

Review

Twin-arginine-dependent translocation of folded proteins

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Twin-arginine translocation (Tat) denotes a protein transport pathway in bacteria, archaea and plant chloroplasts, which is specific for precursor proteins harbouring a characteristic twin-arginine pair in their signal sequences. Many Tat substrates receive cofactors and fold prior to translocation. For a subset of them, proofreading chaperones coordinate maturation and membrane-targeting. Tat translocases comprise two kinds of membrane proteins, a hexahelical TatC-type protein and one or two members of the single-spanning TatA protein family, called TatA and TatB. TatC- and TatA-type proteins form homo- and hetero-oligomeric complexes. The subunits of TatABC translocases are predominantly recovered from two separate complexes, a TatBC complex that might contain some TatA, and a homomeric TatA complex. TatB and TatC coordinately recognize twin-arginine signal peptides and accommodate them in membrane-embedded binding pockets. Advanced binding of the signal sequence to the Tat translocase requires the proton-motive force (PMF) across the membranes and might involve a first recruitment of TatA. When targeted in this manner, folded twin-arginine precursors induce homo-oligomerization of TatB and TatA. Ultimately, this leads to the formation of a transmembrane protein conduit that possibly consists of a pore-like TatA structure. The translocation step again is dependent on the PMF.

Keywords: TatA pore; TatBC receptor; TatA-type protein; TatC-type protein; proofreading chaperones; twin-arginine translocation (Tat)

1. INTRODUCTION

Tat translocases are found in bacteria, archaea and plant chloroplasts. They are distinguished from other protein translocation systems, like the Sec system, by their ability to translocate fully folded proteins across lipid bilayers. The most prominent representatives of bacterial Tat substrates are redox proteins involved in anaerobic respiration, proteins required for biogenesis and remodelling of the cell envelope, and virulence proteins [1–3]. Many of these Tat substrates acquire cofactors by complex cytosolic insertion processes; others are composed of several subunits, of which only one is equipped with a Tat-specific signal sequence. Obviously, these Tat substrates can target their Tat translocases only after they have adopted their final ternary or quaternary structure. For other Tat substrates that remain monomeric or do not contain any cofactor, such as for most Tat substrates directed to the thylakoids of chloroplasts, fast folding kinetics have been hypothesized to necessitate export by the Tat pathway. Owing to the folded state of their substrate proteins, Tat translocases are confronted with a large spectrum of substrate sizes and diameters.

The second remarkable characteristic of Tat protein transport systems is the signal sequence of their

substrate proteins. These signal sequences carry the consensus motif S-R-R-x-F-L-K with the name-giving arginine pair (twin-arginine (RR) precursors) [4]. Other than that, Tat signal sequences, in general, are cleavable and share the canonical tripartite structure with Sec-targeting signal peptides, i.e. an amino-terminal basic n-region, a central hydrophobic domain and a carboxy-terminal region ending with the recognition sequence for signal peptidase I.

Our current knowledge on how Tat translocases are structured and function is still rather limited owing to the scarcity of high-resolution structural information. Nevertheless, recent biochemical data have led to new insights that seem to allow a more detailed picture at least of the first stages of the Tat pathway. The main purpose of this review is to screen old and new facts for their common ground on which a model for membrane targeting of Tat substrates and the formation of a translocase structure can be built. After a brief compilation of the basic features of Tat signal sequences and Tat substrates, homologues of TatC and TatA are introduced as the building blocks of Tat translocases. Their propensity to form homo- and hetero-oligomeric complexes is discussed and, according to the available data, the Tat pathway is pictured in stepwise events. Further aspects, such as energetization of the transport and proofreading of substrates are viewed. Because of the multitude of data collected for the Tat translocation machinery of *Escherichia coli*, we will primarily focus on

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One contribution of 11 to a Theme Issue ‘Bacterial protein secretion comes of age’.

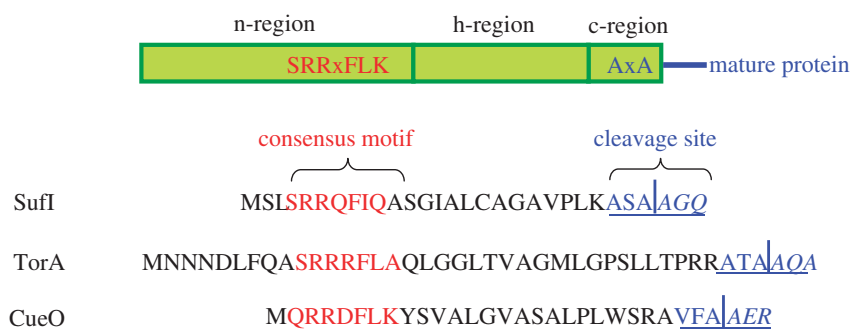


Figure 1. Tat signal sequences. Shown are the general composition of (top) Tat signal sequences and the amino acid sequences of three natural Tat precursor proteins of *E. coli*. SufI and TorA are the most common Tat substrates used for investigations of the Tat pathway and CueO is a copper oxidase, the RR-signal peptide of which comes closest to the consensus motif.

this organism but continuously discuss similarities and dissimilarities with the Tat systems of plant chloroplasts and also Gram-positive bacteria. We refer the interested reader to excellent reviews by our colleagues, each one with an individual main emphasis [1,3,5–7].

2. TAT SIGNAL PEPTIDES AND TAT SUBSTRATES

(a) Specific features of Tat signal sequences

As illustrated in figure 1, the consensus motif S-R-R-x-F-L-K (with x denoting a polar amino acid) is located at the distal end of the n-regions of Tat signal peptides. The RR-pair is almost invariant and a conservative substitution by a KK-pair, in general, abolishes translocation. There seem to be a few natural exceptions having one Arg replaced by a Lys, Asn or Gln [2,8–10] and corresponding mutations introduced into various Tat precursors are partially tolerated [11–14].

However, a mere RR-pair is not sufficient to direct a precursor to the Tat route [7]. Other residues of the extended Tat-specific consensus motif S-R-R-x-F-L-K (figure 1) are also important and accordingly are found conserved in more than 50 per cent of cases [15]. Mutations at these positions often affect the translocation efficiency of a particular substrate, but the effect can vary between different Tat signal sequences [12,16]. Mutational analysis of the consensus sequence further revealed that an increase in the overall hydrophobicity of the n-region can direct a Tat substrate to the Sec pathway [17].

The same effect has been described for increasing the hydrophobicity of the h-region of a Tat signal peptide [18]. A statistical analysis revealed that Tat signal peptides of Gram-negative bacteria are, in general, less hydrophobic than Sec signals owing to more Gly and fewer Leu residues [18].

Besides containing the recognition sequence for signal peptidase I [19,20], the c-region of Tat signal peptides is often enriched in basic residues when compared with classical Sec signal sequences [15,17]. In fact, these basic amino acids were shown to function as a Sec avoidance signal [21,22] and together with the overall charge of the adjacent N-terminus of the Tat client they constitute a Tat specificity determinant [23].

(b) Folding and other characteristics of Tat substrates

Using fusions between Tat signal peptides and alkaline phosphatase of *E. coli*, it could be demonstrated both

in vivo and *in vitro* that Tat-dependent export requires folding of the phosphatase moiety by disulphide-bridge formation [24,25]. Likewise, cofactor insertion into apoproteins proved to be a prerequisite for transport by the Tat translocase [14,26,27]. Attaining a folded state prior to translocation must also occur for hetero-oligomeric protein complexes, of which only a single subunit carries an RR-signal sequence such as in *E. coli* hydrogenases [28] and dimethyl sulphoxide (DMSO) reductase [29]. This mode of transport, in which one Tat substrate carries signal sequence-less partner proteins across the bacterial plasma membrane has been termed the hitchhiker mechanism [28]. In contrast, monomeric and cofactor-less proteins that use the Tat pathway might exhibit fast folding kinetics. Such a situation seems to prevail in halophilic *Archaea*, which in fact export the majority of their secreted proteins via the Tat route [30,31]. High salinity seems to determine the need for Tat-dependent secretion also in the Gram-positive bacterium *Bacillus subtilis* [32].

Most Tat substrates are soluble proteins released after membrane passage, but some remain anchored by either N- or C-terminal transmembrane (TM) domains [33–35]. The number of Tat substrates varies quite dramatically between different organisms and species [3,6,7,31]. Of particular importance are Tat-dependent virulence factors of some pathogenic bacteria because they render the Tat system a potential drug target. Some extracellular Tat substrates involved in the formation and maintenance of the cell envelope are of interest, since their defective export in *tat* strains determines the *tat* phenotype. This is characterized by long, non-separated cell chains and outer membrane permeability defects.

3. THE COMPONENTS OF TAT TRANSLOCASES

(a) Homologues of TatC and TatA

Tat translocases comprise two kinds of membrane proteins, a polytopic TatC-type protein with six predicted TM domains and one or more single-spanning TatA-type proteins denoted TatA, TatB or TatE (figure 2a,c). For a detailed analysis of sequence conservation between TatA- and TatC-type proteins of bacteria, archaea and plant chloroplasts, the reader is referred to a comprehensive review by the Saier group [40].

Gram-negative bacteria require a TatA and a TatB protein, because they fulfil diverse functions in these organisms. The same holds true for plant chloroplast,

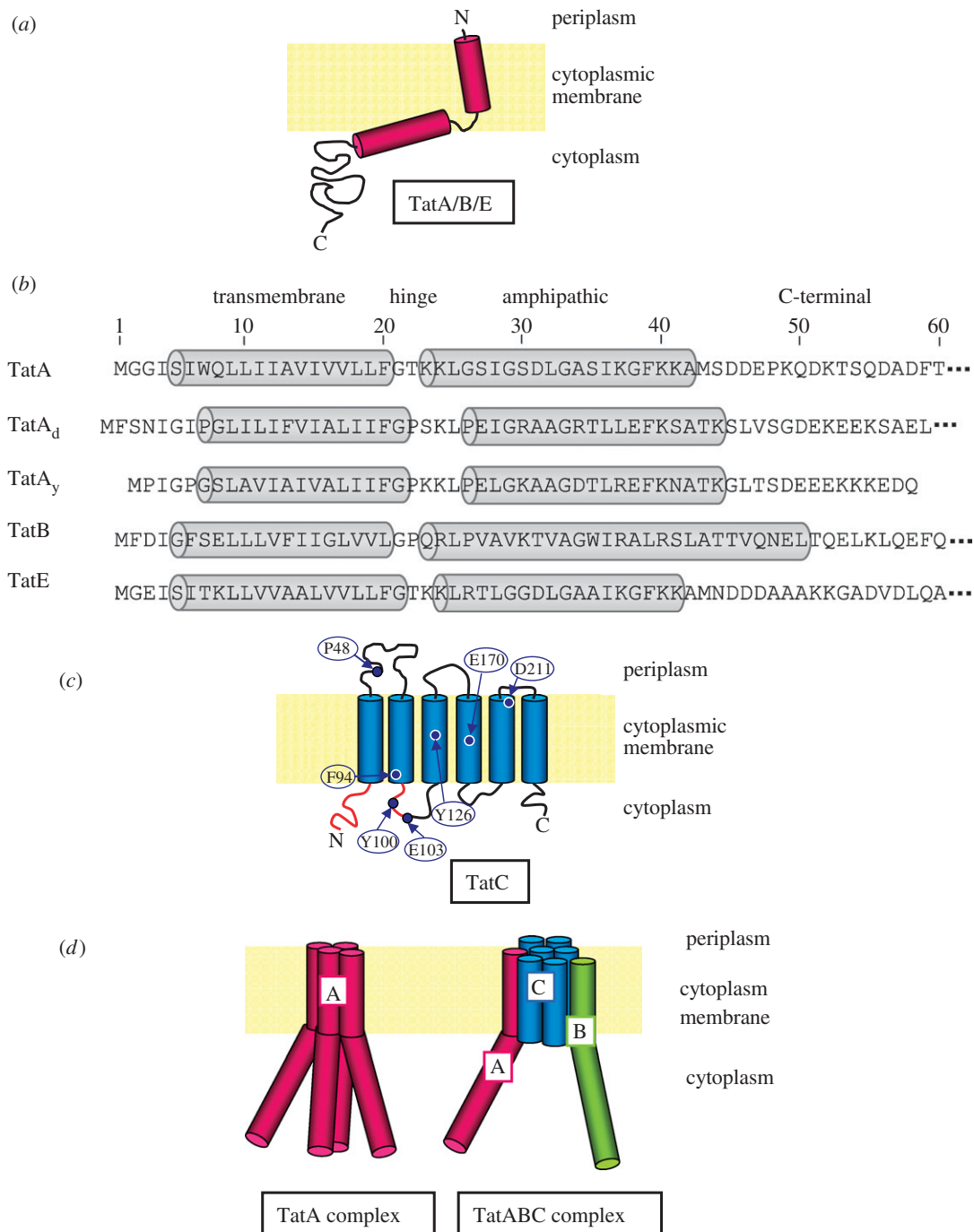


Figure 2. The Tat components. (a) The known representatives of the TatA-protein family, TatA, TatB, TatE are depicted according to a recent nuclear magnetic resonance structure solved for an N-terminal fragment of the *B. subtilis* paralogue TatA_d [36]. (b) Sequence alignment of the conserved N-terminal fragments of TatA, TatE and TatB from *E. coli* and TatA_d and TatA_y from *B. subtilis*. Numbering refers to the residues of *E. coli* TatA. The transmembrane and amphipathic helices and the intervening hinge region are indicated according to references [36–39]. (c) Predicted structure of TatC of *E. coli* with the approximate positions of residues that influence its activity and the putative substrate binding site highlighted in red. (d) The major Tat complexes recovered from TatABC translocases of Gram-negative bacteria and thylakoids of plant chloroplast. TatA forms homo-oligomeric complexes and is represented here by a tetramer. TatB and TatC form stable complexes independently of TatA. When purified from Gram-negative bacteria, not from chloroplasts, TatBC complexes often contain TatA.

where TatA, TatB, TatC are denominated as Tha4, Hcf106, cpTatC, respectively. In addition to the *tatABC* genes, Gram-negative bacteria often contain a *tatD* gene encoding a cytosolic protein, and *tatE*, which is regarded as a gene duplication of *tatA* expressing a functional paralogue of TatA. While *tatE* is monocistronic, *tatABCD* constitute an operon, the major transcript of which comprises *tatABC* [41]. Translational fusions to β -galactosidase revealed that in *E. coli*, TatA is

produced at a 20-fold higher level than TatB, at a 50-fold higher level than TatC and at a 100–200-fold higher level than TatE [42]. Although many *E. coli* Tat substrates are involved in anaerobic respiration, the Tat components are expressed constitutively [42].

In contrast, Gram-positive bacteria express orthologues of TatA and TatC only. The extensively studied *B. subtilis* expresses two paralogues of TatC—denoted TatC_d and TatC_y and three paralogues of

TatA—denoted TatA_d, TatA_y, TatA_c. The *tatA_dC_d* and *tatA_yC_y* genes are organized in operon-like arrangements. They code for two substrate-specific Tat translocases, in which the TatA proteins are bifunctional, replacing the individual TatA and TatB orthologues of Gram-negative bacteria [7,43]. Functional Tat translocases that consist only of TatA and TatC subunits are called minimal Tat translocases.

(b) *Tat* subunits

Homologues of TatA, TatB and TatC are essential components of the Tat translocases of Gram-negative bacteria and plant chloroplasts [44–46].

(i) *TatA*

TatA of *E. coli* consists of 89 amino acids (9.6 kDa). Its N-terminal TM helix (figure 2*b*) is linked via a hinge region to an amphipathic helix and an unstructured C-terminus. Recent solution [47,48] and solid-state [36] NMR structures of TatA orthologues confirmed the previously predicted two α -helices and their L-shaped arrangement in lipid environments that is stabilized by extensive inter-helical contacts. When reconstituted into planar bicelles, the only 14 amino acids long N-terminal TM domain of *B. subtilis* TatA_d was found to be tilted by 13° and deeply inserted in the lipid bilayer. This caused both the hinge region and even the proximal part of the amphipathic helix to be immersed in the lipid bilayer (figure 2*a*). The amphipathic helix showed a slanted alignment [36] rather than a flat orientation in the lipid–water interphase.

The topology of the TM helix of TatA has remained controversial. Studies probing the accessibility of TatA towards proteases [49] and oxidants [37] suggest a periplasmic location of the N-terminus of TatA. This would also be in line with the removal of an N-terminal extension from the TatA of *Providencia stuartii* requiring a membrane protease that is oriented towards the periplasm, as discussed in Palmer *et al.* [3]. On the contrary, using fusions of TatA to topological marker proteins [50] as well as cysteine-accessibility studies [51], an inverse orientation of the TM of TatA was reported. This leaves the possibility that, depending on its functional state, TatA might adopt dual topologies in the membrane.

Another point at issue is whether *in vivo* cytosolic forms of TatA might occur that represent functional intermediates. Particularly from Gram-positive bacteria, soluble TatA species have been isolated [52–56], and *E. coli* TatA upon overexpression was reported to form tube-like structures in the cytosol [57]. Notably, a soluble form of TatA present in the chloroplast stroma is functionally active [58].

Amino acid conservation between TatA orthologues is rather low and restricted to the TM and amphipathic helices [38] (figure 2*b*). Highly conserved residues of the *E. coli* TatA sequence are F20, G21 and F39 [38]. In agreement with the restricted conservation of TatA homologues, mutagenesis studies mapped residues that are important for the activity of *E. coli* TatA to the first 42 amino acids (table 1), in particular to the amphipathic helix [37,38,60,62]. Furthermore, truncation of the C-terminal 40 amino acids of *E. coli* TatA was found not to negatively affect its activity [69]. An F39A mutation completely blocks Tat-dependent

transport [61], but the mutant TatA assembles incorrectly in the membrane [70], whereas the K41A mutation destabilizes TatA [38]. Similarly important are residues F20 and G21 in the hinge region (figure 2*b*) [38,60] and Q8 at the proximal end of the TM helix, where a polar residue seems to be required for helix oligomerization [37]. The acidic D–D–E motif distal of the amphipathic helix of *E. coli* TatA is required for Tat activity and TatA subunit interactions [59].

(ii) *TatB*

Escherichia coli TatB consists of 171 amino acids with a molecular mass of 18.5 kDa. It shares about 20 per cent sequence identity with TatA [38] (figure 2*b*). Despite being structural homologues, TatA and TatB are functionally diverse proteins [44]. Highest homologies between prokaryotic orthologues of TatB are found in the predicted TM and amphipathic helices [38]. In accordance with this, the C-terminal part of *E. coli* TatB beyond the amphipathic helix was found not to be essential for its function [69]. No single mutation has been described that completely inactivates *E. coli* TatB [38,39,62,71] (table 1).

TatB is virtually essential for the transport of endogenous Tat substrates in *E. coli*. Nevertheless, artificial Tat substrates made by fusing the RR-signal sequence of *E. coli* TorA (TMAO reductase) to colicin V [13,72] or maltose-binding protein [73] are slightly exported in the absence of TatB. These findings suggest a low degree of TatB-like activity inherent to *E. coli* TatA. Such a situation prevails in Gram-positive bacteria that express minimal Tat translocases consisting only of TatA-like homologues and their cognate TatC partners. These organisms did not undergo the early gene duplication event of most Gram-negative bacteria that resulted in the functionally unique TatB paralogues [40]. The TatA homologues of Gram-positive bacteria therefore remained bifunctional. Bifunctionality can be restored to *E. coli* TatA by discrete mutations in the first N-terminal residues of TatA [73,74].

(iii) *TatC*

Escherichia coli TatC consists of 258 amino acids with a molecular mass of 28.9 kDa. TatC has six predicted TM (figure 2*c*), which was experimentally confirmed using reporter fusions [75,76] and thiol-labelling techniques [77].

Mutational analysis (table 1) of conserved residues did not always yield unambiguous results. P48A and F94A (figure 2*c*) were unanimously described as inactivating TatC [61,64,65,68], which in the case of P48A might be due to a destabilizing effect on the Tat(A)BC complex [70]. Y100A, Y126A and E170A substitutions show decreased activity [64]. E103A and D211A mutants, however, were found to be inactive in one study [64], but active in others [61], which might be due to different expression levels of the mutant proteins. The P48A, F94A, Y100A, E103A and Y126A mutations all interfere with binding of an RR-precursor protein [68].

(iv) *TatD*

TatD is a 29 kDa cytosolic protein [41]. *Escherichia coli* expresses two additional paralogues of TatD, and orthologues of TatD exist across all kingdoms, often independently of other Tat proteins. A triple deletion strain of

Table 1. Mutagenesis analysis of *E. coli* TatA, TatB and TatC.

amino acid	mutation to	conserved ^a	phenotypes	growth on		periplasmic TMAO activity	references
				SDS	TMAO		
<i>mutations in TatA</i>							
Gln 8	Ala	–	no influence on TatA oligomerization	n.d. ^b	n.d.	+++	[59]
	Cys		defective TatA oligomerization	n.d.	n.d.	–	[37]
Ile 12	Asn	+		±	n.d.	++	[60]
Leu 18	Pro	+		±	n.d.	++	[60]
Phe 20	Ala	+		+	+	++	[38,61]
Gly 21	Ala	+		+	+	+++	[38,60,61]
	Cys			n.d.	n.d.	–	[37]
Thr 22	Pro	+		+	+	+	[38]
	Ala			+	n.d.	++	[60]
Lys 23	Cys	+		n.d.	n.d.	–	[37]
Lys 24	Cys	+		n.d.	n.d.	–	[37]
Leu 25	Cys	+		n.d.	n.d.	–	[37]
	His			+	n.d.	++	[60]
Gly 29	Cys	+		n.d.	n.d.	–	[37]
Asp 31	Cys	+		n.d.	n.d.	–	[37]
Leu 32	Cys	+		n.d.	n.d.	–	[37]
Gly 33	Cys	+		n.d.	n.d.	–	[37]
	Asp			±	n.d.	++	[60]
	Ser			–	n.d.	++	[60]
	Ala		TorA-GFP transport blocked	n.d.	n.d.	++	[62]
Ile 36	Cys	+		n.d.	n.d.	–	[37]
	Thr			+	n.d.	++	[60]
Lys 37	Cys	+		n.d.	n.d.	–	[37]
Gly 38	Cys	+		n.d.	n.d.	–	[37]
	Ser			+	n.d.	++	[60]
Phe 39	Ala	+	dominant mutation	–	–	+	[38,61]
	Cys			n.d.	n.d.	–	[37]
	Leu			+	n.d.	++	[60]
Lys 40	Cys	+		n.d.	n.d.	–	[37]
Lys 41	Ala	+		+	+	+++	[38]
Ala 42	Cys	–		n.d.	n.d.	–	[37]
	Thr			+	n.d.	++	[60]
DDE 45,46,47	NNQ	+		n.d.	n.d.	++	[59]
DDE 45,46,47	LLM	+		n.d.	n.d.	–	[59]
<i>Mutations in TatB</i>							
Glu 8	Ala	+		+	+	+++	[38]
	Lys		restores transport of KQ-TorA-MalE	n.d.	n.d.	n.d.	[63]
Leu 20	Phe	+		+	+	+	[38]
Gly 21	Ala	+	phenotype dependent on expression level	+	+	+++	[38,61]
Pro 22	Leu	+	phenotype dependent on expression level	+	+	+	[38,61]
Leu 25	Ala	+		+	+	+++	[38]
Pro 26	Ala	+	phenotype dependent on expression level	+	+	+++	[38,61,62]
			TorA-GFP transport blocked				
Glu 49	Ala	+		+	+	+++	[38]
Glu 53+Glu 58	Ala	+		+	+	+++	[38]
Glu 49,53,58	Ala	+		+	+	+	[38]
<i>Mutations in TatC</i>							
Leu 9	Phe	+	restores transport of KQ-TorA-MalE	n.d.	n.d.	n.d.	[63]

(Continued.)

Table 1. (Continued.)

amino acid	mutation to	conserved ^a	phenotypes	growth on		periplasmic TMAO activity	references
				SDS	TMAO		
His 12	Ala	+		+	+	+	[64]
Leu 16	Ala	+		n.d.	+	+++	[65]
Arg 17	Ala	+		+	+	+	[61,64,65]
Lys 18	Lys			+	+	+++	[64]
	Glu	-	restores transport of KQ-TorA-MalE	n.d.	n.d.	n.d.	[63]
	Met		restores transport of KQ-TorA-MalE	n.d.	n.d.	n.d.	[63]
Leu 20	Ala	-		n.d.	+	+	[65]
Asn 22	Ile	-	restores transport of KQ-TorA-MalE	n.d.	n.d.	n.d.	[63]
Pro 48	Ala	+	TatBC complex assembly affected	n.d.	+	n.d.	[61,65]
Phe 94	Ala	+		-	-	+	[61,64,66]
	Leu			-	-	+	[64]
	Tyr			+	+	+++	[64]
	Ser		transports KK TorA-GFP but not RR TorA-GFP	n.d.	n.d.	n.d.	[67]
Pro 97	Ala	+		+	+	+	[64]
Leu 99	Ala	+		±	±	+	[64]
	Pro		transports KK TorA-GFP but not RR TorA-GFP	n.d.	n.d.	n.d.	[67]
Tyr 100	Ala	-		+	+	+	[64]
Glu 103	Ala	+	phenotype dependent on expression level	-	-	+	[61,64]
	Asp			-	-	+	[64]
	Gln			-	±	+	[64]
	Arg			-	-	+	[64]
Arg 104	Ala	+		n.d.	+	+++	[65]
Arg 105	Ala	+		n.d.	+	+++	[65]
Try 126	Ala	+		±	±	+	[64]
Pro 142	Ser	-	transports KQ- and RR TorA-substrates	n.d.	n.d.	n.d.	[67]
Tyr 154	Ser	+		n.d.	+	+++	[65]
Glu 170	Ala	+	no phenotype <i>in vitro</i>	+	+	+	[64,68]
Asp 211	Ala	+	phenotype dependent on expression level	+	-	+	[61,64,68]
	Glu			+	+	+++	[64]
	Asn		phenotype dependent on expression level	+	+	++	[64]
Gln 215	Ala	+		+	+	+	[61,64]

^aIdentical in at least 50% of TatAB [38] and TatC [64].

^bNo data.

E. coli lacking all TatD paralogues is not impaired in Tat-dependent protein export [41]. Although TatD of *E. coli* was reported to be required for the degradation of translocation-incompetent, misfolded Tat substrates *in vivo* [78], this effect was not seen under normal expression levels of the RR-precursor [79]. Consistent with its ubiquitous occurrence, TatD has frequently been characterized as a deoxyribonuclease with no obvious functional link to the Tat pathway [41,80,81].

(v) *TatE*

Escherichia coli TatE is a protein of 67 amino acids with 53 per cent sequence identity to TatA [45]

(figure 2b) that likely arose by a rather late gene duplication event [40]. TatE and TatA are functionally interchangeable, because either homologue can complement the *tat* phenotype of a *tatAtatE* double deletion strain [44] and neither protein is by itself essential [45]. TatE seems to be less important than TatA, because more Tat substrates are mislocalized in a Δ *tatA* mutant than in a Δ *tatE* strain [45]. On the other hand, a defective TatE strongly impairs the export of some Tat substrates [45]. These genetic findings would be consistent with TatE being an active member of the Tat translocase in *E. coli*.

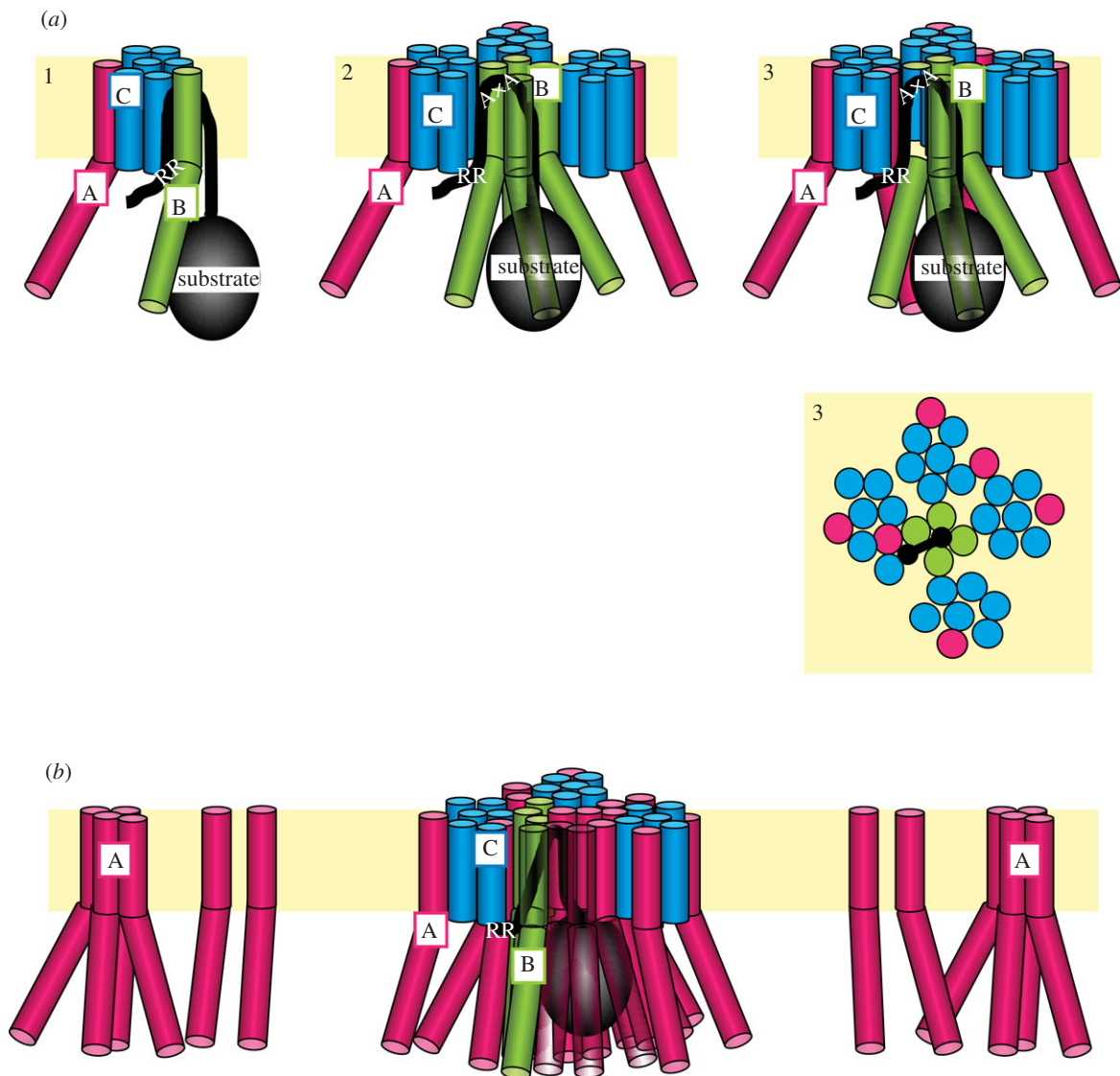


Figure 3. Model of a substrate-induced formation of a functional Tat translocase. (a) Step 1: superficial binding of a Tat signal sequence to the Tat translocase involves the RR-consensus motif and cytosolic parts of TatC. The coordinate recognition of a Tat signal sequence by TatC and TatB leads to its hairpin-like insertion into the plane of the membrane. Step 2: TatB homo-oligomerizes and encapsulates the folded mature domain of the substrate. Step 3: a proton motive-force-dependent step brings monomeric TatA in close contact to the signal sequence. AxA denotes the C-terminal end of the signal sequence. Step 3 is also shown as top view from the periplasmic side of the membrane. (b) More TatA subunits are recruited from a pool of monomeric TatA or more likely in tetrameric units. Processive oligomerization leads to the pavement of a transmembrane path. This might comprise oligomeric TatA pores, hetero-oligomeric TatA(BC) pores as drawn here or some other undefined TatA structure.

(c) Complex formation by Tat subunits

The subunits of Tat translocases have the remarkable property to homo-oligomerize. This has been vastly documented for TatA (see §3e) but was also described for the individually expressed *E. coli* TatB and TatC subunits [82,83]. When expressed together, the TatC and TatA family members also form hetero-oligomeric complexes both *in vitro* and *in vivo*. In *E. coli* and other Gram-negative bacteria, the TatA, TatB and TatC proteins associate to form TatABC and TatA complexes [84,85] (figure 2d). There are no data available as to the possible association of TatE with either complex. The orthologues Tha4 (TatA), Hcf106 (TatB) and cpTatC in the thylakoidal membrane of chloroplasts show a similar association pattern except that a TatBC complex free of TatA can be isolated [86]. To standardize nomenclature between organisms, we will refer to this complex as Tat(A)BC complex. In Gram-positive bacteria, the sole

TatA and TatC subunits can also be recovered from separate TatAC and TatA complexes [87]. Despite the fact that Tat subunits are consistently recovered from these Tat(A)BC and TatA complexes, doubt has been cast on the functional relevance of those isolatable structures. Thus in *E. coli*, TatA variants, which are able to functionally suppress a TatB deficiency, do so without forming complexes with TatC [74].

(d) Tat(A)BC complex, the site where Tat-dependent translocation starts?

The molecular masses of isolated Tat(A)BC complexes vary between 360 and 700 kDa [85,88–90] depending on the isolation procedure and also owing to some intrinsic heterogeneity [83,91]. Even in the absence of TatA, *E. coli* TatB and TatC associate into high molecular mass complexes [82,83,88] suggesting that TatB and

TatC form the core of the Tat(A)BC complex. TatB and TatC associate in a 1 : 1 stoichiometry and a translational fusion of both proteins retains activity [89]. When purified from TatABC-expressing *E. coli* cells, this core TatBC complex is usually found associated with TatA [84,85,88,89] and TatA was actually described to stabilize the TatBC complex [92].

In addition to forming hetero-oligomeric complexes, TatB and TatC have the property to self-interact. Homo-oligomeric assemblies of TatB ranging from dimers up to pentamers were detected by chemical cross-linking [93], disulphide mapping [39], bimolecular fluorescence complementation [94] and a two-hybrid approach [71]. Self-association occurs via both the TM and the amphipathic helix of TatB. Homomeric TatB complexes might not only represent resting states, but also have been characterized as multivalent attachment platforms for RR-precursors [95]. Likewise, interaction between two TatC monomers was revealed by cysteine cross-linking [77]. Furthermore, the finding that in *E. coli*, two inactivating mutations in *tatC* (F94A and E103A) can be cross-complemented by another *tatC* mutation (D211A) (figure 2c) suggested that TatC functions as a dimer [64]. This could in fact be confirmed by demonstrating that a genetically fused tandem TatC molecule retains activity, even if one of the fused TatC monomers carries the inactivating point mutation F94A [66]. Based on the above-mentioned findings that TatB and TatC form a 1:1 complex and that both have the tendency to homo-oligomerize, a functional TatBC core complex should consist of several TatBC dimers. From single-particle electron microscopy of isolated TatBC complexes that were devoid of TatA, a heptameric composition was estimated [96]. An oligomeric array of TatBC dimers is also strongly supported by the finding that multiple precursor molecules bind to a single TatBC-receptor complex [96,97]. It is further suggested by the cooperative-binding behaviour described for a chloroplast RR-precursor [98]. Because of the self-association of TatB monomers, a model of the TatBC complex was put forward, according to which the TatB protomers would form the central core of the TatBC complex and the TatC protomers a peripheral ring [39] (figure 3a).

As already mentioned, TatBC complexes, when purified from Gram-negative bacteria, usually contain TatA protomers. A functional association of TatA with the TatBC complex has thus far not been addressed in much detail. Bimolecular fluorescence complementation suggests a close vicinity of TatA to TatC *in vivo* [94] and recent data from our own laboratory provide evidence for a functional targeting of *E. coli* RR-precursors not only to TatBC, but also to TatA [99].

The major function of the Tat(A)BC complex is to specifically recognize and bind to RR-precursor proteins. This receptor function was verified by manifold experimental strategies, such as (i) interference of precursor binding to the Tat translocase by antibodies directed against TatB and TatC [86], (ii) co-migration of precursor with a TatBC complex on BN-PAGE [91], (iii) co-purification of precursor, TatB, and TatC upon membrane solubilization [96,100], (iv) chemical and site-directed cross-linking [97,101,102], and

(v) identification of TatBC suppressor mutations of export-defective Tat signal peptides [63].

(e) *TatA complex, a protein-conducting structure?*

The size of isolated TatA complexes varies between about 100 and 700 kDa [70,85,100,103]. Homo-oligomers of TatA clearly exist *in vivo* as shown by cysteine cross-linking [37] and fluorescent labelling techniques [94,104], even at wild-type expression levels [104]. Oligomerization of TatA occurs via its TM [49,59,93,105,106] and amphipathic domains [37,105], a conserved acidic motif (DDE in *E. coli*, cf. figure 2b) following the amphipathic helix [59], and the C-terminus (shown for Tha4 of chloroplasts) [105,106]. The finding that on Blue Native-polyacrylamide gel electrophoresis (PAGE) purified TatA resolves into a ladder of bands with a step size of about 40 kDa corresponding to the size of a TatA tetramer [85,103], suggests that a minimal building block of TatA might consist of four protomers (figures 2d and 3b). TatA tetramers might then oligomerize to higher order assemblies [104] of various sizes. Whereas, the formation of TatA dimers and trimers was observed in the absence of TatB and TatC [93], higher order oligomerization usually requires the TatBC subunits [104–106]. Consistent with this, TatA complexes when purified from *E. coli* cells expressing high levels of Tat proteins also contain TatB [84,107]. TatA complexes are less stable when purified from plant chloroplasts [86] and a recent re-evaluation also indicated that the chloroplast thylakoids of *Arabidopsis thaliana* seem to contain the TatA-homologue Tha4 in a considerably smaller abundance over TatBC than bacterial organisms [108].

The fact that oligomerization of TatA also requires contact to substrates [105], could reflect the formation of size-fitting pores that allow TM passage of the folded and therefore differently sized Tat substrates. A pore-like structure of TatA complexes purified from *E. coli* was revealed by single-particle electron microscopy [103]. The size classes of the obtained particles varied between 130 and 390 kDa with estimated subunit numbers of 12–35 and channel diameters of 30–70 Å. This spectrum of diameters would well fit with the size range (20–70 Å) of known folded Tat substrates from bacteria [109]. The walls of the pore-like particles would consist of the TM helices of the TatA protomers, which in fact arrange in a ring-like order as observed by EPR spectroscopy of spin-labelled *E. coli* TatA variants [110]. TatA particles as seen in the electron microscope possess a lid-like density on their presumed cytosolic face, which Gohlke *et al.* suggested represented the amphipathic helices of the circularly arranged TatA protomers functioning as a gating device of the TatA pore [103]. The potential of the amphipathic helices of TatA to adopt different orientations was suggested by studies making use of fusions between TatA and topological markers [50,51] as well as by chemical cross-linking [105]. Following these results, the possibility of the TatA amphipathic helices to at least transiently flip into the membrane to line a hydrophilic pore has been proposed [50,51].

The concept of size-fitting pores that consist of an appropriate number of TatA monomers was recently

challenged by the finding that TatE, the functional paralogue of TatA in *E. coli*, also forms pore-like structures which, however, are considerably smaller than those of TatA [111].

4. THE TAT PATHWAY

(a) Recognition of an RR-signal sequence by TatBC and TatA

It is an open question whether Tat signal sequences depend on cytosolic chaperones for membranetargeting. On the one hand, RR-signal sequences are presumably unfolded and unstructured before they contact the membrane [112,113] and as such conceivably might need protection by chaperones. In fact, DnaK, Trigger factor, SlyD and other FK506-binding proteins were reported to interact with Tat signal sequences [114–116]. However, Tat-dependent export was not negatively affected in the absence of any of these chaperones with the notable exception of DnaK, knock-out of which completely abolished transport of the Tat substrate CueO from *E. coli* [115]. Since the chaperoning effect of DnaK was independent of the Tat signal sequence, it could, however, also be explained by a general stabilizing effect of DnaK on Tat substrates [117]. Finally, Tat-dependent translocation was observed in the *bona fide* absence of any cytosolic chaperone of *E. coli* [114].

An overwhelming wealth of results indicates the specific recognition of the RR-signal by the Tat(A)BC–receptor complex (see below). Nevertheless, RR-precursors were found to insert into protein-less lipid bilayers [118–120] and the conversion of membrane-targeted RR-precursors from a lipid-bound into Tat translocase-bound form was described [121]. Furthermore, a TatABC-independent binding of RR-precursors to membrane vesicles was observed using co-sedimentation and filtration assays [121,122], although in these studies interaction with the membrane lipids was not rigorously addressed. On the contrary, co-elution assays using Sepharose columns did not reveal binding of an RR-precursor to membrane vesicles of *E. coli* [25]. Moreover, in the presence of TatABC-deficient membrane vesicles, RR-signal sequences were found not to cross-link to a non-Tat membrane protein but to a soluble protein exactly as they did in the complete absence of membranes [68]. Thus it is an open question, if lipid-binding is a required step in membrane-targeting of all RR-precursors.

By several experimental approaches, the Tat(A)BC–receptor complex both of chloroplasts and bacteria was identified *in vivo* and *in vitro* as the specific binding site for RR-signal sequences [86,100,101]. Cross-linking studies revealed a close proximity between the consensus RR-motif and TatC [25,101,102]. Signal peptide–TatC cross-links are obtained even in the absence of TatB, whereas cross-linking to TatB strictly depends on the presence of TatC and is also quenched by high amounts of TatC [101]. TatC has therefore been regarded as the primary recognition site for RR-signal sequences. Consistent with such a notion, a fusion protein between RR-signal peptides and *E. coli* alkaline phosphatase (PhoA) was found to mistarget the Sec translocon in the absence of TatC [123]. Further support for TatC specifically recognizing the RR-motif comes from the

finding that KK-mutant signal peptides fail to cross-link to TatC [101,102] and do not associate with a TatBC complex resolved by Blue Native-PAGE [91]. In seeming contrast, KK- and other translocation-deficient variants of the RR-consensus motif were demonstrated to still physically interact with Tat translocases [25,98,100]. This discrepancy is explained by assuming that recognition of Tat signal sequences by TatB and TatC extends beyond the RR-pair. In fact, mutational analyses of residues flanking the RR-pair in the S-R-R-x-F-L-K consensus sequence clearly demonstrate that the Ser, Phe and Leu residues are involved in a productive interaction with the Tat translocase [16,124,125]. Moreover, the above-described finding that KK-signal sequences bind to the Tat(A)BC–receptor complex but fail to cross-link to TatC actually suggests that TatB constitutes another half of the precursor-binding site of Tat translocases (as further detailed in §4c). This assumption could experimentally be verified by the finding that an extragenic suppressor mutation restoring transport to a KQ-variant of an RR-precursor mapped to the Glu residue at position 8 of TatB [63]. Fully consistent with a concerted TatBC-binding site for RR-precursor proteins, Tat signal sequences were not only found cross-linked to TatC but also to TatB [25,101,102].

When site-specific cross-linking was performed to identify contacts between RR-precursors and TatB and TatC, a Tat signal sequence was also found to cross-link with TatA [101]. These cross-links disappeared when the TatABC-containing membrane vesicles had been treated with carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) to dissipate their proton-motive force (PMF) [101]. Recently, we obtained evidence that RR-precursors come into contact with the TM helix of TatA before the actual translocation starts. These contacts likely involve the Tat signal sequence after it has been inserted into a TatBC-binding pocket [99]. Such a scenario would invoke a close proximity between the TM parts of TatA and TatC (figure 3a), which was corroborated by site-specific photo cross-linking [99]. Importantly, the observed contacts between RR-precursors and the TatA TM domain were clearly impaired in the absence of a PMF, suggesting that if TatA in fact joins TatBC in recognizing the signal sequence, it might do so only subsequently to TatB and TatC.

(b) Superficial binding sites for RR-precursors

Five single alanine substitutions at residues P48, F94, Y100, E103 and Y126 of *E. coli* TatC that interfere with translocation both *in vivo* and *in vitro* [61,64, 65,68] were shown to impair membrane-targeting of RR-precursors [68]. These mutations are spread over the N-terminal half of TatC, including periplasmic, cytosolic and TM sites of the molecule (figure 2c). Therefore, they are less likely constituents of a linear-binding epitope but are rather involved in the formation of a sterical precursor-binding site of TatC. Indirect evidence as to the location of binding site(s) on TatC for Tat signal peptides came from the isolation of suppressor mutants in *tatC* that partially alleviate a transport block of KQ- and KK-precursor mutants. Suppressors were isolated that mapped to the predicted cytosolic N-terminus and the first cytosolic domain of TatC [63,67]. The interaction of these areas of TatC with

RR-precursor proteins was recently confirmed by an extensive cross-linker scanning of the *E. coli* TatC molecule [126]. Hence the available data suggest that TatC provides a superficially exposed precursor-binding site as highlighted in red in figure 2c. In full agreement with a superficial attachment site, binding of RR-precursors to the thylakoidal Tat translocase still render signal sequences [124] including their N-termini [127] accessible to proteases.

(c) *Advanced binding of an RR-signal sequence to the Tat translocase*

Whether a first superficial binding of an RR-precursor to cytosolic domains of TatC is immediately followed by a deeper insertion of the signal sequence into the plain of the membrane, is not exactly known. Evidence for a loop-like insertion of an RR-signal peptide into the lipid bilayer of the membrane that is solely executed by TatC was recently obtained [128]. In the following, we summarize the numerous data suggesting a loop-like topology of an RR-signal sequence deeply within a concerted TatBC-binding pocket and discuss the experimental evidence for an advanced step of interaction with the Tat translocase involving the PMF.

As mentioned in §4a, RR-signal peptides seem to be recognized by TatC and TatB in a concerted fashion. Thus, several single-site mutations in *tatC* were described that simultaneously affected cross-linking of RR-precursors to TatC and TatB [68]. Moreover, suppressors of the translocation defect of KQ-variant precursors were not only isolated in *tatC* (see §4a) but also in *tatB*, where they mapped to Glu8 near the *trans*-sided N-terminus of TatB [63]. The location of this mutation suggests that the RR-signal peptide extends far into the membrane. Consistent with a deep insertion of a Tat signal peptide into a TatBC-binding pocket, the Leu residue at position 9 of TatB next to Glu8 was found to strongly cross-link to the precursors of TorA and SufI [95]. Furthermore, the h-region of Tat signal peptides located distal to the RR-consensus motif also cross-links preferentially to TatB [25,101,102]. If such a deep insertion of an RR-precursor protein into a TatBC-binding pocket occurs in a loop-like fashion, also the stretch distal to the signal peptidase cleavage site might become accommodated next to one of the Tat subunits. This was described to be the case for the precursor of SufI and TatB [101,102]. Furthermore, a *tatC* suppressor mutant of a KK-variant precursor was isolated that mapped to Pro142 in a predicted periplasmic loop of TatC [67]. The location of this mutation would corroborate again the idea that *trans*-sided sections of TatB and TatC are involved in the formation of a binding pocket for Tat signal sequences. Finally, preventing the processing of RR-precursors, which *per se* does not interfere with translocation [129], results in the accumulation of a precursor deep inside the membrane [130].

Binding of RR-precursors to thylakoids [131] and *E. coli* inner membrane vesicles [25,132] does not depend on the PMF, nor is Tat signal sequence-dependent cross-linking to TatB and TatC affected when the PMF is dissipated [101]. Moreover, a TatC-mediated insertion of Tat signal sequences was observed

to occur in the absence of the PMF [128]. All together, these results suggest that insertion of Tat signal sequences into the TatBC-binding pocket does not require energy from the PMF. On the contrary, more advanced binding stages were described that are definitely dependent on the PMF. Thus, a protease-cleavage site that had been engineered into a thylakoid-bound RR-precursor was transferred from an accessible to a membrane-protected environment upon implementation of the PMF [124]. Furthermore, cross-linking of the signal peptide to TatA was observed only in the presence of the PMF [101] and recent data suggest that the TM helix of TatA is involved in this PMF-dependent interaction with the signal sequence [99]. In this context, the TM domain of the chloroplast TatA homologue Tha4 contains a conserved Glu residue, which could become protonated following the *trans*-sided accumulation of protons. This glutamate was shown to be essential for recruiting Tha4 to the TatBC-precursor complex [133]. Collectively, these results are compatible with a scenario in which RR-precursor proteins, after their primary binding to TatBC and accommodation in a TatBC-binding pocket, undergo a PMF-dependent advanced interaction with the Tat translocase. This advanced binding/insertion step potentially involves a first contact with TatA.

(d) *Multivalent binding of TatB to the folded moiety of membrane-targeted RR-precursors*

Binding of a whole RR-precursor molecule to the TatBC-receptor complex has been addressed in different studies and thereby revealed multivalent attachment sites. Single-particle electron microscopy of TatA-free TatBC complexes from *E. coli* produced *in vivo* and *in vitro* revealed about 10 nm oval-shaped particles, in which up to seven protomeric TatBC complexes could be fitted [96]. When these complexes were produced in the presence of a natural *E. coli* RR-precursor, one or two extra densities asymmetrically associated with the surface of the particles were obtained. Into each of these densities, a folded precursor molecule could be modelled. These results suggested that a folded precursor binds to discrete superficial binding sites on an oligomeric TatBC assembly and that a functional TatBC complex might accept more than one substrate at a time. Using chemical cross-linking Ma & Cline [97] recently reported that two or four precursor molecules bind to the chloroplast Tat translocase such that even covalently cross-linked precursor molecules were still efficiently translocated. The assembly of a functional Tat translocase might thus involve recruitment of multiple Tat subunits and substrate molecules. Probing the molecular environment of *E. coli* RR-precursors after their targeting to the membrane but prior to the actual translocation event unexpectedly revealed the close proximity of several TatB monomers to the folded domains of the precursors [95]. The authors interpreted these findings such that after binding of an RR-precursor to the Tat(A)BC-receptor complex, TatB assembles into an oligomeric structure capable of transiently accommodating folded precursor domains (figure 3a). Encapsulation would most likely be achieved by multiple amphipathic helices of TatB creating a structure that

might resemble the ones visualized by electron microscopy of isolated TatBC-precursor particles [96]. This multivalent binding of an RR-precursor to TatB occurs independently of the PMF [95] and would therefore precede the advanced, PMF-dependent interaction of the Tat signal peptide with TatA. Recruitment of several TatB monomers to a folded RR-precursor might also be the starting point for triggering further oligomerization of TatABC subunits, ultimately leading to a functional Tat translocase (figure 3a, step 2).

(e) Membrane translocation

It is not yet understood how the Tat translocase enables the translocation of folded substrates without compromising the ionic seal of the membrane. All models proposed are based on the assumption that TatA plays a major role in this process. As shown for the Tat translocases of chloroplasts, after binding of precursor proteins to the TatBC-receptor complex, TatA (Tha4) subunits are recruited in a PMF-dependent manner [105,134]. Both TatBC and precursor are required to induce oligomerization of TatA [104,105]. These findings would be consistent with TatA forming a protein-conducting channel on the periphery of the TatBC-receptor complex. Thus far, the strongest experimental support for such a scenario comes from the pore-like structures of variable diameters that had been visualized by single-particle imaging of homo-oligomeric TatA particles isolated from *E. coli* membranes [103]. As discussed in §3e, these and other studies [110] suggest that the TM helices of TatA line a protein-conducting channel. Depending on the number of TatA monomers, they could thus form pores with different diameters to adjust to differently sized Tat substrates. The size of a Tat substrate does, however, not seem to determine the number of recruited TatA protomers, because oligomerization of Tha4 is initiated merely by an RR-signal peptide [106,134]. Whether a cylinder made from the TM helices of TatA would surround an aqueous interior or instead be filled with membrane lipids is not known. An alternative view suggests that the amphipathic helices of TatA fold into the interior of the TM TatA ring in a trap-door-like manner, so that their hydrophilic faces would line the actual protein-conducting channel [6,37,51,105].

Thus far, no translocation intermediate could be generated that was trapped within a ring-like TatA structure, be it made from its TM or its amphipathic helices. A partially membrane-protected translocation intermediate of a folded RR-precursor was, however, described that was dependent on the presence of TatA and accumulated in the immediate vicinity of TatA [25]. In addition, when a membrane-targeted-folded RR-precursor of *E. coli* was probed for surface contacts by site-specific photo cross-linking, cross-links to TatA that required the PMF were in fact obtained [95]. However, a fusion protein consisting of an RR-precursor, an unstructured linker region and a globular C-terminal domain was found stuck in the thylakoidal membrane without contacting any Tat component [135]. This could, in principle, mean that Tat-dependent translocation does not proceed via pores made from the Tat subunits. Alternatively, pores might quickly disassemble in such a case of a halted translocation event. Since no experimental situation has so far been

established that would transiently arrest an RR-precursor in an oligomeric TatA structure, it is also not clear if the presumed pore would exclusively consist of TatA subunits. Because TatB seems to oligomerize around membrane-targeted RR-precursors [95], it cannot be excluded that TatB oligomers constitute a kind of nucleation centre for the pore formation. If so, TatB monomers may even remain part of the final pore. A lateral access of an RR-precursor from the TatBC subunits to the centre of a presumed TatA pore is invoked by the finding that complete translocation can occur when the signal sequence of an RR-precursor is covalently bound to TatC [102]. It is therefore conceivable that TatB and TatC are even integrated in a pore structure (figure 3b).

At variance with the pore-model, Brüser & Sanders suggested that the local accumulation of TatA monomers in the vicinity of the Tat(A)BC-receptor complex could serve to destabilize the ordered lipid bilayer structure, thereby allowing a direct passage of Tat substrates through the membrane lipids [136].

(f) Cleavage of the signal sequence and dissociation of the Tat complex

RR-signal sequences are cleaved by signal peptidase I [19,20]. It is not clear what the exact temporal relationship between translocation and precursor processing is. Obviously, a premature cleavage leading to the detachment of a translocation-competent Tat substrate from the Tat(A)BC-receptor complex would abolish translocation and therefore needs to be prevented. For the Tat system of plant chloroplasts, a situation was described in which a lack of signal sequence processing can lead to the accumulation of non-translocated precursor in the thylakoidal membrane [130]. We have recently obtained experimental evidence pointing to an important function of TatB in controlling a TatC-mediated access of the cleavage site of Tat signal peptides to the peptidase [128]. On the contrary, signal peptide processing is not a prerequisite for a complete Tat-dependent translocation. This has been repeatedly found *in vivo* and *in vitro* using mutant RR-precursors harbouring non-functional cleavage sites [25,129,137]. The finding that translocation can occur with a signal peptide covalently linked to TatC [102] could indicate that the processing step might be a rather late event only following complete translocation. Taking this idea one step further, one might speculate that the association of the Tat signal peptides with TatBC has to be maintained during the translocation process in order to keep the protein conduit structurally intact. Only after a complete translocation, would the cleavage site become fully accessible to the signal peptidase, and cleavage of the precursor would then initiate the dissociation of the Tat translocase. In fact, dissociation of the TatABC complex after translocation has been documented for the chloroplast Tat translocase [134]. As discussed in §4e, dissociation of the translocating apparatus would also explain why partially translocated RR-chimeras remaining stuck in the membrane were not found in the vicinity of the Tat subunits, even if they had their signal sequences cleaved off [135].

(g) Energetics

A hallmark of Tat systems is that they do not use nucleotide hydrolysis for energetization but solely the PMF consisting of the pH gradient (ΔpH) and the electrical potential ($\Delta\psi$) at the membrane [138]. The dependence of Tat-specific translocation on the PMF has frequently been demonstrated using compounds that dissipate the H^+ -gradient or the electrochemical potential. In addition, the phage-shock protein PspA that is somehow involved in maintaining the PMF under cellular stress conditions [139] was found to improve the efficiency of Tat translocation in bacteria [140,141].

At variance with the preceding statements, processing of natural and model Tat precursor proteins was shown to proceed independently of the PMF in transfected tobacco protoplasts [142]. Although these results do not exclude the possibility that the PMF is involved in completion of translocation, they would at least not be consistent with a PMF-dependent assembly of the Tat translocase. Furthermore, in the green alga *Chlamydomonas reinhardtii*, the Tat pathway was reported to be unaffected by clamping the ΔpH to zero [143]. This result could be explained by assuming that both components of the PMF, the ΔpH and the $\Delta\psi$, can contribute equally to the driving force of the Tat pathway as has been experimentally verified [144]. As a consequence of the equivalency of ΔpH and $\Delta\psi$, a coupling of H^+ -flow and protein transport would have to be postulated [145], which is in good accordance with previous studies by Theg's group [146,147].

With regard to the involvement of the PMF in individual steps of the Tat pathway, targeting and binding to the TatBC-binding pocket occur independently of the PMF. The same holds true for the oligomerization of TatB around a membrane-bound-folded RR-precursor. On the contrary, advanced binding of Tat signal peptides with the Tat translocase, which might encompass a first interaction with TatA and well precede oligomerization steps of TatA, does require the PMF (see §4c).

In a more recent *in vitro* study, Bageshwar & Musser [148] came to the conclusion that transport of an *E. coli* RR-precursor into membrane vesicles requires two $\Delta\psi$ -dependent steps, one that is necessary early and one later during transport.

5. PROOF-READING AND QUALITY CONTROL**(a) Handling of folded and unfolded substrates by Tat translocases**

As mentioned earlier, many Tat substrates can exit the cytosol only after they are completely folded, either because cofactor insertion is confined to the cytosol or because hetero-oligomeric substrates have to associate with a single Tat signal sequence-containing subunit. In contrast, the thylakoidal Tat system of plant chloroplasts has long been known also to accept unfolded proteins [149].

The question of whether or not Tat translocases discriminate between the folded and unfolded conformations of a single Tat substrate was experimentally addressed by the use of model Tat substrates. In one case, disulphide-containing proteins were equipped with Tat signal sequences and shown both *in vivo* and *in vitro* to be exported in a Tat-dependent manner

only when oxidizing conditions allowed disulphide-bridge formation [24,25,91]. Likewise, Tat-mediated export of an RR-signal sequence-containing variant of cytochrome *c* occurred only after incorporation of haem [26]. In addition, mutations and truncations of Tat substrates, even if they seem to have only a small impact on their conformation, have repeatedly been found to impair Tat-dependent transport both in bacteria and chloroplasts [137,150].

The major question arising from these findings is how an improperly folded Tat substrate is rejected by Tat translocases. Translocation-incompetent Tat substrates can still associate with Tat translocases [25,91,151]. Co-purification of an unfolded Tat substrate with the TatBC complex seems to suggest that neither of those Tat subunits possesses an intrinsic quality control function [91]. Furthermore, overexpression of this translocation-incompatible Tat substrate was found to be toxic for *E. coli* cells. This has nourished the idea that it is not the Tat translocase that discriminates against malformed substrates but that an efficient degradation system is required that clears Tat translocases of faulty substrates [91,136]. Degradation of incompletely assembled Fe/S proteins in *E. coli* was in fact demonstrated to occur, provided that they could interact with the Tat machinery [152].

In contrast, site-specific photo cross-linking revealed a perturbed interaction of the signal sequence of an incorrectly folded RR-precursor with TatB and TatC despite the fact that this precursor was physically associated with the TatABC translocase [25]. Thus, improperly folded Tat substrates do not seem to correctly insert into the TatBC-binding pocket, but it is not understood by which measure this is prevented.

Tat translocases of both chloroplasts and bacteria are capable of transporting unstructured peptides up to a certain length of about 100–120 amino acids, provided that they have a Tat signal peptide and do not contain hydrophobic residues [135,153]. Moreover, improperly folded Tat substrates that the *E. coli* Tat machinery does not transport, become partially tolerated when they are C-terminally shortened [137]. Why unstructured polypeptides cannot exceed a certain length in order to be Tat-dependently transported is not clear. A likely parameter is the total surface area of an RR-precursor that, when exceeding certain limits, seems to interfere with Tat-dependent translocation [135].

A yet totally speculative idea as to how a Tat-incompatible structure could be recognized on a molecular level, is that oligomerization of TatB following precursor binding (see §4d) would require correctly folded domains. Such a scenario seems to be at odds with the observation that even a translocation-incompetent substrate shows surface contacts to TatB [95], unless one assumes that in this particular case, the Tat substrate retained some tertiary structure [137].

(b) Proofreading of cofactor-containing Tat substrates

Several Tat-dependent redox proteins of bacteria undergo cofactor insertion, folding and even hetero-oligomerization in the cytosol before they become export-competent by the Tat machinery. Because

specific chaperones (also termed redox enzyme maturation protein (REMP)) are often involved in these maturation steps, these chaperones serve as proof-reading checkpoints that are independent of the TatABC proteins (reviewed in [3,154,155]).

In *E. coli*, the best-characterized proof-reading chaperones are TorD, DmsD, HyaE, HybE and NapD, which are specific for the substrates TMAO reductase (TorA), DmsA subunit of DMSO reductase, HyaA subunit of hydrogenase-1, HybO subunit of hydrogenase-2 and NapA subunit of nitrate reductase, respectively. These chaperones, which often dimerize [156], bind to the signal sequences of their cognate substrates [157–161]. A promiscuous interaction was shown for DmsD and the signal peptide of TorA [159], which might be due to some sequence homology between DmsD and TorD [162], but less-specific interactions between REMPs and redox partners were also reported [163]. In addition to the signal sequence, TorD has a second-binding site on the mature domain of its substrate TorA [157], by which it could assist in cofactor insertion. The finding that TorD is a GTP-binding protein and that its affinity is enhanced by the TorA signal peptide suggests that the chaperone–substrate interaction might be regulated by GTP-hydrolysis [154,164]. Importantly, Jack *et al.* demonstrated that the chaperones TorD and HybE prevent Tat-specific premature targeting and translocation of non-matured substrates [157]. Thus only after complete maturation, would these proof-reading chaperones be released from the RR-signal sequences to allow for a subsequent targeting to the Tat translocase.

6. PERSPECTIVES

Although many details recently became manifest of how RR-signal peptides are recognized by Tat translocases, the exact structure and the composition of the signal sequence-binding sites remain to be revealed. In particular, the individual contributions of the TatABC subunits to the binding and insertion of RR-signal sequences that likely occurs in a stepwise manner need to be further substantiated. Undoubtedly, the major challenge for future studies is elucidating the structure, composition and functioning of a leak-proof protein conduit across the membrane. Information relating to the mode of translocation should also settle the issue of whether translocation is paralleled by a topology change of TatA proteins. If future progress should confirm the model of a Tat pore, the mechanism of its assembly from the participating Tat subunits would be a next step of investigation. A pivotal approach towards many of these objectives would be the generation of true translocation intermediates that freeze the molecular environment during TM passage. Understanding how the proton gradient is transduced into the formation of a protein-conducting structure or even directly into the transmembrane movement of Tat substrates will be a milestone in the investigation of the Tat apparatus. Another key question is the fate of translocation-incompetent Tat substrates, be they rejected or cleared from the Tat components. Obviously, any structural information, even on substructures or intermediate

assemblies of the Tat subunits, would pave a much more direct way to some of these exciting issues.

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