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ProOmpA Is Stabilized for Membrane Translocation by Either Purified E. coli Trigger Factor or Canine Signal Recognition Particle

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Summary

We have isolated large amounts of E. coli outer-membrane protein A precursor (proOmpA). Purified proOmpA is active in membrane assembly, and this assembly is saturable with respect to the precursor protein. A proOmpA–Sepharose matrix allows affinity isolation of trigger factor, a soluble, 63,000 dalton monomeric protein that stabilizes proOmpA in assembly competent form. Comparison of trigger factor's amino-terminal sequence with those in a computer data bank and with those encoded by *sec* genes, as well as *groEL* and heat shock gene *dnaK*, suggests that trigger factor is encoded by a previously undescribed gene. Trigger factor and proOmpA form a 1:1 complex that can be isolated by gel filtration. Purified canine signal recognition particle (SRP) can also stabilize proOmpA for membrane insertion. This post-ribosomal activity of SRP suggests a unifying theme in protein translocation mechanisms.

Introduction

The salient features of protein translocation across membranes have been described during the last decade (reviewed in Wickner and Lodish, 1985). These include the presence of a leader sequence, a requirement for metabolic energy, and conformational change of the precursor proteins. Protein insertion into the mammalian endoplasmic reticulum has been viewed as a strictly cotranslational event (Blobel, 1980), coupled to translation by the action of signal recognition particle (SRP; Walter and Blobel, 1981). Although mammalian and bacterial presecretory proteins share a common leader peptide structure, bacterial secretion is not coupled to translation, and many proteins are exported largely posttranslationally.

Although bacterial membrane assembly has been reconstituted with purified components for one protein, the M13 procoat protein (Ohno-Iwashita and Wickner, 1983), this protein is unusually simple in that its membrane insertion does not require the functioning of *sec*- and *prl*-encoded proteins (Wolfe et al., 1985). More recently, an in vitro translation–translocation reaction has been described for *sec* and *prl*-dependent proteins (Müller and Blobel, 1984; Rhoads et al., 1984). In this reaction, presecretory proteins, synthesized in an Escherichia coli extract, translocate into the lumen of inverted, sealed plasma membrane vesicles. As clearly established in vivo, the in vitro translocation is not coupled to ongoing polypeptide chain growth and requires both ATP hydrolysis (Chen and Tai, 1985) and the membrane electrochemical potential (Geller et al., 1986).

We have undertaken an enzymological approach to resolve and reconstitute the components of this translocation reaction. [^{35}S]proOmpA was purified 1900-fold in 8 M urea from an in vitro protein synthesis reaction (Crooke and Wickner, 1987). Upon dilution, the proOmpA renatures into a form competent for membrane assembly (Crooke et al., 1988). However, it rapidly loses this competence unless a soluble protein, which we have termed trigger factor, is present. Trigger factor stabilizes proOmpA in a form that is competent for membrane assembly. Indirect evidence has been presented which indicates that trigger factor and proOmpA may form a complex (Crooke and Wickner, 1987).

We now report the isolation of homogeneous proOmpA and trigger factor. An important step in the purification of trigger factor is affinity chromatography on proOmpA–Sepharose, confirming our hypothesis that these proteins can form a complex. N-terminal sequence analysis indicates that trigger factor is not one of the proteins encoded by the well-studied *sec*, *prl*, or heat shock genes. Studies reported here and in an accompanying article (Lill et al., 1988) show interactions of trigger factor with ribosomes, proOmpA, and membranes and suggest a cyclic mode of its action. The similarity of this trigger factor cycle to the cyclic action of canine SRP led us to the finding that SRP, which had been thought to act exclusively in the context of the ribosome, can also stabilize purified proOmpA for membrane insertion. These studies suggest that a primary function of trigger factor, and possibly SRP, is to stabilize presecretory proteins for membrane translocation.

Results

Purification of ProOmpA

A first step toward the isolation of large amounts of proOmpA was its overproduction in vivo. Expression of the *omp9* gene under the *trc* promoter in a multicopy plasmid causes substantial overproduction of proOmpA and OmpA (Figure 1, lanes 1 and 2). Cells were disrupted and incubated with Sarkosyl to solubilize the membrane proteins. As described by Freudl et al. (1986), the proOmpA remains aggregated in this detergent extract and can be separated from the other cellular proteins simply by centrifugation. ProOmpA was solubilized from this "washed pellet" fraction with 8 M urea. This procedure resulted in a 5-fold purification and 35% yield of proOmpA. In a typical preparation, 422 mg of purified proOmpA (Figure 1, lanes 3–9) was obtained from 62 g (wet weight) of cells.

The purified proOmpA can be renatured for membrane assembly by dilution of the urea. ProOmpA in 8 M urea was diluted into a reaction mixture that contained ATP and inverted, sealed E. coli plasma membrane vesicles

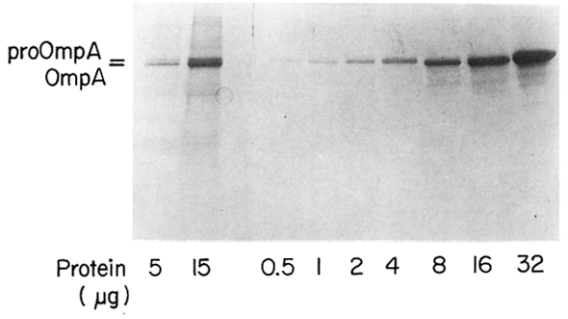

Figure 1. Isolation of ProOmpA from an Overexpressing Strain

Strain W3110 bearing the *trc-ompp9* plasmid was grown at 37°C in M9 minimal medium with 0.5% glucose, 0.4% Casamino Acids (Difco), and 100 μg/ml ampicillin. At an A_{600} of 1.0, isopropyl thiogalactoside was added to 0.5 mM and growth was continued for 2 hr. Cells were collected by centrifugation, suspended in buffer (50 mM Tris–HCl [pH 7.5], 10% sucrose), frozen as small nuggets by pipetting the suspension into liquid nitrogen, and stored at −80°C. To prepare proOmpA, frozen cell suspensions (128 g) were thawed, mixed with lysozyme (0.9 mg/ml), and incubated for 5 min at 23°C. After addition of $MgCl_2$ (5 mM) and DNAase I (4 μg/ml), the suspension was incubated an additional 5 min at 23°C. Extraction buffer (360 ml; 1.5% [wt/vol] Sarkosyl, 50 mM citrate, titrated to pH 6.0 with solid Na_2HPO_4) was added and the mixture subjected to Dounce homogenization. After 30 min at 23°C), the solution was centrifuged (27,000 × g, 15 min at 23°C). This extraction was repeated twice. The final pellet was suspended in 22 ml of buffer C (see Experimental Procedures) by continuous vortexing for 5 min at 23°C), followed by centrifugation for 1 min at 27,000 × g. Samples were analyzed by SDS-PAGE with Coomassie brilliant blue R250 staining. The leftmost two lanes show 5 μg and 15 μg of the unfractionated crude lysate. The other lanes contain the indicated amounts of purified proOmpA.

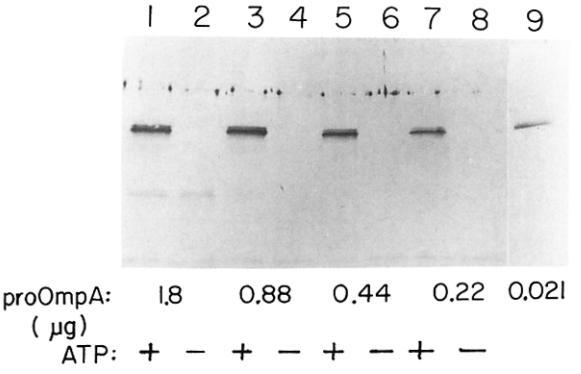

Figure 2. Membrane Assembly of Purified ProOmpA

To present a low background for the assembly assay, inner-membrane vesicles were prepared from E. coli JF699, a strain deficient in OmpA. Each translocation reaction contained 1.5 μg of membrane protein. The indicated amount of proOmpA in 1 μl of buffer C was diluted into an ice-cold translocation mixture (60 μl), allowed to translocate for 20 min at 40°C, and assayed for translocation by protease inaccessibility (see Experimental Procedures). ATP and NADH were omitted from the incubation in even-numbered lanes. Samples were analyzed by SDS-PAGE and by immunoblot with antiserum to OmpA. Lane 9 is a standard of 0.021 μg of proOmpA.

(Rhoads et al., 1984). After incubation at 40°C to allow membrane assembly, samples were chilled on ice and assayed for translocated OmpA by incubating the sealed membrane vesicles with proteinase K. Each reaction mixture was subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with antiserum to OmpA (Figure 2). When 0.22 μg of proOmpA was assayed with 1.5 μg of membrane vesicles (Figure 2, lane 7), approximately 10% was translocated across the membrane and processed to mature OmpA (compare with standard in lane 9). Translocation was dependent on ATP (see lanes 2, 4, 6, and 8; no ATP). The total translocation increased with increasing amounts of proOmpA in the assay (Figure 2, lanes 5 and 3).

At the highest levels of proOmpA, the translocation reached a plateau level, suggesting that this concentration of proOmpA was saturating an essential membrane element. To test this, varying amounts of proOmpA were mixed with a constant amount of [^{35}S]proOmpA in 8 M urea, then diluted with membranes and assayed for translocation (Figure 3). As previously reported (Crooke et al., 1988), [^{35}S]proOmpA translocates into inner-membrane vesicles, where it is cleaved by leader peptidase to OmpA. Both translocated proOmpA and OmpA are inaccessible to digestion by protease (Figure 3, lane 2), and translocation is energy dependent (lane 1). The addition of 0.16, 0.8, or 4.0 μg of proOmpA (Figure 3, lanes 11, 7, and 3) progressively inhibits the translocation of proOmpA. The translocated proOmpA into these vesicles is thus both saturable and energy dependent. As a control, we determined that addition of various amounts of nonradioactive proOmpA to the assembly reactions after translocation of [^{35}S]proOmpA was complete had no effect on the protease protection assay (Figure 3, lanes 5, 9, and 13). As expected, OmpA translocates poorly or not at all under these reaction conditions (data not shown).

Purification of Trigger Factor

We have previously reported indirect evidence that trigger factor forms a complex with proOmpA (Crooke and Wickner, 1987). With large amounts of pure proOmpA now

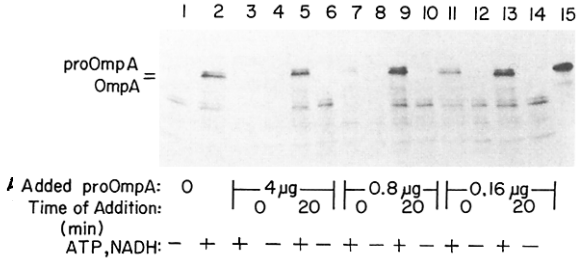

Figure 3. Saturation of ProOmpA Translocation into Plasma Membrane Vesicles

Translocation reactions with [^{35}S]proOmpA renatured by dilution were performed as described in Experimental Procedures and analyzed by incubation with proteinase K and by SDS-PAGE and fluorography. The indicated amounts of purified proOmpA (in 1 μl) were added to each incubation, either from the beginning of the translocation reaction (time 0) or after 20 min of the 23 min incubation. Incubations were performed in either the presence, or absence of ATP and NADH, as indicated. Lane 15 is a standard of proOmpA.

Table 1. Purification of E. coli Trigger Factor

Purification Step	Protein (mg)	Trigger Factor (U)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
I. Cell Lysate (S40)	3,300	440,000	133	1	100
II. Affinity Chromatography	79	243,200	3,078	23	55
III. S-Sepharose Fast Flow	19	180,000	9,474	71	41
IV. Mono Q Anion Exchange	12	240,000	20,000	150	54

See Experimental Procedures for details.

available, we covalently linked proOmpA to a CNB-activated Sepharose column to create an affinity resin for the isolation of trigger factor. The resin was suspended in crude soluble protein extract (S40) from E. coli in the presence of 8 M urea, then dialyzed to remove the urea. These conditions were chosen to mimic those in which the trigger factor in S40 was shown to support renaturation of radiochemically pure [^{35}S]proOmpA (Crooke and Wickner, 1987). The dialyzed suspension was poured into a column, rinsed with buffer, and step-eluted with buffer containing 8 M urea. This affinity step resulted in a 23-fold purification of trigger factor with a 55% yield.

Further purification was achieved by successive ion exchange chromatography with S-Sepharose Fast Flow cation exchange resin and Mono Q anion exchange FPLC. The overall purification, in three steps, is 150-fold with a 54% yield (Table 1; see Experimental Procedures for trigger factor activity assay). Analysis of trigger factor at each step of the purification by SDS-PAGE and silver staining (Figure 4A) shows that the purified protein (lane 5) is a single polypeptide of apparent size 63,000 daltons. It was recovered as a single, sharp, symmetric peak on the final step of ion exchange chromatography (Figure 4B). The 63,000 dalton polypeptide eluted at fraction 28 (Figure 4C), coincident, with the trigger factor activity of promoting proOmpA translocation into membrane vesicles (Figure 4D). While minor bands of lower molecular weight can be detected, these polypeptides are not consistently seen (see, for example, Figure 3A of Lill et al., 1988) and do not form a complex with proOmpA (see Figure 7). Gel filtration analysis shows that the prominent polypeptide (Figure 4C) and trigger factor activity co-elute with an apparent size of 73,000 daltons (Figure 5), indicating that trigger factor is a monomeric protein.

Automated Edman sequence analysis showed that the amino terminus of trigger factor has the sequence Met-Gln-Val-Ser-Val-Glu-Thr-Thr-Gln-Gly-Leu-Gly-. This sequence does not correspond to any of the published *sec* or *prl*-encoded proteins, to heat shock proteins DnaK (Bardwell and Craig, 1984) or C62.5 (Bardwell and Craig, 1987), or to GroEL (Hemmingsen et al., 1988). It does not correspond to any protein currently listed in the 1987 update of the PSQ data base (Dayhoff, 1979), which currently lists approximately 500 E. coli proteins.

Characteristics of Purified Trigger Factor

Aliquots of purified trigger factor were incubated with proOmpA–Sepharose and OmpA–Sepharose and assayed for binding. Trigger factor was also incubated with Sepharose with covalently bound M13 procoat leader peptide, a leader typical in structure (von Heijne, 1983) that functions in vivo to promote OmpA secretion (Kuhn et al., 1987). Trigger factor bound to proOmpA–Sepharose but not to the immobilized OmpA or leader peptide (E. C., unpublished observation), suggesting either that the trigger factor recognizes features of the leader as well as the mature protein or that proper recognition of a domain of proOmpA requires the presence of the intact precursor protein.

We have previously reported that partially purified trigger factor will stabilize proOmpA in a conformation competent for membrane assembly (Crooke et al., 1988). To establish that this is a feature of the purified protein, and to characterize its action, proOmpA was diluted from 8 M to 0.8 M urea and assayed for translocation. Immediately after dilution, proOmpA is competent for translocation (Figure 6, lane 3) and the efficiency of this translocation is not affected by trigger factor (lane 4). However, proOmpA lost competence for membrane insertion after a 3 hr preincubation at 21°C (Figure 6, lane 5). As previously reported (Crooke et al., 1988), proOmpA preincubated in the presence of trigger factor retains full activity for assembly into inverted plasma membrane vesicles from E. coli (Figure 6, lane 7). Addition of trigger factor to proOmpA that had already misfolded during preincubation at 21°C did not restore its competence for assembly (lane 9), suggesting that trigger factor stabilizes the competent form of proOmpA rather than refolding the incompetent protein. Since proOmpA can bind directly to membrane vesicles in the absence of trigger factor, we determined whether membranes would stabilize the translocation-competent form of the precursor protein. ProOmpA preincubated at 21°C with membrane vesicles lost competence for subsequent membrane translocation (Figure 6, lane 11), while preincubation in the presence of membranes and trigger factor, but without ATP and NADH (lane 12), stabilized the assembly-active precursor.

Trigger factor and proOmpA form a 1:1 complex that can be isolated by gel filtration. ProOmpA and trigger factor were mixed in 8 M urea, dialyzed to remove the urea and allow complex formation, and size fractionated on a Superose 12 FPLC column (Figure 7). The complex emerged as a single, symmetric peak (Figure 7, line C). In the absence of trigger factor, proOmpA aggregates and emerges in the void volume (Figure 7, line B), while trigger factor alone (line A) is eluted from the gel filtration column at a

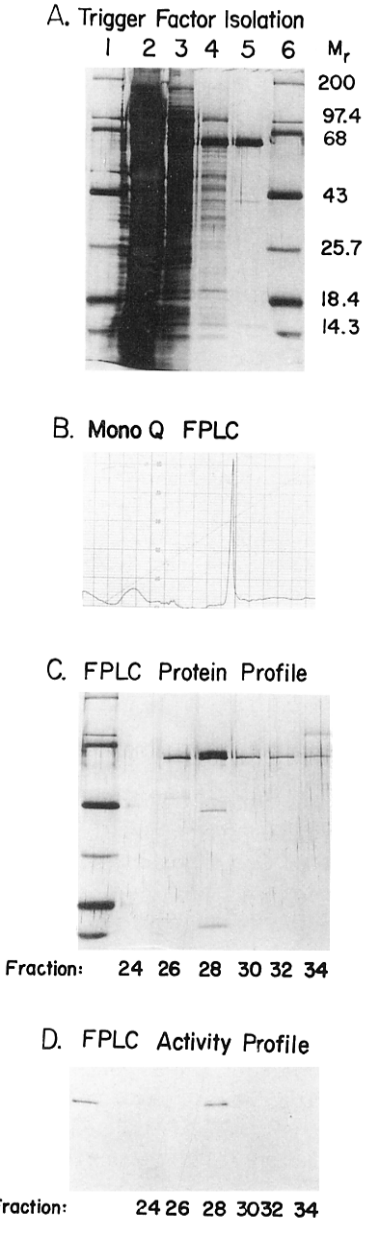

Figure 4. Isolation of Trigger Factor

Trigger factor was purified as described in Experimental Procedures. (A) Samples of trigger factor (10 U) at purification steps I–IV were analyzed by SDS-PAGE and silver staining (lanes 2–5, respectively). Lanes 1 and 6 contain molecular weight markers. (B) Elution of trigger factor from Mono Q FPLC. The vertical axis represents absorbance at 280 nm. The linear gradient is from 0–0.5 M NaCl. (C) Fractions near the Mono Q elution peak were analyzed by SDS-PAGE and silver staining. The first lane contains molecular weight markers. (D) Trigger factor activities of the fractions analyzed in (C) were assayed as described in Experimental Procedures. The leftmost lane is a proOmpA standard.

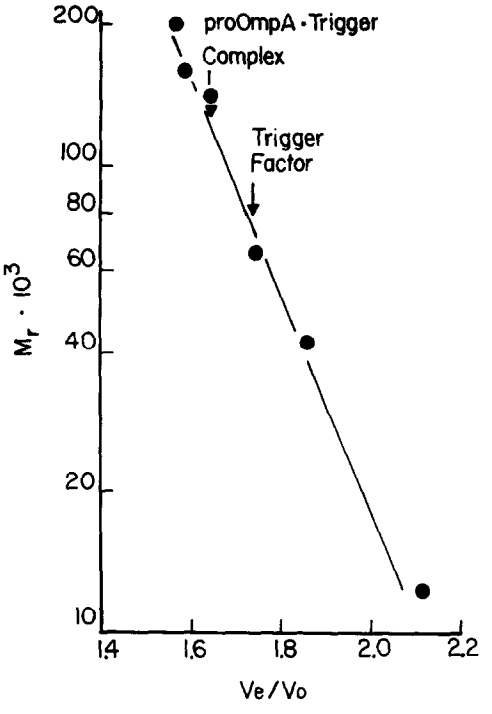

Figure 5. Gel Filtration Analysis of Trigger Factor and ProOmpA–Trigger Factor Complex

Trigger factor (50 μg, 100 μl) was analyzed by gel filtration on a 25 ml Superose 12 FPLC column equilibrated in buffer E (see Experimental Procedures). ProOmpA–trigger factor complex was formed and analyzed as described in Figure 7. Protein elution was detected by absorbance at 280 nm. The column was calibrated with 100 μg (100 μl) each of the following proteins: β-amylase (MW = 200,000), bovine IgG (MW = 160,000), yeast alcohol dehydrogenase (MW = 141,000), bovine serum albumin (MW = 67,000), chicken ovalbumin (MW = 43,000), and cytochrome c (MW = 12,400). The column void volume (V_0) was determined with Blue Dextran 2000.

lower molecular weight than the complex. Analysis of the FPLC fractions by SDS-PAGE confirms that proOmpA and trigger factor elute together at a position distinct from either component alone. Calibration of the column with standards of known molecular mass suggests an apparent molecular mass of 125,000 daltons (Figure 5), consistent with a 1:1 stiochiometry of proOmpA (molecular mass 37,563 daltons; Chen et al., 1980) and trigger factor (apparent molecular mass 73,000 daltons as judged by gel filtration). A 1:1 complex can also be demonstrated by covalent cross-linking with dimethyl suberimidate (data not shown). The purified complex was stable to storage and was fully active for membrane assembly (S. L., unpublished observations). Further studies will be needed to establish the structural features of this complex and the parameters of its formation and dissocation.

SRP Stabilizes ProOmpA for Membrane Translocation

Like trigger factor, canine SRP has been shown to recognize presecretory proteins, although until now its activity has been assayed in the context of a polysome. We therefore asked whether dog pancreas microsomes might contain a trigger factor activity. ProOmpA was diluted from urea and preincubated at 21°C with either a salt extract of dog pancreas microsomes or with salt extract buffer alone. After various preincubation times, the proOmpA was assayed for assembly into E. coli plasma membrane vesicles. The microsomal extract stabilizes proOmpA for membrane insertion (Figure 8), although not as well as

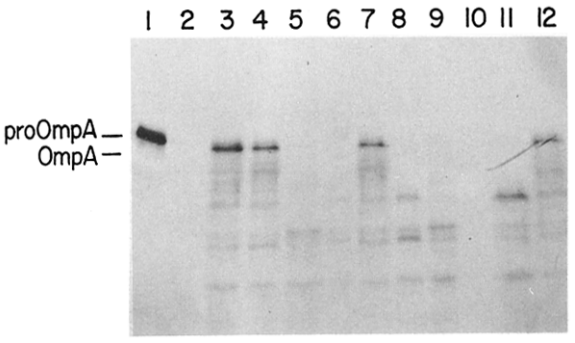

Figure 6. Purified Trigger Factor Stabilizes ProOmpA for Membrane Assembly

[^{35}S]proOmpA was renatured by dilution into translocation mixtures (as described in Experimental Procedures, except membranes were omitted). Membrane vesicles were immediately added to the mixtures in lanes 3 and 4, and the samples were assayed for translocation in either the absence (lane 3) or presence (lane 4) of trigger factor (100 ng). Samples in lanes 5–12 were preincubated at 21°C for 3 hr before addition of membrane vesicles and assaying of membrane assembly. The samples in lanes 5 and 6 were preincubated in the absence of trigger factor (lane 6 also lacked ATP and NADH during the preincubation). Samples in lanes 7 and 8 were preincubated in the presence of trigger factor (lane 8 had no ATP or NADH during preincubation or during membrane assembly). The sample in lane 9 was preincubated for 3 hr without trigger factor, then mixed with trigger factor and assayed for membrane assembly. Samples in lane 11 and 12 were preincubated in the presence of membrane vesicles for 3 hr at 21°C but without ATP or NADH; after the 3 hr preincubation, ATP and NADH were added and samples were assayed for translocation. The sample in lane 12 had trigger factor present during preincubation. Lane 1 contains a proOmpA marker. Lanes 2 and 10 had no sample.

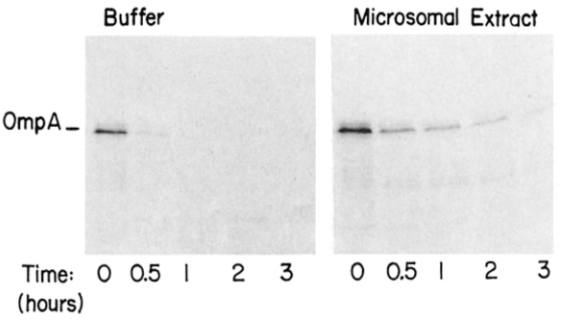

Figure 8. Canine Microsomal Salt Extract Stabilizes ProOmpA for Membrane Assembly

[^{35}S]proOmpA was renatured by dilution into translocation mixtures (as described in Experimental Procedures, except membranes were omitted). The translocation mixtures (60 μl) analyzed at left contained buffer H (2 μl), while mixtures analyzed at right contained canine microsomal salt extract (2 μl). Following the addition of proOmpA, samples were maintained at 21°C for the indicated times before addition of membranes and incubation at 40°C for 20 min. Translocation was assayed by protection from proteinase K (see Experimental Procedures).

trigger factor (Figure 6; see also Crooke et al., 1988). This may in part reflect the instability of SRP itself in the absence of detergent (Walter and Blobel, 1980). To stabilize the SRP and to allow its purification, we added 0.01% Nikkol to our buffers and isolated SRP by published procedures (Walter and Blobel, 1980). Nikkol does not inhibit the membrane assembly of proOmpA that has already formed a complex with trigger factor (Figure 9, lane 3, no detergent; lane 4, detergent added), and thus has no effect on the membrane translocation "machinery" per se or on the protease protection assay. However, it strongly inhibits the membrane assembly of proOmpA in the absence of stabilizing trigger factor (Figures 10A and 10B, lanes 3).

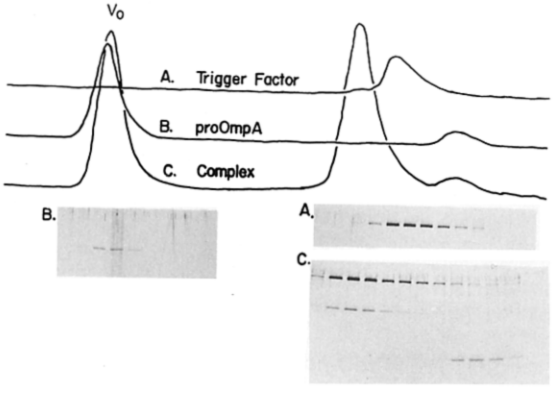

Figure 7. Isolation of a Trigger Factor–ProOmpA Complex by Gel Filtration

ProOmpA (300 μg; 25 mg/ml in buffer C), trigger factor (100 μg), or a mixture of the two was diluted to 250 μl in buffer C and dialyzed overnight against 1.0 liter of 66 mM Tris–HCl (pH 7.6), 25 mM NH_4Cl, 10 mM Mg acetate, 0.5 mM β-mercaptoethanol. Aliquots (200 μl) were applied to a 25 ml Superose 12 FPLC column (equilibrated in the dialysis buffer) and eluted at a flow rate of 0.25 ml/min. Fractions (250 μl) were collected, and 5 μl aliquots of each were analyzed by SDS-PAGE and silver staining. The A_{280} tracing and corresponding gel analysis are shown for trigger factor (A) proOmpA (B) and trigger factor–proOmpA complex (C). V_0 indicates the column void volume.

SRP and the activity of stabilizing proOmpA copurified by either ω-aminopentyl agarose chromatography or sucrose gradient sedimentation. SRP prepared by each of these methods (Figure 10) stabilizes proOmpA against the detergent denaturation (lanes 4 and 5) and allows a level of assembly comparable to that seen in the absence of detergent (lanes 8). This SRP-dependent membrane assembly requires ATP (lanes 6 and 7, no ATP). To assess the relative potency of trigger factor and SRP in stabilizing proOmpA, comparable molar amounts of each were assayed (Figure 10C). While the trigger factor is clearly somewhat superior in stabilizing proOmpA, this is hardly surprising since these proteins evolved in the same organism, while SRP was isolated from canine pancreas.

Discussion

The membrane assembly reaction of proOmpA with trigger factor and inner-membrane vesicles requires ATP, the membrane potential, and the leader sequence of proOmpA. It therefore represents an authentic reconstitution of translocation. The proOmpA is translocated across an intact membrane and, as in vivo, it is processed to OmpA after translocation. It is likely that other soluble proteins, such as those encoded by the *secA* and *secB* genes, would bind to our preparations of plasma membrane vesicles or, if added to the in vitro translocation

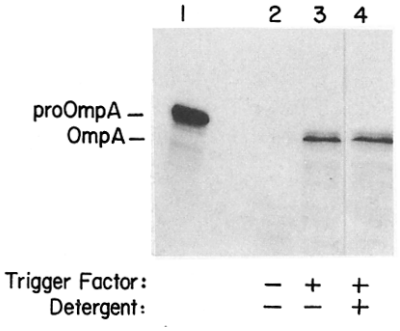

Figure 9. Low Concentrations of Nikkol Do Not Affect Translocation of ProOmpA Complexed with Trigger Factor

[^{35}S]proOmpA was renatured by dilution into translocation mixtures (without membranes) in the absence (lane 2) or presence (lanes 3 and 4) of trigger factor (100 ng). Samples were maintained at 21°C for 3 hr. Membrane vesicles were added to each reaction, and samples were incubated at 40°C for 20 min. Buffer J (3 μl, contains 0.01% Nikkol) was added to the sample in lane 4 immediately before addition of membranes. Translocation was assayed by protease protection. The marker in lane 1 represents 33% translocation efficiency.

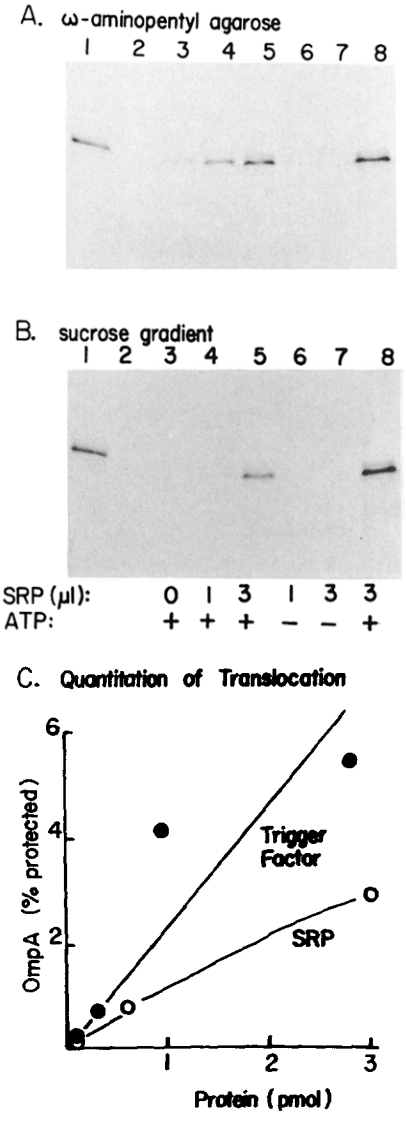

Figure 10. Purified SRP Stabilizes ProOmpA for Membrane Assembly

[^{35}S]proOmpA was renatured by dilution into translocation mixtures (without membranes) containing varying amounts of purified SRP (lanes 3–7). Each sample in lanes 3–7 contained the equivalent of 3 μl of buffer J. ATP was omitted where indicated. Membranes were immediately added, and samples were incubated for 20 min at 40°C. Purified SRP (in 3 μl of buffer J) was added to the sample in lane 8 after the translocation reaction. All samples (lanes 3–8) were assayed for translocation by incubation with proteinase K. The marker in lane 1 represents 10% translocation; lane 2 contains no sample. SRP was purified from canine microsomal salt extract by elution from an ω-aminopentyl agarose column (A) or by sucrose gradient centrifugation (B). (C) [^{35}S]proOmpA was renatured by dilution into translocation mixtures (without membranes) that contained the indicated amounts of purified trigger factor or ω-aminopentyl agarose–isolated SRP. Membrane vesicles were immediately added to reactions that contained 0.0003% Nikkol and SRP. The reactions were assayed for membrane assembly. Reactions that contained trigger factor were preincubated for 3 hr at 21°C before addition of membrane vesicles and assaying of membrane assembly. Protease-protected [^{35}S]OmpA was analyzed by SDS-PAGE. The resulting fluorography bands were quantitated by densitometric scanning.

reaction, would enhance either the rate or extent of the assembly reaction. The SecB protein (Collier et al., 1988) may have the same function as trigger factor, with different, but overlapping, substrate specificity. Further experiments with these proteins will be essential to settle these questions.

The purification of proOmpA has provided several insights into the mechanisms of its assembly across the E. coli plasma membrane. It can spontaneously fold into a form competent for membrane assembly (Crooke et al., 1988). ATP is the only other essential soluble component. In the absence of other proteins, proOmpA loses this competence (Crooke et al., 1988). At physiological temperatures, proOmpA misfolds within minutes (E. C., unpublished observations), suggesting a physiological role for trigger factor in stabilizing proOmpA by forming a stoichiometric complex.

The isolation of these proteins and the demonstration of their activity in translocation across a membrane raise several important questions. Perhaps foremost is the question of whether trigger factor has a role in vivo similar to that deduced from its action in vitro. This may be addressed by isolating the gene for trigger factor and establishing control of its synthesis in the cell. A second question is whether trigger factor is related to the heat shock, ATP-dependent unfolding activities required for protein translocation into endoplasmic reticulum and mitochondria in yeast (Deshaies et al., 1988; Chirico et al., 1988). We note, however, that the cellular concentration of trigger factor is not affected by heat shock (E. C. and S. L., unpublished observations). Finally, it will be important to understand how trigger factor is released from proOmpA as the proOmpA crosses the membrane. The availability of milligram quantities of pure trigger factor and proOmpA should facilitate studies of these questions.

A close analogy can be drawn between trigger factor and the SRP. Either may associate with both the ribosome

(Walter and Blobel, 1981; Lill et al., 1988) and with proteins bearing a leader sequence. Each can allow a presecretory or membrane protein to translocate across a membrane. While the interaction of SRP with precursor proteins was initially thought to be strictly coupled to an early stage of polypeptide chain growth (Walter and Blobel, 1981), recent studies have revealed that it can promote the translocation of nearly full-length polypeptides (Rottier et al., 1985; Ainger and Meyer, 1986; Perara et al., 1986). We have found that SRP can interact with purified proOmpA to stabilize it in a form competent for membrane transit. This raises the question of whether SRP also functions in vivo to stabilize presecretory and membrane proteins in a conformation that allows transit across the endoplasmic reticulum.

The subunit structures of SRP and trigger factor are, of course, completely different. SRP consists of six polypeptides and a 7S RNA, while trigger factor is simply a monomeric protein. This difference in structural complexity is reminiscent of the leader peptidases of these two organisms: the E. coli enzyme is a single polypeptide (Wolfe et al., 1983), while that from dog pancreas has six polypeptide subunits (Evans et al., 1986). In addition to its affinity for leader sequences and ribosomes, SRP has a specific membrane receptor (Meyer et al., 1982). Studies in the accompanying paper (Lill et al., 1988) suggest that trigger factor may also bind to a specific site on the plasma membrane. Our observations lead to a working model of the cyclic action of trigger factor in promoting preprotein translocation (Lill et al., 1988).

Experimental Procedures

Materials and Bacterial Strains

Sucrose and urea (ultrapure) were from Schwartz/Mann. E. coli RNA polymerase, proteinase K, and DNAase I were from Boehringer Mannheim. Fatty acid–free bovine serum albumin, lysozyme, phenylmethylsulfonyl fluoride, sodium N-lauroylsarcosine, dithiothreitol (DTT), and gel filtration molecular weight markers were from Sigma. Cyanogen bromide was from Aldrich. Blue Dextran 2000, Sepharose CL-4B, S-Sepharose Fast Flow, and FPLC Mono Q and Superose 12 were from Pharmacia. "Translabel," a mixture of 85% [^{35}S]methionine and 15% [^{35}S]cysteine (1000Ci/mmol; 1 Ci = 37 GBq), was from ICN.

Ribosomes, S100 (Gold and Schweiger, 1971), and S40 were prepared from E. coli strain D10 (*rna-10 relA1 spoT1 metB1*) grown in L-broth at 37°C. Inverted inner-membrane vesicles were prepared from D10 (Rhoads et al., 1984) and, where indicated, JF699 (*proC24 ompA252 his-53 purE41 ilv-277 met-65 lacY29 xyl-14 rpsL97 cycA1 cycB2 tsx-63* λ^-). ProOmpA was isolated from wild-type E. coli W3110 carrying the pTRC-Omp9 plasmid. Cells for S40, S100, ribosomes, and proOmpA were harvested from a 150 liter fermenter using a Sharples centrifuge. Cell pastes were suspended in an equal weight of 50 mM Tris–HCl (pH 7.5), 10% (wt/vol) sucrose, then frozen as small nuggets by rapid pipetting into liquid nitrogen and stored frozen at −80°C (Wickner et al., 1972).

Preparation of ProOmpA–Sepharose Resin

ProOmpA (475 mg of the washed pellet, Figure 1) was suspended in 30 ml of 10 mM potassium phosphate (pH 8.0), then centrifuged for 10 min at 41,000 × g. This suspension and centrifugation procedure was repeated three times to remove any residual Tris–HCl buffer. The final pellet was dissolved by adding 1.0 ml of 10% (wt/vol) SDS and heating at 65°C for 20 min with occasional vortexing. The solubilized proOmpA was covalently attached to 150 ml of CNBr-activated Sepharose CL-4B by the method of Kadonaga and Tjian (1986).

Purification of Trigger Factor

Step I, S40

Nuggets of frozen D10 cell suspension (300 g; A_{600} = 1.0 at harvest) were thawed without warming. Lysozyme (6 ml, 10 mg/ml) was added with gentle stirring. The suspension was immediately poured into centrifuge tubes and maintained on ice for 30 min. Tubes were heated at 37°C for 3 min; care was taken not to subject the sample to hydrodynamic shear forces. The lysate was centrifuged for 60 min at 41,000 × g at 0°C. The clear amber supernatant (S40) was pooled.

Step II, Affinity Chromatography

S40 (110 ml) was made 8 M in urea by the addition of 66 g of urea. This was mixed with 110 ml of settled Sepharose CL-4B resin with covalently attached proOmpA, equilibrated in 50 mM Tris–HCl (pH 8.0), 2 mM DTT, 8 M urea (buffer C). The S40 and resin slurry was dialyzed at 2°C with vigorous stirring against three 6 liter portions of 66 mM Tris–HCl (pH 7.6), 25 mM NH_4Cl, 10 mM Mg acetate, 2 mM DTT (buffer B). The resin was harvested by low-speed centrifugation (10 min at 150 × g). The supernatant was removed, and the resin was resuspended in 100 ml of fresh buffer B and poured into a 2.5 × 22.5 cm column. Unbound proteins were eluted with 600 ml of buffer B. Bound proteins were eluted with buffer C.

Step III, S-Sepharose Fast Flow

Step II trigger factor (18 ml) was dialyzed at 2°C against two 1 liter portions of 50 mM sodium phosphate (pH 6.0), 1 mM DTT, 10% (vol/vol) glycerol (buffer D). The dialyzed sample was applied to a 15 ml (1.5 × 9.0 cm) S-Sepharose Fast Flow column equilibrated in buffer D. The column was washed with 75 ml of buffer D. Trigger factor was eluted with 0.5 M NaCl in buffer D.

Step IV, Mono Q FPLC

The pool (8 ml) of Step III trigger factor was dialyzed against two 500 ml portions of 50 mM Tris–HCl (pH 8.0), 1 mM DTT, 10% (vol/vol) glycerol (buffer E). The dialyzed sample was applied to an FPLC Mono Q 10/10 column (approximately 8 ml) equilibrated in buffer E. The column was washed with 40 ml of buffer E. Trigger factor was eluted with a 0–0.5 M NaCl linear gradient (120 ml) in buffer E. Trigger factor eluted at approximately 275 mM NaCl.

Isolation of Trigger Factor–free [^{35}S]proOmpA

[^{35}S]proOmpA was synthesized in a cell-free synthesis reaction (4.0 ml, 10 mCi ^{35}S-Translabel) as previously described (Bacallao et al., 1986). SDS (0.25%) was added following the synthesis reaction. The sample was heated at 100°C for 10 min, then cooled to 21°C. Triton X-100 (15% in 2.5 ml of buffer B) was added to 5.7% final concentration. [^{35}S]proOmpA was immediately isolated on a 15 ml proOmpA affinity column as previously described (Crooke and Wickner, 1987).

In Vitro Translocation of ProOmpA Renatured by Dilution

ProOmpA (6 μl, 100,000 cpm) in 8 M urea was diluted into 60 μl translocation reaction mixtures as previously described (Crooke et al., 1988). Translocation of proOmpA into E. coli inverted inner-membrane vesicles was assayed by accessibility to added protease (Bacallao et al., 1986).

Trigger Factor Activity

Trigger factor was assayed for its ability to maintain proOmpA in a translocation-competent state following renaturation by dialysis (Crooke and Wickner, 1987). For each sample, a 2 μl aliquot was mixed with trigger factor–free [^{35}S]proOmpA (100,000 cpm in 98 μl of buffer C with 300 μg/ml bovine serum albumin). Each mixture was dialyzed against two 100 ml portions of buffer B at 2°C for 8 hr. Aliquots of the dialyzed samples were added to translocation reaction mixtures (60 μl) that contained [^{35}S]proOmpA, 50 mM Tris–HCl (pH 7.6), 37 mM KCl, 16 mM NH_4Cl, 8.2 mM Mg acetate, 1.4 mM DTT, 1 mM spermidine chloride, 8 mM putrescine chloride, 330 μg of protein per ml of inverted inner-membrane vesicles, and (unless indicated) 1 mM ATP and 5 mM NADH. Translocaiton was assayed by the inaccessibility of proOmpA and OmpA to added protease (Bacallao et al., 1986). One unit (Table 1) of activity is defined as the amount of trigger factor that causes 10% of the added proOmpA to be inaccessible to protease following translocation.

Preparation of Microsomal Extract and Purification of SRP

A salt extract of dog pancreas microsomes was prepared, and SRP

was purified following published procedures (Walter and Blobel, 1980) with the following minor modifications: Buffer H (50 mM triethanolamine [pH 7.5], 500 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT) was used for the extraction of microsomes. SRP was purified by chromatography on ω-aminopentyl agarose using buffer H as the loading buffer and buffer J (50 mM triethanolamine [pH 7.5], 1 M KCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.01% Nikkol) as the elution buffer. Alternatively, purification was achieved by ultracentrifugation on a 5%–20% linear sucrose gradient in buffer H containing 0.01% Nikkol.

Other Methods

ProOmpA purification and translocation were analyzed by SDS-PAGE as described by Ito et al. (1980). Trigger factor and ribosome protein profiles were analyzed by SDS-PAGE using 15% acrylamide, 0.12% N,N'-methylene bis-acrylamide, and 6 M urea (Ito et al., 1981). Silver staining was performed by the method of Ansorge (1983). Radiolabeled proteins were visualized by fluorography (Chamberlin, 1979). Immunoblotting was according to the method of Towbin et al. (1979).

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