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Detergent disruption of bacterial inner membranes and recovery of protein translocation activity

(secretion/membrane assembly/SecY/pro-OmpA)

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ABSTRACT **Isolation of the integral membrane components of protein translocation requires methods for fractionation and functional reconstitution. We treated inner-membrane vesicles of *Escherichia coli* with mixtures of octyl β-D-glucoside, phospholipids, and an integral membrane carrier protein under conditions that extract most of the membrane proteins into micellar solution. Upon dialysis, proteoliposomes were reconstituted that supported translocation of radiochemically pure [^{35}S]pro-OmpA (the precursor of outer membrane protein A). Translocation into these proteoliposomes required ATP hydrolysis and membrane proteins, indicating that the reaction is that of the inner membrane. The suspension of membranes in detergent was separated into supernatant and pellet fractions by ultracentrifugation. After reconstitution, translocation activity was observed in both fractions, but processing by leader peptidase of translocated pro-OmpA to OmpA was not detectable in the reconstituted pellet fraction. Processing activity was restored by addition of pure leader peptidase as long as this enzyme was added before detergent removal, indicating that the translocation activity is not associated with detergent-resistant membrane vesicles. These results show that protein translocation activity can be recovered from detergent-disrupted membrane vesicles, providing a first step towards the goal of isolating the solubilized components.**

Protein export in *Escherichia coli* has been investigated by both genetic and biochemical approaches. Numerous *sec* mutants that possess pleiotropic defects in protein export have been isolated. These include mutants in the *secA* (1), *secB* (2), *secD* (3), *secE* (4), and *secY* (*prlA*) (5, 6) genes. Analysis of these genes and their mutant alleles has indicated that their products play a role in the export of many, though not all, precursor proteins (7). Strains bearing *secY* (*prlA*) or *secA* (*prlD*) mutant alleles can suppress specific defects in the leader peptides of pre-LamB, suggesting that the proteins encoded by these genes may physically interact with the precursor (5, 8). The *secY24* temperature-sensitive allele causes thermosensitive pro-OmpA translocation *in vivo* (6) and *in vitro* (9, 10). There is indirect evidence that suggests SecY (PrlA) interacts with SecA (11, 12). These studies suggest that SecA, a peripheral membrane protein, and SecY, an integral membrane protein (13), participate directly in the translocation of pro-OmpA.

Cell-free translocation of many precursor proteins across inverted inner membrane vesicles has been achieved (14, 15). Efficient protein translocation requires ATP hydrolysis and is enhanced by the presence of a transmembrane electrochemical potential (16, 17). Fractionation of this system has led to the identification of several soluble and membrane-bound factors that stimulate the translocation of the precursor protein pro-OmpA. Trigger factor was identified and purified based on its ability to bind pro-OmpA and maintain the precursor in a conformation that is competent for translocation (18–20). Trigger factor is associated with the large subunit of ribosomes (21) and may interact with the nascent pro-OmpA polypeptide chain during synthesis. It has been demonstrated that other soluble factors such as SecB and GroEL can also stabilize precursor proteins for posttranslational translocation *in vitro* (22–24). The purified SecA protein is essential for translocation of pro-OmpA *in vitro* (25, 26) and functions as the catalytic subunit of the pro-OmpA translocation ATPase (12). Recent data indicate that a receptor specific for the leader region of pro-OmpA functions in *E. coli* membrane vesicles to activate the translocation ATPase (27).

Despite this progress, it has not yet been possible to determine whether the identified proteins are sufficient to catalyze the entire translocation process or which other proteins may be required. It will be important for the study of the bacterial translocation reaction, as well as its eukaryotic counterparts, to be able to extract the membranes with detergent, fractionate the extract, and reconstitute proteoliposomes that function faithfully for protein translocation. The major difficulties in these studies, in addition to the complexity of the overall reaction, are the limitations of the translocation assay. The current assays employ limiting amounts of radiolabeled precursor proteins as translocation substrates and excess membrane vesicles, making it difficult to quantify the levels of enzyme present. Improving the assay while seeking conditions for reconstitution of translocation activity may best be approached for bacterial protein export, where the plasma membrane can be isolated to high purity and where there is a powerful combination of genetics and biochemistry.

In this report we show that authentic protein translocation activity can be recovered in proteoliposomes after detergent treatment of bacterial membranes. Although the conditions of detergent extraction employed here will solubilize most proteins in the membrane (such as leader peptidase), translocation activity and SecY protein are recovered in the pellet fraction after ultracentrifugation. This work represents a necessary first step in the biochemical dissection of protein translocation at the membrane level.

MATERIALS AND METHODS

Bacterial Strains and Reagents. *E. coli* strain D10 (*rna-10, relA1, spoT1, metB1*) was grown at 37°C in TYE medium in 150-liter batches (9). Cells were harvested by centrifugation at 4°C, resuspended to 50% (wt/vol) in 50 mM Tris·HCl, pH 7.5/10% (wt/vol) sucrose, frozen as nuggets in liquid nitrogen, and stored at −70°C until use.

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Abbreviation: ATP[γS], adenosine 5′-[γ-thio]triphosphate.

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Inner membranes were prepared from frozen D10 cells as described (28) except that the cell suspension was passed twice through the French pressure cell at 8000 psi (1 psi = 6.89 kPu) and that Hepes·KOH (pH 7.5) was substituted for Tris·HCl (pH 7.5) throughout the procedure. The membranes were sedimented in a Beckman Airfuge (150,000 × *g*, 15 min, 4°C) and resuspended in buffer prior to detergent extraction. A cytosolic fraction of *E. coli*, termed S100, was prepared from frozen D10 cells (9).

Radioactive [^{35}S]pro-OmpA was synthesized *in vitro* as described (9). Radioactive OmpA was synthesized in a parallel reaction containing 0.1% Triton X-100 and 0.1 mg of leader peptidase per ml, resulting in processing of 80% of the pro-OmpA to OmpA. Pro-OmpA and OmpA were purified by affinity chromatography as described (20), mixed with S100 to 0.2 mg/ml, and dialyzed against buffer A (50 mM Hepes·KOH, pH 7.5/50 mM KCl/5 mM $MgCl_2$/1 mM dithiothreitol) at 0°C for 16 hr.

Phospholipids were purified from *E. coli* strain D10 as described (29) and were further purified by ether extraction and acetone precipitation (30). The dried phospholipids were suspended at 50 mg/ml in 50 mM Hepes·KOH, pH 7.5/2 mM dithiothreitol and bath-sonicated to form liposomes. Leader peptidase was purified as described (31). M13 coat protein was isolated from the bacteriophage (32) and exchanged into buffer A containing octyl β-D-glucoside (15 mg/ml; Calbiochem) by dialysis.

Antiserum that crossreacts with the SecY protein was prepared by immunizing New Zealand White rabbits with a conjugate of keyhole limpet hemocyanin (Sigma) and a synthetic peptide corresponding to amino acid residues 2–22 of the cytoplasmic, amino terminus of SecY (33, 34). The synthetic peptide AKQPGLDFQSAKGGLGELKRRC contained an additional cysteine residue at the carboxyl terminus for crosslinking to hemocyanin with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (35).

Detergent Extraction of Inner Membranes and Reconstitution of Proteoliposomes. The procedure of Newman and Wilson (30) for solubilization of lactose permease in bacterial membranes was modified slightly for our purposes. Inner membranes at a final protein concentration of 0.5 mg/ml (equivalent to 1 absorbance unit at 280-nm in 1% SDS) were treated at 0°C with extraction buffer [consisting of octyl β-D-glucoside (15 mg/ml), phospholipids (3.75 mg/ml), and bacteriophage M13 coat protein (0.1 mg/ml) in 50 mM Hepes·KOH, pH 7.5/50 mM KCl/5 mM $MgCl_2$/1 mM dithiothreitol]. M13 coat protein was included because it routinely increased the recovery and stability of reconstituted translocation activity. The mixture was incubated at 0°C for 15 min, and then a portion was centrifuged at 150,000 × *g* for 30 min at 4°C in a Beckman Airfuge. The supernatant was recovered and the pellet was resuspended in the same volume of extraction buffer. All fractions were dialyzed against excess buffer A at 0°C for 16 hr with a change of buffer after 6–10 hr. This procedure yielded sealed proteoliposome vesicles as judged by the ability of these vesicles to generate and maintain an electrochemical potential upon addition of ATP (data not shown). Proteoliposomes were recovered and stored at 0°C until assayed for protein translocation.

Translocation Assays. Translocation assays were performed on native membrane vesicles or reconstituted proteoliposomes at 0.05 mg/ml in 50 μl of buffer A. Where indicated, the sodium salt of ATP, GTP, or adenosine 5′-[γ-thio]triphosphate (ATP[γS]) (Boehringer Mannheim) was added to 2.5 mM. The reaction mixtures were incubated at 40°C for 4 min prior to addition of 10 μl of [^{35}S]pro-OmpA (200,000 cpm per reaction). After 16 min at 40°C, the samples were chilled to 0°C. Aliquots (45 μl) were transferred to fresh tubes (to remove aggregated pro-OmpA) containing 5 μl of 1% (wt/vol) proteinase K (Boehringer Mannheim) and incubated at 0°C to degrade all untranslocated proteins. After 15 min, protected proteins were precipitated by addition of 50 μl of 25% (wt/vol) trichloroacetic acid, collected by centrifugation for 3 min at 4°C in a Brinkman Microfuge, suspended in 1 ml of ice-cold acetone, collected by centrifugation, and analyzed by SDS/PAGE and fluorography (36). Translocation efficiencies were estimated relative to standards of known amounts of radiolabeled pro-OmpA included in each gel. Because the translocation efficiency was low with reconstituted proteoliposomes, it was necessary to minimize the background of nontranslocated pro-OmpA by including M13 coat protein during dialysis, by preincubating the reaction mixtures at 40°C prior to pro-OmpA addition, and by transferring the mixtures after the reaction to fresh tubes before protease digestion.

RESULTS

Since the goal of our studies was to study precursor protein translocation into reconstituted proteoliposomes, we began by determining whether proteoliposomes inhibit translocation into inverted, sealed, inner-membrane vesicles of *E. coli*. Translocation of [^{35}S]pro-OmpA into these membrane vesicles was assayed by its inaccessibility to added proteinase K. Native membrane vesicles translocated ≈25% of the input pro-OmpA during our standard reaction; however, the translocation of pro-OmpA was dramatically inhibited by the addition of proteoliposomes (Fig. 1). This inhibition may be due to the substantial affinity of pro-OmpA for lipid vesicles (37). Detection of translocation activity under these conditions relied on the use of radiochemically pure [^{35}S]pro-OmpA to eliminate the background of contaminating proteins.

Proteoliposomes containing membrane proteins were prepared by suspending native membrane vesicles (0.5 mg/ml) in buffer A containing octyl glucoside and phospholipid at 0°C for 30 min, followed by dialysis. Approximately 1% of the input pro-OmpA was translocated into these proteoliposomes when ATP was included in the reaction mix (Fig. 2, lane 4). When ATP was omitted from the reaction, pro-OmpA translocation was reduced to background levels at <0.1% (lane 3). This ATP-dependent translocation of pro-OmpA was abolished when a nonhydrolyzable ATP analogue (ATP[γS]) was also included (lanes 5 and 6), and GTP could not efficiently substitute for ATP (lane 7). As expected for translocation across the inner membrane, mature OmpA (without a leader sequence) did not show energy-dependent translocation (lanes 1 and 2). Pro-OmpA translocation was not detected when membrane proteins were omitted from the

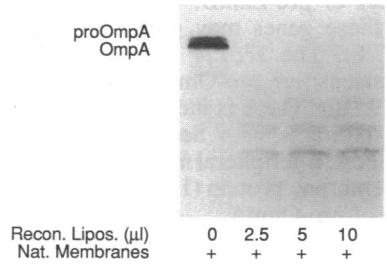

FIG. 1. Proteoliposomes inhibit translocation of pro-OmpA into native membrane vesicles. Translocation reaction mixtures (50 μl) contained native membrane vesicles (0.05 mg/ml) and the indicated volumes of proteoliposomes prepared by dialysis of octyl glucoside (15 mg/ml), *E. coli* phospholipids (3.8 mg/ml), and M13 coat protein (0.1 mg/ml) against buffer A. The reactions were performed as described in *Materials and Methods*, using [^{35}S]pro-OmpA (200,000 cpm per reaction) and ATP (2.5 mM) as substrates. The translocated pro-OmpA and OmpA that were inaccessible to external proteinase K were visualized by SDS/PAGE and fluorography.

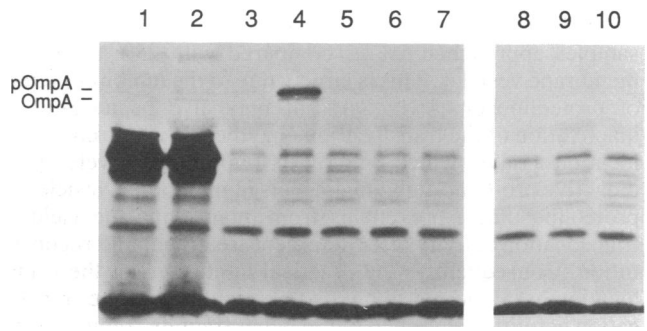

FIG. 2. Recovery of energy-dependent translocation activity from membranes treated with octyl glucoside. Proteoliposomes reconstituted with (lanes 1–7) or without (lanes 8–10) incorporated membrane proteins were assayed for their ability to translocate OmpA (lanes 1–2) or pro-OmpA (lanes 3–10). The 50-μl reaction mixtures contained 10 μl of proteoliposomes (final membrane protein concentration, 0.1 mg/ml) and various nucleoside triphosphates: ATP (lanes 2, 4, 5, 9, and 10), ATP[γS] (lanes 5, 6, and 10), or GTP (lane 7). Samples in lanes 1, 3, and 8 received no nucleotide. Translocated pro-OmpA (pOmpA) and OmpA were visualized by SDS/PAGE and fluorography.

reconstitution (lanes 8–10). Thus, by several well-established criteria, the translocation activity observed after detergent treatment and reconstitution is that of the bacterial inner membrane.

To determine whether the inner membrane proteins and the translocation activity were truly solubilized, the crude detergent extract was fractionated by ultracentrifugation. While most proteins were recovered quantitatively in the supernatant, some proteins were found predominantly in the pellet as detected by stained gels (Fig. 3*A*). Almost all of the leader peptidase was found in the supernatant (Fig. 3*B*), showing that this integral membrane protein was efficiently solubilized. In contrast, we estimate that only 30% of the SecY protein was retained in the supernatant, while the remainder was recovered in the pellet (Fig. 3*C*). More stringent centrifugations resulted in the sedimentation of up to 90% of the total SecY protein, suggesting that it is not stably soluble under these conditions (data not shown). The two fractions were reconstituted and assayed for translocation of pro-OmpA. The translocation efficiency of each fraction closely correlated with the amount of SecY protein in the sample (Fig. 3*D*), the pellet fraction (lane 5) having approximately twice as much protected pro-OmpA as the supernatant fraction (lane 2) as quantified by densitometer scanning of the original fluorographs. As predicted from the observed distribution of leader peptidase (Fig. 3*B*), there was no detectable processing of the translocated pro-OmpA to OmpA in the reconstituted pellet fraction, whereas 10% of pro-OmpA translocated into the vesicles reconstituted from the supernatant was processed to OmpA (as judged by quantitation of other fluorographs). Due to the assay limitations mentioned above and the inhibition of translocation by proteoliposomes, we cannot state with certainty that the specific activity of the pellet fraction is greater than that of the unfractionated membranes. It is evident that the membranes were disrupted, since leader peptidase could be functionally removed from the translocation activity by sedimentation.

To be assured that the reconstituted translocation activity was not associated with a small amount of native membranes that survived the detergent treatment, we devised a test that took advantage of the diminished processing of the translocated pro-OmpA by leader peptidase in the reconstituted samples. Addition of purified leader peptidase to the detergent extracts should complement the processing deficiency only if it can functionally assemble into the vesicles that contain the translocation activity. Active leader peptidase

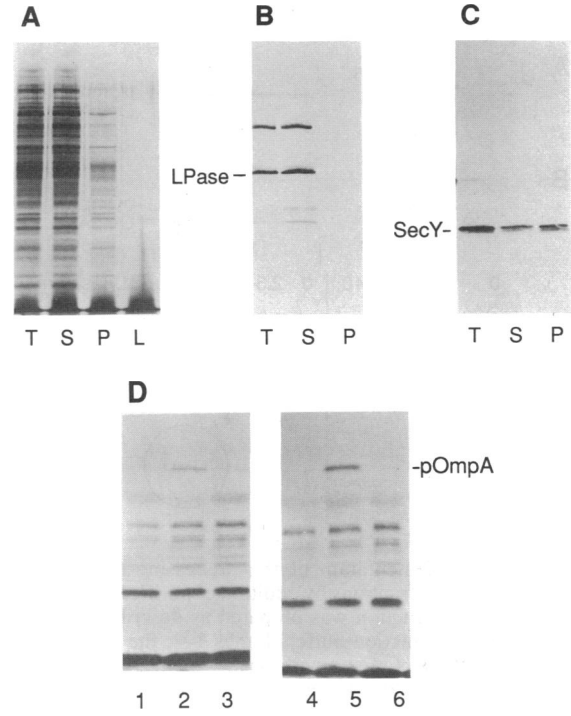

FIG. 3. Fractionation of the membrane detergent extract by ultracentrifugation. A suspension of membranes (0.5 mg/ml) in extraction buffer was centrifuged at 150,000 × *g* for 30 min in a Beckman Airfuge. The supernatant fraction was collected and the pellet was resuspended in extraction buffer to the starting volume. Equivalent volumes of the unfractionated extract (T), supernatant fraction (S), pellet fraction (P), and extraction buffer alone (L) were dialyzed and 10-μl aliquots were analyzed by SDS/PAGE. The gels were silver-stained for total protein (*A*) or electroblotted onto Zeta-Probe membranes (Bio-Rad) and immunostained for leader peptidase (LPase) (*B*) or for SecY protein (*C*) as described (38). Pro-OmpA (pOmpA) translocation activity was determined (*D*) using 10 μl of either the reconstituted supernatant fraction (lanes 1–3) or in the reconstituted pellet fraction (lanes 4–6), with no nucleotide present (lanes 1 and 4) or with ATP (lanes 2 and 5) or ATP plus ATP[γS] (lanes 3 and 6) present during the reaction.

presumably cannot insert into intact membrane vesicles, although tiny quantities can assemble cotranslationally into native membrane vesicles (39). When a pellet fraction was resuspended in extraction buffer and mixed with purified leader peptidase prior to dialysis, the resulting proteoliposomes processed a large fraction of the translocated pro-OmpA to OmpA (Fig. 4*A*, lanes 1–4). When leader peptidase that was deliberately reconstituted into separate proteoliposomes and subsequently mixed with proteoliposomes containing only the translocation activity, there was no increase in the processing of translocated pro-OmpA (lanes 5–8). An aliquot of each translocation reaction mixture was removed before protease treatment and analyzed for the total conversion of pro-OmpA to OmpA (Fig. 4*B*). Equivalent levels of leader peptidase activity were present in the separate reconstitution and mixed reconstitution experiments, since the processing of pro-OmpA was indistinguishable between the two types of vesicles. At the highest levels of leader peptidase used (lanes 4 and 8), the majority of the input pro-OmpA was converted to OmpA that remained accessible to external protease. Since OmpA cannot translocate efficiently (Fig. 2), premature processing can explain the observation that overall translocation efficiency decreases with increasing leader peptidase concentration (Fig. 4*A*, lanes 1–4). Thus, it is clear that leader peptidase can interact productively with the translocation activity during the detergent treatment and become coreconstituted into vesicles containing both activ-

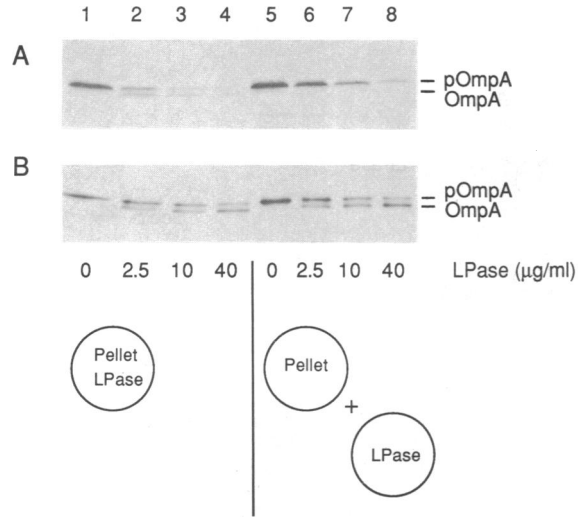

FIG. 4. Complementation of the processing deficiency in the reconstituted pellet fractions by addition of purified leader peptidase (LPase). A pellet fraction was prepared as described in Fig. 3 and resuspended in extraction buffer. Lanes 1–4, the pellet fraction in extraction buffer was mixed with equal volumes of purified leader peptidase (0, 5, 20, or 80 μg/ml) in extraction buffer and then dialyzed for vesicle reconstitution as described in *Materials and Methods*. Lanes 5–8, the pellet fraction and the four suspensions of leader peptidase were dialyzed separately and the resulting proteoliposomes were mixed in equal volumes to yield mixtures equivalent in leader peptidase concentration to those of lanes 1–4, except that the added leader peptidase and the pellet fraction were segregated into separate proteoliposomes (see diagram). A portion (10 μl) of each mixture (lanes 1–8) was assayed for translocation and processing of pro-OmpA in the presence of 2.5 mM ATP. (*A*) Pro-OmpA (pOmpA) and OmpA species that had translocated after the reactions (i.e., became protected from protease) into the mixtures of proteoliposomes. (*B*) Total pro-OmpA and OmpA species that were produced during the reactions (i.e., without the addition of protease) as a measure of the amount of leader peptidase activity present in the mixture. The fluorographs in *A* and *B* were exposed for different lengths of time to produce bands of similar intensity. By comparison with pro-OmpA standards, ≈0.5% of the input pro-OmpA was translocated in *A*, lane 1.

ities. Even though much of the translocation activity (and SecY protein) is recovered in the pellet fraction after centrifugation, it is unlikely that the activity is associated with detergent-resistant membrane vesicles, since leader peptidase can be functionally removed or restored.

DISCUSSION

The treatment of *E. coli* plasma membrane vesicles with octyl glucoside and phospholipids effectively solubilized leader peptidase and most other membrane proteins. Dialysis was used to reconstitute the total detergent suspension, the soluble fraction, or the insoluble fraction into proteoliposomes. These proteoliposomes had *bona fide* protein translocation activity, as judged by their ability to sequester pro-OmpA from exogenous protease in a manner that requires ATP hydrolysis (14, 15, 40) and additional membrane components. Further, the activity was specific for a competent precursor, as OmpA was not translocated. To rule out the possible contamination of our fractions with nondisrupted membrane vesicles, we showed that the translocation activity associated with the detergent-insoluble pellet had been depleted of leader peptidase (Fig. 3) and that the translocated protein could be processed when additional enzyme was added prior to dialysis/reconstitution (Fig. 4). These results strongly suggest that the insoluble activity was not associated with intact membrane vesicles.

The maximum translocation efficiency of the reconstituted samples approached 1% as compared with 25% for native membrane vesicles. This is largely due to the inhibitory effect of proteoliposomes (Fig. 1) but may also be a result of inactivation of the translocation enzymes. The current translocation assays, which employ extremely low levels of radioactive pro-OmpA and high levels of membrane vesicles or proteoliposomes, prevent us from quantifying the yield of translocation activity after detergent treatment and reconstitution. Consequently, the practical limitations of the translocation assay do not allow us to conclude that the translocation activity is enriched in the pellet fraction, though some correlation exists between translocation efficiency and the amount of SecY protein (Fig. 3).

The SecY (PrlA) protein resembles lactose permease in hydrophobicity, transmembrane topology, and solubility in the presence of SDS (13). The first step in the isolation of lactose permease was the selective detergent extraction of other, less hydrophobic membrane proteins (41). Our current studies suggest that this may also be possible for the SecY protein, resulting in an enrichment in the pellet fraction (Fig. 3). Even though SecY is not stably soluble in mixed micelles of octyl glucoside, selective extraction with this detergent may be followed by solubilization with other detergents. Our current procedure allows complementation during the formation of liposomes (Fig. 4) and may provide a means to separate and develop assays for other membrane components that are important in protein translocation.

Note Added in Proof. Recently, buffer conditions have been found that allow extraction of SecY protein into micellar solution and reconstitution of translocation into proteoliposomes (A. Driessen and W.T.W., unpublished data).

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