Structure of TatA Paralog, TatE, Suggests a Structurally Homogeneous Form of Tat Protein Translocase That Transports Folded Proteins of Differing Diameter

Jacopo Baglieri, Daniel Beck, Nishi Vasisht, Corinne J. Smith, and Colin Robinson

From the School of Life Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

Background: The Tat system transports folded proteins in bacteria.
Results: Unlike TatA, the paralogous TatE is found as small, homogeneous complexes.
Conclusion: TatE cannot form a variable translocation channel as suggested for TatA.
Significance: This work suggests a new model in which a structurally homogeneous form of translocase uses a flexible channel.

The twin-arginine translocation (Tat) system transports folded proteins across bacterial and plant thylakoid membranes. Most current models for the translocation mechanism propose the coalescence of a substrate-binding TatABC complex with a separate TatA complex. In Escherichia coli, TatA complexes are widely believed to form the translocation pore, and the size variation of TatA has been linked to the transport of differently sized substrates. Here, we show that the Tat paralog TatE can substitute for TatA and support translocation of Tat substrates including AmiA, AmiC, and TorA. However, TatE is found as much smaller, discrete complexes. Gel filtration and blue native electrophoresis suggest sizes between ~50 and 110 kDa, and single-particle processing of electron micrographs gives size estimates of 70–90 kDa. Three-dimensional models of the two principal TatE complexes show estimated diameters of 6–8 nm and potential clefts or channels of up to 2.5 nm diameter. The ability of TatE to support translocation of the 90-kDa TorA protein suggests alternative translocation models in which single TatA/E complexes do not contribute the bulk of the translocation channel. The homogeneity of both the TatABC and the TatE complexes further suggests that a discrete Tat translocase can translocate a variety of substrates, presumably through the use of a flexible channel. The presence and possible significance of double- or triple-ring TatE forms is discussed.

The twin-arginine translocation (Tat) pathway system transports folded proteins across the chloroplast thylakoid membrane and the plasma membranes of many bacteria (reviewed in Refs. 1 and 2). Tat substrates bear cleavable N-terminal signal peptides containing a critical and highly conserved twin-arginine motif within the consensus sequence (3, 4). In Escherichia coli, the minimal set of components required for Tat-dependent translocation are three integral membrane proteins, TatA, TatB, and TatC, encoded by the tatABC operon, with molecular masses of 10, 18 and 30 kDa, respectively (5–7). At the steady state, these subunits are found in two types of complex within the plasma membrane: a substrate-binding TatABC complex of ~370 kDa and separate TatA homo-oligomeric complexes ranging from ~50 kDa to over 500 kDa (8–10). There is good evidence that the TatABC complex is responsible for the initial binding of substrate molecules, and the TatBC subunits appear to be particularly important in this respect (9). It is also believed that TatA complexes assemble with the TatABC complex to form a transient “supercomplex,” with TatA forming the translocation channel. It has been proposed that the variable size of the TatA complex could be a key feature that enables the system to generate a channel of appropriate diameter for a given substrate (10, 11), and electron microscopy studies have shown that TatA complexes do indeed contain potential pores of widely differing diameter (11). Accordingly, TatA is generally considered to form the bulk of the translocation pore. Studies on the Tat system in thylakoids suggest a similar mechanism in which the binding of substrate to the TatBC subunits (denoted Hcf106-cpTatC in plants) initiates assembly with the separate TatA complex (Tha4 in plants) to form the active translocon (12).

Although the TatABC subunits appear to form the primary translocation system, E. coli also contains a separate TatA paralog, TatE, that is encoded by a monocstronic tatE gene. The tatE gene is found only in enterobacteria (13) with the exception of the Gram-positive bacteria Corynebacterium glutamicum and Corynebacterium efficiens, which also have a Tat system containing the tatE gene in addition to tatA, tatB, and tatC (14). The E. coli tatE gene encodes a 67-amino acid predicted membrane protein that exhibits greater than 50% sequence identity with TatA (5). Overexpression of TatE results in complementation of a tatA mutant, indicating a similar role (15), but TatE is normally produced at levels that are much lower than those of TatA (16). Nevertheless, there is evidence that TatE plays a distinct role. In E. coli, tatE is induced in biofilms, and Pseudomonas stutzeri tatE expression is associated with denitrification, perhaps pointing to an important role for TatE proteins under specific growth conditions or possibly as an adaptation for handling specific substrates (17, 18).
In this study, we report for the first time the purification and characterization of E. coli TatE complexes following expression in the presence of native levels of the TatB and TatC components. TatE supports efficient translocation of Tat substrates, but the data reveal major differences between TatA and TatE complexes. Most importantly, the TatE complexes are far smaller and more homogeneous than TatA complexes. Indeed, single TatE complexes are smaller than one of the best studied Tat substrates. The results are consistent with a different model for Tat action in which a limited number of discrete Tat complexes are sufficiently flexible to transport a wide range of substrates. In this model, single TatE/TatA complexes would not provide the bulk of the translocation pore.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—All of the strains and plasmids used are listed in Table 1. E. coli MC4100 was used as the parental strain (19). ΔtatABCDE, ΔtatAE, and ΔtatA (5, 20) have been described previously. Arabinose-resistant derivatives were used as described (8). E. coli was grown aerobically in Luria broth (LB). E. coli was grown anaerobically in Luria broth supplemented with 0.5% glycerol, 0.5% trimethylamine N-oxide (TMAO), and 1 μM ammonium molybdate. The media were supplemented with ampicillin to a final concentration of 100 μg/ml. The media were supplemented with ampicillin to a final concentration of 100 μg/ml. The media were supplemented with ampicillin to a final concentration of 100 μg/ml.

**DNA Techniques**—All of the enzymes used were from Roche Applied Science or Fermentas Life Sciences. The polymerase chain reaction (PCR) was performed using Expand DNA polymerase (Roche Applied Science). For arabinose-inducible overproduction of E. coli TatE, plasmid pBAD-E-Strep was constructed as follows. The tatE gene was amplified from E. coli MC4100 chromosomal DNA with primers tatE_NcoI_for (ataccATGGGTGAGATTAGTATTAC; nucleotides identical to genomic DNA are capitalized, and restriction sites are underlined) and tatEs_PstI_rev (tttttc-ctcttttttatgagagA; nucleotides in bold specify the Strept-II™ tag peptide). The resulting product was digested with NcoI and PstI and ligated into plasmid pBAD24, generating pBAD-E-Strep.

**Expression and Purification of the TatE complex and TatA Complex**—E. coli ΔtatAE cells containing plasmid pBAD-E-Strep or pBAD-A-Strep were grown anaerobically to mid-exponential phase with the induction of tatE on plasmid pBAD-E-Strep and tatA on plasmid pBAD-A-Strep using 1 mM arabinose. The membranes were isolated as described previously (8) and solubilized in 2% dodecyl maltoside (DDM). The solubilized membranes were loaded on a Q-Sepharose column, and the proteins were eluted as described in Ref. 8. Avidin (2 μg/ml) was added to the sample to block any biotin-containing proteins, and the sample was loaded on a 2-ml Strept-Tactin-Sepharose (Institut für Bioanalytik). The column was washed with 8 column volumes of equilibration buffer containing Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% DDM. Bound protein was eluted from the column in 10 0.5-ml fractions using the same buffer as above but containing 2.5 mM desthiobiotin (Institut für Bioanalytik). The eluted peak fraction was loaded onto a Superdex™ 700 HR 10/30 gel filtration column (Amersham Biosciences) and was eluted with the same equilibration buffer as described above but containing 0.02% DDM.

**SDS-PAGE and Western Blotting**—The proteins were separated using SDS-polyacrylamide gel electrophoresis and immunoblotted using specific antibodies to TatA and TatB (8) and goat anti-rabbit IgG horseradish peroxidase conjugate. The Strept-II™ tag on TatE and TatA was detected directly using a Strept-Tactin-horseradish peroxidase conjugate (Institut für Bioanalytik). An ECL detection kit (Amersham Biosciences) was used to visualize the proteins.

**Blue Native Polyacrylamide Gel Electrophoresis**—Blue native polyacrylamide gel electrophoresis of Tat complexes was performed as described previously (10). Solubilized membranes and purified protein were loaded and separated on a polyacrylamide gradient gel (5–13.5%). The proteins were detected by immunoblotting as described above.

**TMAO Reductase Activity Assay**—TMAO reductase activity assay was performed as described previously (8, 23). E. coli cells were grown anaerobically until mid-exponential growth phase prior to fractionation into periplasmic, cytoplasmic, and membrane fractions. The cell fractions were loaded and separated on a 10% native polyacrylamide gel (5–13.5%). The proteins were detected by immunoblotting as described above.

**Protease Accessibility Experiments**—For the protease accessibility experiments, E. coli ΔtatAE cells containing plasmid pBAD-E-Strep, pBAD-A-Strep (21), and pRD8 (22) were used to express TatE, TatA, and leader peptidase, respectively. Spheroplasts were prepared by resuspending the cell pellet in 2.5 ml of 33 mM Tris-HCl (pH 8.0), 40% sucrose, and 5 mM Na₂EDTA (0.1 mg/ml). Cells were incubated at 4 °C for 30 min and then harvested at 4000 × g for 15 min. The resulting spheroplast pellet was resuspended in 1 ml of ice-cold 33 mM Tris-HCl and 40% sucrose. Aliquots (50 μl) of the spheroplasts were subjected to SDS-PAGE, and Western blot analysis was performed as described above.

**TABLE 1**

Plasmids and strains used in this work

| Plasmids       | Relevant properties                                                                 | Reference/source |
|----------------|------------------------------------------------------------------------------------|------------------|
| pBAD-E-Strep   | pBAD24 derivative containing the E. coli tatE gene with a C-terminal Strept-II™ tag | This study       |
| pBAD-A-Strep   | pBAD24 derivative containing the E. coli tatA gene with a C-terminal Strept-II™ tag | (21)             |
| pBAD-ABC-Strep | pBAD24 derivative containing the E. coli tatABC operon; with a C-terminal Strept-II™ tag | (8)              |
| pRD8           | pING1 derivative containing the E. coli Lep gene; Amp’                           | (22)             |

| Strains of E. coli | Relevant properties       | Reference/source |
|--------------------|--------------------------|------------------|
| MC4100             | F′ ΔlacU169 araD139 rpsL150 relA1 ptsF rbs flbB5301 | (19)             |
| MC4100 ΔtatABCDE    | tat deletion strain      | (5)              |
| MC4100 ΔtatAE       | tatA and tatE deletion strain | (5)            |
| MC4100 ΔtatA        | tatA deletion strain     | (5)              |
preparations were incubated at 25 °C for 30 min in the presence of 0.5 mg/ml proteinase K (Invitrogen) and/or 2% (v/v) Triton X-100 (BDH Laboratory Supplies).

Microscopy—Images were recorded with a Leica DMRE microscope equipped with a Leica TCS SP2 confocal unit and an argon laser. The natural light was selected, and the images were recorded with a photomultiplier using the Leica confocal software. The samples were visualized with a 63× oil immersion objective (numerical aperture, 1.4; Leica). The image size is 512 × 512 pixels, and the images were averaged from four successive scans.

Electron Microscopy—A sample of the TatE complex taken from the peak fraction of the gel filtration run was diluted 1:2 with the gel filtration sample buffer + 0.02% DDM. 4 μl of this sample was applied to freshly glow discharged (negatively) carbon-coated copper grids (300 mesh). The grid was then washed and stained following the touching drop method (40) 10-s washes in water followed by 2 washes in gel filtration buffer minus detergent and 2 washes in 0.2% (v/v) uranyl acetate. After blotting, the grid was then left to air-dry. Micrographs were taken on a 200-kV JEOL 2011 FEG TEM operating a 4k Gatan UltraScan CCD camera with a pixel size of 15 μm. Tilt pairs of images were taken at a magnification of ~86,000× under low dose conditions, with the first at an angle of 45° and the second at 0°. The average defocus of the 10 (40) selected tilt pairs was 1.8 μm.

Image Processing—Image processing was performed using the SPIDER/WEB software (24) managed through the SPIRE GUI interface (25). Many of the batch files used were adapted from those provided with the SPIDER tutorial package. Micrograph quality and defocus of the untilted images were estimated using both CTFFIND3 and the TF ED SPIDER operation. Particles were then picked from the best micrographs interactively using the JWEB pair-wise picking option. CTFFILT3 (26) was used to verify the tilt angle of the tilted images and calculate the defocus across these images. Particles were normalized and then contrast transfer function-corrected by phase flipping. Clear top-down views of the untilted particles were selected and shift-aligned and then cross-correlated to the whole dataset to find more. They were then assessed for size variation using the approach described previously (27) and classified using a hierarchical ascendant classification multivariate statistical analysis method into two size classes. The remaining particles were separated by the same method into potential side views and multiple-ringed structures. The untilted particles were then aligned both translationally and rotationally using a reference-free approach to provide the in-plane rotation angles needed to align the images. The tilted particles were centered only (no rotational alignment). With the required Euler angles defined (in-plane rotation from the untilted images and out-of-plane tilt from the tilted images), three-dimensional reconstructions were generated by back projection using the Simultaneous Iterative Reconstruction Technique algorithm (28). After refinement of the structures by projection matching, these models were then filtered around the nominal resolution as determined by the 0.5 Fourier shell correlation.

RESULTS

TatE Expression Restores Export of TorA in ΔtatAE Mutant—E. coli TatE is a 67-residue membrane protein with predicted secondary structural elements that are shown in Fig. 1A. TatE is strongly predicted to contain a single transmembrane α-helix at the amino terminus (helix α-1) followed by a segment containing an amphipathic α-helix (helix α-2) similar to TatA. For comparison, the predicted secondary structure of E. coli TatA is illustrated in Fig. 1B. In general, the structure of TatE resembles those of E. coli TatA and Bacillus subtilis TatAd, which have been studied by NMR (30, 31).

Previous experiments performed with Tat proteins expressed at native levels have shown TatA and TatE to have overlapping functions in the Tat pathway (5). We set out to test whether overexpressed TatE can complement the previously characterized E. coli ΔtatAE cells by restoring export activity in our strains. First, we assessed the activity of TatE in E. coli fol-

In Fig. 2A, the left-hand panels show control tests in which wild-type E. coli MC4100 cells (WT) were fractionated into...
membrane, cytoplasm, and periplasm samples (lanes M, C, and P, respectively). The data show that the TatA activity is found in the periplasm as expected, with TatA activity also present in the cytoplasm as is often observed (8). As expected in the E. coli ΔtatAE double mutant, no periplasmic activity is detected because Tat export is blocked. As found previously (5), TorA export proceeds in the E. coli ΔtatA single mutant because TatE is able to substitute for TatA. Importantly, overexpression of TatE in E. coli ΔtatAE cells (ΔtatAE + TatE panel) leads to efficient export of TorA. This experiment confirms that the TatE is active under these conditions and able to function effectively in the absence of TatA, in combination with TatB and TatC components expressed at native levels. The effectiveness of the cell fractionation protocol was positively assayed as shown in supplemental Fig. 1. Expression of TatE was routinely confirmed by immunoblotting in this and subsequent experiments (data not shown).

TatE Restores Export of AmiA and AmiC in ΔtatAE Mutant—E. coli amidases AmiA and AmiC are periplasmic proteins that are translocated by the Tat pathway and involved in cytokinesis (32). These substrates are mislocalized in an E. coli ΔtatABCDE strain causing a distorted cell division phenotype, such that the cells grow in long chain-like filaments (33). This phenotype is shown in Fig. 2B by the E. coli ΔtatAE cells. Overexpression of TatE in E. coli ΔtatAE cells restores the wild-type cell division phenotype (ΔtatAE + TatE panel), and the cells resemble wild-type cells, indicating that AmiA and AmiC are being correctly exported by the Tat pathway. The cells appear identical to wild-type cells (WT). These data, taken in conjunction with the TatA export data, show that TatE is able to effectively substitute for TatA and form an active translocon in this strain.

Characterization of TatE Complexes Formed after Overexpression of tatE Gene—To study the nature of TatE complexes, E. coli ΔtatAE cells expressing TatE from the plasmid pBAD-E-Strep (with a Strept-II™ tag fused to the C terminus of TatE) were fractionated, and membranes were isolated. Total membranes were solubilized in 2% DDM and first subjected to anion exchange chromatography using a Q-Sepharose resin. Eluted protein was further purified on a Strept-Tactin affinity column as described under “Experimental Procedures.” All column fractions were immunoblotted using antibodies to the Strept-II™ tag on TatE. The data presented in Fig. 3A show that a proportion of TatE was detectable in the column wash fractions, but most of the protein bound to the column and was specifically eluted from the column across elution fractions 5–10.

The TatE complexes were then applied to a calibrated Superdex™ 200 HR 10/30 gel filtration column, and elution fractions were immunoblotted using antibodies to the Strept-II™ tag on TatE (Fig. 3B). The immunoblots show TatE to elute across fractions 20–26 with a peak in fraction 23. This represents a relatively tight peak, suggesting that TatE is rather homogeneous, and calibration of the column shows the complex to be ~130 kDa in mass on average, including the detergent micelle. This result is in marked contrast to previous findings on the E. coli TatA complex, which was found to be remarkably heterogeneous and present as complexes ranging in size from ~50 kDa to over 500 kDa (10, 11). To confirm this point under similar experimental conditions, we expressed E. coli TatA from the plasmid pBAD-A-Strep, where TatA is likewise tagged with a C-terminal Strept-II™ tag, and we purified the protein by Q-Sepharose and Strept-Tactin column using the same protocol (data not shown). When subjected to gel filtration chromatography under identical conditions, E. coli TatA elutes across a much broader range of fractions (Fig. 3B), confirming the large range of size forms for this complex. TatA elutes with a sharper peak, which indicates the presence of a complex that is both smaller and far more homogeneous than the TatA complex.

The purified TatE complexes were further analyzed by blue native (BN) polyacrylamide gel electrophoresis. BN gels have been widely used to study hydrophobic membrane-bound protein complexes because they often provide a higher level of resolution when compared with gel filtration, enabling more accurate determination of molecular mass and complex composition (34, 35). The TatE gel filtration fractions 21–26 from Fig. 3B were loaded onto a BN gel, and the gel was subjected to immunoblotting with antibodies against the Strept-II™ tag on TatE. Fig. 4 shows the presence of two major bands corresponding to TatE complexes that migrate with, or close to, the 66-kDa marker. A minor third band runs above them. The sizes of these TatE complexes are consistent with those estimated by gel filtration, where the detergent micelle contributes to the apparent size of complexes. Again, we analyzed TatA under identical conditions, and the data are shown in Fig. 4. TatA gel filtration elution fractions 18–25 were subjected to BN gel electrophoresis, and the TatA complexes formed the characteristic ladder of bands observed in an earlier study (10). These bands correspond to separate complexes with estimated sizes ranging from ~100 kDa to over 500 kDa, with the gel filtration fractions containing distinct size classes. Thus, the BN gel results confirm that TatE subunits are organized as relatively small, homogeneous complexes that differ considerably from TatA complexes generated under the same conditions.

**Figure 2.** E. coli TatE complements ΔtatAE. E. coli mutant. A, the figure shows a native polyacrylamide gel stained for TorA activity. Periplasm, membrane, and cytoplasm samples (P, M, and C, respectively) were prepared and analyzed from wild-type E. coli MC4100 cells (WT), from the E. coli ΔtatAE and ΔtatA cells, and from E. coli ΔtatAE cells expressing E. coli TatE from plasmid pBAD-E-Strep (ΔtatAE + TatE). The mobility of active TorA is indicated. B, TatE expression can complement the filamentous phenotype of E. coli ΔtatAE cells. Confocal microscope images of wild-type E. coli MC4100 cells (WT), E. coli ΔtatA cells, and E. coli ΔtatAE cells expressing TatE from the pBAD-E-Strep plasmid (ΔtatAE + TatE) are shown. The scale bar is 1.5 μm.
The properties of TatE complexes were further analyzed as shown in Fig. 5. In Fig. 5A, we expressed TatE in ΔtatAE cells as above but subjected the solubilized membranes to BN gel analysis without further purification. The results (TatE lane) show that TatE complexes run as the same two primary forms as observed in Fig. 4 (~50 and 70 kDa). As a control to confirm that the gel resolved larger complexes, we expressed TatABC in the same cells, with a C-terminal Strep-II™ tag present on TatC. The blot (ABC lane) shows that TatC is present in the ~400-kDa TatABC complex previously described in Ref. 10. In a second experiment, we expressed TatE in the same manner and solubilized samples of the membranes in digitonin and C12E9 as well as DDM. The solubilized membranes were then subjected to gel filtration as in Fig. 3, and the elution profiles of the TatE complexes are shown in Fig. 5B. The data show that TatE complexes elute in a generally similar manner in all three cases, with similarly sharp elution peaks and a relatively small average size (under 150 kDa in all cases, including the micelle).

Electron Microscopy of TatE Complexes—Examination of TatE complexes using electron microscopy of negatively stained samples confirmed the relatively homogeneous nature of the TatE complexes.

The properties of TatE complexes were further analyzed as shown in Fig. 5. In Fig. 5A, we expressed TatE in ΔtatAE cells as above but subjected the solubilized membranes to BN gel analysis without further purification. The results (TatE lane) show...
of the sample in comparison with reported images of TatA. We observed small ring-shaped structures lying in a preferred orientation on the grid. In addition, we observed larger structures composed of two or more rings, which appeared to adopt a wider array of orientations. We analyzed these structures using single-particle image processing techniques and obtained a three-dimensional reconstruction using a random conical tilt strategy. Fig. 6 shows an area of a micrograph untilted (A) and tilted to ~45° (B).

The raw micrographs and class averages both revealed that most of the complexes formed small ring-shaped structures with a maximum diameter of ~8 nm and a dark central area indicating the possibility of a pore or well in the center. Approximately 1000 initial particles were picked from 10 tilt pairs of images, and clear top-down views of the untilted particles were selected for further analysis. Multivariate statistical analysis of the untilted particles revealed a moderate variation in particle size of around 2 nm. The particles were separated into two size classes (containing 226 and 288 particles, respectively) using the strategy described in Ref. 27 and aligned using a reference-free method (28) to generate the class averages shown in Fig. 6.

As well as the single-ring structures described above, a number of particles contained multiple rings and appear as potential dimers or trimers of the single-ringed structures. These multimers were picked separately from the single-ringed particles as the obvious difference in structure made them easily distinguishable; they were then processed and aligned using a refer-
ence-free alignment. This alignment was then refined using the class average as a final reference.

These larger particles appear to sit in two preferred orientations, a clear top-down view (as seen for the single-ringed particles) and a potential side view where the central pore or cleft is occluded. These larger structures were observed across a range of samples and grids and appear to represent a significant subset of the particles (~20% of the initial particle set). The majority of this set consisted of two rings with a small number showing three potential rings. Dilution of the sample (1:2 and 1:5) did not remove these multiple-ringed structures, suggesting that they are not attributable to crowding on the grid. The class average of the most homogeneous set of particles with two-ringed structures shows a complex of 6 by 10.5 nm, as shown in Fig. 8. Crude size estimations based on a low resolution three-dimensional model of this class, contoured to the dimensions of the two-dimensional average, give a molecular mass of ~120 kDa. This correlates with the uppermost band seen using BN gel electrophoresis (Fig. 4) and is approximately twice the size of the single-ring structures (68–90 kDa). The existence of multiringed forms of TatE may be relevant for the mechanism of pore formation because they show that the purified TatE complexes are capable of associating to form larger structures (see below). Interestingly, these larger structures still show two small and distinct pores. The cellular relevance of this phenomenon remains to be established in future studies.

Topological Orientation of TatE—As mentioned above, the TatE three-dimensional density map shows a cylindrical ring with additional protein density forming a potential lid covering the central channel at one end only. To assess whether the lid is located at the cytoplasmic or periplasmic side of the membrane, we attempted to determine the topological organization of TatE using protease accessibility measurements. Spheroplasts were prepared from *E. coli* ΔtatAE cells expressing TatE from the plasmid pBAD-E-Strep and were subjected to proteinase K treatment. The fate of TatE was assessed by immunoblotting. TatE was insensitive to proteinase K treatment (Fig. 9), which suggests that TatE is not accessible from the periplasmic side of the membrane. To assess whether the lid is located at the cytoplasmic or periplasmic side of the membrane, we attempted to determine the topological organization of TatE using protease accessibility measurements. Spheroplasts were prepared from *E. coli* ΔtatAE cells expressing TatE from the plasmid pBAD-E-Strep and were subjected to proteinase K treatment. The fate of TatE was assessed by immunoblotting. TatE was insensitive to proteinase K treatment (Fig. 9), which suggests that TatE is not accessible from the periplasmic side of the membrane.

---

**FIGURE 7. Three-dimensional density maps of TatE complex assemblies.** The small and large TatE complexes are shown filtered to 29 Å and contoured at ~5 σ (S.D. above mean density). Dimensions shown from top to bottom are: complex diameter, complex height, and pore width. Molecular masses were estimated based on a protein density of 0.844 Da/Å³. The figure was produced using the program UCSF Chimera.

**FIGURE 8. Montage and class averages of multiringed TatE complex structures.** A range of multiringed structures was observed across multiple grids, and the majority of these structures consisted of two rings. Representative raw images are shown in A. Of these, the most homogeneous particles were averaged (B) to provide a top-down view (132 particles) and potential side view (95 particles). In the top-down view, two small but distinct pores are present, and these are occluded in the side view. The top-down view is ~6 nm wide, reduced to 5 nm for the side view. Both averages measure 10.5 nm in their longest dimension. The scale bar is 10 nm.

**FIGURE 9. Topological organization of TatE probed by protease accessibility assay.** Spheroplasts were prepared from *E. coli* ΔtatAE cells expressing TatE and TatA (from pBAD-E-Strep and pBAD-A-Strep plasmid, respectively) and leader peptidase (Lep) from (pRD8 plasmid) and subjected to proteinase K accessibility assay as described under “Experimental Procedures.” Spheroplasts were incubated in presence of proteinase K and/or Triton X-100 where indicated. Samples were subjected to precipitation with trichloroacetic acid and then analyzed by immunoblotting using antibodies to the Strep-tag II™ on TatE and TatA (panels TatE and TatA) or an antiserum directed against the leader peptidase (Lep panel). Lep* indicates the characteristic degradation product formed by digestion of the periplasmic domain of membrane-embedded leader peptidase by proteinase K. Mobilities of molecular mass markers (in kDa) are indicated on the left. Mobilities of TatE, TatA, and leader peptidase are also indicated.
Structure of TatE Complexes

the membrane. As has been shown previously (36), TatA is not accessible from the periplasmic side of the membrane either (Fig. 9, center panel). Control experiments demonstrate that permeabilization of the spheroplast membrane with the detergent Triton X-100 renders both TatE and TatA susceptible to digestion by proteinase K. As a control for the efficacy of the proteinase K treatment, we tested for degradation of the periplasmic loop of leader peptidase as demonstrated previously (37). The data in Fig. 9 show the appearance of characteristic degradation products when proteinase K is incubated with the spheroplasts. These results suggest that the bulk of TatE is present in the cytoplasm, as shown for TatA, which would place a potential lid in the TatE structure on the cytoplasmic side of the membrane. However, this point requires further analysis because we cannot be certain that TatE does not contain a periplasmic domain that is insensitive to proteinase K in the absence of Triton.

DISCUSSION

The mechanism of the Tat system is still poorly understood in many respects, but a series of studies indicates the possibility of distinct roles for the separate TatABC and TatA complexes that are found at the steady state. There is clear evidence that, in both plant thylakoid and E. coli systems, the TatABC complex has a role in substrate binding. These subunits have been shown to cross-link to in vitro-synthesized substrates under conditions that promote binding but not translocation (9), and a TatBC complex has been purified with one or two substrate molecules bound to it after overexpression of substrates in the absence of TatA (38). In contrast, the TatA complex appears only to become involved after this initial substrate binding step (12).

Several lines of evidence have suggested that the TatA complex either forms the entire translocation channel, or at the very least, contributes the bulk of the channel. EM images of E. coli TatA complexes show the presence of potential channels (11) with a possible lid structure that could serve to seal the channel until the appropriate time. E. coli TatA complexes are furthermore present in a large range of sizes when purified (10, 11), and it was suggested that this could facilitate the transport of a wide range of substrate sizes. On the basis of these data, it has become widely accepted that the function of TatA is to generate the translocation pore for folded proteins, and this general model has been discussed in several reviews (e.g. Ref. 2). Key factors in the above model are the size and heterogeneity of the apparent channels in the EM images. The largest class of TatA complex had a diameter of 13.5 nm and a potential channel diameter of 6.5–7 nm, large enough to accommodate the largest Tat substrates, including TorA. The heterogeneity, on the other hand, appeared to provide a means of transporting Tat substrates of widely varying mass and shape. Thus, at the point of translocation, the full Tat translocase would exist in a series of size forms, dictated by the size of the TatA complex that was involved.

Our studies on TatE complexes are not consistent with this model. Previous studies have shown that TatE can complement the ΔtataA mutant (15), and in this study, we have confirmed that overexpressed TatE facilitates the efficient transport of several Tat substrates, including TorA, in the absence of TatA. However, the physicochemical/structural properties of isolated TatE complexes are very different from those of TatA complexes. Gel filtration studies show that TatE elutes as a smaller, more homogeneous set of complexes with an average size of ~130 kDa. However, this includes the detergent micelle, and more accurate BN studies suggest the presence of 2–3 complex types with sizes in the 50–110-kDa range. EM analysis has enabled us to derive three-dimensional models for the two primary TatE forms, although we stress that the small size of the complexes places them on the lower limit for accurate characterization by EM. Overall, single-ring TatE complexes are even smaller than the very smallest class of TatA complex analyzed to date (class 1 in Ref. 11), which had a diameter of 8.5–9 nm and a potential channel diameter of 3–3.5 nm. It should be emphasized that although TatE is overexpressed in this study, the pBAD vector directs only a moderate level of overexpression, and exactly the same expression system was previously used to characterize the large range of TatA complexes under essentially identical conditions (10). Moreover, the TatE complexes have exhibited the same characteristics after expression for short times or after purification in a range of different detergents. There has been no evidence for the presence of larger TatE complexes under any of these conditions, and we have therefore concluded that the smaller size of solubilized/purified TatE is likely to reflect the natural state of the complex.

TatE complexes do resemble smaller TatA complexes in some respects, and they likewise appear to possess a central cavity that is accessed from one side of the membrane. The N-terminal periplasmic region of TatA (and TatE) comprises only a few residues, and it is therefore highly likely that the potential lid is on the cytoplasmic face of the complex, where it may function to prevent leakage of small molecules. Proteinase K digestion studies support this idea. Overall, however, two important points emerge from the combined biochemical/structural studies.

The first is that the small sizes of TatE complexes appear to preclude a role in forming the entire translocation channel, or even the bulk thereof. One alternative possibility is that the TatABC complex forms the primary translocation channel and that TatA/E serves to activate it. The size of the TatABC complex, ~370 kDa in E. coli (10), is such that combination with even a small TatE complex could generate an active supercomplex of over 400 kDa, which may be more capable of forming a translocation channel for substrates of 90 kDa or more. Moreover, there is evidence from the thylakoid Tat system that TatC is actively involved in the actual translocation event because substrate molecules that are covalently cross-linked to TatC on the stromal side can be subsequently transported to the luminal side (39).

More generally, the data are consistent with a model for Tat function that is radically different from currently favored models. The observed heterogeneity of TatA has prompted models that involve recruitment of one variant of TatA complex, from a spectrum of size classes, to match the size of the substrate being translocated. In contrast, the observation of such a limited range of TatE forms (only two major classes) is consistent with a model in which a much more discrete form of Tat trans-
locase is sufficiently flexible to transport a wide range of substrates. Further study is of course required to test this model.

A second model involves a different scenario; multiple TatA/E complexes bind to the TatABC complex once substrate has been bound. This would have the advantage of providing flexibility of pore size, with differing numbers of TatA/E complex generating translocation channels of differing diameters (the same role proposed for the variability of TatA complexes). The multiringed structures of TatE that we have observed would support this modular interaction of smaller rings to produce a larger superstructure. The simpler scenario, involving the assembly of only one or two defined translocases, may be more logical, but this model again deserves further consideration.

Acknowledgments—We are grateful to Ian Portman for technical help with electron microscopy and to the Wellcome Trust for generous support (Grant 055663/Z/98/Z) to the Imaging Suite at the University of Warwick. We thank Dr. James P Barnett for advice and useful discussions and Professor Gunner Von Heijne for providing the leader peptidase expression plasmid and anti-leader peptidase antisemur.

REFERENCES

1. Robinson, C., Matos, C. F., Beck, D., Ren, C., Lawrence, J., Vasiht, N., and Mendel, S. (2011) Transport and proofreading of proteins by the twin-arginine translocation (Tat) system in bacteria. Biochim. Biophys. Acta 1808, 876–884
2. Müller, M., and Klösgen, R. B. (2005) The Tat pathway in bacteria and chloroplasts (review). Mol. Membr. Biol. 22, 113–121
3. Chaddock, A. M., Mant, A., Karnauchov, I., Brink, S., Herrmann, R. G., Klösgen, R. B., and Robinson, C. (1995) A new type of signal peptide: central role of a twin-arginine motif in transfer signals for the delta pH-dependent thylakoidal protein translocase. EMBO J. 14, 2715–2722
4. Stanley, N. R., Palmer, T., and Berks, B. C. (2000) The twin-arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in Escherichia coli. J. Biol. Chem. 275, 11591–11596
5. Sargent, F., Bogsch, E. G., Stanley, N. R., Wedler, M., Robinson, C., Berks, B. C., and Palmer, T. (1998) Overlapping functions of components of a bacterial Sec-independent protein export pathway. EMBO J. 17, 3640–3650
6. Bogsch, E. G., Sargent, F., Stanley, N. R., Berks, B. C., Robinson, C., and Palmer, T. (1998) An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. J. Biol. Chem. 273, 18003–18006
7. Weiner, J. H., Blous, P. T., Shaw, G. M., Lubitz, S. P., Frost, L., Thomas, G. H., Cole, J. A., and Turner, R. J. (1998) A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. Cell 93, 93–101
8. Bolhuis, A., Mathers, J. E., Thomas, J. D., Barrett, C. M., and Robinson, C. (2001) TatB and TatC form a functional and structural unit of the twin-arginine translocase from Escherichia coli. J. Biol. Chem. 276, 20213–20219
9. Alami, M., Lüke, I., Deitersmann, S., Eisner, G., Koch, H. G., Brunner, J., and Müller, M. (2003) Differential interactions between a twin-arginine signal peptide and its translocase in Escherichia coli. Mol. Cell 12, 937–946
10. Oates, J., Barrett, C. M., Barnett, J. P., Byrne, K. G., Bolhuis, A., and Robinson, C. (2005) The Escherichia coli twin-arginine translocation apparatus incorporates a distinct form of TatABC complex, spectrum of modular TatA complexes and minor TatAB complex. J. Mol. Biol. 346, 295–305
11. Gohlke, U., Pullan, L., McDevitt, C. A., Porcelli, I., de Leeuw, E., Palmer, T., Saibil, H. R., and Berks, B. C. (2005) The TatA component of the twin-arginine protein transport system forms channel complexes of variable diameter. Proc. Natl. Acad. Sci. U.S.A. 102, 10482–10486
12. Morì, H., and Cline, K. (2002) A twin-arginine signal peptide and the pH gradient trigger reversible assembly of the thylakoid APh/Tat translocase. J. Cell Biol. 157, 205–210
13. Yen, M. R., Tseng, Y. H., Nguyen, E. H., Wu, L. F., and Saier, M. H., Jr. (2002) Sequence and phylogenetic analyses of the twin-arginine targeting (Tat) protein export system. Arch. Microbiol. 177, 441–450
14. Ikeda, M., and Nakagawa, S. (2003) The Corynebacterium glutamicum genome: features and impacts on biotechnological processes. Appl. Microbiol. Biotechnol. 62, 99–109
15. Sargent, F., Stanley, N. R., Berks, B. C., and Palmer, T. (1999) Sec-independent protein translocation in Escherichia coli: a distinct and pivotal role for the TatB protein. J. Biol. Chem. 274, 36073–36082
16. Jack, R. L., Sargent, F., Berks, B. C., Sawers, G., and Palmer, T. (2001) Constitutive expression of Escherichia coli tat genes indicates an important role for the twin-arginine translocase during aerobic and anaerobic growth. J. Bacteriol. 183, 1801–1804
17. Heikila, M. P., Honisch, U., Wunsch, P., and Zumft, W. G. (2001) Role of the Tat transport system in nitrous oxide reductase translocation and cytochrome cd 1 biosynthesis in Pseudomonas stutzeri. J. Bacteriol. 183, 1663–1671
18. Beloin, C., Valle, J., Loutour-Lambert, P., Faure, P., Kzreminski, M., Balestrino, D., Haagensen, J. A., Molin, S., Prensier, G., Arbeille, B., and Ghigo, J. M. (2004) Global impact of mature biofilm lifestyle on Escherichia coli K-12 gene expression. Mol. Microbiol. 51, 659–674
19. Casadaban, M. J., and Cohen, S. N. (1979) Lac- tase genes fused to exogenous promoters in one step using a Mu-lac bacteriophage: in vivo probe for transcriptional control sequences. Proc. Natl. Acad. Sci. U.S.A. 76, 4530–4533
20. Wedler, M., Sargent, F., Jack, R. L., Stanley, N. R., Bogsch, E. G., Robinson, C., Berks, B. C., and Palmer, T. (2000) TatD is a cytoplasmic protein with DNase activity: no requirement for TatD family proteins in sec-independent protein export. J. Biol. Chem. 275, 16717–16722
21. Warren, G., Oates, J., Robinson, C., and Dixon, A. M. (2009) Contributions of the transmembrane domain and a key acidic motif to assembly and function of the TatA complex. J. Mol. Biol. 388, 122–132
22. Dalbey, R. E., and Wickner, W. (1985) Leader peptidase catalyzes the release of exported proteins from the outer surface of the Escherichia coli plasma membrane. J. Biol. Chem. 260, 15925–15931
23. Silvestro, A., Pommier, J., Pascal, M. C., and Giordano, G. (1989) The inducible trimethylamine N-oxide reductase of Escherichia coli K12: its localization and inducers. Biochim. Biophys. Acta 999, 208–216
24. Frank, J., Radermacher, M., Penczek, P., Zhu, J., Li, Y., Ladjadi, M., and Leith, A. (1996) SPIDER and WEB: processing and visualization of images in three-dimensional electron microscopy and related fields. J. Struct. Biol. 116, 199–199
25. Shaikh, T. R., Gao, H., Baxter, W. T., Asturias, F. J., Boisset, N., Leith, A., and Frank, J. (2008) SPIDER image processing for single-particle reconstruction of biological macromolecules from electron micrographs. Nat. Protoc. 3, 1941–1974
26. Mindell, J. A., and Grigorieff, N. (2003) Accurate determination of local defocus and specimen tilt in electron microscopy. J. Struct. Biol. 142, 334–347
27. White, H. E., Saibil, H. R., Ignatiou, A., and Orrlova, E. V. (2004) Recognition and separation of single particles with size variation by statistical analysis of their images. J. Mol. Biol. 336, 453–460
28. Penczek, P., Radermacher, M., and Frank, J. (1992) Three-dimensional reconstruction of single particles embedded in ice. Ultramicroscopy 40, 33–53
29. Jones, D. T. (1999) Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. 292, 195–202
30. Hu, Y., Zhao, E., Li, H., Xia, B., and Jin, C. (2010) Solution NMR structure of the TatA component of the twin-arginine protein transport system from gram-positive bacterium Bacillus subtilis. J. Am. Chem. Soc. 132, 15942–15944
31. Walther, T. H., Grage, S. L., Roth, N., and Ulrich, A. S. (2010) Membrane alignment of the pore-forming component TatA of the twin-arginine translocase from Bacillus subtilis resolved by solid-state NMR spectro-
Structure of TatE Complexes

32. Bernhardt, T. G., and de Boer, P. A. (2003) The *Escherichia coli* amidase AmiC is a periplasmic septal ring component exported via the twin-arginine transport pathway. *Mol. Microbiol.* **48**, 1171–1182

33. Ize, B., Stanley, N. R., Buchanan, G., and Palmer, T. (2003) Role of the *Escherichia coli* Tat pathway in outer membrane integrity. *Mol. Microbiol.* **48**, 1183–1193

34. Schägger, H., and von Jagow, G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* **199**, 223–231

35. Schägger, H., Cramer, W. A., and von Jagow, G. (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* **217**, 220–230

36. Porcelli, I., de Leeuw, E., Wallis, R., van den Brink-van der Laan, E., de Kruijff, B., Wallace, B. A., Palmer, T., and Berks, B. C. (2002) Characterization and membrane assembly of the TatA component of the *Escherichia coli* twin-arginine protein transport system. *Biochemistry* **41**, 13690–13697

37. de Gier, J. W., Mansournia, P., Valent, Q. A., Phillips, G. J., Luirink, J., and von Heijne, G. (1996) Assembly of a cytoplasmic membrane protein in *Escherichia coli* is dependent on the signal recognition particle. *FEBS Lett.* **399**, 307–309

38. Tarry, M. J., Schäfer, E., Chen, S., Buchanan, G., Greene, N. P., Lea, S. M., Palmer, T., Saibil, H. R., and Berks, B. C. (2009) Structural analysis of substrate binding by the TatBC component of the twin-arginine protein transport system. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 13284–13289

39. Gérard, F., and Cline, K. (2006) Efficient twin-arginine translocation (Tat) pathway transport of a precursor protein covalently anchored to its initial cpTatC-binding site. *J. Biol. Chem.* **281**, 6130–6135

40. Rubinstein, J. L. (2007) Structural analysis of membrane protein complexes by single particle electron microscopy. *Methods* **41**, 409–416