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Translocation can drive the unfolding of a preprotein domain

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Precursor proteins are believed to have secondary and tertiary structure prior to translocation across the *Escherichia coli* plasma membrane. We now find that preprotein unfolding during translocation can be driven by the translocation event itself. At certain stages, translocation and unfolding can occur without exogenous energy input. To examine this unfolding reaction, we have prepared proOmpA-Dhfr, a fusion protein of the well studied cytosolic enzyme dihydrofolate reductase (Dhfr) connected to the C-terminus of proOmpA, the precursor form of *o*uter *m*embrane *p*rotein A. At an intermediate stage of its *in vitro* translocation, the N-terminal proOmpA domain has crossed the membrane while the folded Dhfr portion, stabilized by its ligands NADPH and methotrexate, has not. When the ligands are removed from this intermediate, translocation occurs by a two-step process. First, 20-30 amino acid residues of the fusion protein translocate concomitant with unfolding of the Dhfr domain. This reaction requires neither ATP, $\Delta\mu_H+$ nor the SecA subunit of translocase. Strikingly, this translocation accelerates the net unfolding of the Dhfr domain. In a second step, SecA and ATP hydrolysis drive the rapid completion of translocation. Thus energy derived from translocation can drive the unfolding of a substantial protein domain.

Key words: preprotein translocase/translocation energetics/protein unfolding

Introduction

Newly made proteins that do not remain in the cytosol must cross a membrane during or soon after their synthesis. How are folded proteins, with their polar surfaces, accommodated during membrane transit? For mammalian endoplasmic reticulum, the inhibition of preprotein synthesis in the cytosol by signal recognition particle and the release of this inhibition by docking protein at the membrane surface may serve to couple translation and membrane translocation (Walter *et al.*, 1984). By this means, preproteins may be prevented from folding in the cytosol and thus are able to cross the membrane as an extended polypeptide chain as they emerge from the ribosome. For the membranes of mitochondria and chloroplasts in eukaryotes and the plasma membrane in prokaryotes such as *Escherichia coli*, protein translocation is largely post-translational and is not coupled to protein synthesis (Verner and Schatz, 1988). Proteins that are substantially folded may have to undergo major conformational changes at the membrane to allow translocation (Wickner, 1979).

The basic outlines of protein export in *E.coli* are now established, allowing a close examination of its mechanism (Bieker *et al.*, 1990; Mizushima and Tokuda, 1990; Schatz and Beckwith, 1990; Wickner *et al.*, 1991). Exported proteins, synthesized with an N-terminal leader sequence (Gierasch, 1989; Randall and Hardy, 1989), are believed to have secondary and tertiary structure in the cytosol (Lecker *et al.*, 1990) and form complexes with chaperones such as SecB (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Weiss *et al.*, 1988; Lecker *et al.*, 1989; Randall *et al.*, 1990). Their transit across the plasma membrane begins after they reach a large 'critical molecular weight' or after synthesis is complete (Randall, 1983). The preprotein-SecB complex binds to the membrane at the SecA subunit of preprotein translocase (Hartl *et al.*, 1990), as a result of the affinity of SecA for the SecB chaperone (Hartl *et al.*, 1990) and for the leader and mature domains of the preprotein (Cunningham *et al.*, 1989; Lill *et al.*, 1990). In addition to SecA, translocase has integral membrane components of acidic phospholipid (de Vrije *et al.*, 1988; Lill *et al.*, 1990; Hendrick and Wickner, 1991) and SecY/E, a multimer of membrane-embedded, hydrophobic polypeptides (Brundage *et al.*, 1990, 1992; Akimura *et al.*, 1991). ATP binding to SecA drives the translocation of an N-terminal loop of the preprotein across the plasma membrane (Schiebel *et al.*, 1991). Both nucleotide hydrolysis and the proton electrochemical gradient ($\Delta\mu_H+$) are needed for maximal rates of translocation. Cleavage by leader peptidase completes the translocation reaction and allows selected proteins to proceed to the outer membrane (Dalbey and Wickner, 1985).

Little is known of the structures of preproteins prior to translocation. The reducing environment of the cytosol, presence of a leader sequence and association with chaperones prevent attainment of the final, 'tightly folded' structures that are characteristic of periplasmic proteins. Leader peptides have been shown to retard the folding rate of the precursor form of maltose and ribose binding protein (Park *et al.*, 1988). Furthermore, in the absence of SecB, maltose binding protein folds into its compact, mature structure, which prevents its translocation (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Weiss *et al.*, 1988). Mutations in the mature domain of maltose binding protein that inhibit this folding enhance the export of this protein (Liu *et al.*, 1988). However, the absence of mature structure by no means indicates that preproteins are fully unfolded, i.e. random coil, prior to translocation. Polypeptide chains assume secondary structure in milliseconds or less (Kim and Baldwin, 1982; Jaenicke, 1991) and, in general, tertiary structure formation requires only seconds (Kim and Baldwin, 1982; Ptitsyn, 1987). Preproteins typically transit the *E.coli* plasma membrane with half-lives of 3-12 s (Randall, 1983). ProOmpA, isolated in a form competent for translocation,

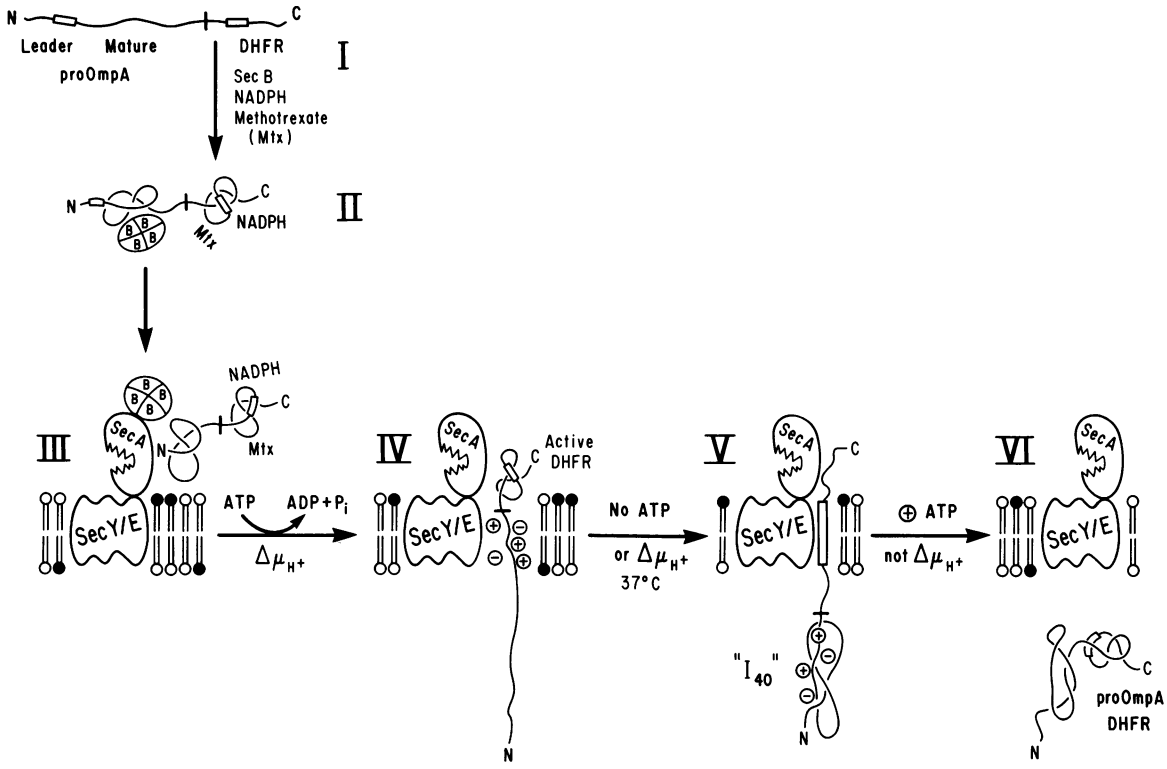

Fig. 1. Working model of translocation-driven unfolding of the Dhfr domain of proOmpA−Dhfr. Step I−II: the Dhfr domain of proOmpA−Dhfr, a fusion protein consisting of the N-terminus of dihydrofolate reductase connected to the C-terminus of proOmpA, folds correctly upon dilution from urea into a solution containing SecB, NADPH and Mtx. This fusion protein is maintained in a translocation competent state, as is proOmpA itself (Lecker *et al.*, 1989, 1990), by interactions with SecB. Step II−III: the proOmpA−Dhfr−SecB complex binds the SecA subunit of translocase by virtue of the affinities of SecA for the leader and mature domains of the precursor (Cunningham *et al.*, 1989; Lill *et al.*, 1990) and for SecB (Hartl *et al.*, 1990). Step III−IV: the energy of nucleotide binding (Schiebel *et al.*, 1991), subsequent hydrolysis and the proton electrochemical gradient ($\Delta\mu_H+$) supports the translocation of the proOmpA domain of the proOmpA−Dhfr up to the folded, ligand-stabilized Dhfr domain (I_{37}). Step IV−V: in the absence of an exogenous energy source, the Dhfr domain of I_{37} is unfolded concomitant with the translocation of ~3 kDa of the fusion protein, resulting in I_{40}. In this intermediate the Dhfr domain is globally unfolded. Step V−VI: I_{40} undergoes ATP-dependent completion of translocation. The proton electrochemical gradient is insufficient to drive this reaction. The spatial relationships between the translocation intermediates, SecY/E and lipid are not yet known.

has been shown to have substantial secondary and tertiary structure (Lecker *et al.*, 1990). Its binding to SecB does not grossly alter this structure (Lecker *et al.*, 1990).

Both *in vivo* and *in vitro* studies have demonstrated that large, stably-folded structures inhibit or block bacterial preprotein translocation. Fusion proteins containing β-galactosidase (Silhavy and Beckwith, 1985; Ferenci and Silhavy, 1987; Freudl *et al.*, 1988) or dihydrofolate reductase (Freudl *et al.*, 1988) block the export pathway when expressed at high levels *in vivo*. The inability to translocate these fusion proteins has been ascribed to the folded structures of β-galactosidase and dihydrofolate reductase. In contrast, Freudl *et al.* (1988) observed that production of small amounts of such fusion proteins allows transit of the plasma membrane *in vivo*. More recently, Reed and Cronan (1991) have shown that a protein that was biotinated, and therefore presumably folded, could subsequently translocate into the periplasmic space. While disulfide bridges can be translocated *in vitro* (Tani *et al.*, 1990), large covalently-folded structures stabilized by disulfide bonds, such as bovine pancreatic trypsin inhibitor, block translocation and thereby form arrested transmembrane intermediates (Schiebel *et al.*, 1991). We now address the question of whether the translocation process actually promotes protein unfolding of a stably folded protein domain, as epitomized by the enzyme dihydrofolate reductase (Dhfr).

The import of Dhfr fusion proteins into mitochondria has been studied in considerable detail (Eilers and Schatz, 1986; Rassow *et al.*, 1989). The genes for proOmpA and Dhfr, a protein of established structure and known ligands (Volz *et al.*, 1982), were fused in-frame and the fusion protein purified and studied. We now report that translocation drives the rapid unfolding of the Dhfr domain, even in the absence of SecA or exogenous energy sources. Our results are presented in the working model (Figure 1).

Results

ProOmpA−Dhfr forms a translocation intermediate in the presence of Dhfr ligands

To investigate how translocation affects the folding of precursor proteins, we constructed a fusion protein with mouse Dhfr connected to the C-terminus of proOmpA (Figure 1, I). This chimera (proOmpA−Dhfr) was overproduced in *E.coli*, purified to homogeneity and labeled with tritium by reductive methylation (Means and Feeney, 1968). We examined whether this chimeric protein would display characteristics of both proOmpA and Dhfr. Dihydrofolate reductase, when properly folded, is resistant to protease digestion (Eilers and Schatz, 1986). The Dhfr portion of the fusion protein became resistant to proteinase K treatment when the protein was diluted from urea into a

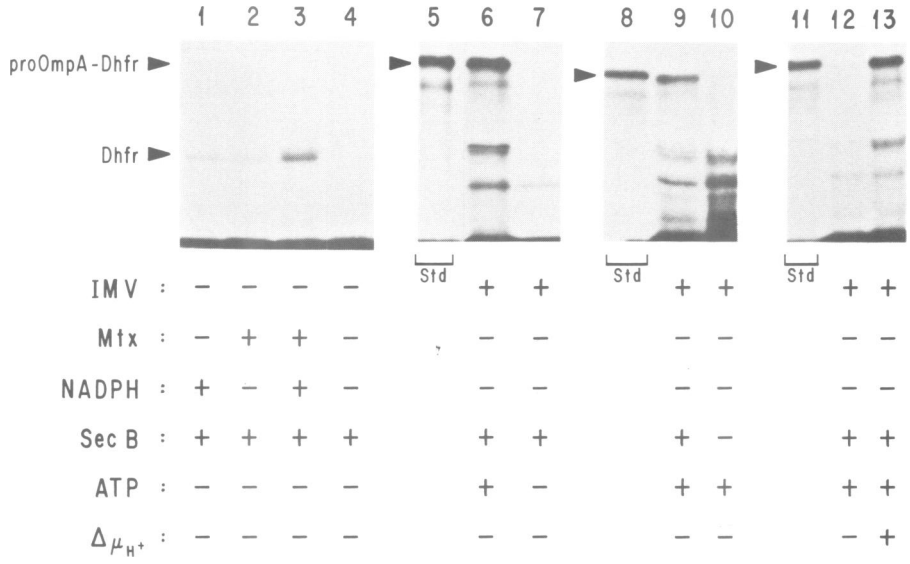

Fig. 2. ProOmpA−Dhfr folding and translocation. [^{3}H]proOmpA−Dhfr (lanes 1−4) was diluted from 6 M urea into buffer A with 120 μg/ml SecB, 10 mM NADPH and 10 μM Mtx where indicated. Samples were incubated for 20 min at 37°C followed by proteinase K treatment on ice. Each sample was then concentrated by trichloroacetic acid precipitation and analyzed by SDS−PAGE and fluorography (see Materials and methods). [^{3}H]proOmpA−Dhfr (lanes 6 and 7, and 12 and 13) was diluted from 6 M urea into a 100 μl reaction mixture containing buffer A, 25 μg membrane vesicles, 60 μg/ml SecB, 200 μg/ml SecA, 0.1 mg/ml BSA, 2 mM DTT, 5 mM creatine phosphate and 10 μg/ml creatine kinase (for ATP regeneration). Where indicated, 2 mM ATP (lane 6), 100 μM ATP (lanes 12,13) or 5 mM succinate (for $\Delta\mu_H$+ generation) were present. [^{3}H]proOmpA−Dhfr (lanes 9 and 10) was diluted from 6 M urea into a 100 μl reaction mixture containing buffer A and 60 μg/ml SecB where indicated. These samples were incubated at 0°C for 30 min. An equal volume of translocation buffer containing 25 μg membrane vesicles, 400 μg/ml SecA, 4 mM DTT, 10 mM creatine phosphate, 20 μg/ml creatine kinase, 0.1 mg/ml BSA and 4 mM ATP was added to both reactions. All translocation reactions were initiated by warming to 37°C and incubated for 10 min. Samples were chilled, treated with proteinase K and analyzed (see above). Standards (lanes 8 and 11) represent 10 and 20% (lane 5) of the added [^{3}H]proOmpA−Dhfr.

solution with SecB, NADPH (a Dhfr cofactor) and methotrexate (a dihydrofolate transition state analog), indicating correct folding (Figure 2, lane 3). In the presence of SecB and either Dhfr ligand alone, less fusion protein formed proteinase K-resistant Dhfr domains (Figure 2, lanes 1 and 2). Furthermore, when proOmpA−Dhfr was diluted from urea into a solution containing SecB, dihydrofolate and NADPH, Dhfr activity (20% the specific activity of rat liver Dhfr) was observed photometrically. As expected, this activity was inhibited by methotrexate. These data demonstrate that the Dhfr domain of the fusion protein folds correctly upon dilution from denaturant (Figure 1, II).

To determine whether proOmpA−Dhfr can translocate into inverted *E.coli* inner membrane vesicles, the labeled preprotein was diluted from 6 M urea into a reaction mixture containing inverted *E.coli* inner membrane vesicles and SecA. Translocation of [^{3}H]proOmpA−Dhfr was routinely assayed at a concentration of 70 nM, sufficient to occupy approximately one-half of the translocase. Incubation was at 37°C in either the presence or absence of 2 mM ATP. Samples were treated with proteinase K at 0°C to digest untranslocated precursor protein, then analyzed by SDS−PAGE and fluorography. ProOmpA−Dhfr translocation required ATP (Figure 2, lanes 6 and 7) and translocation was strongly stimulated by $\Delta\mu_H$+ in the presence of 100 μM ATP (lanes 12 and 13). Under the conditions used, the fusion protein was translocated at approximately the same rate and to the same extent as [^{3}H]proOmpA (data not shown). ProOmpA forms a stoichiometric 1:1 complex with SecB (Lecker *et al.*, 1990), maintaining the precursor in a translocation-competent state by preventing misfolding and aggregation. SecB can also stabilize proOmpA−Dhfr. ProOmpA−Dhfr alone can become incompetent for translocation (Figure 2, lane 10), but is stabilized by SecB interactions (lane 9), presumably through the proOmpA domain.

We investigated whether proOmpA−Dhfr with a tightly folded Dhfr domain, formed by dilution into a mixture of SecB, NADPH and methotrexate, could translocate into *E.coli* inner membrane vesicles. In the presence of either Dhfr ligand alone, a substantial amount of precursor protein was translocated as shown by its proteinase K inaccessibility (data not shown). However, when both ligands were included in both the preincubation and in subsequent translocation reactions, little proOmpA−Dhfr underwent complete translocation. The arrested translocation intermediate was sedimented through a sucrose solution to remove the untranslocated proOmpA−Dhfr. This intermediate yielded three products upon treatment with proteinase K (Figure 3A, lane 6): full-length proOmpA−Dhfr (due to the limited ability to digest the junction between the proOmpA domain and the Dhfr domain), a doublet of polypeptides of the size of proOmpA and OmpA, characteristic of leader peptidase cleavage, and a Dhfr-sized polypeptide. We designate this intermediate I_{37}, because 37 kDa of protease-inaccessible, translocated polypeptide were observed. Each polypeptide species seen in lane 6, though labeled by apparent molecular weight, is derived from I_{37} (Figure 1, IV) by proteinase K treatment. The proOmpA−Dhfr translocation intermediate I_{37} spans the inner membrane with the Dhfr domain on the cytoplasmic side, the proOmpA portion inaccessible to protease, and the leader-mature junction proOmpA accessible to leader peptidase on the lumenal side (Figure 1, IV). Sedimentation of these proteinase K-treated membrane vesicles confirmed that the proOmpA domains had translocated into the lumen while the Dhfr portion remained

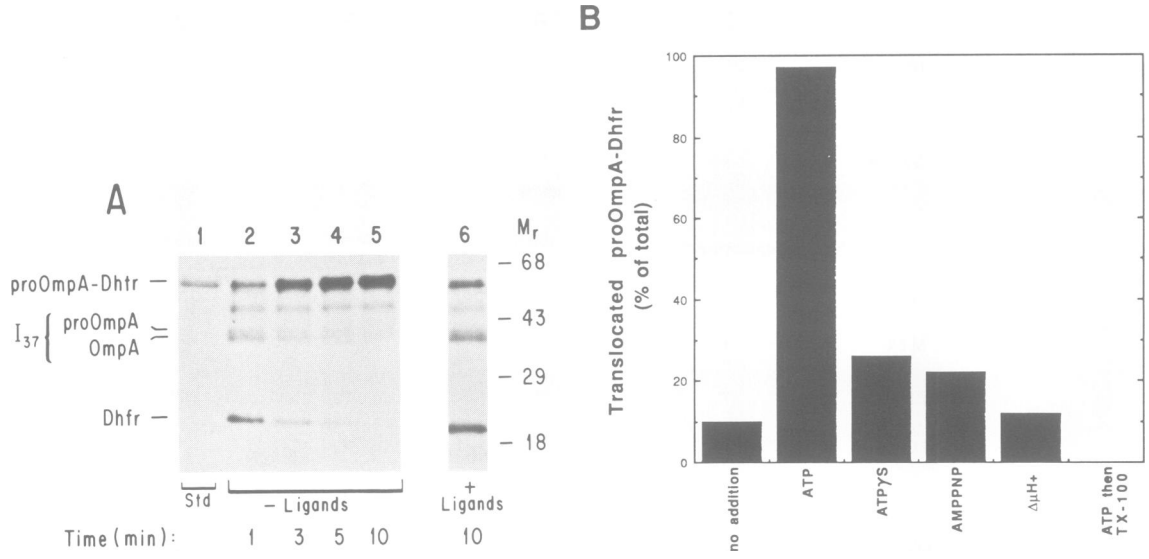

Fig. 3. A translocation intermediate is formed by a folded Dhfr domain. (**A**) Removal of ligands from I_{37} allows ATP-dependent completion of translocation of the Dhfr domain. Inner membrane vesicles bearing I_{37} were generated by incubation of a mixture of 3.5 μg [^{3}H]proOmpA−Dhfr, 30 μg/ml SecB, 1 mM NADPH, 1 μM Mtx and 5.3 mM ATP in 0.75 ml buffer A for 20 min at 37°C. This was added to a 0.25 ml reaction containing 0.8 mg membrane vesicles, 8 mM DTT, 200 μg/ml SecA, 20 mM creatine phosphate, 40 μg/ml creatine kinase, 20 mM succinate, 0.4 mg/ml BSA, 1 mM NADPH, 1 μM Mtx and buffer A. The translocation reaction was allowed to proceed for 15 min at 37°C. Membranes bearing I_{37} were collected by sedimentation (210 000 *g* for 45 min at 4°C) through 3 ml of buffer S (0.2 M sucrose, 50 mM Tris−Cl pH 7.9 and 50 mM KCl) plus 1 mM NADPH and 1 μM Mtx. The sediment was resuspended in 1/20 the initial volume of buffer B (buffer A plus 2 mM DTT and 0.1 mg/ml BSA) and the solution was bath sonicated for 10 s at 0°C. NADPH and Mtx were removed by two subsequent sedimentation steps in an airfuge (for 30 min at 30 psi and 4°C) through buffer S and resuspended in the same volume of buffer B. Bath sonication was for 10 s at 0°C following each resuspension. Based on scintillation counting, the recovered membrane vesicles contained 0.5 μg proOmpA−Dhfr (I_{37}). The translocation reaction (lanes 1−5) was in 250 μl under standard conditions with 10 μM ATP. A reaction of 1/5 the volume had 1 mM NADPH, 1 μM Mtx and 2 mM ATP (lane 6). The reactions were incubated at 37°C for 2 min prior to the addition of vesicles bearing I_{37} (10 μl). Samples (50 μl) were withdrawn at the indicated times, chilled, treated with proteinase K and analyzed as described in Figure 2. Std represents 20% of added membranes bearing I_{37}. (**B**) Requirements for the completion of I_{37} translocation. Membranes bearing I_{37} were isolated and ligands were removed as described as in (A). Translocation reactions were incubated for 10 min at 37°C under standard translocation conditions with 10 μM ATP, 2 mM ATPγS, 2 mM AMPPNP or 5 mM succinate to generate a $\Delta\mu_H+$, added where indicated. Samples were chilled on ice and treated with proteinase K, ATP then TX-100: the sample was incubated for 30 min at 37°C in the presence of 2 mM ATP. It was then chilled, mixed with TX-100 (1%), treated with proteinase K and analyzed as described in Figure 2. Fluorographs were quantified by densitometry and reported relative to standards.

in the supernatant, resistant (though not inaccessible) to the protease (data not shown). The tightly folded Dhfr portion of proOmpA−Dhfr (stabilized by binding both ligands) blocks the subsequent translocation of added [^{35}S]proOmpA (data not shown), indicating that translocase can be saturated with the proOmpA−Dhfr protein. A different translocation intermediate consisting of BTPI cross-linked to the cysteine of proOmpA also blocks subsequent translocation of added proOmpA (Schiebel *et al.*, 1991).

I_{37} can complete translocation

To establish that I_{37} is a true translocation intermediate, we examined the completion of its translocation upon removal of ligands. Membrane vesicles containing I_{37} were sedimented through a sucrose solution containing NADPH and methotrexate (Mtx) and resuspended in the presence of these ligands. This procedure removed ATP and most of the untranslocated proOmpA−Dhfr. To remove the ligands from the Dhfr portion of I_{37}, the membrane vesicles were then sedimented twice through sucrose solutions in the absence of ligands. Control experiments with [^{3}H]Mtx or [α-^{32}P]ATP confirmed that >90% of the methotrexate and 99.9% of the ATP were removed by this treatment. Upon incubation at 37°C with ATP, ~85% of the unliganded I_{37} rapidly completed translocation (Figure 3A, lanes 2−5). In contrast, I_{37} could not translocate if ligands were added prior to the second incubation at 37°C (lane 6). Thus, the transmembrane intermediate formed by a tightly folded Dhfr domain can complete translocation upon ligand removal at a rate, and to an extent that is consistent with the overall translocation of proOmpA−Dhfr.

The identification of I_{37} allowed us to investigate the roles of ATP binding, ATP hydrolysis and $\Delta\mu_H+$ in the completion of translocation of this intermediate. Membrane vesicles with I_{37} were warmed to 37°C in the presence or absence of ATP. The production of full-length protected proOmpA−Dhfr was dependent on ATP (Figure 3B). Translocated proOmpA−Dhfr became fully accessible to protease upon membrane disruption. We have previously noted that the binding of non-hydrolyzable ATP analogs to SecA causes a limited translocation (~20 amino acid residues) of proOmpA translocation intermediates (Schiebel *et al.*, 1991). Neither a transmembrane electrochemical gradient generated with succinate nor the ATP analogs ATP-γ-S and AMPPNP were sufficient for the complete chase of I_{37} (Figure 3B), yet each resulted in further limited translocation of this intermediate (see below). These data establish the necessity of ATP hydrolysis for the overall completion of translocation of I_{37} and demonstrate that $\Delta\mu_H+$ has little effect on this reaction. Schiebel *et al.* (1991) showed previously that $\Delta\mu_H+$ alone could drive the completion of translocation of certain proOmpA translocation intermediates. Our studies support this finding, as $\Delta\mu_H+$ stimulates the overall translocation of proOmpA−Dhfr at low ATP concentrations (Figure 2, lanes 12 and 13). However, the ability of a $\Delta\mu_H+$ to complete the

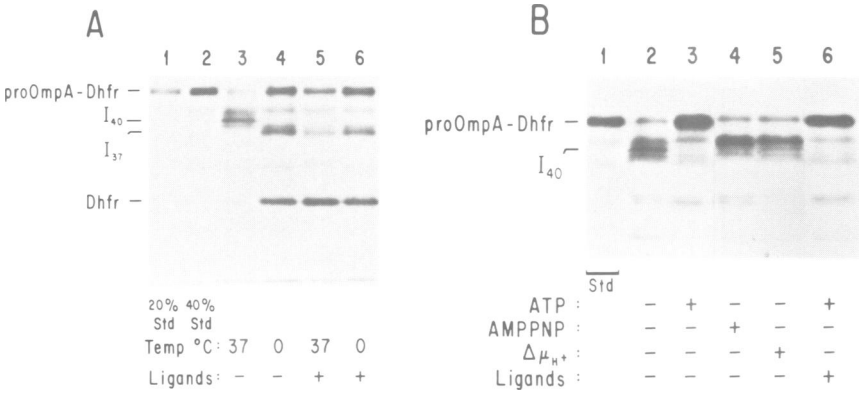

Fig. 4. Further translocation of I_{37} to I_{40}. **(A)** I_{40} formation occurs concomitantly with unfolding of the Dhfr domain. Membranes bearing I_{37} free of ligands were prepared as described in Figure 3A. Samples were incubated under standard translocation conditions for 30 min at the specified temperature. Where indicated, 1 mM NADPH and 1 μM Mtx (+ ligands) were added to the reactions. Samples were then chilled, treated with proteinase K and analyzed as described in Figure 2. Std represents the indicated amounts of added membranes bearing I_{37}. **(B)** I_{40} is an authentic translocation intermediate. Membranes, bearing I_{37} from which the ligands had been removed, were isolated as described in Figure 3A. I_{40} was generated by incubation of these vesicles (22 μl) under standard translocation conditions (reaction volume 450 μl) in the absence of nucleotides and succinate for 10 min at 37°C, followed by chilling on ice. Aliquots (50 μl) of membranes bearing I_{40} were analyzed for further translocation by incubation (100 μl for 3 min at 37°C) under standard translocation conditions with 2 mM ATP, 2 mM AMPPNP, 5 mM succinate or 1 mM NADPH and 1 μM Mtx (+ ligands) where indicated. Samples were then chilled, treated with proteinase K and analyzed as described in Figure 2. Std represents 40% of added membranes bearing I_{40}.

translocation of an intermediate is dependent on the precursor protein domain remaining to translocate, just as different precursor proteins themselves exhibit different extents of $\Delta\mu_H+$ stimulation (Daniels *et al.*, 1981; Yamada *et al.*, 1989). The membrane potential is apparently unable to promote the translocation of the C-terminal domain of Dhfr.

Partial translocation of I_{37} in the absence of an energy source or SecA

The junction between the folded, exposed Dhfr domain and the translocated proOmpA domain was difficult to fully digest with proteinases. Analysis of I_{37} by proteinase K treatment therefore reveals three prominent species (Figure 4A, lane 4); undigested full-length transmembrane proOmpA−Dhfr, protected proOmpA and released protease-resistant Dhfr. When membrane vesicles with I_{37} were warmed to 37°C without ATP or $\Delta\mu_H+$, we observed a striking disappearance of the protease-inaccessible, proOmpA-sized polypeptide concomitant with the appearance of a new protease-inaccessible species at ~40 kDa (termed I_{40}) and the disappearance of protease-resistant Dhfr (Figure 4A, lane 3). I_{40} appears as a cluster of species in a narrow molecular weight range.

This limited translocation of I_{37} to form I_{40} is accompanied by unfolding the Dhfr domain (Figure 1, IV−V). The full-length proOmpA−Dhfr observed upon proteinase K treatment of I_{37} (lane 4) also disappeared upon I_{40} formation (lane 3), consistent with this full-length proOmpA−Dhfr representing I_{37} in which the junction between the Dhfr domain and the proOmpA domain is resistant to proteinase K. I_{40} (Figure 4B, lane 2) is an authentic translocation intermediate, as it is quantitatively and rapidly converted to full-length, translocated fusion protein upon addition of ATP (lane 3). In addition, the rapid and quantitative completion of translocation of I_{40} was observed with as little as 10 μM ATP present. AMPPNP caused only limited further translocation of I_{40} (Figure 4B, lane 4). Similar results have been observed upon AMPPNP addition to intermediates of proOmpA translocation (Schiebel *et al.*, 1991). Imposition of a $\Delta\mu_H+$ also resulted in a limited translocation of I_{40} (lane 5), although this energy source could not drive translocation to completion. Although the readdition of Dhfr ligands to I_{37} prevented I_{40} formation (Figure 4A, lane 5), Dhfr ligands did not prevent the completion of translocation of I_{40} to full-length protected proOmpA−Dhfr (Figure 4B, lane 6), confirming that the Dhfr domain was largely unfolded (Figure 1, step V). Thus the unfolding of the Dhfr domain accompanies and is essential for the conversion of I_{37} to I_{40}.

Because the I_{37} to I_{40} conversion is the first reported 'forward' translocation reaction to not require exogenous energy input, further experiments were performed to test this feature. To rule out the possibility that the formation of I_{40} was due to residual ATP, membranes bearing I_{37} were incubated with apyrase, then twice sedimented to remove apyrase and ligands. After this procedure, < 16 pmol of ATP remained, though 20−30 pmoles of I_{37} were recovered. This treatment had no effect on the formation of I_{40}; 95% of the protease-resistant Dhfr domain of I_{37} (Figure 5A, lane 2) was lost upon formation of I_{40} (lane 3). The apyrase-treated I_{37} was also incubated at 0°C with the proton electrochemical gradient uncouplers valinomycin and nigericin. This treatment dissipated both ΔpH and $\Delta\Psi$, as determined by oxonol VI and 9-amino-acridine fluorescence (Klionsky *et al.*, 1984; Apell and Bersch, 1987). I_{40} formation was still observed in the absence of a potential (lane 6); only 5% of the protease-resistant Dhfr remained after incubation at 37°C. Experiments in which CCCP was used to collapse any remaining $\Delta\mu_H+$ gave similar results. Not only is the 40 kDa species observable upon proteinase K treatment of I_{40}, but the loss of protease-resistant Dhfr is also readily apparent (lanes 3 and 6). Neither pretreatment of I_{37} with apyrase nor with a mixture of apyrase and uncouplers prevented the subsequent completion of translocation upon readdition of ATP; 80−100% of the I_{37} was converted to fully-translocated proOmpA−Dhfr (lanes 4 and 7). These results indicate that the treatments have no significant effects on completion of translocation.

Since ATP was not required for the conversion of I_{37} to I_{40}, it seemed possible that SecA is also dispensable.

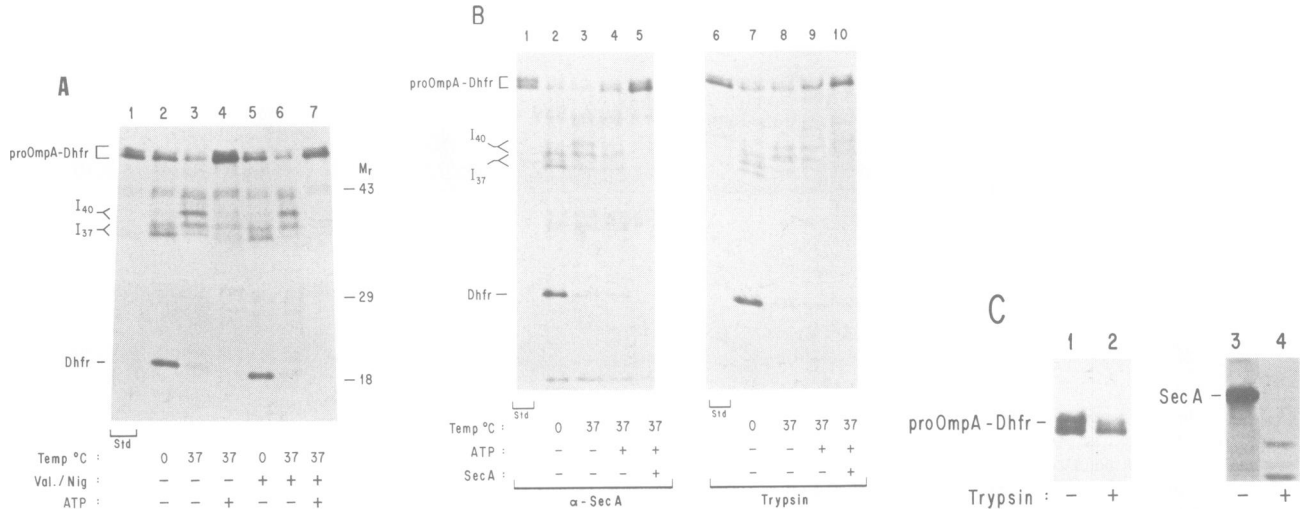

Fig. 5. ATP, $\Delta\mu_H+$ and SecA are not required for I_{40} Formation. (**A**) Membranes bearing I_{37} with ligands were isolated as described in Figure 3A. After centrifugation, the vesicles bearing I_{37} were resuspended in buffer B containing 1 mM NADPH and 1 μM Mtx. The membrane suspension (90 μl) was incubated with 5 units of potato apyrase at 37°C for 5 min followed by chilling on ice. Ligands and apyrase were removed by twice sedimenting (190 000 *g* for 45 min at 4°C) through 0.5 ml of buffer S. Sedimented membranes were resuspended in the same volume buffer B with bath sonication for 10 s at 0°C following each centrifugation. Control I_{37} samples that were not treated with apyrase (data not shown), isolated under identical conditions, also showed full conversion to I_{40} (as in Figure 4A). Membranes bearing I_{37} (5 μl, 100 000 c.p.m., 25 ng intermediate) were used in each reaction. Where indicated, 0.5 μM valinomycin and 0.1 μM nigericin were added to the translocation reactions, which were then preincubated on ice for 10 min. The reactions in lanes 4 and 7 had 2 mM ATP. Other translocation reactions (10 min at 37°C) were without ATP. Samples were then chilled, treated with proteinase K and analyzed as described in Figure 2. Std represents 50% of added membranes bearing I_{37}. (**B**) SecA is not required for I_{40} formation. Membranes bearing I_{37} with ligands were isolated as described in Figure 3A except that two reactions, each of double the volume, were used. Following sedimentation, vesicles bearing I_{37} (lanes 1–5) were resuspended in buffer B containing 1 mM NADPH and 1 μM Mtx and bath sonicated for 10 s at 0°C. Aliquots (80 μl, 2.3×10^6 c.p.m., 470 ng I_{37}) were mixed with 0.42 ml buffer B containing 1.7 mg/ml α-SecA IgGs. This reaction was incubated at 4°C with continuous mixing for 2 h. Ligands and α-SecA IgGs were subsequently removed by two sedimentation steps (210 000 *g* for 45 min at 4°C) through 3.5 ml buffer S. Membranes bearing I_{37} without functional SecA were resuspended in 100 μl buffer B and bath sonicated for 10 s at 0°C. Translocation reactions (15 μl I_{37}, 120 000 c.p.m., 24 ng intermediate) had 2 mM ATP and 200 μg/ml SecA where indicated. Each sample was incubated for 10 min at 37°C. Samples were then chilled, treated with proteinase K and analyzed by SDS–PAGE and fluorography. Std (lane 1) represents 40% of added membranes bearing I_{37}. Membranes bearing I_{37} (lanes 6–10) with ligands removed were isolated as described in Figure 3A except that removal of ligands was carried out by two successive sedimentations at 190 000 *g*, 45 min, 4°C through 0.5 ml buffer S. These membranes (40 μl, 1×10^6 c.p.m., 220 ng intermediate) were then incubated (300 μl buffer B, 30 min at 0°C) with 100 μg/ml TPCK-trypsin. The reaction was quenched by the addition of TLCK to 30 mM and incubated for 10 min on ice. Translocation reactions (38 μl of membranes bearing I_{37}, 130 000 c.p.m., 10 min at 37°C) had 2 mM ATP and 200 μg/ml SecA where indicated. Each sample was incubated for 10 min at 37°C. Samples were then chilled, treated with proteinase K and analyzed. Std (lane 6) represents a reaction that was not proteinase K treated (100%). I_{40} formation (lanes 3 and 8) resulted in 93–99% loss of the protease resistant Dhfr (determined by densitometry). (**C**) Trypsin treatment of I_{37} leaves [^{3}H]proOmpA–Dhfr intact but degrades SecA. Trypsin-treated I_{37} was from the reaction described in Figure 5B. Both trypsin-treated I_{37} and untreated I_{37} (19 μl) were chilled, precipitated with trichloroacetic acid and analyzed by SDS–PAGE and fluorography (lanes 1 and 2). Two samples (100% of the reaction size Figure 5B) were also analyzed for SecA content by immunoblot, using α-SecA sera, with [^{125}I]protein A and autoradiography.

Membrane vesicles bearing the liganded I_{37} were incubated with anti-SecA IgG to inactivate SecA (Schiebel *et al.*, 1991). These membranes were sedimented to remove excess antibody and the sedimentation was then repeated twice to remove the Dhfr ligands. This treatment largely inactivated SecA, as ATP addition yielded far less completion of translocation (16%; Figure 5B, lane 4) than was observed when both ATP and SecA were added (65%; lane 5). Even though SecA had been inactivated, 93% of the I_{37} (lane 2) was converted to I_{40} (lane 3).

An independent experiment confirmed the ability of I_{37} to form I_{40} in the absence of SecA. Quantitative immunoblot analysis confirmed that the three centrifugation steps used in the isolation of I_{37}-bearing membranes left only the high affinity-bound SecA with the inner membrane vesicles, as observed previously (Hartl *et al.*, 1990). We then exploited the fact that the cytosolic Dhfr portion of I_{37} is resistant to proteases; ~50% of the intermediate remained intact with trypsin treatment (Figure 5C, lanes 1 and 2), whereas the protease had removed 92–100% of the SecA (lanes 3 and 4). Upon warming the trypsin-treated I_{37} (lane 7) to 37°C, it was converted to I_{40} (lane 8). In agreement with the immunoblot analysis, the addition of ATP to these proteolyzed membrane vesicles only supported 33% completion of translocation (Figure 5B, lane 9), whereas the addition of both SecA and ATP resulted in the quantitative completion of translocation (lane 10). Thus, although SecA is normally present during I_{37} to I_{40} conversion (Figure 1, IV–V), neither the addition of an energy source (ATP or $\Delta\mu_H+$) nor SecA are required for the formation of I_{40}. Rather, the relative energies inherent in the initial and final states, I_{37} and I_{40}, are driving this partial translocation event.

To understand better the structures of I_{37} and I_{40}, we compared their protease sensitivities. I_{37} is only partially sensitive to trypsin (Figure 5C, lanes 1 and 2) and proteinase K (Figure 6). Complete digestion at the junction between the proOmpA and Dhfr domains is observed at proteinase K concentrations of >50 μg/ml (Figure 6, I_{37} lanes), resulting in a 37 kDa protected species and a Dhfr-sized resistant species. In contrast, I_{40} is exquisitely sensitive to proteinase treatment, with cleavage even at 2 μg/ml

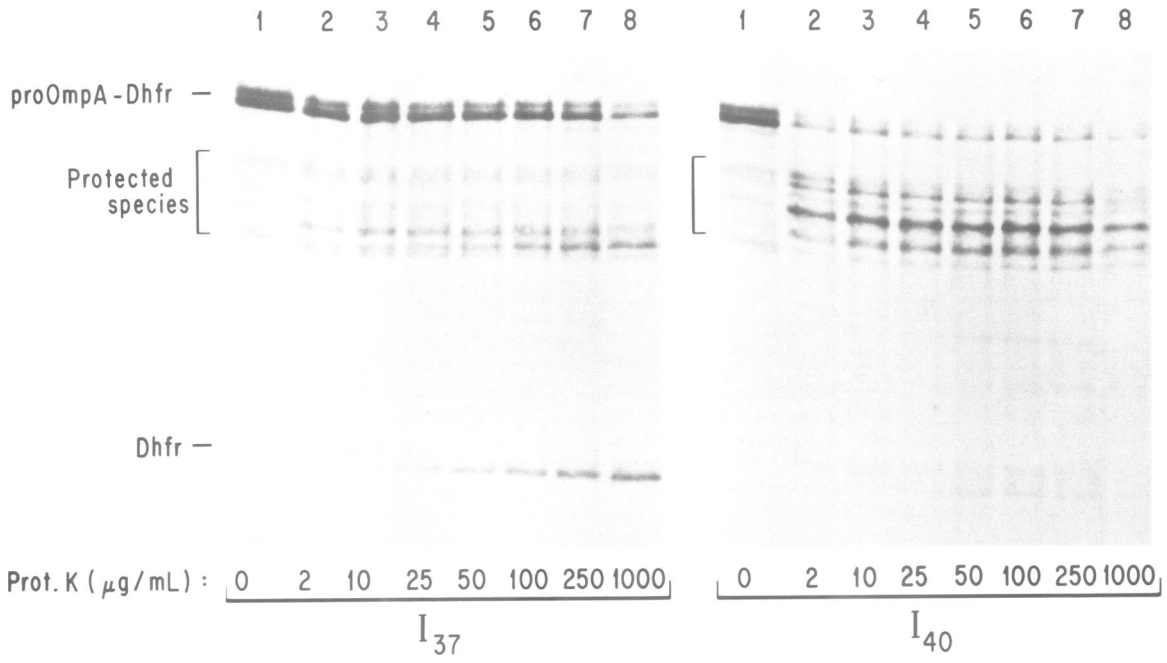

Fig. 6. Protease sensitivities of I_{37} versus I_{40}. Membranes bearing I_{37} with ligands removed were isolated as described in Figure 3A except that removal of ligands was carried out by sedimentation at 190 000 *g* for 45 min at 4°C through two successive 0.5 ml buffer S solutions. Aliquots (20 μl, 1.4×10^6 c.p.m., 280 ng intermediate) were mixed with 680 μl buffer B and 200 μg/ml SecA. Reactions were kept on ice (I_{37}) or incubated for 10 min at 37°C (I_{40}) followed by chilling on ice. Aliquots (50 μl) were incubated with proteinase K (20 min, 100 μl). Proteolysis was terminated by addition of 10 μl of 100 mM PMSF in ethanol with a subsequent 10 min incubation on ice. Reactions were precipitated with an equal volume of chilled 50% trichloroacetic acid and analyzed by SDS−PAGE and fluorography as described in Materials and methods.

proteinase K (Figure 6, I_{40} lanes) yielding the characteristic 40 kDa protected species and no protease-resistant Dhfr domain. Dhfr ligands did not prevent the completion of translocation of I_{40} in the presence of ATP (Figure 4B, lane 6) and, in the absence of ATP, no proteinase K resistant Dhfr domain is observed when Dhfr ligands are added (data not shown). Readdition of ligands to I_{37}, I_{40} and fully-translocated proOmpA−Dhfr in the presence of detergent resulted in the formation of tightly folded Dhfr domains as determined by proteinase K resistance (data not shown). These results show that the active site of Dhfr is destroyed as the I_{40} intermediate is formed (Figure 1, step IV−V). As an independent assessment of the conformations of the Dhfr domain, the Dhfr activity of I_{37} was assayed. I_{37} has essentially the same Dhfr specific activity as the fusion protein in solution, 8.3 ± 2.0 U (μmol protein)$^{-1}$ and 13.8 ± 1.5 U (μmol protein)$^{-1}$, respectively. This enzymatic activity is inhibited by methotrexate and becomes undetectable upon either conversion to I_{40} or chase to fully-translocated proOmpA−Dhfr. Taken together, these results demonstrate that the Dhfr domain of I_{40} is unfolded and lacks an intact active site.

Translocation drives unfolding of the Dhfr domain

While translocation occurs concomitant with unfolding the Dhfr domain, these results do not differentiate between, on the one hand, translocation driving or catalyzing the unfolding of Dhfr or the possibility that the rate limiting step in the translocation reaction might be the spontaneous unfolding of Dhfr. We compared the time-course of loss of the folded Dhfr domain from I_{37} with the appearance of fully translocated proOmpA−Dhfr (Figure 7A). When ~50% of the Dhfr domain had disappeared, 50% of the fully translocated proOmpA−Dhfr was observed. Thus the loss of the proteinase K resistance of the Dhfr domain occurs during its translocation. However, to determine whether translocation itself was driving the unfolding of Dhfr, it was important to compare the time-course of loss of protease resistance of the Dhfr domain from I_{37} during translocation and in the absence of translocation. If translocation were driving the unfolding of the Dhfr domain, then one would expect the loss of protease-resistant Dhfr during translocation to be substantially faster than in the absence of translocation. To block translocation, membrane vesicles with I_{37} were treated with thermolysin at 0°C and the protease was then quenched with EDTA. Although this protease treatment left the fusion protein intact, the ATP-dependent chase of the intermediate, and the formation of I_{40}, were inhibited due to proteolysis (data not shown). Aliquots were removed from a 37°C incubation of membranes bearing I_{37} or thermolysin-treated membranes with I_{37} and quenched by chilling to 0°C in the presence of proteinase K. These samples were analyzed by SDS−PAGE and fluorography to determine the amount of folded Dhfr remaining as a function of time. The Dhfr domain unfolds significantly faster when translocation occurs than in the translocation-inactivated intermediate (Figure 7B, compare thermolysin treated with untreated). Unfolding of the Dhfr domain is thus dependent on translocation itself. As another independent measurement of the rate of Dhfr unfolding in the absence of translocation, we examined soluble Dhfr itself (not as a fusion protein) for both the loss of proteinase K resistance of [^{125}I]Dhfr and Dhfr enzymatic activity (Figure 7C) in the presence of membrane vesicles bearing unlabeled I_{37} and SecA. Under these conditions, the amount of the Dhfr enzymatic activity and protease-resistant Dhfr decreased ~20% during the incubation, whereas in the presence of translocation (Figure 7B) the amount of protease-resistant

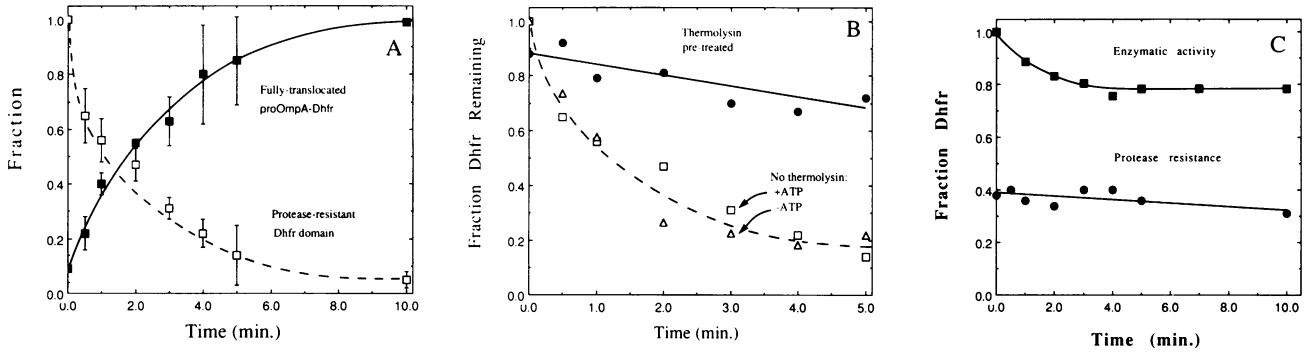

Fig. 7. Translocation drives the unfolding of the Dhfr domain of proOmpA−Dhfr. (**A**) The completion of translocation of I_{37} occurs concomitant with the loss of protease resistance of the Dhfr domain. Membranes bearing I_{37} with ligands removed were isolated as described in Figure 3A except that the removal of ligands was carried out by sedimentation at 190 000 *g* for 45 min at 4°C through two successive 0.5 ml buffer S solutions. Membranes bearing I_{37} (22 μl, 1.1−1.7×10^6 c.p.m., 310−340 ng intermediate) were suspended at 0°C in 1280 μl of buffer B, 4 mM ATP, 10 mM creatine phosphate, 20 μg/ml creatine kinase and 400 μg/ml SecA. The zero time point represents a 10 μl aliquot removed from the reaction on ice and added to 40 μl of 1.25 mg/ml proteinase K at 0°C. The reaction was initiated by mixing with an equal volume of H_2O prewarmed to 37°C. Aliquots (20 μl) were removed and added to 30 μl of 1.7 mg/ml proteinase K at 0°C. Proteinase digestions were for 20 min on ice and were quenched with an equal volume of chilled 50% TCA. Samples were analyzed by SDS−PAGE and fluorography. The intensities of Dhfr band (□) and the proOmpA−Dhfr band (■) were quantified by densitometry (Bio-Rad video densitometer model 620). The maximum intensity from each time-course was set to 1.0. All intensities were corrected for differences in loading by normalization to total lane intensities. Values represent the average of three separate experiments. (**B**) The loss of protease-resistant Dhfr domain of I_{37} is accelerated by translocation. Membranes bearing I_{37} without ligands were isolated as described above. Incubations were with (□) or without (△) ATP. A third reaction (●) was pretreated with 1 mg/ml thermolysin (60 U/mg) and 2 mM $CaCl_2$ at 0°C for 30 min. The thermolysin was quenched with 10 mM EDTA and the reaction was incubated for 10 min on ice. This treatment left ~50% of the fusion protein intact and did not cleave at the proOmpA−Dhfr junction. All three reactions were initiated as described above and aliquots were removed at indicated times and analyzed as above. (**C**) Soluble Dhfr is stable in the presence of I_{37}. Membranes bearing I_{37} were prepared as described above except that the [^{3}H]proOmpA−Dhfr used to form I_{37} was diluted 50-fold with unlabeled proOmpA−Dhfr. In addition to the I_{37} present, either [^{125}I]Dhfr (15 μl, 1.1×10^6 c.p.m., 270 ng) (●) or Dhfr (24 μl, 29 μg) (■) was added. The reaction was initiated by warming to 37°C and samples were removed at indicated times. The [^{125}I]Dhfr samples were treated with proteinase K as described above. The intensities of the Dhfr bands were quantified by densitometry and values are reported relative to standards. For determination of enzymatic activity, the removed aliquots were immediately diluted 25-fold into assay buffer and absorbance at 340 nm was monitored at 25°C for 90 s. Values represent the average of two experiments.

Dhfr domain (of the proOmpA−Dhfr fusion protein) decreased by 80%. We conclude that the translocation of I_{37} actually drives the unfolding of the Dhfr domain.

Discussion

Preprotein translocation can drive the net unfolding of precursor proteins. We have investigated this unfolding reaction using proOmpA−Dhfr, a fusion protein consisting of Dhfr connected to the C-terminus of proOmpA. The specific steps of the translocation and unfolding of proOmpA−Dhfr are outlined in Figure 1. A correctly folded Dhfr domain (II) is formed by dilution of the precursor protein out of urea into a solution containing SecB, NADPH, and Mtx (I−II). When added to inner membrane vesicles, the proOmpA domain binds to the SecA domain of translocase (III) and crosses the membrane (IV). The stabilized, folded Dhfr structure blocks further translocation, forming a stable transmembrane intermediate (IV). Upon removal of ligands from this intermediate, the translocation drives the net unfolding of the Dhfr domain by a two step process (IV−VI). Initially 20−30 amino acid residues of the fusion protein translocate, driving the unfolding of the Dhfr domain (IV−V). This limited translocation reaction requires neither an exogenous energy source nor SecA. The ability of translocation, in the absence of energy input, to drive the net unfolding of proOmpA−Dhfr shows that the relative energies of the initial and final states of a partial translocation reaction can contribute significantly to the driving force, sufficient to unfold a protein domain during post-translational translocation. The conversion of I_{37} to I_{40} is an example of a translocation reaction driven by the potential energy of the system and suggests that SecA and ATP are not essential for this 'unfoldase' activity. In a subsequent step (V−VI), SecA and ATP hydrolysis drive the rapid completion of translocation. A transmembrane electrochemical gradient is not sufficient for the completion of translocation.

Translocase-dependent precursor protein unfolding in *E.coli* has many mechanistic parallels to the unfolding of precursor proteins during mitochondrial import. In mitochondrial protein import, Mtx blocks translocation by stabilizing a tightly folded Dhfr domain of a fusion protein, resulting in the accumulation of a transmembrane intermediate (Eilers and Schatz, 1986; Rassow *et al.*, 1989). Removal of Mtx from the Dhfr on the cytoplasmic face of the mitochondria allows completion of translocation (Rassow *et al.*, 1989). Pfanner *et al.* (1990) have shown that drastic reductions of the ATP level did not affect the unfolding of the Dhfr domain or the completion of translocation. These data are consistent with our results that energy-independent unfolding can occur during translocation and is perhaps a common theme in membrane translocation. In contrast to preprotein translocation in *E.coli*, both biochemical (Schleyer and Neupert, 1985; Schwaiger *et al.*, 1987; Ostermann *et al.*, 1989, 1990; Scherer *et al.*, 1990) and genetic evidence (Cheng *et al.*, 1989; Kang *et al.*, 1990) strongly support the role of matrix ATP-dependent chaperones (mHsp60 and mHsp70) in mitochondrial protein import. These matrix chaperones have been shown to interact directly with transmembrane protein intermediates (Schleyer and Neupert, 1985; Schwaiger *et al.*, 1987; Ostermann *et al.*, 1989, 1990; Scherer *et al.*, 1990). It has been suggested that the energy for translocation into mitochondria may be

derived from interactions with these mHsps and their ATP hydrolysis (Neupert *et al.*, 1990). Kang *et al.* (1990) have elegantly shown that import of a Dhfr fusion protein was defective in yeast mitochondria with mutant Hsp70 (Ssc1p). This translocation defect could be overcome if urea denatured fusion protein was used. These results suggest that in mitochondria, the differences in free energies of folding can also play an important role in translocation. Presently, no bacterial Hsp60 or 70 analog has been found in the periplasmic space. Furthermore, due to the low level of ATP in the periplasmic space, a role for ATP hydrolysis seems unlikely. Genetic evidence suggests that SecD and SecF may participate in the late stages of bacterial translocation (Gardel *et al.*, 1990). It is attractive to speculate that the interactions of translocating preproteins with SecD and SecF are in some sense analogous to the functioning of mHsp60 and 70.

The intrinsic energy of translocation is most likely composed of the energies of unfolding the precursor protein on the cytoplasmic membrane surface relative to refolding of the protein in the lumen, as suggested by von Heijne and Blomberg (1979), and the transfer of residues into and out of the membrane. Upon examination of the junction region of proOmpA−Dhfr, it became apparent that the last C-terminal 20 amino acid residues of proOmpA are quite hydrophilic, containing eight charged residues. Furthermore the first 20 residues of Dhfr are hydrophobic, displaying the high average hydrophobicity of a transmembrane helix (Eisenberg *et al.*, 1989). This stretch of polar residues followed by apolar residues suggests that one possible explanation for the independence of the conversion of I_{37} to I_{40} from exogenous energy is the transfer of polar residues out of the membrane and non-polar residues into the membrane environment of the transiting polypeptide chain. This possibility cannot be tested directly by deleting the apolar domain of Dhfr, since this region is essential for forming the folded, active structure of Dhfr, and thereby creating the arrested intermediate I_{37}. An alternative explanation for this conversion could be the refolding of the lumenal proOmpA portion of I_{37} relative to the unfolding of the Dhfr domain on the cytoplasmic membrane surface. However, the following observations make the latter interpretation less likely. A tripartite fusion protein consisting of proOmpA, a duplication of the 162 C-terminal amino acid residues of proOmpA and Dhfr is strikingly similar to proOmpA−Dhfr in its translocation characteristics. This chimera also forms an arrested translocation intermediate in the presence of Dhfr ligands. After the ligands are removed, the intermediate still undergoes the energy independent translocation of 20−30 residues and concomitant unfolding of the Dhfr domain (R.Arkowitz, J.Joly and E.Schiebel, unpublished data). This result shows that increasing the length of the region between Dhfr and proOmpA in the fusion protein has no apparent effect on the observed energy-independent translocation. If refolding of the lumenal proOmpA portion of I_{37} were driving the conversion to I_{40}, the addition of a 162 amino acid region between the proOmpA and Dhfr domains would be expected to have allowed the fully translocated proOmpA portion of the tripartite fusion protein to complete its folding. In addition, a translocation intermediate can be formed by oxidizing the two cysteines of the proOmpA portion of proOmpA−Dhfr, similar to the previously-described proOmpA translocation intermediate I_{29} (Tani *et al.*, 1990; Schiebel *et al.*, 1991). Upon reduction of the disulfide block in proOmpA−Dhfr, energy-independent translocation is not observed (unpublished data). This result suggests that it is not merely the release of a physical block that provides the driving force for partial translocation. Rather, these results indicate that the residues near the blocking structure of the proOmpA−Dhfr I_{37} intermediate are involved in the I_{37} to I_{40} conversion.

Although our results show that translocation can accelerate the rate of unfolding of the Dhfr domain, the molecular mechanism of this unfolding reaction is unknown. We envision that the translocation reaction unfolds Dhfr by 'pulling' on the N-terminus of the globular Dhfr domain, analogous to pulling on the end of a ball of string. Consistent with this notion, the crystal structure of Dhfr reveals that residues within the first 20 amino acids of the N-terminus form a significant portion of the NADPH binding site (Volz *et al.*, 1982; Stammers *et al.*, 1987). As a result, it appears reasonable that when these 20−30 N-terminal Dhfr residues are translocated in the I_{37} to I_{40} conversion, the global structure of Dhfr is destroyed. We cannot yet differentiate between the following two mechanisms of translocation-driven precursor unfolding. The integral domain of translocase, SecY/E and acidic phospholipids, might catalyze the unfolding of Dhfr by lowering the barrier of the transition state to the unfolded species. In this mechanism, translocase is directly involved in the unfolding reaction. Alternatively, the translocation reaction could kinetically compete with the refolding of Dhfr on the cytoplasmic side due to the irreversible nature of the translocation event. Either of these mechanisms would result in an overall increase in the rate of net unfolding during translocation.

Three energy sources can drive individual steps in the translocation process: ATP binding and hydrolysis, the membrane protonmotive force $\Delta\mu_H+$ and the intrinsic energy of translocation. This intrinsic energy of translocation is itself comprised of the folding and unfolding energies of preprotein domains and the potential energy of the polypeptide in the ill-defined environment through which the preprotein traverses the membrane itself. Studies of intermediates in proOmpA and proOmpA−Dhfr translocation have revealed defined experimental conditions for studying each of these three energy contributions in isolation. (i) The energy of ATP binding uniquely drives the initial insertion of a loop of the preprotein across the membrane and can drive a limited translocation of 20−30 residues at every stage of transit examined (Schiebel *et al.*, 1991). The energy of ATP binding and hydrolysis is the only energy source sufficient to drive I_{40} to fully translocated proOmpA−Dhfr. (ii) $\Delta\mu_H+$ prevents reverse translocation and drives forward translocation in the absence of SecA/ATP association (*ibid*). (iii) The intrinsic energy of translocation drives I_{37} to I_{40} in the absence of SecA, ATP or $\Delta\mu_H+$. I_{37} and I_{40} constitute a defined model system for studying the contribution of this separate energy source. These results demonstrate one mechanism by which translocation can result in the net unfolding of a protein domain. This is particularly relevant under conditions where protein translation and translocation across the membrane are uncoupled processes (Verner and Schatz, 1988). The relative contribution of translocation-driven protein unfolding compared with unfolding promoted by ATP dependent chaperones (Rothman, 1989) will have to await further

studies of secreted proteins. The intrinsic energy of translocation can make the unfolding of folded domains an energetically favorable process. This energy source includes at least the energy of unfolding preprotein domains and the potential energies of distinct preprotein regions occupying the membrane transiting site.

The availability of a proOmpA−Dhfr translocation intermediate, arrested at a unique point in translocation, also provides a powerful tool for exploring the molecular nature of the environment of the membrane-transiting polypeptide chain. In the accompanying article (Joly and Wickner, 1992), this intermediate is shown to traverse the membrane through the SecY subunit of translocase.

Materials and methods

Biologicals

Inverted *E.coli* inner membrane vesicles were prepared from *E.coli* KM9 (*unc*$^-$::Tn*10*, *rel*A1, *spo*T1, *met*B1; Klionsky *et al.*, 1984) as described by Chang *et al.* (1978). The following proteins were purified according to published methods: SecA protein (Cunningham *et al.*, 1989), [^{35}S]proOmpA (Crooke and Wickner, 1987) and SecB [Weiss *et al.*, 1988, as modified by Lecker *et al.* (1989)]. ProOmpA−Dhfr was purified following the procedure of Crooke *et al.* (1988) for proOmpA. Anti-SecA immunoglobulin G fractions were purified according to Lill *et al.* (1989). TPCK-treated bovine pancreatic trypsin (>180 U/mg) was purchased from Cooper Biomedical. Thermolysin (type X, 60 U/mg), dihydrofolate reductase (rat liver, 4 U/mg) and potato apyrase (grade VI, 250 U/mg) were purchased from Sigma Chemicals. The apyrase was dialyzed versus 20 mM sodium citrate pH 5.5 prior to use. ^{14}C molecular weight markers were purchased from BRL. [α-^{32}P]ATP (800 Ci/mmol) was obtained from NEN.

Construction and expression of proOmpA−Dhfr fusion protein

PCR-directed mutagenesis was used to modify the murine Dhfr cDNA (kindly provided by Dr G.Schatz, Basel) adding a *Sal*I restriction site at the N-terminus (GCATC*GTCGAC*ATGGTTCGACCATTGAACTGA) and a *Hin*dIII site in the 3′ noncoding sequence (AGCTC*AAGCTT*TTAGTCT-TTCTTCTCGTAGAC). Newly created restriction sites are italicized. After digestion with these restriction enzymes, the fragment was ligated into the *Sal*I and *Hin*dIII sites of pUC19 (Yanisch-Perron *et al.*, 1985). PCR-directed mutagenesis was used to introduce a *Sal*I restriction site at the C-terminus of proOmpA (AATAT*GTCGAC*AGCCTGCGGCTGAGTTACAAC) in conjunction with a primer from the *Eco*RI site (TCACACAGGAAACAG-AATTC) from the proOmpA overexpression plasmid pTrcOmp9 (Crooke *et al.*, 1988). After digestion with these enzymes, this fragment was ligated into the pUC-Dhfr plasmid cut with *Eco*RI and *Sal*I. The *Eco*RI−*Hin*dIII fragment from the resulting plasmid was ligated into the *Eco*RI and *Hin*dIII sites of the pTrcOmp9 plasmid and the fusion protein expressed in JM109 (Yanisch-Perron *et al.*, 1985). The fusion protein contains a two amino acid junction of Val-Asp due to the *Sal*I restriction site between the C-terminus of proOmpA and the N-terminus of Dhfr.

In vitro translocation of proOmpA−Dhfr

Translocation into inverted inner membrane vesicles was performed in buffer A (50 mM HEPES−KOH pH 8.0, 50 mM KCl, 5 mM $MgCl_2$) containing 2 mM DTT, 0.1 mg/ml fatty acid free BSA (Sigma) and 200 μg/ml SecA. An ATP regenerating system consisting of 5 mM creatine phosphate and 10 μg/ml creatine kinase was used when ATP was present. Translocation reactions (50 μl) typically contained membranes bearing 10 000 c.p.m. of [^{3}H]proOmpA−Dhfr translocation intermediate. Samples were incubated for the indicated times and rapidly chilled. Translocation reactions were treated with 1 mg/ml proteinase K for 20 min at 0°C. Digestions were stopped by the addition of an equal volume of ice-cold trichloroacetic acid. After incubation at 0°C for at least 1 h, precipitated protein was collected by centrifugation (Eppendorf centrifuge, 10 min at 4°C). The sediment was resuspended in 1 ml ice cold acetone and centrifuged as above. The acetone was removed by aspiration followed incubation at 37°C to remove residual acetone. The sediment was resuspended in 50 μl SDS sample buffer and heated for 3 min at 95°C. Samples were analyzed by SDS−polyacrylamide gel electrophoresis followed by immunoblotting or fluorography.

Dhfrase activity

The activity of dihydrofolate reductase was determined at 25°C in 50 mM Tris−Cl pH 7.4, 1 mM DTT, 50 μM EDTA, 50 μM NADPH and 100 μM dihydrofolate, modified from the method of Pastore *et al.* (1974). Reactions were initiated by addition of NADPH or enzyme and monitored at 340 nm. One unit of enzyme activity reduces 1 μmol dihydrofolate/min under these conditions (based on an extinction coefficient of 12 000 M^{-1} cm^{-1}; Blakley, 1969). Measurements of the Dhfr activity of proOmpA−Dhfr were performed in the presence of 10 μg/ml SecB. Activity measurements of the fusion protein were from 3−5 determinations.

Protein labeling

Dhfr was labeled with [^{125}I]NaI (50 mCi/μmol, NEN) (Markwell and Fox, 1978) to a specific activity of 100 000 c.p.m./μg. Free iodine was removed by chromatography on Sephadex G-25 (Pharmacia). The iodinated Dhfr retained full activity. ProOmpA−Dhfr was tritiated by reductive methylation (Means and Feeney, 1968). Protein was labeled in 10 μg batches in 50 μl reactions containing 100 mM borate pH 9.0 and 6 M urea. Formaldehyde was added to final concentration of 0.04% followed by 1 mCi of [^{3}H]$NaBH_4$ (50 mCi/μmol, NEN). The reaction was incubated on ice for 1 min followed by 25% trichloroacetic acid precipitation, centrifugation, suspension in acetone and resedimentation as described above. Protein was labeled to a specific activity of $\sim 3.0\times10^6$ c.p.m./μg. Radioactivity was quantified by liquid scintillation counting in a Beckman LS-100C counter and ^{125}I was quantified in a Beckman 8000 gamma counter.

Measurements of $\Delta\Psi$ and ΔpH

$\Delta\Psi$, inside positive, was determined with 2 μM oxonol VI(*bis*[3-propyl-5-oxoisoxazol-4-yl]pentamethine oxonol, Molecular Probes) (Apell and Bersch, 1987) at 25°C. Excitation and emission were at 599 and 634 nm with slit widths of 5 nm on a Hitachi fluorescence spectrometer. ΔpH, inside acidic, was measured with 2 μM 9-amino-chloro-2methoxyacridine (Molecular Probes) (Klionsky *et al.*, 1984). Excitation and emission were at 409 and 474 nm with slit widths of 5 nm. Measurements were carried out in buffer A (diluted to half concentration for ΔpH measurements) containing 2 mM DTT and 0.1 mg/ml BSA with membrane vesicles bearing I_{37} (20 μl, 500 ng I_{37} for $\Delta\Psi$ and 5 μl, 125 ng I_{37} for ΔpH). Valinomycin (0.1 μM) or nigericin (0.1 μM) was added either prior to or subsequent to $\Delta\mu_H$+ generation.

Other methods

Protein was determined by Bradford reagent (Bio-Rad) with BSA as standard. SDS−PAGE and electrophoretic transfer to PVDF was carried out according to Ito *et al.* (1980) and Towbin *et al.* (1979). The ATP content of membrane vesicles bearing I_{37} was determined by addition of 1×10^8 c.p.m. [α-^{32}P]ATP to a translocation reaction in which I_{37} was generated. Membrane vesicles bearing I_{37} were isolated and incubated with apyrase as described in Figure 5A. Protein was precipitated from membrane vesicles bearing I_{37} with 20% perchloric acid and 20 nmol ATP was added as carrier. The supernatant was neutralized with KOH and analyzed on cellulose PEI-F thin layer chromatography plates (J.T.Baker) developed in 0.5 M potassium phosphate, pH 7.5. TLCs were visualized by UV and autoradiography followed by quantification by scintillation counting.

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