DnaK Promotes the Selective Export of Outer Membrane Protein Precursors in SecA-deficient Escherichia coli*

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In bacteria, many presecretory proteins that travel through the “general secretory pathway” are first targeted to the inner membrane (IM)† either co-translationally or post-translationally by molecular chaperones (reviewed in Refs. 1 and 2). Chaperones do not recognize signal peptides and do not necessarily mediate direct interactions with membrane proteins, but they invariably play two critical roles in the export process. First, they maintain their cargo in a loosely folded conformation that is required for transport across the membrane (3). In addition, they keep signal peptides accessible for interactions with components of the membrane-bound translocation machinery. A subset of Gram-negative bacteria produce a chaperone called SecB whose only known function is to maintain the transport competence of presecretory proteins. In Escherichia coli, SecB is required for the efficient export of many outer membrane proteins (OMPs) as well as a few periplasmic proteins such as maltose-binding protein (MBP) (4). SecB is a tetramer that interacts with its ligands via an ATP-independent kinetic partitioning mechanism (5, 6). Several studies have indicated that highly abundant molecular chaperones such as DnaK and GroEL, which play essential roles in protein folding, can also maintain the transport competence of presecretory proteins (7–10). DnaK and GroEL are structurally unrelated to SecB and bind polypeptides in an ATP-dependent cycle that is regulated by co-chaperones (11, 12). Moreover, the substrate specificity and the constraints on substrate binding for each chaperone differs considerably (11–14). Nevertheless, available evidence suggests that chaperones have overlapping functions in the protein export pathway. Indeed the functional redundancy of chaperones may explain why SecB is not essential for cell viability.

A great deal of evidence indicates that a single translocation complex or “translocase” plays a predominant role in the subsequent transport of presecretory proteins across the IM (reviewed in Ref. 15). The core of the translocase (the “SecY complex”) consists of a heterotrimer of integral membrane proteins (SecY, SecE, and SecG) and a homodimer (SecA) that is found in both soluble and membrane-associated forms. The importance of the E. coli secY, secE, and secA genes was initially suggested by genetic studies. Each gene was isolated in multiple independent screens for mutations that affect secretion and was shown to be essential for viability (16). Subsequently, inactivation or depletion of any of the core translocase subunits was shown to severely impair the export of a wide variety of proteins in vivo and in vitro (17–21). The SecY complex is thought to be the pore through which proteins traverse the membrane. Consistent with this view, EM reconstruction studies have indicated that the Bacillus subtilis SecY complex forms an oligomeric ring structure (22). The SecY complex is universally conserved throughout evolution, and its eukaryotic counterpart (the “Sec61p” complex) facilitates transport of proteins into the endoplasmic reticulum. The SecA protein, which is unique to bacteria, functions as a molecular motor that uses the energy of ATP hydrolysis to drive proteins across the IM. Starting at the signal peptide, SecA promotes translocation of ~20 amino acid loops of presecretory proteins in a ratchet-like fashion (23). Together, the SecY complex and SecA are both necessary and sufficient for protein translocation into lipid vesicles in a reconstituted system (24).

Despite the apparent centrality of the SecY/SecA translocation machinery, the possibility that alternate transport pathways also exist in E. coli has been raised by several different studies. Most of these studies show that the translocation of OMPs, but not periplasmic proteins, can be observed under conditions where the Sec machinery is severely impaired. OMPs are relatively hydrophobic, but unlike typical periplasmic proteins...
they are comprised entirely of β-strands (or, as in the case of OmpA, contain a large all β-strand domain) that forms a “β-barrel” upon integration into the outer membrane. In translocation assays performed using inverted membrane vesicles prepared from E. coli containing <5% of the wild-type level of SecY, SecE, or SecG, OMPs such as LamB and OmpA were translocated at an appreciable level (25, 26). In these experiments little or no alkaline phosphatase (AP), a periplasmic protein, was translocated into the vesicles. Likewise, about 25% of the OmpA synthesized after substantial SecA depletion was still properly localized in intact cells but no export of two different periplasmic proteins was detected (27). Under conditions where overexpression of AP inhibited protein export (possibly by jamming translocation pores), significant translocation of OmpA was observed but β-lactamase (Blal) transport was completely blocked (28). These results are all difficult to interpret, however, because the possibility that export of some proteins requires only very low levels of Sec activity cannot be ruled out.

In this study we examined the phenomenon of differential protein export in Sec-deficient cells in more detail. We observed slow but significant export of every OMP that we examined after SecA depletion. By contrast, we could not detect export of any periplasmic protein under the same conditions. OMAP export was absolutely dependent, however, upon the presence of a low level of SecA activity. Taken together, several lines of evidence suggested that the selective translocation of OMPs in SecA-depleted cells is due to the ability of DnaK to maintain them in a prolonged translocation-competent state. The data show how protein export in Sec-impaired cells can be explained without invoking the existence of Sec-independent transport pathways and also provide novel insights into the function of DnaK in vivo.

EXPERIMENTAL PROCEDURES

Reagents, Bacterial Strains, and Media—Polyclonal antisera were obtained from 5 Prime-3 Prime Inc., Boulder, CO (bla and AP), New England Biolabs (MBP), Covance (influenza virus hemagglutinin [HA] epitope HA.11), Dr. Greg Phillips (OmpC and OmpF), and Dr. Tom Silhavy (LamB). A monoclonal antibody against DnaK was obtained from Stressgen. Normal mouse serum and normal rabbit serum were the kind gifts of Dr. Chung-shan Shi. The bacterial strains used in this study and their genotypes are BA12 (MC1000 supF trp am secA13am), BA13 (secA13am supFts trp am secA13am), BA14 (secA13am), BA15 (secA13am supF trp am), BA16 (secA13am supF trp am), and BA17 (secA13am supF trp am). BA13 (secA13am supF trp am) was generated by first introducing L164P and V166D mutations into pRB11-OmpA-3 (New England Biolabs). The BA14 (secA13am) strain was obtained by introducing a point mutation (A2785T) into pMAL-p2X (New England Biolabs). BA15 (secA13am supF trp am) was generated by first transducing the amber suppressor and depleting almost all of the SecA. As previously observed (27), BA13 grew as well as BA25 during the SecA depletion phase (data not shown). Cells were then pulse-labeled and incubated for variable chase periods, and the export of individual proteins was analyzed by immunoprecipitation. The accumulation of the unprocessed precursor form of

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GCAGAGCTATGAA-3' and its complement, which encode an HA tag, into the BamHI site of RB11-OmpA. NdeI and EagI restriction sites were introduced into pJH3 at the start of ompA and at the end of the signal peptide to create pJH34. Oligonucleotides encoding the AP signal peptide (5'-TATGAAAATGTAACAAGACATGTACGATGCACRTTACGTCATGTTAAACA-3' and 5'-GGCCCTTGTGCAACAGGGGTTAATAAGCAGTATCAGTGGCAGTAATGCTGTTGTGGTTTTTCACTTTCA-3') were then ligated to pJH34 to generate pJH35 (which produces AP-ompA(32)). To construct pJH36, a DNA fragment to facilitate cloning, an Xmal site was introduced at the end of the β-barrel domain in the PCR reaction. The PCR product was then digested with Xmal and XhoI and cloned into the cognate sites of pHL34. Subsequently, the oligonucleotides 5'-AGCTT TACGCTCGAATCTACGACATGCACATGCAGATGTTG-3' and 5'-CCGGCATATGGCGTGAAGT-3' were ligated to the Xmal and HindIII sites of pHL36 to generate pHL37 (which encodes pro-OmpA(Δ3)). The AP gene was amplified using oligonucleotides 5'-GCCGTGGTCTTACGCTCTTCACTGTTGCGCTGATCAGTAATGTTGCTGACACATGTC-3' and either 5'-GGCTGTGCAGATGTTGACACTGACACATGTC-3' or 5'-GGTTGCGAAAACGCCGTCGTCAAGCGTAGATGTGATCGTACGACACATGTC-3' and pHL1 (33) as a template and cloned into the BamHI site or Spal and BamHI sites of pHL34, respectively, to construct pHL38 and pHL39 (encoding pro-OmpA(Δ3)-AP and pro-OmpA(Δ5)-AP). Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Gel Electrophoresis—Protein samples were analyzed by SDS-PAGE on 8–16% minigels (Novex) unless otherwise noted. NuPAGE gels (Novex) were run using the MOPS buffer recommended by the manufacturer. Immunoprecipitated proteins were visualized using a Fuji BAS 2500 phosphorimeter.

RESULTS

OMPs Are Exported at a Moderate Level after SecA Depletion—We previously found that depleting ~95% of the SecA from E. coli completely blocks the export of AP and ribose-binding protein, two periplasmic proteins, but less severely impairs the export of OmpA, an OMP (27). To determine whether the fate of a protein in SecA-deficient cells correlates with its intracellular destination, we analyzed the export of a larger set of proteins after SecA depletion. BA13 (secA13am supF trp am) and control DO251 (secA13am supF trp am) cells were grown at 30 °C and the cultures were shifted to 41 °C for 3 h to inactivate the amber suppressor and deplete almost all of the SecA. As previously observed (27), BA13 grew as well as DO251 during the SecA depletion phase (data not shown). Cells were then pulse-labeled and incubated for variable chase periods, and the export of individual proteins was analyzed by immunoprecipitation. The accumulation of the unprocessed precursor form of

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a protein indicated an export block. In BA13 cells, only the precursors of the periplasmic proteins AP, Bla, DegP, ribose-binding protein, and MBP were detected even after a 30-min chase (Fig. 1, lanes 1–3). Most of the precursors were stable or degraded slowly during the chase period. By contrast, between ~10 and 50% of the newly synthesized OmpA, OmpC, OmpF, and LamB molecules were exported within 2 min. A larger fraction of each protein was converted to the mature form after a 10-min chase, and the export of LamB continued even longer. As expected, all of the proteins were completely exported from DO251 cells within 2 min (Fig. 1, lanes 4–6). These results demonstrate that SecA deletion differentially affects the export of periplasmic proteins and OMPs, two biochemically distinct classes of proteins.

**OMP Export in SecA-deficient Cells Is SecA-dependent**—The observation that OMPs are selectively exported after SecA depletion suggested that either they can be translocated across the IM via a SecA-independent mechanism or that they require much less SecA to traverse the IM than periplasmic proteins. To distinguish between these two possibilities, we depleted most of the SecA from BA13 cells as described above and then inhibited the activity of the residual protein by adding sodium azide. We expected that the SecA inhibitor would affect the fate of OMPs only if their export remains SecA-dependent. Interestingly, the translocation of both OmpA and OmpC was completely abolished by the addition of azide; only the precursor form of both proteins was observed even after a 30-min chase (Fig. 2, lanes 1–3). Consistent with previous results indicating that azide is a relatively weak inhibitor of SecA activity (36), the export of OmpA and OmpC was only delayed or slightly inhibited in azide-treated DO251 cells (Fig. 2, lanes 4–6). Thus, it is unlikely that the complete translocation block observed in BA13 cells was due to a nonspecific toxic effect of the azide. These results strongly suggest that OMP export is strictly dependent on the availability of at least a low concentration of SecA.

**Structural Requirements for Protein Export after SecA Depletion**—To identify the structural features of OMPs that explain their continued export after SecA depletion, we constructed a variety of plasmids that produce deleted or modified forms of pro-OmpA (Fig. 3A). BA13 and DO251 were transformed with each plasmid and the export of the pro-OmpA derivative was examined after SecA depletion. Initial experiments demonstrated that only the first two-thirds of the protein, which consists of the 170-amino acid β-barrel domain and about 60 amino acids of the C-terminal periplasmic domain, are required for translocation. In BA13 cells nearly 50% of a pro-OmpA derivative that lacked the last ~100 amino acids (pro-OmpA(Δ2)) was exported within 2 min (Fig. 3, A and B, top panel, lane 1). OmpA(Δ2) was relatively unstable in both BA13 and DO251 and was degraded more rapidly than untranslocated pro-OmpA(Δ2) (Fig. 3B, top panel). These observations confirmed that cleavage of the precursor in SecA-depleted cells was due to transport across the IM. In light of evidence that SecA binds to the N terminus of presecretory proteins and then promotes the transport of successive 20-amino acid segments, it is conceivable that OMPs are selectively exported in SecA-deficient cells because SecA binds with particularly high affinity to their signal peptides. This explanation is unlikely, however, because replacement of the pro-OmpA signal peptide with the AP signal peptide did not abolish translocation of the protein after SecA depletion (Fig. 3, A and B, second panel). Moreover, no export of pro-OmpA(Δ3), which contains the entire N-terminal β-barrel domain, was observed (Fig. 3, A and B, third panel). Thus, it is unlikely that SecA preferentially associates with any sequence near the N terminus of the protein. Because pro-OmpA variants containing a deletion in the β-barrel domain (pro-OmpA(Δ1)) or a double point mutation (pro-OmpA*) that abolishes β-barrel formation (37) were exported in SecA-deficient cells (Fig. 3A), translocation was not contingent on the ability of the protein to form a β-barrel. Complete deletion of the β-barrel domain, however, created unstable variants that appeared to remain in the cytoplasm (data not shown). Taken together, the results of the mutational analysis suggest that both a significant portion of the β-barrel domain and the first 60 amino acids of the periplasmic domain of pro-OmpA are required for export after SecA depletion.
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Fig. 3. Export of OmpA derivatives after SecA depletion. A, BA13 and DO251 transformed with a plasmid expressing OmpA or the indicated OmpA derivative were shifted to 41 °C and radiolabeled as described in the legend to Fig. 1. OmpA was immunoprecipitated with anti-HA, and OmpA derivatives were immunoprecipitated with anti-OmpA or anti-AP antisera as appropriate. The presence (+) or absence (−) of mature OmpA protein in BA13 cells after SecA depletion is indicated. B, selected results from the experiments described in part A are shown. After pulse labeling, cells were incubated for the indicated chase period; cells containing AP−-OmpA(Δ2), however, were incubated for a chase period of 2, 5, or 10 min. OmpA(Δ2), AP−-OmpA(Δ2), and OmpA(Δ3)-AP and their precursors were resolved on 10% NuPage gels. OmpA(Δ2)-AP and its precursor were resolved on 4–12% NuPage gels. Lanes 1–3, BA13 cells; lanes 4–6, DO251 cells.

Interestingly, experiments with OmpA-AP fusions revealed that periplasmic proteins can be exported in SecA-limited cells when linked covalently to OmpA. BA13 and DO251 were transformed with a plasmid containing an AP domain fused to either pro-OmpA(Δ2) or a slightly truncated version of pro-OmpA(Δ3), and SecA was depleted as described above. The export properties of each fusion protein correlated with those of the corresponding pro-OmpA deletion mutant. About half of the pro-OmpA(Δ2)-AP was transported across the IM after SecA depletion, whereas all of the pro-OmpA(Δ3)-AP was retained in the cytoplasm even after a long chase (Fig. 3, A and B, fourth and fifth panels). The observation that OmpA(Δ2)-AP was stable in BA13 cells (unlike the precursor form of the protein) suggested that it was properly translocated across the IM. Taken together, the results indicate that the quantitative block of periplasmic protein export observed after SecA depletion is not due to the presence of sequence or structural elements that hinder translocation across the IM when SecA is limiting.

OMP Translocation after SecA Depletion Is Promoted by an Interaction with DnaK—Because the pattern of OMP and periplasmic protein translocation after SecA depletion could not be easily attributed to differences in SecA binding affinity, it is likely that the fate of the two classes of proteins diverged during or prior to targeting. Given that OMP export was observed over a period of at least several minutes, the simplest interpretation of the data is that OMPs were released from ribosomes and remained in a conformation in which they are competent both for interaction with SecA and for transport across the IM. It is formally possible, however, that a fraction of each OMP was rapidly targeted to the IM as translationally arrested nascent chains that were completed only after translocation began. To distinguish between these two explanations, SecA was depleted from BA13, and translation elongation was inhibited by the addition of chloramphenicol 1 min after pulse labeling. The same amount of OmpA was exported from chloramphenicol-treated cells and untreated control cells (Fig. 4, lanes 1–3). These results indicate that pro-OmpA is translocated post-translationally, and suggest that fully synthesized polypeptide chains persist in the cytoplasm until they encounter a SecA molecule.

Because tightly folded proteins cannot be transported across the IM by the Sec machinery, it is likely that a molecular chaperone is required to preserve OMPs in a loosely folded conformation after SecA depletion. A reasonable candidate is SecB, the chaperone that targets most OMPs to the IM under normal growth conditions. Available evidence suggests, however, that SecB promotes OMP export only very early after protein synthesis is complete. Within less than a minute, alternative targeting mechanisms begin to compensate for the loss of SecB, and as a result OMP export is merely delayed in secB− strains (see, for example, Refs. 9, 10, and 38 and Fig. 5A, lanes 4–6). Moreover, SecB normally targets a subset of periplasmic proteins such as MBP that are not translocated after SecA depletion. Thus, protein export in SecA-deficient cells cannot merely be a consequence of interaction with SecB. To directly examine the role of SecB in the export of OMPs after SecA depletion, we constructed secB− derivatives of BA13 and DO251 (HDB108 and HDB109, respectively) and incubated the cells at high temperature. Although the lack of SecB slowed the rate of export, about the same amount of OmpA was eventually exported in HDB108 as in BA13 (Fig. 5A, lanes 1–3). These data suggest that a chaperone other than SecB preserves the export competence of OMPs in SecA-deficient cells.

The first evidence that OMP export in SecA-deficient cells is promoted by DnaK emerged from experiments with a mutant form of the chaperone. Previous studies have shown that DnaK can serve as an alternative targeting factor for at least some presecretory proteins including OmpA and LamB (9, 10). Presumably because DnaK plays a key role in cell physiology, we found that significant perturbation of the DnaK pathway in BA13 cells resulted in severe growth defects (data not shown). BA13 transformed with a multicopy plasmid that constitu-
tively overexpresses the weak recessive dnaK A174 allele (pS368 [DnaK A174T]), however, grew nearly as well as cells containing the cloning vector pHDB3 (34) at 30 °C and after a shift to 41 °C. Several lines of evidence indicate that the A174T mutation only mildly impairs DnaK function (39, 40). The mutant protein has relatively normal peptide binding and ATPase activities, but shows clear defects in an assay that measures the simultaneous interaction of DnaK with the co-chaperones GrpE and DnaJ. Moreover, overproduction of the mutant protein has relatively normal peptide binding and mutation only mildly impairs DnaK function (39, 40). The shift to 41 °C containing the cloning vector pHDB3 (34) at 30 °C (pS368 [DnaK A174T]), however, grew nearly as well as cells.

To obtain evidence that DnaK plays a direct role in promoting the translocation of OMP precursors after SecA depletion, we tested for the presence of pro-OMP-DnaK complexes using co-immunoprecipitation assays. BA13 and DO251 cells incubated at 41 °C were pulse-labeled and cytoplasmic extracts were prepared after a 2-min chase. In some experiments cells were first transformed with a plasmid that produces pro-OmpA Δ3. A significant amount of a 39-kDa protein that co-migrated with pro-OmpA was immunoprecipitated with anti-DnaK antibodies from both transformed and untransformed BA13 cells (Fig. 6, A and B, lanes 1 and 2). The observation that little or none of this protein was immunoprecipitated by non-immune sera (Fig. 6, A and B, lanes 3 and 4) suggested that its isolation was because of the formation of a specific complex with DnaK. None of the 39-kDa protein was immunoprecipitated from DO251 cells, which contain essentially no untranslocated OmpA precursor (Fig. 6, A and B, lanes 5 and 6).

Immunoprecipitation followed by Western blot analysis confirmed that the 39-kDa protein is pro-OmpA (data not shown). Although no DnaK was immunoprecipitated with an anti-OmpA antisera (Fig. 6A, lane 1), it is possible that the binding of the antibodies caused dissociation of pro-OmpA-DnaK complexes. Because relatively few other proteins were isolated in the co-immunoprecipitations, it is likely that pro-OmpA binds particularly tightly to DnaK. A few of the other proteins may correspond to less abundant OMPs (e.g. OmpC, which runs slightly slower than OmpA). Interestingly, pro-OmpA Δ3, an OmpA derivative that is completely retained in the cytoplasm of SecA-deficient cells, was not immunoprecipitated with anti-DnaK antibodies (Fig. 6B, lanes 1 and 2). Taken together, the results strongly suggest that DnaK associates physically with proteins that are exported after SecA depletion.
DISCUSSION

In this report we show that the selective export of OMPs in cells that contain only $\sim 5\%$ of the wild-type level of SecA is not due to their utilization of a Sec-independent transport pathway, but rather to their preservation in a prolonged translocation-competent state. Initially we found that our previous observations on protein export in SecA-deficient cells could be generalized. After SecA depletion, E. coli were completely inactive in the export of every periplasmic protein that we tested, but were partially active in the export of several different OMPs. Whereas most proteins are exported from wild-type cells either co-translationally or within a few seconds after synthesis, OMPs were exported post-translationally over a period of $>10$ min after SecA depletion. OMP export was abolished by inhibiting the activity of the remaining SecA protein, and therefore must have required a minimal level of SecA function. Experiments with hybrid proteins and truncated versions of OmpA ruled out the possibility that SecA binds to the $N$ terminus of OMPs with particularly high affinity and facilitates their entry into the secretory pathway even at low concentrations. These experiments implied that the difference in the fate of OMPs and periplasmic proteins is determined at an early stage. Consistent with this conclusion, OMP export was abolished by perturbing the function of DnaK, a chaperone that has previously been implicated in protein targeting and that could be isolated in a complex with OmpA in SecA-depleted cells. The simplest interpretation of the data is that DnaK binds selectively to OMPs (presumably after they interact transiently with SecB) so as to maintain their transport competence until a relatively scarce SecA molecule becomes available.

Although our experiments directly address the export of OMPs only in SecA-depleted cells, they also provide a plausible explanation for the preferential export of OMPs under other conditions where Sec components are limiting. Because the SecY complex works in concert with SecA, it is likely that inactivation or depletion of any of the Sec components would have a similar effect on protein export. Regardless of the method used to block protein translocation, even a very low level of functional Sec translocase may be sufficient to transport proteins that remain export-competent for an extended period. Because mutation, depletion, and chemical inhibition affect each component of the Sec machinery in unpredictable ways, however, it is difficult to determine whether a given subunit is completely inactivated. Thus the observation that significant amounts of OMPs are translocated into inverted vesicles containing relatively little SecY, but not those treated with sodium azide (26), may simply indicate that the chemical reagent is a particularly potent inhibitor of the Sec machinery in vitro. In addition, whereas DnaK appeared to play the predominant role in maintaining the export competence of OMPs in the experiments described here, there is evidence that a variety of highly abundant chaperones including GroEL and trigger factor can stabilize OMPs in cell-free extracts (7). Indeed the availability of multiple mechanisms to maintain OMPs in a transport-competent state may explain why they are particularly good substrates for in vitro translocation assays in which the Sec machinery is almost certainly less active than it is in an intact cell.

Our results also yield novel insights into the relationship between the structure of a protein and its interaction with DnaK under physiological conditions. The substrate specificity of DnaK has previously been investigated by probing large libraries of random peptides displayed on phage (41) or cellulose-bound peptides derived from known protein sequences (42). In these studies a consensus DnaK binding motif consisting of a short hydrophobic core flanked by positively charged amino acids was identified. The crystal structure of a DnaK-peptide complex confirmed the biological relevance of this motif and showed that the chaperone binds peptides only in an extended conformation (43). The structural data also suggested that DnaK only binds peptides within substantially unfolded proteins. Interestingly, the majority of high affinity binding sites that were identified in complete protein sequences correspond to $\beta$-strands in the folded structure. Although the OMPs that we examined in our experiments are comprised entirely of or contain a large all-$\beta$-strand domain, they do not appear to be particularly enriched in sequences containing the proposed binding motif. They also do not contain a region of high sequence identity that might serve as a common high affinity DnaK binding site. Moreover, strong DnaK binding sites have been identified empirically in MBP and AP (42). Thus, our results suggest that the presence of putative DnaK binding sites is not sufficient to predict the fate of exported proteins in SecA-depleted cells. In light of our data, it is conceivable that the distinctive secondary structure of OMPs at least partly accounts for the interaction with DnaK that we observed. Perhaps protein domains composed entirely of $\beta$-strands acquire secondary and tertiary structures particularly slowly under the conditions of our experiments. If so, then the DnaK binding sites in OMPs may simply be more accessible than those of periplasmic proteins. The observation that DnaK can be co-immunoprecipitated with full-length pro-OmpA but not with pro-OmpA(D3), however, suggests that in some cases sequences outside the $\beta$-barrel domain may also be required to maintain the accessibility of DnaK binding sites.

Our results highlight profound differences in the chaperone function of SecB and DnaK. The data suggest that only DnaK can maintain presecretory proteins in an unfolded conformation for a prolonged period of time. Our observations are consistent with the results of co-immunoprecipitation experiments indicating that DnaK interacts with a subset of newly synthesized cytoplasmic proteins for $>10$ min (35, 44). Although it is conceivable that DnaK can sequester substrates for a longer time than SecB by utilizing a highly regulated mode of substrate release, it is unclear whether translocation-competent OMPs remain continuously bound to DnaK in SecA-depleted cells until they interact with the Sec machinery. Indeed the observation that most cytoplasmic proteins associate with DnaK only transiently (35) suggests that the inherent rate of substrate release is relatively rapid and that long term binding is due to repeated reassociation. The differential ability of SecB and DnaK to preserve the translocation competence of presecretory proteins may reflect differences in the length of their respective ligands more than differences in the rate of substrate release. Both chaperones bind to unfolded polypeptide chains, but unlike DnaK, which binds to short peptides, SecB associates with a contiguous stretch of $\sim 150$ amino acids and probably requires simultaneous interactions with multiple subsites to bind effectively (45, 46). Interestingly, the crystal structure of SecB reveals the presence of a 70-Å long groove on each side of the tetramer (12). It has been proposed that a long polypeptide segment sits in this channel and in effect wraps around the chaperone. When a substrate dissociates from DnaK, at least one potential binding site may often remain exposed. By contrast, when a substrate dissociates from SecB, the probability that some portion of the long polypeptide ligand will fold or adopt an unfavorable conformation that prevents rebinding may be relatively high.

Finally, our results suggest that the export of heterologous proteins produced in E. coli may be enhanced by fusing them to OMPs. This strategy might be particularly useful in cases where protein export is inhibited by rapid folding (47). The
observation that AP translocation in SecA-limited cells is partially rescued by fusing it to OmpA suggests that a protein that would otherwise fold into a transport-incompetent conformation remains sufficiently unfolded in the context of the protein chimera to be translocated across the IM. If the recognition sequence for a site-specific protease were engineered downstream of the OMP moiety, then the protein of interest could be recovered by cleaving it from the fusion protein.

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