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# Specific recognition of the leader region of precursor proteins is required for the activation of translocation ATPase of *Escherichia coli*

(SecA protein/SecY protein/secretion/membrane assembly)

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**ABSTRACT** **The ATP-hydrolytic activity of SecA protein is stimulated up to 100-fold by the translocation-competent precursor to outer membrane protein A (pro-OmpA) in conjunction with inner-membrane vesicles bearing active SecY [Lill, R., Cunningham, K., Brundage, L., Ito, K., Oliver, D. & Wickner, W. (1989) *EMBO J.* 8, 961–966]. This reaction is saturable, with Michaelis–Menten kinetics for an enzyme with two substrates, ATP and pro-OmpA, and is defined as translocation ATPase. Another precursor protein, pre-PhoE, is also a substrate for this translocation ATPase. Neither OmpA nor its synthetic leader peptide are effective substrates for translocation ATPase, suggesting that both domains of the complete precursor are necessary for the reaction. The leader peptide is a potent inhibitor and apparently competes with pro-OmpA for necessary binding sites on translocation ATPase. After a brief preincubation, the activity of translocation ATPase becomes resistant to inhibition by leader peptide, suggesting that the leader peptide is recognized at an early step in the protein translocation pathway. Our enzymological studies show that translocation ATPase recognizes and functionally binds the leader region of precursor proteins.**

Several features of translocation are conserved between bacterial protein export and protein import into mitochondria, chloroplasts, and endoplasmic reticulum. For each membrane, the information for translocation resides in the primary protein sequence (1). Prior to membrane transit, proteins must avoid folding into their final, stable conformation (2). Instead, they can complex with cytosolic proteins termed "chaperones," which inhibit aggregation and stabilize them for membrane transit (3). Translocation across all membranes, whether bacterial or organellar (endoplasmic reticulum, mitochondria, chloroplast, nucleus, or peroxisome), requires the hydrolysis of ATP (4).

In *Escherichia coli*, genetic strategies have identified several proteins involved in translocation. Genetic and biochemical evidence suggests that the SecA and SecY proteins interact with each other (5–7) and with precursor proteins (8, 9). Recently, SecA has been purified to homogeneity and shown to be necessary for translocation *in vitro* (10, 11). Active SecY is also required *in vitro* (6, 12), but the functional protein has not been isolated. A previous report (7) showed that the isolated SecA hydrolyzes the $\gamma$ phosphate of ATP. The ATPase activity of SecA protein is stimulated dramatically by the presence of both translocation-competent pro-OmpA and membrane vesicles bearing functional SecY. This "translocation ATPase" is composed of SecA and SecY and possibly other protein and lipid components which remain to be identified.

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Several lines of evidence (7) demonstrate that the translocation ATPase is required for translocation. Not only is the SecA protein essential for translocation (10), but membranes that have been inactivated for translocation by photolysis with $N_3ATP$ are fully restored by the addition of pure SecA protein (figure 3 in ref. 7). Derivatization of pure SecA protein by $N_3ATP$ causes a parallel inactivation of the ATPase activity inherent in SecA, of its ability to support translocation ATPase, and of its ability to support pro-OmpA translocation (figure 4 in ref. 7). The translocation ATPase activity is specifically inhibited by antibody to SecY (table 3 in ref. 7) and requires pro-OmpA that is in its translocation-competent form (figure 1 in ref. 7).

Since ATP hydrolysis is crucial for protein translocation across all organelle membranes (13), detailed characterization of the substrates, products, and reaction mechanisms of the bacterial translocation ATPase will greatly extend our understanding of these processes. Measurements of translocation ATPase activity are sensitive, rapid, and quantitative, providing a powerful tool for the analysis of protein translocation. In this report, we show that both the leader and mature regions of pro-OmpA are necessary for activation of translocation ATPase and that the leader region is recognized by membrane-bound factors at an early step in the export process.

## MATERIALS AND METHODS

*E. coli* plasma membrane vesicles were prepared from strain KM9 (*unc*::Tn*10*, *rna-10*, *relA1*, *spoT1*, *metB1*) and treated with 6 M urea as described (7). Few proteins other than SecA are removed by this treatment (data not shown). Though this treatment inactivates the membranes for translocation and for translocation ATPase, each of these activities is fully restored to urea-treated membranes by the addition of SecA protein (7, 11). [$^{35}$S]Pro-OmpA was synthesized *in vitro*, purified, and dissolved in 8 M urea/1 mM dithiothreitol/50 mM Tris·HCl, pH 8.0 (14). Pro-OmpA was prepared as described (15) and further purified on Pharmacia FPLC Mono Q resin (in 5.4 M urea/1 mM 2-mercaptoethanol/50 mM Tris·HCl, pH 7.6), where the protein was eluted at 0.2 M KCl. OmpA was prepared from the sarkosyl (*N*-lauroylsarcosine) supernatant obtained during the isolation of pro-OmpA, and was further purified by FPLC Mono Q chromatography (in 5.4 M urea/1 mM 2-mercaptoethanol/50 mM Tris·HCl, pH 7.6) followed by Mono S chromatography (in 6 M urea/1 mM 2-mercaptoethanol/10 mM potassium acetate, pH 4.9). The OmpA protein was eluted from the Mono S column at 5 mM KCl in this buffer. Purified pre-PhoE (in 8 M urea/50 mM Tris·HCl, pH 8.0/1 mM dithiothreitol) was a generous gift from B. de Kruijff (Utrecht, The Netherlands).

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Table 1. Sequences of synthetic peptides

| Description* | Sequence† | Function‡ |
|---|---|---|
| Pro-OmpA leader | MKKTAIAIAVALAGFATVAQA | +++ |
| Procoat leader | MKKSLVLKASVAVATLVPMLSFA | +++ |
| Procoat Δ(−8,−14) | MKKSLVLKA VPMLSFA | − |
| Procoat, −8R | MKKSLVLKASVAVATRVPMLSFA | + |
| Procoat, −14R | MKKSLVLKARVAVATLVPMLSFA | +++ |
| Consensus leader | KKSALLALMYVCPGKADKE | ? |

*Procoat, precursor of the coat protein of bacteriophage M13.
†Underlined residues differ from the natural polypeptide sequence. These leader sequences are from proteins studied *in vivo* (16–19).
‡Relative ability of the indicated leader peptide to promote procoat and/pro-OmpA translocation *in vivo* (17, 20).

Synthetic peptides used in this study were prepared by Dennis Olshefski (University of California, San Diego), using solid-phase synthesis and dicyclohexylcarbodiimide coupling. They were purified by chromatography on a reverse-phase $C_{18}$ HPLC column with a water/acetonitrile eluant. They were routinely solubilized in 1 mM HCl/2 M urea. These peptides showed no tendency to aggregate at neutral pH at the final concentrations employed in our studies. Their sequences are listed in Table 1.

ATPase activity was monitored by the release of radioactive orthophosphate from [$\gamma$-$^{32}$P]ATP (7). Reaction mixtures (25 $\mu$l) containing urea-treated membrane vesicles, SecA, ATP, and synthetic peptides in buffer C (50 mM Hepes·KOH, pH 7.5/50 mM KCl/1 mM $MgCl_2$) were prepared at 0°C as described in the figure legends. The reactions were initiated by dilution of protein substrates from stock solutions and, after incubation at 40°C for 10 min, were terminated by addition of 0.2 ml of 1 M perchloric acid/1 mM sodium phosphate. For each reaction condition, the amount of ATP hydrolyzed by the components without added protein substrate was subtracted from the total (with substrate), yielding the net translocation ATPase activity. Units of ATPase activity are expressed as picomoles of orthophosphate released per minute.

## RESULTS

Pro-OmpA is competent for translocation during a short interval after it is diluted from solutions of concentrated urea (21). During this interval, pro-OmpA can also stimulate the activity of translocation ATPase (7). The ATPase reaction is saturable by excess pro-OmpA or by another outer membrane precursor, pre-PhoE (22), though the latter substrate is somewhat less effective under these assay conditions (Fig. 1). These data indicate that the translocation ATPase enzyme utilizes a variety of precursor protein substrates and is amenable to detailed enzymological analyses.

To further characterize the translocation ATPase, the initial reaction kinetics were monitored as the concentrations of ATP and pro-OmpA were varied. Lineweaver–Burk plots of the data are linear and can be approximated by a simple formula for a two-substrate reaction (Fig. 2). The straight lines drawn through the data points are calculated from the rapid-equilibrium derivation for a random single-displacement reaction (23), where the apparent Michaelis–Menten constants ($K_m$) for ATP and pro-OmpA were 0.24 mM and 0.19 $\mu$M (6.9 $\mu$g/ml), respectively, and the interaction factor ($\alpha$) was 0.82. The maximum velocity (at infinite substrates) ($V_{max}$) was calculated to be 20 pmol of ATP hydrolyzed per minute per microliter of reaction (containing 50 ng of SecA and 50 ng of membrane vesicles). The data presented in Fig. 2 are consistent with a single-displacement model reaction, though additional studies are necessary to establish this kinetic model.

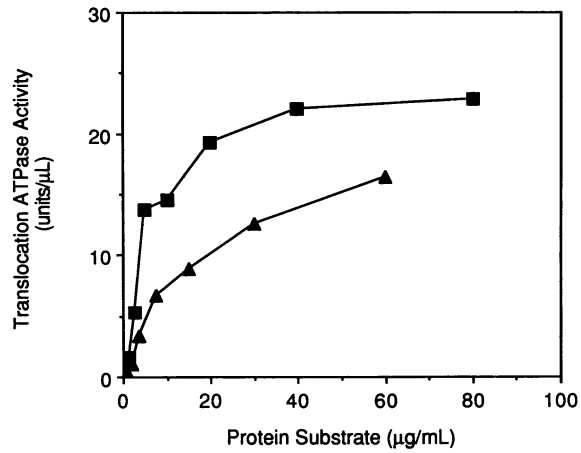


FIG. 1. Translocation ATPase is saturable with pro-OmpA and pre-PhoE protein substrates. Purified pro-OmpA (■) and pre-PhoE (▲) dissolved in buffer Q (5.4 M urea/50 mM Hepes·KOH, pH 7.5/1 mM 2-mercaptoethanol) were diluted 25-fold into reaction mixtures (24 $\mu$l) containing urea-treated membrane vesicles from *E. coli* KM9 (100 $\mu$g/ml), SecA (100 $\mu$g/ml), and [$\gamma$-$^{32}$P]ATP (1 mM, 350,000 cpm per reaction) and immediately incubated at 40°C. After 10 min, the reactions were quenched and the mixtures were processed for assay of translocation ATPase activity (1 unit is defined as 1 pmol of ATP hydrolyzed per minute). A small correction was made for hydrolysis of ATP in the absence of the protein substrates.

The ability of OmpA to participate in the translocation ATPase was examined to determine whether the leader peptide is necessary for this reaction. OmpA was at least 80-fold less efficient as a substrate than pro-OmpA (Fig. 3). The small amount of stimulation associated with OmpA is most likely due to residual pro-OmpA contamination, since the proteins are only partially resolved by FPLC Mono S chromatography (data not shown). Thus the leader peptide is

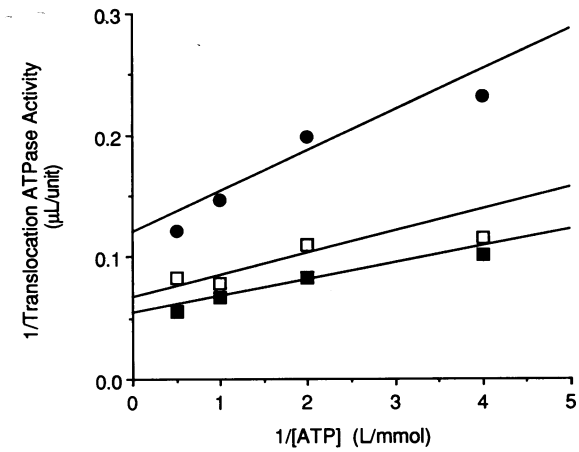


FIG. 2. Lineweaver–Burk plots of initial reaction kinetics. Reaction mixtures (25 $\mu$l) containing membrane vesicles (50 $\mu$g/ml), SecA (50 $\mu$g/ml), and [$\gamma$-$^{32}$P]ATP (0.25–2 mM; 350,000 cpm per reaction) were initiated with various amounts of pro-OmpA diluted from urea (■, 80 $\mu$g/ml; □, 20 $\mu$g/ml; ●, 5 $\mu$g/ml) and assayed for translocation ATPase activity as described in Fig. 1. The data were plotted as the reciprocal of the reaction velocity ($\mu$l·min/pmol of ATP hydrolyzed) versus the reciprocal of ATP concentration ($mM^{-1}$). Straight lines drawn through the data were calculated from the rapid-equilibrium Michaelis–Menten derivation for a random single-displacement reaction (23), $v = (V_{max}[ATP][pOA])/\{(K_{m(ATP)}K_{m(pOA)}/\alpha) + [ATP]K_{m(pOA)} + [pOA]K_{m(ATP)} + [ATP][pOA]\}$, where [ATP] and [pOA] are the initial concentrations of ATP and pro-OmpA, respectively, $K_{m(ATP)}$ and $K_{m(pOA)}$ are the apparent Michaelis–Menten constants for ATP and pro-OmpA (0.24 mM and 0.19 $\mu$M, respectively), the interaction factor $\alpha$ was 0.82, and the $V_{max}$ was 20 units/$\mu$l.

an important characteristic of protein substrates for translocation ATPase.

Since OmpA was not a suitable substrate for translocation ATPase, we tested synthetic leader peptides as potential substrates. Peptides corresponding to the pro-OmpA and M13 procoat leaders were completely ineffective at stimulating hydrolysis of ATP at levels up to 500 $\mu$M (data not shown). Mixtures of leader peptides and OmpA were also inactive in stimulation of translocation ATPase (data not shown). Synthetic leader peptides are known to be potent inhibitors of translocation (24) and of leader peptidase *in vitro* (25). Several synthetic leader peptides also inhibit translocation ATPase (Fig. 4*A*). The leader peptide of pro-OmpA was slightly more potent than that of procoat, and a consensus leader peptide (16) was weaker still. Mutations in the leader peptide of procoat that slow or abolish translocation *in vivo* (17) were analyzed as well (Fig. 4*B*). The potency of these mutant peptides as inhibitors of translocation ATPase correlated well with their ability to function *in vivo* (Fig. 4*B*). Although procoat does not require *secY* or *secA* function for export (26), genetic exchange of its leader with that of pro-OmpA (20) has confirmed that the functions of these leaders are interchangeable.

Double-reciprocal plots of ATPase reaction data obtained when the concentrations of pro-OmpA and its synthetic leader peptide were varied simultaneously were analyzed to determine the mechanism of leader peptide inhibition (Fig. 5*A*). The apparent $V_{max}$ of the reaction was unchanged by leader peptide, whereas the $K_m$ for pro-OmpA was significantly increased. These results suggest a competitive mechanism for leader peptide inhibition. Straight lines were drawn according to a model of competitive inhibition where the $K_i$ for pro-OmpA leader peptide (6 $\mu$M) is $\approx$30 times greater than the apparent $K_m$ for pro-OmpA (0.2 $\mu$M). The leader peptide of procoat was also competitive, with a $K_i$ of 16 $\mu$M (data not shown). If synthetic leader peptides are indeed nonfunctional analogs that compete for a precursor binding site, the $K_i$ is equivalent to the dissociation constant ($K_d$), which at about 10 $\mu$M predicts that the putative receptor is of relatively low specificity.

These data do not distinguish whether the leader peptide competes with pro-OmpA for a site on the translocation ATPase or simply interacts directly with pro-OmpA, inactivating it as a substrate. To distinguish between these possibilities, pro-OmpA concentrations were fixed at 10 $\mu$g/ml and the ATP concentrations were varied at different leader peptide concentrations. The leader peptide inhibition was significantly reversed by increasing the concentration of ATP

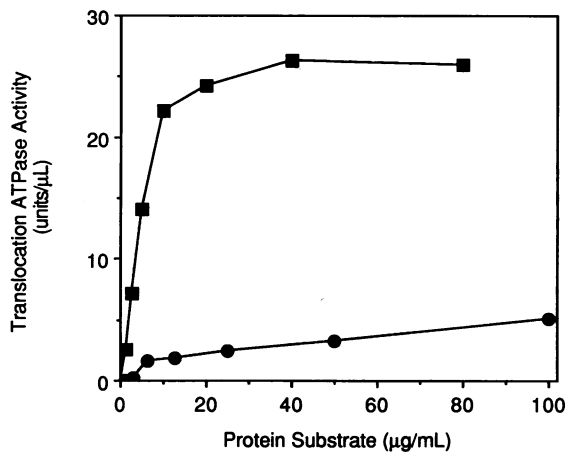


FIG. 3. OmpA is a poor substrate for translocation ATPase. Translocation ATPase was assayed as described in Fig. 1 with the indicated amounts of pro-OmpA (■) or OmpA (●).

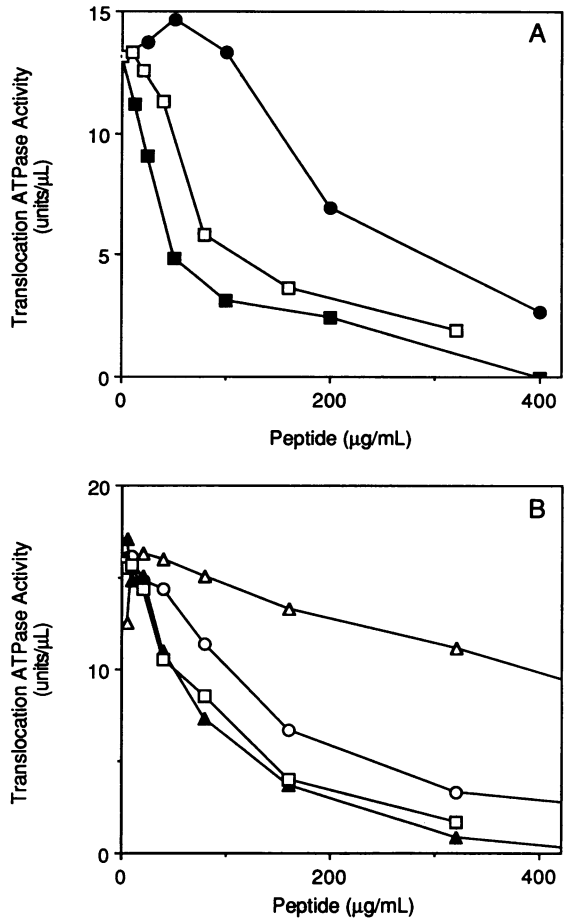


FIG. 4. Synthetic leader peptides inhibit translocation ATPase. (*A* and *B*) Reaction mixtures (25 $\mu$l) were prepared as described in Fig. 1 with the indicated amounts of synthetic peptides (sequences listed in Table 1) corresponding to a consensus leader (●) or to the leader of pro-OmpA (■) procoat (□), procoat Δ(−8,−14) (△), procoat −8R (○), or procoat −14R (▲). Pro-OmpA (20 $\mu$g/ml) was added to initiate the reactions and translocation ATPase activity was determined as described in Fig. 1.

(Fig. 5*B*). The simplest interpretation of these results is that there is interaction between the sites on the translocation ATPase that hydrolyze ATP and those that bind leader peptide. The inability of either leader peptide or mature OmpA to activate the ATPase, and the inhibition of translocation ATPase by synthetic leader peptides, suggests that the leader region of pro-OmpA binds to a saturable site on translocation ATPase as a necessary, but not sufficient, step for its activation. The ATPase activity of pure SecA in the absence of membrane vesicles was unaffected by the synthetic leader peptides (at concentrations <500 $\mu$M; data not shown) or by pro-OmpA (7), suggesting that SecY and other integral membrane components are important for functional recognition.

An activated state of the translocation ATPase can be achieved that is no longer sensitive to leader peptide inhibition. When a complete reaction mixture containing ATP, pro-OmpA, SecA, and membrane vesicles was incubated for 3 min at 40°C, most of the translocation ATPase activity became resistant to the subsequent addition of high levels of leader peptide (Fig. 6). Acquisition of resistance reached a maximum after 3 min of preincubation and required the concurrent presence of heat, ATP, SecA, and membrane vesicles (data not shown). Thus, the synthetic leader peptide of pro-OmpA must act within a narrow kinetic window in

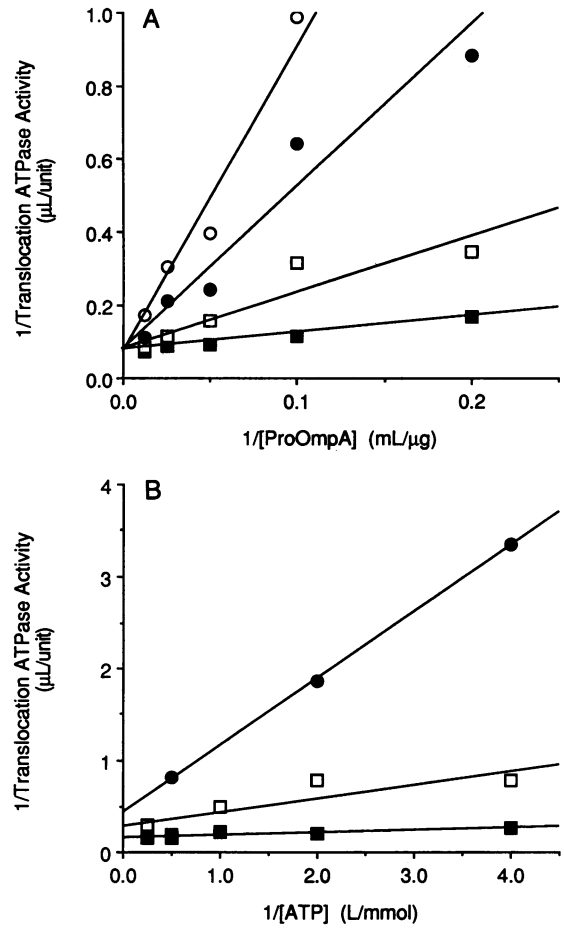


FIG. 5. Double-reciprocal plots of translocation ATPase reaction kinetics in the presence of pro-OmpA leader peptide. (*A*) Reaction mixtures were prepared as described in Fig. 4 containing synthetic pro-OmpA leader peptide (■, 0 μM; □, 24 μM; ●, 48 μM; ○, 96 μM). Translocation ATPase activity was determined for each peptide concentration after the reactions were initiated with pro-OmpA (5–80 μg/ml). The reciprocal of the reaction velocity (μl·min/pmol of ATP hydrolyzed) was plotted versus the reciprocal of the pro-OmpA concentration (ml/μg) at different concentrations of leader peptide. Straight lines drawn through the data intersect at the ordinate, indicating a competitive type of inhibition due to the peptide. The calculated $K_i$ for competitive inhibition by leader peptide was $6 \times 10^{-6}$ M (apparent $K_{m_{(pOA)}} = 0.2$ μM = 7 μg/ml). (*B*) Reaction mixtures were prepared as described in Fig. 2 containing various concentrations of ATP (0.25–4 mM; 400,000 cpm per reaction) and synthetic pro-OmpA leader peptide (■, 0 μM; □, 25 μM; ●, 50 μM) and a fixed concentration of pro-OmpA (20 μg/ml). The reciprocal of translocation ATPase activity (μl·min/pmol of ATP hydrolyzed) was plotted versus the reciprocal of ATP concentration ($mM^{-1}$).

order to achieve inhibition of pro-OmpA-activated translocation ATPase.

## DISCUSSION

In this report, we have defined a translocation ATPase that is amenable to straightforward enzymological analyses. Translocation ATPase utilizes outer membrane precursor proteins such as pro-OmpA or pre-PhoE as substrates (Fig. 1). Effective substrates must be translocation-competent (7), though further studies are needed to establish more precisely the conformational features that are important for translocation ATPase or translocation. The translocation ATPase reaction is linear for at least an hour (unpublished observation) even though pro-OmpA translocation *per se* is complete

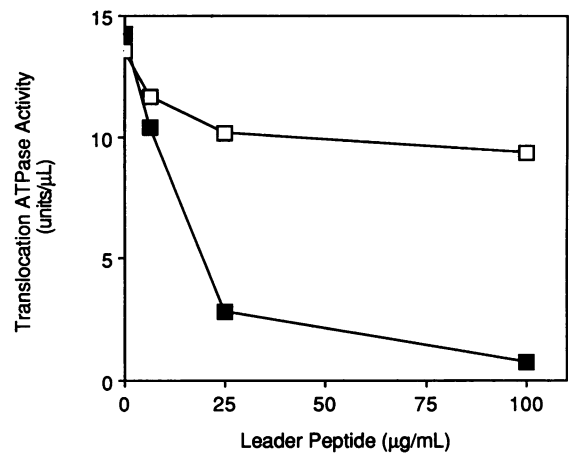


FIG. 6. The activated translocation ATPase becomes resistant to leader peptide inhibition. Reaction mixtures containing membrane vesicles (100 μg/ml), SecA (100 μg/ml), and ATP (1 mM; 300,000 cpm per reaction) were incubated for 3 min at 40°C with (□) or without (■) pro-OmpA (20 μg/ml), then divided into 25-μl aliquots and assayed for translocation ATPase activity in the presence of the indicated concentrations of pro-OmpA leader peptide (with the pro-OmpA added, in the latter case, at the start of the second incubation).

within the first 10 min of incubation (27). This results in an inefficient stoichiometry of ≈5000 ATP molecules hydrolyzed per molecule of pro-OmpA translocated in our standard reactions (7). We suspect that, due to improper folding, partially translocated pro-OmpA can become stuck in the active site of translocation ATPase, allowing ATP hydrolysis to persist and causing the translocation ATPase to become uncoupled from translocation. This reaction, once established, is resistant to inhibition by leader peptide. In this regard, it will be important to seek additional components, such as chaperone proteins (15, 28, 29) and the transmembrane electrochemical potential (27, 30), or other unidentified factors, that more efficiently couple ATP hydrolysis with translocation of pro-OmpA through the membrane. Detailed characterization of the translocation ATPase may ultimately lead to a better understanding of the essential components of protein export and their roles and mode of action in the process. We have begun this characterization by assessing the role of the leader region in activation of translocation ATPase.

There has been great interest in the roles of leader peptides during protein translocation (31). Leader peptides are known to function in several ways. (*i*) They confer on the passenger protein an unfolded conformation (32), which is a prerequisite for translocation (2). (*ii*) Leader peptides can promote the insertion of precursors through the membrane. This concept is clearly supported by studies of the biogenesis of bacteriophage M13 coat protein (33). This protein does not require the function of SecA or SecY proteins for translocation *in vivo* (26) nor does it require the function of any membrane proteins for assembly *in vitro* into liposomes (33, 34). The leader of procoat is typical in structure (35) and is functionally interchangeable with the leader of pro-OmpA (20). The leaders of all precursor proteins may promote penetration into the membrane. (*iii*) Leader peptides are important for recognition of the precursor by proteins that are essential for translocation. These include chaperone proteins, which help to maintain the translocation-competent conformation and membrane-bound receptors that sort and localize the precursor to the correct membrane. Leaders are essential for translocation. For many aspects of translation, regions of the mature portion also play positive roles, though this is little understood. Here we show that neither the leader region of

pro-OmpA nor the mature protein, nor even a mixture, is an effective substrate of the enzyme. Prior studies have shown that the mature region, rather than the leader, determines whether the precursor can bypass the functions of SecA and SecY (20).

It has been demonstrated previously that synthetic leader peptides can inhibit translocation of many precursors (18). We have extended this observation by showing that synthetic leader peptides are competitive inhibitors of translocation ATPase activity in a manner that correlates with their ability to direct translocation of the intact precursor protein *in vivo* (17). These observations predict the existence of a specific receptor on the membrane for the leader region of precursors. Because the synthetic leader peptide also affects the apparent $K_m$ for ATP, we suggest that the leader binding domain interacts with the catalytic ATPase subunit within the translocation ATPase. Based on genetic studies (8, 36), SecY and SecA are good candidates as the leader-binding subunits. However, we cannot rule out other components, including SecD, SecE, PrlC, lipids, or unidentified factors as participants in translocation ATPase. So far, we have established that translocation ATPase requires the functions of SecA, SecY, and the leader and mature domains of intact precursor proteins. Establishing the identity and roles of all the components of translocation ATPase, and of the entire protein export pathway, will require solubilization, fractionation, and reconstitution of the entire system.

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