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ARTICLE *in* BIOCHEMISTRY · JUNE 2009

Impact Factor: 3.02 · DOI: 10.1021/bi900520d · Source: PubMed

5096 *Biochemistry* **2009,** *48,* 5096–5105
DOI: 10.1021/bi900520d

BIOCHEMISTRY
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Twin-Arginine-Dependent Translocation of SufI in the Absence of Cytosolic Helper Proteins[†]

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Received March 26, 2009; Revised Manuscript Received May 6, 2009

ABSTRACT: The twin-arginine translocation (Tat) machinery present in bacterial and thylakoidal membranes is able to transport fully folded proteins. Folding of some Tat precursor proteins requires dedicated chaperones that also sequester the signal sequence during the maturation process. Whether or not signal sequence-binding chaperones are a general prerequisite for all Tat substrate proteins is not known. Here, we have studied the propensity of Tat signal sequences of *Escherichia coli* to interact with general chaperones and peptidyl-prolyl-*cis*, *trans*-isomerases. Site-specific photocross-linking revealed a clear specificity for FK506-binding proteins. Nevertheless transport of the Tat substrate SufI into inverted inner membrane vesicles of *E. coli* was found to occur in the *bona fide* absence of any cytosolic chaperone. Our results suggest that in *E. coli*, cytosolic chaperones are not essential for the twin-arginine-dependent export of cofactor-less substrates.

Twin-arginine translocation (Tat[1]) denotes a distinct protein transport pathway found in bacteria, archaea, and plant chloroplasts. Its outstanding features are (i) a consensus motif with an almost invariant pair of arginines in the signal sequences of Tat precursor proteins and (ii) the fact that many of the Tat substrates are transported across their hosts' membranes in a completely folded conformation. In Gram-negative bacteria such as *Escherichia coli*, three functionally distinct membrane proteins named TatA, TatB, and TatC enable Tat-dependent transport across the cytoplasmic membrane. TatB and TatC build a 1:1 complex (*1*) that forms high molecular mass oligomeric structures (*2*, *3*). The TatBC complex functions in the recognition of twin-arginine signal peptides (*4*, *5*). TatA prevails in the form of homomultimeric complexes both *in vitro* and *in vivo*, which when isolated have the shape of pore-like structures (*6*−*9*). The actual protein-conducting device of the Tat apparatus has, however, remained elusive. Likewise, the precise mechanism by which the indispensable H^+-motive force is transduced into vectorial protein movement is not understood (*10*, *11*). Although the Tat machinery can export unfolded protein domains (*12*, *13*), genuine Tat substrates, which normally undergo folding in the cytosol prior to export, do not progress along the Tat pathway if not folded (*14*−*16*).

Typical for the latter group of Tat substrates are many bacterial redox proteins, which acquire cofactors, fold, and even assemble into larger protein complexes in the cytosol before they are translocated into the periplasm. These maturation steps often involve substrate-specific chaperones. One of the best studied examples is TorD, which binds to the mature part of the *E. coli* Tat protein TorA (trimethylamine *N*-oxide reductase) and thereby probably assists in the incorporation of the molybdenum cofactor (*17*, *18*). A second binding site of TorD was localized to the signal sequence of TorA (*18*). Therefore, TorD was suggested to function as an antitargeting factor, preventing membrane targeting until the protein is correctly folded, and TorD is released, probably in a GTP-dependent manner (*19*). In a similar manner, HybE (*18*, *20*), NapD (*21*), and DmsD (*22*) seem to function as substrate-specific chaperones in the maturation and targeting of hydrogenase-2, nitrate reductase, and dimethylsulfoxide reductase of *E. coli*, respectively.

Because not every *E. coli* Tat substrate contains cofactors, the involvement of general chaperones in the biogenesis of cofactor-less proteins has been investigated. DnaK and SlyD were found associated with several Tat substrates of *E. coli* (*23*, *24*), but it has remained unclear whether or not these interactions reflect a functional involvement of those chaperones in the Tat export pathway. In this article, we have pursued this question by combining site-specific cross-linking of Tat signal sequences for

[†]This work was supported by Grant LSHG-CT-2004-005257 of the European Union (to E.H. and M.M.), grants form the Deutsche Forschungsgemeinschaft (Collaborative Research Centres 388 and 746, and Research Training Group GRK 434 to E.H. and M.M.), and a grant from the Human Frontier Science Program (to T.U.).

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[1]Abbreviations: ARS, amino acyl-tRNA synthetase; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; EF, translation elongation factor; FKBP, FK 506-binding protein; IF, translation initiation factor; INVs, inverted inner membrane vesicles; IPTG, isopropyl-*β*-D-thiogalactopyranoside; MTF, methionyl-tRNA transformylase; PPIase, peptidyl-prolyl *cis*, *trans*-isomerase; PK, proteinase K; RF, translation release factor; RRF, ribosome-recycling factor; Tat, twin-arginine translocation; TCA, trichloroacetic acid; TMAO, trimethylamine *N*-oxide; Tmd-Phe, L-4′-(3-[trifluoromethyl]-3*H*-diazirin-3-yl); TorA, TMAO reductase.

 Published on Web 05/11/2009

detecting specific interaction partners with studying Tat-dependent transport in a highly purified, i.e., *bona fide* chaperone-free environment.

EXPERIMENTAL PROCEDURES

Plasmids. Plasmid pKSMSuf-RR (*5*) was used for the *in vitro* synthesis of wild type pSufI, pSufL16-RR (*5*) for the TAG stop codon mutant at position 16 of pSufI, and pBluSK-T23K-L31 (*25*) for the TAG stop codon mutant at position 31 of TorA-23K. Plasmid pKSM-SufI-6xHis was constructed similarly to plasmid pKSMSufI-RR using as template p60-sufI, which encodes *sufI* with a C-terminal RSHHHHHH extension (obtained from Frank Sargent). Plasmid pASK-IBA3C-FkpA encodes a Strep-tagged version of FkpA under control of the *tet* promoter. For its construction, the *fkpA* gene was amplified by PCR using genomic *E. coli* DNA and the forward primer 5′ATG GTA GGT CTC AAA TGG CTG AAG CTG CAA AAC CTG CTA C 3′ and the reverse primer 5′ATG GTA GGT CTC AGC GCT TTT TTT AGC AGA ATC TGC GGC TTT C 3′, both introducing *Bsa*I restriction sites for ligating the PCR product into vector pASK-IBA3C (IBA GmbH, Gottingen, Germany). Plasmid pAAF3 (*19*) containing *torD* in vector pQE60 was obtained from Frank Sargent. The *E. coli* strains JW5746 (fklB), JW0026 (fkpB), JW0514 (ppiB), JW3748 (ppiC), and JW3311 (slyD) expressing the indicated PPIases with N-terminal His-tags from vector pCA24N under *lac* promoter control (*26*) in host strain BW25113 (*27*) were obtained from the NIG collection (Shizuoka, Japan). Plasmids expressing the individual genes for the components of the PURE system are described in ref (*28*).

Preparation of Membrane Vesicles. Inverted membrane vesicles (INVs) were prepared from strain MC4100 (F^-, Δ*lacU169*, *araD139*, *rpsL150*, *relA1*, *ptsF*, *rbsR*, *flbB5301*) (*29*) (wt INV), strain DADE (MC4100, Δ*tatABCDtatE*) (*30*) (ΔTat-INV), and strain DADE transformed with plasmids pQE60-TatABC and pREP4 (*25*) (Tat^+-INV). The preparation procedure has been detailed elsewhere (*31*).

Protein Synthesis in Vitro. Cell-free synthesis of pSufI for assaying transport into INVs (Figure 5C) and of pSufI-L16 for photocross-linking to INVs (Figure 1) was performed as described (*31*) using an S-135 cell extract prepared from *E. coli* strain SL119 (*31*).

In vitro transcription/translation using the PURE system was performed in 20 μL reactions containing 50 mM HEPES-KOH at pH 7.3, 10 mM magnesium acetate, 170 mM potassium acetate, 2 mM spermidine, 1 mM dithiothreitol, 3.5 mM ATP, 0.7 mM each of GTP, CTP, and UTP, 20 mM creatine phosphate, 9.375 A_{260} units/mL *E. coli* tRNA, 10 μg/mL 5-formyl-5,6,7,8-tetrahydrofolic acid, 0.1 mM each of 18 amino acids (pooled without methionine and cysteine), 40 μg/mL creatine phosphokinase, 3 μg/mL myokinase (Sigma, St. Louis, MO), 1.1 μg/mL nucleoside 5′-diphosphate kinase (Sigma), 2 μg/mL pyrophosphatase (Sigma), and 5−10 units of T7 RNA polymerase, 0.4 units of placental RNase inhibitor (RNasin), and 0.4−1.0 μM salt-washed ribosomes prepared as described (*32*) except that prior to sucrose gradient centrifugation ribosomes were freed of most of the cosedimenting membranes by scratching off the yellowish layer on top of the translucent ribosomal pellet, 20 μg/mL IF1, 40 μg/mL IF2, 15 μg/mL IF3, 70 μg/mL EF-G, 135 μg/mL EF-Tu, 70 μg/mL EF-Ts, 45 μg/mL RF1, 45 μg/mL RF3, 45 μg/mL RRF, 20 μg/mL each of 20 ARS and MTF, 4−6 μCi[^{35}S]-labeled methionine and cysteine, and ∼1 μg of DNA. The reaction was started by incubation at 37 °C, continued for 1 h, and terminated on ice.

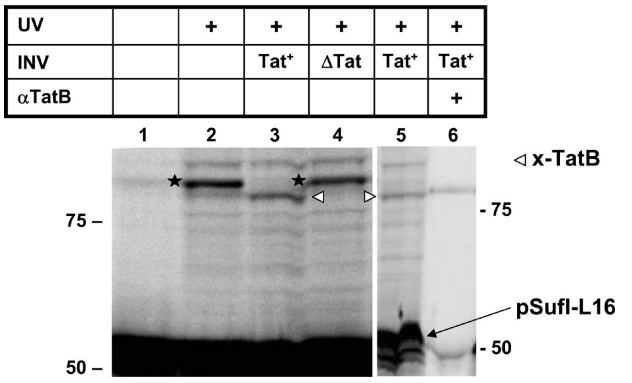

FIGURE 1: In the absence of TatABC, the twin-arginine signal peptide of *in vitro* synthesized pSufI cross-links to a 30 kDa protein. The precursor protein pSufI carrying Tmd-Phe at position L16 of its Tat signal sequence (pSufI-L16) was synthesized by an S-135 *E. coli* cell extract-based transcription/translation system. [^{35}S]-labeled translation products were separated by SDS−PAGE and are visualized by phosphorimaging. UV-irradiation generated specific adducts (marked with symbols). In the absence of membrane vesicles (INV), pSufI-L16 was cross-linked to an ∼30 kDa protein (star). In the presence of INVs, pSufI-L16 comes into contact with TatB (open arrowhead, x-TatB) as verified by coimmunoprecipitation using antibodies against TatB (αTatB; a slightly reduced electrophoretic mobility of the SufI-TatB adduct was occasionally observed following immunoprecipitation).

Transport into Membrane Vesicles. To assay transport of Tat precursor proteins, INVs were added 10 min (S-135) or 30 min (Pure system) after starting the synthesis reaction and incubated for 30 min at 37 °C. Together with the vesicles ATP, DTT, creatine phosphate, and creatine phosphokinase were added again at their initial concentrations, and reactions were made 0.5 M in potassium acetate, which was found to stimulate Tat-dependent translocation into *E. coli* INVs (*25*). Transport into INVs was monitored by resistance toward proteinase K (*31*).

Photocross-Linking. For the site-specific incorporation of Tmd-Phe (*5*), amber stop codon mutants of pSufI and TorA-23K were synthesized either by the S-135-based system or the PURE system, which for this purpose did not contain RF 1. The concentration of magnesium ions was individually adjusted in order to allow sufficient suppression by the Tmd-Phe-charged suppressor tRNA and to simultaneously prevent read-through of the stop codon.

Purification of FkpA. The 30 kDa protein cross-linking to the signal peptide of pSufI was purified from 4 L of an *E. coli* MC4100 culture. A postribosomal supernatant (500 mg protein) was applied to a Hydroxyapatite column in 20 mM potassium phosphate buffer at pH 7, and the 30 kDa cross-linking protein was eluted with 1 M potassium phosphate buffer at pH 7. The concentrated eluate was loaded on a Superose 12 column and fractionated using 50 mM HEPES-KOH at pH 7.6. Active fractions were combined and further purified by passage through a Mono Q column equilibrated with 20 mM NaCl in 25 mM Tris-HCl at pH 7.5. An SDS−PAGE of the flow through of the MonoQ column is depicted in Figure 2 after staining with Coomassie Blue. After blotting onto a PVDF membrane, the major 30 kDa protein was subjected to automated Edman degradation.

Purification of Tagged Proteins. The expression of FkpA-Strep from plasmid pASK-IBA3C-FkpA was induced by 200 μg/L anhydrotetracyline, and the protein was purified

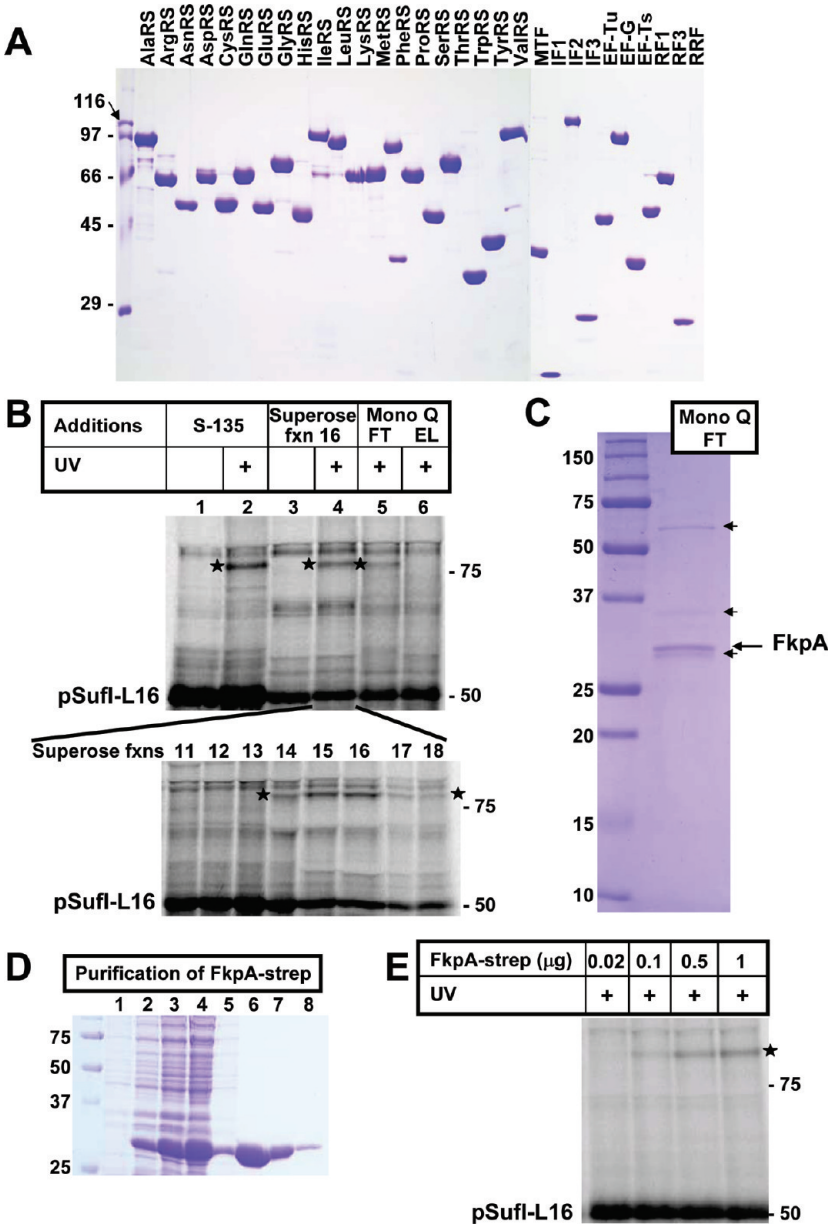

FIGURE 2: Purification and identification of the 30 kDa protein that cross-links to the signal sequence of pSufI. (A) The individual components of the PURE system purified by metal affinity chromatography and displayed by SDS−PAGE and staining with Coomassie Blue. (B) When pSufI-L16 was synthesized by the PURE system, the cross-link to the 30 kDa protein (star) appeared only upon addition of soluble protein fractions, such as S-135 or subfractions of an S-150 (for details on the purification see Experimental Procedures). FT, flow through; EL, eluate. Experimental conditions were as in Figure 1, except for the use of the PURE system. (C) Protein pattern (arrows) after the final step of purification (flow through of Mono Q) displayed by SDS−PAGE and staining with Coomassie Blue. The major 30 kDa protein was identified by N-terminal sequencing as FkpA. (D) Overexpression and purification of a Strep-tagged version of FkpA as follwed by SDS−PAGE and staining with Coomassie Blue. Lane 1, uninduced cells; lane 2, cells induced with anhydrotetracycline; lane 3, extract of induced cells; lane 4, proteins not retrained by the Strep-Tactin matrix; lane 5, wash fluid; lanes 6−8, eluate steps. (E) Cross-linking of pSufI synthesized by the PURE system to increasing amounts of pure FkpA-strep. Experimental conditions were as in panel B.

using Strep-Tactin Spin columns (IBA GmbH). His-tagged proteins were purified from *E. coli* S-30 cell extracts via Co^{2+}-affinity chromatography (Talon, BD Biosciences, Clontech). Proteins were eluted with steps of 50 mM, 100 mM, and 200 mM imidazole in 20 mM HEPES-KOH at pH 7.6 containing 1 M ammonium acetate, 10 mM magnesium acetate, and 7 mM β-mercaptoethanol, and active fractions were pooled. Trigger factor was purified as described (*32*).

Pulse-Chase Experiments. The MC4100 derivative SR1458 (*33*) and the isogenic transductant NS16 (*fkpA::cam*) were transformed with plasmids pKSM-SufI-6xHis and pREP4 (Qiagen). Overnight cultures were diluted 1:50 into M63 minimal media containing 10 mg/L thiamine hydrochloride, 100 μM each of 18 amino acids except methionine and cysteine, and the appropriate antibiotics, and grown at 37 °C. At A_{600} = 0.5, aliquots were transferred to prewarmed microfuge tubes, IPTG was added to 1 mM, and cultivation continued for various times in an Eppendorf Thermomixer. Cells were pulsed for 30−60 s with 4 μL/mL [^{35}S]-containing methionine and cysteine (11−15 μCi/μL) and then chased with unlabeled methionine and cysteine (500 μg/mL final). Samples (250 μL) were removed as indicated, mixed with TCA (5% final), and incubated on ice for 30 min. Precipitates were collected by centrifugation (12 500*g* for 10 min) and dissolved in 50 μL of DTT-less SDS−PAGE sample buffer. After the addition of 1 mL of 8 M Urea in 0.1 M Tris-HCl at pH 8 (urea buffer), the sample was mixed with 50 μL of Ni-Sepharose 6

Fast Flow (GE Healthcare) and incubated under shaking for 60 min at 25 °C. After washing the matrix twice with urea buffer, bound proteins were eluted with 25 μL of SDS−PAGE sample buffer and heating to 56 °C for 10 min.

Spheroplasts were prepared from SR1458 (pKSM-SufI-6xHis, pREP4) cells grown in M63 medium to an A_{600} = 0.5 as described (*34*) with the following modifications. M63 was the minimal medium used; after incubating the freshly prepared spheroplasts for 15 min at 37 °C, 1 mM IPTG and 3 min later [^{35}S]-methionine/cysteine were added. The labeling period was stopped after 30 s by adding unlabeled methionine and cysteine (500 μg/mL final each). Samples were subjected to Ni-Sepharose chromatography and prepared for SDS−PAGE as detailed above.

Anaerobic Growth on TMAO. Cells were grown on minimal (*35*) agar plates in the presence of 0.4% glycerol, 10 mg/L thiamine hydrochloride, and 50 mM TMAO in a BBL Gaspack System.

RESULTS

In the Absence of TatABC, the Signal Sequence of in Vitro Synthesized SufI Preferentially Interacts with the Periplasmic Chaperone FkpA. Most of the experiments described herein were performed with the cofactor-less *E. coli* Tat substrate SufI (*36*). In order to identify molecular binding partners of its signal sequence, we replaced the leucine at position 16 within the hydrophobic core of the signal sequence by a photoreactive derivative of phenylalanine (Tmd-Phe, L-4′-(3-[trifluoromethyl]-3*H*-diazirin-3-yl). This was achieved by *in vitro* transcription/translation of a *sufI* mutant containing an amber stop codon instead of the leucine codon at position 16. In the presence of an amber suppressor tRNA that had been chemically acylated with Tmd-Phe, the full-size precursor of SufI carrying the photoprobe Tmd-Phe at this position (pSufI-L16) was synthesized *in vitro* (Figure 1). As shown previously (*5*, *25*), when pSufI-L16 was allowed to bind to inside-out inner membrane vesicles (INVs) of *E. coli*, UV-irradiation leads to a cross-link of the signal sequence to TatB (Figure 1, lanes 3, 5, and 6, open triangle). In contrast, in the absence of membranes a slightly larger adduct was obtained (lane 2, star), representing cross-linking of the 52 kDa pSufI-L16 to an ∼30 kDa protein. This 80 kDa adduct was also obtained in the presence of INVs that had been prepared from a *tatABCDE* deletion mutant (lane 4), suggesting that when no functional Tat translocase was available, the signal sequence of pSufI preferentially interacted with the 30 kDa protein.

Because the 30 kDa protein was found to associate with the signal sequence of pSufI even if no INVs were added, it must be contained in the membrane-free *E. coli* extract that was used as the cell-free translation system. In order to identify the 30 kDa cross-linking partner, we therefore switched to a highly purified translation system (PURE system) (*28*). This system consists of the 20 *E. coli* amino acyl-tRNA synthetases and all of the translation factors displayed in Figure 2A. These 30 components were purified by metal affinity chromatography (Figure 2A). When pSufI-L16 was now synthesized by the PURE system, the 80 kDa cross-link could be detected if the PURE system was supplemented with preparations of soluble proteins (Figure 2B). Thus, the cross-linking partner of the SufI signal sequence was recovered from the total protein pool of an S-135 extract of *E. coli* (Figure 2B, lane 2, star). Upon further purification, it was found to bind to hydroxyapatite (not shown) and eluted from Superose 12 as a defined peak (Figure 2B, lane 4 and inset). After passage through an anionic exchange column (Mono Q) (lanes 5 and 6), the cross-linking activity displayed one major 30 kDa protein upon staining with Coomassie Blue, in addition to three faintly stained bands (Figure 2C, arrows). The 30 kDa material was subjected to automated N-terminal Edman degradation and yielded the amino acid sequence AEAAKPATAADSKAA. This sequence is identical to the N-terminal sequence of FkpA that has been characterized as chaperone and peptidyl-prolyl *cis*,*trans*-isomerase (PPIase). FkpA is, however, a periplasmic protein and as such was not expected to interact with a signal sequence. In order to therefore confirm the identity of the 30 kDa cross-linking partner of the SufI signal sequence as FkpA, we subcloned the fkpA gene in front of a Strep-tag and purified the tagged version to apparent homogeneity (Figure 2D). When this material was added to the PURE system synthesizing pSufI-L16, the 80 kDa adduct in fact appeared in a concentration-dependent manner (Figure 2E, star).

In Vivo FkpA Is Not Essential for Twin-Arginine Translocation. The presence of the periplasmic FkpA within the pool of soluble proteins used for *in vitro* synthesis of pSufI was most likely due to a contamination of cytosolic with periplasmic proteins occurring during the disruption of the *E. coli* cells. Nevertheless, the site-specific cross-linking of the signal sequence of pSufI with FkpA was rather selective. We therefore examined whether *in vivo* FkpA exerted any effect on Tat-specific translocation (Figure 3). *E. coli* cells expressing a His-tagged version of SufI under *lac* promoter control were induced for 3 min with 1 mM IPTG and pulse-labeled for 30 s with ^{35}[S]-methionine/cystein. Samples were withdrawn after different chase periods, the precursor and mature form of SufI were purified by adsorption to Ni-Sepharose, displayed by SDS−PAGE, and visualized by phosphorimaging (Figure 3A). Within 60 min of chase, 75% of the precursor (pSufI) synthesized during the pulse period became processed to the mature form (SufI). The same type of experiment was repeated with an isogenic pair of wild type and *fkpA* deletion mutant cells. In the absence of FkpA, pSufI was processed by virtually the same kinetics as those in wild type cells (Figure 3B), indicating that FkpA did not influence the efficiency of SufI translocation. This was true although these cells had purposely been induced by IPTG for longer periods of time than in the experiment shown in Figure 3A, in order to challenge the Tat translocase with a higher load of precursor of SufI.

Increased sensitivity toward growth in the presence of SDS is a typical phenotype of cells lacking a functional Tat system (*37*, *38*). This is illustrated in Figure 3C, in which cell growth in the presence of increasing concentrations of SDS is compared between wild type cells (wt), the *fkpA* deletion mutant (Δ*F*kpA), and a *tatABCDE* deletion mutant (ΔTat). Whereas the ΔTat cells show the reported increased sensitivity toward SDS, FkpA-deprived cells were as resistant as wild-type cells. Tat-deficient cells further fail to grow anaerobically on minimal media with the nonfermentable carbon source glycerol and trimethylamine N-oxide (TMAO) as the sole electron acceptor. This is due to the impaired translocation of the Tat substrate TMAO reductase (TorA) to the periplasmic space in *tatABCDE* cells (Figure 3D, ΔTat). Again, Δ*fkpA* cells did not show reduced growth under these conditions indicating that FkpA is not an essential component of the Tat pathway. Finally, we examined whether periplasmic chaperones could globally be shown to exert an effect on the Tat-dependent protein export. To this end, the rate of processing of pSufI as a reflection of transmembrane

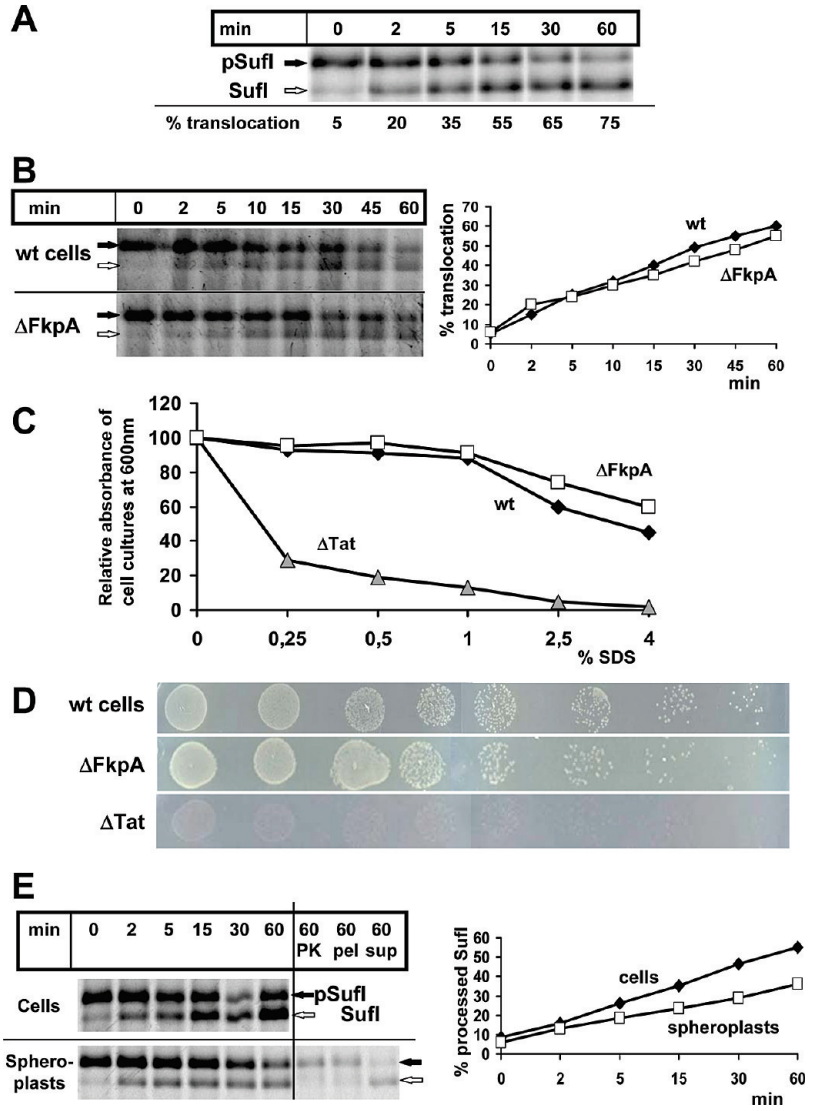

FIGURE 3: FkpA is not essential for the Tat export *in vivo*. (A) Wild-type *E. coli* MC4100 cells harboring plasmid pKSM-SufI-6xHis were induced for the synthesis of pSufI for 3 min with 1 mM IPTG, pulse-labeled with [^{35}S]-methionine/cysteine for 30 s, and chased as detailed in Experimental Procedures. Samples were withdrawn at the indicated times, and precursor (pSufI) and mature form (SufI) were isolated via Ni-affinity chromatography and are displayed following SDS−PAGE and phosphorimaging. The conversion of pSufI to SufI was quantitated and is indicated as the relative percentage of translocation. (B) Maturation of pSufI in an isogenic pair of MC4100 wild type (wt) and Δ*fkpA* mutant cells. Experimental conditions are as in panel A, except that pSufI was induced with IPTG for a longer period of time (15 min) prior to a 1 min labeling period. (C) *E. coli* cell cultures of strains SR1458 (wt), NS16 (Δ*F*kpA), and DADE (ΔTat) were grown in the presence of the indicated concentrations of SDS, and the absorbance at 600 nm was measured after 3 h of growth. (D) Cell cultures of the strains specified in panel C were adjusted to an A_{600} = 1 with LB medium and then serially diluted 10-fold each, applied to minimal agar plates containing TMAO, and grown anaerobically. (E) As in panel B, except that the maturation of pSufI was compared between intact cells and spheroplasts (see Experimental Procedures). Control lanes show proteinase K-resistant pSufI indicating the intactness of the spheroplasts (PK) and the separation of the spheroplast-associated pSufI (pel) from the secreted, i.e., released, SufI (sup).

translocation was compared between intact cells and spheroplasts that *bona fide* lack the major pool of periplasmic proteins (Figure 3E). Whole cells and spheroplasts in parallel were induced for the synthesis of pSufI and pulse-labeled as described above. The success of spheroplasting was examined by treatment with proteinase K, which, as expected, left the cytosolic precursor of SufI undigested but degraded most of the translocated and therefore released SufI (PK). Furthermore, centrifugation of the pulse-labeled spheroplasts showed the expected separation of the soluble SufI (Sup) from the spheroplast-associated, pelletable precursor (Pel). Both the rate of translocation of SufI and its maximal efficiency after 60 min were lower for the spheroplasts as compared to those of intact cells. The defect could, however, not be repaired by adding a fraction of periplasmic proteins to the spheroplasts during the period of SufI synthesis (data not shown). It is therefore unlikely that the absence of FkpA and other periplasmic chaperones is the cause for the reduced translocation of SufI from spheroplasts, which might rather be due to a global metabolic deficiency following the procedure of spheroplasting. Collectively, the results summarized in Figure 3 could not disclose any physiological involvement of the periplasmic chaperone and PPIase FkpA in the Tat export pathway of *E. coli*.

Tat Signal Sequences Are Preferentially Recognized by FKBP-Type Chaperones. The pronounced interaction of the SufI signal sequence with FkpA nevertheless suggested a free accessibility of the signal sequence prior to its binding to the Tat translocase and indicated some kind of affinity for an FkpA-type chaperone/PPIase. Besides the ribosome-associated Trigger factor, the cytoplasm of *E. coli* contains the three FK 506-binding

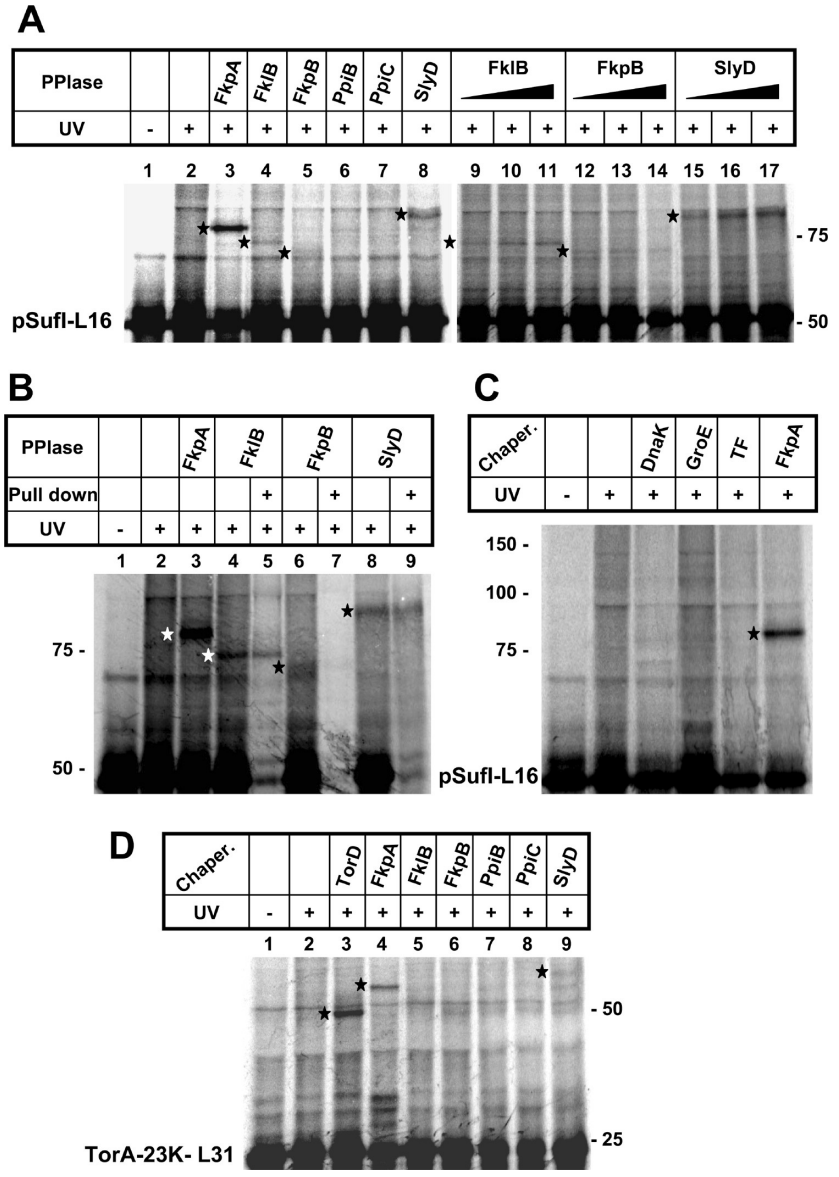

FIGURE 4: Tat signal sequences preferentially bind FK 506-binding proteins. (A) The Tat precursor pSufI-L16 was synthesized in the PURE system in the presence of the indicated chaperones. UV-irradiation led to distinct cross-links (stars). Purification of FkpA-Strep as well as of His-tagged PPIases and TorD is described in Experimental Procedures. Lanes 9−17: the amounts of the purified PPIases added are 1, 2, and 3 μg each. For further experimental details, see legends to Figures 1 and 2. (B) As in panel A, except that where indicated cross-linking products were purified via Ni-affinity chromatography (pull down). (C) As in panel A, except for the addition of DnaK (1.5 μg DnaK + 0.7 μg DnaJ + 0.35 μg GrpE), GroE (1.5 μg GroEL + 1 μg GroES), Trigger factor (TF, 1 μg), and FkpA (1 μg FkpA-Strep). DnaK and GroE chaperones and cochaperones were obtained from Stressgen. (D) As in panel A, except that the Tat precursor protein TorA-23K-L31 was probed for contacts to the indicated chaperones.

proteins (FKBPs) SlyD, FklB, and FkpB, in addition to the cyclophilin PpiB and the parvulin PpiC. In order to find out if any of these PPIases showed a similar strong interaction with the signal sequence of SufI, we purified His-tagged versions of these five cytosolic PPIases of *E. coli* by cobalt-affinity chromatography and added them during the synthesis of pSufI-L16 by the PURE system. As illustrated in Figure 4A (stars), the strongest cross-link was obtained with the periplasmic FkpA (lane 3), but the FKBPs FklB and SlyD also gave clearly visible adducts (lanes 4 and 8), whereas that of FkpB is hardly visible (lane 5), and none could be detected for the cyclophilin PpiB and the parvulin PpiC (lanes 6 and 7). Because the apparent electrophoretic mobility of the individual adducts did not match the calculated sum between the molecular mass of pSufI (52 kDa) and those of FklB (30 kDa), FkpB (17 kDa), and SlyD (21 kDa), additional experiments were performed to confirm the identity of the cross-links obtained. Thus, at least for FklB and SlyD, the intensity of the adducts increased with increasing amounts of PPIases added (Figure 4A, lanes 9−17), and the FklB- and SlyD-adducts of pSufI-L16 were clearly retained by Ni-Sepharose (Figure 4B, lanes 5 and 9) indicating that those adducts in fact contained a His-tagged FKBP. In contrast, no cross-linking of the signal sequence to the cytosolic *E. coli* chaperones DnaK-DnaJ-GrpE, GroEL-GroES, and Trigger factor was detected under conditions in which FkpA strongly reacted (Figure 4C). The affinity of a twin-arginine signal sequence for *E. coli* FKBPs was finally corroborated by use of the signal sequence of TorA. Figure 4D depicts the result obtained with the fusion protein TorA-23K, in which the TorA signal sequence is fused to the 23 kDa subunit of the photosystem II oxygen-evolving complex from spinach chloroplasts (*39*). When this composite Tat substrate harboring the photoreactive cross-linker in the hydrophobic core of its

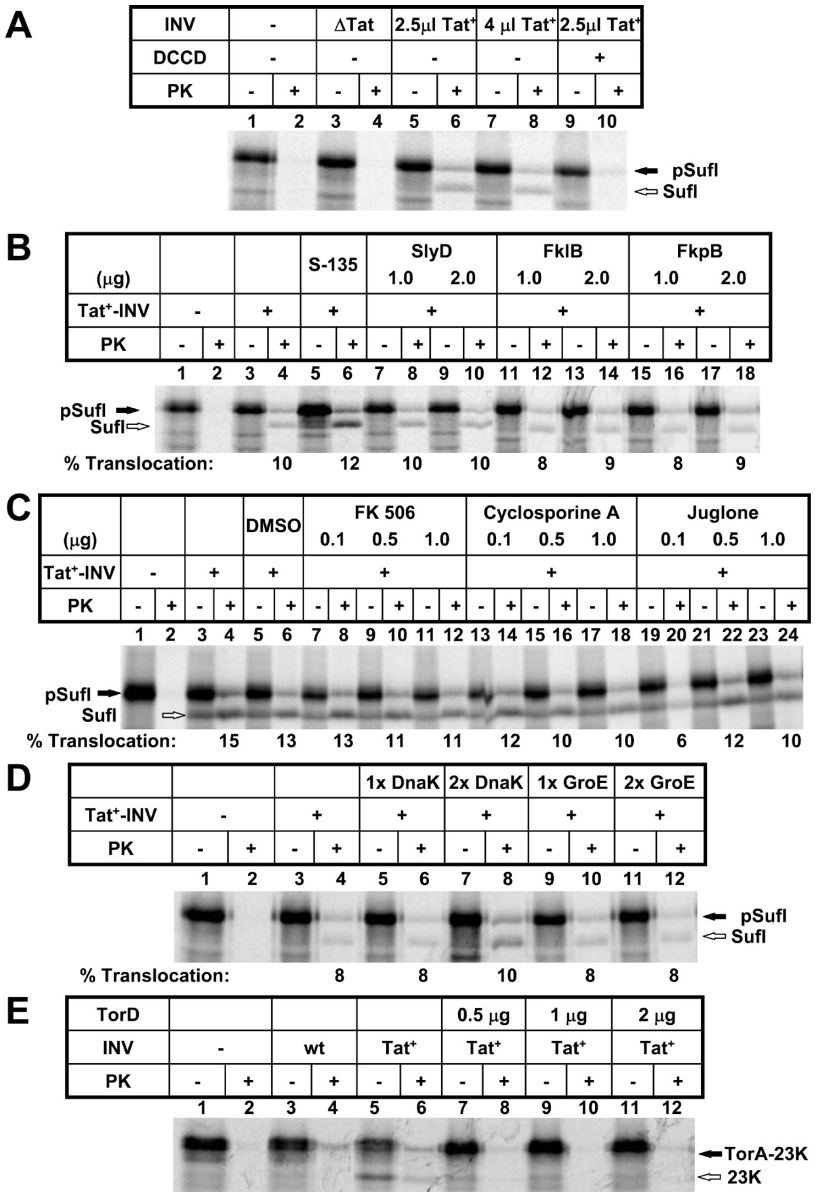

FIGURE 5: *In vitro* twin-arginine-dependent translocation of pSufI is not influenced by cytosolic chaperones. (A) SufI was synthesized by the PURE system, and transport into INVs was measured via the resistance of precursor (pSufI) and mature SufI against added proteinase K (PK; 0.5 mg/mL final concentration for 20 min at 25 °C). INVs used were prepared from a *tatABCDE* deletion strain (ΔTat) and a Tat-overproducer (Tat^+). DCCD was prepared as described (*53*). (B) As in panel A, except for the addition of either S-135 extract or purified FKBPs. Translocation efficiency was calculated as described (*32*). (C) SufI was synthesized by an S-135-based transcription/translation system in the presence of the indicated PPIase inhibitors, which had been dissolved in DMSO. (D) As in panel B except for replacing FKBPs by the DnaK and GroE chaperone/cochaperone systems (for details see the legend to Figure 4C, with 1x denoting the amounts indicated there). (E) Synthesis of the Tat precursor TorA-23K by the PURE system in the presence of wild-type (wt) and TatABC-enriched (Tat^+) INVs. PK-resistance reflecting transport into INVs was only observed for Tat^+-INV and was prevented by the TorA-specific chaperone TorD. The medium translocation efficiencies for pSufI between all experiments shown in panels A−D amounted to 10% (also confirmed by the experiments depicted in panel A, lanes 6 and 8; not shown). The small increases and decreases in translocation efficiency that were observed for individual additions are regarded as not significant.

signal sequence (TorA-23K-L31) (*25*) was synthesized in the PURE system and in the presence of purified chaperones, the strongest cross-link was obtained with TorD (lane 3), which is the dedicated chaperone of TMAO reductase (*17*, *18*). As with the signal sequence of SufI, FkpA yielded a prominent adduct also with the TorA signal sequence (lane 4). Except for SlyD (lane 9), no other PPIase visibly cross-linked to the TorA signal sequence.

Twin-Arginine-Dependent Translocation of pSufI into E. coli Membrane Vesicles Does Not Require Cytosolic Transport Factors. The results presented so far suggest that in the absence of the cytoplasmic membrane, Tat signal sequences preferentially interact with FKBP-type chaperones. In order to find out if this interaction had a functional impact, pSufI was synthesized in the *bona fide* chaperone-free environment of the PURE system (Figure 5). As depicted in Figure 5A, pSufI synthesized in the absence of INVs was completely susceptible to digestion by proteinase K (PK, lane 2) but gained PK-resistance in the presence of INVs prepared from a TatABC-overexpessing *E. coli* strain (Tat^+-INV, lanes 6 and 8). PK-protection by the INVs was observed for the precursor of SufI but more revealingly also for the signal sequence-less mature SufI (lanes 6 and 8) indicating that some pSufI had been fully translocated across the membrane of the vesicles where it gained access to the INV-associated signal peptidase. Moreover, translocation into INVs was seen only with vesicles containing TatABC (compare lanes 4 and 6) and was largely abolished by

dissipating the H^+-motive force of the vesicles by the F_1F_O inhibitor DCCD (lane 10). Collectively, these findings indicate that pSufI even when synthesized in the chaperone-free environment of the PURE system nevertheless is translocated via the Tat pathway into the lumen of INVs, where it becomes inaccessible to externally added PK.

Consistent with a principal independence of chaperones, the translocation efficiency of pSufI could not be increased when the PURE system was supplemented with the purified signal sequence-binding FKBPs, SlyD, FklB, and FkpB (Figure 5B). Nor was this the case when the individual chaperones were replaced by a total cytosolic *E. coli* extract (S-135) (lane 6). However, we could not rule out the formal possibility that the PURE system that we used contained some residual contaminating PPIase activities, which were sufficient to sustain the observed levels of Tat-specific translocation. We therefore performed the synthesis of pSufI in a total cytosolic S-135-based translation system, i.e., in the presence of all cytosolic chaperones, and added inhibitors specific for each class of PPIases (Figure 5C). Translocation into Tat^+-INV was, however, not visibly affected either by the FKBP inhibitor FK506 (lanes 7−12) or the cyclophilin inhibitor cyclosporine A (lanes 13−18), nor the parvulin inhibitor juglone (lanes 19−24).

Because the FKBPs did not improve the twin-arginine-specific translocation into INVs in a chaperone-deprived environment despite their obvious capability to interact with Tat signal sequences, we next tested the two general chaperone/cochaperone systems DnaK/DnaJ/GrpE and GroEL/GroES under the same experimental conditions. Again, no stimulation of the efficiency of the transport of pSufI into INVs was observed (Figure 5D). On the contrary, TorD specifically interacting with the signal sequence of TorA and thereby preventing a premature membrane-targeting (*18*) clearly interfered with the transport of TorA-23K into Tat^+-INV when synthesized in the PURE system (Figure 5E, compare lane 6 with lanes 8, 10, and 12). This finding suggests that our experimental system was sensitive toward chaperones that are functionally involved in the Tat pathway. Thus, the failure to detect any stimulating influence of cytosolic chaperones on the Tat-dependent translocation of pSufI into INVs in fact suggests that this precursor can pass through the Tat pathway largely unassisted by general cytosolic chaperones and PPIases.

DISCUSSION

When the Tat substrate pSufI was synthesized by a cell-free *E. coli* translation system consisting of highly purified amino acyl-tRNA synthetases and translation factors as well as high salt-washed ribosomes, the only requirement for its translocation into membrane vesicles was the presence of the TatABC proteins in these vesicles. An obvious conclusion of this finding is that this twin-arginine precursor protein of *E. coli* does not seem to require any cytosolic transport factor for translocation. We cannot entirely rule out the possibility that our preparation of the PURE system, although being composed of highly purified components (Figure 2A), contains residual amounts of ribosome- and vesicle-associated cytosolic proteins that might be involved in the Tat pathway. We regard this possibility, however, unlikely as these potential translocation factors should be present only at contaminating low levels.

However, the translocation efficiency of our PURE system barely exceeded 10%, which could be indicative of a missing transport factor. This explanation would, however, not be consistent with the finding that previously cell-free translation systems, which are based on total cell extracts of *E. coli* rather than being reconstructed from isolated components, did not yield much higher translocation efficiencies for pSufI or other Tat precursors (Figure 5 and refs *40* and *41*). The limiting parameter of these *in vitro* systems has largely remained elusive but may conceivably be linked to the INVs used. This can be inferred from the above-mentioned fact that only when membrane vesicles are prepared from *E. coli* strains vastly overproducing the TatABC proteins do they allow detection of Tat-dependent transport. Likewise, it was suggested that the considerably higher transport efficiency recently reported for pSufI in a novel *in vitro* system was due to a different manner of preparing INVs (*11*). These results point toward the membrane vesicles as the limiting parameter in Tat-specific *in vitro* systems rather than to some missing cytosolic factor.

Results obtained here with the PURE system suggest that pSufI can be translocated via the Tat pathway in the absence of any cytosolic factor. Despite this finding, we cannot rule out the possibility that *in vivo* chaperoning proteins are involved in, and required for, optimizing the kinetics and efficiency of the transport of SufI and other cofactor-less Tat substrates. In this context, we obtained conspicuous cross-links between the signal sequence of pSufI and FK506 binding proteins. Of note, these cross-linking partners were detected by the same experimental strategy that led to the discovery of TatC and TatB as the functional signal sequence recognition site of the bacterial and chloroplast Tat systems (*5*, *42*). Typically, the FkpA adduct is obtained in the complete absence of the Tat translocase but was also obtained with INVs carrying inactive *tatC* mutants (*25*). Thus, *in vitro*, FkpA-SufI adducts accumulate whenever targeting to the Tat translocase is abrogated, as if FkpA were a soluble predecessor of TatBC in recognizing the Tat signal sequence of pSufI. Because *in vivo*, FkpA is synthesized itself as a precursor protein and exported to the periplasm, it is difficult to imagine how it could recognize Tat signal sequences in the cytoplasm. The affinity of FkpA for the presumably unfolded and unstructured (*43*, *44*) signal sequence observed here *in vitro* might simply reflect FkpA's known chaperoning activities. Thus, FkpA was shown to interact with early folding intermediates, to reactivate inactive proteins, and to prevent aggregation of proteins at stoichiometric amounts independently of its PPIase activity (*45*−*47*). Its flexible V-shaped structure might be predestined to interact with unfolded substrates or domains (*48*).

SlyD consists of an N-terminal FKBP-type PPIase domain and a C-terminal, histidine-rich metal-binding domain involved in regulating the PPIase activity (*49*). Because of its metal-binding domain, it frequently was described as a contaminant of nondenaturing immobilized metal-affinity chromatography. Probably because of its chaperone properties, SlyD stabilizes the lysis protein E of bacteriophage ΦX174 (*50*), and permanently unfolded proteins bind with high affinity to SlyD (*51*). This could easily explain why here and in a recent report (*23*) SlyD was found as a binding partner of Tat signal sequences that remain unstructured prior to a first membrane contact. Similarly PceT, a Trigger factor orthologue of *D. hafniense*, was recently described to interact with the Tat signal sequence of the dehalogenase PceA (*52*).

DnaK was found to associate with several different Tat signal sequences (*23*) and was also shown to stabilize overexpressed Tat substrates (*24*). In addition, the Tat transport of the multicopper oxidase CueO was found to be completely abolished in *dnaK*

knockout strains (*23*). However, the interaction between DnaK and the signal sequence of this Tat substrate does not reflect an essential involvement of DnaK in the Tat pathway, as evidenced by the fact that the CueO signal sequence fused to another substrate allows transport without the need for DnaK. Likewise, overexpressed substrates without a fully functional Tat signal peptide can also associate with DnaK (*24*), suggesting that it is the mature part of the protein to which DnaK binds. Except for the essential effect on CueO export, no evidence for a specific participation of DnaK in the Tat pathway of other substrates has so far been described, and all other influences reported could be ascribed to the general chaperone function of DnaK.

Collectively, no indication thus far exists for general chaperones playing a similar antitargeting role during the Tat export as previously demonstrated for the specific chaperones TorD, HybE, and NapD. However, it cannot be excluded that molecular crowding in the bacterial cytosol necessitates protection of the unstructured Tat signal peptides against aggregation and proteolysis in order to guarantee high export efficiency. Such protective measures would not be essential for sustaining a basal transport activity in dilute *in vitro* systems.

ACKNOWLEDGMENT

We thank Dr. Frank Sargent for providing *sufI* DNA subcloned into pQE60 and plasmid pAAF3, Dr. Iris Luke for constructing plasmid pKSM-SufI-6xHis, and Dr. Enguo Fan for a critical reading of the manuscript. We acknowledge the National BioResource Project (NIG, Japan): *E. coli* for sending strains JW5746, JW0026, JW0514, JW3748, and JW3311.

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