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Review

Transport and proofreading of proteins by the twin-arginine translocation (Tat) system in bacteria☆

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ABSTRACT

The twin-arginine translocation (Tat) system operates in plant thylakoid membranes and the plasma membranes of most free-living bacteria. In bacteria, it is responsible for the export of a number of proteins to the periplasm, outer membrane or growth medium, selecting substrates by virtue of cleavable N-terminal signal peptides that contain a key twin-arginine motif together with other determinants. Its most notable attribute is its ability to transport large folded proteins (even oligomeric proteins) across the tightly sealed plasma membrane. In Gram-negative bacteria, TatABC subunits appear to carry out all of the essential translocation functions in the form of two distinct complexes at steady state: a TatABC substrate-binding complex and separate TatA complex. Several studies favour a model in which these complexes transiently coalesce to generate the full translocase. Most Gram-positive organisms possess an even simpler "minimalist" Tat system which lacks a TatB component and contains, instead, a bifunctional TatA component. These Tat systems may involve the operation of a TatAC complex together with a separate TatA complex, although a radically different model for TatAC-type systems has also been proposed. While bacterial Tat systems appear to require the presence of only a few proteins for the actual translocation event, there is increasing evidence for the operation of ancillary components that carry out sophisticated "proofreading" activities. These activities ensure that redox proteins are only exported after full assembly of the cofactor, thereby avoiding the futile export of apo-forms. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

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1. Introduction

The twin-arginine translocation protein transport system, or Tat system, has attracted a great deal of interest in recent years. Most protein translocases transport their substrate proteins in an unfolded

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conformation, and there are good reasons for doing it this way; the translocation channel can be kept to a minimal diameter, and a variety of substrates can be transported using the same basic channel. In a sense, this broad type of mechanism offers a "one size fits all" advantage, although there is now good evidence that most protein translocases are rather flexible and able to adjust to the type of substrate being translocated. Few protein translocases are able to transport fully folded proteins and the Tat system appears to be unique in its ability to transport relatively massive folded proteins–even oligomeric proteins–across energy-transducing membranes. In this review we discuss recent studies on the structure and mechanism of this remarkable system. While much of the previous work has been carried out on the *Escherichia coli* Tat system, we have sought to highlight the properties of the Tat system from Gram-positive organisms in order to consider bacterial (and plant) Tat systems in a wider sense.

2. The Tat system subunits

The subunits of the Tat transport machinery in thylakoids and Gram-negative bacteria all share a high degree of sequence homology, and this has helped to identify the presence of Tat proteins in a wide range of bacteria [1–3]. The essential members of the Tat translocation apparatus in *E. coli* are the TatABC subunits, and their counterparts in plants are Tha4, Hcf106 and cpTatC respectively [1–6]. However, most Gram-positive bacteria contain only TatA and TatC-type proteins; these thus comprise a minimal Tat translocation system [7,8] that will be discussed in more detail below. Tat subunits are resident in the inner membrane of Gram-negative bacteria and their homologues in plants are located in the thylakoid membrane, where they mediate the transport of proteins into the lumen.

In *E. coli*, TatA and B are small proteins of 9.6 and 18.4 kDa, respectively, and each contains a single transmembrane span. The two subunits are homologous, and each is predicted to contain an N-terminal transmembrane (TM) helix followed by a short hinge region and an amphipathic helix ending in an unstructured and charged C-terminus [1,2,4]. Studies using circular dichroism (CD) and oriented CD (OCD) have largely confirmed the presence of these secondary structures, and there is now good evidence that the N-terminal TM α-helix does traverse the membrane with the amphipathic helix lying along the surface of the membrane [9,10]. Further studies using solid state NMR have shown the TM helix to be 14–16 residues long with a 17° tilt compared to normal in the membrane [11]. However, it should be pointed out that while most studies have proposed an N-out topology for TatA, this is not universally accepted and a study using thiol-reactive reagents concluded that the N terminus is in the cytoplasm rather than the previously assumed periplasm [12]. TatA and TatB share 25% sequence identity and the two subunits have a similar secondary structure; TatB is, however, significantly longer as it has an extended C-terminal domain and a key point is that the two subunits cannot substitute for each other, even when over-expressed [13].

The best-studied Tat system is that of *E. coli*, and there is clear evidence that the TatABC subunits carry out the key functions in protein translocation. However, this organism also contains a TatE subunit, encoded by a monocistronic gene that is outside the main *tatABC* operon. This subunit is thought to result from a cryptic gene duplication of *tatA* and the proteins share up to 50% amino acid sequence homology. Expression of the *tatE* gene partially complements *tatA* null mutants, but the TatE subunit is present at much lower levels than TatA and appears to be largely redundant [2,13].

TatC is the largest subunit of the Tat machinery (28.9 kDa in *E. coli*) and has been predicted to contain six TM domains from its primary sequence [14]. CD and OCD performed on TatCd (from *Bacillus subtilis*) showed a high helical content (~50%), with the helices apparently exhibiting significant tilt in the bilayer. However, it could not be determined whether this was due to the six helices lying at an angle in the membrane or, for example, because two of the helices lie on the surface of the membrane [15]. The latter case would signify the presence of only four TM helices (also suggested in a previous study [16]). Other work, using introduced cysteine residues along the length of TatC and membrane-permeant and non-permeant thiol reactive reagents, pointed to the presence of six TM helices [17], and this appears to be the most likely scenario at present.

None of the Tat subunits shares real sequence homology with other proteins in the database. The Tat system is thus unrelated to other known transport systems and this point, together with the apparently unique reaction mechanism (below) makes the Tat system both interesting and challenging. The basic structures of Tat subunits are shown in Fig. 1.

3. Structures of Tat complexes

3.1. Tat sub-complexes in Gram-negative bacteria

Many studies have focused on the structures and compositions of Tat complexes, and the results have been used to help build models for the Tat mechanism. In Gram-negative bacteria, two separate complexes are found at steady state: a TatABC complex and homo-oligomeric TatA complex [18,19]. This is a key point that has been instrumental in building the mechanistic models described below. The TatABC complex has a mass of about 370 kDa according to Blue-native electrophoresis studies [20,21] and within this complex, TatB and TatC form a functional unit, with the two subunits present in a 1:1 ratio [18]. The TatBC subunits are furthermore functional when translationally fused together [18]. Each subunit contacts other cognate TatB/C subunits in a larger complex, but there is also evidence that TatB and TatC form autonomous units within the TatABC complex [22]. Some TatA co-purifies with the TatBC subunits, and several Gram-negative bacteria are believed to contain a TatABC complex. TatB and TatC play important roles in substrate binding (see below) but the role of TatA in the complex is unclear, although the complex appears to be less stable in its absence [23,24]. The corresponding complex in plants (Hcf106-TatC) does not contain the TatA homologue (Tha4) suggesting that the TatA subunits in the bacterial TatABC complex may play a structural role, or perhaps a role in interaction

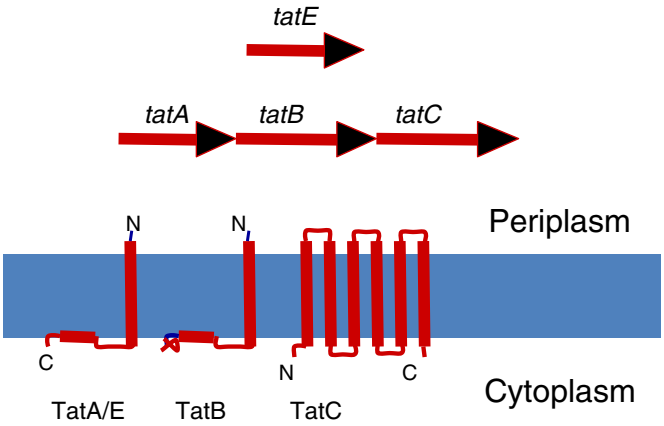

Fig. 1. Overall structure of subunits in the *E. coli* Tat system. Gram-negative bacteria usually contain 3-component Tat systems with TatABC subunits. In *E. coli*, these proteins are encoded by the *tatABC* operon as shown, but this organism also contains a TatA paralogue, TatE, which is encoded elsewhere in the genome. Most Gram-positive bacteria lack *tatB* genes and the TatA subunit is bifunctional. The diagram shows the proposed overall structure for the subunits of the TatABC system; the TatA and TatB proteins contain a single TM span, a very short N-terminal section in the periplasmic space and an amphipathic helix lying along the cytoplasmic face of the plasma membrane. TatC is believed to contain 6 TM spans with the N- and C-termini in the cytoplasm.

with the separate TatA complex. Importantly, the majority of TatA did not co-purify with the TatABC complex in the purification studies carried out to date [e.g. 18], and instead exists as separate complexes.

TatA complexes have very unusual properties: they are remarkably heterogeneous and range in size from 50 kDa to well over 500 kDa [20,21]. On Blue-native gels they run as a striking ladder of bands, with the "rungs" of the ladder differing by about 34 kDa. Given that *E. coli* TatA has a mass of 9.6 kDa, this has prompted the suggestion that the TatA is present in modules of 3 or 4 subunits [20,21]. Since the TatABC and TatA complexes show little or no propensity to associate after isolation, it is generally accepted that, at steady state, the Tat system comprises two distinct complexes, one containing TatABC and the other containing only TatA.

3.2. Tat complexes in Gram-positive organisms

With the exception of the actinomycetes [25] Gram-positive organisms contain only *tatAC* genes [7,8] indicating a fundamental difference when compared with Tat systems from Gram-negative bacteria and plants. To date, *B. subtilis* has been shown to contain two such AC-type Tat systems with differing substrate specificities (TatAdCd and TatAyCy) and a lone TatA subunit (TatAc), the function of which is not known [7]. Expression of the *B. subtilis tatAdCd* operon in an *E. coli tat* null mutant led to the presence of an active Tat system capable of transporting several Tat substrates. Analysis of the Tat complexes in the membrane showed the presence of a ~230 kDa TatAdCd complex and a separate, rather discrete ~270 kDa TatAd complex [8]. The TatAd protein was shown to be capable of complementing *E. coli tatA* and *tatB* null mutants, confirming that it is indeed truly bifunctional. In general, these results have interesting implications. First, the TatAdCd system was able to translocate a wide range of native *E. coli* substrates using two apparently stable, discrete complexes. The *E. coli* TatA complex is normally very heterogeneous indeed, and it was originally suggested that this may reflect the need to transport a wide variety of substrates. However, the *B. subtilis* TatAdCd data cast doubt on this possibility and suggest instead that a primary form of Tat translocon may be rather flexible in terms of accepting substrates of different sizes.

The above study also suggested that, despite the lacking of a TatB component in most Gram-positive organisms, Tat systems are generally conserved in nature, comprising a TatC-containing complex and a separate TatA complex. This overall structure has been confirmed in solution NMR studies on detergent-solubilised TatAd [26] although it should be noted that the protein was monomeric under the conditions used but is probably part of an oligomeric complex in reality. As stated earlier, TatC is usually predicted to contain 6 TM spans with the N- and C-termini on the cytoplasmic face of the membrane.

4. The Tat mechanism

4.1. The roles of the two Tat complexes

It is widely accepted that the Tat system transports large proteins in a folded form, but the actual mechanism is still poorly understood. Early work on the thylakoid system showed that the system is dependent on the thylakoid ΔpH but not nucleoside triphosphate hydrolysis [27–29]. These are unusual requirements; all other mainstream protein transporters rely on either ATP or GTP hydrolysis at some stage. Efficient *in vitro* assays have been developed using inverted *E. coli* membrane vesicles, and there again appears to be a requirement for the proton motive force [30]. It is thus possible that this is the sole driving force for the translocation by the Tat system—possibly indicating an antiport-type system in which proteins are exchanged for protons.

Several studies have used cross-linking techniques to specifically probe both the initial substrate-translocase interaction and the subsequent translocation process. Using isolated thylakoids, it was shown that substrates bind to Hcf106 and cpTatC subunits under energy-depleted conditions that prevented further translocation [5]. These are homologues of bacterial TatB and TatC subunits, respectively, and subsequent work using *E. coli* inverted membrane vesicles gave similar results in that the substrate pre-protein cross-linked to TatB and TatC [30]. A recent electron microscopic study into the TatBC complex has provided direct evidence that this complex represents the substrate binding site and how this binding occurs [31]. Overproduction of TatBC and the SufI substrate in the absence of TatA led to the formation of distinct TatBC-SufI complexes with one or two substrate molecules bound peripherally to TatBC. Interestingly, when two substrate molecules were seen to bind, they do so only at adjacent peripheral positions around TatBC, implying some structural or functional disparity between TatC protomers or negative cooperativity of substrate binding. The TatBC complex appears to lack the lidded channel shown for TatA (see below) and appear more roughly spherical with a small central cavity that does not alter size or shape upon substrate binding. A comparison with unliganded TatBC also revealed a slight reduction in the TatBC complex diameter upon substrate binding (~12 nm reduced to ~10 nm).

It therefore appears clear that the TatBC heterodimer contains at least one substrate-binding site (probably more), and this would appear to be one key function of the ~370 kDa TatABC complex discussed above. It also has been shown by cross-linking that Tha4 (the thylakoidal TatA homologue) was only found cross-linked to the Hcf106-cpTatC complex in the presence of both substrate and a ΔpH across the thylakoid membrane [32]. This finding provides evidence that the separate Tha4 complex is only recruited to the substrate-binding complex after binding of substrate to Hcf106-cpTatC in the presence of the proton motive force. Assuming that the same basic mechanism operates in Gram-negative bacteria, substrates would bind to the TatB and TatC components of the ~370 kDa TatABC complex, and this would trigger association of the separate TatA complex to generate the full translocon. In this model, TatA would form the bulk of the translocation pore in a transient "super-complex." This has become a widely accepted basic model for the Tat system, at least in Gram-negative bacteria and plants.

These striking TatA complexes have also been studied by electron microscopy [21] and shown to form channel-like complexes of variable diameter that correlate well with the modular organisation model described above. Using single particle EM in negative stain and a random conical tilt reconstruction strategy a number of electron density maps were produced showing a channel with a lid present on one side; the results of protease accessibility studies of the TatA complex in a membrane environment suggest this lid to be exposed to the cytoplasmic face [33]. These models ranged from 85 to 130 Å wide and 45 to 55 Å high, comparable to the thickness of a membrane bilayer. At 30–70 Å wide, the size range of the observed central pore matches well with the diameters of known *E. coli* Tat substrates [34,35], in support of the dominant–but still unproven–theory that TatA complexes represent the translocon channel unit.

4.2. The translocation event

While the above model for Tat operation has support from cross-linking data, Tat complex purification studies and electron microscopy, the essential details are unfortunately lacking. Most importantly, we have very little information on the proposed "super-complex"—it has never been isolated or characterised in a meaningful sense. TatA complexes do indeed have properties consistent with a role in channel formation, but no real data are available to confirm this. It has been shown [36] that Tha4 (the thylakoid TatA homologue) forms oligomers during transport by the Tat machinery and the data

suggested that this involved alignment of the amphipathic helices and C-terminal domains. On the basis of these data it was proposed that the aligned oligomers fold into the membrane, in concert, to allow passage of the translocating protein. If TatA does contribute to the channel, it seems clear that it does not act alone; it has been shown that a Tat passenger protein can be transported across the thylakoid membrane after crosslinking of the signal peptide to the TatBC complex [37]. This would imply that these subunits at least contribute to the translocation channel. These ideas are illustrated in Fig. 2, which outlines the basic steps in the overall translocation pathway for redox proteins. In the translocation step, it is envisaged that the amphipathic helices of the TatA subunits form an interlocked "basket" which accepts the substrate and allows it to be transported through the bilayer by virtue of the membrane-interactive properties of these helices. The structures of TatA-type subunits are, to an extent, consistent with this model, and Fig. 3 illustrates this point. The sequence alignment shows that the conserved residues are primarily found in the C-terminal region of the transmembrane span and a "hinge" region that contains a highly-conserved Phe–Gly pair in TatA subunits. This links to a predicted amphipathic helix (AMP in Fig. 3). The lower diagram depicts the TatAd subunit from *B.* subtilis and, on the basis of numerous mutagenesis studies [34,35] several key residues have been highlighted in black (these include the conserved Phe-Gly pair). Nevertheless, further work is required to define the steps involved and this model must be regarded as speculative at the present time. One of the long-standing problems has been that the thylakoid system is amenable to *in vitro* studies but very poor for genetics and structural studies, while the bacterial systems have diametrically opposite strengths and weaknesses and are particularly good for mutagenesis studies. However, advances in the use of bacterial membrane *in vitro* translocation assays [38] suggest that these systems may offer advantages in the longer term.

4.3. Tat operation in Gram-positive organisms

As outlined above, most Gram-positive bacteria lack a *tatB* gene and the TatB-type function appears, at least in *B. subtilis,* to be carried out by a bifunctional TatA subunit. The first identified Tat substrate in the Gram-positive *B. subtilis* was PhoD, a phosphodiesterase, and it has been shown [39–42] that the *tatAd* and *tatCd* genes, which colocalize with *phoD* in an operon, were essential and sufficient to export PhoD. In *B. subtilis* a second set of genes, *tatAy* and *tatCy,* encode a second TatAC translocase mediating the export of YwbN [7]. Two minimal Tat translocases are thus active in *B. subtilis*, each composed of specific TatA and TatC molecules. It is not clear why two are needed; the TatAdCd system appears to be used for export of PhoD in phosphate-starvation conditions, but it remains unclear why the 'housekeeping' TatAyCy system is not used [7].

Several studies point to similarities, both structural and functional, between the Tat systems of Gram-negative and -positive organisms. As outlined above, the TatAC subunits of *B. subtilis* are present in the form of two types of complex that are reminiscent of those found in Gram-negative bacteria. In these studies, it was proposed [7,8] that a membrane-bound TatAd subunit carries out both TatA-like and TatB-like roles, as both subunits are believed to be membrane-bound in *E. coli*. This scenario is more plausible in the light of a study [43] which selected for TatA mutants that were capable of carrying out a TatB function (in that they could complement *E. coli tatB* mutants). Most of the mutated TatA proteins contained only single substitutions near the N-terminus, providing clear evidence that TatA and TatB are similar in many respects. However, other studies have suggested that the Tat systems of Gram-positive organisms operate by a completely different mechanism. In the alternative model, TatAd acts as a soluble, cytoplasmic receptor that shuttles substrate to the cell membrane [44,45]. In one of these studies TatAd was analysed using negative stain and freeze-fracture EM. TatAd complexes of 150–250 kDa were purified in the absence of detergent by sucrose density gradient centrifugation and the electron micrographs revealed particles that were heterogeneous in both size and shape ranging from 12 nm to 100 nm in diameter. It is most likely that such micelle type structures represent newly synthesised TatAd on its way to the membrane. Freeze-fracture analysis of proteoliposomes revealed membrane-integrated TatAd complexes in the absence of TatCd, and immunogold labelled substrate prePhoD was used to show co-localisation to these complexes. From this it was inferred that TatAd represents the substrate recognition complex. It was further proposed that TatCd serves as a receptor for the soluble TatAd-prePhoD complex, stabilizing TatAd in the membrane and assisting the formation of the protein-conducting channel to mediate prePhoD transport [46]. Clearly, this model differs in fundamental respects from that proposed

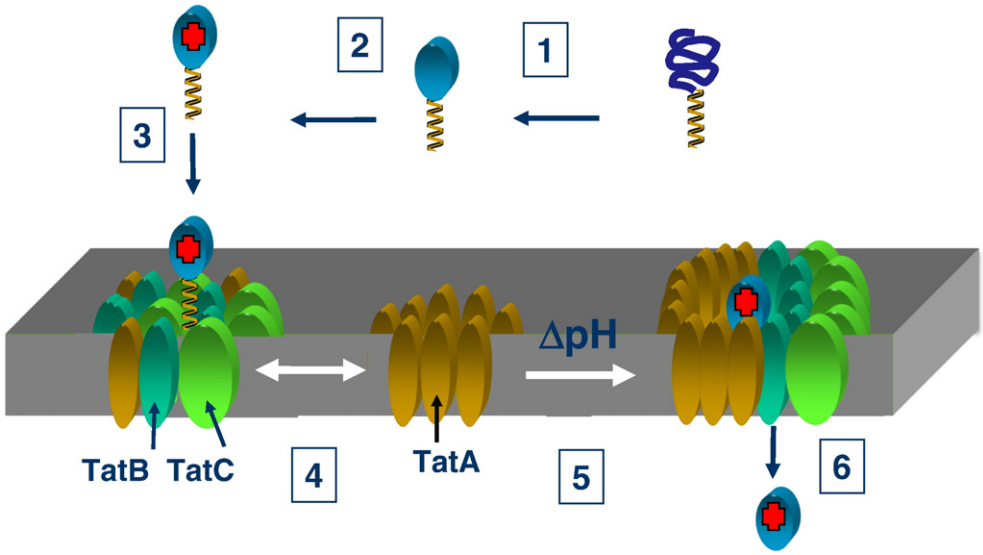

Fig. 2. Generalised model for the Tat-dependent export of cofactor-containing substrates. Tat substrates are exported post-translationally in a process mediated by N-terminal signal peptides (helix). After synthesis and initial folding (step 1), many bacterial Tat substrates acquire redox cofactors in the cytoplasm (step 2), often with the aid of chaperones and substrate-specific guidance factors (not shown). After this point, the substrate binds to a TatABC complex (in Gram-negative bacteria; step 3). This triggers association of the separate TatA complex (step 4) to form the active translocon, and the substrate is transported by a mechanism requiring the proton motive force (step 5), after which it is processed to the mature size (6).

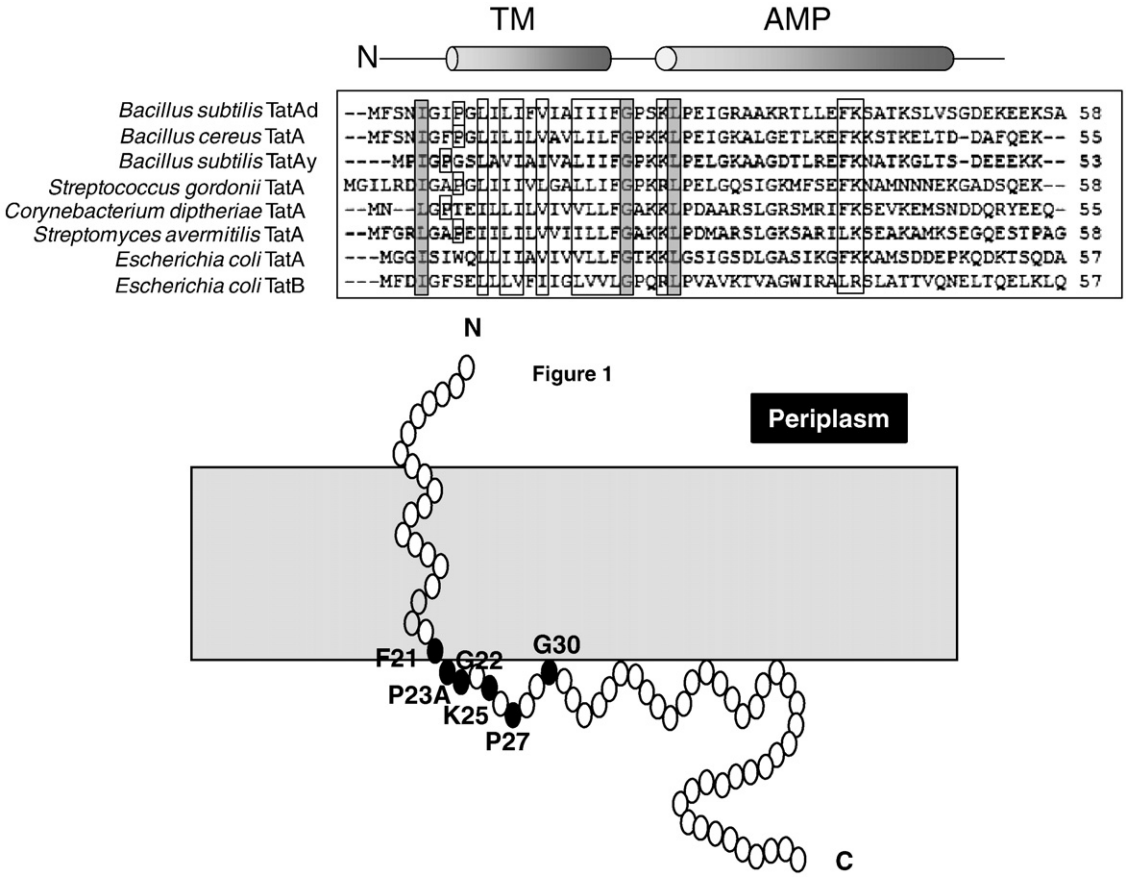

Fig. 3. Structure and proposed function of TatA. TatA subunits contain an N-terminal transmembrane span and a predicted amphipathic helix (APH), followed by a more unstructured C-terminal domain. Alignment of bacterial TatA sequences shows that conserved residues cluster in the C-terminal half of the TM span and an adjacent putative "hinge" region. These residues may be important for a concerted TatA-driven membrane-insertion process that has been proposed to involve the amphipathic helices. The lower diagram shows the overall structure for *B. subtilis* TatAd, with the key conserved residues shown in black.

for Tat operation in Gram-negative organisms and plant thylakoids, and further studies are required to clarify this area.

5. Tat signal peptides

Tat signal peptides have been reviewed in the past [34,35] but it is useful to consider them in the light of the different models mechanistic proposed (above) and their emerging role in proofreading activities (see below). Substrates for the Tat pathway bear N-terminal signal peptides that contain three distinct domains; a polar N-terminal domain, hydrophobic core domain and C-terminal domain terminating in an Ala–Xaa–Ala consensus motif specifying cleavage by signal peptidase. A twin-arginine motif is almost invariably present at the junction of the N- and C-domains of *E. coli* Tat signal peptides [34,35]. While the Sec-type signal peptides and the Tat substrates have the same basic three-domain structure and similar Ala–Xaa–Ala motif at the C-terminus, studies on Tat signal peptides have revealed a consensus SRRxFLK motif (where x is a polar amino acid) around the arginine motif [34,35]. Mutagenesis studies have shown that while the twin arginine motif is absolutely critical in chloroplast Tat signals (substitution of either Arg to Lys results in a complete block in translocation [47]) the twin-arginine is less critical in bacterial Tat signal peptides. In *E. coli* mutation of both arginines completely abolishes Tat-specific export but the conservative substitution of a single Arg usually affects only the rate of translocation [48–50]. Of the two arginines, the second appears to be more important [49]. The basic structure of bacterial Tat signal peptides is shown in Fig. 4, with several thylakoid Tat signal peptides shown for comparison.

The TatAdCd system from *B. subtilis* recognizes targeting determinants that are very similar to those characterized in *E. coli* system; signal peptide mutations have broadly similar effects on export by both the *E. coli* TatABC and the *B. subtilis* TatAdCd systems [51]. Thus, Tat signal peptides have a generally similar structure in both Gram-positive and Gram-negative bacteria. Three determinants appear to be particularly important, although there is evidence that their importance varies between signal peptides: a hydrophilic −1 residue, relative to the twin-arginine motif (typically serine, threonine, aspartate or asparagine), a conserved twin-arginine motif, in which at least one arginine is a requirement, and a subsequent hydrophobic determinant centred around the +2 residue and perhaps the +3 residue. These determinants appear equally important for export by the *E. coli* TatABC and *B. subtilis* TatAdCd systems [51]. The results of other studies have already pointed to the presence of important targeting information immediately after the RR-motif, and altering the overall hydrophobicity can also affect the export efficiency of Tat substrates *in vivo* [52]. In fact, hydrophobicity was shown to be potentially important in other respects: *E. coli* Tat signal peptides are, on average, less hydrophobic than Sec-type signal peptides and this appears to be functionally significant. Increasing the hydrophobicity of one Tat signal peptide enabled it to be recognised by the Sec machinery, suggesting that this may be an important sorting determinant to prevent mis-targeting by signal peptides that are otherwise rather similar. Perhaps surprisingly, this interesting study has not been followed up in detail.

While the essential targeting determinants in Tat signal peptides have been studied in some detail, more recent studies have shown

E. coli

HyaA MNNEETFYQAMRRQGV**TRR**S**FLK**YCSLAATSLGLGAGMAPKIAWA
FdnG MDVS**RR**Q**FFK**ICAGGMAGTTVAALGFAPKQALA
DmsA MKTKIPDAVLAAEV**SRR**G**LVK**TTAIGGLAMASSALTLPFSRIAHA
TorA MNNNDLFQA**SRR**R**FL**AQLGGLTVAGMLGPSLLTPRRATAAQA
SufI MSL**SRR**Q**FI**QASGIALCAGAVPLKASA

Consensus: S/TRRxFLK -------- H domain ----------AxA

B. subtilis

YwbN MSDEQKKPEQIH**RR**D**ILK**WGAMAGAAVAIG
PhoD VQKLKEESFQNNTFD**RR**K**FI**QGAGKIAGLSLGLTIAQSVGAFEVNA

Thylakoid

Sp OE23 AQ**K**QDDNEANVLNSGV**SRR**LALTVLIGAAAVGSKVSPADA
Sp OE16 **A**QQVSAEAET**SRR**AMLGFVAAGLASGSFVKAVLA
Ar PsbT TPSLEVKEQSST**TRR**DLMFTAAAAAVCSLAKVAMA

Consensus: S/TRRxL/V/F/M------- H domain ----------AxA

Fig. 4. Structures of Tat signal peptides. Bacterial Tat signal peptides contain three domains: an N-terminal domain containing a key twin-arginine motif, a central hydrophobic domain and a more polar C-terminal domain which ends with the consensus motif of Ala–Xaa–Ala (denoted A–X–A; specifies cleavage by signal peptidase after transport). The twin-arginine motif is often found in the midst of an S/TRRxFLK consensus motif, although the lysine is absent in many cases. A selection of *E. coli* Tat signal peptides is shown, with the consensus motif highlighted. The figure also shows the sequences of two Tat signal peptides from *B. subtilis,* a Gram-positive organism; the signal peptides are those of PhoD (exported by the TatAdCd system) and YwbN (exported by the separate TatAyCy system). Finally, the figure shows thylakoid Tat signal peptides from spinach (sp) OE23 and OE16, and from Arabidopsis PsbT. These signal peptides contain the invariant twin-arginine motif and preceding hydroxylated residue, but tend to lack the FLK motif found in Gram-negative bacteria.

that some of these signal peptides also carry out a second important function, namely interaction with proofreading machinery. This issue is considered below.

6. Proofreading and quality control of Tat substrates

6.1. A preference of the Tat translocon for folded substrates?

The Tat pathway's ability to translocate fully folded proteins is one of its most intriguing aspects, but this ability also poses real problems for the cell. The Tat system's best-known substrates are redox proteins that bind their cofactors *before* being exported to the periplasm. These cofactors include FeS, molybdopterin and NiFe centres (and others) and the key point is that these are inserted by components that operate only in the cytoplasm. Thus, the Tat system has to achieve the difficult task of exporting these proteins only after assembly is complete. This necessitates the operation of effective proofreading systems that prevent the futile export of apo-proteins.

Several studies have shown that the Tat system preferentially exports even heterologous proteins if they are in a correctly folded state. For example, Tat-mediated export of cytochrome *c* into the periplasm occurs only if maturation and folding in the cytoplasm are allowed [53] and disulfide bond-containing proteins, like PhoA and other multi-disulphide proteins, were only translocated by the Tat pathway if oxidative protein folding and disulfide bond formation could take place in the cytoplasm prior to export [54]. These results led the authors to conclude that the Tat translocase itself, proofreads its passenger proteins and is thus predisposed to recognize and export folded globular proteins. A more recent study has suggested that incorrectly folded precursors may reach the Tat translocon, but then fail to undergo full translocation [55] although these results require further exploration to determine how well such unfolded substrates are generally recognised. In a similar vein, recent work in *E. coli* showed that the Tat-dependent translocation of 29 eukaryotic proteins (that were fused to a Tat signal peptide) correlated, to an extent, with the molecular weight of these proteins, with smaller proteins exhibiting superior export efficiencies [56].

The above work suggests that the Tat system is predisposed to reject unfolded proteins and one possible mechanism for this has been suggested by mutagenesis studies in which the *E. coli* Tat pathway was shown to exclude unfolded polypeptides containing hydrophobic patches. In the same study, however, small, unfolded hydrophilic polypeptides fused to Tat signal sequences were translocated [57]. On the basis of these studies, it is possible that Tat substrates must mask hydrophobic domains to permit translocation. However, it should be noted that Tat-dependent export could only be achieved by overexpression of the TatABC components from a multicopy plasmid; without overexpression of the *tat* genes, transport was hardly detectable which suggests that these proteins, while translocated, may be relatively poor substrates.

Other mutagenesis studies also favour some form of quality control system operating at the Tat translocase. A Tat signal peptide-PhoA fusion was only successfully translocated into *E. coli* membrane vesicles via the TatABC translocase when presented to the translocase in a folded state [58]. Along similar lines, misfolded/misassembled FeS Tat substrate proteins were completely blocked for export [59]. Clearly, the issues of substrate proofreading/quality control are key aspects of the Tat field and there is much to do. Some of the general principles relating to proofreading have yet to be properly elucidated before we can understand what the Tat system needs to 'see' in a substrate for it to be transported.

There is also debate over the ability of the chloroplast Tat system to transport both folded and unfolded passenger proteins. A carboxyl-terminal truncation of the cpTat substrate OE23, and insertion of a proline into a helical region of the cpTat substrate OE17 were shown to block cpTat-dependent translocation [60], suggesting that the overall folding of the protein substrate is important for transport. However, the Tat translocase in chloroplasts is able to translocate unstructured peptides of 120 amino acids and certain precursors with

both folded and unstructured domains [61]. In general, the chloroplast Tat pathway is poorly characterised in this respect and the issue of substrate 'proofreading' has received little attention [62]. This may reflect the fact that very few chloroplast Tat substrates bind cofactors prior to translocation—perhaps there is less pressure to avoid any futile transport of incorrectly assembled proteins.

6.2. Tat-associated chaperones

While the Tat system appears to have general preferences in terms of substrate folding and properties, there is now good evidence that more complex and stringent quality control systems operate for some substrates. Some bacterial Tat-dependent redox proteins have specific cytosolic chaperones that associate with their signal peptides to assist in folding, cofactor insertion and oligomerization with partner proteins. These chaperones effectively act as sophisticated quality control sensors/check points, and they mediate the difficult task of slowing down export until the entire assembly process is complete. The genes encoding these cytosolic chaperones were termed REMPs (redox enzyme maturation proteins), and they are often found in the same operon as the gene encoding Tat substrate with which they interact [63,64]. Examples include the TorA, and NapA Tat substrates which are encoded by the *torCAD* and *napFDAGHBC* operons, respectively, where TorD and NapD are the REMPs aiding the maturation of these Tat substrates [65,66].

The *E. coli* DmsD protein was the first REMP to be described and is required for dimethyl sulphoxide (DMSO) reductase (DmsA) biogenesis prior to its export to the periplasm [67]. *E. coli* DMSO reductase is a membrane-bound molybdoenzyme which functions as a terminal reductase during anaerobic growth on various sulphoxide and N-oxide compounds [68]. The operon coding for the molybdoenzyme has been designated *dmsABC*. This molybdoenzyme consists of a periplasmic DmsAB complex and DmsC, which acts as a membrane anchor. The DmsA subunit binds a molybdopterin cofactor and has a Tat signal peptide, which targets DmsA in a complex with DmsB (in a so-called hitchhiker mechanism) to the Tat translocase [69]. This illustrates a remarkable property of the Tat pathway, namely the ability to export not only folded proteins, but pre-formed protein *complexes*, across the tightly sealed plasma membrane. In *E. coli*, DmsD is a REMP [67] that associates not only with the DmsA signal peptide but also to an extent with the TorA signal peptide. Furthermore, DmsD was found associated with the inner membrane in the presence of TatB and TatC, suggesting a possible role in Tat-dependent transport [70], although DmsD is not required for Tat-dependent export of simple fusion proteins bearing a DmsA signal peptide [71]. More recently, DmsD was shown to bind general chaperones (such as DnaK, DnaJ and GroEL) as well as proteins involved in the molybdenum cofactor biosynthesis pathway. The authors proposed that the DmsD chaperone escorts its substrate through a cascade of chaperone assisted protein-folding maturation events [72].

The *E. coli* TorD protein is possibly the most well characterized REMP and is required for the Tat substrate TorA (trimethylamine N-oxide reductase subunit A) maturation prior to export via the Tat pathway [65]. These proteins are encoded by the *torCAD* operon where TorA and TorC constitute the trimethylamine N-oxide (TMAO) reductase. The TorA protein contains a molybdopterin guanine dinucleotide (MGD) cofactor and TorD was shown to recognise the TorA signal peptide [65,73], protecting it against proteolysis [74] and aiding cofactor insertion [75]. Besides binding the TorA signal peptide, TorD was also found associated with both the mature part of the TorA protein [65] and with enzymes involved in the last step of MGD cofactor synthesis [76]. Moreover recent studies revealed an affinity of TorD for GTP that is enhanced by initial signal peptide binding. The authors suggested that GTP could govern the Tat signal peptide interactions with the TorD chaperone [77,78]. The interactions between REMPs and Tat signal peptides of known and predicted Tat-specific redox enzyme subunits were recently analysed. The study demonstrated that some REMPs are specific to a redox enzyme(s) of similar function, whereas others are less specific and able to interact with signal peptides of related enzymes [79].

While the specific chaperones discussed above generally bind the signal peptides of their target protein, in order to assist in the folding of the protein and insertion of cofactors, this is not the case for all Tat passenger proteins. It must therefore be considered that, for Tat passenger proteins which do not have specific chaperones, the general folding machinery of the cytosol plays a role. In one recent study the chaperones DnaK and SlyD were found to associate with several different Tat signal sequences. In the absence of DnaK or SlyD, other cytosolic chaperones bind in their place [80].

Overall, some of the above studies suggest that the chaperones interact with the signal peptide, triggering a cofactor-insertion, folding and assembly process. The interaction would simultaneously shield the signal peptide from the Tat translocase and prevent premature interaction. However, it should be pointed out that this type of general quality control mechanism is brought into question by the finding that unprotected Tat signal peptides are prone to proteolysis [18]. This might imply that chaperone binding simply stabilises the signal peptide while the passenger protein folds. In any event, the specific nature of at least some REMP–signal peptide interactions highlights the point that Tat signal peptides carry a variety of determinants that mediate interaction with both the translocon and any associated quality control systems. However, it should be pointed out that even though real advances have been made in understanding the proofreading/quality control of the Tat passenger proteins, at the present time it is still not clear how "decide" whether simple passenger proteins are correctly folded.

7. Concluding remarks

The Tat system continues to fascinate and frustrate in equal measure. Its abilities are now more impressive than ever and it is fairly well established that it can transport large, folded proteins (and even protein complexes) across energy-transducing membranes. It even appears to have an inbuilt ability to sense when globular proteins are indeed folded, and this may help to ensure that relatively simple globular substrates are not exported until ready. With more complex redox enzymes, the emerging evidence suggests that a general proofreading/quality control system does not operate, at least in the well-studied *E. coli* system. Instead, a range of substrate-specific systems are in place to ensure that each substrate is properly folded and assembled before export.

More frustratingly, the translocation mechanism continues to be an enigma. There is now widespread agreement that two Tat types of complex are present in the resting state, and there is some evidence that they coalesce at the point of translocation. However, we have little information beyond this point and this has to be a priority for future studies.

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