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An Essential Role for the DnaK Molecular Chaperone in Stabilizing Over-expressed Substrate Proteins of the Bacterial Twin-arginine Translocation Pathway

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All secreted proteins in *Escherichia coli* must be maintained in an export-competent state before translocation across the inner membrane. In the case of the Sec pathway, this function is carried out by the dedicated SecB chaperone and the general chaperones DnaK-DnaJ-GrpE and GroEL-GroES, whose job collectively is to render substrate proteins partially or entirely unfolded before engagement of the translocon. To determine whether these or other general molecular chaperones are similarly involved in the translocation of folded proteins through the twin-arginine translocation (Tat) system, we screened a collection of *E. coli* mutant strains for their ability to transport a green fluorescent protein (GFP) reporter through the Tat pathway. We found that the molecular chaperone DnaK was essential for cytoplasmic stability of GFP bearing an N-terminal Tat signal peptide, as well as for numerous other recombinantly expressed endogenous and heterologous Tat substrates. Interestingly, the stability conferred by DnaK did not require a fully functional Tat signal as substrates bearing translocation defective twin lysine substitutions in the consensus Tat motif were equally unstable in the absence of DnaK. These findings were corroborated by crosslinking experiments that revealed an *in vivo* association between DnaK and a truncated version of the Tat substrate trimethylamine *N*-oxide reductase (TorA502) bearing an RR or a KK signal peptide. Since TorA502 lacks nine molybdo-cofactor ligands essential for cofactor attachment, the involvement of DnaK is apparently independent of cofactor acquisition. Finally, we show that the stabilizing effects of DnaK can be exploited to increase the expression and translocation of Tat substrates under conditions where the substrate production level exceeds the capacity of the Tat translocase. This latter observation is expected to have important consequences for the use of the Tat system in biotechnology applications where high levels of periplasmic expression are desirable.

Keywords: molecular chaperone machinery; heat shock protein; inner membrane transport; periplasm; protein secretion

Introduction

In bacteria, most proteins with extra-cytoplasmic function are translocated across the inner cytoplasmic membrane by either the general secretory (Sec) pathway or the twin-arginine translocation (Tat) pathway, both of which recognize their

Abbreviations used: REMP, redox enzyme maturation protein; TF, trigger factor; MF, mean cell fluorescence; TMAO, trimethylamine *N*-oxide; MBP, maltose-binding protein.

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substrates, in part, by cleavable N-terminal signal peptides. In the case of the Sec pathway, the translocation pore formed by SecYEG is a narrow (5–8 Å) conduit that precludes passage of folded proteins.[1] Thus, proteins that transit the Sec pathway must be maintained in a largely extended conformation, a feat that is accomplished with assistance from the cytoplasmic chaperone SecB[2–9] and the general molecular chaperones DnaK-DnaJ-GrpE and GroEL-GroES.[10–12] Whereas the Sec system processes proteins that are unfolded, the hallmark of the bacterial Tat machinery is the ability to post-translationally translocate proteins that have undergone folding in the cytoplasm, before export.[13,14] One of the most remarkable features of the bacterial Tat system is its ability to selectively transport only substrates that are correctly folded and assembled,[15–19] leading to speculation that a folding quality control mechanism governs Tat transport.

In light of such a quality control system, a two-tiered mechanism to prevent premature export of incompletely folded and/or assembled substrates was proposed[20] in which the late stages of substrate folding are "sensed" by the Tat machinery directly while earlier stages of folding are proofread by cytoplasmic chaperones. For instance, a large proportion of Tat substrate proteins in *Escherichia coli* acquire complex redox-active multi-atom cofactors (e.g. Fe-S clusters, molybdopterin) in the cytoplasm and require correct assembly before transport can proceed.[21] How such complex cofactor-containing proteins are prevented from engaging the translocation machinery before cofactor attachment has been resolved partially by the discovery of Tat system-specific chaperones such as redox enzyme maturation proteins (REMPs).[20,22,23] REMPs are cytoplasmic chaperones that are involved in the maturation of redox enzymes. A subset of REMPs are postulated to bind and sequester Tat signal peptides until their cognate Tat-targeted redox enzyme is fully folded, assembled and competent for transport, a mechanism known as the "shelter" model.[13] The best-characterized REMP is *E. coli* DmsD, which has been shown to bind to the Tat signal peptides of DmsA[24] and TorA,[25] and plays an important role in DmsA maturation and localization.[24,26] Numerous other REMPs have been identified, such as the TorD protein that pairs with TorA,[23] and it has been suggested recently that all Tat substrates may possess a cognate leader-binding chaperone.[27]

It is noteworthy, however, that not all Tat substrates attach complex cofactors. Notable examples include *E. coli* AmiA, AmiC, CueO and SufI,[28] as well as numerous Tat-competent heterologous substrates such as alkaline phosphatase,[15,29] β-lactamase,[16] green fluorescent protein[30–32] and maltose-binding protein.[29,33] Without the insertion of such cofactors or a corresponding REMP to monitor the progression of substrate maturation, it is unclear how the folding and transport of heterologous proteins lacking cofactors is coordinated so as to prevent membrane targeting of incompetent substrates. In these instances, it is envisioned that general folding catalysts may participate in the folding and proofreading of Tat substrates by sequestering misfolded proteins from the translocon until correct folding or proteolytic degradation has occurred.[34] Several lines of evidence implicate a role for general molecular chaperones in bacterial Tat transport including: (1) the chaperone trigger factor (TF) binds extensively to the TorA and SufI signal peptides, although depletion or over-expression of TF had little effect on the kinetics and efficiency of the Tat export process;[35] (2) GroEL was found to affect Tat-specific hydrogenase-1 maturation and assembly,[36] and was found to associate with the Tat-specific amidase AmiA;[37] (3) DnaK binds specifically to the Tat leader peptide of DmsA;[25] (4) members of the chaperone cascade including TF, DnaK-DnaJ-GrpE and GroEL interact with the Tat-specific REMP DmsD[38]; and finally, (5) over-expression of DnaK-DnaJ-GrpE, GroEL-GroES and TF has been shown to enhance the transport of the Tat substrate preproPAC.[39]

In addition to the direct involvement of cytoplasmic chaperones, a number of indirect observations suggest that factors other than TatABC are involved in Tat transport. For instance, two separate groups have reported that translocation of the Tat substrate SufI could not be observed in inside-out inner membrane vesicles (INVs) prepared from wt *E. coli* cells.[40,41] Only in membrane vesicles that had been prepared from cells over-expressing TatABC from a strong T7 promoter could translocation be detected, albeit at a relatively low yield (at most 20%). Along similar lines, a number of native Tat substrates expressed *in vivo* have been observed to transit the inner membrane with efficiencies well below 100%.[42–44] Collectively, these observations suggest that cytoplasmic factors involved in substrate proofreading and/or targeting (akin to SecB) might help maintain export competence and enhance translocation efficiency *in vivo*. To test this hypothesis, we have systematically evaluated the effect of well-characterized *E. coli* chaperone and protease mutant strains for their ability to translocate GFP *via* the Tat pathway. Overall, our results indicate that the molecular chaperone DnaK is essential for maintaining the stability of highly expressed Tat substrates in the cytoplasm before export. Consistent with this role, we observed that DnaK associates *in vivo* with at least one Tat-targeted substrate, a truncated variant of the Tat-specific enzyme trimethylamine *N*-oxide reductase (TorA). In addition, DnaK is able to promote efficient transport under conditions where the Tat machinery is saturated by high levels of substrate, a finding that is expected to have important consequences for the use of the Tat system in biotechnology applications.