Separate Analysis of Twin-arginine Translocation (Tat)-specific Membrane Binding and Translocation in *Escherichia coli* 

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The twin-arginine translocation (Tat) pathway exports those precursor proteins to the periplasmic space of bacteria that harbor a twin-arginine (RR) consensus motif in their signal sequences. We have reproduced translocation of several Tat substrates into inside-out plasma membrane vesicles from *Escherichia coli*. Translocation proceeding at an efficiency of up to 20% occurs specifically via the Tat pathway as indicated by (i) its requirement for elevated levels of the TatABC proteins in the membrane vesicles, (ii) competition by an intact twin-arginine signal peptide, and (iii) susceptibility toward dissipation of the transmembrane H⁺ gradient. The latter treatment, while blocking translocation, still allows for functional membrane association of Tat precursors. This is shown by the finding that translocation of isolated membrane-bound Tat precursor is restored upon re-energization of the vesicles.

Bacteria export proteins from their cytoplasm to the cell envelope, which consists of the cytoplasmic membrane, the surrounding periplasmic space and, in the case of Gram-negative organisms, also the outer membrane. Whereas the majority of these proteins are exported via the classical Sec pathway (1), several periplasmic proteins use the more recently discovered twin-arginine translocation (Tat) pathway, a homologue of which is also operative at the thylakoidal membrane of chloroplasts, as shown to be constituents of the Tat pathway (for references, see the indicated reviews). The Tat translocase of *Escherichia coli* appears to be represented by ~600-kDa complexes that, depending on the detergent used for solubilization, contain varying amounts of TatA, TatB, and TatC (11–13). In addition, one RR signal sequence-binding protein, unrelated to TatABC, has been described (14). By use of a cell-free system with unprecedented high efficiency, we show here that in *E. coli*, translocation of Tat substrates across the cytoplasmic membrane follows a targeting step that is independent of the H⁺ gradient but requires an intact RR signal.

EXPERIMENTAL PROCEDURES

*Plasmids*—The pre-SufI and pre-SufI-R5K,R6K (SufI-KK) coding regions, subcloned from derivatives of pNR14 (15) into pBluescript SK (Stratagene) using the EcoRI-XhoI sites of the polylinker, were kindly provided by Dr. Tracy Palmer (John Innes Centre, Norwich, UK). The TorA-P2 fusion (16) cloned into pBAD18 was kindly provided by Drs. Jan Willem de Gier and Gunnar von Heijne (Stockholm University, Sweden). The TorA-P2 coding sequence was amplified by PCR using Fwo DNA polymerase (Roche Molecular Biochemicals) and 5’-GCT-CAAGAAGGATCCAAAATAATGAAC-3’ and 5’-CGACCCGGGCTAT-TAATTGGATGCC-3’ as forward and reverse primers, respectively. The amplified fragment was digested with BamHI and Smal and subcloned into the pKSM717 polylinker (17) to give plasmid p117TorP2. The inserted DNA was verified by sequencing. Plasmids pMW18 encoding the fusion protein TorA-23K (18) and pDMB encoding pre-OmpA (19) have been described.

The TatABCD gene cluster was cloned from the chromosome of *E. coli* strain TG1 by PCR using the primers 5’-GGCAGGTGTCTGATCGTCTAGATTGTCGG-3’ and 5’-TATTTACCGATGATGATGG-TGGGGTCGGCGTCGCT-3’ with the Expand High Fidelity PCR System according to the manufacturer’s instructions (Roche Molecular Biochemicals). The amplified 2621-bp fragment, which encompasses the entire *tat* operon and about 126 bp upstream of *tatA* and 95 bp downstream of *tatD*, was digested with XbaI and cloned into the same site of plasmid pET22b (+) (Novagen) behind the phage T7 promoter. The structure of the resulting plasmid p8737 was verified by EcoRV, HindIII, BamHI, and XhoI endonuclease digestion. Insertion of the plasmid p8737 into the mutants MCMTA (::Kn) (20) and B1LK0 (tatC) (18) restored the growth of these strains on trimethylamine N-oxide under anaerobic conditions.

In Vitro Transcription/Translation—An S-135 was prepared according to Ref. 21 from *E. coli* strain SL119 (BL21; recD::Ts10, F−, hsdS, gal, OmpT-) kindly provided by Dr. Richard Burgess (University of Wisconsin, Madison, WI). Cells were grown in a medium containing 9 g of bacterotryptone, 0.8 g of yeast extract, 5.6 g of NaCl, 0.8 g of glucose, and 1 ml of 1 M NaOH/liter. After growth, cells were resuspended at 1 g/ml in 10 mM triethanolamine acetate (pH 8, 14 mM Mg(OAc)2, 60 mM KAc, and 1 mM dithiothreitol) and passed twice at 8,000 p.s.i. through a French pressure cell. An S-30 was obtained by centrifugation of the homogenate for 30 min at 30,000 × g.

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750-μl aliquots of membrane-free S-135 were obtained from 1-ml aliquots of S-30 by centrifugation at 90,000 rpm for 13 min at 4 °C in a TLA 100.2 rotor (Beckman).

Coupled transcription/translation from plasmid DNA was performed similarly as described in Ref. 21. The reaction mixture (25 μl) contained 40 μM TeoAOac, pH 7.5, 70 mM KOAc, 9.5 mM Mg(OAc)₂, 0.8 M spermidine, 3.2% (v/v) polyethylene glycol 6000–8000, 2 mM DTT, 2.5 mM ATP, 0.5 mM each of GTP, UTP, and CTP, 10 mM KOH to neutralize the NTP, 8 mM phosphoenolpyruvate, 8 mM putrescine, 8 mM creatine phosphate, 40 μg/ml creatine phosphokinase, 40 μM each of 18 amino acids, 10 μCi of ³⁵S-labeled methionine and cysteine, 3–4 μl of S-135, 1 μg of plasmid DNA, 10 μg/ml rifampicin to inhibit the E. coli RNA polymerase, and 3 units of T7 RNA polymerase. Incubation was for 45 min at 37 °C. Where appropriate, inside-out inner membrane vesicles (INV) were added 5 min after the start of the incubation to a final concentration of 1–2 A₂₆₀ units/ml. Reactions were stopped as detailed in the legend of Fig. 1.

Preparation of Membrane Vesicles—INV were prepared from E. coli strains MC4100 (F-, lacU169, araD139, rpsL150, araC130, relA1, ptsI, rbsB, βbB5301 (22)), and DADE (MC4100, ΔtatABCΔrtaE) (23). A crude membrane pellet obtained as described (21) was loaded on a three-step sucrose gradient (12 ml of 0.77 M sucrose, 12 ml of 1.44 M sucrose, and 10 ml of 2.2 M sucrose in 50 mM TrisOAc, pH 7.5, 1 mM EDTA, 1 mM DTT) and centrifuged for 16 h at 25,000 rpm at 4 °C in a SW27 rotor (Beckman). INV were withdrawn from the 0.77/1.44 M sucrose interface, pelleted by centrifugation at 30 p.s.i. for 10 min. The supernatant above the sucrose cushion was used for the following experiments. INV were prepared from wild-type vesicles had not occurred. Similar to a previous finding (9), proteolytic processing and protease resistance of pre-SufI were completely abolished by the dissipation of the H⁺ gradient of the Tat⁺-INV using the uncoupler carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) at 100 μM (lanes 7 and 8), these phenomena cannot be due to unspecific cleavage or inherent protease resistance but rather reflect true translocation of pre-SufI into Tat⁻-INV. This was further confirmed by the abolishment of protease resistance in the presence of a membrane-disrupting detergent (not shown). Fig. 1C illustrates the overproduced amounts of TatB and TatC in Tat⁻-INV, and similar results were obtained for TatA (not shown).

In three independent experiments, the extent of translocation of SufI into Tat⁻-INV was determined to be 20.5% ± 3.2. A similar value (18.4% ± 5.1) was found for another Tat substrate, TorA-23K (Fig. 1A), a hybrid protein consisting of the TatABCD-overproducing strain (Fig. 1A, lane 4), the 23-kDa subunit of the photosystem II oxygen-evolving complex from spinach chloroplasts (27). The 23-kDa protein is a substrate of the thylakoidal Tat apparatus. Another hybrid made of the TorA signal sequence and the other...
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Fig. 2. In vitro translocation of Tat precursors requires an intact twin-arginine signal sequence. A, the indicated precursor proteins were synthesized in vitro for 45 min. Subsequently, samples were made 0.4 mM in puromycin using a neutralized stock solution of 11 mM, incubated for 5 min at 37 °C and then for another 45 min at 37 °C in the presence of Tat^-INV with buffer, K^+ and Mg^{2+} salts, ATP, DTT, creatine phosphate, and creatine phosphokinase added to give the same concentrations as during the initial synthesis reaction. RR^wild, signal peptide of SufI; KK^mut, same but with the two consensus arginines exchanged against lysines. Peptides dissolved in H_2O were added at 400 μM together with INV, PK, proteinase K, B. the precursor of the KK mutant of SufI was synthesized in vitro as described in the legend of Fig. 1.

wise Sec-dependent periplasmic P2 domain of E. coli leader (signal) peptidase (TorA-P2) (16) was also translocated into Tat^-INV albeit less efficiently (Fig. 1A; 5.1% ± 1.9).

In contrast to SufI, the two TorA hybrid precursors were proteolytically processed also by wild-type INV (lane 3, asterisks). These cleavage products, however, were not translocated into INV as they remained totally proteinase K-sensitive (compare lanes 4 and 6) and appeared even in the presence of CCCP (panel B), which otherwise effectively inhibited both the Tat (panel A, lane 7) and the Sec pathway (panel B, OmpA). Non-Tat-related processing appears to be a peculiarity of TorA hybrid proteins (16).

To confirm that the three Tat precursors were in fact translocated into the vesicles via the Tat pathway, competition experiments were performed (Fig. 2) using the signal sequence peptides of SufI (RR^wild) and a mutant peptide in which the two invariant arginines had been replaced by lysines (KK^mut). Translocation into Tat^-INV of all three Tat precursors was completely inhibited by the wild-type SufI signal peptide (Fig. 2A, lane 4). Inhibition was specific since it was not obtained with the mutant KK peptide (lane 6) nor was it observed for the Sec-dependent OmpA. Thus translocation of Tat substrates into Tat^-INV involves specific recognition of the RR signal sequence. Accordingly, the KK mutant precursor of SufI was not translocated into Tat^-INV (Fig. 2B).

Separating Functional Membrane Binding from Translocation—To analyze the membrane targeting of Tat substrates, we determined the amount of INV-bound precursor by flotation centrifugation. Under the conditions employed (Fig. 3A), INV float predominantly to fraction 2 of the sucrose gradient and to some degree also to fraction 3, whereas non-membrane-associated material is recovered mostly from the pellet P. This is exemplified in the upper panel of Fig. 3A for OmpA, which, when synthesized in the absence of INV, largely stays in the pellet P. On the contrary, the majority of precursor and mature form of OmpA obtained in the presence of Tat^-INV is found in fraction 2. Likewise, in vitro-synthesized SufI and TorA-23K quantitatively floated with Tat^-INV (Fig. 3A). In contrast to OmpA, the Tat precursors did, however, not exhibit any significant flotation with wild-type INV (not shown) consistent with the low level of TatABC proteins of these vesicles being limiting for translocation.

To analyze membrane binding independently of transmembrane translocation, we employed de-energized INV. Removal of the F_1-ATPase from INV by low salt stripping impairs translocation of Sec precursors by the dissipation of the H^+ motive force and is reversed by the readdation of F_1-ATPase or the low salt extract (LSE) (28). Likewise (Fig. 3B), when Tat^-INV were treated with low salt (wTat^-INV), processing and translocation of SufI and TorA-P2 were completely abolished (compare lanes 3 and 4 with lanes 5 and 6). The low salt-stripped vesicles, however, regained RR translocation activity by the addition of the LSE (lanes 7 and 8).

Next, the flotation behavior of Tat precursors in the presence of wTat^-INV was analyzed (Fig. 3A). As found for Tat^-INV, the majority of SufI and TorA-23K floated with the low salt-extracted vesicles (cf. fractions 2 of the Tat^- and wTat^- gradients). The only difference between the two vesicle populations was the lack of signal sequence cleavage by wTat^-INV that, however, was restored by the low salt extract. Evidently, inactivation of the Tat machinery by the dissipation of the H^+ gradient does not prevent the precursor from binding to the vesicles.

Translocation via the Tat Translocase Follows RR Signal Sequence-Dependent Membrane Binding—If the Tat precursors floating with low salt-stripped INV reflect populations of functional transport intermediates, translocation of the accumulated precursors should proceed once the H^+ gradient has been restored. Pre-SufI was therefore synthesized in the presence of low salt-stripped Tat^-INV to allow for membrane targeting. Subsequently, the low salt extract was added (Fig. 3C, LSE^mot), either directly or after isolating pre-SufI bound to low salt-stripped INV by flotation centrifugation (SufI-wTat^-). In both cases, the low salt extract did restore processing and translocation of SufI (lanes 3, 4, 7, and 8), clearly indicating that the precursor had been targeted to the translocation-deficient vesicles in a functional manner.

To find out whether functional targeting requires an intact RR signal sequence, we investigated the flotation behavior of the translocation-defective SufI-KK. As summarized in Table I, the sedimentation pattern of pre-SufI-KK obtained after synthesis in the absence of INV hardly changed upon the addition of Tat^-INV with only a minor part of the pre-SufI-KK being recovered from the prime vesicle fraction 2. This was in clear contrast to the results observed with translocation-proficient precursors (cf. Fig. 3A). Moreover, sedimentation of pre-SufI-KK was only marginally influenced by de-energizing Tat^-INV with CCCP or by low salt stripping. These results therefore suggest that a specific targeting of Tat precursors requires an intact RR signal sequence.

DISCUSSION

We have separated membrane targeting of bacterial twin-arginine preproteins from their translocation into inside-out plasma membrane vesicles. The cell-free system employed here does not grossly differ from previous ones described for the Sec-dependent protein export except that the usage of membrane vesicles prepared from a TatABCD overproducer was imperative. Although wild-type vesicles allow for efficient Sec-dependent translocation, they lack RR translocase activity for...
reasons that are not yet evident. However, the TatABC proteins have also been shown to be limiting for the Tat pathway in vivo (9, 13).

The efficiency of our in vitro system (up to 20% for at least two different Tat substrates) is by far higher than that of a similar one (0.4% for pre-SufI only) that was recently described (9) in which INV were routinely added after synthesis of pre-SufI. We have repeatedly made the observation that the later the vesicles are added after starting synthesis of the Tat precursor, the lower the efficiency of transport into these vesicles.

...cross-linking methods.

This strategy should now enable us to characterize both precursors and cursors. This strategy should now enable us to characterize both precursors and cursors.

FIG. 3. Membrane targeting of Tat substrates precedes transmembrane translocation. A, precursors were synthesized in vitro in the presence of the indicated INV, wTat, low salt-washed INV. Samples were subjected to flotation gradient centrifugation. The four fractions sequentially withdrawn from the top of the gradient were precipitated with trichloroacetic acid and together with the pelleted material (P), which was directly dissolved in SDS-PAGE sample buffer, analyzed by SDS-PAGE and phosphorimaging. The intensities of the bands corresponding to precursor and mature forms of OmpA, SufI, and TorA-23K were quantified with the sum of the five fractions each set at 100%. The given numbers are the means of two independent experiments, only one of which is shown. Note that in contrast to the two Tat precursors, signal sequence cleavage of OmpA is only partially sensitive toward low salt-stripping of INV (cf. panel A, fractions 2), reflecting the H⁺-gradient-independent initial step of translocation of this Sec precursor (30). B, SufI and TorA-P2 were synthesized in vitro. Their translocation into the indicated INV was tested by proteinase K (PK) protection. C, SufI was synthesized in the presence of wTat⁻-INV. Lanes 3 and 4, samples were made 0.4 mM in puromycin, incubated for 5 min at 37°C and then for another 45 min at 37°C in the presence of LSE and the additions described in the legend for Fig. 2 (LSE⁺inv), Lanes 5–8, SufI translation products derived from 800 μl of puromycin-treated reaction mix were separated by flotation centrifugation on 20 gradients; the combined fractions 2 containing membrane-targeted SufI (SufI-wTat⁻) were divided in half and incubated as described for lanes 3 and 4 in the absence or presence of LSE.

TABLE I

Membrane targeting of SufI requires an RR signal sequence

Flotation centrifugation of SufI-KK synthesized in the absence or presence of the indicated INV is shown. Numbers indicate the percentage of distribution between the four fractions of the sucrose gradients and are the means derived from the number of parallel experiments given in parentheses.

| Fraction | 1 | 2 | 3 | 4 | P |
|----------|---|---|---|---|---|
| No INV (n = 3) | 5 | 27 | 31 | 35 |
| Tat⁻-INV (n = 4) | 18 | 31 | 19 | 27 |
| Tat⁻-INV/CCCP (n = 3) | 12 | 24 | 22 | 41 |
| wTat⁻-INV (n = 2) | 8 | 32 | 19 | 39 |

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