DnaK Plays a Pivotal Role in Tat Targeting of CueO and Functions beside SlyD as a General Tat Signal Binding Chaperone

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The Tat (twin-arginine translocation) system from Escherichia coli transports folded proteins with N-terminal twin-arginine signal peptides across the cytoplasmic membrane. The influence of general chaperones on Tat substrate targeting has not been clarified so far. Here we show that the chaperones SlyD and DnaK bind to a broad range of different Tat signal sequences in vitro and in vivo. Initially, SlyD and GroEL were purified from DnaK-deficient extracts by their affinity to various Tat signal sequences. Of these, only SlyD bound Tat signal sequences also in the presence of DnaK. SlyD and DnaK also co-purified with Tat substrate precursors, demonstrating the binding to Tat signal sequences in vivo. Deletion of dnaK completely abolished Tat-dependent translocation of CueO, but not of DmsA, YcdB, or HiPIP, indicating that DnaK has an essential role specifically for CueO. DnaK was not required for stability of the CueO precursor and thus served in some essential step after folding. A CueO signal sequence fusion to HiPIP was Tat-dependently transported without the need of DnaK, indicating that the mature domain of CueO is responsible for the DnaK dependence. The overall results suggest that SlyD and DnaK are in the set of chaperones that can serve as general Tat signal-binding proteins. DnaK has additional functions that are indispensable for the targeting of CueO.

Folded proteins can be translocated across the bacterial cytoplasmic membrane if they are synthesized with an N-terminal signal peptide that is recognizable by the Tat (twin-arginine translocation) system (1). Signal sequences of these proteins have a polar N terminus (n-region), which is followed by an uncharged and more or less hydrophobic sequence (h-region). If the signal sequence is cleavable, a recognition site for a leader peptidase marks the end of the signal sequence (c-region). Importantly, Tat signal sequences typically carry a sequence motif that includes two conserved arginine residues, the eponymous "twin arginine motif" (2). The Tat signal sequence of the high potential iron-sulfur protein (HiPIP) from Allochromatium vinosum is the best studied cleavable Tat signal. It has been shown not to fold per se, even when the connected mature domain is completely folded (3). Tat signal sequences are therefore in principle sensitive toward proteolytic attacks. Accordingly, proteolytic degradation up to the mature folded domain is often observed with recombinant Tat substrates in vivo (4). For this reason, it is likely that there exist signal sequence binding chaperones that ensure the integrity of the signal sequence prior to the recognition by the Tat apparatus under physiological expression levels. In the case of transported Me2SO reductase subunits (DmsAB), periplasmic trimethylamine N-oxide reductase (TorA), and hydrogenase 2 (HybOC), specific signal sequence binding chaperones have been identified, which are likely to play important roles in the folding or cofactor assembly of the corresponding mature domain (5–7). The TorA signal-binding chaperone TorD has also been demonstrated to prevent degradation of the TorA signal sequence in recombinant TorA signal-green fluorescent protein fusion proteins (8). However, no such specific chaperones have been found for Tat substrates that contain only [4Fe-4S] clusters, such as NapG, NrfC, or YdhX (1). In line with this, the [4Fe-4S] cluster containing HiPIP from A. vinosum has been found to be efficiently translocated by the heterologous Escherichia coli Tat system, demonstrating that Tat substrates do exist that can be translocated without the presence of a specific signal-binding chaperone (9). Further, no specific signal-binding chaperones have been identified so far for Tat substrates with single metal ion cofactors or without predicted cofactors (1).

We therefore searched for more general Tat signal-binding proteins, which could serve to bind many different signal sequences without being adapted to certain specific Tat substrates. In our approach, we profited from our previously established efficient in vitro folding procedure for the HiPIP precursor (9). This method allowed us to use fully folded HiPIP and derivatives with exchanged Tat signal sequences for affinity purification of general Tat signal sequence-binding proteins. As a result, we identified the chaperones SlyD, GroEL, and DnaK. The data suggest that especially SlyD and DnaK are both functioning as broad range Tat signal sequence binding chaperones in E. coli. An essential function could be specifically assigned to DnaK for the targeting of the Tat substrate CueO.

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2 The abbreviation used is: HiPIP, high potential iron-sulfur protein.
MATERIALS AND METHODS

Strains and Growth Conditions—E. coli MC4100 (10) and its DnaK-deficient derivative BB1042 (11) or E. coli BW25113 (12) and its mutant derivatives JW3311 (ΔslyD::Km), JW0013 (ΔdnaK::Km), or JW3815 (ΔtatC::Km) (13) were used for physiological experiments, E. coli XL1-Blue Mfr' Kan (Stratagene) was used for cloning, and E. coli BL21 DE3 was used for inclusion body production of HiPIP derivatives. The bacteria were grown at 37 °C or when indicated at 30 °C aerobically on LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract) or anaerobically on LB medium supplemented with 0.5% glycerol and 0.4% nitrate, using antibiotics as required (100 μg/ml ampicillin, 20 μg/ml chloramphenicol, 50 μg/ml kanamycin).

Plasmids and Genetic Methods—The hip gene, which encodes the full-length HiPIP precursor, was expressed from the T7 promoter in pEXH5 (14) or from the tac promoter in pEXH5-tac (15). T7 promoter constructs were used for inclusion body production, whereas tac promoter constructs were used for translocation assays. For exchange of the signal peptides, a BamHI site was introduced at the end of the HiPIP signal sequence coding region in pEXH5 and pEXH5-tac with QuickChange™-directed mutagenesis (Stratagene) using the primers hip-BamHI-F (5'-GGT TTC GGC ACC GCG GAT CCC TCC GCT CCC GC-3') and hip-BamHI-R (5'-GGC GAG GAG GAG TCG GGC CGC AAA CC-3'), resulting in the plasmids pEXH5-BamHI and pEXH5-tac-BamHI. The signal sequence-encoding regions of AmiA and CueO were amplified with genomic DNA as template, using the primer pairs AmiA-NdeI-F (5'-CAG GGC ATCA TGA GCA CCTT TTA AAC CAT TAA AAA AAC CAC-3') and AmiA-BamHI-R (5'-TGC TGG GAT CCT TC TG GTT TTT TTA AAA GGT CG-3') or CueO-NdeI-F (5'-GAA ATA CAT ATG CAA CGT CGT GAT TTC TTA-3') and CueO-BamHI-R (5'-AAT CAG GGC GAT CCG GTA ACG TTT GGC GTT CTG-3'), respectively. The PCR products with the amiA and cueO signal sequence coding regions were digested with Ndel and BamHI and cloned into the corresponding sites of pEXH5-BamHI, resulting in the expression plasmids pEX-amia(ss)-hip or pEX-cueo(ss)-hip, respectively. For in vivo analyses, the signal sequence coding region of cueO was amplified using the primer pair, CueO-Ndel-F (as above) and CueO-BamHI-R2 (5'-TGG GAT CCG CCG CAA ATA CTG CGG GCC GC-3') and CueO-BamHI-R1 (5'-GAA TCC TAT CAC CAT ATC ATC CCC GTA-3'), resulting in the expression plasmid pEX-cueo(ss)-hip. The plasmid pEX-mat-hip, which served for the expression of mature untagged HiPIP, was generated by amplification of the mature region of hip in pEXH5 using the primer pair mat-hip-Ndel-F (5'-CCG GCC ATA TGT CCG CTC CGG CCA ATG CGG-3') and hip-Xhol-R (5'-GTG CTC GAG TGC CGG CGC AAG-3'), followed by a restriction with Ndel and Xhol and ligation into the corresponding sites of pEXH5.

The hip gene encoding StreP-tagged HiPIP was expressed from pBW-hip-strep, and the signal sequence-deficient mature domain was produced from pBW-mat-hip-strep. For construction of pBW-hip-strep, hip was amplified by PCR with pEXH5 as template using the primer pair hip-Ndel-F (5'-GGG GAT CTT CAT ATG CCC GAT AGG-3') and hip-BamHI-R (5'-AAC GGG GAT CCG CCG GCC TTC AGG GTC CAG-3'), and the PCR product was introduced into the Ndel and BamHI sites of a StreP tag derivative of pBW22 (16) to allow a rhamnose-inducible production of a C-terminal StreP tag II fusion of HiPIP. The vector pBW-mat-hip-strep was analogously constructed, but the signal sequence coding region was excluded by using the forward primer hip-mat-Ndel-F (5'-CCG CCC ATA TGT CCG CTC CGG CCA ATG CGG-3') in the PCR. Similarly, also cueO could be expressed with this system. Since cueO contains a natural Ndel site, cueO was cloned with two fragments using the primer pairs cueO-Ndel-F (5'-GAA AATA CAT ATG CGG GAC CAA CGT GTT TTC TTA-3')/cueO-Clal-R (5'-ACG TAA TGC ATA CCC CAC TGT TTG G-3') and cueO-Clal-F (5'-GAA GAA TCG ATG TTC CGG GTA TGA CGG-3')/cueO-BamHI-R (5'-GAG TTG TTG CCT ACC GTA AAC CCT AAC ATC ATC-3'). The fragments were restricted with Ndel/Clal or Clal/BamHI, respectively, and cloned into the Ndel/BamHI sites of the vector. For mature CueO, the signal sequence coding region was omitted by use of the alternative forward primer matcueO-Ndel-F (5'-GCA GTA ATG CAA GGC CGC CCG ACC TTA C-3') and the same cloning strategy.

The cueO gene was also expressed from pEX-cueO-H4-tac, which encodes a hexahistidine-tagged CueO under control of the tac promoter. The cueO gene was amplified using the primer pair cueO-Ndel-F (5'-GAA AATA CAT ATG CAA CGT GTT TTC TTA-3') and cueO-Xhol-R (5'-TAT ACC TCG AGC CCT AAC ATC CCC GTA TC-3'), resulting in the expression plasmid pEX-cueO-H4-tac. The PCR product was restricted with Ndel and Xhol, and the full-length fragment was cloned into the corresponding sites of pEXH5 (14), resulting in pEX-cueO-H4-tac. The cueO-H4-containing fragment of this vector was excised with Xbal and Scal and cloned into the corresponding sites of pTB-DG (17), resulting in pEX-cueo-H4-tac.

The ycdB gene was expressed from pBAD-ycdB-strep (4). The vector pBAD-mat-ycdB-strep was analogously constructed, but the signal sequence coding region was excluded by using the forward primer ycdB-mat-NcoI-F (5'-GGG TAC ATG GAA AAA ACG CAA AGT-3') in the PCR. All constructs were confirmed by restriction analyses and sequencing.

The ΔdnaK ΔslyD strain was generated from JW3311 by removal of the kanamycin cassette according to the protocol of Datsenko and Wanner (12) and P1 transduction from the ΔdnaK::Km strain JW0013 (E. coli BW25113 ΔdnaK::Km). The gene deletions were confirmed by PCR and by immunoblots using specific antibodies against DnaK (Acris antibodies; Hidenhausen) and SlyD (a kind gift of C. Schiene-Fischer, Halle, Germany).

Biochemical Methods—Inclusion bodies of HiPIP precursor variants were produced and folded as described previously (9). Mature HiPIP was purified after recombinant expression in BL21 DE3 from the soluble fraction as follows: 2-liter cultures of BL21 DE3 pEXH5-mat were aerobically grown and induced with 1 mM isopropyl-β-D-galactopyranoside at an OD600 nm of 0.8. After a 2-h induction, cells were harvested, resuspended in five volumes of 50 mM Tris acetate, pH 7.4, and disintegrated by two French pressure cell passages at 130 MPa.
RESULTS

Identification of SlyD as a General Tat Signal Sequence-binding Protein in Vitro—HiPIP from A. vinoseum is a Tat substrate that can be efficiently translocated by the E. coli Tat system (9). The signal sequence of HiPIP has been shown to be per se unfolded in solution and highly susceptible to proteolytic degradation in vitro (3, 9). However, the signal sequence of HiPIP is cytoplasmically only degraded at higher expression levels, when translocation becomes limiting. Similar observations have been made with other recombinant Tat substrates, such as YcdB (4). It was therefore likely that a titratable factor exists that is able to bind and protect Tat signal sequences and that could be involved in targeting of Tat substrates. Since HiPIP is a heterologous Tat substrate in E. coli, this titratable factor was likely to be a general signal sequence binding chaperone with affinity to many signal sequences. We thus focused on the identification of proteins with Tat signal sequence binding activities that could serve that purpose.

In the past, we identified DnaK as a chaperone that binds to HiPIP precursor (9), and others found that DnaK bound the signal sequence of DmsA (5). However, DnaK-deficient strains were not significantly affected in the translocation of the Tat substrates HiPIP or Sul (9, 21).

Since DnaK is an abundant chaperone that may compete with alternative chaperones for binding sites on Tat signal sequences, we avoided this problem by using extracts of a dnaK-deficient strain for affinity purifications. Homogeneous HiPIP precursor was coupled to an Aminolink™ (Pierce) matrix, and membrane-free crude extract was loaded on the column. After a washing step, HiPIP-binding proteins were eluted with buffer containing 0.5 M NaCl (Fig. 1A). One dominant protein band near 28 kDa and two faint bands (near 55 and 90 kDa) were detectable in the elution fractions. As identified by mass spectrometric analyses of peptides from the individual Coomassie-stained bands, the dominant band represented the chaperone SlyD, whereas the faint bands represented the chaperone GroEL and the endopeptidase MepA. MepA is periplasmic and thus no reasonable candidate for a signal sequence binding chaperone. In a control experiment, mature HiPIP was shown not to bind any soluble protein under these experimental conditions, indicating that all identified proteins bound HiPIP precursor in a signal sequence-dependent mode (Fig. 1B).

Since HiPIP is not an E. coli protein, these data were promising in that they pointed to general signal sequence binding activities of the detected chaperones. We tested this hypothesis by use of the two completely unrelated Tat signal sequences of CueO and AmiA. Both sequences were fused to the mature domain of HiPIP. This allowed us to exclude any unspecific binding and was very convenient, since fully folded and pure precursor fusion proteins could be obtained in large amounts by the same method as described for native HiPIP precursor (9). As with native HiPIP, the affinity chromatographies with AmiAs-HiPIP and CueOas-HiPIP resulted in the isolation of the chaperones SlyD and GroEL from DnaK-free extracts (Fig. 2, A and B). With CueOas-HiPIP there was a broader stained area below 70 kDa in which we could only detect GroEL. MepA was not present in these elutions, indicating that MepA does not generally bind signal sequences. In all cases, SlyD was the predominant Tat signal sequence-interacting chaperone in the absence of DnaK. Importantly, SlyD and DnaK, but not GroEL, were identified as signal sequence-binding proteins when DnaK-containing extracts were used for affinity purification (Fig. 2C and supplemental Fig. S1). SlyD was the predominant signal sequence-binding protein also in these cases. We therefore tested which proteins bind Tat signal sequences when extracts without SlyD are used for affinity purification (supplemental Fig. S2). With the HiPIP precursor, we found DnaK as a strongly interacting chaperone. Interestingly, with AmiAs-
HiPIP, we identified YcfD, an essential protein of unknown function (22), and SlpA (SlyD-like protein) (23).

Together, the affinity screen for Tat signal sequence-binding proteins identified SlyD as a new chaperone that generally binds to Tat signal sequences in addition to DnaK in vitro. Moreover, YcfD and SlpA can bind the signal sequence of AmiA. GroEL can also bind signal sequences, but this chaperone is most likely less important for signal sequence binding under conditions when DnaK is present, because it was not detectable in affinity chromatographies with extracts containing DnaK.

**DnaK Is Essential for the Tat Transport of CueO** — Having identified DnaK and SlyD as the most likely general Tat signal sequence binding chaperones in *E. coli*, we analyzed the effect of their absence on the transport and stability of Tat substrates. At first, we chose for this analysis the Tat substrate CueO, for which specific signal sequence chaperones are not predicted (1). CueO is a multicopper oxidase that is involved in copper homeostasis in *E. coli* (24, 25). The transport of recombinant CueO was analyzed by subcellular fractionation and Western blot detection in wild type *E. coli*, in strains with single or double deletions in the *slyD* and *dnaK* genes, and in a *tatC* deletion strain. In the Δ*slyD*Δ*dnaK* strain, the transport was blocked, and no processed CueO was anymore detectable in the periplasm, whereas full-length CueO accumulated inside the
cytoplasm (Fig. 3). The absence of the chaperones thus affected translocation without compromising the stability of the precursor in the cytoplasm. When we analyzed the effects that were already caused by single deletions in the slyD or dnaK genes, we found that the transport of CueO was not inhibited in the ΔslyD strain, whereas in the ΔdnaK strain no mature CueO was detectable in the periplasm (Fig. 3). Due to the block of translocation, CueO precursor clearly accumulated in the cytoplasm of the ΔdnaK strain. Traces of precursor and intermediate size CueO were reproducibly released into the periplasm by some cytoplasmic leakage due to an increased sensitivity toward the osmotic shock, but no mature size CueO was generated. The leakage of cytoplasmic proteins was supported by the detection of small amounts of cytoplasmic biotin carrier protein in the periplasm of this strain (Fig. 3). We found that the expression of CueO itself caused the leaking, since the ΔdnaK strain fractionated normally in other cases (Fig. 4). It is interesting that the additional absence of SlyD resulted in a normal fractionation behavior. Together, the data indicated that the block of CueO translocation in the ΔslyD/ΔdnaK strain was caused by the absence of DnaK alone and not of SlyD. There was a rather positive effect of the slyD deletion on membrane interaction and translocation of CueO. Without SlyD, CueO appeared more abundant in the membrane and periplasmic fractions. However, as the ΔslyD/ΔdnaK strain was blocked in translocation, the lack of DnaK was dominant over any positive effect of SlyD absence.

Together, the CueO data show that DnaK is essential for targeting of CueO at a late stage after an initial folding inside the cytoplasm. Full-length precursor CueO clearly accumulates in the double deletion strain, indicating that this protein folds to a protease-resistant conformation in the absence of DnaK or SlyD. Importantly, the signal sequence of CueO is not degraded in the double or single deletion mutants, suggesting that other chaperones can bind the CueO signal sequence in the absence of DnaK or SlyD. This is not surprising, considering the finding that, for example, GroEL binds the Tat signal sequence of CueO when DnaK is absent (see Fig. 2).

DnaK Dependence of CueO Targeting Is Not Only Conferred by Signal Sequence Characteristics—Since SlyD and DnaK act on CueO in vivo, we asked the question whether the chaperone dependence of CueO targeting was only conferred by signal sequence characteristics. To test this, we fused the signal sequence of CueO to the mature domain of HiPIP and assessed the transport of this CueOss-HiPIP fusion protein (Fig. 4). Precursor and mature forms of this fusion protein migrated very closely in SDS-PAGEs. Importantly, CueOss-HiPIP transport was completely blocked in a TatC-deficient strain, underlining a strict Tat dependence of CueOss-HiPIP targeting. CueOss-HiPIP was Tat-dependently targeted in all chaperone deletion mutants, and there was no significant difference in targeting efficiency detectable. Together, these data indicate that characteristics of the mature domain must be the basis of the requirement of DnaK for targeting of CueO.

DnaK and SlyD Have No Effects or Have Only Minor Effects on the Tat-dependent Translocation of DmsA, HiPIP, and YcdB—After having established a targeting-related phenotype for the absence of DnaK in the case of CueO, we addressed the question of whether or not DnaK or SlyD is generally important for Tat transport. We thus tested three further Tat substrates, two of which do not have known specific signal sequence chaperones.

Me2SO reductase is Tat-dependently targeted to the membrane as a DmsAB heterodimer. In the membrane, it associates with DmsC to form a functional membrane integral DmsABC trimmer (26, 27). DmsA signal sequences are bound by a specific chaperone, DmsD, which has been the first specific Tat substrate chaperone reported (5). We analyzed the presence of DmsA in membranes from the above described single and double mutant strains (Fig. 5A). DmsA was membrane-targeted in the presence or absence of the chaperones SlyD and DnaK. As expected, there was no membrane targeting in the absence of...
SlyD and DnaK Copurify with Precursor Tat Substrates—SlyD and DnaK bound Tat signal sequences in vitro, but in vivo these chaperones appeared to be essential only in the exceptional case of CueO. We thus tested the above described soluble Tat substrates YcdB, CueO, and HiPIP for their chaperone binding activity in vivo. Strep-tagged precursor proteins were purified by affinity chromatography, and the co-elution of DnaK or SlyD was assessed by immunoblot analyses (Fig. 6). DnaK co-eluted strongly with the precursor forms of these three Tat substrates. A small amount of DnaK was also detectable in wash fractions, indicating that some DnaK was released from the Tat substrates during washes. In contrast to the co-elution with precursor Tat substrates, there was no co-elution of DnaK with mature YcdB and almost no co-elution with mature CueO and HiPIP. The data therefore clearly show that DnaK binds the signal sequences of all three tested Tat substrates. In the case of SlyD, co-elutions were exclusively detectable with Tat substrate precursors. SlyD was especially strongly binding to and co-eluting with the HiPIP precursor (Fig. 6C, elution fractions 2–4). In this case, almost all SlyD from the crude extract was bound to the affinity column, and hardly any SlyD was present in the flow-through. The amount of SlyD co-eluting with YcdB or CueO precursor was lower but still significant (Fig. 6, A, elution fraction 1, and B, elution fraction 2).

Together, these data document the in vivo interaction of SlyD and DnaK with the signal sequences of the Tat substrates YcdB, CueO, and HiPIP. In agreement with the in vitro binding, these results underline that a broad range of Tat signal sequences is recognized by the two chaperones.

Since SlyD and DnaK have overlapping functions in E. coli with regard to their binding to Tat signal sequences, other func-
FIGURE 6. DnaK and SlyD interact with the signal sequences of HiPIP, CueO, and YcdB in vivo. Cultures of strain MC4100 containing either pBAD-ycdB-strep (A, upper three blots), pBAD-mat-ycdB-strep (A, lower three blots), pBW-cueO-strep (B, upper three blots), pBW-mat-cueO-strep (B, lower three blots), pBW-hip-strep (C, upper three blots), or pBW-mat-hip-strep (C, lower three blots) were grown aerobically at 37 °C and induced for 2 h with 0.1% arabinose (A) or rhamnose (B and C). Soluble proteins were prepared, and Strep-tagged YcdB, CueO, or HiPIP was purified by affinity chromatography using a Strep-tactin Superflow matrix (IBA, Göttingen). Fractions from the purification were analyzed by SDS-PAGE and Western blots, using Strep-tactin horseradish peroxidase conjugate (IBA, Göttingen) or sera specific for SlyD or DnaK. CE, crude extract (soluble proteins); FT, flow-through (nonbound material); W6, last wash fraction; E, elution fractions. Molecular mass markers (in kDa) are indicated on the left, and proteins of interest are indicated on the right, pre, precursor; mat, mature protein.

There Are Several General Chaperones That Can Interact with Tat Signal Sequences—This study sheds some light on the obscure role of chaperones in Tat-dependent transport. One of the central Tat-related questions is how unfolded Tat signal sequences are targeted to the translocon. For a large subset of redox-Tat substrates, specific chaperones are recruited by the signal sequence, thus allowing a protection of the signal sequence inside the cytoplasm (1). The scenario has not been clarified so far for those Tat substrates that do not have their own specific chaperone.

We approached this problem by a combination of in vitro and in vivo techniques. In a screen for binding proteins, we could identify with SlyD, DnaK, and GroEL several chaperones that bind to a broad range of unrelated Tat signal sequences. DnaK is a well known chaperone for Sec substrates that can even substitute SecB under some conditions (28, 29). This is not surprising, since DnaK binds hydrophobic sequences with positively charged flanking regions, just like those typically found in signal sequences of Sec or Tat substrates or distributed across unfolded proteins that expose their hydrophobic cores (30). In the past, attempts to identify Tat signal sequence binding chaperones resulted in the identification of DnaK (5, 9). However, the physiological relevance of these findings remained unclear, since a binding of DnaK has not been shown in vivo, and translocation of some substrates was not affected in dnaK deletion strains (9, 21, 31).

SlyD is less well known. It has been originally identified as a gene required for sensitivity to lysis (sly) upon infection with a spherical phage (32) and turned out to protect the phage lysis protein E from degradation (33). SlyD has been shown only recently to act as a general chaperone (34). It has an additional prolyl isomerase activity that has been well described earlier (23). The chaperone activity of SlyD resembles that of DnaK (34), and our data suggest some cooperative function of the two chaperones, making a similar recognition of exposed hydrophobic sequences likely.

GroEL is an important folding chaperone (35). It forms a cavity in which folding is promoted and which can be covered by GroES. The set of GroEL substrates has been identified recently (36). Notably, there is one Tat substrate, AmiA, that requires GroEL for folding. Since we also detected GroEL as an interaction partner of an AmiA-HiPIP fusion, the signal sequence might be one of the natural AmiA binding sites of GroEL. However, we did not detect GroEL with DnaK-containing extracts, and SlyD was much more prominent in the elutions than GroEL and DnaK. When SlyD was absent from the extracts, a so far unknown protein, YcfD, bound predominantly to the AmiA signal sequence, and a small amount of the SlyD-like protein SlpA (supplemental Fig. S2). YcfD is reported to be...
an essential protein (22). Our data indicate that the 42-kDa protein YcfD has a chaperone activity that has not been recognized so far. Future studies may reveal the exact role of YcfD.

Most importantly, the results reveal that there is not only one general Tat chaperone for those Tat substrates that do not have their specific chaperone; Tat signal sequences are chemically not fundamentally different from denatured protein domains or any peptide that exposes hydrophobic patches to its surface. Chaperones like DnaK, SlyD, or GroEL bind such peptides. DnaK and SlyD appear to bind signal sequences more efficiently than other chaperones.

**DnaK Functions in the Tat Pathway with CueO**—Only with CueO, we found that DnaK plays a key role in allowing this substrate to be targeted by the Tat pathway. The DnaK dependence of CueO transport is not conferred by its signal sequence alone, since a CueO<sub>Δ<sub>514</sub></sub>−HiPIP fusion was Tat-dependently targeted even in an slyD/dnaK double deletion strain. The requirement for DnaK thus must be caused by the mature domain of CueO.

Since DnaK is not essential for the targeting of the other tested Tat substrates, the exceptional case of CueO deserves much attention. What causes the DnaK dependence of CueO targeting? CueO folds in the absence of DnaK to a protease-resistant conformation, and its signal sequence does not require DnaK for stability. Therefore, DnaK must be involved in an essential step after folding. One might speculate that such a step could be related to the occupancy of copper sites in CueO. As an alternative, DnaK might play a unique role as a factor that is required to mediate the interaction of CueO with the Tat translocon. In ongoing studies, we now focus on these aspects in order to reveal the biochemical basis for the requirement of DnaK by CueO. Together, the data obtained with CueO suggest some active role of DnaK that adds to a simple signal sequence binding function.

**What Is the Function of SlyD?**—SlyD bound all tested signal sequences. However, the deletion of the slyD gene alone had no significant effect on the targeting of Tat substrates. In conjunction with a dnaK deletion, the slyD deletion had minor effects on DmsA targeting and processing or on the abundance of HiPIP precursor in the cytoplasm. One cannot exclude the possibility that SlyD has more prominent roles in the case of so far untested Tat substrates. The most striking phenotype was the slower growth of the slyD/dnaK double deletion strain, which points to a more important interplay of DnaK and SlyD in other cellular functions (supplemental Fig. S3). The absence of SlyD might be compensated by other chaperones in ΔslyD strains, making a functional assignment difficult. The situation resembles that of SecB, a prominent chaperone for post-translational translocation via the Sec pathway; SecB functionally overlaps with general chaperones, such as DnaK or GroEL, which have been implicated to be involved in chaperoning of Sec and Tat substrates (37). In fact, the general chaperone model of Randell and Hardy (37) is very attractive, and we would like to extend it only by the essential need of individual chaperones in the case of certain Tat substrates.

**How Do SlyD and DnaK Act on Signal Sequences?**—The chaperone activities of SlyD and DnaK are the basis for the observed signal sequence binding. DnaK is known to cycle between a substrate-bound and a free state (38). This cycling depends on ATP, ATP hydrolysis to ADP, and an ADP-ATP exchange. ATP hydrolysis and ADP-ATP exchange may be enhanced by specific co-chaperones. Whether this occurs also with Tat signal-bound DnaK has not been analyzed so far. An ATP-dependent binding/release cycle such as that of DnaK is not known for SlyD. SlyD is much smaller and does not appear to require additional helper proteins for efficient function. ATP binding of SlyD has been reported, but no physiological relevance could be demonstrated (39). In contrast, the chaperone function of SlyD has been demonstrated in the absence of nucleotides (34). The conditions under which SlyD efficiently releases bound proteins are unclear. This is probably the most striking difference between DnaK and SlyD functions. In this context, it is interesting to note that the SlyD-binding φX174 phage lysis protein E (MutE) is a protein that interacts with the cytoplasmic membrane (33). MutE consists of 91 residues. The protein has at its N terminus a hydrophobic leucine-rich sequence at positions 10–28 that most likely mediates the membrane interaction. At the membrane, MutE inhibits MraY, which catalyzes the initial step of the lipid carrier cycle for the transfer of N-acetyl-muramoyl pentapeptides across the cell membrane for cell wall biosynthesis (40). SlyD protects MutE from degradation and allows MraY inhibition and cell lysis. These considerations suggest a function of SlyD as a chaperone that can target hydrophobic sequences to the membrane. Membrane interactions have been demonstrated with Tat substrates (9, 41, 42), and the astonishing parallel between membrane targeting of MutE in the φX174 life cycle and the membrane targeting of Tat signal sequences might not be coincidental. These ideas are surely interesting hypotheses that have to be addressed experimentally.

**Perspectives**—We explicitly searched for chaperones that generally bind Tat signal sequences. For that, we initially used signal sequences of HiPIP, AmiA, and CueO, all of which do not have any obvious specific chaperone. We expected that the most prominent signal sequence binding chaperones could be essential for the stabilization of Tat signal sequences. Our search ended up in the understanding that the only really essential function of an identified signal binding chaperone was again related to the mature domain of a Tat substrate. DnaK thus acts like a “specific” chaperone on CueO. Although we definitely found two general Tat signal binding chaperones, these chaperones appear not to be the only ones that can contribute to the Tat-dependent targeting. We presume that SlyD and DnaK compete for signal sequences, and when both are absent, other chaperones perform the same function. We already have evidence that GroEL can bind various signal sequences in the absence of DnaK (Figs. 1 and 2). Overlapping functions of DnaK and GroEL are also supported by the observation that GroEL/ES overproduction can allow survival of a trigger factor and DnaK-deficient strain at low temperatures (43). Preliminary data of ongoing studies in our laboratory indicate that also EF-Tu and possibly further chaperones can interact with Tat signal sequences (data not shown). The amount of general chaperones that can bind unfolded signal sequences in the cell is therefore likely to be sufficient under most conditions. However, since it is well known that signal sequences of highly abun-
Tat Signal Sequence Binding Chaperones

dant recombinant Tat substrates are proteolytically attacked in the cytoplasm, the amount of chaperones must be insufficient under certain conditions of overexpression.

The binding of general chaperones to Tat signal sequences is a phenomenon that is likely to be very important for the understanding of the targeting mechanism. Tat signal sequences clearly bind general chaperones in the cytoplasm, which explains how the corresponding proteins can have the time to fold with only a minimal risk of losing their targeting signal prior to a translocon interaction.

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