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Kinetics and Energetics of the Translocation of Maltose Binding Protein Folding Mutants

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Protein translocation in *Escherichia coli* is mediated by the translocase that, in its minimal form, comprises a protein-conducting pore (SecYEG) and a motor protein (SecA). The SecYEG complex forms a narrow channel in the membrane that allows passage of secretory proteins (preproteins) in an unfolded state only. It has been suggested that the SecA requirement for translocation depends on the folding stability of the mature preprotein domain. Here we studied the effects of the signal sequence and SecB on the folding and translocation of folding stabilizing and destabilizing mutants of the mature maltose binding protein (MBP). Although the mutations affect the folding of the precursor form of MBP, these are drastically overruled by the combined unfolding stabilization of the signal sequence and SecB. Consequently, the translocation kinetics, the energetics and the SecA and SecB dependence of the folding mutants are indistinguishable from those of wild-type preMBP. These data indicate that unfolding of the mature domain of preMBP is likely not a rate-determining step in translocation when the protein is targeted to the translocase via SecB.

Keywords: protein translocation; folding; SecA; SecY; maltose binding protein

Introduction

In bacteria, translocation of the majority of proteins across the cytoplasmic membrane is mediated by the SecYEG translocase complex (see for a review: Ref. 1). Translocase consists of the integral membrane proteins SecY, SecE and SecG, which form a protein-conducting channel, and SecA, an ATP-dependent motor protein. The cytosolic chaperone SecB guides unfolded secretory proteins to the translocase, where they bind to SecA. Proteins are translocated as preproteins with an N-terminal extension, the so-called signal sequence. The signal sequence retards the folding of the mature domain of preproteins, thereby increasing the time window for interaction with SecB.[2,3] SecB maintains preprotein in a translocation-competent state, a state that has been suggested to correspond to a stabilized unfolded conformation of the preprotein.[4] In addition, SecB targets the preprotein to the translocase by direct association with the SecYEG-bound SecA.[5,6] Upon transfer to SecA, the preprotein is translocated through the SecYEG channel using the energy from ATP hydrolysis and the proton motive force (PMF).[7]

During translocation, preproteins are almost completely unfolded and threaded through the SecYEG channel in steps of discrete polypeptide lengths.[7–9] Recent studies have shown that the translocase is also able to translocate stably folded structures, such as the folded titin domain fused at the C-terminus of the preprotein proOmpA.[10] However, translocation of the folded titin domain is preceded by an unfolding event that results in an increased energy demand for translocation. Whereas the translocation and folding of the titin domain occur independent of SecB, native preproteins, such as the precursor of maltose binding protein (MBP), strictly depend on this molecular chaperone.[11,12] An *in vitro* analysis of the SecB-dependent translocation of two preMBP derivatives with mutations known to destabilize the folded state of the mature domain

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Abbreviations used: MBP, maltose binding protein; PMF, proton motive force; GuHCl, guanidine hydrochloride; IMV, inner membrane vesicle.

suggested a reduced SecA requirement for translocation and an increased translocation rate relative to the wild type.[13] These were taken as evidence that stable tertiary structural elements in preproteins exist even in the SecB-bound state and that these are responsible for the observed slower kinetics of the translocation of the wild type. However, a recent study with optical tweezers has demonstrated that SecB-bound MBP lacks any stable tertiary structure.[14] Because of this apparent discrepancy, we reinvestigated the translocation of folding mutants of MBP, but we included an expanded set with mutations that were shown to either destabilize or stabilize the fold of the mature MBP. The precursors of these MBP mutants were purified and used in protein folding and translocation studies. The data suggest that the presence of SecB and the signal sequence drastically overrule the effects of the mutations on the overall fold of the mature MBP domain. Also, in kinetic translocation assays, the folding mutants are indistinguishable from the wild type, showing the same SecA dependence. It is therefore concluded that during the SecB-dependent translocation of preMBP, unfolding of the mature domain is not a major rate-determining step.

Results

Mutagenesis and purification of folding variants of preMBP

To investigate to which extent the folding status of a preprotein affects translocation kinetics and energetics, we fused the native MBP signal sequence to MBP derivatives containing a mutation that either destabilizes (V8G, A276G and Y283D) or stabilizes (T345I) the folded state of the mature domain (Fig. 1a). The ΔG for urea-induced unfolding of isolated mature MBP has been reported to be 44 kJ/mol. The V8G, A276G and Y283D mutations decrease ΔG for unfolding by 4.6, 6.3 and 13.4 kJ/mol, respectively. On the other hand, the T345I mutation increases the stability of the folded state of MBP by 2.9 kJ/mol.[15] A unique cysteine residue at the surface-exposed position 51 of the mature domain was introduced to allow for fluorescent labelling (Fig. 1a) to fluorescently monitor the translocation with saturating amounts of preMBP.[16] Control experiments showed that the introduced cysteine residue and its labelling with fluorescein maleimide did not affect the folding and translocation of (pre) MBP (data not shown). Therefore, all folding mutations were combined with the single cysteine mutant of (pre)MBP, which will be referred to as wild-type preMBP. Proteins were overproduced in the *Escherichia coli* SecA mutant strain BL21.1, and the precursor forms were isolated and purified by anion exchange chromatography. The preMBP Y283D mutant showed a slightly altered migration on SDS-PAGE (Fig. 1b).

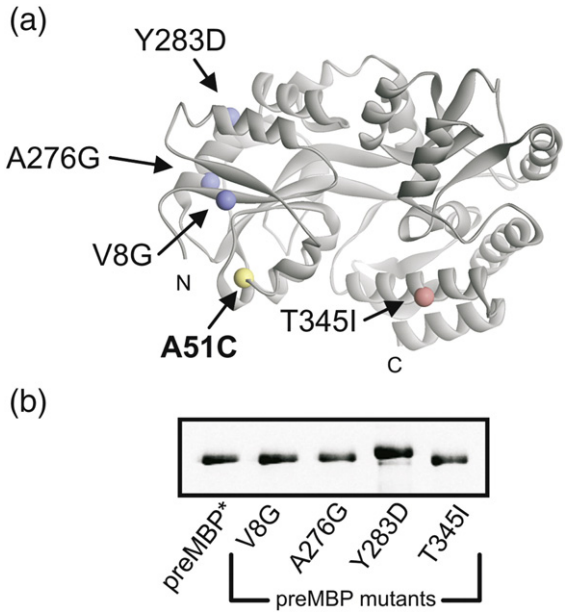

Fig. 1. Location of folding stabilizing and destabilizing mutations in MBP and purification of precursor derivatives. (a) Model of the structure of the *E. coli* MBP (Protein Data Bank code 3MBP). The location of introduced mutations is indicated by spheres and arrows. Blue indicates folding destabilizing mutations; red, folding stabilizing mutation; yellow, cysteine mutation. (b) Fluorescent image of purified fluorescein-labelled wild-type and mutant preMBPs analyzed by 12% SDS-PAGE.

Folding of preMBP mutants

Purified preMBP was diluted from urea into a translocation buffer or into a guanidine hydrochloride (GuHCl) solution and the tryptophan fluorescence emission spectrum was recorded to analyze the folding of the precursor form of MBP. MBP contains eight tryptophan residues (positions 36, 88, 120, 155, 184, 256, 258 and 366)[17] that can be used to monitor folding of MBP.[18] In the GuHCl-denatured state, preMBP and mature MBP exhibit a fluorescence emission maximum at 350 nm (Fig. 2a, lines 3 and 4). Identical spectra were obtained for the GuHCl-denaturated preMBP mutants (Fig. 2b). Upon dilution into the translocation buffer, the emission maximum for the mature MBP showed a blue shift to 344 nm and a sevenfold increase in intensity (Fig. 2a, compare lines 1 and 3). The fluorescence spectrum resembles that of native MBP as the renatured protein was found to bind to an amylase resin (data not shown). A similar blue shift in emission maximum was recorded for preMBP diluted from urea into a translocation buffer, but only a twofold increase in intensity was observed (Fig. 2a, compare lines 2 and 4). Likewise, the refolded preMBP mutants showed the typical blue shift, but their final fluorescence intensities differed and were markedly lower as compared with refolded preMBP (Fig. 2c). Interestingly, the T345I mutation that stabilizes the mature MBP folded state

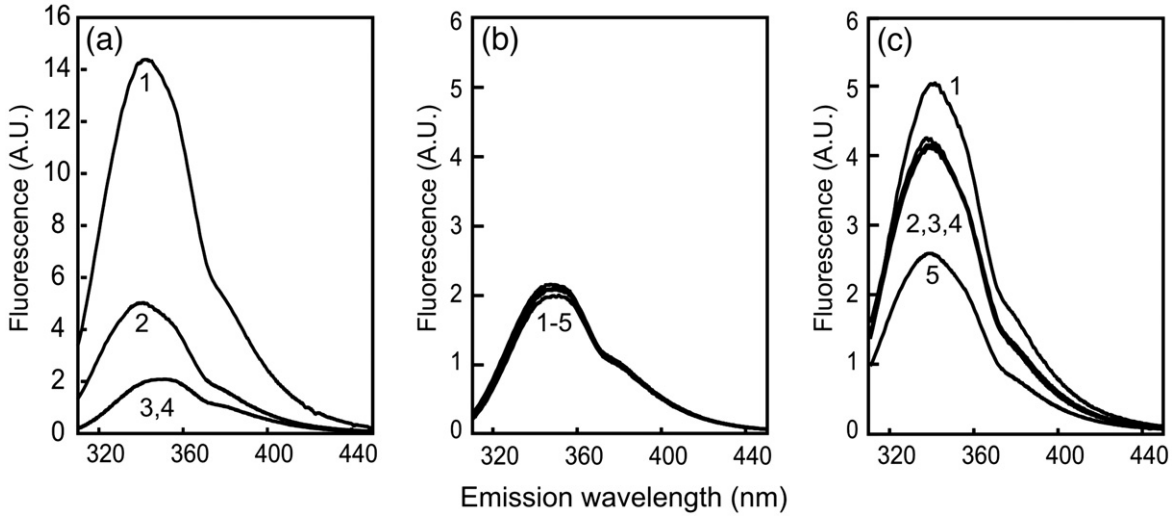

Fig. 2. Tryptophan fluorescence emission spectra of (pre)MBP derivatives. (a) Emission spectra of the refolded (lines 1 and 2) and GuHCl-denaturated (lines 3 and 4) mature and precursor forms of MBP, respectively. (b) Emission spectra of preMBP derivatives denaturated in 2.5 M GuHCl. (c) Emission spectra of refolded preMBP derivatives in 50 mM Hepes/KOH, pH 7.4, 50 mM KCl and 2.5 mM $MgCl_2$. Line 1, preMBP; line 2, preMBP(V8G); line 3, preMBP(A276G); line 4, preMBP(Y283D); and line 5, preMBP(T345I).

showed the lowest fluorescence intensity of all the mutants (Fig. 2c, line 5). These data demonstrate that the preMBP derivatives are all partially unfolded.

(Re)Folded mature MBP is resistant to degradation by proteinase K[19] (Fig. 3a). In contrast, preMBP is highly susceptible to proteinase K digestion as compared with MBP. The preMBP variants with folding destabilizing mutations (V8G, A276G and Y283D) were even more sensitive to proteinase K digestion than preMBP, while the derivative that is predicted to fold more stably (T345I) exhibits a proteinase sensitivity close to that of wild-type preMBP (Fig. 3a). The proteinase sensitivity decreased in the following order: A276G > V8G > Y283D ≫ T345I ≈ preMBP ⋙ MBP. When (pre)MBP was diluted into a buffer containing the molecular chaperone SecB, the precursor forms of the wild-type and mutant proteins showed an even further increase in proteinase sensitivity, while the protease resistance of the mature MBP was hardly affected (Fig. 3b). In

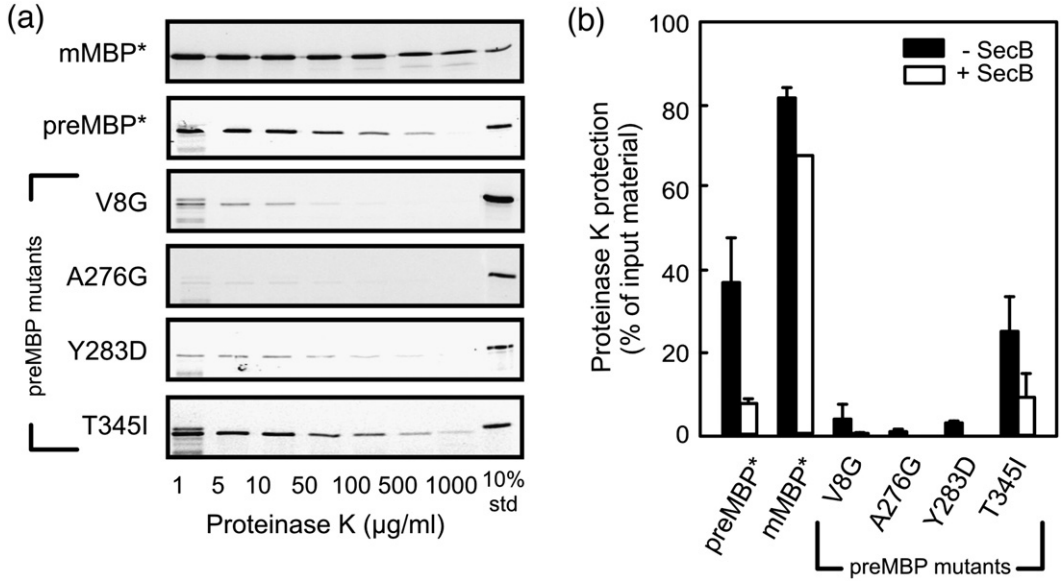

Fig. 3. Proteinase K sensitivity of refolded (pre)MBP derivatives. (a) Urea (6 M)-denaturated fluorescein-labelled proteins were diluted in 50 mM Hepes/KOH, pH 7.4, 50 mM KCl and 2.5 mM $MgCl_2$, incubated for 15 min and subsequently treated with proteinase K for 30 min at 4 °C. Protease-resistant (pre)MBP was analyzed by 12% SDS-PAGE and fluorescent imaging. Std: 10% of the input preMBP without protease K. (b) Quantification of the proteinase K (10 μg/ml)-resistant (pre)MBP derivatives in the presence (black bars) or absence (white bars) of SecB.

summary, the introduced mutations in the MBP mature domain affect folding of the precursor form, but these effects are relatively small as compared with the impact of the signal sequence and SecB on the folding. Moreover, in contrast to the effect on the folding in mature MBP, the T345I mutation does not stabilize the fold of preMBP but rather seems to destabilize it to some extent.

Translocation kinetics of folding mutants of the preMBP mature domain

To investigate the effect of the folding mutations on the kinetics and energetics of protein translocation, we performed *in vitro* translocation experiments with the purified fluorescein maleimide-labelled preMBP derivatives (Fig. 1b). To this end, preMBP was diluted 50 times from 6 M urea into a reaction mixture containing SecA, SecB and *E. coli* inner membrane vesicles (IMVs). Next, preMBP was allowed to fold for 5 min at 37 °C, and translocation was initiated by the addition of ATP in the absence or presence of NADH/succinate to generate a PMF. Non-translocated preprotein was removed by sedimentation and proteinase K treatment. Translocated (pre)MBP was monitored by SDS-PAGE and in-gel fluorescent detection. As noted previously, preMBP translocation is strictly dependent on the PMF[20] (Fig. 4a) and SecB[11,21] (Fig. 4b). A similar strict dependence was observed for the preMBP folding mutants. Next, the kinetics of translocation was monitored with a saturating concentration of preMBP (25 μg/ml; approximately four times above the apparent K_m value) in the presence of SecB, SecA and a PMF. Under these conditions, wild-type preMBP is rapidly translocated into IMVs (Fig. 4c). Strikingly, the folding mutants were translocated with kinetics essentially indistinguishable from that of wild-type preMBP (Fig. 4d). We therefore conclude that under substrate-saturating conditions, the folding status of preMBP is not rate determining for SecB- and PMF-dependent translocations.

SecA requirement for translocation of folding mutants of the preMBP mature domain

Previously, it has been suggested that more tightly folded preproteins require more SecA for translocation as compared with unfolded preproteins.[13] Translocation experiments with the various preMBP folding mutants were performed in the presence of different concentrations of SecA to further test this hypothesis. Below 100 nM SecA, the translocation rate of preMBP is limited by the SecA (Fig. 5) and decreases almost linearly with the SecA concentration. We did not detect any significant difference in the SecA dependence of preMBP and the various folding mutants (Fig. 5). Taken together, these data demonstrate that folding mutations in the mature

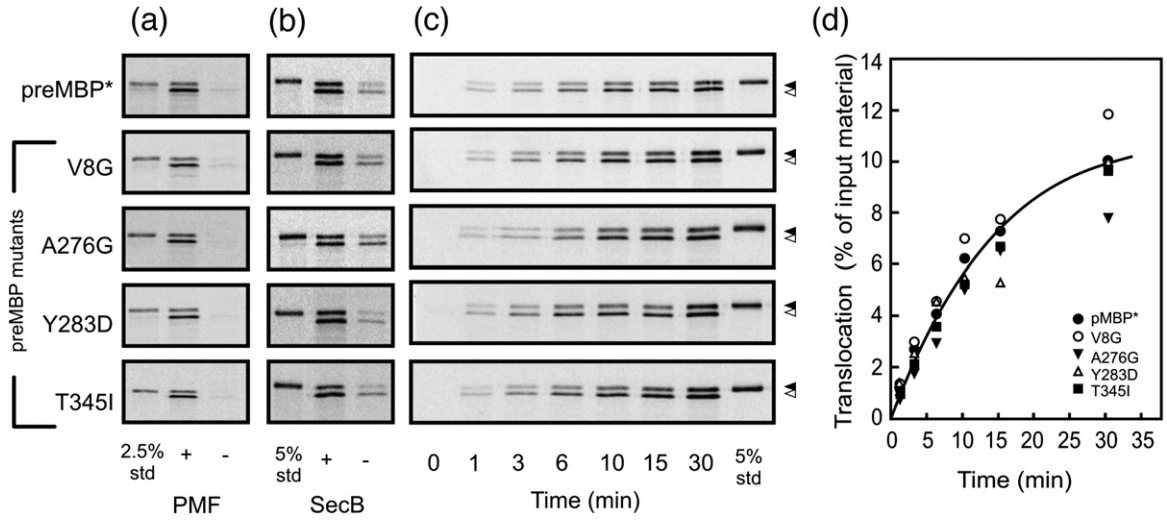

Fig. 4. *In vitro* translocation of wild-type and mutant (V8G, A276G, Y283D and T345I) preMBP derivatives into IMVs derived from *E. coli* strain SF100 with overexpressed levels of SecYEG. (a) PMF dependence of translocation. Translocation reactions were performed for 10 min at 37 °C with an ATP-regenerating system and in the absence (−PMF) or presence (+PMF) of oxidizable substrates (5 mM NADH and 10 mM succinate). Valinomycin and nigericin (2 μM each) were added to the mixture in the absence of a PMF to ensure its complete absence. (b) SecB dependence of translocation. Wild-type and mutant preMBPs were diluted into translocation buffer in the absence (−) or presence (+) of SecB. After 30 min at 37 °C, IMVs, SecA, ATP and a regenerating system and NADH/succinate were added. The samples without SecB were supplemented with SecB protein, and translocation was followed for 10 min at 37 °C. (c) Kinetics of translocation. Translocation reactions of preMBP derivatives (25 μg/ml) were performed in the presence of a PMF and 140 and 53 μg/ml of SecA and SecB, respectively. Black and white triangles represent the precursor form and the mature form of MBP, respectively. (d) Quantification of translocation kinetics as shown in (c). Wild-type preMBP (●), V8G (○), A276G (▼), Y283D (Δ) and T345I (■). The translocation efficiency is expressed as a percentage of input material. Std: 5% or 2.5% of the input preMBP. Translocation reactions were performed as described under Materials and Methods.

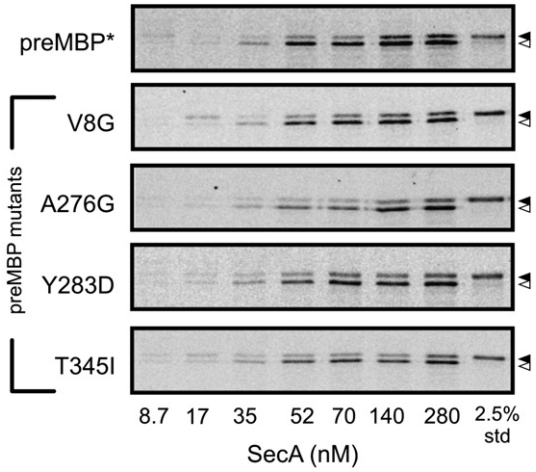

Fig. 5. SecA requirement for the translocation of wild-type and mutant preMBP proteins. Reactions were performed with IMVs pretreated with anti-SecA immunoglobulin G to remove the endogenously bound SecA. Translocation reactions were performed in the presence of various concentrations of SecA protein (0–280 nM) and initiated by the addition of ATP and NADH/succinate. Std: 2.5% of the input material. Black and white triangles represent the precursor form and the mature form of MBP, respectively.

domain of MBP have little impact on the SecA dependence of translocation.

Discussion

Preproteins are generally assumed to be translocated through the SecYEG channel in an extended conformation.[4,19,22] Two main strategies are utilized to promote and ensure an unfolded state prior to translocation. First, the signal sequence that specifies the targeting information slows the rate folding of the mature preprotein domain.[2] Second, the molecular chaperone SecB stabilizes preproteins in an unfolded state prior to translocation.[12,19] This unfolded state is characterized by a native-like secondary structure and a non-native-like tertiary structure, possibly reflecting a molten globule.[4,23] It has been suggested that SecB-bound preproteins still contain some stable tertiary structure and that this affects the kinetics of translocation, thus resulting in an increased requirement for protein unfolding by SecA.[13] This implies that the folding characteristics of the mature domain would be a dominant determinant over folding counteracting factors, such as the signal sequence, and molecular chaperones, such as SecB. However, a recent study with optical tweezers demonstrated that SecB-bound MBP has a structure that contains no stable tertiary element and is unfolded with virtually zero force.[14] Here we reinvestigated the folding behaviour and translocation kinetics of a set of mutants of MBP but, in addition to folding destabilizing mutations, also included a folding stabilizing mutant. The impact of the mutations in MBP on its folding has only been studied for the isolated mature domain[15] and not in the context of an associated signal sequence and/or in the presence of SecB. Our data now demonstrate that these mutations also affect the folding of preMBP but that these are overshadowed by a strong folding destabilizing effect of the signal sequence and SecB. Remarkably, a mutation that stabilizes the fold in the mature domain (T345I) seems to destabilize the fold in preMBP. Currently, we do not have an explanation for this phenomenon, but this observation is consistent with the notion that the signal sequence can have a major effect on the folding characteristics of the mature preprotein domain. Interestingly, despite their unfolded state, the preMBP mutants remained strictly SecB dependent for translocation. The latter may be a result of a critical role of SecB in targeting of preproteins to the translocase.[5,6] We did not observe any significant effect of the mature domain mutations on the kinetics of translocation. Likewise, the various mutants also did not differ significantly in their PMF and SecA dependence of translocation. This is at variance with the early observations of de Cock and Randall,[13] who suggested that the preMBP mutants Y283D and A276G are translocated much faster under SecA-limiting conditions as compared with wild-type preMBP. However, in this *in vitro* system, the wild-type and mutant proteins were synthesized in a cell-free translation system at different concentrations. Because of the complexity of the cytosolic lysate, one cannot exclude that chaperones other than SecB contribute to the translocation reaction. Moreover, instead of translocation, the rate of signal sequence processing was followed in time that does not necessarily have to match the kinetics of full translocation. Finally, the reduced translocation kinetics of the radiochemical amounts of wild type could be due to a more rapid loss of translocation competence as suggested by the reduced half-life of the SecB–preMBP complex in the absence of translocation. When saturating concentrations of the purified precursor are used, the translocase functions at maximal velocity and no difference in the kinetics of translocation are observed (Fig. 4d).

Our data on the folding of the precursor forms provide an explanation as to why stabilizing and destabilizing of the mature domain have little impact on the kinetics and energetics of preMBP translocation *in vitro*. Clearly, the presence of the signal sequence and that of SecB overrule the effects of the point mutations on the folding of the mature MBP domain. As the *in vitro* translocation of preMBP absolutely requires a functional signal sequence and SecB, it seems that for preMBP, unfolding of the mature domain is not a rate-determining step in the *in vitro* translocation reaction. Likewise, these mutants show an ATP requirement similar to that in wild-type preMBP.[14] It is of interest to note that the initial genetic screen to isolate translocation-restoring mutations in the mature domain was performed with preMBP mutants with a defective signal sequence.[15,24] Apparently, *in vivo*, this secretion defect can partially be overcome by mutations in the

mature domain that slow folding. It should be stressed, however, that the genetic screen scored for rescuing the growth-defective phenotype of cells grown on maltose as a carbon source. Since only very small amounts of translocated MBP suffice to compensate for this growth-negative phenotype, the exact impact of such mutations may be difficult to access both *in vivo* and *in vitro*. Indeed, an analysis of the effect of the signal sequence mutations used to isolate the MBP folding mutants (i.e., A14E and A14P) showed little effect on the folding of preMBP (unpublished data). Also, the introduction of the translocation defect-suppressing mutation in the mature domain (A276G) in combination with the two defective signal sequence mutants did not restore translocation *in vitro* (unpublished data). This indicates that the mechanism of suppression is complex and possibly subtle, which makes it difficult to reproduce these *in vitro*.

Taken together, our data show that the signal sequence and the binding of the molecular chaperone SecB largely determine the folding characteristics of preMBP. In this context, folding stabilizing and destabilizing mutations in the mature domain seem to contribute only marginally to the folding of preMBP, although they have pronounced effects on the folding of the mature MBP. Overall, these mutations do not significantly influence the kinetics and energetics of the *in vitro* translocation of preMBP. In contrast to our *in vitro* experiments, *in vivo*, the translocase might not function at maximum speed. Under such sub-saturating conditions, some preMBPs might be translocated co-translationally by the translocase; in this context, the folding status of the mature domain might influence the translocation kinetics. It should be stressed, however, that also *in vivo*, translocation of preMBP is strongly dependent on SecB. Therefore, unfolding of the mature MBP domain prior to translocation through the SecYEG channel in the posttranslational SecB-dependent targeting route does not seem to be a major rate-determining step during translocation. Since not all secretory proteins are dependent on SecB for translocation, the question on whether translocation of SecB-independent precursors is more energy demanding than SecB-dependent translocation arises. In this respect, the SecB-independent translocation of a fusion protein of proOmpA with the stably folded titin domain showed an increased demand for ATP hydrolysis and slower translocation kinetics as compared with the stably unfolded titin domain.[10] Therefore, it would be of interest to analyze the effects of folding mutations on the translocation of SecB-independent substrates.

Materials and Methods

Bacterial strains and materials

E. coli strain BL21.1 [BL21(λDE3) *leu*::Tn10 *secA51* (Ts)][25] was used to overproduce wild-type preMBP and derivatives. SecA[26] and His-tagged SecB[27] were purified as previously described. IMVs were isolated from *E. coli* strain SF100 [F-, Δ*lacX74*, *galK*, *thi*, *rpsL*, *strA* Δ*phoA*(*pvuII*), Δ*ompT*][28] or NN100 [SF100, Δ*(uncB–C) zid*::Tn10][29] as previously described.[30] SF100 and NN100 cells were transformed with plasmid pET605[30] and grown in the presence of 0.5 mM IPTG to obtain IMVs containing high levels of SecYEG. IMVs (200 μg) were incubated for 1 h with 30 μl of polyclonal SecA antibody under constant mixing to remove endogenously bound SecA from the membrane. Subsequently, IMVs were sedimented (20 min, 80,000 rpm, TLA 120.1 rotor) through a sucrose cushion (25% sucrose, 50 mM Tris–Cl, pH 8.0, and 1 mM DTT), washed with 50 mM Tris–Cl, pH 8.0, and after a second centrifugation step resuspended into 50 mM Tris–Cl, pH 8.0, and 20% glycerol. SecA-stripped IMVs were stored in small aliquots at −80 °C.

Table 1. Plasmids used in this study

Plasmid	Mutation	Codon	Name
pNN226	–	–	WT
pNN227	A51C	GCG/TGC	WT*
pEK215	A51C, V8G	GTA/GGC	V8G
pEK216	A51C, A276G	GCG/GGG	A276G
pEK217	A51C, Y283D	TAT/GAT	Y283D
pEK218	A51C, T345I	ACC/ATC	T345I

Introduction of single mutations into the *malE* gene

The *malE* gene was cloned by PCR with the addition of an NdeI site at the N-terminus (CCAACAAGGACCATAG-CATATGAAAATAAAAACAGGTGC) and an HindIII site at the C-terminus (CGCATCCGGCATTTCACAAGCTT-ACTTGGTGATACGAGTC). Newly created restriction sites are underlined. After digestion with NdeI and HindIII, the *malE* gene was ligated into the NdeI/HindIII sites of pET3a (pNN226). A unique cysteine residue was introduced at position 51 of the mature domain using a QuikChange™ site-directed mutagenesis kit (Stratagene), resulting in plasmid pNN227. This plasmid was used to introduce point mutations into the MBP mature domain (Table 1).

Purification and labelling of preMBP and its derivatives

PreMBP and derivatives were overproduced at 30 °C in *E. coli* BL21.1 [BL21(λDE3) *leu*::Tn10 *secA51*(Ts); a kind gift of Dr. D.B. Oliver, Wesleyan University].[25] After harvesting the cells, we isolated inclusion bodies as previously described[31] and solubilized them in buffer A (50 mM Tris–Cl, pH 9.0, and 6 M urea). Proteins were purified by anion exchange chromatography (HiTrap Q Sepharose column, Amersham Pharmacia Biotech) in buffer A. PreMBP-containing fractions were combined and dialysed against buffer B (50 mM bis-Tris propane, pH 7.4, and 6 M urea). Mature MBP was overproduced at 30 °C in *E. coli* BL21(DE3), isolated from the periplasm and purified by means of an amylose resin according to the manufacturer's instructions (New England BioLabs). Fractions containing pure mature MBP were combined and concentrated using a Centriprep YM-50 column (Amicon Bioseparations).

Fluorescent (pre)MBP derivatives were obtained by labelling the unique cysteine residue (C51) with fluorescein maleimide (Molecular Probes).[16] Proteins were stored in small aliquots at −80 °C.

Tryptophan fluorescence and protease digestion

Purified (pre)MBP or derivatives thereof in buffer B were diluted 100 times to a final concentration of 0.6 μM into buffer C (50 mM Hepes/KOH, pH 7.4, 50 mM KCl and 2.5 mM $MgCl_2$) with or without 2.5 M GuHCl (Fluka). Following 7 min of equilibration at room temperature, the tryptophan fluorescence emission spectra were recorded (excitation wavelength, 295 nm; band pass, 4 nm) using an SLM Aminco Bowman® Series 2 luminescence spectrometer. Absorbance spectra of the same samples were recorded on a Varian Cary 100 UV-visible spectrophotometer and used to correct for small differences in protein content (tryptophan emission spectra of all samples were standardized to an OD_{280} of 0.05).

Protease digestion of fluorescein-labelled (pre)MBP derivatives (25 μg/ml) was performed in buffer C with or without SecB (53 μg/ml). After 15 min of preincubation at 37 °C, proteinase K (final concentration, 0–1 mg/ml) was added and the reaction mixture was incubated for 30 min on ice. Reactions were stopped by adding PMSF; samples were trichloroacetic acid precipitated and analyzed by 12% SDS-PAGE and in-gel fluorescence using a Roche Lumi Imager F1 (Roche Molecular Biochemicals).

In vitro translocation

Translocation into IMVs was performed in buffer D (50 mM Hepes/KOH, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 2 mM DTT and 0.1 mg/ml of bovine serum albumin) containing 53 μg/ml of SecB, 140 μg/ml of SecA and 500 μg/ml of IMVs. When necessary, the translocation mix was supplemented with an energy mix (10 mM creatine phosphate and 50 μg/ml of creatine kinase), 10 mM NADH and 5 mM succinate. Typically, urea-denatured preMBP (25 μg/ml) was added to the above mixture; after 5 min at 37 °C, translocation was started by addition of ATP (final concentration, 1 mM). Reactions were stopped by chilling in an ice-water bath. The reaction mixture was layered on a 200-μl sucrose cushion (0.2 M sucrose, 50 mM Hepes/KOH, pH 7.5, 50 mM KCl and 2.5 mM $MgCl_2$) and centrifuged for 30 min at 70,000 rpm in a TLA 120.1 rotor at 4 °C to remove protease-resistant non-translocated preMBP. The IMVs were resuspended in 50 μl of buffer C and treated with proteinase K (0.1 mg/ml). After 30 min on ice, the protease was inactivated with PMSF (final concentration, 1 mM) and the protease-protected material was precipitated with 5% trichloroacetic acid, washed with ice-cold acetone and analyzed by 12% SDS-PAGE and in-gel fluorescence using a Roche Lumi Imager F1 (Roche Molecular Biochemicals).[16] Nigericin and valinomycin (final concentration, 2 μM) were added to the translocation reaction to dissipate the PMF.

Other techniques

Protein concentrations were determined using a DC protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

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