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Review

Protein transport by the bacterial Tat pathway ☆

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ABSTRACT

The twin-arginine translocation (Tat) system accomplishes the remarkable feat of translocating large – even dimeric – proteins across tightly sealed energy-transducing membranes. All of the available evidence indicates that it is unique in terms of both structure and mechanism; however its very nature has hindered efforts to probe the core translocation events. At the heart of the problem is the fact that two large sub-complexes are believed to coalesce to form the active translocon, and 'capturing' this translocation event has been too difficult. Nevertheless, studies on the individual components have come a long way in recent years, and structural studies have reached the point where educated guesses can be made concerning the most interesting aspects of Tat. In this article we review these studies and the emerging ideas in this field. This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.

1. Introduction

The twin arginine translocase (Tat) is a protein transport pathway that exists in Archaea, bacteria and plant chloroplasts. In bacteria, it exports proteins across the plasma membrane and is important for many processes including energy metabolism, formation of the cell envelope, biofilm formation, heavy metal resistance, nitrogen-fixing symbiosis, bacterial pathogenesis and others [1,2]. What makes this protein transport system unusual compared to other transport systems (such as the general secretory, or Sec pathway) is its ability to transport fully folded proteins across membranes. This remarkable feat has no requirement for ATP as an energy source, and relies solely on the proton motive force (PMF) [3–5].

The mechanism of translocation remains poorly understood, in part due to a lack of high resolution structural information on this complex and its individual components. That said, a number of recent biophysical and structural studies have provided a more detailed picture of the action and composition of this translocase, particularly with respect to the early events prior to the actual translocation event. This review discusses the key information from each of these studies. Much of this article will focus on the *Escherichia coli* (*E. coli*) Tat system, but relevant data on the Gram-positive homologs from *Bacillus subtilis* (*B. subtilis*) and the chloroplast Tat system are also mentioned. A more detailed analysis of Gram-positive Tat systems is given elsewhere in this volume by Goosens et al. [6].

2. The Tat system's substrates

The extent to which different organisms utilise the Tat pathway varies significantly. Gram-positive bacteria such as *Staphylococcus aureus* or *B. subtilis* have few predicted substrates [7–9], whereas enteric bacteria typically possess around 20–30 substrates [10]. Whilst the rationale for using this translocase remains unclear for some Tat substrates, three key underlying factors have been identified. The first is a requirement for the enzymatic insertion of complex cofactors in the cytoplasm prior to transport, thereby bypassing the requirement for extra mechanisms to firstly, separately export the cofactor and then subsequently catalyse its insertion in the periplasm [1]. The second motive is avoidance of metal ions that compete for insertion into the active site, and lastly, the transport of hetero-oligomeric complexes that optimally assemble in the cytoplasm [11,12]. The latter is achieved through proteins forming complexes with other proteins that possess an N-terminal Tat signal peptide [13].

Navigation to the Tat translocase is dictated by the presence of an N-terminal signal peptide that possesses an overall tripartite architecture of: a polar amino terminal (N) domain, hydrophobic core (H) region and a polar carboxyl (C) domain (Fig. 1). Despite the Sec- and Tat-type signal peptides having the same basic structure and a similar terminal Ala-X-Ala motif, studies on Tat signals revealed a highly conserved SRRxFLK motif [14,15] located at the junction of the N- and H-domains. The twin-arginine motif gives this translocase its name. Both arginines are critical in chloroplast Tat signals [16], but less so in bacteria, where mutation of a single arginine in bacteria only affects the rate of translocation, whereas mutation of both completely abolishes transport [17–19]. Within the SRRxFLK motif, three determinants are important: the twin-arginine pair, the hydrophilic −1 residue

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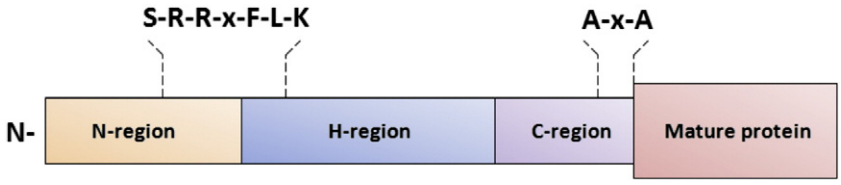

Fig. 1. The Tat signal peptide. A polar amino domain (N-region), hydrophobic core (H-region), and polar carboxyl domain (C-region) comprise the tripartite structure of a Tat signal peptide, which is located at the N-terminus of the substrate protein. On average they are less hydrophobic than Sec-specific signals, as well as being longer (on average 38 to 24 amino acids, respectively). Tat signal peptides are distinguished by their conserved twin-arginine motif in the N-region. The C-terminal region houses an A-x-A motif, which is a consensus cleavage site for removal of the signal peptide by signal peptidase.

and the hydrophobic +2 residue (+/− relative to the twin-arginine pair). However, while Tat and Sec signal peptides share a similar overall architecture, it is not the twin arginine pair alone that prevents mistargeting to the Sec pathway. Tat signal peptides are less hydrophobic than those used in Sec-targeting [20], and the C-region of certain Tat signal peptides houses basic residues, which are seldom found in the same region of Sec signal peptides. The latter is believed to hinder engagement with Sec machinery [21–23].

3. Tat Translocase components and complexes

3.1. The Tat subunits

Three integral membrane proteins form the minimal set of components for the assembly of the Tat translocase in *E. coli*: TatA, TatB and TatC. These proteins are expressed from the *tatABC* operon and reside in the cytoplasmic membrane arranged as a Tat(A)BC substrate binding complex and a separate TatA complex (Fig. 2). TatA is an 89 amino acid protein (9.6 kDa) that consists of a short periplasmic N-terminal region, a transmembrane helix that is linked via a hinge region to a cytosolically exposed amphipathic helix (APH), and a highly unstructured, cytoplasmically-exposed C-terminal region [24–26]. This arrangement is supported by spectroscopy studies, which indicate that the APH lies along the surface of the membrane [27,28]. Additionally, solid-state NMR has shown the TMH to cross the cytoplasmic membrane at a 17° tilt [29]. An N-out topology is the favoured orientation of TatA in the cytoplasmic membrane and is supported by recent NMR data of the TatA component of a Gram-positive homolog, TatAd [30,31]. Some studies have predicted that TatA may also have a dual topology, on the basis of data that suggest the N-terminal region of TatA can also be accessed from the cytoplasm [32]. Moreover, there is evidence of soluble TatA in bacteria and chloroplasts [33–39]; however the functional relevance of this soluble TatA pool remains controversial.

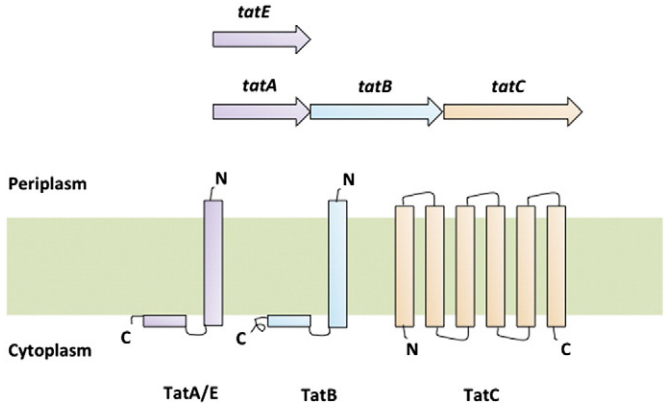

Fig. 2. Component organisation of the *E. coli* Tat system. In Gram-negative bacteria the Tat translocase system is usually made up of three integral membrane proteins, encoded by the *tatABC* operon. Both TatA and TatB are single-span transmembrane proteins that possess: a short periplasmic N-terminal region; single-span transmembrane helix; hinge region; amphipathic helix lying along the cytoplasm-membrane interface and a highly charged, unstructured C-terminus. In contrast, TatC is a polytopic protein that is predicted to contain 6 transmembrane spans, with both the N- and C- termini in the cytoplasm. In *E. coli* a TatA paralog exists, TatE, which is encoded elsewhere in the genome.

TatB consists of 171 amino acids with a molecular mass of 18.5 kDa. Despite sharing a 20% sequence similarity with TatA [40] and a very similar predicted secondary structure (Fig. 2), TatB and TatA carry out functionally distinct roles within the Tat translocase [41].

TatC consists of 258 amino acids with a molecular mass of 28.9 kDa. As predicted by its secondary structure, this protein traverses the membrane 6 times, possessing an N-in C-in topology [42]. The *tatABC* gene products form two distinct membrane complexes at steady state: a TatBC-containing substrate binding complex and a separate TatA complex. It is in this former 370 kDa Tat(A)BC complex where most of TatB and -C are found at a 1:1 stoichiometric ratio [43]. TatA (found at ~ 25 and 50 fold higher concentrations than TatB and TatC, respectively [44]), is present as highly heterogeneous complexes ranging from 100–500 kDa [43,45,46] and is not required for TatBC complex assembly [47].

The composition of the Tat system differs significantly in most Gram-positive bacteria; all except Streptomycetes contain only *tatAC* genes [48,49]. In those examples studied to date, the TatA protein is bifunctional [50]. The best characterised Tat system in this type of bacteria is found in *B. subtilis* — a non-pathogenic soil bacterium, which contains two discrete Tat systems that operate in parallel, yet possess different substrate specificities [48]. The first of the two is TatAdCd, whose only substrate identified at present is the phosphodiesterase, PhoD [49]. The second translocase is TatAyCy, which exports YwbN, an iron-dependent DyP-peroxidase [49]. There is a third *tatA* gene encoding the TatAc protein, which like TatAd, was recently shown to form small homogeneous complexes and restore export of TorA in a Δ*AE* mutant [51].

Reminiscent of the situation in *E. coli*, the TatAyCy system is composed of two types of membrane protein complexes: TatAyCy and TatAy that have been reported to form ~200 kDa complexes (as judged by gel filtration chromatography) [37,52]. Likewise, TatAdCd exists as a ~230 kDa complex, alongside a separate and discrete TatAd complex of ~160 kDa[50,53]. The TatAd and TatAy proteins are bifunctional fulfilling the role of the TatB protein that would otherwise be present in Gram-negative bacteria [50,54].

In addition to these bacterial TatAC-containing complexes being smaller than their *E. coli* counterparts (TatABC is ~370 kDa on BN gels [43,45,55]), the lack of a *tatB* gene and TatA heterogeneity appear to be conserved features of Gram-positive bacteria. This is an important point because the remarkable heterogeneity of *E. coli* TatA complexes has been considered to be a key element of current translocation models (see below)[53].

Finally, *E. coli* also possesses a TatA paralogue, TatE. This 67 amino acid protein possesses 57% sequence identity to TatA [25] and is thought to have arisen from a gene duplication of *tatA* [56]. Whilst it can fulfil TatA activity if overexpressed [41], there is no evidence for a specific role for this protein, and indeed many Gram-negative bacteria lack a *tatE* gene [46,57].

3.2. The TatBC complex

3.2.1. Structural characterisation of the TatBC complex

Early cross-linking studies conducted in chloroplasts demonstrated that a Tat-dependent substrate could be cross-linked to the Hcf106-cpTatC complex (equivalent to TatBC in bacteria) [58], demonstrating that this is the initial receptor for the Tat pathway. Similar binding characteristics were observed for the TatBC complex of *E. coli* [59]. In both instances full translocation was prevented by the absence of a PMF, and these studies represent good evidence that the TatBC complex does indeed carryout the binding of a substrate [58]. These complexes were initially shown to be large (370–500 kDa), multi-subunit entities using Blue Native Polyacrylamide Gel Electrophoresis [43,46,60].

Electron microscopy characterisation of the TatBC complex presented a hemispherical complex ranging from 11–17 nm in diameter [60]. It was inferred by Tarry et al. that the size of the complex is large enough to accommodate 7 copies of TatB and TatC in equimolar ratio [43,60]. The central cavity within the hemisphere was not large enough to accommodate the substrate; although it was shown to expand upon substrate binding [60]. The functional role of this cavity is not clearly understood, however it is predicted to be implicated in signal peptide insertion once the precursor protein has been recognised by Tat [61,62]. The substrate itself is shown to bind to the periphery of the TatBC complex with a maximum of two substrates bound per complex, which possibly indicates a negatively cooperative binding event. In this review we have presented a feasible assembly for the TatBC complex, using the structure of TatC recently resolved by Ramasamy et al. [63]. In this hypothetical assembly, the substrate binding would have a knock on effect on the adjacent TatC monomers by "closing off" binding sites to neighbouring TatC proteins. The second substrate binding would therefore be most favourable on the opposite side of the complex, similar to that observed by Tarry et al. [60].

Recent structural characterisation of the individual TatC components has hugely contributed to the understanding of its functional role. TatC is the largest and most conserved component of the Tat machinery in both bacteria and chloroplasts [54]. It plays a central role in the translocation event, ranging from substrate recognition and binding, to the recruitment of other Tat components [36]. The predicted topology of TatC shows the protein to form 6 transmembrane helices with the N- and C-termini residing in the cytoplasm (Fig. 3) [64]. Recent 3D crystallisation studies on TatC from *Aquifex aeolicus* present a "glove-like structure" [63,65], where TatC appeared to assemble into a concave structure as a result of transmembrane helix (TMH) TMH5 and TMH6 being shorter in length relative to the remaining helices (Fig. 3)[65]. Data showing that TMH5 could interact with the TMH of TatB suggests that a functional role for the "groove" of TatC is possible: it could accommodate a single TatB protein- or at least in part (Fig. 3B). Additionally, the interaction of TatC's periplasmic loops with TMHs of neighbouring TatC proteins, suggests they most likely possess a role of maintaining the stability of the complex (Fig. 3)[65].

The residues that line the groove of this glove-like structure are predominantly aromatic with the potential to bind the lysine on the amphipathic helix of the signal peptide [63]. This characteristic shape and residue environment may imply a possible site for signal peptide insertion into the bilayer [61]. In compliment, mutation within the N terminus of TMH1 and the first cytoplasmic loop are involved in substrate binding. Compilation of these studies and recent studies suggest the substrate is bound inside the TatC protein with the linker region forming a hairpin around TMH4 of TatC. The possible association of the TatBC unit with the substrate bound is shown in Fig. 4.

3.2.2. Recent mechanistic studies on the TatBC complex

It has been well documented that the Tat substrate-binding site in *E. coli* consists of a hetero-oligomeric complex of TatB and TatC (Hcf106 and cpTatC in the thylakoid Tat system). Whilst it is known that the Tat-dependent precursor binds to this complex for subsequent translocation into the periplasm/thylakoid lumen, the precise details of this interaction have only started coming to light in recent years. This information is vital for elucidating the most ambiguous topic of this translocase — its mechanism of translocation.

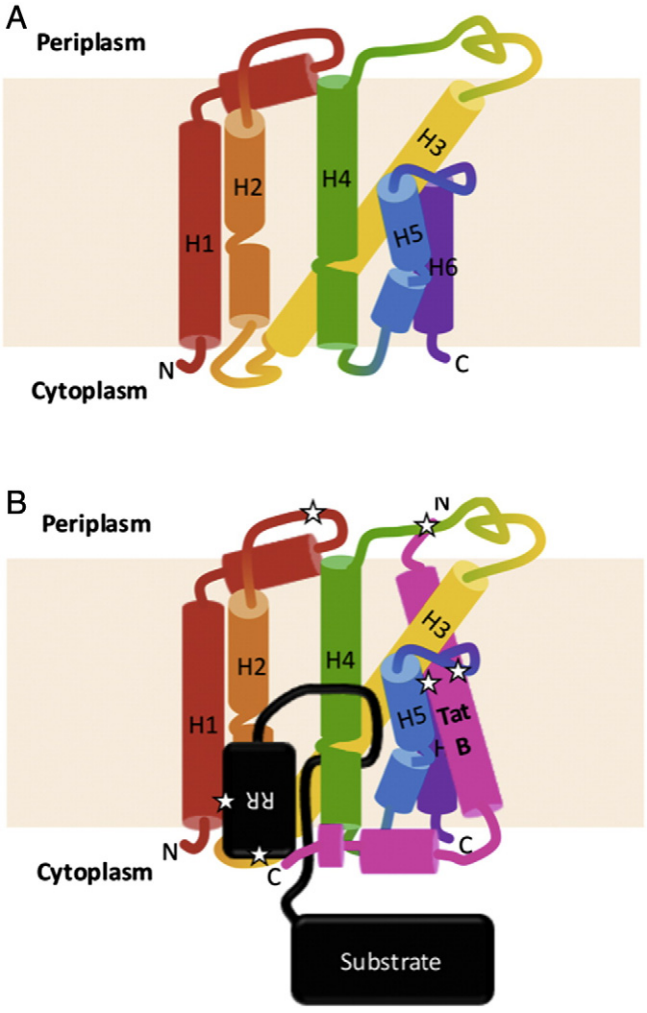

Fig. 3. The topology of TatC within the cytoplasmic membrane +/− the presence of TatB and precursor protein. A. Shows the N-in C-in arrangement of the multi-spanning TatC protein within the cytoplasmic membrane. B. Shows how the TatBC complex may arrange itself within the cytoplasmic membrane upon precursor binding. Critical interactions identified from cross linking studies are starred. TatB makes three vital contacts to TatC. Namely: to TMH 5 of TatC (blue) and its second periplasmic loop (green/yellow). TatB (pink) could be accommodated within the groove of TatC which was shown to exist from recent crystallisation data (§3.2.1). The first periplasmic loop (red/orange) fails to make contact with TatB, yet is in close proximity to a neighbouring TatC; lending to the idea that this loop is implicated in self-assembly and maintaining the stability of the TatBC complex. Recent data allude to a hairpin-like insertion of the precursor signal peptide (shown in black). The signal peptide makes vital contacts to the extreme N-terminus of TatC and its first cytoplasmic loop (orange/yellow).

TatC is known to be the primary site of interaction between a Tat RR-precursor protein and the substrate-binding complex [59,60,62,66–71]. Site specific cross-linking has elucidated that RR-precursors make contact to the cytosolically exposed extreme N-terminus and first cytosolic loop of TatC (Fig. 3B) [72]. This data is in agreement with results demonstrating that the signal sequences of membrane-bound Tat substrates are still accessible to proteases and can be detached by disrupting electrostatic interactions [73,74].

To gain further insight into this interaction between TatC and RR-precursor proteins, a random mutagenesis study was performed with the aim of identifying regions within TatC that are essential for protein transport [75]. Mirroring the results mentioned previously, conserved residues of TatC that are essential for its activity were identified in its

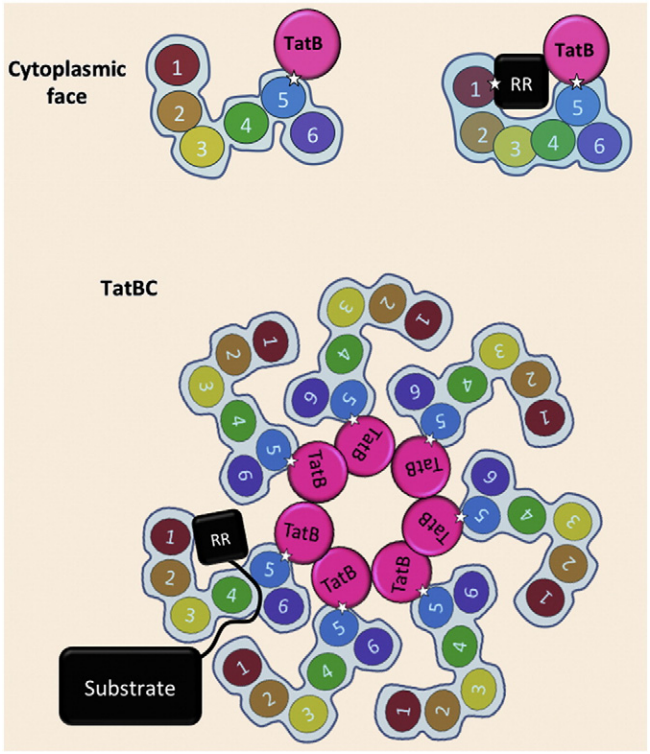

Fig. 4. Cytoplasmic-view of a Tat RR-precursor protein binding to the TatBC substrate-binding complex. This schematic amalgamates available structural and cross-linking data on the TatBC complex to hypothesise how TatB and TatC could arrange themselves in the cytoplasmic membrane upon precursor binding. We hypothesise that for Tat precursors to bind an individual binding site, whilst the complex still accommodates 7 copies of TatBC in equimolar ratio, the TatBC complex could arrange itself as shown. Not only would this satisfy the aforementioned cross-linking data, an arrangement such as this would be in agreement with EM data of SufI-bound TatBC; in that the Tat precursor would bind to the periphery of the TatBC complex. It is plausible that precursor binding to the shown position could alter the accessibility of neighbouring binding sites and therefore result in the Tat translocase exhibiting negative cooperativity.

first cytoplasmic loop. However an abundance of this study's inactivating mutations localised in the first two periplasmic loops of TatC (P1 and P2). Whilst additional insertion and deletion analysis supported the idea that these regions are essential for transport, it remains unclear as to what roles they may fulfil. Inactivating mutations in P1 and P2 did not affect insertion of TatC into the membrane or make it unstable, nor did they inhibit the formation of TatBC substrate-binding complexes. More specifically, the P1 loop fails to make contact with TatB, yet is in close proximity to a neighbouring TatC (Fig. 3B) [75]. The P2 loop makes contact with neighbouring TatC [42], TatB and TatA [72]. Zoufaly et al. [72] inferred that the P2 loop of TatC may be involved in maintaining the stability and functional interaction between TatB and TatC, since a P142S substitution mutation in this region suppressed the defective transport of a mutant KK-precursor protein [76]. Mutational studies on the TatC protein in chloroplasts revealed the functional significance of the stromal loops, where the 1st and 2nd loops are involved in self-assembly as well as interaction with other Tat components [77].

Despite it being well documented that TatB and TatC form a stable, substrate-binding complex, there is a deficiency of molecular details on specific sites of contact between the two proteins and the precursor protein. In the aforementioned study [75], in addition to the N-terminal region of TatC making contact with the RR-signal peptide, cross-linking analysis also revealed that this region comes into close contact with TatB; reinforcing the molecular neighbourhood of these two proteins [75]. In light of the data showing that TatB possesses the capability to directly interact with RR signal peptides [59,68,70,71,78], including residues flanking the RR-pair [59,68], it is suggested that TatB functionally cooperates with TatC by forming part of the signal binding pocket [72].

Lausberg et al. found evidence for a tight cooperation of TatB and TatC during recognition of Tat signal peptides. To analyse which regions are involved in the substrate binding event, this study searched for suppressor TatB or TatC mutants that successfully supressed the translocation defect of mutant precursor TorA(D^{2+})-MalE [79]. Three mutations were found (TatC K18E, TatB L**9**P and TatB L**9**Q) which each displayed clear synergies with a L9F mutation of TatC i.e. restored export of TorA(D^{2+})-MalE; adding strong genetic evidence for a tight cooperation of TatB and TatC during signal peptide recognition, and for the participation of both components in the formation of a specific signal peptide binding site [79].

According to the topology model of TatB, position **9** is located at the periplasmic-oriented end of the TatB TMH. Such a location reflects one of two situations. Either the effects of these mutations are long-range conformational ones in that they are transmitted via the TMH of TatB to a binding pocket located on the cytoplasmic face of the TatBC receptor complex. Or, they could affect precursor binding at a stage when the signal peptide and the early mature part of the precursor had been transferred to an advanced-stage binding site that would reach out as far as the periplasmic end of the TMH of TatB.

Beyond recognition of the RR-precursor, it has remained questionable as to which component of the TatBC substrate-binding complex mediates the transmembrane insertion of the RR-signal peptide; commencing translocation of the protein. Frobel et al. showed that despite a lack of TatB, the signal sequence of translocation-incompetent substrates was removed, in addition to the premature cleavage of the signal sequence from a substrate capable of translocation [80]. This lends itself to the idea that when a precursor protein interacts with TatB, it prevents the premature cleavage of the signal peptide; revealing a potential insertase activity of TatC in addition to further evidence for the concerted activity of TatB and TatC in recognising Tat substrates.

Although it is well established that TatB is part of the substrate binding site, its precise function in the substrate-binding process has remained elusive. Maurer et al. demonstrated that upon precursors being targeted to the cytoplasmic membrane via their RR-motif they become surrounded by TatB and make contact to its TMH and APH [62]. This interaction is then lost upon translocation, lending itself to the idea that the close vicinity of TatB and precursor is an intermediate binding step that occurs prior to translocation [62]. This same study found homooligomeric complexes of TatB, containing potentially 2 or 3 TatB monomers, bound to a single precursor molecule. Dimerisation of TatB has previously been demonstrated [81], and this suggests that TatB may assemble into an oligomeric binding site that is capable of transiently accommodating large parts of the folded precursor protein [62]. A model of TatB-precursor protein interaction is speculated in Maurer et al., which is in line with a hairpin-like insertion of Tat signal peptides [62].

In recent years there has been frequent mention of a hairpin-like insertion of Tat-dependent signal sequences into the cytoplasmic and thylakoid membrane (Fig. 3B). It is proposed that once the RR-signal sequence is recognised by the TatBC substrate-binding complex, it is threaded deep into the receptor complex reaching out as far as the periplasmic end of the TatB TMH; resulting in the formation of a hairpin loop topology of the signal peptide and early mature region of the precursor protein. This hypothesis is experimentally supported by numerous studies. In *E. coli*, the N-terminus of TatB was found to cross-link to precursors TorA and SufI prior to translocation completion [62]. For the same region of TatB, suppressor mutations were found to compensate the translocation defect of mutant TorA precursors [70,79]. Finally, translocation can progress to completion despite the N-terminus of an RR signal peptide being covalently bound to TatC in thylakoids [68].

Until recently there had been a lack of information regarding the precise logistics of substrate binding. A recent study by Ma and Cline gained insight into the topology of bound precursors [82]. They found that Cys residues inserted into the signal peptide failed to produce dimers, proposing that each signal peptide is bound to an individual

binding site (Fig. 4). It was also identified that the Tat substrate binding complex has the capability of binding more than one precursor protein. More interestingly, they identified that cross-linked dimer and tetramer were readily transported into the lumen without breaking the linkage, concluding that multiple occupied receptor units operate coordinately to transport the oligomer [82].

A later study conducted by Celedon and Cline performed binding analyses on both intact membranes and purified thylakoid Tat complexes in an attempt to further elucidate the stoichiometry of binding to this complex [83]. Their results very much mirrored that of the aforementioned study in that they found the Tat complex bound multiple precursor proteins [82]. More specifically, each Tat substrate-binding complex had the capability of binding 8 precursor proteins that were each independently functional for transport. With sufficient Tha4, all sites were active for simultaneous transport [83]. Despite evidence for simultaneous transport of Tat substrates occurring [82], it is not certain whether this occurs for all Tat substrates; perhaps each site has the same probability of undertaking translocation [83].

On a different note, it still remains to be determined whether this protein transport system displays any form of cooperativity during substrate binding i.e. does Tat's affinity for its RR-precursor proteins alter upon a substrate already having bound the translocase? In agreement with Cline et al., Celedon and Cline found no evidence of cooperativity when examining the binding step [83,84]. However, Tarry et al. inferred that Tat displayed a negative cooperativity for the *E. coli* Tat system in light of evidence that TatBC complexes possessed only one or two bound precursors [60]. Again in contrast, Alder and Theg described a positive cooperativity for the translocation of a Tat precursor. This study kinetically characterised cpTat-mediated protein translocation, where the protein transport displayed a sigmoidal rate-substrate relationship; indicating allosteric proteins. The measured Hill coefficient of 1.8 was postulated to result from 2 binding sites per translocation with strong cooperativity. However, it could not be determined whether such characteristics were exhibited at the binding or translocation step [85]. Clearly more research needs to be employed in this area to absolutely identify which form of cooperativity is displayed during substrate-binding to the Tat translocase.

Once the precursor protein has been recognised by and subsequently bound to TatBC, it is still unclear as to how the Tat-dependent substrate traverses the membrane. A real-time FRET experiment analysed the kinetics of precursor interactions with the Tat translocase [86]. Results showed that once the membrane becomes energised there is a delay in cargo migration away from the binding site; suggesting that the Δ is not directly responsible for promoting migration of the Tat precursor across the membrane [86]. Moreover, it was found that TatA increases the affinity of the TatBC receptor complex for the precursor in the presence of a membrane potential [86]. These data are in agreement with the hypothesis that TatBC and TatA oligomerisation occurs in the presence of a Δ with or without the cargo. It is plausible that this oligomerisation process is responsible for the lag phase observed. The subsequent loss of FRET signal after such phase is rapid, signifying that migration of the mature domain from TatABC is fairly quick post-oligomerisation [86]. Despite this, it still remains unclear whether the mature domain of the Tat-dependent protein proceeds directly across the membrane after oligomerisation of TatABC or whether there is an additional kinetic intermediate e.g. the protein residing within a pore [86].

Whilst a wealth of information regarding substrate binding focuses on the TatBC complex, there is evidence for the involvement of TatA during the early stages of translocation. Since *E. coli* TatBC complexes contain TatA when purified [46,87], it is unsurprising that Frobel et al. reported TatA to be an early interacting partner of the TatBC receptor complex [88]. The finding that cross-links between TatA and Tat-dependent precursors were not obtained in the absence of TatBC, not only highlights the intramembrane proximity of TatBC and TatA, but also strongly suggests that a functional hierarchy exists between Tat precursors and Tat subunits i.e. the interaction with TatA requires that the precursor has already been recognised by the TatBC substrate binding complex [88]. Moreover, the precursor contacts to TatA were sensitive to the dissipation of the PMF. Hence, this interaction with TatA does not directly correspond with the precursor binding to the TatBC but instead represents a subsequent targeting event that is dependent on the presence of a PMF [88].

More specifically, the RR-precursors made contact to the N-proximal region of the TMH of TatA. Considering data that showed the precursor protein to make contact with the N-terminal region of TatB [62], a possible explanation for this interaction would be the deep insertion of a Tat signal sequence prior to translocation. Furthermore, once a TatA monomer is recruited to the TatBC receptor, it is possible it could serve as a 'nucleation point' for the subsequent recruitment of additional TatA to form the active translocase [88]. This is supported by the finding that oligomerisation of Tha4 (the thylakoid TatA homolog) occurred upon binding of a Tat signal peptide [89].

3.3. The TatA complex

Vital to elucidating the mechanism of the twin arginine translocase is gaining structural insight into the individual Tat components. TatA is one such component, which through oligomerisation with other TatA protomers has been predicted to form the 'pore' of this translocase in the cytoplasmic membrane, permitting the passage of fully-folded proteins into the periplasm.

Whilst electron microscopy structures of TatA showed ring-like complexes, there is a lack of detail on the arrangement of TatA oligomers within this ring. Nor is there any information on how TatA facilitates the transport of fully-folded proteins with such an arrangement [90]. Through the use of solution NMR, Rodriguez at al. generated a model for the TatA oligomer, which was subsequently utilised to propose a structure of the oligomeric complex [91]. Combination of these structures with molecular dynamic simulations permitted predictions as to how TatA might mediate the translocation of Tat-dependent precursors across the membrane.

The precise oligomeric state of TatA varies in cell membranes and detergent micelles [60,90,92,93]. The interfacial contacts obtained between TatA TMHs and dodecylphosphocholine (DPC) micelles in Rodriguez et al. are consistent with previous EPR studies of TatA in $C_{12}E_9$ [27]. For the 9-mer repeat of a TatA oligomer, the crossing angle of each subunit's TMH is 4.2°, meaning there is a narrow contact surface whereby large increase in subunit numbers results in small changes in intersubunit contacts. However, the APH extend out from the pore axis meaning that the varying subunit number in this instance would avoid APHs making contact. These components of TatA may provide an upper limit to oligomer size in light of the fact that inter-APH distances decrease upon the addition of subunits.

On a different note, TatA proteins possess a conserved polar residue at the N-terminal end of the TMH [40]. Through molecular dynamics conducted in this study it is postulated that when TatA oligomerises, this residue resides in the centre of a TatA pore and is water accessible [94]. The position of this residue results in a pore that is only hydrophobic for 3 turns of the TMH; approximately half that of a typical membrane bilayer [94]. Further simulations indicated that this pore can house lipids, but they are distorted relative to those in the surrounding membrane. It is predicted that these lipids could provide an energetic barrier to the loss of protons during transport [94]. This thinned and distorted membrane within the pore of a TatA oligomer would provide a clear pathway for the translocation of the substrate protein, giving a reasonable explanation for how substrates traverse the cytoplasmic membrane through the pore of TatA.

Additionally, when TatA oligomerises during translocation, it must reorient in the cytoplasmic membrane to generate the 4.2° shallow crossing angles for TMH packing. Such movement would result in the entire APH of TatA lying along the surface of the membrane, which is in agreement with data that found changes in accessibility of Tha4

APH upon substrate binding [95]. This relocalisation would pull the TMH of TatA into the cytoplasmic leaflet of the membrane, reducing the length of the TMH. Further shortening occurs due to the presence of a conserved polar residue at position 8 in the N-terminal region of the TMH. As a consequence, a hydrophobic mismatch is generated within the membrane bilayer resulting in its thinning and lipid disordering within the pore of TatA. Therefore, to add to the model of translocation for Tat, this study suggests that a bound substrate is located over a thinned and disordered patch of membrane that is susceptible to rupture [94].

To continue the theme of subunit organisation of TatA within the lipid bilayer, a site-directed spin labelling study shed light on how the TMHs of TatA are arranged in the membrane [27]. As already mentioned, single-molecule electron microscopy of detergent-solubilised TatA has shown TatA to exist as ring-shaped structures with variable diameters [90]. The largest of these complexes contained cavities that could accommodate large, folded Tat substrates, leading to models that proposed TatA complexes to provide the translocation pore in the assembling translocase. With the assumption that the TatA ring has a transmembrane orientation, it is likely that the TMH of TatA forms part of the ring wall. However, the extent to which the APH and C-terminal tail of TatA contribute to this wall structure as opposed to the cap is unclear. It also remains unknown as to how the TMH of this Tat component may pack together to form rings of variable diameter, nor do we know which residues within the TMH lie at the inter-helical binding surfaces [27].

White et al. hypothesise that the TMH of TatA are positioned side-by-side during pore formation. Their EPR study detected interactions between Ile112 on one side of each TMH and Val14 on the other, suggesting a Ille112-Val14 arrangement of helices for TatA during pore formation [27]. Of course, other regions of TatA could be positioned within the ring or on either side of the wall of TMHs.

However, the exact nature and functionality of TatA-type complexes was made more confusing by recent structural studies that showed the TatE component of *E. coli*, which can functionally substitute for TatA, to form complexes that are much smaller and more homogeneous than TatA complexes [93]. More specifically, the solubilised TatE forms ring-shaped complexes of 6–8 nm in diameter that are much too small to accommodate the larger Tat substrates like TorA that is 90 kDa. The small size of TatE complexes would appear to preclude this protein from possessing a pore-forming role, suggesting another mechanism of Tat translocation whereby TatABC contributes the bulk of the translocation channel and TatA/E activates it [93]. This is supported by data illustrating that the thylakoidal TatC is actively involved in the translocation event as opposed to solely being the primary binding site for the substrate protein [68].

Another possibility is that multiple TatA or TatE complexes bind to the TatABC complex once the substrate has bound, with flexibility in pore size arising from different number of TatA/E protomers [93]. This model is supported by the identification of multi-ringed structures of TatE i.e. modular interaction of smaller rings to produce a larger super-structure [93]. In agreement with the inferences from TatE electron microscopy data, ultrastructural characterisation of TatAd complexes from *B. subtilis* suggests that these too are smaller and more homogeneous than *E. coli* TatA complexes, with no indication of a pore that would be large enough to translocate large fully-folded Tat substrates. In this study, it was shown that TatAd, which can also substitute for TatA, forms a ring-shaped structure of ~7.5–9 nm in diameter, possessing a potential cavity or pore of 2.5–3 nm that is occluded at one end by a lid-like structure [51]. The authors speculate that this lid structure is comprised of the APH and C-terminal tails of TatAd since these are the most flexible regions of the protein. It is predicted that this lid serves to seal the translocation channel, maintaining the integrity of the membrane.

As for TatE, the small size of the pore has major implications on the mechanism of translocation and supports the above model of the coalescence of relatively homogeneous Tat(A)BC and multiple TatA or TatE complexes. It also questions the functional relevance of TatA heterogeneity in *E. coli*. Simply put, additional functional studies are required to understand the nature and role of TatA-type complexes with respect to the overall Tat mechanism.

4. Mechanism of translocation — summary and emerging ideas

There appears to be a general agreement that the substrate binds initially to TatBC (Hcf106-cpTatC in thylakoids), and this binding is independent of other Tat components [58]. Once the precursor is bound to the substrate-binding TatBC complex, the TatA complex associates with the TatBC complex in the presence of a ΔpH [66]. It is likely that the initial binding of the substrate requires only TatB and TatC, while the TatA is recruited once substrate binding has occurred. It is at this point that uncertainty starts in earnest: the active translocon has never been 'captured' in any sense apart from cross-linking studies and our understanding of the core translocation event is accordingly vague, but the recent structural data have led to plausible models for the mechanism of translocation (Fig. 5): the translocation pore model and the membrane destabilisation model [96].

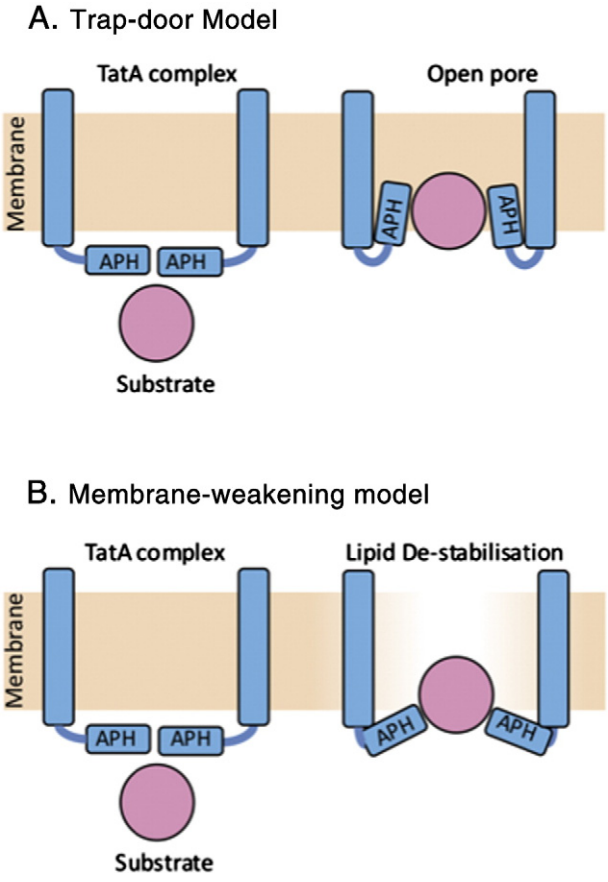

Fig. 5. Mechanism of Tat translocation. A substrate bearing a twin-arginine signal peptide is recognised by the TatBC complex in a resting membrane. The figure shows two proposed models for the subsequent translocation event involving the TatA complex. A. Trap-door model: the amphipathic helix (APH) of the TatA protein has dual topology. In a resting membrane the APH lies along the cytoplasmic face, to form a one-sided lid complex. Upon substrate recognition the APH flips into the bilayer, providing a tightly regulated pore for the translocation of the substrate in the presence of a membrane potential. B. Membrane-weakening model: The APH of TatA in the resting state aligns parallel to the cytoplasmic membrane. The recognition of a substrate induces a topological change in the APH so that it partially perturbs the lipid bilayer, leading to the de-stabilisation of the lipid bilayer. Subsequently, this permits the translocation of the substrate in a less regulated manner.

4.1. Trapdoor model

The "trapdoor mechanism" is based on the hypothesis that the topology of TatA has the ability of to flip its amphipathic helix (APH) domain into the membrane from its resting position along the cytoplasmic face of the membrane [97]. In *E. coli*, TatA is a highly abundant protein relative to TatB and TatC and its ability to form complexes of variable size suggested a possible role as translocation pore [46,98].

The idea of a "Trap door" gained prominence with the first 3D model of the TatA complex constructed by single particle electron microscopy. This study showed two main points: the presence of a cupped pore-like structure was generated and the presence of a range of complex sizes [46,90,99].

In a more recent study, Walther et al. explored the concept of a "Charge Zipper mechanism", portraying a simulated model where the C-terminus region of TatA permits a hairpin fold between the C-terminus and APH using complementary charge interactions [100]. This theoretical work is more inclined to the pore-forming model where the APH C-terminus hairpin flips into the membrane, to provide an internal hydrophilic coating of the pore.

In addition, the substrate itself has been shown to carry a motif which is capable of interacting with TatA [62]. This study suggests the binding conformation between the substrate and TatA is what is expected if TatA was to surround the substrate as if it were a pore. This suggests that TatA may be interacting with the substrate which then leads to a seeding of monomeric TatA to form a pore. In a recent review this substrate interaction and seeding process of TatA is interpreted as mode of pore formation using the PMF [61].

4.2. Membrane weakening model

The concept of membrane weakening was first presented by Bruser and Sanders, who hypothesised that TatA's role is not to form a pore but instead aggregate in an unordered manner, which would result in the formation of Tat complexes large enough to destabilise the membrane [101]. One reason for proposing an alternative mechanism is due to the relatively low abundance of Tha4 (TatA ortholog) found in the membrane of chloroplast, as opposed to the excessive level detected in *E. coli*. As a result, making it more difficult for Tha4 to bind to substrate and rapidly form a membrane-inserting structure round it.

As structural data on the Tat components has mounted over the recent years, this mode of transport is now considered more feasible. Walther et al. resolved the structure of TatAd using NMR, where the amphipathic helix was shown to partially incorporate into the membrane bilayer during a conformational change [31]. In contrast to the otherwise predicted flip topology in the pore model, these NMR studies suggest that the topology of TatA may not be as flexible during the conformation change as initially anticipated [31,97,102]. The swing of the APH from the cytoplasmic face into the bilayer would imply a 'lipid disrupting property' [31].

Another structural characterisation of the TatAd complex performed 3D reconstruction using single particle electron microscopy. Mirroring the results of Gohlke et al., this study also identified the one-sided lid structure in TatA [90]. However, the structural characterisation of this TatAd pore, suggests it may not be able to accommodate substrate [103]. This implies that the pore-like structure assembled from TatA may not actually function as a pore and instead function in membrane destabilisation or active translocon stabilisation after it has been recruited to the substrate-bound complex [104].

When membrane destabilisation is considered as a possible mode of mechanism it must be emphasised that the specificity of substrate transport is also compromised. Therefore for this mechanism to function correctly there must be a maintenance system in place to counteract the destabilisation and prevent leakage of ions. It was proposed that this is mediated by a phage shock protein PspA in *E. coli* and its homolog VIPPI in chloroplasts [105,106]. From the evidence of phylogenetic conservation and ability of TatA to interact with PspA, this method of membrane stabilisation during export could conceivably be a core element of this proposed membrane weakening model [107].

In summary, we are still a long way from understanding Tat. Structural advances have helped a great deal, but the transient nature of the elusive 'super-complex' has presented a huge barrier to more rapid progress. Studies on other protein translocases were revolutionised by experimental tricks to trap substrates within the translocation channel. Unfortunately, the Tat system has resolutely refused to play this game and substrates are either rapidly translocated or completely rejected. Nevertheless, real advances have been made in the last few years and some of the most essential pieces of the jigsaw are coming into place. Further efforts in this direction may enable us to catch this remarkable system *in flagrante* and understand its unique mechanism.

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