°C with a glass homogenizer and centrifuged as before. Pellets were then suspended in 200 ml of buffer E, membranes were collected by centrifugation, and these final pellets were suspended in 18 ml of buffer E and frozen in liquid nitrogen.

Other Biochemicals

Purified E. coli inner membrane vesicles were prepared from frozen D10 cells by isopycnic centrifugation (Chang et al., 1979). SecA (Cunningham et al., 1989), SecB (Lecker et al., 1989), ^{35}S-labeled proOmpA (Crooke and Wickner, 1987), and unlabeled proOmpA (Crooke et al., 1988) were prepared as described. Polyclonal antiserum to the N-terminal peptide of SecY (Watanabe and Blobel, 1989) was prepared as in Lill et al. (1989). Acetone/ether-washed E. coli phospholipids were purchased from Avanti polar lipids (Pelham, AL).

Proteoliposomes

E. coli phospholipids (50 mg/ml in 10 mM Tris–HCl [pH 7.0], 1 mM dithiothreitol) were bath sonicated. Lipid suspension (7 μl) was mixed with 25–100 μl of protein fractions and diluted with 4 ml of 50 mM Tris–HCl [pH 8.0], 50 mM KCl, and 1 mM dithiothreitol. $CaCl_2$ (40 μl of 1 M) was added and, after 1 hr, the proteoliposomes were collected by centrifugation (41,000 × g, 30 min). Proteoliposomes were suspended in 200 μl of 50 mM Tris–HCl (pH 8.0), 50 mM KCl, and 2.5 mM EGTA by bath sonication. All steps were performed at 0°C–4°C. Translocation ATPase activity supported by proteoliposomes was measured as described by Lill et al. (1989). Typically, 5 μl of proteoliposomes was incubated in a 50 μl assay with 50 mM Tris–HCl (pH 8.8), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 10 μg/ml SecA, and 40 μg/ml proOmpA for 30 min at 40°C. ATP hydrolysis was quantified using malachite green (Lanzetta et al., 1978) with 0.1% Triton X-100 as detergent. One unit of membrane translocation ATPase activity stimulates SecA to hydrolyze 1 nmol ATP per min in the presence of proOmpA. Translocations of ^{35}S-labeled proOmpA into proteoliposomes were assayed by protease accessibility (Cunningham et al., 1989), except that digestion was with 0.8 mg/ml proteinase K. Approximately 50,000 cpm of ^{35}S-labeled proOmpA (10,000 cpm/ng) was added to each translocation reaction. One unit of translocation activity is the amount of membranes or reconstituted proteoliposomes that translocates 1 pg of proOmpA per min in our standard assay.

Purification of the SecY/E Protein

The SecY/E protein was assayed by reconstituting fractions into proteoliposomes and measuring their ability to support the translocation ATPase. Crude E. coli membranes (400 mg) were solubilized in 80 ml of 20 mM Tris–HCl (pH 7.9), 1.25% (w/v) n-octyl-β-D-glucopyranoside, 20% (v/v) glycerol, and 3.75 mg/ml E. coli phospholipids. After 30 min on ice, the mixture was centrifuged (40 min, 250,000 × g, 4°C). The supernatant was applied at 8 ml/cm^2·hr to a 3.1 cm (d) × 10.4 cm (h) column of DE-52 resin (Whatman) equilibrated in buffer A (20 mM Tris–HCl [pH 7.9], 20% glycerol, 1.25% [w/v] octylglucoside, 0.5 mg/ml E. coli phospholipids, 1 mM dithiothreitol). Buffer A (80 ml) was applied to the column and proteins were eluted with a 280 ml linear gradient of 0 to 100 mM KCl in buffer A. Fractions (10 ml) were collected and immediately mixed with 5 ml of glycerol and 0.5 ml of 12.5% octylglucoside to stabilize the SecY/E protein. Peak fractions were pooled (80 ml). Portions of the DE-52 pool (50 ml) were exchanged into buffer C (20 mM ethanolamine–HCl [pH 9.4], 40% glycerol, 1 mM dithiothreitol, 0.5 mg/ml E. coli phospholipids, 1.25% octylglucoside) by filtration through a column of Sephadex G-25 (Pharmacia; 7 cm [d] × 6 cm [h], 4 ml/cm^2·hr) and applied to a Q Sepharose Fast-Flow column (Pharmacia; 1 cm [d] by 7.5 cm [h], 20 ml/cm^2·hr), which was eluted with 5 ml of buffer C and a linear gradient of 0 to 125 mM KCl in buffer C. Fractions of peak specific activity were pooled, mixed with 1/50th vol of 1 M Tris–HCl (pH 7.9), and applied to a column of hydroxyapatite (Ultrogel-HA, LKB, 0.7 cm [d] × 2 cm [h]) equilibrated with buffer D (20 mM Tris–HCl [pH 7.9], 50% [v/v] glycerol, 1.25 % [w/v] octylglucoside, 0.5 mg/ml E. coli phospholipids, 1 mM dithiothreitol). The column was developed with 5 ml of buffer D, then 10 ml of buffer D containing 0.5 M KCl. Fractions of peak activity were pooled, mixed with E. coli phospholipids (2 mg/ml final concentration), and stored at −20°C.

Uncleaved SecY/E was purified through the Q-Sepharose Fast-Flow step by the same method, except that membranes were prepared from E. coli UT5600, a strain deleted for the protease OmpT (Elish et al., 1988). All buffers were supplemented with 5 mM p-aminobenzamidine, and buffer salt concentrations were correspondingly decreased by 5 mM.

Other Procedures

SDS–PAGE was performed as described (Ito et al., 1980). In the "high Tris" system, the separating gel contained 19.6% acrylamide, 0.216% bisacrylamide, 0.7 M Tris–HCl (pH 8.85), 0.5 M NaCl, the stacking gel contained 5% acrylamide, 0.07% bisacrylamide, 125 mM Tris–HCl (pH 6.8), and the "running buffer" contained 1.4% glycine, 0.6% Tris–HCl, and 0.1% SDS. Gels of 15% acrylamide were as described by Crooke et al. (1988). Samples for SDS–PAGE were mixed with an equal volume of 4% SDS, 100 mM Tris–HCl (pH 6.8), 20% glycerol, 1.5% β-mercaptoethanol, and 0.02% bromophenol blue. Samples were heated to 37°C prior to electrophoresis (Ito, 1984). Protein molecular weight standards were purchased from Bethesda Research Laboratories, Gaithersburg, MD.

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Note Added in Proof

The work referred to throughout as B. Guthrie and W. Wickner, submitted, can now be updated as Guthrie, B., and Wickner, W. (1990). Trigger factor depletion or overproduction causes defective cell division but does not block protein export. J. Bacteriol., in press.