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Bacterial Sec-translocase Unfolds and Translocates a Class of Folded Protein Domains

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It is generally assumed that preprotein substrates must be presented in an unfolded state to the bacterial Sec-translocase in order to be translocated. Here, we have examined the ability of the Sec-translocase to translocate folded preproteins. Tightly folded human cardiac Ig-like domain I27 fused to the C terminus of proOmpA is translocated efficiently by the Sec-translocase and the translocation kinetics are determined by the extent of folding of the titin I27 domain. Accumulation of specific translocation intermediates around the fusion point that undergo translocation progress upon ATP binding suggests that the motor protein SecA plays an important and decisive role in promoting unfolding of the titin I27 domain. It is concluded that the bacterial Sec-translocase is capable of actively unfolding preproteins.

Keywords: protein translocation; folding; SecA; SecY

Introduction

The function of proteins is determined by their native folded conformation. In bacteria, proteins that act outside the cytosol need to be translocated across (at least) the cytoplasmic membrane to reach their final destination. The major route for protein translocation in these organisms is the so-called Sec-pathway.[1] Secretory proteins are synthesized in the cytosol as precursors (preproteins) with an N-terminal extension, the signal sequence. During or shortly after the synthesis of the preprotein in the Gram-negative bacterium *Escherichia coli*, the molecular chaperone SecB binds to the mature region of some polypeptide chains, thereby stabilizing them in an unfolded conformation. In addition, SecB targets a subset of preproteins post-translationally to the Sec-translocase in the cytoplasmic membrane. The central part of the translocase consists of a protein-conducting channel, SecYEG, and a motor protein, SecA, located in the cytoplasm. Upon binding of the SecB-precursor complex to SecYEG-bound SecA, the ATPase activity of SecA is activated and multiple cycles of ATP binding and hydrolysis by SecA result in a stepwise translocation of the preprotein through the SecYEG pore. During or shortly after translocation, the signal sequence is cleaved off by leader peptidase, allowing the release of the mature protein into the periplasm.

In vivo and *in vitro* studies in *E. coli* have demonstrated that the presence of large stably folded (sub-) domains in preproteins inhibit or block translocation. Unless directed to the co-translational translocation route, fusion proteins containing β-galactosidase block the translocase when expressed at high levels *in vivo*.[2–4] *In vitro*, preproteins containing disulfide-bridged loops of up to 16 amino acid residues can be transported,[5] but larger loops or C-terminally fused stably folded proteins such as bovine pancreatic trypsin inhibitor and methotrexate/NADPH-bound dihydrofolate reductase (DHFR) block translocation, resulting in the generation of arrested translocation intermediates.[5–7] On the basis of these findings it is generally accepted that preproteins need to be presented to the Sec-translocase in an almost completely unfolded conformation in order to be threaded through the SecYEG channel as a linear polypeptide chain.

Most newly synthesized and ribosome-released polypeptides or polypeptides unfolded in urea and diluted into buffer assume secondary structure within

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Abbreviations used: AFM, atomic-force microscopy; DHFR, dihydrofolate reductase; IMV, inner membrane vesicle; PMF, proton motive force.

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a time-frame of milliseconds, while formation of tertiary structure requires often only seconds.[8] *In vitro*, preprotein substrates are usually first unfolded by a high concentration of urea, whereupon they are translocated readily post-translationally upon dilution from urea. Nevertheless, it is assumed that preproteins remain unstructured until they are translocated across the cytoplasmic membrane. First, signal sequences have been shown to retard the folding rate of preproteins.[9,10] Second, binding of SecB to the precursor form of maltose binding protein (MBP) has been shown to prevent folding of MBP into a compact, protease-resistant, native-like structure that cannot be translocated.[11,12] Third, mutations in the mature domain of MBP that inhibit folding enhance the translocation of this protein.[13] However, in contrast with the traditional paradigm, preproteins in complex with SecB have been shown to contain substantial secondary and tertiary structure,[14,15] while many secretory proteins do not utilize the SecB chaperone for targeting and stabilization of the unfolded state. This indicates that these proteins are at least partially folded before translocation.

To address to what extent the translocase is able to unfold and translocate folded proteins, we fused the immunoglobulin-like domain I27 from human cardiac titin to the extreme C terminus of the model preprotein proOmpA. We chose the titin I27 domain, as its mechanical unfolding characteristics have been studied extensively by atomic-force microscopy (AFM),[16,17] and because the I27 domain can readily and reversibly be unfolded and re-folded into the native structure *in vitro*.[18] Moreover, the folding state of the I27 domain can be simply distorted by chemical modification of two cysteine residues in the polypeptide chain.[19] Here, we show that the translocase is able to efficiently translocate the folded titin Ig-like domain I27 and that the translocation kinetics are determined by the extent of folding of the domain as presented to the translocase. These data demonstrate that the translocase is capable of actively unfolding preprotein substrates.

Results

The titin Ig-like domain I27 fused to proOmpA is translocated *in vivo*

To investigate to what extent the Sec-translocase is able to translocate tightly folded proteins, we constructed a fusion protein in which the human cardiac titin I27 domain is fused to the C terminus of proOmpA. The I27 domain has a simple immunoglobulin fold (Figure 2(a)) with a buried single tryptophan residue that can be used as a fluorescent measure of the folding state. To monitor folding of the I27 domain in the chimeric protein, the domain was fused to a proOmpA derivative in which the five endogenous tryptophan residues were replaced by tyrosine.[20] Moreover, the two cysteine residues in proOmpA were replaced by serine. Consequently, the two cysteine residues and the single tryptophan residue in the I27 domain are unique in the chimeric proOmpA-I27 protein. To facilitate purification, a His_6 tag was added to the C terminus of the I27 domain. When expressed in *E. coli* strain UH203, proOmpA-I27 co-fractionates with the outer membrane fraction in a sucrose density gradient (Figure 1, lane 3). Treatment of proOmpA-I27 in the outer membrane fraction with trypsin resulted in the formation of a ~20 kDa and ~14 kDa protease-protected fragment (Figure 1, lane 4), corresponding to the membrane inserted β-barrel domain of OmpA and the folded I27 domain, respectively. This indicates that OmpA-I27 is assembled correctly into the outer membrane and, consequently, that the titin I27 domain is translocated across the cytoplasmic membrane *in vivo*.

The titin Ig-like domain I27 fused to proOmpA folds correctly upon dilution from urea

In vivo the titin I27 domain could be translocated co-translationally or chaperones present in the cytoplasm could prevent the folding of the titin I27 domain before translocation across the cytoplasmic membrane. As these possibilities are difficult to address *in vivo*, we decided to analyse the folding

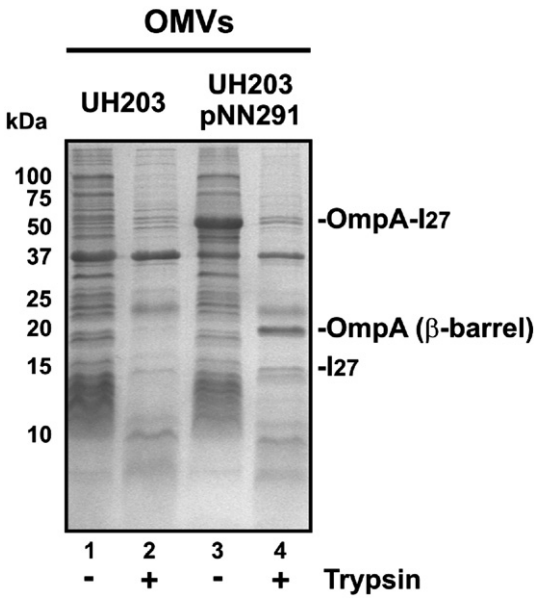

Figure 1. ProOmpA-I27 is assembled into the outer membrane *in vivo*. *E. coli* UH203 cells expressing proOmpA-I27 were lysed by French press and outer membrane vesicles were isolated by sucrose density centrifugation. Outer membrane vesicles were incubated for 15 min at 37 °C in the presence or in the absence of 1.25 mg/ml of trypsin. After inactivation of the protease with PMSF (0.1 mM final concentration) the samples were resuspended in SDS-PAGE sample buffer. Samples were analysed by SDS-PAGE (12% (w/v) polyacrylamide gel) and staining with Coomassie brilliant blue. Outer membrane vesicles from wild-type *E. coli* UH203 cells were used as a control.