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ProOmpA contains secondary and tertiary structure prior to translocation and is shielded from aggregation by association with SecB protein

Stewart H.Lecker, Arnold J.M.Driessen[1] and William Wickner[2]

Molecular Biology Institute and Department of Biological Chemistry, University of California, Los Angeles, CA 90024–1737, USA

[1]Permanent address: Department of Microbiology, University of Groningen, Kerklaan 30, 9751NN, The Netherlands
[2]To whom correspondence should be addressed

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***Escherichia coli* protein export involves cytosolic components termed molecular chaperones which function to stabilize precursors for membrane translocation. It has been suggested that chaperones maintain precursor proteins in a loosely folded state. We now demonstrate that purified proOmpA in its translocation component conformation contains both secondary and tertiary structure as analyzed by circular dichroism and intrinsic tryptophan fluorescence. Association with one molecular chaperone, SecB, subtly modulates the conformation of proOmpA and stabilizes it by inhibiting aggregation, permitting its translocation across inverted *E.coli* inner membrane vesicles. These results suggest that translocation competence does not simply result from the maintenance of an unfolded state and that molecular chaperones can stabilize precursor proteins by inhibiting their oligomerization.**

Key words: E.coli secretion/molecular chaperone

Introduction

Secreted and organellar proteins may cross a membrane either during, or after, their synthesis. However, a coupling of synthesis to translocation has only been found for large proteins traversing the mammalian rough endoplasmic reticulum. There, continued association of a growing polypeptide with both a signal recognition particle and a ribosome is essential for translocation of even full length polypeptide chains (Perara *et al.*, 1986). Translocation across yeast endoplasmic reticulum (Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Hansen *et al.*, 1986), *Escherichia coli* plasma membrane (Ito *et al.*, 1980; Randall, 1983; Zimmermann and Wickner, 1983) and the membranes of cytoplasmic organelles such as mitochondria (Harmey *et al.*, 1977), chloroplasts (Chua and Schmidt, 1978) and peroxisomes (Imanaka *et al.*, 1987) need not be coupled to ongoing translation. These studies have raised the question of whether polypeptide conformation and stabilization occur prior to the translocation event. Goodman *et al.* (1981) showed that M13 procoat is competent for membrane transit for a few minutes after its synthesis but loses this competence during subsequent incubation *in vitro*. This phenomenon, a marked lability of translocation competence, has since been reported for many secreted and membrane protein precursors. Crooke *et al.* (1988a) showed that an *E.coli* precursor protein, proOmpA, is competent for *in vitro* translocation immediately after dilution from urea, but loses translocation competence during incubation without membranes. A relatively unfolded or 'open' precursor conformation has been implicated in the translocation process (Randall and Hardy, 1986; Eilers and Schatz, 1986). For example, prior to its insertion across the plasma membrane, *E.coli* leader peptidase is in a form which is 100-fold more protease sensitive than its mature, trans-membrane structure (Wolfe and Wickner, 1984). Eilers and Schatz (1986) showed that a fusion protein which joined a mitochondrial leader sequence to dihydrofolate reductase was folded but had to be unfolded to be imported into mitochondria. Randall and Hardy (1986) have shown that pre-maltose binding protein (pre-MBP) loses its translocation competence when it folds into its compact, mature structure. They also used the intrinsic fluorescence of tryptophan residues in pre-MBP to demonstrate that the leader peptide retards folding (Park *et al.*, 1988).

A group of proteins termed 'molecular chaperones' (Hemmingsen *et al.*, 1988) have been implicated in the maintenance of precursor proteins in the translocation competent state (Meyer, 1988; Zimmermann *et al.*, 1988; Deshaies *et al.*, 1988b). Deletion of the yeast SSA genes, which encode the hsp70 family of proteins, impairs both mitochondrial import and translocation across the endoplasmic reticulum (Deshaies *et al.*, 1988a; Chirico *et al.*, 1988). Mitochondrial hsp60, a GroEL homolog, is associated with newly-imported precursor proteins and catalyzes their folding (Cheng *et al.*, 1989; Ostermann *et al.*, 1989). Several molecular chaperones are involved in bacterial export. GroEL is needed for efficient secretion of pre-β-lactamase (Bochkareva *et al.*, 1988; Kusukawa *et al.*, 1989). SecB promotes the efficient export of maltose binding protein and other precursor proteins by modulating their conformation (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Weiss *et al.*, 1988). Trigger factor was isolated as a protein which can maintain proOmpA in a translocation competent conformation (Crooke and Wickner, 1987; Crooke *et al.*, 1988b). Trigger factor and GroEL can be isolated from ribosomes, suggesting that they bind to nascent polypeptides (Neidhardt *et al.*, 1981; Lill *et al.*, 1988). Trigger factor, SecB and GroEL can each form specific stoichiometric complexes with proOmpA *in vitro* and are active in maintaining precursor competence (Lecker *et al.*, 1989).

Little is known about the basis of the physical interaction between precursor proteins and chaperones. Binding may be mediated through hydrophobic interactions, thus preventing non-specific protein aggregation. Rothman and coworkers have shown that hsp70 can bind to small peptides and is released in an ATP dependent manner (Flynn *et al.*, 1989), suggesting that chaperones could bind to 'unfolded' polypeptide chains and stabilize them in their translocation

competent state. In accordance with this proposal, SecB and trigger factor maintain precursor proteins in a protease-sensitive conformation (Crooke *et al.*, 1988a; Weiss *et al.*, 1988). We have previously shown that SecB, trigger factor and GroEL can each form a soluble 1:1 stoichiometric complex with proOmpA, while such stable complexes are not formed with any of several cytoplasmic proteins (Lecker *et al.*, 1989).

Using purified precursor and chaperone proteins, we have now sought to assess directly the structural motifs in a translocation-active precursor protein. We find that translocation competent proOmpA in complex with SecB has significant tertiary structure. Upon binding to SecB, proOmpA undergoes a subtle conformational change as detected by fluorescence energy transfer. Loss of proOmpA translocation competence is due to aggregation, and SecB inhibits this aggregation event. The contrast between these results and those obtained by Randall and colleagues for preMBP suggests an underlying mechanism of SecB action.

Results

We have previously reported the purification of proOmpA, the precursor form of a major bacterial outer membrane protein, in solutions of 6–8 M urea. Upon dilution from the urea, proOmpA can translocate into inverted inner membrane vesicles prepared from *E.coli* (Crooke *et al.*, 1988a; Lecker *et al.*, 1989). To ensure complete unfolding, the denaturant guanidine-HCl can also be employed. To characterize the structure of the translocation-competent form of proOmpA, we measured the circular dichroism and intrinsic tryptophan fluorescence spectra of this protein (Figure 1). ProOmpA is a 346 amino acid polypeptide with five tryptophan residues in the amino half of the molecule (residues 30, 36, 78, 123 and 164; Chen *et al.*, 1980). The circular dichroism spectrum of proOmpA in 3 M guanidine-HCl lacks ellipticity minima in the 210–240 nm range (Figure 1A, dashed line). This is characteristic of a random coil structure. The proOmpA tryptophan residues show a low fluorescence intensity and high emission wavelength maximum (~355 nm), suggesting that they are accessible to solvent (Figure 1B, dashed line). Higher concentrations of guanidine do not further perturb either the circular dichroism or tryptophan fluorescence spectra (data not shown). In contrast to the random coil structure of proOmpA in 3 M guanidine, proOmpA which has been diluted into aqueous buffer has both secondary and tertiary structure (Figure 1A and B, solid lines). Analysis of the circular dichroism data in Figure 1A using secondary structure algorithms (Greenfield and Fasman, 1969; Siegel *et al.*, 1980; Wu *et al.*, 1981) predicts that proOmpA contains 18% α-helix and 44% β-sheet structure. This is consistent with other structural analyses of OmpA in which the N-terminal half is thought to form a β-barrel in the outer membrane while the C-terminal half resides in the periplasmic space (Vogel and Jahnig, 1986). The tryptophans of translocation-competent proOmpA are buried within the protein, as demonstrated by their increased fluorescence intensity and decreased emission wavelength maximum.

Once proOmpA has been removed from denaturant, it undergoes a time-dependent loss of competence for membrane translocation unless it is complexed with a chaperone protein (Crooke *et al.*, 1988a and b; Lecker *et al.*, 1989). The nature of the translocation-incompetent state was analyzed by sucrose gradient sedimentation. ProOmpA that had been rendered incompetent by removing the guanidine-HCl by dialysis was sedimented on sucrose gradients for 1.25 h (Figure 2A). Although this material remained soluble through dialysis and low speed centrifugation procedures (data not shown), it sedimented as oligomers or aggregates upon ultracentrifugation. Soluble proteins (SecA, bovine serum albumin and ovalbumin) centrifuged under identical conditions to those in Figure 2A were found in the top two fractions of the gradient. In the presence of SecB, however, proOmpA avoided aggregation by forming a 1:1 complex with SecB (Figure 2B, top panel, and Lecker *et al.*, 1989). ProOmpA which was diluted from denaturant and incubated to permit aggregation prior to addition of SecB was incapable of forming such a complex (Figure 2B, lower panel). These results suggest that the role of SecB protein in this system is to inhibit the formation of the higher order proOmpA structures.

To analyze further the structure of proOmpA in complex

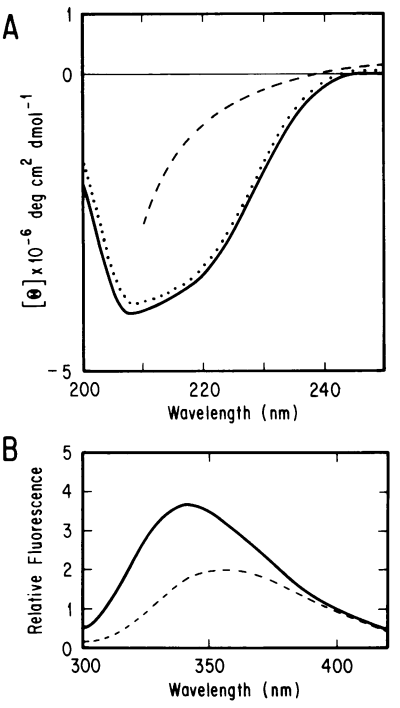

Fig. 1. Renatured proOmpA has secondary and tertiary structure. **A.** circular dichroism. ProOmpA solutions were prepared at 0.15 mg/ml in buffer C (dashed line), by dialysis of proOmpA (1.0 mg/ml) in buffer C into 20 mM Tris–Cl, pH 7.6 and dilution in that buffer to 0.15 mg/ml (dotted line), or by 110-fold dilution of proOmpA (17 mg/ml) in buffer C into 20 mM Tris–Cl, pH 7.6 (solid line). Data were acquired on a Jasco J600 spectropolarimeter using the following parameters: scan speed, 50 nm/min; sensitivity, 20 mdeg; time constant, 2 s; path length, 1 mm; temperature, 25°C. 4 scans were averaged for each spectrum. Far UV wavelengths <210 nm could not be penetrated in the spectrum containing guanidine-HCl due to high absorbance of the denaturant in that region. Data are expressed as total protein molecular ellipticity. For conversion to mean residue molecular ellipticity (used for secondary structure estimation), these values were divided by 346 (the number of amino acid residues in proOmpA). **B.** Tryptophan fluorescence. ProOmpA (10 mg/ml) in buffer G was diluted 200-fold to a final concentration of 50 μg/ml in buffer G (dotted line) or buffer TL (solid line). ProOmpA tryptophan fluorescence spectra were obtained at 37°C with stirring as described in Materials and methods.

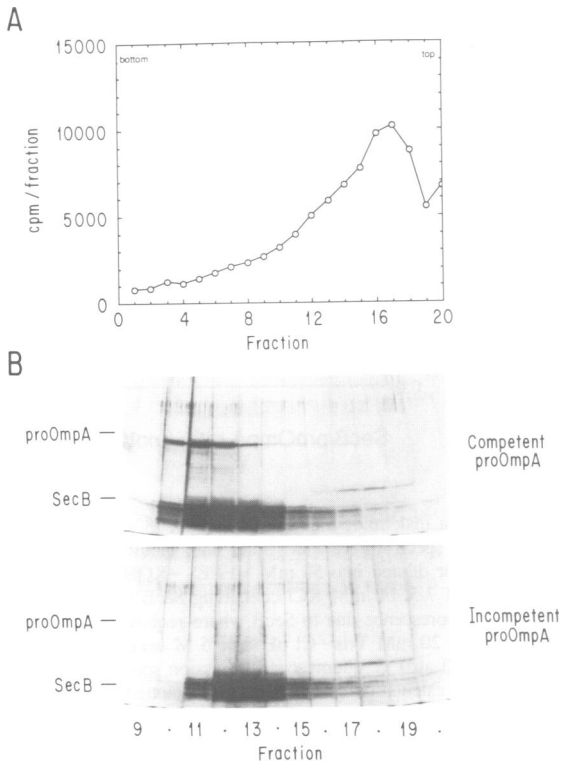

Fig. 2. Incompetent proOmpA is an aggregate and does not bind to SecB. **Panel A.** Translocation-incompetent [^{35}S] proOmpA was prepared by dialysis as in Figure 1. [^{35}S]proOmpA (0.3 mg/ml, 100 μl, 50 kc.p.m./μg) was layered onto a sucrose gradient as described in Materials and methods and sedimented (4°C, 1.25 h, 36 000 r.p.m.). Fractions were analyzed by liquid scintillation counting. Fraction 20 is the top of the gradient. **Panel B.** ProOmpA in buffer G (3 μl, 10 mg/ml) or translocation-incompetent proOmpA (46 μl, 0.65 mg/ml) was added to 68 μg SecB in a final volume of 100 μl (buffer TL). The samples were layered on sucrose gradients, sedimented (4°C, 45 h, 36 000 r.p.m. in an SW40 rotor) and fractionated as described in Materials and methods. Fractions were analyzed by SDS−PAGE and silver staining (Ansorge, 1983). The top 12 fractions are shown (21 fractions were collected). The remaining bottom nine fractions were completely free of protein.

with SecB, the tryptophan fluorescence of proOmpA was measured in the presence and absence of chaperone (Figure 3). SecB monomer contains a single tryptophan which is accessible to solvent and thus makes only a small contribution to the fluorescence of the SecB−proOmpA complex (data not shown). The SecB contribution to the total complex fluorescence was subtracted, resulting in the solid line in Figure 3. SecB clearly has little effect on the tertiary structure of proOmpA in the region of the tryptophan residues.

We further studied proOmpA−SecB interactions by using coumarin-derivatized proOmpA. Coumarin maleimide was specifically coupled to the two unique cysteine residues at the C-terminus of proOmpA (residues 311 and 323). This conjugated proOmpA was fully active for membrane translocation and translocation ATPase (Cunningham *et al.*, 1989) (data not shown). The derivatized proOmpA was then analyzed using fluorescence energy transfer. Upon excitation of the proOmpA tryptophan residues at 280 nm, emission could be measured at the coumarin wavelength (463 nm), signifying close proximity of the N-terminal tryptophans and the C-terminal coumarin (Figure 4A, solid line). There was

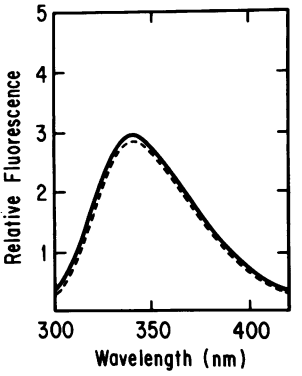

Fig. 3. ProOmpA in complex with SecB has similar tryptophan fluorescence characteristics to translocation competent proOmpA. 5 μl of proOmpA (10 mg/ml) in buffer G was diluted into 1.0 ml buffer TL in the presence (solid line) or absence (dashed line) of 114 μg/ml SecB. Fluorescence emission spectra of proOmpA tryptophan residues were measured at 25°C as described in Materials and methods. The background fluorescence due to SecB was subtracted from the combined spectra.

far less transfer of energy from tryptophan to coumarin if the proOmpA was denatured in 3 M guanidine (Figure 4A, dashed line). In the presence of SecB, the energy transfer was diminished (Figure 4A, dotted line), suggesting that SecB induces a conformational change in proOmpA upon binding.

Other chaperone proteins were tested for their ability to inhibit proOmpA fluorescence transfer. Neither trigger factor nor GroEL had effects on the energy transfer (data not shown). As further evidence for the specificity of the effect, a strict correspondence between the reduction of energy transfer and amount of SecB added was found with saturation at one proOmpA per SecB (Figure 4B). This is in agreement with previous studies which showed that SecB forms a tighter complex with proOmpA than does trigger factor or GroEL, and that such complexes have a 1:1 stoichiometry (Lecker *et al.*, 1989).

ProOmpA was tested for its ability to translocate into *E.coli* inner membrane vesicles (Figure 5). ProOmpA denatured in guanidine was, upon dilution, capable of translocating into vesicles with 70% efficiency in an energy dependent reaction (lanes 1 and 2). Sucrose gradient purified proOmpA−SecB complex was imported with similar efficiency (lanes 3 and 4). In an experiment in which the remaining untranslocated proOmpA−SecB complex was separated from inner membranes, 41% could be imported in a second incubation with fresh membranes (data not shown). This high efficiency of this translocation confirms that our physical measurements are of translocation competent molecules.

Discussion

Establishing the structural features which comprise the translocation competent state of precursor proteins is critical to understanding the translocation process. It has been suggested that only unfolded proteins can translocate across membranes. We show here that competent OmpA precursor contains both secondary and tertiary structure. ProOmpA, though initially folded, may unfold at a later step in the translocation process. This is suggested by studies which show that stabilization of the native, folded conformation

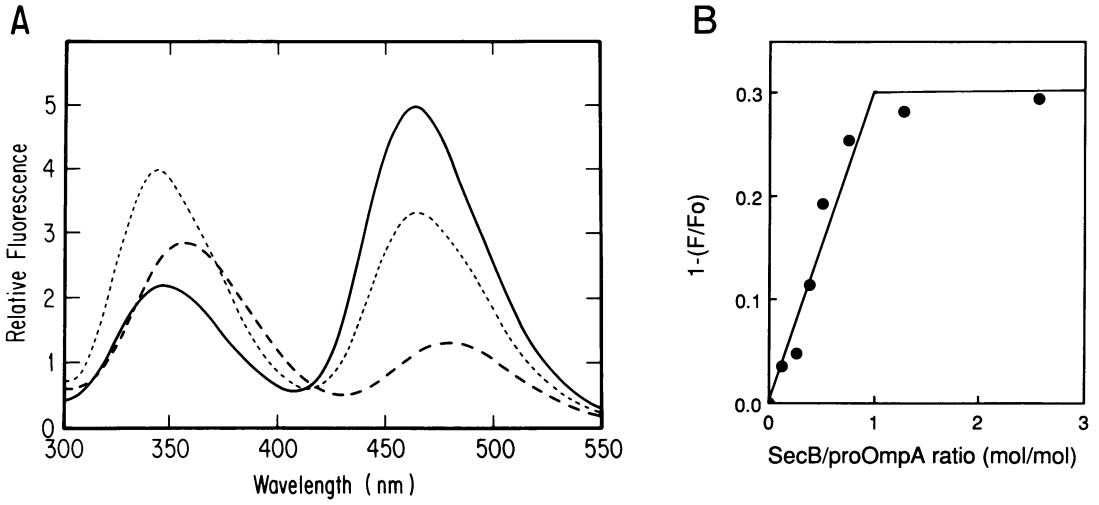

Fig. 4. Fluorescence energy transfer of coumarin maleimide-modified proOmpA. **A**. Coumarin maleimide-modified proOmpA (8 μg, 0.21 nmol) in 20 mM Tris−Cl, pH 8.0, 6 M urea was diluted into a 1.5 ml stirring solution at 0°C in the spectrofluorimeter. Solid line, modified precursor diluted in 50 mM HEPES−KOH pH 7.5, 50 mM KCl; heavy dashed line, modified precursor diluted into 50 mM HEPES−KOH pH 7.5, 50 mM KCl, 3 M guanidine HCl; thin dashed line, proOmpA diluted into 50 mM HEPES−KOH pH 7.5, 50 mM KCl containing SecB (41.6 μg, 0.49 nmol). Fluorescence emission spectra were recorded and corrected for the tryptophan fluorescence due to SecB where required. Excitation was at 280 nm using a slit width of 5 nm. **B**. Coumarin-modified proOmpA (8 μg, 0.21 nmol) in 20 mM Tris−Cl pH 8.0, 6 M urea was diluted into stirring solutions of SecB (0−41.6 μg, 0−0.49 nmol) in 50 mM HEPES−KOH, 50 mM KCl at 0°C. Fluorescence emission spectra were obtained as described above. The emission of coumarin maleimide (λ_{max} = 463 nm) was used for the calculation of ΔF. Data were plotted as reduction in energy transfer [1-F/F_0)] versus SecB−proOmpA ratio. The line represents the expected outcome for a 1:1 complex with a rapid association and slow dissociation rate.

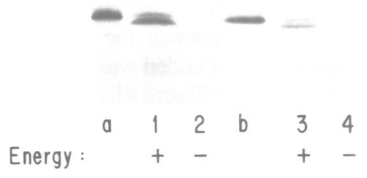

Fig. 5. ProOmpA diluted from denaturant or in complex with SecB translocates with high efficiency. ProOmpA (25 ng, 25 kc.p.m./μl, 1 μl) in buffer G (**lanes 1** and **2**) or sucrose gradient purified proOmpA−SecB complex (30 μl, 300 ng proOmpA, **lanes 3** and **4**) was added to 50 μl translocation reactions in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 5 mM ATP, 5 mM NADH, 20 mM creatine phosphate and 100 μg/ml creatine kinase. Reactions were performed in buffer TL containing 0.5 mg/ml BSA, 50 μg/ml SecA and 0.468 mg/ml D10 inner membrane vesicles, at 37°C for 30 min. Samples were placed on ice and transferred to fresh microfuge tubes containing 5 μl of 10 mg/ml proteinase K. Digestion was for 15 min at 4°C to reveal translocated, protease-inaccessible OmpA. Protein samples were then precipitated with two volumes of ice-cold 20% TCA, collected by centrifugation, and rinsed with 1 ml of ice-cold acetone. Protein pellets were solubilized in sample buffer, heated for 3 min at 95°C, and subjected to SDS−PAGE and fluorography as described by Ito *et al.* (1980). **Lane a** is a 50% standard for lanes 1 and 2, **lane b** is a 50% standard for lanes 3 and 4. Lane 1 contains 70.5% translocation efficiency, lane 3 contains 40.6% translocation efficiency as determined by densitometry of the autoradiograms. Sucrose gradient purified complex was prepared as follows: a 100 μl mixture of proOmpA (13.3 μg, 46 kc.p.m./μl, 0.36 nmol, 28 μl) in buffer U and SecB (15.3 μg, 0.18 nmol) in buffer TL were sedimented into a 3.5 ml sucrose gradient in buffer TL (SW56, 37 kr.p.m., 21 h, 4°C). 0.3 ml fractions were collected from the bottom and the peak fraction of proOmpA−SecB complex (1000 c.p.m./μl, 10 ng proOmpA/μl) was used for translocation assays.

of a precursor protein inhibits mitochondrial import (Eilers and Schatz, 1986; Rassow *et al.*, 1989). Methotrexate, a drug which binds tightly to dihydrofolate reductase, blocks the mitochondrial import of a fusion protein between a mitochondrial matrix targeting peptide and this cytoplasmic enzyme. Presumably, the fusion protein is initially folded, which allows interaction with the methotrexate. With this native conformation stabilized, the fusion protein might be unable to unfold for subsequent translocation. Similarly, folding of the bacterial pre-maltose binding protein into its stable, sugar-binding structure prevents its export (Randall and Hardy, 1986).

Our data do not establish whether proOmpA diluted from denaturant or in complex with SecB is fully folded into the final conformation of OmpA. Renatured proOmpA may undergo 'breathing' reactions like those described for the 'molten globule' state (Ptitsyn, 1987). In this physical state of protein folding, the polypeptide is highly compact with fluctuating tertiary structure. The circular dichroism spectra of the active and inactive proOmpA are identical. This suggests that the relative amounts of α-helix and β-sheet structure are similar in the two forms, making large conformational differences unlikely.

SecB stabilizes proOmpA by preventing its aggregation. SecB is required for efficient *in vivo* export of a number of secreted proteins (Kumamoto and Gannon, 1988; Collier *et al.*, 1988). Association with SecB can modulate the protease sensitivity of preMBP (Collier *et al.*, 1988; Weiss *et al.*, 1988). Purified SecB can bind to proOmpA, prePhoE and preMBP to form specific 1:1 complexes (Lecker *et al.*, 1989). Our data show no measurable changes in the tertiary structure in the region surrounding the tryptophan residues of proOmpA upon such interaction with SecB. SecB binding does induce a subtle conformational change in proOmpA as seen by fluorescence energy transfer. SecB does not slow the folding of proOmpA as deduced from the lack of changes in the tryptophan fluorescence intensity with time (S.L., unpublished). In similar studies, the intramolecular folding of preMBP was dramatically slowed by SecB (Liu *et al.*,

1989). It has been suggested (Hemmingsen *et al.*, 1988) that chaperones such as SecB bind to apolar domains. SecB binding might either prevent apolar domains of an otherwise soluble protein precursor such as preMBP from being internalized during folding, or it may shield the apolar surface of a folded, integral membrane protein precursor such as proOmpA. Although SecB clearly acts to inhibit aggregate formation, it also subtly alters the conformation of proOmpA, which may help to maintain its translocation competence. Since OmpA is hydrophobic and resides in the outer membrane as an oligomer, the requirements for a chaperone protein might be different from those for the more soluble, periplasmic MBP. Molecular chaperones have also been shown to catalyze the correct formation of quaternary structure. GroEL is required for correct assembly of phage head structures (Georgopoulos and Hohn, 1978; Hemmingsen *et al.*, 1988) and, in a heterologous system, ribulose bisphosphate carboxylase (Golubinoff *et al.*, 1989).

Accumulated evidence from diverse studies is consistent with the hypothesis that molecular chaperones act to stabilize precursors by preventing the formation of a structure which cannot undergo translocation. This can be performed by a number of different mechanisms. For rapidly folding soluble proteins such as preMBP, the chaperones may slow the normal folding kinetics of the polypeptide. For hydrophobic proteins such as proOmpA, chaperones may inhibit aggregation without preventing the formation of tertiary structure. To test these ideas, it will be important to determine directly whether the folded proOmpA described here unfolds again as part of the translocation process.

Materials and methods

Bacterial strains and materials

Inverted inner membrane vesicles used in the translocation assay were prepared from *E.coli* strain D10 (Crooke *et al.*, 1988b). Urea (ultra pure), sucrose and guanidine-HCl (ultra pure) were form Schwarz/Mann and proteinase K was from Boehringer Mannheim. 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (coumarin maleimide) was from Molecular Probes.

Buffers

Buffer TL is 50 mM Tris−Cl, pH 7.6, 35 mM KCl, 25 mM NH_4Cl, 10 mM $MgOAc_2$, 1 mM DTT. Buffer U is 50 mM Tris−Cl, pH 8.0, 8 M urea, 2 mM DTT. Buffer C is 20 mM Tris−Cl, pH 7.6, 3 M guanidine-HCl. Buffer G is 50 mM K HEPES, pH 7.5, 3 M guanidine-HCl, 2 mM DTT.

Biochemicals

[^{35}S]proOmpA was prepared according to Crooke *et al.* (1987). ProOmpA was isolated from cells overproducing the protein as described in Crooke *et al.* (1988b). SecA protein was prepared as in Cunningham *et al.* (1989). SecB protein was prepared as in Lecker *et al.* (1989). Protein concentrations were assayed according to Bradford (1976) using bovine serum albumin as a standard.

ProOmpA modification

The sulfhydryl groups of proOmpA (7 mg/ml in 20 mM Tris−Cl pH 8.0, 6 M urea) were reduced in the presence of 200 μM DTT and subsequently reacted with 5 mM coumarin maleimide (added as a 100 mM stock solution in dimethylformamide) for 2 h at 0°C. The reaction was terminated by the addition of DTT to 10 mM. The proOmpA was precipitated with TCA (15% w/v final concentration, 30 min, 0°C), and collected by centrifugation (microfuge, 15 min, 4°C). Nonreacted coumarin maleimide was removed by suspending the proOmpA pellet twice in acetone (1.5 ml, 0°C) followed by centrifugation (microfuge, 15 min, 4°C) to retrieve the protein. Modified proOmpA was resuspended in 20 mM Tris−Cl pH 8.0, 6 M urea at a concentration of 2 mg/ml. The coumarin maleimide content was determined by measuring the absorbance at 388 nm, and standardized by the use of the 2-mercaptoethanol adduct of coumarin maleimide dissolved in methanol. The modified precursor protein contained 1.05 ± 0.15 mol coumarin maleimide/mol of proOmpA.

Intrinsic tryptophan fluorescence measurements

Fluorescence measurements were made using a Hitachi F-3010 fluorescence spectrophotometer equipped with a temperature-controlled cuvette holder with stirring. Excitation wavelength was 290 nm (5 nm bandpass). Emission wavelength was either held constant at 344 nm (5 nm bandpass) for time course measurements or varied between 300 and 420 nm for wavelength scans. Unless stated otherwise, the data presented is uncorrected. No buffers had background fluorescence.

Sucrose gradient centrifugation

Twelve ml linear 10−30% sucrose gradients in buffer TL were prepared in SW40 ultracentrifuge tubes. Samples (0.1 ml) were layered on the gradients and subjected to ultracentrifugation (4°C, 45 h, 35 000 r.p.m.). Gradient fractions were collected from the bottom (20 fractions, 0.5 ml each).

Acknowledgements

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