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Subunit interactions in the twin-arginine translocase complex of *Escherichia coli*

Albert Bolhuis, Erik G. Bogsch, Colin Robinson*

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

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Abstract A subset of *Escherichia coli* proteins, in particular cofactor-binding proteins with so-called twin-arginine signal peptides, is transported to the periplasm via the twin-arginine translocation (Tat) pathway. The *tatA* and *tatB* genes encode important components of the export system and we have analysed whether the proteins encoded by these genes physically interact. Using co-immunoprecipitation experiments, we show that TatA and TatB do indeed associate with each other. Gel filtration chromatography demonstrates that both proteins are present in a large complex with an apparent molecular mass of approximately 600 kDa, indicating the presence of other components and/or several TatA and TatB subunits. Finally, we show that TatA is stable in the absence of TatB and may participate in a separate complex lacking TatB in wild-type cells.

Key words: Protein translocation; Twin-arginine translocation pathway; Protein interaction; *Escherichia coli*

1. Introduction

In prokaryotes, most proteins that are translocated across the cytoplasmic membrane utilise the Sec machinery [1,2]. The translocase involved comprises a membrane-bound complex, consisting minimally of SecY, SecE, and SecG, and the peripheral membrane protein SecA that functions as the translocation motor. Translocation through this membrane complex is by a threading mechanism that excludes proteins with significant tertiary structure.

Recently, a novel translocation pathway was discovered in bacteria [3–5], and termed the twin-arginine translocation (Tat) pathway. This pathway is structurally related to the thylakoidal ΔpH pathway [6]. A characteristic feature of substrates transported by the Tat pathway is that they contain unusually long signal peptides with a conserved twin-arginine motif directly upstream of the hydrophobic domain, with a consensus sequence S/TRRxFLK [7]. In prokaryotes, most proteins with twin-arginine signal peptides bind complex redox cofactors, such as iron–sulfur clusters or molybdopterin cofactors [7]. These cofactors are believed to be inserted in the cytoplasm, which would require proper folding before translocation. Therefore, it is likely that cofactor-containing proteins with a twin-arginine signal peptide are translocated in a folded state [6,7].

In *Escherichia coli*, four genes encoding Tat components have thus far been identified. These are the *tatA*, *tatB*, and *tatC* genes, which are located in one operon, and the unlinked *tatE* gene [3–5]. The *tatA*, *tatB*, and *tatE* genes are homologues of the *hcf106* and *tha4* genes from maize, which were shown to be components of the thylakoidal ΔpH-dependent translocation system [8–10]. Recently, it was shown that TatA and TatE are functionally interchangeable, whereas TatB is functionally distinct [11]. The TatA, TatB, and TatE proteins are predicted to be type I membrane proteins, with a single membrane-spanning helix at the amino-terminus followed by a cytoplasmic domain. TatC is predicted to be an integral membrane protein containing six membrane-spanning domains with the amino- and carboxyl-termini in the cytoplasm.

Although the *tatABCE* gene products are clearly involved in Sec-independent protein export in *E. coli*, little is known about the composition of the Tat translocase or the roles of TatA, TatB, TatC, and TatE. Thus far, it has only been shown that TatC is rapidly degraded in the absence of TatB [11], suggesting that TatB forms a complex with TatC. The composition of the corresponding thylakoidal ΔpH-dependent translocase is similarly obscure at present, although there is evidence for the presence of a large complex during the translocation process. In pea thylakoids, a chimeric precursor protein (denoted '16/23') was found to be arrested within the membrane during translocation [12], and the translocation intermediates were found in two distinct complexes with apparent molecular masses of 560 and 620 kDa, respectively. These data suggested the presence of a large translocase in which these intermediates were trapped.

In the present study, we have sought to analyse subunit interactions in the Tat translocase, focusing on the two major Hcf106 homologues of *E. coli*, TatA and TatB. We show that these proteins physically interact with each other and that both proteins are present in a large complex of approximately 600 kDa. Our data furthermore indicate that TatB is largely unstable in the absence of TatA. In contrast, TatA is stable in mutants lacking either TatB or TatC, and can be detected as a separate pool in large complexes lacking TatB.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

E. coli strain MC4100 (F-Δ*lacU169 araD139 rpsL150 relA1 ptsF rbs flbB5301*) [13] was the parental strain. ELV15 (MC4100 Δ*tatA*), JARV15 (MC4100 Δ*tatAE*), B0D (MC4100 Δ*tatB*), and B1LK0 (MC4100 Δ*tatC*) have been described before [4,5,11]. Arabinose is toxic for MC4100 and its derivatives, due to the presence of the *araD139* mutation [14]. To be able to use the pBAD vectors [15], which contain an arabinose-inducible promoter, strains that were resistant to arabinose, but unable to use arabinose as carbon source, were isolated from MC4100 and its derivatives as described [14]. *E. coli* was grown aerobically in TY medium, containing tryptone (1%),

*Corresponding author. Fax: (44)-24-76523701.
E-mail: cg@dna.bio.warwick.ac.uk

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yeast extract (0.5%) and NaCl (1%), or anaerobically in M9 minimal salts [16] supplemented with glycerol (0.5%), TMAO (0.4%), $CaCl_2$ (0.1 mM), $MgSO_4$ (2 mM), thiamine (0.001%), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (1 μM), and K_2O_3Se (1 μM). Ampicillin was used at 100 μg/ml.

To construct TatA with a hexahistidine-tag at the carboxyl-terminus, a DNA fragment containing the *tatA* gene was amplified with the primers AB.tatA1 (ataccATGGGTGGTATCAGTATTTG; nucleotides identical to genomic template DNA are printed in capital letters and restriction sites used for cloning are underlined) and AB.tatA3 (ataaagcttagtgatggtgatggtgatgCACCTGCTCTTTATCGTGG; the sequence specifying the hexahistidine-tag is indicated in bold) using MC4100 chromosomal DNA as template. The resulting product was digested with *Nco*I and *Hin*dIII and cloned into plasmid pBAD24, resulting in plasmid pBAD-tatAhis. The gene encoding TatBhis was cloned in a similar way, using the primers AB.tatB1 (ataccatgG-TGTTTGATATCGGTTTTAG) and AB.tatB3 (ataaagcttagtgatggtgatggtgatgCGGTTTATCACTCGACGAAG). The resulting plasmid was denoted pBAD-tatBhis. As the *tatB* gene starts with a GTG start codon, an additional ATG start codon was introduced to enable ligation into the *Nco*I restriction site of pBAD24.

2.2. Antibodies

The *tatABC* operon was cloned into the vector pFAT75 and overexpressed in *E. coli*; the membrane fraction was then solubilised and subjected to gel filtration. The TatA and TatB proteins elute in the high molecular mass fraction (F. Sargent, B.C Berks and T. Palmer, manuscript in preparation). These samples were run on SDS–polyacrylamide gels and the TatA and TatB bands were electro-eluted; antibodies to TatA and TatB were then raised in rabbits. Antibodies to TatB were purified by the methods described [17], using TatB coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech).

2.3. SDS–PAGE and Western blot analysis

Proteins were separated by SDS–PAGE, as previously described [18]. Western blotting was performed using a semi-dry system as described by Kyhse-Andersen [19]. After separation by SDS–PAGE, proteins were transferred to polyvinylidene difluoride membranes (Roche Molecular Biochemicals). Proteins were visualised with specific antibodies and horseradish peroxidase anti-rabbit IgG conjugates, using the ECL detection system of Amersham.

2.4. Isolation of membranes and immunoprecipitation

Cells were grown to a mid-exponential growth phase. Cells were collected by centrifugation and sphaeroplasted as described [20]. Sphaeroplasts were collected by centrifugation, resuspended in buffer A (50 mM Tris–HCl pH 8.0, 20% glycerol, 100 mM KCl), and disrupted by sonication. Intact cells and cellular debris were removed by centrifugation (5 min at $10\,000\times g$). Membranes were separated from the cytoplasmic contents by centrifugation (30 min at $250\,000\times g$, 4°C). Membranes were resuspended in buffer B (50 mM Tris–HCl pH 8.0, 200 mM KCl, 10% glycerol) at a final concentration of 0.5 mg/ml protein and solubilised by the addition of 1% (v/v) digitonin. Insoluble material was removed by centrifugation (15 min at $250\,000\times g$). Proteins were incubated with anti-TatA or anti-TatB serum for 3 h at 4°C. For control immunoprecipitations, an equal amount of irrelevant antiserum (raised against spinach photosystem II subunit W) was included. Next, 10 mg of protein A Sepharose was added, and the samples were incubated for an additional 2 h. Immunoprecipitates were isolated by centrifugation, washed four times with buffer B containing 0.5% digitonin, and resuspended in SDS sample buffer. Immunoprecipitated proteins were visualised by SDS–PAGE and Western blotting. His-tagged proteins were specifically visualised using an antiserum to the hexahistidine-tag (Invitrogen).

3. Results and discussion

3.1. TatB levels are affected in cells lacking TatA

The subunit composition of the translocase involved in the Tat pathway has not been addressed in previous studies, and we investigated this issue using wild-type cells or cells expressing the Tat components at levels comparable to that of wild-type cells. To facilitate the detection of TatA or TatB in wild-type cells, antibodies were raised against these proteins as detailed in Section 2. Fig. 1, upper panel shows that the TatA antiserum recognises an 18 kDa protein in wild-type cells and that this protein is absent in extracts of the mutant strain lacking the *tatA* gene (lane ΔA). The antiserum also reacts with a lower molecular mass protein, denoted by an asterisk; this may well be due to non-specific interactions, although this remains to be confirmed. The TatB antiserum recognises a single 30 kDa protein in wild-type cells that is absent in the Δ*tatB* mutant (lane ΔB).

In order to achieve plasmid-borne expression at wild-type levels, genes encoding hexahistidine-tagged TatA ($TatA_{His}$) or TatB ($TatB_{His}$) were cloned into the vector pBAD24 [15]. This vector contains the arabinose-inducible P_{BAD} promoter, the activity of which can be modulated by varying the concentration of arabinose. To test whether $TatA_{His}$ and $TatB_{His}$ were functional proteins, pBAD-tatAhis and pBAD-tatBhis were transformed to the Δ*tatAE* and Δ*tatB* mutant strains, respectively. The latter two strains are unable to grow anaerobically in minimal medium containing glycerol as the carbon source and trimethylamine *N*-oxide (TMAO) as the final electron acceptor [3,4], as TMAO reductase is dependent on the Tat pathway for its export to the periplasm. Both $TatA_{His}$ and $TatB_{His}$ were able to restore the growth defect, showing that the His-tagged derivatives are functional proteins (data not shown). As expected, $TatA_{His}$ and $TatB_{His}$ showed a slightly reduced mobility in SDS–PAGE gels compared to the proteins without the His-tags (Fig. 1, upper and lower panel). The presence of arabinose at even low concentrations (between 0 and 2 μM) results in expression of $TatA_{His}$ in Δ*tatA* (pBAD-tatAhis) cells at levels comparable with those of TatA in wild-type cells. In contrast, wild-type TatB levels were significantly below that of Δ*tatB* (pBAD-tatBhis) cells without arabinose induction. $TatA_{His}$ or $TatB_{His}$ were not absent in

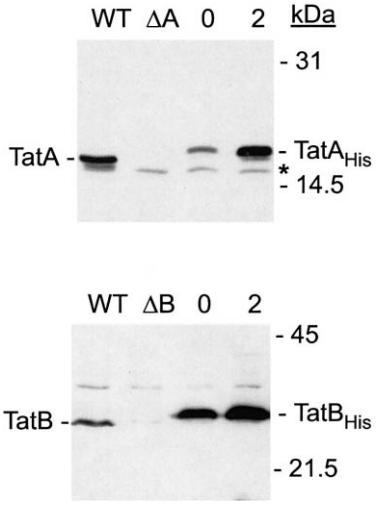

Fig. 1. Detection of TatA and TatB. Cells of *E. coli* MC4100 (WT), Δ*tatA* (ΔA), Δ*tatB* (ΔB), all containing pBAD24 (vector without insert), were grown in TY medium until the end of the exponential growth phase. Cells were collected by centrifugation, and TatA or $TatA_{His}$ was visualised by SDS–PAGE and Western blotting. Similarly, cells of *E. coli* Δ*tatA* containing pBAD-tatAhis (vector containing *tatAhis* gene), or Δ*tatB* containing pBAD-tatBhis (vector containing *tatBhis* gene) were grown in the presence of 0 or 2 μM arabinose (lanes indicated as 0 and 2) in TY medium, and analysed by Western blotting using antisera raised against TatA (upper panel) or TatB (lower panel). Mobilities of molecular mass markers (in kDa) are indicated.

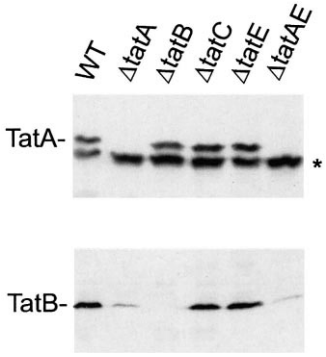

Fig. 2. TatA and TatB levels in *tat*-deficient strains. Cells of *E. coli* MC4100, Δ*tatA*, Δ*tatB*, Δ*tatC*, Δ*tatE*, or Δ*tatAE* were grown in TY medium until the end of the exponential growth phase. Cells were collected by centrifugation and TatA or TatB were visualised by SDS–PAGE and Western blotting as in Fig. 1. Equal amounts of cells were loaded in each lane (standardised on the basis of optical density).

cells grown without arabinose, due to a low basal level of expression from the P_{BAD} promoter.

To test whether the levels of TatA or TatB were affected in the strains lacking known *tat* genes, Western blotting experiments were carried out using wild-type cells and cells lacking functional *tatA*, *tatB*, *tatC*, or *tatE* genes. As shown in Fig. 2, upper panel, the level of TatA was not affected in *tatB*, *tatC*, or *tatE* mutant strains, indicating that this protein is stable in the absence of a functional Tat translocase. However, TatB was present in much-reduced amounts in the Δ*tatA* strain and Δ*tatAE* strains, but not in the *tatC* or *tatE* mutant strains (Fig. 2, lower panel). This strongly suggests that TatB is unstable in the absence of TatA. An alternative possibility is that *tatB* expression is reduced in cells lacking TatA, but it is improbable to have polar effects affecting expression, since the Δ*tatA* mutant strain contains an in-frame deletion of the *tatA* gene. Thus, it is likely that TatB interacts, either directly or indirectly, with TatA and is unstable in its absence. The level of TatA does not depend on the presence of TatB, showing that TatA is not prone to degradation in the absence of TatB. Our results are contradicted by recent observations of Sargent et al. [11], who did not see an effect on the stability of TatB in a strain lacking TatA. However, it has to be noted that in this paper the experiments differed on two major points. Firstly, Sargent et al. analysed TatB levels in a strain overexpressing the entire *tatABCD* operon on a plasmid, and secondly, they used pulse-chase experiments to analyse the stability of the various proteins. In contrast, in our experiments the wild-type level of proteins was analysed in steady-state conditions. As under these conditions TatB was still present in low amounts in a Δ*tatA* strain, it can be anticipated that the kinetics of TatB degradation are rather slow, and not detectable by pulse-chase analysis.

3.2. TatA interacts with TatB

To test more directly for an interaction between TatA and TatB, co-immunoprecipitation experiments were performed under native conditions. Membranes were isolated from *E. coli* MC4100, and the Δ*tatA* and Δ*tatB* strains, solubilised with digitonin, and immunoprecipitated with anti-TatA or anti-TatB. As shown in Fig. 3A, TatA could be effectively immunoprecipitated from wild-type or Δ*tatB* cells (lanes WT, ΔB) using antibodies raised against TatA ('IP α-TatA' panel). The lower molecular mass contaminating protein, observed in the Western blots in Fig. 1, is also immunoprecipitated by these antibodies (asterisk). This protein is also immunoprecipitated from Δ*tatA* cells, but the TatA protein is absent as expected. Significantly, TatA is also immunoprecipitated from wild-type cells by the TatB antiserum (panel 'IP α-TatB'). No co-immunoprecipitation of TatA is observed from Δ*tatA* cells (confirming that the protein detected on the blot is indeed TatA) or from strains lacking TatB. The latter result confirms that the anti-TatB antiserum does not recognise the native TatA protein, and TatA must therefore be immunoprecipitated by virtue of its interaction with TatB. No immunoprecipitation of either TatA or the lower molecular mass protein is observed in control experiments using an irrelevant antiserum raised against a thylakoid membrane protein ('IP control' panel). These data demonstrate that the two proteins are present within a single complex. In this and other experiments, we have found that approximately 5% of the TatA can be immunoprecipitated using the TatB antiserum. This might reflect the difficulty in immunoprecipitating the entire TatB population but we believe that a more likely explanation is that only a relatively small proportion of TatA is actually in a complex with TatB (see below).

Parallel tests using the anti-TatA serum demonstrated co-immunoprecipitation of TatB together with TatA, but a high

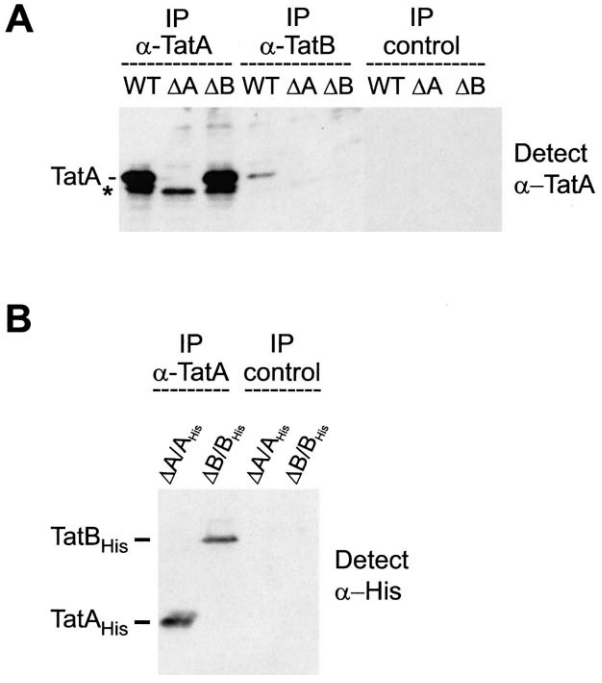

Fig. 3. Co-immunoprecipitation of TatA with TatB. A: Membranes of *E. coli* MC4100 (WT), Δ*tatA* (ΔA), or Δ*tatB* (ΔB) were solubilised with digitonin, and immunoprecipitation was performed using anti-TatA or anti-TatB antibodies (IP α-TatA or α-TatB, respectively) or with an antiserum raised against PsbW, a thylakoid membrane protein (IP control). TatA was visualised by SDS–PAGE and Western blotted using the antiserum to TatA. B: Membranes of *E. coli* Δ*tatA* containing pBAD-tatAhis (ΔA/A_{His}), or Δ*tatB* containing pBAD-tatBhis (ΔB/B_{His}) were solubilised with digitonin and immunoprecipitation was performed using anti-TatA antibodies. TatA and TatB were visualised by SDS–PAGE and Western blotting, using monoclonal antibodies recognising the hexahistidine-tag ('detect α-His'). His-tagged proteins were expressed using an arabinose concentration of 1 μM in each case.

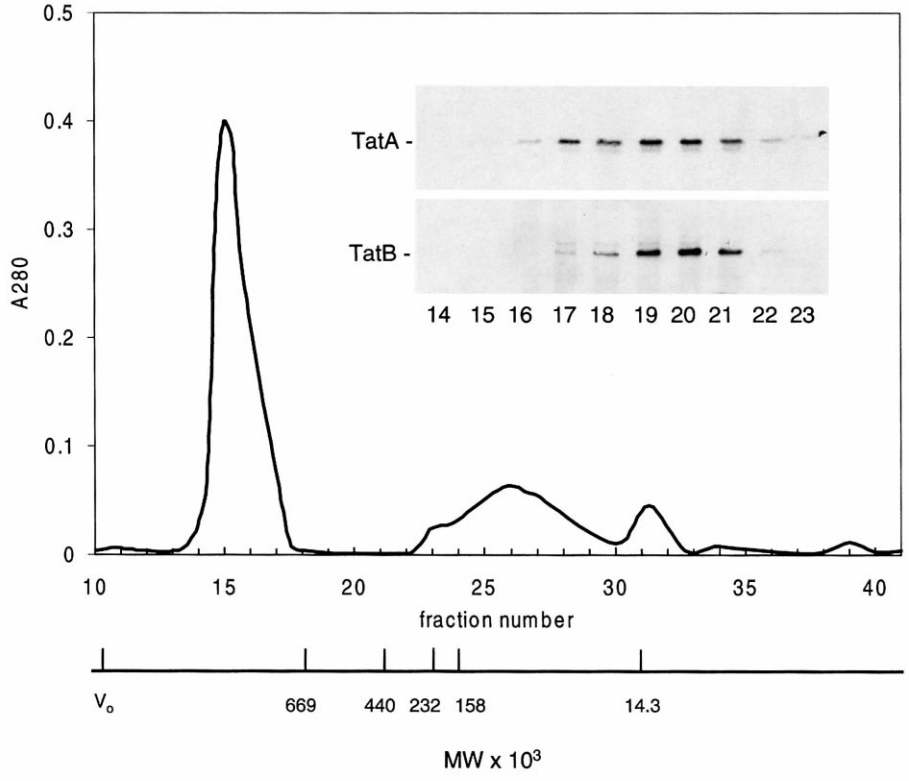

Fig. 4. TatA and TatB participate in a complex of ca. 600 kDa. *E. coli* MC4100 membranes were solubilised in a buffer containing 50 mM Tris–HCl pH 8.0, 200 mM KCl, 10% glycerol, and 1% Nonidet P-40. Solubilised proteins (250 μl; corresponding to 5 ml culture with an OD_{600} of 0.7) were fractionated on a Superose 6HR column (Amersham Pharmacia Biotech). Elution was with a buffer 50 mM Tris–HCl pH 8.0, 200 mM KCl, 10% glycerol, and 0.1% Nonidet P-40, at a flow rate of 0.2 ml/min. Fractions of 0.6 ml were collected, and protein elution was monitored by absorbance (*A*) at 280 nm. Fractions were precipitated with 10% trichloroacetic acid, and TatA or TatB were detected by Western blot analysis; fractions containing TatA or TatB are shown in the inset. The column was calibrated with the following proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and lysozyme (14.4 kDa). MW, molecular weight; V_o, void volume.

level of background reaction was observed, apparently with the IgG proteins that migrate in the TatB region of the gel (data not shown). This problem was circumvented by expressing plasmid-encoded, His-tagged TatB ($TatB_{His}$; see Fig. 1) in the Δ*tatB* strain lacking chromosome-encoded TatB. In these experiments, TatB was detected using antibodies that specifically recognise the His-tag; these antibodies were found to generate much lower background reactions in Western blots of immunoprecipitates (data not shown). For control purposes, His-tagged TatA ($TatA_{His}$) was also expressed in Δ*tatA* strains. Fig. 3B shows the results obtained when cell extracts were immunoprecipitated with TatA antibodies and then Western blotted with the His-tag antibodies (to detect $TatA_{His}$ or $TatB_{His}$ in the immunoprecipitates). The data confirm that $TatA_{His}$ is immunoprecipitated from Δ*tatA* cells expressing $TatA_{His}$ (lane $\Delta A/A_{His}$). Importantly, the adjacent lane ($\Delta B/B_{His}$) shows a clear $TatB_{His}$ signal, demonstrating that this protein was co-immunoprecipitated using anti-TatA antibodies and providing further evidence that TatA and TatB are located within the same complex. Neither TatA nor TatB are immunoprecipitated by control antibodies.

3.3. *TatA and TatB are subunits of a complex with a molecular mass of 600 kDa*

Attempts to isolate a complex containing TatA and TatB using His-tagged proteins were not successful, as the complex appeared to be unstable during nickel affinity chromatography. Therefore, gel filtration chromatography was used to investigate the complex in which TatA and TatB participate in more detail. To this purpose, *E. coli* MC4100 membranes were solubilised using Nonidet P-40 and the extracted proteins were separated on a Superose 6HR column. The amounts of TatA and TatB in the various fractions were determined by Western blotting. Fig. 4 shows that TatB elutes in a single

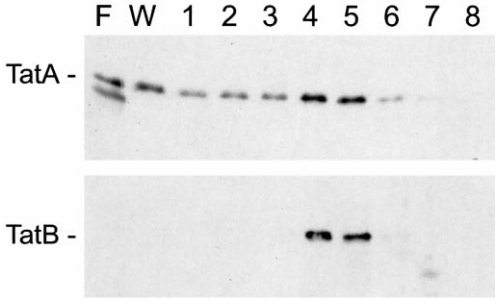

Fig. 5. Ion-exchange chromatography of TatA and TatB. Fractions 19–21 from the Superose eluate shown in Fig. 4 were pooled, diluted in an equal volume of 50 mM Tris–HCl, pH 8.0, 10% glycerol, 0.1% (v/v) Nonidet P-40 and loaded on a 1 ml mono-Q column equilibrated in the same buffer containing 100 mM KCl. Samples were collected of the flow-through (lane F) and fractions eluted during washing of the column (lane W) with 5 ml of the above buffer containing 100 mM KCl. Elution was then performed using a KCl gradient of 100–400 mM (lanes 1–8). Samples of each fraction were Western blotted using antisera to TatA and TatB as indicated.

peak corresponding to a molecular mass of approximately 600 kDa. TatA also elutes in a single peak and, given the co-immunoprecipitation data, at least some of the TatA molecules must be bound to TatB. The observed size of this complex is very similar to that of the putative thylakoid Tat complex [12]. However, in this and other experiments we have found that the TatA and TatB elution peaks differ, with TatA elution consistently observed to elute in a wider range of fractions. This observation strongly suggests that either the TatA:TatB ratio varies considerably in Tat complexes, or that some TatA molecules are in the form of a different high molecular mass complex that possibly does not contain TatB. In either case, the size of the TatB/TatA-containing complex suggests the presence of other subunits and/or the presence of several copies of TatA, TatB or TatC. No TatA or TatB was detected in other column fractions corresponding to the lower molecular mass proteins (not shown), strongly suggesting that under these conditions most or all of the TatA and TatB is present in large complexes.

The fractionation characteristics of TatA and TatB were further analysed by subjecting the Superose eluate to ion-exchange chromatography. Fig. 5 shows that the elution of the two proteins differs significantly. TatB elutes as a sharp peak at KCl concentrations of approximately 250 mM (fractions 4 and 5). In contrast, TatA is detected in the flow-through and wash fractions (lanes F, W) as well as in most of the salt gradient fractions. However, it is notable that TatA elution peaks in fractions 4 and 5, which is consistent with this population of the molecules being in a complex together with TatB. Although we can not exclude the possibility that TatA is removed from the Tat complex during fractionation, we believe that these data, together with those shown in Fig. 4, support the proposal that TatB is almost entirely in the form of a large complex whereas TatA is present as two distinct populations, one of which is complexed with TatB (and presumably other Tat components) whereas the other is in a separate complex lacking TatB.

3.4. Implications for the Tat translocase

In conclusion, we have shown for the first time that two of the Tat proteins, namely TatA and TatB, interact within the same complex. This was shown through different lines of evidence. Firstly, TatB appears to be unstable in cells lacking TatA. Secondly, TatA and TatB can be co-immunoprecipitated by antibodies to either protein. Thirdly, TatA and TatB co-elute from a gel filtration column in a complex of approximately 600 kDa. It is not yet clear whether the interaction of TatA and TatB is direct, or mediated via another protein (an obvious candidate being TatC). Interestingly, it has recently been shown that TatB stabilises TatC [11], suggesting that TatC is also present in the TatA–TatB complex. However, the lack of antibodies recognising TatC at wild-type levels prevented us thus far from demonstrating the presence of TatC in this complex. The TatE protein seems not to be of major importance, as a *tatE* mutant strain shows only mild defects in the translocation of Tat substrates [4]. However, TatA and TatE were shown to be functionally interchangeable [11].

Interestingly, the results obtained indicate that TatA could be present in a complex even in the absence of TatB, as: (i) TatA levels seem to be significantly higher than those of TatB; (ii) the stability of TatA was not affected in strains lacking TatB; (iii) only a minor fraction of TatA could be immunoprecipitated using anti-TatB antibodies; and (iv) chromatographic analysis indicated that a portion of TatA may elute without TatB. Thus, it is conceivable that there is a heterogeneous population of Tat complexes which vary in their subunit composition. As TatB is required for the translocation of Tat substrates [3,11], complexes lacking TatB might be in an inactive state.

A major challenge for the future is to establish which other subunits are present in the Tat complex, and to determine the stoichiometry of these. Candidates are obviously the TatC and TatE proteins, but it is also conceivable that other, still unknown, components are required.

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