Inhibition of Translocation of $\beta$-Lactamase into the Yeast Endoplasmic Reticulum by Covalently Bound Benzylpenicillin*

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We found recently that $\beta$-lactamase folds in the yeast cytosol to a native-like, catalytically active, and trypsin-resistant conformation, and is thereafter translocated into the ER and secreted to the medium. Previously, it was thought that pre-folded proteins cannot be translocated. Here we have studied in living yeast cells whether $\beta$-lactamase, a tight globule in authentic form, must be unfolded for ER translocation. A $\beta$-lactamase mutant (E166A) binds irreversibly benzylpenicillin via Ser\textsuperscript{36} in the active site. We fused E166A to the C terminus of a yeast-derived polypeptide having a post-translational signal peptide. In the presence of benzylpenicillin, the E166A fusion protein was not translocated into the endoplasmic reticulum, whereas translocation of the unmutated variant was not affected. The benzylpenicillin-bound protein adhered to the endoplasmic reticulum membrane, where it prevented translocation of BiP, carboxypeptidase Y, and secretory proteins. Although the 321-amino acid-long N-terminal fusion partner adopts no regular secondary structure and should have no constraints for pore penetration, the benzylpenicillin-bound protein remained fully exposed to the cytosol, maintaining its signal peptide. Our data suggest that the $\beta$-lactamase portion must unfold for translocation, that the unfolding machinery is cytosolic, and that unfolding of the remote C-terminal $\beta$-lactamase is required for initiation of pore penetration.

Depending on the hydrophobicity of the signal peptide, newly synthesized polypeptides are translocated into the yeast endoplasmic reticulum (ER)\textsuperscript{1} either during translation or after completion of translation and release from the ribosomes (1). Post-translational and co-translational translocation occur through heterotrimeric Sec61p complexes, which are composed of subunits Sec61p, Sbh1p, and Sss1p. Sec61p spans the ER membrane 10 times and forms an aqueous pore. In addition to the heterotrimeric translocon complex, post-translational translocation requires also the Sec62–63 subcomplex (7). The release of the translocation substrate from the subcomplex is mediated by BiP and its co-chaperone Sec63p, and requires ATP (8). Thereafter, the signal peptide intercalates into transmembrane domains 2 and 7 of Sec61p, perhaps opening the pore, whereafter passage of pre-pro-$\alpha$-factor proceeds in an ATP- and BiP-dependent manner (9).

Events preceding these steps have been much less studied. It has been thought that cytosolic Hsp70s bind to completed pre-pro-$\alpha$-factor proteins to prevent them from folding and to keep them in a translocation-competent form (10, 11). However, we showed recently that newly synthesized Escherichia coli RTEM-1 $\beta$-lactamase folded to a native-like, catalytically active, and trypsin-resistant conformation in the cytosol of Saccharomyces cerevisiae. Thereafter, it was translocated into the ER lumen and secreted in active form to the medium (12). $\beta$-Lactamase was expressed as a chimeric protein, fused to a yeast-derived polypeptide (Hsp150\textsubscript{37}) having a signal peptide conferring post-translational translocation. The crystal structure of compact RTEM-1 $\beta$-lactamase is a tight two-domain globule measuring $32 \times 37 \times 53$ Å (13), whereas the 321-amino acid-long N-terminal Hsp150\textsubscript{37} fragment occurs as a random coil (14). As the translocon pore has been estimated to be able to enlarge to a maximal width of 60 Å (15), folded $\beta$-lactamase could traverse the pore without unfolding. Here we show that benzylpenicillin, which was irreversibly bound to a mutated $\beta$-lactamase portion, prevented ER translocation of the fusion protein at a stage preceding signal peptide cleavage, suggesting that unfolding by a cytosolic machinery was required for initiation and completion of pore penetration.

EXPERIMENTAL PROCEDURES

Strain Construction—The DNA fragment coding for the $\beta$-lactamase mutants E166A or E166D was PCR-amplified with $\phi 29$ polymerase (Stratagene) using wild type $\beta$-lactamase gene in plasmid pKTH4539 (16) as a template, and oligonucleotides MS2 (5'-TTCTGAGCAGGCTACCTC), 92395 (5'-GACCGTGGCCAGGAGCT), 92396 (5'-AGCTCGGGTCCCAACGA), and 92397 (5'-ATCGTTGGGCACCGGAGCT), 92398 (5'-GCAAGACGTGGAGTAACTTGGTCTGACAG) for the E166A mutant, and oligonucleotides MS2, 92397 (5'-GACGGTGCTCCACGAGCT), 92398 (5'-TCTGTTTGGACCGGAGCGT), and 82629 (5'-AGCTCCG-TTCCTTCGCGAC) for the E166D mutant. The mutations were digested with KpnI-HindIII and cloned into plasmid pKTH4539 to replace the wild type gene. The plasmids were named pKTH4825 (E166D) and pKTH4826 (E166A) and the mutations verified by sequencing. The BamHI fragments containing the mutated HSP150-$\beta$-lactamase-ADIC\textsubscript{1} terminator cassettes were cloned into plasmid pFL34, designated pKTH4828 (E166D) and pKTH4830 (E166A), and into plasmid pFL26, designated pKTH4829 (E166A). The mutant $\beta$-lactamase gene (E166A) with an His\textsubscript{6} tag was created by PCR using plasmid pKTH4829 as a template, and MS2 and C2500 (5'-

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1 The abbreviations used are: ER, endoplasmic reticulum; PCR, polymerase chain reaction; CPR, carboxypeptidase Y; CAPS, (3-cyclohexyl-aminopropanesulfonic acid; PenG, penicillin G; CLX, claxocin; CHX, cycloheximide; PAGE, polyacrylamide gel electrophoresis.

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\textsuperscript{2}We named Kar2p in yeast (2–6). By chemical cross-linking of the yeast endoplasmic reticulum (ER), plus the soluble ER chaperone BiP, also
TTAAGCCTTAGTGAATGATGTAGTTGCAATCCTAATCAGT) as primers. The PCR fragments were digested with KpnI and HindIII and ligated to pKT4539. The resulting plasmid pKT4956 was verified by sequencing. The BamHI fragment containing the HSP150β-lactamaseE166A-His6-ADC1 terminator cassette was cloned into pFL26, resulting in plasmid pKT4960, which was transformed to yield strain H1248. The DNA fragment of HSP150β-lactamase-lactamase lacking the signal peptide codons was PCR-amplified using pKT4539 as template, and oligonucleotides 82629 and 82239 (5'-ATAAGCTTAGTGATGGTGATGGTGATGCCAATGCTTAATCAGT) as primers. The product was digested with NsiI and HindIII (Promega) and ligated to plasmid pKT4700 containing the HSP150 promoter and the ADH1 terminator to produce plasmid pKT4716. The XhoI-Hael fragment of pKT4716 with the truncated Δ1-18HSP150β-lactamase fragment with promoter and terminator sequences was cloned into pFL26 to produce plasmid pKT4757, which was transformed to Sey2101a (R. Schekman) to produce strain H977. The Nhel-KpnI fragment derived from pKT4716 containing the truncated version of the HSP150 gene lacking the signal sequence and flanked by the HSP150-terminator cassette and with the pKTH4539 fragment of pKTH4828 containing the β-lactamaseE166A mutant gene flanked by the ADC1 terminator, were successively cloned into pFL34, to create pKT4996 containing the signal sequence-less Hsp150β-lactamase E166A mutant (Δ1-18E166A), pKT4544 (16), pKT4830, pKT4828, and pKT4995 were transformed into CJY004 (17) to produce strains H987, H1045, H1046, and H1376, respectively (Table I). Yeast cells were grown overnight, in synthetic complete medium lacking Ura, Trp, His, Ade, and Thr, and mature (145 kDa) forms (12).

N-terminal Sequencing of E166A-His6—Cells (2 liters, optical density of 1) treated with benzyloxycarbonyl (5 mg/ml) were lysed with a Glass-Beater (BioSpec) in 30 ml of Tris-HCl, pH 8.0, containing 300 mM NaCl, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 100 µl of Yeast Protease Inhibitor Mixture (Sigma). The lystate was clarified by centrifugation at 10,000 × g for 50 min at 4 °C, and the supernatant was added to 1 ml of 250 mM NaCl and the pH adjusted to 8.0. The lystate (80 ml) was mixed with 1 ml of Ni2+-nitrilotriacetic acid-agarose (Qiagen) overnight at 4 °C. Further procedures were at room temperature. The resin was loaded into a 2-ml column and washed successively with 5-ml buffers B, C, D, and E, which consisted of 8 mM urea, 100 mM NaH2PO4, and 20 mM Tris, the pH of which was 8.0, 6.3, 5.9, and 4.5, respectively. The final wash was with 2 ml of discharging buffer (100 mM EDTA, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0). Samples of the flow-through and 1-ml fractions were analyzed by SDS-PAGE (7.5%) and Western blotting using His6 antibody (Qiagen). The second flow-through and the 1-ml fractions were analyzed by SDS-PAGE (8%) gels if not otherwise stated. Penicillin G (PenG), cloxacinil (CLX), and cycloheximide (CHX) (Sigma) were used at final concentrations of 2, 2, and 0.1 mg/ml, respectively, unless otherwise stated. Labeling with benzyl[14C]penicillin (57 mCi/mmol; Amersham Biotech) was with 2 µCi/0.5 ml of cell suspension.

**RESULTS**

**Covalently Bound Penicillin G Prevents Translocation of Hsp150Δ-β-lactamaseE166A—**E. coli RTEM-1 β-lactamase was fused to the C terminus of Hsp150Δ, an N-terminal portion of 321 amino acids of the secretory yeast glycoprotein Hsp150 (18). The Hsp150 signal peptide of 18 amino acids confers post-translational translocation (12). The fusion protein is designated Hsp150Δ-β-lactamase (or βla for short). The Hsp150Δ fragment has 106 serine and threonine residues, most of which acquire in the ER single mannose residues that are elongated in the Golgi (14, 10), allowing distinction of the cytoplasmic (66 kDa), ER (110 kDa), and mature (145 kDa) forms (12). Hsp150Δ-β-lactamase consists mostly of 11 repeats of a 19-aminoc acid peptide, which do not adopt any regular secondary structure, as determined for the glycosylated protein by CD spectroscopy and for the synthetic unglycosylated consensus peptide by NMR spectrometry (14). Thus, conformational restrictions that would require unfolding before ER translocation should be due to the β-lactamase portion only.

When glutamate 166 of RTEM-1 β-lactamase is exchanged...
into alanine, the enzyme becomes deacylation-defective, and PenG is covalently and irreversibly bound to Ser270 of the active site (21). We fused the E166A-\(\beta\)-lactamase variant (data not shown), as shown previously for native cytosolic \(\alpha\la\) samples, indicated collectively by asterisks. \(\beta\la\), \(\beta\)-lactamase.

![Fig. 2. \(\beta\)-Lactamase activity. Strains H335 (A, Hsp150\(\Delta\)-\(\beta\)-lactamase in WT background), H987 (B and D), Hsp150\(\Delta\)-\(\beta\)-lactamase in \(\Delta\)erg6 background), and H1045 (C, E166A variant in \(\Delta\)erg6 background) were incubated at 37 °C in the absence (circles) or presence (squares) of PenG as indicated. Intracellular (IN, open symbols) and extracellular (EX, filled symbols) \(\beta\)-lactamase activity was determined and plotted against incubation time. In C, no activity was detected in any of the samples, indicated collectively by asterisks. \(\beta\la\), \(\beta\)-lactamase.

![Fig. 3. Localization of PenG-bound E166A. A, the strains (H1045 in lanes 1 and 2, H482 in lanes 3 and 4, H393 in lanes 5 and 6) were incubated for 1 h at 37 °C. The cytosolic (c) and microsomal (m) fractions were separated and subjected to SDS-PAGE and Western analysis using \(\beta\)-lactamase (\(\beta\la\)) antiserum. Molecular mass markers (M) are indicated on the left and fusion protein variants on the right (c, cytosolic fraction; m, microsomal fraction). B, strains H1045 (a and b) and H1376 (c; signal peptide-less E166A in \(\Delta\)erg6 background) were incubated for 30 min at 37 °C in the presence of PenG, followed by immunofluorescent staining with \(\beta\)-lactamase (a and c) or Lhs1p (b) antiserum.

\(\beta\)-lactamase completely (Fig. 2C, asterisks). In WT (Fig. 2A) or \(\Delta\)erg6 (Fig. 2B) cells expressing native Hsp150\(\Delta\)-\(\beta\)-lactamase, catalytic activity increased in the medium (EX) and some remained intracellular (IN) similarly in the absence (circles) and presence (squares) of PenG. Thus, PenG did not effect translocation, folding, or secretion of native Hsp150\(\Delta\)-\(\beta\)-lactamase. We conclude that, when bound to PenG, the E166A fusion protein was unable to translocate into the ER lumen.

**ER Association of PenG-bound E166A**—Next we studied whether cytosolic PenG-bound E166A was attached to the ER membrane. PenG-bound E166A was accumulated for 30 min in H1045 cells (E166A/\(\Delta\)erg6), the cells were lysed under mild detergent conditions, and the microsomal membranes were isolated. The cytosolic and membrane fractions were subjected to SDS-PAGE and Western analysis using \(\beta\)-lactamase antiserum. Most of the 66-kDa form was detected in the microsomal fraction (Fig. 3A, lane 2) and some in the soluble fraction (lane 1). The sec63–1 and sec18–1 mutants expressing native
A mature CPY; pre-CPY, untranslocated CPY. In the case of Aci, precipitated with antiserum against BiP (lane 1), and the precipitate resolved by SDS-PAGE, followed by autoradiography.

Hsp150Δ-β-lactamase served as controls. At 37 °C in sec63–1 an early step of translocation is blocked, leading to cytosolic accumulation of pre-proteins and in the mutant membrane traffic is halted before arrival in the Golgi (22, 23). In both mutants most of the 66-kDa form was pelleted with the microsomes (lanes 4 and 6). In sec18–1 part of the Hsp150Δ-β-lactamase pool had been translocated and could be visualized as the glycosylated 110-kDa form (lane 6). The 62-kDa form probably was an artifactual degradation product rather than a biosynthetic intermediate, because the signal peptide-less form runs like a 64-kDa protein and no 62-kDa form was found after metabolic labeling (see below). Moreover, the 62-kDa form was found in lysates of the sec63–1 mutant, where no signal peptide cleavage should occur, and even in the cytosolic fraction (lane 1). H1045 cells (E166A/Δerg6) were then incubated with PenG and subjected to immunofluorescent staining using β-lactamase antiserum. Mostly the plasma membrane was stained (Fig. 3B, a). In yeast cells the ER is mostly located beneath the plasma membrane, as shown in Fig. 3B (b), where another sample of H1045 cells was stained with antiserum against Lhs1p, an ER-resident protein (24). As control we used the E166A mutant lacking a signal peptide, expressed in a Δerg6 background (strain H1376) in the presence of PenG. This variant was not membrane-associated, as it stained the entire cytosol (Fig. 3A, c). Thus, PenG-bound E166A was mostly attached to the ER membrane in a signal peptide-dependent fashion.

PenG-bound E166A Is Associated with Translocons—Next we showed that PenG-bound E166A molecules blocked translocation of other precursor proteins. First we used as markers BiP and CPY, mostly translocated during and after translation, respectively (1). Unlabeled PenG-bound E166A was allowed to accumulate for different times in H1045 cells (E166A/Δerg6), whereafter the cell samples were 35S-labeled, lysed, and immunoprecipitated with BiP antiserum. Before PenG treatment, only mature BiP was detected (Fig. 4A, lane 1). With PenG preincubation, pre-BiP started to accumulate (lane 2), until after 60 min less than half of the newly synthesized molecules were mature, and thus translocated (lane 3). The sec18–1 mutant, where mature BiP accumulates (lane 4), and the kar2–159 mutant, where translocation of pre-BiP is partially blocked (lane 5) (2), served as controls. In the H1045 cells, the ER form of vacuolar carboxypeptidase Y (pro-CPY or p1) could be immunoprecipitated after a 5 min pulse (Fig. 4B, lane 1), and mature CPY (m) after a 30-min chase (lane 3). After preincubation with PenG of the same cells, mostly untranslocated pre-CPY, and some pro-CPY form were detected (lane 2). In control cells (H1) in the absence of PenG, a 2-min pulse revealed pre-CPY and pro-CPY (lane 4), which were converted to the golgi form p2 and mature CPY upon a 15-min chase (lane 5). In the sec63–1 mutant, CPY fails to be translocated, revealing pre-CPY (lane 6), and pro-CPY (p1) accumulates in the sec18–1 mutant (lane 7).

Next, H1045 cells (E166A/Δerg6) were preincubated with PenG for different times, followed by labeling with [35S]methionine/cysteine for 1 h in continuous presence of the drug (Fig. 5A). The culture supernatants were trichloroacetic acid-precip-
itated and resolved in SDS-PAGE. In the absence of the drug, E166A together with several other proteins were detected (lane 1). The identity of E166A was confirmed by immunoprecipitation of a parallel sample β-lactamase antiserum prior to SDS-PAGE analysis (lane 4). After 30 min with PenG, very little if any E166A was detected, whereas other proteins still appeared in the medium (lane 2). After 1 h of PenG preincubation, no 35S-labeled proteins could be detected in the medium (lane 3). This must have been due to inhibition of their translocation, as PenG did not decrease protein synthesis. Incorporation of [35S]methionine/cysteine into trichloroacetic acid-precipitable material was after 3 h as efficient as in the absence of the drug (Fig. 5B). Since pre-accumulated PenG-bound E166A inhibited translocation of an ER-resident protein (BiP), a vacuolar enzyme (CPY), and a number of secretory proteins, it must have been engaged with ER components required for both co-translational and post-translational translocation.

Cytosolic Exposure of Ligand-bound E166A—We then asked how far the Hsp150Δ portion of PenG-bound E166A had advanced into the ER lumen, by examining signal peptide cleavage. The signal peptide appeared not to be cleaved, since PenG-bound E166A migrated in SDS-PAGE (Fig. 6, lane 1) like native pre-Hsp150Δ-β-lactamase blocked in the cytosol before pore penetration in the sec63Δ mutant (lane 3). The cytosolic signal peptide-less Hsp150Δ-β-lactamase variant served as a control; it migrated slightly faster (lane 2) than PenG-bound E166A. These data were confirmed by direct amino acid sequencing. An E166A fusion protein variant with a C-terminal histidine tag, E166A-His6, was expressed in a Δerg6 mutant (H1248). The cells were incubated with PenG at 37 °C for 2 h and lysed by glass beads in the presence of Triton X-100. The lysate was subjected to affinity chromatography over a nickel column as described under "Experimental Procedures." Fractions that, according to SDS-PAGE and Western blotting using His6 antibody, contained the E166A-His6 protein of 66 kDa were subjected to SDS-PAGE, blotting onto a polyvinylidene difluoride filter, and N-terminal amino acid sequencing. The sequence was that of the signal peptide of Hsp150 (18). We conclude that the ER-attached PenG-bound E166A fusion protein was not translocated far enough to reach the signal peptidase, though the Hsp150Δ fragment should have no structural constraints for pore penetration, and authentic Hsp150 is translocated extremely rapidly (12). This suggests that, in normal conditions, unfolding of the C-terminal β-lactamase portion has to occur before pore penetration can be initiated.

Reversible Binding of Ligand to the β-Lactamase Portion Allows Translocation—Finally we examined the Hsp150Δ-β-lactamaseE166D mutant (E166D), which also binds PenG, but reversibly (21). We anticipated that E166D molecules should be translocated immediately when PenG dissociates from the active site. As the crystal structure of PenG-bound molecules is almost identical to that of the native unmodified β-lactamase molecules (25), E166D molecules binding PenG in the ER lumen after translocation should be competent for ER exit and secretion. The Δerg6 mutation allows penetration of drugs also...
across the ER membrane. Pulse-chase experiments showed that E166D was translocated and secreted similarly in the presence (Fig. 7A) and absence (Fig. 7B) of PenG. As no more ER form could be detected after chase in the presence of PenG (Fig. 7A, lane 4) than in its absence (Fig. 7B, lane 4), E166D apparently exited the ER as rapidly in free and PenG-bound form. Since the E166D mutation inactivated the enzyme similarly as shown in Fig. 2C for the E166A mutation, and PenG had no effect on the fate of the E166D fusion protein, we needed to confirm that the drug bound to the reporter protein. To this end, parallel E166D/Δerg6 cell samples (H1046) were incubated with [35S]PenG and [3H]methionine/cysteine for 30 min at 37 °C. Immunoprecipitation of the medium with β-lactamase antiserum and SDS-PAGE analysis revealed a 14C-labeled protein comigrating with [35S]-labeled E166D (data not shown).

The results on the E166D mutant were complemented using native, enzymatically active Hsp150Δ-β-lactamase and the penicillinase inhibitor CLX, which is bound to authentic β-lactamase, hydrolyzed, and released (26). CLX inactivated Hsp150Δ-β-lactamase, confirming binding (Fig. 7E). The control experiment demonstrating secretion of active molecules in the absence of CLX is shown in Fig. 2B (circles). Pulse-chase experiments showed that CLX had no effect on translocation and secretion of Hsp150Δ-β-lactamase (Fig. 7, C and D). These data suggest that release of the ligand allowed unfolding, which in turn allowed translocation of native Hsp150Δ-β-lactamase as well as the E166D variant.

**DISCUSSION**

Here we show that prefolded β-lactamase with a covalently bound ligand could not be translocated into the yeast ER. Our reporter protein was E. coli RTEM-1 β-lactamase, which in authentic form is a tight globule (13). It was fused to the C-terminus of a 321-amino acid fragment (Hsp150Δ) of the yeast secretory glycoprotein Hsp150. Hsp150Δ-β-lactamase is translocated post-translationally, but before that, the β-lactamase portion folds in the cytosol to a native-like catalytically active conformation (12). Here we introduced to the β-lactamase portion a point mutation (E166A), which causes covalent and irreversible binding of PenG to the active site residue Ser70 (21). In addition, Glu166, Lys233, Ser190, Asn192, Lys243, and Ala237 are involved in substrate binding, and the crystal structure of the PenG-bound mutant protein is nearly identical to that of the native unmodified protein (25). In the presence of PenG, the Hsp150Δ-β-lactamase/E166A protein (designated E166A) was unable to translocate and remained cytosolic with a trypsin-resistant β-lactamase portion. PenG and another penicillin derivative, cloxacinil, which bind reversibly to variant E166D and native β-lactamase, respectively, did not prevent translocation of the respective fusion proteins. We suggest that irreversibly PenG-bound E166A molecules could not penetrate the translocon because the ligand prevented unfolding, whereas release of the reversibly bound ligands allowed unfolding and translocation.

PenG-stabilized E166A was attached in a signal peptide-dependent fashion to the ER membrane, where it inhibited translocation of BiP, CPY, and a number of secretory proteins. This shows that the PenG-bound molecules were on a productive translocation pathway, normally shared by co- and post-translationally translocated polypeptides. Whether the binding site was Sec63p or Sec61p (7), or perhaps an as yet unknown receptor upstream of these components, remains to be studied. Anyhow, PenG-bound E166A did not penetrate deep enough into the translocon pore to be processed by the signal peptide. The failure of the 321-amino acid-long Hsp150Δ fragment to penetrate into the ER lumen is surprising, since most of it adopts no regular secondary structure as determined by NMR spectroscopy and CD spectroscopy (14), and should thus have no structural constraints for translocation. Moreover, authentic Hsp150 translocates so rapidly that no cytosolic form can be detected even after a 1-min 35S pulse (12). The cytosolically exposed, signal peptide-containing, ER-attached PenG-bound E166A fusion protein must have represented a proper translocation intermediate, because the E166D variant binding PenG reversibly was readily translocated. It appears that unfolding of the C-terminal β-lactamase portion had to occur before translocation of the Hsp150A portion could be initiated or advanced significantly. Pore opening is carefully controlled, and completion of the unfolding process may somehow trigger pore opening. The scenario is different from what has been suggested for mitochondrial import. Stably folded precursor proteins cannot traverse mitochondrial import sites (27, 28). However, the N-terminal F0-ATPase subunit of 86 amino acids was fully translocated, and only the fusion partner dihydroflorurate reductase, when stabilized by methotrexate, remained stalled against the mitochondrial outer membrane (29).

As PenG-bound molecules were exposed to the cytosol, the machinery unfolding the remote β-lactamase portion must be cytosolic. BiP has been shown not to actively pull precursor proteins, but to trap them passively by binding and preventing backwards sliding (30). This does not exclude the possibility that BiP exerted a pulling function on our reporter protein, but pulling as well as trapping could occur only after destabilization of the β-lactamase portion by cytosolic factors, and sufficient advancement of the polypeptide into the ER lumen for BiP to be able to grab it. It has been suggested that pore penetration becomes BiP-dependent after the signal peptide has intercalated into transmembrane domains 2 and 7 of Sec61p (9). Unfolding of cytosolic prefolded proteins for mitochondrial import has been proposed to occur as matrix Hsp70 actively pulls the polypeptide through import sites. In this scenario mtHsp70 is viewed as a motor, which interacts with the inner membrane protein Tim44 to generate pulling force acting on the translocation substrate (29, 31). Other data suggest that mtHsp70, anchored to Tim44, acts like BiP as a ratchet minimizing retrograde movements, that unfolding occurs spontaneously, and that forward movement is driven by Brownian motion (32, 33). Import of polypeptides into mitochondria requires a high degree of unfolding. Only 50 amino acids were sufficient to span both outer and inner membrane, demonstrating that the passenger polypeptide is imported into the matrix in an extended state (34). According to equilibrium measurements with 8 M urea, pre-β-lactamase folds in vitro via a molten globule state, which retains native-like secondary structure but lacks catalytic activity, and whose compactness is between those of native and completely unfolded forms (35). Whether the translocation-competent β-lactamase portion retains secondary structure in yeast cells remains to be determined.

Pre-pro-α-factor was post-translationally translocated, in the absence of cytosolic components, into reconstituted proteoliposomes, which contained the heptameric translocon complex in the membrane and BiP in the lumen (30). However, pre-pro-α-factor was urea-denatured before dilution and the translocation assay. Moreover, it is not known whether it folds prior to translocation in vivo. Loosely folded molecules may not require unfolding, and evolution may have selected such proteins for post-translational translocation, and directed tightly folded proteins to the co-translational pathway, which allows folding only on the luminal side of the ER membrane. Nevertheless, our data unravel new activities in the yeast cytosol, unfolding of tightly folded protein for post-translational ER translocation, and a connection between unfolding of the translocation...
substrate and pore opening. Such events were not anticipated, as it was thought that polypeptides do not fold to native-like conformation prior to translocation. Our experiments were performed on living cells, confirming the physiological relevance of the findings.

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