TatB and TatC Form a Functional and Structural Unit of the Twin-arginine Translocase from *Escherichia coli*

Albert Bolhuis, Joanne E. Mathers, Joanna D. Thomas, Claire M. L. Barrett, and Colin Robinson ∗
From the Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

In *Escherichia coli*, a subset of periplasmic proteins is exported via the twin-arginine translocation (Tat) pathway. In the present study, we have purified the Tat complex from *E. coli*, and we show that it contains only TatA, TatB, and TatC. Within the purified complex, TatB and TatC are present in a strict 1:1 ratio, suggesting a functional association. This has been confirmed by expression of a translational fusion between TatB and TatC. This Tat(BC) chimera supports efficient Tat-dependent export, indicating that TatB and TatC act as a unit in both structural and functional terms. The purified Tat complex contains varying levels of TatA, suggesting a gradual loss during isolation and a looser association. The molecular mass of the complex is ~600 kDa, demonstrating the presence of multiple copies of TatA, B, and C. Co-immunoprecipitation experiments show that TatC is required for the interaction of TatA with TatB, suggesting that TatA may interact with the complex via binding to TatC.

In bacteria, the vast majority of extracytoplasmic proteins are transported across the cytoplasmic membrane by the Sec apparatus (for review, see Ref. 1). In addition to several specialized protein export systems, another major protein export apparatus is able to transport folded proteins. This translocation pathway, denoted the twin-arginine translocation (Tat) pathway, is closely related to the ΔH-dependent pathway of thylakoid membranes (reviewed in Ref. 2). Substrates for this pathway are characterized by an essential twin-arginine motif in their signal peptides (3, 4).

In *Escherichia coli*, four genes have been shown to encode components of the Tat pathway (5–9). Three of these, tatA, tatB, and tatC, are located in one operon, whereas the fourth gene, *tatE*, is monocistronic. TatA, TatB, and TatE are homologous proteins that are predicted to contain a single transmembrane helix at their amino termini followed by a cytoplasmic domain. TatB and TatC are present in a strict 1:1 ratio, suggesting a functional association. This has been confirmed by experiments with TatC, suggesting that TatA may interact with the complex via binding to TatC.

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**RESULTS**

**TatA Requires TatC for Interaction with TatB**—Previously, we have shown TatA and TatB co-immunoprecipitate with each other and participate in a large complex of ~600 kDa (11). However, the complex was not purified during this work, and we did not establish whether TatC was associated with this complex. To address these points, we first tested whether TatC is required for interaction of TatA and TatB. Membranes were isolated from cells grown aerobically in TY medium were solubilized with 1% digitonin, and proteins were immunoprecipitated with anti-TatA or anti-TatB serum as described before (11). M. Molecular mass reference marker (indicated in kilodaltons).

**TMAD Reductase Activity Assay**—Cells were grown anaerobically in TY-GT medium until the mid-exponential growth phase, and periplasm and spheroplasts were prepared by the EDTA/lysozyme/cold osmoshock procedure (14). Spheroplasts were lysed by sonication, and intact cells and cellular debris were removed by centrifugation (5 min at 10,000 × g). Membranes were separated from the cytoplasmic fraction by centrifugation (30 min at 250,000 × g). Protein fractions were separated on a 10% nondenaturing polyacrylamide gel, and TMAD reductase activity was visualized in the gel using a methyl-violeten-linked TMAD reduction as described before (15).

**DISCUSSION**

**Purification of the TatABC Complex**—Cells were grown anaerobically in TY-GT medium to the end of the exponential growth phase and then spheroplasted and sonicated, and the membranes were isolated as described before (11). Membranes were solubilized in buffer I (20 mM Tris-HCl, pH 8.0, 20% glycerol) plus 50 mM KCl and 1% digitonin (Calbiochem). To prevent degradation of proteins, a protease-inhibitor mixture (Complete, Roche Molecular Biochemicals) was added to buffers for spheroplasting, membrane isolation, and membrane solubilization. Solubilized membranes were loaded on a Q-Sepharose column. The column was washed with 1 column volume buffer I containing 100 mM KCl and 0.1% digitonin, and proteins were eluted with 2 column volumes of buffer I containing 300 mM KCl and 0.1% digitonin. Avidin (2 μg/ml) was added to the sample to block any biotin-containing proteins, and the sample was loaded on a biotin-pancreatin-Sepharose (Institut für Bioanalytik, Göttingen, Germany).

**Isolation of Membranes and Immunoprecipitation**—Membranes isolated from cells grown aerobically in TY medium were solubilized with 1% digitonin, and proteins were immunoprecipitated with anti-TatA or anti-TatB serum as described before (11). Peak fractions from the MonoQ column of radiolabeled TatABC-s complex were immunoprecipitated using anti-TatA, anti-TatB, or an irrelevant serum (raised against spinach photosystem II subunit W) and visualized by SDS-PAGE and fluorography.

**Analysis of the TatABC Complex**—Cells were grown anaerobically in TY-GT medium until the mid-exponential growth phase, and periplasm and spheroplasts were prepared by the EDTA/lysozyme/cold osmoshock procedure (14). Spheroplasts were lysed by sonication, and intact cells and cellular debris were removed by centrifugation (5 min at 10,000 × g). Membranes were separated from the cytoplasmic fraction by centrifugation (30 min at 250,000 × g). Protein fractions were separated on a 10% nondenaturing polyacrylamide gel, and TMAD reductase activity was visualized in the gel using a methyl-violeterlinked TMAD reduction as described before (15).

**Controls Expression of the tatABC Operon**—To analyze the composition of the Tat complex more directly, an expression vector was constructed encoding TatC with a Streptag II fusion on the carboxyl terminus (denoted TatC-s). The Streptag

**Fig. 1. Co-immunoprecipitation of TatA using anti-TatB serum.** Membranes of *E. coli* MC4100 (WT), ΔtatAE (ΔAE), ΔtatB (ΔB), or ΔtatC (ΔC) were solubilized with digitonin, and immunoprecipitation (IP) was performed using anti-TatB serum. As a control, TatA was immunoprecipitated from *E. coli* MC4100 using anti-TatA serum. TatA was visualized by SDS-PAGE and Western blotted using anti-TatA serum. Because anti-TatB serum co-immunoprecipitates only a small fraction of the total TatA pool (11), the exposure time of the film was longer for the left panel. $M$, molecular mass reference marker (indicated in kilodaltons).
II peptide enables detection using Streptactin (an engineered streptavidin; Ref. 16)-HRP conjugate and purification on Streptactin-Sepharose affinity columns. A major advantage of the latter system is that proteins can be purified using very mild conditions, because elution can be achieved by simply adding low concentrations of a biotin derivative, desthiobiotin, to a physiological buffer. Because the stability of TatB and TatC depends on the presence of TatA and TatB, respectively (8, 11), the expression vector (pABC-s) encoded TatA and TatB as well as TatC-s. All three tat genes were under the control of the arabinose-inducible $\beta_{BAD}$ promoter.

To test whether the plasmid-borne expression of TatA, TatB, and TatC-s was fully functional, pABC-s was transformed into an E. coli strain lacking the tatABC operon and $\beta_{BAD}$. The latter strain is unable to grow anaerobically in minimal glycerol/TMAO medium (5, 7, 8). Plasmid-borne $\text{tatABC-s}$ was able to restore this growth defect of E. coli $\Delta\text{tatABCDE}$, showing that $\text{tatA, tatB, and tatC-s}$ are expressed and, moreover, that the Strep-tagged derivative of TatC is functional (data not shown).

To verify the expression level of the plasmid-borne $\text{tatABC}$ genes, cells of E. coli strain $\Delta\text{tatABCDE}$ containing plasmid pABC-s were grown in the presence of 5 or 100 $\mu\text{M}$ arabinose, and TatB levels were compared with those in wild-type cells (E. coli MC4100) by Western blotting. In the presence of 5 $\mu\text{M}$ arabinose, the expression level of TatB is similar to that of wild-type cells (Fig. 2). In the presence of 100 $\mu\text{M}$ arabinose, TatB was overproduced ~50-fold. Similar values were obtained for the cellular levels of TatA (data not shown).

**Purification of a TatABC-s Complex**—To identify the proteins that co-purify with TatC-s, digitonin-solubilized membranes (Fig. 3A, lane 1) isolated from cells grown in the presence of 100 $\mu\text{M}$ arabinose were subjected to Q-Sepharose chromatography. Proteins eluting in a buffer with 300 mM KCl (Fig. 3A, lane 2) were further purified on a Streptactin column. The eluate from this step contains two major bands of ~27 and 31 kDa (Fig. 3A, lane 3). Western blotting showed that these bands are TatB and TatC-s, respectively (Fig. 3, B and C). Furthermore, a band running at ~17 kDa was visible, which, as confirmed by Western blotting, is TatA. Finally, a number of other bands were visible that are multimers of TatC (indicated by $C^*$) and a degradation product of TatB (indicated by $B^*$), because these bands were also detected with Streptactin-HRP and anti-TatB, respectively (data not shown).

The TatABC proteins were further purified on a Superose 6HR gel filtration column. TatABC eluted in a single peak corresponding to a molecular mass of ~600 kDa (Fig. 4), which is in agreement with the molecular mass found for the solubilized TatA/B-containing complex in our earlier studies (11). A final purification step was performed using anion-exchange chromatography. Silver staining of peak fractions revealed only the presence of TatB and TatC-s (Fig. 3A, lane 5), but Western blotting showed that these fractions also contained TatA protein (Fig. 3D). Concentration of peak fractions using a Centricon filter (100,000 molecular weight cut-off) and analysis by SDS-PAGE and Coomassie staining showed a similar result: only TatB and TatC-s were detectable; the levels of TatA are once again too low for visualization (not shown).

In the experiments described above, the TatABC-s proteins were overproduced to prepare sufficient quantities of material. However, it was important to purify the complex from cells expressing wild-type levels of the Tat system to test whether the complex contains any other proteins (because these would not be overexpressed from the $\text{tatABC}$ plasmid). This was achieved by purifying the complex from cells grown on low levels of arabinose (5 $\mu\text{M}$), which corresponds to wild-type levels of TatA and TatB, using the first three steps of the purification strategy outlined above. After these purification steps, only bands corresponding to TatB and TatC were visible (data not shown). The presence of TatA was again confirmed by Western blotting. No other bands were detectable, strongly suggesting that the core components of the twin-arginine translocase complex are TatA, TatB, and TatC.

**The Tat Complex Contains TatB/C in a Fixed Ratio Together with Varying Amounts of TatA**—The data shown above indicate
that TatB and TatC are easily detected in the purified complex, but TatA is difficult to detect and quantify using these procedures. We have found that this protein stains aberrantly with Coomassie and very poorly with silver (not shown). We therefore used an alternative strategy to obtain a clearer picture of the subunit ratios in the purified complex. The Tat complex was isolated from cells grown in the presence of [35S]methionine, and subunit ratios in the purified complex. The Tat complex was isolated from cells grown in the presence of [35S]methionine, and Fig. 5A shows the elution of the radiolabeled complex from the penultimate (gel filtration) and final ion-exchange chromatography stages. Only three bands are visible, corresponding to TatA, TatB, and TatC-s. Using this procedure, TatA is now clearly detected, and this is clear evidence that this subunit is indeed an integral component of the purified complex. To analyze the subunit ratios in the various fractions, the band intensities were quantified using a PhosphorImager. Furthermore, to be able to compare the ratios accurately, amino-terminal sequencing was performed to determine whether the amino-terminal methionines were present in the TatA, TatB, and TatC proteins. The sequences obtained were MGGISI, MF

The TatABC-s complex has a molecular mass of ~600 kDa. A chromatogram of the gel filtration of the TatABC-s complex is shown. A sample concentrated to 0.5 ml after purification of the Tat complex on a Streptactin column was loaded on a Superose 6HR gel filtration column, and the proteins were eluted as described under "Experimental Procedures." Protein elution was monitored by absorbance (A) at 280 nm. The column was calibrated with the following proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). MW, molecular weight; Vr, void volume; Ve, elution volume.

To examine expression levels of the tat(BC) gene, cells of E. coli strain ΔtatABCDE containing plasmid pJDT12 were grown in the presence of varying amounts of arabinose, and the protein was visualized by Western blotting using an anti-TatB serum. The Tat(BC) protein has a mobility corresponding to its predicted molecular mass of 48 kDa and was readily detected in samples prepared from whole cells cultured with increasing concentra-
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The periplasmic enzyme TMAO reductase (TorA), which is required for growth on minimal TMAO/glycerol medium, is a Tat-dependent substrate (5, 17). Therefore, a final test on the functionality of the Tat(BC) fusion protein was performed by analyzing whether active TMAO reductase was present in the periplasm, using a methyl-viologen-linked TMAO reduction assay on a nondenaturing gel (Fig. 9). The results show that, in wild-type cells containing pBAD24, TMAO reductase activity is found in the periplasm as expected. Substantial activity is also found in the cytoplasm because not all of the TMAO reductase is exported under these conditions, as shown in other studies (17). No periplasmic activity is detected in the ΔtatABCDE cells containing this vector, again as expected because the export of this protein is completely dependent on the Tat pathway (5, 17). Significantly, TMAO reductase is found in the periplasm of ΔtatABCΔ cells expressing the tatA(BC) operon (Fig. 9, lanes pJDT12) confirming that the Tat pathway is operational. The export efficiency is low in cells grown without arabinose, but upon induction by arabinose the export efficiency is clearly increased to the point where export efficiency is comparable with that found in wild-type cells. In conclusion, our data show that the Tat(BC) fusion protein is active in cells lacking TatB and TatC, supporting the hypothesis that TatB and TatC form a functional unit within the Tat complex.

The Tat(BC)-s Chimera Has a Molecular Mass of ~600 kDa—To test whether the Tat(BC) chimera forms large molecular mass complexes on its own, the Tat(BC) chimera was modified to contain a carboxyl-terminal Strep tag (as added to wild-type TatC in the purification work described above). Addition of the Strep tag does not affect activity because the chimera is active in ΔtatB and ΔtatC cells (not shown). The construct was expressed in the absence of TatA (in ΔtatABCΔ cells), and Tat(BC-s) was purified using the first three steps of the purification protocol as described for the TatABC-s complex. The results of the last gel filtration step are shown in Fig. 10. The Tat(BC-s) protein elutes in a peak corresponding to a molecular mass of ~600 kDa, demonstrating that the Tat(BC-s) protein forms a large complex even in the absence of TatA.

DISCUSSION

In the present study we have purified a complex from E. coli containing all three of the major known Tat components, namely TatA, TatB, and TatC. We find no evidence for the presence of novel membrane-bound proteins that would represent the products of hitherto unidentified tat genes. We emphasize, however, that the purified complex has not been shown to be active in an in vitro assay, and we cannot therefore rule out the possibility that further subunits may remain to be identified.

An important point to emerge from this study is that TatB and TatC are clearly present in a fixed 1:1 ratio, suggesting a close structural association between these subunits. This suggestion is reinforced by the finding that cells lacking tatB and tatC genes but containing instead a single polypeptide in which TatB and TatC domains are functional individually but that the Tat(BC) fusion protein is also functional as a unit.

The control strain E. coli ΔtatABCDE (pBAD24) did not grow at all under these conditions. These results demonstrate that, despite the low levels of Tat(BC) as compared with TatB in wild-type cells, Tat(BC) is able to restore the growth defect of a strain lacking both TatB and TatC. This shows not only that both TatB and TatC domains are functional individually but that the Tat(BC) fusion protein is also functional as a unit.

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These results show that TatB and TatC form a functional unit within the Tat complex, and we conclude from these data that TatB and TatC must act in concert.

These data have implications for the Tat translocation mechanism. At present we do not know which subunits act as either the initial receptor or as the translocation channel. However, our data indicate that TatB and TatC are likely to carry out one or more particular functions together, and this in turn suggests
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that TatA may serve a different function. This idea is supported by the observation that the amount of the third component of the translocase, TatA, varies much more in the purification steps. This may be significant in terms of the translocation mechanism. One possibility is that the “core” TatB/C complex contains low amounts of TatA and that additional molecules are recruited to form the full, active complex. The stably bound TatA molecules might, for example, serve as a nucleation point for the binding of further TatA molecules. We have shown before that only a minor fraction of the total TatA pool is in a complex with TatB (11), and this study has confirmed the following point: the majority of the TatB(C) is efficiently bound by the affinity column, but the vast majority of the TatA is not. However, it is important to stress that the fraction of TatA that is in a complex with TatB(C) is fairly tightly bound, because TatA was still present after purification using a variety of columns and an immunoprecipitation step with anti-TatB serum. Co-immunoprecipitation experiments furthermore demonstrated that TatC is required for the co-immunoprecipitation of TatA using anti-TatB serum, suggesting that TatA and TatB may not interact directly, but only through TatC (although this point remains to be tested more directly, because TatC may simply affect the conformations of TatA or TatB).

The apparent molecular mass of the purified Tat complex is ~600 kDa, indicating that multiple TatA, B, and C subunits must be present. An exact calculation of the number of subunits is difficult to make, because the molecular mass determined is that of the TatABC complex in digitonin micelles, and the detergent could contribute to the size estimation. Thus, for an accurate measurement of the Tat complex, other methods must be used. Further studies are also required to characterize the complex in other respects. First, it has to be established whether the purified complex is active in a reconstituted system, because this would help to determine whether the TatABC proteins are indeed the only components required for Tat-dependent transport and whether the unbound TatA subunits must be recruited for activity. Finally, it is presently completely unclear how the Tat complex is able to transport large folded proteins without compromising membrane impermeability. Structural analysis of the purified complex is clearly required for a more detailed understanding of its organization and mechanism.

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