The Twin-Arginine Protein Translocation Pathway

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Abstract

The twin-arginine translocation (Tat) system, found in prokaryotes, chloroplasts, and some mitochondria, allows folded proteins to be moved across membranes. How this transport is achieved without significant ion leakage is an intriguing mechanistic question. Tat transport is mediated by complexes formed from small integral membrane proteins from just two protein families. Atomic-resolution structures have recently been determined for representatives of both these protein families, providing the first molecular-level glimpse of the Tat machinery. I review our current understanding of the mechanism of Tat transport in light of these new structural data.

Contents

INTRODUCTION

The twin-arginine translocation (Tat) pathway is a protein export system found in the cytoplasmic membrane of prokaryotes. It operates in parallel with the well-characterized Sec pathway (1), but whereas the Sec system transports proteins in an unstructured state, the Tat system has the specialized and unusual function of transporting proteins that have already folded. Substrates of the Tat pathway have an N-terminal signal peptide that is used to target the substrate protein to the Tat apparatus. These signal peptides contain a pair of adjacent arginine residues (the twin arginines), from which the Tat pathway derives its name. Tat translocation is an active transport process that is driven by the transmembrane proton motive force (PMF) (2, 3).

The Tat system is found in ~80% of the bacteria for which genome sequences are available (4) and is also found in many archaea. The Tat pathway plays a key biosynthetic role in a range of cellular processes, including photosynthetic and respiratory energy metabolism, iron and phosphate nutrition, resistance to heavy metals and antimicrobial peptides, cell separation, and symbiotic nitrogen fixation (reviewed in References 5 and 6). The Tat system is also normally required for the virulence of those bacterial pathogens in which it is present (reviewed in Reference 7).

The Tat system is evolutionarily conserved in plant chloroplasts as a thylakoid import pathway that is essential for formation of the photosynthetic apparatus (8, 9). A Tat pathway can also be found in the mitochondria of Jakobid protists (10). However, in the mitochondria of other protists, plants, and the Oscarellid sponges, the Tat pathway has been reduced to a single mitochondrially encoded Tat component (11), and the Tat system has been completely lost in the mitochondria of other animals and fungi.

The Tat pathway differs from almost all other protein translocating systems found in ion-impermeable membranes in transporting folded, rather than unfolded, proteins. Indeed, the only other currently identified examples of pathways that move folded proteins across such membranes are the protein import pathways of peroxisomes (12) and the type VII (or ESX) secretion system of gram-positive bacteria (13). The mechanism of transport in both cases is unclear, but neither pathway shows detectable similarity to the Tat system.

The key practical issue faced by the Tat system is that folded proteins are both larger and more variable in size than unfolded polypeptides. For example, known Tat substrates have minimal

diameters ranging from 25 to 70 Å (14), compared with the ~12-Å diameter of a peptide strand. This variation in substrate diameter makes it challenging for the Tat apparatus to provide a tight seal around the substrate during transport. In addition, the large size of folded proteins raises the question of how their correspondingly large transport pathway can be effectively sealed between transport steps.

Tat transport is accomplished by complexes containing small membrane proteins from just two structural families, TatA [possessing a single transmembrane helix (TMH)] and TatC (possessing six TMHs). Minimal Tat systems contain only one type of TatA protein and one type of TatC molecule. Such systems are typically found in the Firmicutes (low G + C gram-positive bacteria) and in archaea. However, most Tat systems, including the best-studied Tat systems found in *Escherichia coli* and plants, possess a second, functionally distinct member of the TatA family called TatB. Additional TatA paralogs may also be present. For example, *E. coli* possesses a third TatA family member called TatE, which appears to be functionally interchangeable with TatA but is present at a much lower concentration in the cell (15, 16).

High-resolution structures have recently been determined for representatives of both the TatA and TatC families, providing the first molecular-level view of the Tat machinery. This review describes these major advances and discusses our current understanding of the mechanism of Tat transport in the context of the new structural information. Other recent reviews of the Tat system include References 6, 9, 17, and 18.

OVERVIEW OF THE ORGANIZATION AND OPERATION OF THE TAT PATHWAY

Experiments carried out on isolated pea thylakoids or *E. coli* inner-membrane vesicles have determined the basic characteristics of the Tat translocation cycle. The Tat pathways in these experimental systems contain both TatA and TatB but are assumed to have the same general operating principles as Tat systems that contain only TatA. In thylakoids the TatA and TatB proteins are named Tha4 and Hcf106, respectively, but for simplicity I refer to them here as chloroplast TatA and TatB. Similarly, when referring to the compartments on either side of the membrane containing the Tat apparatus, I use the generic terms cytoplasm and periplasm, with the understanding that these correspond, respectively, to the stromal and lumenal compartments in chloroplasts and the cytoplasmic and extracellular compartments of gram-positive bacteria and archaea.

Tat transport is initiated when the signal peptide of a substrate protein is recognized and bound by a multisubunit TatBC complex located in the membrane (**Figure 1*a***) (19, 20). This binding event triggers the PMF-dependent recruitment and oligomerization of TatA protomers from a pool in the membrane to form the active TatABC-containing translocation site (20, 21). The dynamic oligomerization of TatA was initially inferred from cross-linking experiments (22, 23) but has now been directly visualized in living bacteria by use of fluorescent protein–tagged TatA (**Figure 1*b***) (24, 25). Transport of the folded passenger domain across the lipid bilayer is assumed to be mediated by the assembled TatA oligomer. Upon completion of transport, the substrate is released from the translocation site, the signal peptide is cleaved from the substrate mature domain by the enzyme signal peptidase (3, 26), and the translocation site disassembles (21). The order of these posttransport events, and whether they are interdependent, has not been established.

A currently unresolved issue is whether a small subset of TatA molecules are permanently associated with the TatBC complex, rather than participating in the substrate-induced recruitment cycles. Increasing evidence that this is the case comes from the observation that TatA copurifies with TatBC when *E. coli* Tat proteins are overproduced (27, 28), the detection of cross-linking

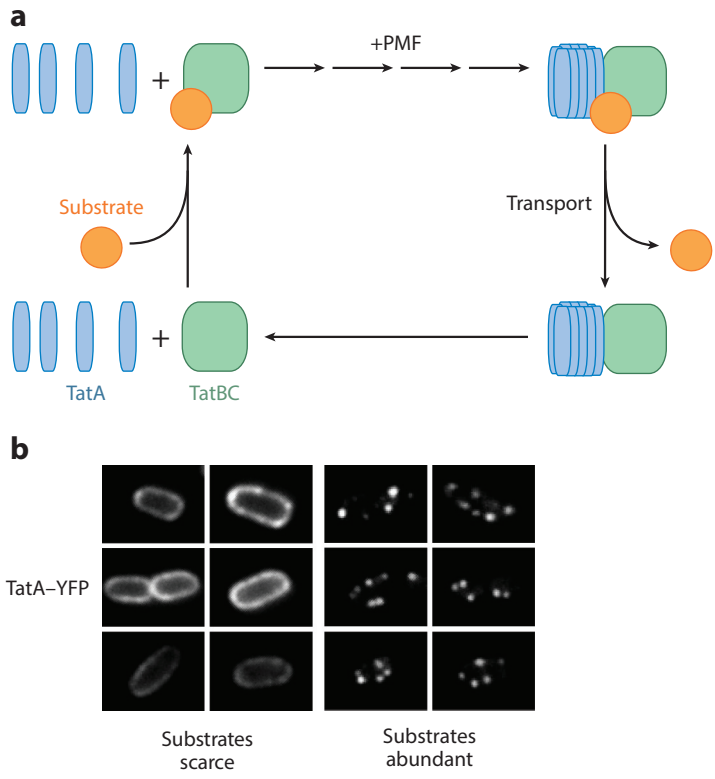


Figure 1

Tat transport involves changes in the oligomeric state of TatA. (*a*) Model for the Tat translocation cycle in organisms containing both TatA and TatB proteins. All Tat components are located within the membrane. The binding of substrate to a TatBC complex (*left*) triggers formation of the active translocation site through stepwise recruitment of TatA protomers. Following transport, the substrate protein is released from the Tat apparatus, which then disassembles. The number of steps shown in the TatA assembly process and the number of subunits in the assembled TatABC complex are arbitrary and were selected for illustrative convenience. (*b*) TatA assembly in live *Escherichia coli* cells. In these fluorescence images, the native TatA protein has been replaced with a TatA–YFP (yellow fluorescent protein) fusion (24). When substrates are scarce (*left*), few of the Tat components are actively involved in transport, and TatA is found predominantly in low-oligomeric-state protomers ($n \leq 4$ subunits). By contrast, if substrate protein is overproduced so that the Tat pathway is working at full capacity (*right*), TatA is found in large oligomers (mean $n \sim 25$ subunits). Abbreviation: PMF, proton motive force. Images courtesy of Felicity Alcock and Matthew Baker.

interactions between TatA and TatC in membranes under resting conditions (29, 30), and the inference that TatA is present in two functional environments (24). The mechanistic significance of such "substrate-independent" binding of TatA molecules to the TatBC complex is unclear.

TAT COMPONENT STRUCTURES

TatC

The structure of the TatC protein from the hyperthermophile *Aquifex aeolicus* has recently been determined by X-ray crystallography (31, 32). Essentially identical structures were obtained for the protein in three distinct crystal environments, suggesting that TatC has limited conformational

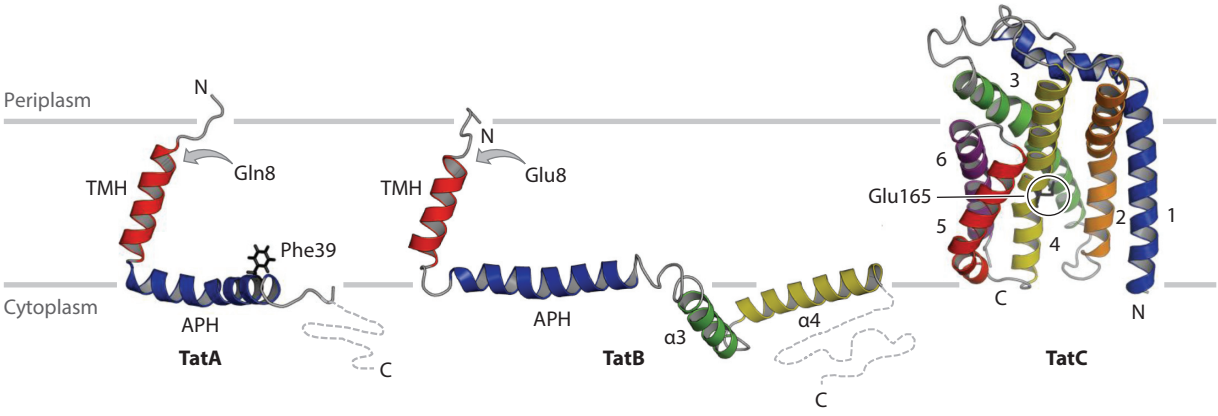


Figure 2

Structures of the Tat components. Solution NMR structures of the *Escherichia coli* TatA and TatB proteins (37, 39) and the highest-resolution X-ray structure of *Aquifex aeolicus* TatC (31) are shown as ribbons. The natively unstructured C-terminal tails of TatA and TatB are represented by dashed lines. The transmembrane helices (TMHs) and amphipathic helices (APHs) of TatA and TatB are indicated, together with the TMH numbering in TatC. Selected residues are depicted. The TatA and TatB TMH polar residue is on the far side of the helix in this view. Figure provided by Philip Stansfeld.

flexibility. TatC possesses a previously unreported fold in which the six TMHs create a structure that resembles a cupped hand or glove (**Figure 2**). The "palm" of the hand forms a cavity, which is overhung on the periplasmic side of the membrane by a rigid capping structure constructed from a surface helix and two parallel surface loops. The last two TMHs of TatC are too short to fully cross the membrane, resulting in a gap between the helix ends and the capping structure. This gap provides a potential route between the cavity interior and the periplasm. Molecular dynamics (MD) simulations suggest that very little of TatC is exposed outside the membrane bilayer (31, 32). The simulations also indicate that the membrane bilayer is distorted around the ends of the two short C-terminal helices, causing thinning of the bilayer.

A notable feature of the TatC structure is that a glutamate residue (Glu165 in *A. aeolicus* TatC) lies in the middle of the cavity, with its polar side chain exposed to the hydrophobic interior of the membrane bilayer (**Figure 2**). This residue is strictly conserved as either a glutamate or a glutamine in the TatC molecules of other organisms. Replacement of this amino acid significantly compromises Tat transport in *E. coli* (33, 34), although the equivalent substitution has only a limited effect on Tat-dependent import into pea thylakoids (35). MD simulations from one group suggest that the glutamate in *A. aeolicus* TatC is at the end of a water-filled cavity extending from the cytoplasm (32). In simulations performed by another group, the glutamate is permanently hydrated but forms only rare and transient aqueous connections with the cytoplasm (31). Although these studies vary in detail, both suggest that the glutamate creates a polar environment in the center of the bilayer and that this environment is at least occasionally contiguous with the cytoplasmic phase. Possible roles for this buried polar residue are considered below.

TatA Family Proteins

Solution NMR structures have now been obtained for each of the three subclasses of TatA proteins. The structure of *Bacillus subtilis* TatAd (*Bs*TatAd) (36) is representative of Tat systems that use a single type of TatA polypeptide, whereas the *E. coli* TatA (*Ec*TatA) (37, 38) and TatB (*Ec*TatB)

(39) structures provide models for systems possessing both TatA and TatB proteins. In each case, the protein was kept in a monomeric state for structural analysis by solubilization in the presence of high concentrations of detergent.

All three TatA family proteins have a similar core structure in which a short hydrophobic N-terminal helix is arranged approximately at right angles to an amphipathic helix (APH) (**Figure 2**). Solid-state NMR analysis (40) and MD simulations (37) suggest that the N-terminal hydrophobic helix has a transmembrane orientation, whereas the APH lies approximately along the membrane surface. The N-terminal TMH is too short to fully span the bilayer by itself, and both MD and solid-state studies suggest that this causes tilting of the TatA molecule that pulls the proximal end of the APH below the membrane surface. The junction between the TMH and the APH is composed of a short (three- to five-residue) loop containing an invariant glycine residue. The relative interhelix orientations are similar in all three structures and are defined by hydrophobic stacking interactions around the helix junction. These stacking interactions are unlikely to provide a significant energy barrier to rotation around the helix junction.

All TatA family molecules have a highly charged, natively unstructured C-terminal tail (36, 37, 41). Removal of the tail impedes, but does not abolish, Tat transport activity (42–44), and the tail is not required for substrate-induced oligomerization of TatA (22). Accessibility studies show that TatA family proteins are oriented in the membrane such that the APH and C-terminal tail are located at the cytoplasmic side of the membrane (45–49).

Almost all TatA family molecules contain a polar residue at the N-terminal end of the TMH (**Figure 2**). This residue is normally one of glutamate, glutamine, histidine, or lysine, and the residue at this position has been shown to be functionally important in chloroplast TatA, chloroplast TatB, and *Ec*TatA (44, 50, 51). Research on the thylakoid Tat system suggests that this residue is likely to be involved in mediating protein–protein interactions with other Tat components because it is essential for the interaction between TatB and TatC (50) and for the substrate-induced oligomerization of TatA onto the TatBC complex (22, 23, 29, 44). Nevertheless, it is worth noting that some TatA proteins, including *Bs*TatAd, lack this "conserved" polar residue and that substitution of the polar residue in *Ec*TatB is functionally tolerated (52). Thus, the "conserved" polar amino acid may not play a universally required role in Tat transport.

It has been difficult to define specific structural features that can be used to distinguish TatA proteins from TatB proteins. Indeed, the TatA and TatB classes are best discriminated by their biochemical behavior: TatB proteins form constitutive, equimolar complexes with TatC, whereas TatA proteins oligomerize and increase their association with TatC in response to substrate binding to TatC. The very close structural relationship between TatA and TatB is illustrated by the 50% sequence identity (to the end of the APH) between the two proteins in pea thylakoids, by the ability of a TatA protein from a Tat system lacking TatB to complement either *E. coli tatA* or *tatB* mutants (53), and by the selection of single–amino acid variants of *E. coli* TatA that enable Tat transport in a strain lacking TatB (54). The most plausible TatA-specific sequence feature is the presence of a conserved phenylalanine residue that is located at the C terminus of the APH and faces into the lipid bilayer (**Figure 2**). This residue is essential for TatA function in *E. coli* (24, 52, 55) but is not well conserved in TatB proteins. It is also known that different regions of the TatA family polypeptide are important for TatA- and TatB-specific function, although the exact structural requirements are unclear. Genetic studies have shown that the N-terminal tail before the TMH is important for TatB activity (54, 56), whereas in *E. coli* essentially the whole of the TatA APH is sensitive to amino acid substitutions (51, 55), but substitutions in the TatB APH have little effect on function (45). TatB proteins normally have a longer tail following the APH than do the TatA proteins from the same organism. In *E. coli* TatB, the additional polypeptide length is, in part, associated with two helices inserted between the APH and the natively unstructured

tail (**Figure 2**) (39). These helices have highly polar surfaces and are linked to one another, and to the APH, through flexible linkers. These features suggest that the C-tail helices are likely to be highly mobile and located in the cytoplasmic phase. Although deletion of the C-tail helices reduces the speed of Tat transport (42), they contain no conserved amino acids, and sequence analyses suggest that they are not a general feature of TatB proteins.

STRUCTURAL ORGANIZATION OF THE TAT COMPLEXES

Tat transport is catalyzed by a dynamically assembled complex of multiple TatA and TatC family proteins. Although the structures of the isolated TatA and TatC proteins are now available, elucidating the mechanism of Tat transport requires establishing how these proteins are put together in the presence of a substrate protein to form the transient Tat translocation site. This section reviews our knowledge of subunit organization in both the resting and substrate-activated Tat complexes and examines the extent to which we are now able to map interprotein contact sites onto the individual Tat subunit structures.

Substrate Recognition by the TatBC Complex

The functional affinity of the Tat system for native substrates is of the order of 100 nM (57–60). Substrates interact with the TatBC complex exclusively through their signal peptides (61). Two modes of signal peptide binding to the TatBC complex have been detected for the thylakoid Tat system (61). In the peripheral-binding mode, signal peptides have a weak, electrostatically driven interaction with the TatBC complex that leaves the signal peptide accessible to proteolysis from the stromal phase. In the deep-insertion mode, the signal peptide–receptor interactions are greatly strengthened and the signal peptide becomes buried in the TatBC complex and inaccessible to stromally located proteases. The PMF drives conversion from peripheral binding to the deep-insertion mode, although this requirement for the proton gradient can be bypassed by certain, empirically identified, nonnative substrates. Substrate binding to the *E. coli* TatBC complex has not been demonstrated to be influenced by the PMF. Nevertheless, both weak binding regimes (in which the substrate is exchangeable) (59, 62) and strong binding regimes (in which the substrate survives copurification with TatBC) (60) have been reported for the same Tat substrate. These two regimes may correspond to the peripheral- and deep-binding modes observed in thylakoids.

Tat signal peptides are normally at least 30 amino acids in length. They have a tripartite structure in which a polar n-region is followed by a hydrophobic h-region and then a second polar c-region bearing the signal peptidase recognition site (**Figure 3**). The n-region contains a consensus sequence motif that includes the twin-arginine residues. For bacterial proteins the consensus motif is Ser_1–Arg_2–Arg_3–Xaa_4–Phe_5–Leu_6–Lys_7, where the arginine residues are essentially invariant, *X*aa is a polar amino acid or glycine, and the other amino acids are present at frequencies exceeding 50% (63, 64). Within this motif, the arginine residues are normally essential for Tat targeting; Phe_5 is the next most important position (reviewed in Reference 5). A similar consensus sequence of Arg_2–Arg_3–Xaa_4–Hyd_5–Leu/Met_6, where Hyd is a hydrophobic amino acid, has been determined for pea thylakoid Tat signal peptides (65). Several programs are available to identify Tat signal peptides in prokaryotic proteins, including Tatfind (**http://signalfind.org/tatfind.html**), TatP (**http://www.cbs.dtu.dk/services/TatP/**), and PRED-TAT (**http://www.compgen.org/tools/PRED-TAT/**) (66–68).

The n-region of the signal peptide binds to a surface patch on the cytoplasmic face of TatC, which encompasses the N-terminal tail and the TM2–TM3 surface loop (**Figure 4**). This interaction surface was defined on the basis of sequence conservation (31), calorimetric binding studies

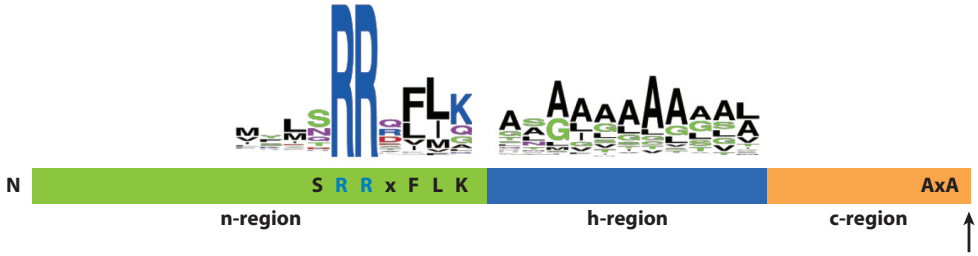


Figure 3

Organization of Tat signal peptides. The arrow indicates the site of signal peptide cleavage following substrate transport. A weblogo plot of sequence variation within the signal peptide is shown above the schematic. The weblogo was constructed from the Tat signal peptide sequences annotated in the UniProt database and then aligned against the signal peptide of the *Escherichia coli* Tat substrate SufI. Consensus sequences for the n-region Tat motif and the c-region signal peptidase recognition site are displayed on the signal peptide schematic. Modified with permission from Reference 17.

(31), site-specific cross-linking experiments (30, 35), and genetic evidence (69–71). An explicit model for the interactions at this contact site proposes that the twin-arginine residues of the signal peptide interact with two conserved glutamic acid residues on TatC, and that Phe_5 of the signal peptide consensus motif stacks with a conserved TatC phenylalanine (17). Ramasamy et al. (32) have suggested that the n-region-binding site may extend into the interior cavity of TatC, with the exposed polar glutamate or glutamine residue in the center of the cavity (introduced above in the section titled TatC) forming bonding interactions with Lys_7 at the end of the n-region Tat

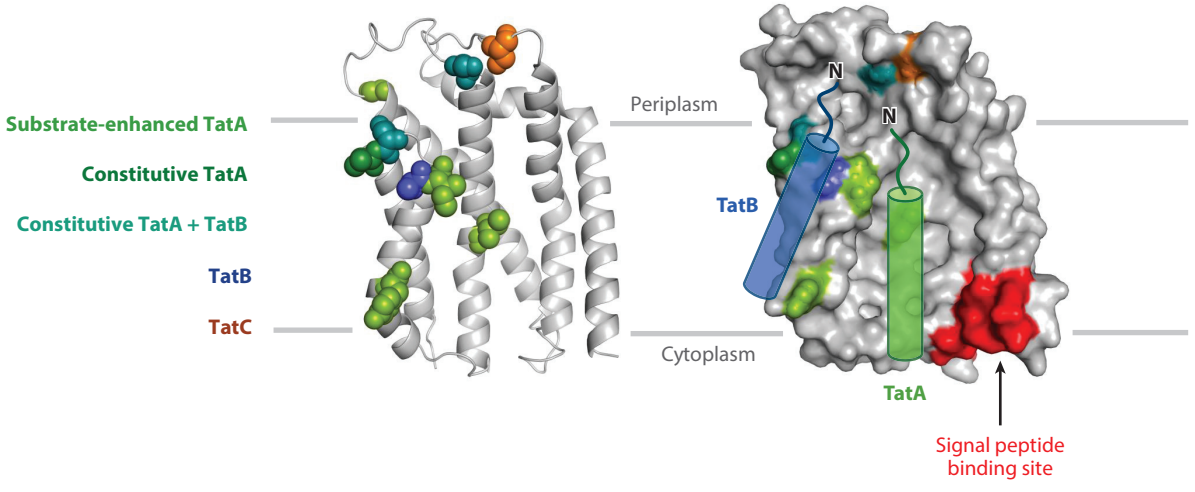


Figure 4

TatC contacts with the transmembrane helices of partner proteins and with the n-region of the substrate signal peptide. (*Left*) Cartoon representation of the *Aquifex aeolicus* TatC protein. (*Right*) Surface representation of the same protein. Positions in the transmembrane or periplasmic parts of TatC that cross-link to other Tat components are colored as indicated (data are from References 29, 30, and 82). Those TatA contacts that are significantly enhanced in the presence of both a substrate and a proton motive force are labeled substrate-enhanced. Contact sites for the TatA and TatB amphipathic helices on the cytoplasmic face of TatC are not shown as it is not clear that these represent defined binding sites. In the structure on the right, the inferred positions of the transmembrane helices of TatB (31) and of substrate-enhanced TatA (29) are indicated by cylinders. The signal peptide n-region-binding site defined in Reference 31 is colored red.

consensus motif. Binding of the n-region in this position is plausible. However, the proposed Lys_7 interaction is unlikely to be a crucial part of the Tat mechanism because many bacterial signal peptides, and all thylakoid signal peptides, lack a polar residue at consensus position 7. Furthermore, removal of the TatC cavity glutamate or glutamine residue has no detectable effect on substrate binding (34, 35).

The signal peptide n-region-binding site lies on the rim of the TatC central cavity. Therefore, it is logical to propose that in deep-insertion mode the remainder of the signal peptide is buried within this cavity (29, 32). Evidence in support of this idea comes from the observation that a disulfide bond can be generated between a position at the end of the signal peptide h-region and a residue on TatC TM5 at the periplasmic edge of the cavity (29). However, site-directed photoaffinity cross-linking studies indicate that the h-region of the signal peptide has minimal exposure to TatC and is, instead, adjacent to TatB (20, 72). The possibility that TatB lines the TatC central cavity has not been excluded. Nevertheless, current evidence is most consistent with the transmembrane portion of TatB being located outside the cavity (see the below section titled The TatBC Complex). It is conceivable that the TatB APH lies over the cytoplasmic entrance to the TatC cavity and that the observed cross-links to the signal peptide h-region are with this part of the TatB molecule.

Fröbel et al. (73) recently made the remarkable discovery that isolated TatC can catalyze the PMF-independent insertion of signal peptides across the membrane bilayer. This observation supports the idea that TatC provides the route by which the signal peptide traverses the membrane during transport. TatB is proposed to block access to this route at early stages of the transport process because the transport-independent movement of the signal peptide mediated by TatC does not occur when TatB is present (73). If the signal peptide initially binds in the central cavity of TatC, as discussed above, then the c-region of the signal peptide might reach the periplasm through the gap between the periplasmic cap and TM5 and TM6 (17).

The TatBC Complex

The TatBC complex contains equimolar amounts of the TatB and TatC proteins (27). TatBC complexes from *E. coli* and pea thylakoids have apparent molecular masses in blue native–polyacrylamide gel electrophoresis (BN-PAGE) of ~400 kDa and ~700 kDa, respectively (19, 74–76). The difference in mass of the two TatBC complexes is in part attributable to the presence of an additional N-terminal stromal domain on the chloroplast TatC protein (77). Molecular mass determination by BN-PAGE is subject to considerable uncertainty, and estimates for the composition of the TatBC complex ranging from four to eight copies of each subunit are plausible (60, 78). A multisubunit TatAC complex has been identified in a *B. subtilis* Tat system that lacks a TatB protein (53), and this complex is probably analogous to the TatBC complexes of *E. coli* and plant chloroplasts.

Within the TatBC complex, the TatC components appear to form an autonomous subcomplex, with TatB subunits then binding to this TatC core (24, 75, 76). This means that there must be specific protein–protein interactions between TatC subunits. These interactions can be inferred to be both limited in extent and weak, both because most detergents cause fragmentation of the TatBC complex (e.g., 19) and because no highly conserved contact points have been identified by evolutionary covariance analyses (31). Such weak intersubunit interactions suggest that the TatC subunits need to move relative to each other during the transport cycle. The observation that the TatBC complex is stabilized by substrate binding (29, 35, 50) provides possible evidence that the complex undergoes a significant conformational change as part of the Tat mechanism, and a comparison between low-resolution electron microscopy structures of TatBC and TatBC–substrate

complexes has been interpreted in the same way (60). Nevertheless, the TatBC complex is almost certainly compositionally stable under physiological conditions, given that TatC subunits do not exchange between complexes (35) or change oligomeric state in response to substrate binding (24). The crystallographic analysis of *A. aeolicus* TatC afforded no clues as to how TatC molecules contact each other because no physiologically plausible TatC oligomer is present in any of the three different crystal forms from which the structures were determined (31, 32). A 40-Å-resolution structure of the *E. coli* TatBC complex has been determined by electron microscopy (60), but at this low resolution it provides no information about how the TatC subunits are arranged within the complex. Likewise, it is difficult to form a clear picture of TatC–TatC contacts from published disulfide cross-linking experiments (29, 79). However, a TatC–TatC contact has been reported for a photoaffinity cross-linker placed in the periplasmic cap above the cavity opening (**Figure 4**) (30).

The multiple TatC subunits within the TatBC complex are all functional for substrate binding and transport (78). Whether these TatC subunits act independently or collaboratively is an interesting and not fully resolved question. Kinetic data are ambiguous and have been interpreted as both for (58) and against (78) cooperativity between sites. However, cross-complementation has been observed between nonfunctional *E. coli* TatC variants (33), strongly suggesting that TatC molecules collaborate to form a translocation site. Multiple TatC subunits have also been inferred to cooperate in the collective transport of either artificially cross-linked substrate oligomers (80) or native heterodimeric substrates with two signal peptides (81). Indeed, the role of TatBC oligomerization may be to provide multiple signal peptide–binding sites for homo-oligomeric substrate proteins (81). A tentative interpretation of the available data is that all TatC sites are functionally equivalent but cooperatively build and access a single translocation site.

Up to four substrate molecules bound to the same TatBC complex can be cross-linked together through cysteine residues located in the passenger domain (80). However, no cross-links are observed if the cysteine residues are, instead, placed in the signal peptide. These observations suggest that the signal peptide–binding sites in different TatC subunits may be physically isolated from each other, rather than sharing a common chamber as has been proposed elsewhere (29). At this stage, the ability of the Tat system to transport multivalent substrate oligomers does not provide strong constraints on the possible structural organization of the translocation site because of the uncertainty in how closely and flexibly the folded passenger domains are linked to the signal peptide–binding sites and to each other. However, low-resolution electron microscopy structures of TatBC–substrate complexes show adjacent substrate molecules on the periphery of the TatBC particle (60), and it is intuitively likely that the TatA molecules that form the translocating element are recruited to an external site.

The TMH of TatB has been inferred to bind near the periplasmic end of TatC helices TM5 and TM6 based on the results of cross-linking studies and genetic analyses (30, 31, 33, 35, 82). The TatB TMH has been explicitly modeled as lying within the external groove between TM5 and TM6 (**Figure 4**) (31) by analogy to a helix-packing interaction found as a crystal contact in all three *A. aeolicus* TatC crystal forms (31, 32). A satisfying feature of this structural model is that TM5 and TM6 are well matched in length to the unusually short TatB TMH. The structural model places the TatB TMH at the opposite end of the TatC molecule from the signal peptide n-region-binding site (**Figure 4**). Nevertheless, connections between TatB and signal peptide binding have been observed. Cross-links have been detected between TatB and the h-region of the signal peptide (discussed above in the section titled Substrate Recognition by the TatBC Complex) and between TatB and the signal peptide n-region-binding site on TatC (30).

The most likely scenario is that the APH of TatB can reach from the TMH-binding site on TatC as far as the n-region-binding site and that it is these contacts to the TatB APH that are being detected in the cross-linking experiments. An additional connection between TatB and

signal peptide binding is the observation that mutations allowing the export of substrates with defects in the signal peptide Tat consensus motif map to the N terminus of TatB (69, 70). Amino acid substitutions in this part of TatB cannot operate by physical contact with the signal peptide consensus motif because that motif is bound at the opposite side of the membrane. There may, therefore, be an allosteric connection between the two sites. Alternatively, the N terminus of TatB may exert its functional effects through a different part of the signal peptide, perhaps by controlling access from the TatC central cavity to the periplasm through the adjacent cap/TM5 gap.

Disulfide cross-linking studies suggest that the TMHs of TatB molecules are in contact with each other within the TatBC complex and that they form a bundle of at least four TatB subunits (45). Given the position of the proposed TatB TMH-binding site on TatC, these cross-linking results suggest a model for the organization of the TatBC complex in which TatC molecules surround a TatB core like petals of a flower. However, this organization is difficult to reconcile with the above observations that TatB oligomerization requires TatC and not vice versa.

TatA–TatC Interactions

Recent cross-linking studies have started to provide information about where the TMH of TatA contacts TatC (**Figure 4**). These investigations suggest that the constitutively bound TatA molecules are positioned close to the site at which TatC binds the TatB TMH (**Figure 4**) (29, 30). Under transport conditions, additional cross-links between TatA and TatC are detected that are assumed to arise from contacts between the assembled TatA oligomer and TatC (29). First, there is increased cross-linking between the TatA TMH and TatC in the vicinity of TM5. This is near the TatB-binding site and on the edge of the TatC central cavity. Second, the TMH of TatA has been detected in the central cavity of TatC by a cross-link close to the conserved glutamate or glutamine residue. A binding site for the TatA TMH within the central cavity had previously been predicted from in silico docking experiments, and it had been speculated that the cavity polar residue forms a bonding interaction with the conserved polar residue in the TatA TMH (31). The TatA TMH polar residue is, indeed, necessary for the observed TatA–TatC cross-link (29), although a requirement for the proposed partner residue in the cavity was not tested. The tentative conclusion from these cross-linking studies is that TatA oligomerization takes place on the cavity side of TatC and extends from the TatB TMH-binding site at TM5 into the cavity.

The Assembled TatA Oligomer

The structure of the assembled TatA oligomer is crucial to understanding the mechanism of transport because it is thought to form the substrate translocation pathway. Each Tat translocation site has been estimated to require ~25 TatA molecules on the basis of the TatA:TatC stoichiometry at which TatA availability becomes limiting for transport (78). A similar mean number of TatA subunits was measured for the fluorescent protein–labeled TatA oligomers formed in *E. coli* cells (83), although in this case it is not certain that this number corresponds to a single translocation element due to the multiple substrate-binding sites present in each TatBC complex. The oligomeric state of the TatA pool from which the TatA complex is formed is highly uncertain, although some data have been interpreted as suggesting that the TatA oligomer is assembled from tetrameric protomers (22, 83). Formation of the TatA oligomer requires the TatA TMH and APH but not the C-terminal tail or the functionally essential phenylalanine residue at the end of the APH (22, 24). Thus, interactions between TatA protomers within the oligomer may be mediated through the TMH, the APH, or both. Cross-linking studies show that within the oligomer both

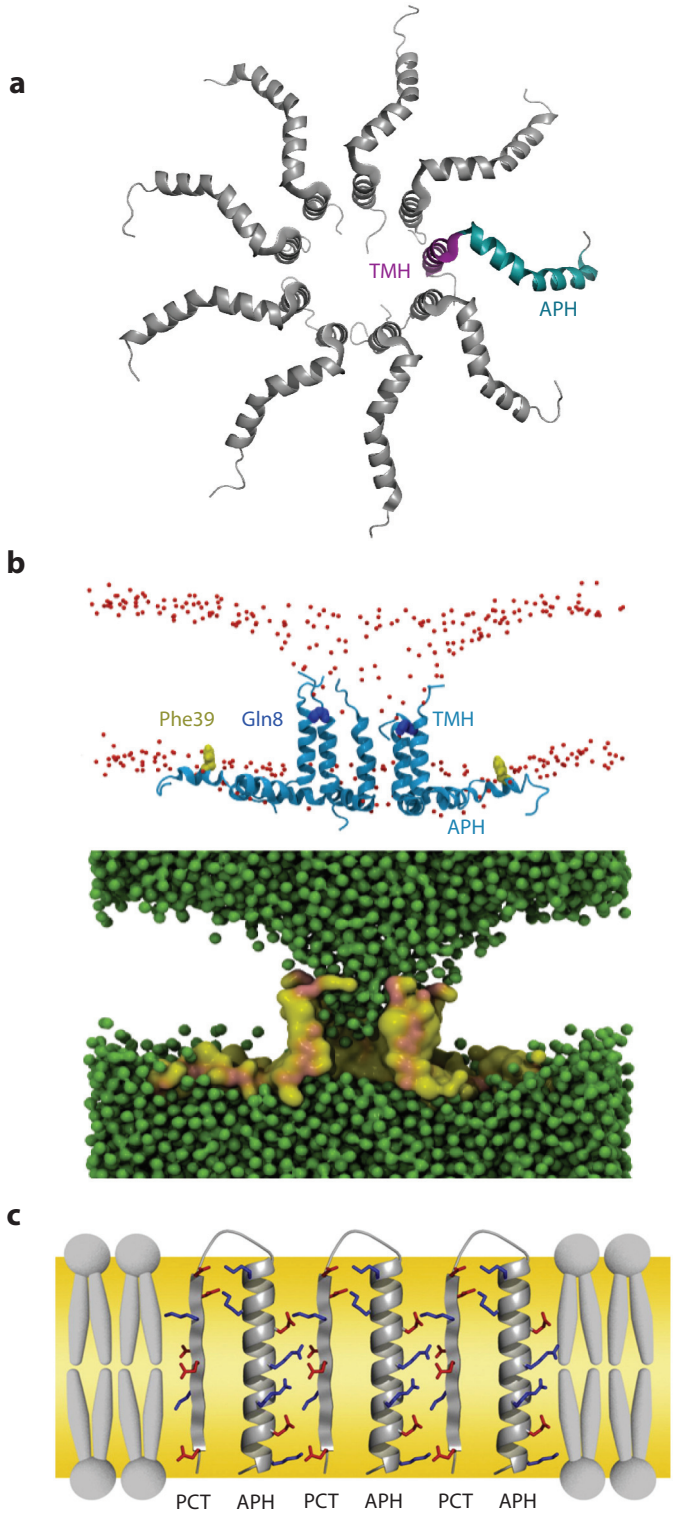
helices are close to the equivalent helices in neighboring TatA molecules (22, 23), suggesting, but not proving, the existence of specific protein–protein contacts between these structural elements. TatA assembly does not require the substrate passenger domain (23), eliminating the possibility that TatA oligomerization occurs by binding to the surface of the substrate.

The molecular structure of the TatA oligomer at active translocation sites has not been established due to the difficulties in undertaking structural analyses on a transient membrane-bound complex. However, TatA is also able to form large oligomers when extracted from its native membrane environment using detergents. What relationship these solubilization-induced structures have to the substrate-dependent oligomers formed under physiological conditions is unclear. Nevertheless, detergent-extracted TatA complexes have been extensively studied as possible models for the physiological TatA oligomer under the assumption that the physiological intersubunit contacts will still be favored when the complex is assembled under artificial conditions. Single-particle electron microscopy of TatA family proteins solubilized in a range of detergents shows ringlike structures (37, 60, 84–86). In some cases, the rings vary in size, indicating differences in the number of TatA polypeptides in different complexes. This variation may correspond to TatA complexes at different stages in the oligomerization process (84). The larger complexes have internal cavities that would be sufficiently wide to accommodate folded protein substrates (84). TatA oligomer size can be controlled by varying the protein-to-detergent ratio, and this method has been used to produce TatA oligomers that are small enough for solution NMR methods to determine the conformation of individual subunits and to identify intersubunit contacts (**Figure 5*a***) (37). The TatA molecules in these small oligomers contact one another solely through their TMHs. These contact sites closely match those inferred for larger detergent-extracted TatA oligomers by spin-labeling methods, suggesting that the subunit arrangement is similar in detergent-solubilized TatA oligomers of different sizes (87). Spin-labeling experiments show that the APHs radiate out from the central TMH bundle (37). Modeling studies find that the NMR-derived structure is scalable to the physiological oligomer size of 25 subunits (37). However, MD simulations suggest that oligomers built this way are unstable in a bilayer environment if they are larger than a tetramer. Indeed, it would be very difficult to build a stable, large TatA oligomer solely through TMH contacts. It is more likely that the structure of the physiological TatA oligomer, whether related to the NMR-derived structure or not, is stabilized through its interaction with TatC molecules and through APH contacts, as suggested by biochemical experiments.

In an alternative approach to producing a TatA oligomer that is small enough for solution NMR analysis, two TatA molecules were linked through a disulfide bond between their flexible N termini

Figure 5

Models for the structure of the TatA oligomer. (*a*) Model for the TatA oligomer in detergent solution, based on NMR and spin-labeling data (37). The model constructed from nine TatA subunits is shown in cartoon representation viewed from the cytoplasmic side of the membrane. The amphipathic helix (APH) and the transmembrane helix (TMH) of one subunit are colored cyan and purple, respectively. The TatA construct used to produce this structure lacks the unstructured C-terminal tail. (*b*) Coarse-grained molecular dynamics simulation of the TatA nonamer in an *Escherichia coli* lipid membrane (37). In both panels, the four closest TatA subunits have been removed to reveal the central pore. (*Top*) A cartoon representation of the protein (*cyan*). Gln8 (*blue*) and Phe39 (*yellow*) residues are shown in space-filling representation. The phosphate particles of the lipid head groups are shown as red spheres. (*Bottom*) The TatA molecules are shown in surface representation (*yellow and orange*). Water particles are colored green. Lipids and ions have been omitted for clarity. (*c*) Zipper model for TatA oligomerization (88). Positively charged residues (*blue*) in the APH of TatA are proposed to form ion pairs with negatively charged residues (*red*) in the proximal C-terminal tail (PCT) of the same and adjacent TatA subunits. Panel *b* modified from Reference 37. Panel *c* modified from Reference 88.


a
TMH
APH
b
Phe39
Gln8
TMH
APH
c
PCT
APH
PCT
APH
PCT
APH

and then analyzed at high detergent concentration to suppress higher-order oligomerization (38). The structure determined by this approach shows the same TMH contacts that were identified in the earlier NMR and spin-label studies (**Figure 4*a***) but, in addition, the APHs are in contact along their entire length. These APH contacts are weaker than the TMH contacts, suggesting that they represent transient interactions.

Walther et al. (88) have proposed a radically different model for TatA oligomerization that is based on the distribution of charged residues along the TatA molecule. In this so-called zipper model, basic residues in the distal APH interact with acidic residues in the proximal C-terminal tail of an adjacent TatA molecule to form a ladder of salt bridges between the TatA subunits (**Figure 5*c***). Although these authors presented experiments on detergent-solubilized TatA variants that they interpreted as being consistent with their zipper model, there are arguments against the plausibility of the model. First, the zipper structure is not observed in the NMR structure of the disulfide-linked *E. coli* TatA dimer (38). Second, the zippering mechanism is incompatible with the structure of the *E. coli* TatA monomer without significant unwinding of the distal end of the APH (38). Third, the zipper residues and their sequence positions are not conserved. Fourth, the asymmetric charge distribution in TatA can equally well be explained as arising from a requirement for the (basic) APH to interact with the acidic head groups of the membrane phospholipids and the (acidic) C-terminal tail to be repelled from the membrane surface. I also note that an analogous prediction of a zipper structure in the protein dermcidin (88) proved incorrect when the structure of the protein was subsequently determined (89). These considerations suggest that the TatA zipper model requires further testing in the native membrane environment.

THE ROLE OF THE PROTON MOTIVE FORCE IN TAT TRANSPORT

The transmembrane PMF is the driving force for Tat transport. How the Tat apparatus uses this energy source is still poorly understood, with both the number and the identity of the transport steps affected by the PMF not yet established. Based on an examination of how transport responds to pulses of PMF, it has been argued that the PMF is required at two different steps in the Tat transport process, each with a different electrical potential ($\Delta\psi$) threshold (90). However, this model requires the assumption that each pulse of PMF drives a single transport cycle, and it is difficult to exclude the more parsimonious interpretation that the PMF pulses in this study were restoring the PMF above a single low $\Delta\psi$ threshold. Initial substrate binding to the translocation machinery is PMF independent (57). However, as discussed above, the PMF drives a more deeply inserted signal peptide–binding mode in thylakoids. This deep-insertion step is likely to be distinct from, and precede, other PMF-dependent steps in Tat transport because certain engineered substrates can attain this state in the absence of a proton gradient yet still require the PMF for completion of transport (61). The substrate-triggered oligomerization of TatA clearly involves the PMF (21, 23–25). What is less certain is whether the PMF is required to maintain the TatA oligomer once assembled, or for the subsequent physical transport of the substrate across the membrane. Substrate-induced TatA oligomers disperse within seconds if the proton gradient is removed (24, 25), an observation that is consistent with a simple model in which TatA oligomerization is reversible but biased in favor of the assembled state by the PMF. However, TatA oligomers persist in the absence of a PMF in some transport-inactive strains, showing that TatA can reach an assembled state that is stable in the absence of a proton gradient (24, 83). If functional translocases form equivalent stable TatA assemblies, then the observation that TatA oligomers disperse when the PMF is abolished is best explained as disassembly of the active site following successful completion of transport. The implication of such a model is that the PMF is not necessary during the transport steps that follow assembly of the stable TatA oligomer.

A distinctive feature of the Tat system is that it can operate at PMFs that are considerably smaller than the physiological proton gradient (29, 90–92). Intriguingly, the threshold PMF for transport differs significantly between proteins (93). This observation implies that the proton gradient has to do different amounts of work to translocate different substrates. The Tat system can use either the chemical (ΔpH) or electrical ($\Delta\psi$) components of the proton gradient (2, 90, 94), indicating that the energy-dependent steps in the Tat pathway are mechanistically linked to the transmembrane movement of protons (which is affected by both components of the PMF) rather than being driven by the electrical field across the membrane. Experiments with plant thylakoids indicate that each Tat transport event is associated with the transmembrane movement of ~80,000 protons (93). Such a large number of proton movements cannot arise from the release of translocase-bound protons in a single mechanistic step. Instead, the high proton stoichiometry suggests either that Tat operates by a multistep motor mechanism or that a significant proton leak is associated with productive transport.

To move protons across the membrane the Tat apparatus must be able to assemble a proton-conducting pathway composed of protonatable amino acid side chains and buried water molecules. A prime candidate for the cytoplasmic half of this pathway is the transient aqueous channel, which has been inferred to extend into the central cavity of TatC as far as the conserved glutamate or glutamine residue (see the above section titled TatC). MD simulations suggest that water molecules can also ingress into TatC from the periplasmic side of the membrane to solvate the periplasmic ends of short helices TM5 and TM6 (31, 32). However, no polar route within TatC links the central cavity to either this location or other locations on the periplasmic side of the protein. Instead, proton movements in the periplasmic half of the membrane might be mediated by TatA family proteins using the conserved TMH polar residue and other hydrophilic amino acids that are located close to the periplasmic side of the membrane. Indeed, MD simulations suggest that water could penetrate the periplasmic side of a TatA oligomer (**Figure 5*b***) (37). Although these proposals for the route of proton transport are highly speculative, it is clear that the proton-conducting pathway is not contained within a single Tat component but is an emergent property of the assembled translocation machinery.

MECHANISM OF TAT TRANSPORT

The duration of the Tat transport cycle is in the range of 1 to 3 min as estimated by kinetic analyses (78, 95) or the time of the first appearance of the transported protein (21, 90, 96). The kinetics of Tat transport can be satisfactorily fitted by a two-step mechanism (78, 95, 96). The first step in this mechanism almost certainly corresponds to TatA oligomerization because its duration is sensitive to the TatA concentration in the membrane (78). The second step then represents substrate transport across the membrane by the assembled TatABC complex. This mechanistic interpretation is consistent with the observation that TatA recruitment to the TatBC complex precedes substrate translocation (21) and that the first kinetic phase occurs on a similar timescale to TatA oligomerization (21, 24). The second kinetic step has an inferred time constant of ~1.5 min under conditions of TatA sufficiency (78), meaning that substrate transport occurs slowly even after TatA assembly is complete. Because the physical movement of the substrate protein across the membrane is likely to require only milliseconds at most, the minute timescale of the second kinetic step may indicate that the activation barrier to transport is high and only infrequently overcome. Alternatively, the TatABC–substrate complex may undergo substantial reorganization after assembly before transport can take place (78).

What is the function of the TatA oligomerization cycle in the Tat mechanism? One possibility is that the assembled translocation site has deleterious consequences for the cell, such as continuously

consuming the PMF, allowing ion leaks, or compromising the ability of the membrane to withstand environmental stresses. Exposure to these effects may be minimized if the site is assembled only when transport is needed (24). An alternative possibility is that TatA oligomerization transduces the PMF into stored conformational energy that can subsequently be used to drive Tat transport (24). Although current data are insufficient to distinguish between these two possibilities, they make distinct predictions about the behavior of the Tat system when substrates are plentiful. If TatA oligomerization drives transport, then a full cycle of TatA assembly and disassembly must accompany each translocation event, whereas if TatA dispersal is a safety mechanism, an assembled translocation site will be able to undertake multiple transport events as long as substrate remains available.

An intriguing mechanistic feature of the Tat apparatus is that it fails to transport improperly folded substrates (97). Two observations show that this behavior does not arise from an inherent inability of the Tat machinery to translocate unstructured proteins. First, the Tat system translocates natively unstructured polypeptides (98, 99). Second, *E. coli* Tat variants have been identified that allow the transport of incompletely folded proteins (100). The existence of translocase variants with relaxed substrate-folding requirements is most readily rationalized as arising from the inactivation of a hypothetical "proofreading" site, which scans substrates for folding state before permitting their transport (100). However, it is also possible that these variants prevent either the stalling of misfolded proteins within the Tat apparatus during transport (101) or the mistargeting of hydrophobic regions of improperly folded polypeptides into the membrane interior (6).

The central challenge in understanding Tat transport is to determine how the folded substrate is transferred across the membrane bilayer. Conventional protein transporters contain an aqueous transmembrane channel through which the unfolded substrate protein is threaded (102). The paradigm of such a transport system is the Sec apparatus (1). The Sec protein-conducting channel is opened only when a substrate is present, and a hydrophobic constriction at the midpoint of the channel packs around the translocating polypeptide to prevent ion leakage. A directly analogous channel mechanism for Tat transport is unlikely, both because the much larger channel needed to transport folded proteins would be difficult to seal when not in use, and because there are mechanical problems associated with sealing a channel around proteins of very different sizes. Consistent with these considerations, the transmembrane parts of the individual Tat proteins show no evidence of being components of a large aqueous channel. A channel might be lined by the APHs of the TatA oligomer (14, 84, 88). However, this model would require the two helices of TatA to adopt a transmembrane hairpin structure, which appears unlikely on the basis of the NMR structures of TatA (**Figure 2**) and the lack of evidence that the APH is ever accessible from the periplasmic side of the membrane (47, 49).

A popular alternative to the channel model proposes that Tat transport may work by a nonconventional mechanism in which concentration of TatA molecules causes a local weakening of the membrane. Substrate movement is envisaged to occur through transient bilayer rupture or once mechanical force has been exerted on the substrate to drive it through the weakened bilayer. In a membrane-weakening model the membrane remains intact, and thus impermeable to ions, except at the moment of transport. The cell would probably be able to tolerate the brief ion leak that occurs during this transient opening of the bilayer. Alternatively, lateral membrane pressure might cause the disordered phospholipids to pack around the substrate protein as it moves through the membrane. Tat transport is strongly influenced by the membrane phospholipid composition, which would be consistent with a mechanism involving protein–phospholipid interactions (103–105). Possible mechanisms of membrane weakening include the interaction between the TatA APH and the cytoplasmic leaflet of the membrane (51, 106) or the mismatch between the length of the TatA TMH and the width of the bilayer (37), which may be augmented by a similar mismatch arising

from TM5 and TM6 of TatC (31, 32). MD simulations of the detergent-solubilized TatA oligomer structure inserted into a membrane suggest that the mismatch mechanism might be capable of locally thinning the bilayer by ~80%, with the phospholipids in the thinned membrane showing high disorder (**Figure 5*b***) (37). Nevertheless, these mechanisms for substrate transport remain very speculative, and other possibilities have been advanced (reviewed in References 6 and 14).

FUTURE PROSPECTS

Now that structures of individual Tat components are available the molecular basis of Tat transport has started to come into focus. However, understanding the Tat mechanism will require us to establish how these components are assembled in the active translocation site. This is a formidable experimental challenge given the size, membranous nature, transience, low stability, and probable conformational and compositional heterogeneity of the translocation complex. It is likely that multiple methodologies will be needed to solve this problem, including less-traditional techniques that can be used on proteins in a membrane environment, such as solid-state NMR or spin labeling. A stepwise assembly approach in which stable subcomplexes containing a few Tat components are targeted for crystallographic analysis and then used as building blocks to model the full complex structure is an attractive strategy. It will also be important to ascertain whether the structures already obtained for TatA oligomers in detergent solution replicate the TatA organization found in the active translocation site. Certainly MD simulations will be crucial to exploring mechanistic details once sufficient structural data are available. It is encouraging for structural analysis that recent research suggests that the translocase can be held in an extended-duration "idling mode" if assembly is induced by signal peptides rather than full substrates (29). The possibility that certain inactive Tat variants stabilize Tat proteins in a transport-analogous state is also worth pursuing (24). The goal of understanding Tat transport remains an exciting challenge that will continue to be underpinned by structural analysis.

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LITERATURE CITED

1. Park E, Rapoport TA. 2012. Mechanisms of Sec61/SecY-mediated protein translocation across membranes. *Annu. Rev. Biophys.* 41:21–40
2. Mould RM, Robinson C. 1991. A proton gradient is required for the transport of two lumenal oxygen-evolving proteins across the thylakoid membrane. *J. Biol. Chem.* 266:12189–93
3. Yahr TL, Wickner WT. 2001. Functional reconstitution of bacterial Tat translocation in vitro. *EMBO J.* 20:2472–79

4. Simone D, Bay DC, Leach T, Turner RJ. 2013. Diversity and evolution of bacterial twin arginine translocase protein, TatC, reveals a protein secretion system that is evolving to fit its environmental niche. *PLOS ONE* 8:e78742
5. Berks BC, Palmer T, Sargent F. 2003. The Tat protein translocation pathway and its role in microbial physiology. *Adv. Microb. Physiol.* 47:187–254
6. Palmer T, Berks BC. 2012. The twin-arginine translocation (Tat) protein export pathway. *Nat. Rev. Microbiol.* 10:483–96
7. De Buck E, Lammertyn E, Anne J. 2008. The importance of the twin-arginine translocation pathway for bacterial virulence. *Trends Microbiol.* 16:442–53
8. Barkan A, Miles D, Taylor WC. 1986. Chloroplast gene expression in nuclear, photosynthetic mutants of maize. *EMBO J.* 5:1421–27
9. Celedon JM, Cline K. 2013. Intra-plastid protein trafficking: how plant cells adapted prokaryotic mechanisms to the eukaryotic condition. *Biochim. Biophys. Acta* 1833:341–51
10. Burger G, Gray MW, Forget L, Lang BF. 2013. Strikingly bacteria-like and gene-rich mitochondrial genomes throughout Jakobid protists. *Genome Biol. Evol.* 5:418–38
11. Pett W, Lavrov DV. 2013. The twin-arginine subunit C in *Oscarella*: origin, evolution, and potential functional significance. *Integr. Comp. Biol.* 53:495–502
12. Hasan S, Platta HW, Erdmann R. 2013. Import of proteins into the peroxisomal matrix. *Front. Physiol.* 4:261
13. Sysoeva TA, Zepeda-Rivera MA, Huppert LA, Burton BM. 2014. Dimer recognition and secretion by the ESX secretion system in *Bacillus subtilis*. *PNAS* 111:7653–58
14. Berks BC, Sargent F, Palmer T. 2000. The Tat protein export pathway. *Mol. Microbiol.* 35:260–74
15. Sargent F, Bogsch EG, Stanley NR, Wexler M, Robinson C, et al. 1998. Overlapping functions of components of a bacterial Sec-independent protein export pathway. *EMBO J.* 17:3640–50
16. Jack RL, Sargent F, Berks BC, Sawers G, Palmer T. 2001. Constitutive expression of *Escherichia coli tat* genes indicates an important role for the twin-arginine translocase during aerobic and anaerobic growth. *J. Bacteriol.* 183:1801–4
17. Berks BC, Lea SM, Stansfeld PJ. 2014. Structural biology of Tat protein transport. *Curr. Opin. Struct. Biol.* 27:32–37
18. Kudva R, Denks K, Kuhn P, Vogt A, Müller M, Koch HG. 2013. Protein translocation across the inner membrane of gram-negative bacteria: the Sec and Tat dependent protein transport pathways. *Res. Microbiol.* 164:505–34
19. Cline K, Mori H. 2001. Thylakoid ΔpH-dependent precursor proteins bind to a cpTatC–Hcf106 complex before Tha4-dependent transport. *J. Cell Biol.* 154:719–29
20. Alami M, Luke I, Deitermann S, Eisner G, Koch HG, et al. 2003. Differential interactions between a twin-arginine signal peptide and its translocase in *Escherichia coli*. *Mol. Cell* 12:937–46
21. Mori H, Cline K. 2002. A twin arginine signal peptide and the pH gradient trigger reversible assembly of the thylakoid ΔpH/Tat translocase. *J. Cell Biol.* 157:205–10
22. Dabney-Smith C, Cline K. 2009. Clustering of C-terminal stromal domains of Tha4 homo-oligomers during translocation by the Tat protein transport system. *Mol. Biol. Cell* 20:2060–69
23. Dabney-Smith C, Mori H, Cline K. 2006. Oligomers of Tha4 organize at the thylakoid Tat translocase during protein transport. *J. Biol. Chem.* 281:5476–83
24. Alcock F, Baker MA, Greene NP, Palmer T, Wallace MI, Berks BC. 2013. Live cell imaging shows reversible assembly of the TatA component of the twin-arginine protein transport system. *PNAS* 110:e3650–59
25. Rose P, Fröbel J, Graumann PL, Müller M. 2013. Substrate-dependent assembly of the Tat translocase as observed in live *Escherichia coli* cells. *PLOS ONE* 8:e69488
26. Luke I, Handford JI, Palmer T, Sargent F. 2009. Proteolytic processing of *Escherichia coli* twin-arginine signal peptides by LepB. *Arch. Microbiol.* 191:919–25
27. Bolhuis A, Mathers JE, Thomas JD, Barrett CM, Robinson C. 2001. TatB and TatC form a functional and structural unit of the twin-arginine translocase from *Escherichia coli*. *J. Biol. Chem.* 276:20213–19
28. de Leeuw E, Granjon T, Porcelli I, Alami M, Carr SB, et al. 2002. Oligomeric properties and signal peptide binding by *Escherichia coli* Tat protein transport complexes. *J. Mol. Biol.* 322:1135–46

29. Aldridge C, Ma X, Gerard F, Cline K. 2014. Substrate-gated docking of pore subunit Tha4 in the TatC cavity initiates Tat translocase assembly. *J. Cell Biol.* 205:51–65
30. Zoufaly S, Fröbel J, Rose P, Flecken T, Maurer C, et al. 2012. Mapping precursor-binding site on TatC subunit of twin arginine specific protein translocase by site-specific photo cross-linking. *J. Biol. Chem.* 287:13430–41
31. Rollauer SE, Tarry MJ, Graham JE, Jääskeläinen M, Jäger F, et al. 2012. Structure of the TatC core of the twin-arginine protein transport system. *Nature* 492:210–14
32. Ramasamy S, Abrol R, Suloway CJ, Clemons WM Jr. 2013. The glove-like structure of the conserved membrane protein TatC provides insight into signal sequence recognition in twin-arginine translocation. *Structure* 21:777–88
33. Buchanan G, de Leeuw E, Stanley NR, Wexler M, Berks BC, et al. 2002. Functional complexity of the twin-arginine translocase TatC component revealed by site-directed mutagenesis. *Mol. Microbiol.* 43:1457–70
34. Holzapfel E, Eisner G, Alami M, Barrett CML, Buchanan G, et al. 2007. The entire N-terminal half of TatC is involved in twin-arginine precursor binding. *Biochemistry* 46:2892–98
35. Ma X, Cline K. 2013. Mapping the signal peptide binding and oligomer contact sites of the core subunit of the pea twin arginine protein translocase. *Plant Cell* 25:999–1015
36. Hu Y, Zhao E, Li H, Xia B, Jin C. 2010. Solution NMR structure of the TatA component of the twin-arginine protein transport system from gram-positive bacterium *Bacillus subtilis*. *J. Am. Chem. Soc.* 132:15942–44
37. Rodriguez F, Rouse SL, Tait CE, Harmer J, De Riso A, et al. 2013. Structural model for the protein-translocating element of the twin-arginine transport system. *PNAS* 110:e1092–101
38. Zhang Y, Hu Y, Li H, Jin C. 2014. Structural basis for TatA oligomerization: an NMR study of *Escherichia coli* TatA dimeric structure. *PLOS ONE* 9:e103157
39. Zhang Y, Wang L, Hu Y, Jin C. 2014. Solution structure of the TatB component of the twin-arginine translocation system. *Biochim. Biophys. Acta* 1838:1881–88
40. Walther TH, Grage SL, Roth N, Ulrich AS. 2010. Membrane alignment of the pore-forming component TatAd of the twin-arginine translocase from *Bacillus subtilis* resolved by solid-state NMR spectroscopy. *J. Am. Chem. Soc.* 132:15945–56
41. Porcelli I, de Leeuw E, Wallis R, van den Brink–van der Laan E, de Kruijff B, et al. 2002. Characterization and membrane assembly of the TatA component of the *Escherichia coli* twin-arginine protein transport system. *Biochemistry* 41:13690–97
42. Lee PA, Buchanan G, Stanley NR, Berks BC, Palmer T. 2002. Truncation analysis of TatA and TatB defines the minimal functional units required for protein translocation. *J. Bacteriol.* 184:5871–79
43. Weiner JH, Bilous PT, Shaw GM, Lubitz SP, Frost L, et al. 1998. A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. *Cell* 93:93–101
44. Dabney-Smith C, Mori H, Cline K. 2003. Requirement of a Tha4-conserved transmembrane glutamate in thylakoid Tat translocase assembly revealed by biochemical complementation. *J. Biol. Chem.* 278:43027–33
45. Lee PA, Orriss GL, Buchanan G, Greene NP, Bond PJ, et al. 2006. Cysteine-scanning mutagenesis and disulfide mapping studies of the conserved domain of the twin-arginine translocase TatB component. *J. Biol. Chem.* 281:34072–85
46. Mori H, Summer EJ, Ma X, Cline K. 1999. Component specificity for the thylakoidal Sec and ΔpH-dependent protein transport pathways. *J. Cell Biol.* 146:45–56
47. Koch S, Fritsch MJ, Buchanan G, Palmer T. 2012. *Escherichia coli* TatA and TatB proteins have N-out, C-in topology in intact cells. *J. Biol. Chem.* 287:14420–31
48. Fincher V, McCaffery M, Cline K. 1998. Evidence for a loop mechanism of protein transport by the thylakoid ΔpH pathway. *FEBS Lett.* 423:66–70
49. Aldridge C, Storm A, Cline K, Dabney-Smith C. 2012. The chloroplast twin arginine transport (Tat) component, Tha4, undergoes conformational changes leading to Tat protein transport. *J. Biol. Chem.* 287:34752–63
50. Fincher V, Dabney-Smith C, Cline K. 2003. Functional assembly of thylakoid ΔpH-dependent/Tat protein transport pathway components in vitro. *Eur. J. Biochem.* 270:4930–41

51. Greene NP, Porcelli I, Buchanan G, Hicks MG, Schermann SM, et al. 2007. Cysteine scanning mutagenesis and disulfide mapping studies of the TatA component of the bacterial twin arginine translocase. *J. Biol. Chem.* 282:23937–45
52. Hicks MG, de Leeuw E, Porcelli I, Buchanan G, Berks BC, Palmer T. 2003. The *Escherichia coli* twin-arginine translocase: conserved residues of TatA and TatB family components involved in protein transport. *FEBS Lett.* 539:61–67
53. Barnett JP, Eijlander RT, Kuipers OP, Robinson C. 2008. A minimal Tat system from a gram-positive organism: A bifunctional TatA subunit participates in discrete TatAC and TatA complexes. *J. Biol. Chem.* 283:2534–42
54. Blaudeck N, Kreutzenbeck P, Müller M, Sprenger GA, Freudl R. 2005. Isolation and characterization of bifunctional *Escherichia coli* TatA mutant proteins that allow efficient Tat-dependent protein translocation in the absence of TatB. *J. Biol. Chem.* 280:3426–32
55. Hicks MG, Lee PA, Georgiou G, Berks BC, Palmer T. 2005. Positive selection for loss-of-function *tat* mutations identifies critical residues required for TatA activity. *J. Bacteriol.* 187:2920–25
56. Barnett JP, Lawrence J, Mendel S, Robinson C. 2011. Expression of the bifunctional *Bacillus subtilis* TatAd protein in *Escherichia coli* reveals distinct TatA/B-family and TatB-specific domains. *Arch. Microbiol.* 193:583–94
57. Ma X, Cline K. 2000. Precursors bind to specific sites on thylakoid membranes prior to transport on the ΔpH protein translocation system. *J. Biol. Chem.* 275:10016–22
58. Alder NN, Theg SM. 2003. Protein transport via the cpTat pathway displays cooperativity and is stimulated by transport-incompetent substrate. *FEBS Lett.* 540:96–100
59. Whitaker N, Bageshwar UK, Musser SM. 2012. Kinetics of precursor interactions with the bacterial Tat translocase detected by real-time FRET. *J. Biol. Chem.* 287:11252–60
60. Tarry MJ, Schafer E, Chen S, Buchanan G, Greene NP, et al. 2009. Structural analysis of substrate binding by the TatBC component of the twin-arginine protein transport system. *PNAS* 106:13284–89
61. Gerard F, Cline K. 2007. The thylakoid proton gradient promotes an advanced stage of signal peptide binding deep within the Tat pathway receptor complex. *J. Biol. Chem.* 282:5263–72
62. Bageshwar UK, Whitaker N, Liang FC, Musser SM. 2009. Interconvertibility of lipid- and translocon-bound forms of the bacterial Tat precursor pre-SufI. *Mol. Microbiol.* 74:209–26
63. Berks BC. 1996. A common export pathway for proteins binding complex redox cofactors? *Mol. Microbiol.* 22:393–404
64. Stanley NR, Palmer T, Berks BC. 2000. The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in *Escherichia coli*. *J. Biol. Chem.* 275:11591–96
65. Peltier JB, Friso G, Kalume DE, Roepstorff P, Nilsson F, et al. 2000. Proteomics of the chloroplast: systematic identification and targeting analysis of lumenal and peripheral thylakoid proteins. *Plant Cell* 12:319–41
66. Bendtsen JD, Nielsen H, Widdick D, Palmer T, Brunak S. 2005. Prediction of twin-arginine signal peptides. *BMC Bioinform.* 6:167
67. Bagos PG, Nikolaou EP, Liakopoulos TD, Tsirigos KD. 2010. Combined prediction of Tat and Sec signal peptides with hidden Markov models. *Bioinformatics* 26:2811–17
68. Rose RW, Brüser T, Kissinger JC, Pohlschröder M. 2002. Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. *Mol. Microbiol.* 45:943–50
69. Kreutzenbeck P, Kröger C, Lausberg F, Blaudeck N, Sprenger GA, Freudl R. 2007. *Escherichia coli* twin arginine (Tat) mutant translocases possessing relaxed signal peptide recognition specificities. *J. Biol. Chem.* 282:7903–11
70. Lausberg F, Fleckenstein S, Kreutzenbeck P, Fröbel J, Rose P, et al. 2012. Genetic evidence for a tight cooperation of TatB and TatC during productive recognition of twin-arginine (Tat) signal peptides in *Escherichia coli*. *PLOS ONE* 7:e39867
71. Strauch EM, Georgiou G. 2007. *Escherichia coli tatC* mutations that suppress defective twin-arginine transporter signal peptides. *J. Mol. Biol.* 374:283–91
72. Gerard F, Cline K. 2006. Efficient twin arginine translocation (Tat) pathway transport of a precursor protein covalently anchored to its initial cpTatC binding site. *J. Biol. Chem.* 281:6130–35

73. Fröbel J, Rose P, Lausberg F, Blummel AS, Freudl R, Müller M. 2012. Transmembrane insertion of twin-arginine signal peptides is driven by TatC and regulated by TatB. *Nat. Commun.* 3:1311
74. Oates J, Barrett CM, Barnett JP, Byrne KG, Bolhuis A, Robinson C. 2005. The *Escherichia coli* twin-arginine translocation apparatus incorporates a distinct form of TatABC complex, spectrum of modular TatA complexes and minor TatAB complex. *J. Mol. Biol.* 346:295–305
75. Orriss GL, Tarry MJ, Ize B, Sargent F, Lea SM, et al. 2007. TatBC, TatB, and TatC form structurally autonomous units within the twin arginine protein transport system of *Escherichia coli*. *FEBS Lett.* 581:4091–97
76. Behrendt J, Lindenstrauss U, Brüser T. 2007. The TatBC complex formation suppresses a modular TatB multimerization in *Escherichia coli*. *FEBS Lett.* 581:4085–90
77. Martin JR, Harwood JH, McCaffery M, Fernandez DE, Cline K. 2009. Localization and integration of thylakoid protein translocase subunit cpTatC. *Plant J.* 58:831–42
78. Celedon JM, Cline K. 2012. Stoichiometry for binding and transport by the twin arginine translocation system. *J. Cell Biol.* 197:523–34
79. Punginelli C, Maldonado B, Grahl S, Jack R, Alami M, et al. 2007. Cysteine scanning mutagenesis and topological mapping of the *Escherichia coli* twin-arginine translocase TatC component. *J. Bacteriol.* 189:5482–94
80. Ma X, Cline K. 2010. Multiple precursor proteins bind individual Tat receptor complexes and are collectively transported. *EMBO J.* 29:1477–88
81. James MJ, Coulthurst SJ, Palmer T, Sargent F. 2013. Signal peptide etiquette during assembly of a complex respiratory enzyme. *Mol. Microbiol.* 90:400–14
82. Kneuper H, Maldonado B, Jäger F, Krehenbrink M, Buchanan G, et al. 2012. Molecular dissection of TatC defines critical regions essential for protein transport and a TatB–TatC contact site. *Mol. Microbiol.* 85:945–61
83. Leake MC, Greene NP, Godun RM, Granjon T, Buchanan G, et al. 2008. Variable stoichiometry of the TatA component of the twin-arginine protein transport system observed by in vivo single-molecule imaging. *PNAS* 105:15376–81
84. Gohlke U, Pullan L, McDevitt CA, Porcelli I, de Leeuw E, et al. 2005. The TatA component of the twin-arginine protein transport system forms channel complexes of variable diameter. *PNAS* 102:10482–86
85. Beck D, Vasisht N, Baglieri J, Monteferrante CG, van Dijl JM, et al. 2013. Ultrastructural characterisation of *Bacillus subtilis* TatA complexes suggests they are too small to form homooligomeric translocation pores. *Biochim. Biophys. Acta* 1833:1811–19
86. Baglieri J, Beck D, Vasisht N, Smith CJ, Robinson C. 2012. Structure of TatA paralog, TatE, suggests a structurally homogeneous form of Tat protein translocase that transports folded proteins of differing diameter. *J. Biol. Chem.* 287:7335–44
87. White GF, Schermann SM, Bradley J, Roberts A, Greene NP, et al. 2010. Subunit organization in the TatA complex of the twin arginine protein translocase: a site-directed EPR spin labeling study. *J. Biol. Chem.* 285:2294–301
88. Walther TH, Gottselig C, Grage SL, Wolf M, Vargiu AV, et al. 2013. Folding and self-assembly of the TatA translocation pore based on a charge zipper mechanism. *Cell* 152:316–26
89. Song C, Weichbrodt C, Salnikov ES, Dynowski M, Forsberg BO, et al. 2013. Crystal structure and functional mechanism of a human antimicrobial membrane channel. *PNAS* 110:4586–91
90. Bageshwar UK, Musser SM. 2007. Two electrical potential-dependent steps are required for transport by the *Escherichia coli* Tat machinery. *J. Cell Biol.* 179:87–99
91. Braun NA, Theg SM. 2008. The chloroplast Tat pathway transports substrates in the dark. *J. Biol. Chem.* 283:8822–28
92. Brock IW, Mills JD, Robinson D, Robinson C. 1995. The ΔpH-driven, ATP-independent protein translocation mechanism in the chloroplast thylakoid membrane. Kinetics and energetics. *J. Biol. Chem.* 270:1657–62
93. Alder NN, Theg SM. 2003. Energetics of protein transport across biological membranes. A study of the thylakoid ΔpH-dependent/cpTat pathway. *Cell* 112:231–42
94. Braun NA, Davis AW, Theg SM. 2007. The chloroplast Tat pathway utilizes the transmembrane electric potential as an energy source. *Biophys. J.* 93:1993–98

95. Musser SM, Theg SM. 2000. Characterization of the early steps of OE17 precursor transport by the thylakoid ΔpH/Tat machinery. *Eur. J. Biochem.* 267:2588–98
96. Teter SA, Theg SM. 1998. Energy-transducing thylakoid membranes remain highly impermeable to ions during protein translocation. *PNAS* 95:1590–94
97. DeLisa MP, Tullman D, Georgiou G. 2003. Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. *PNAS* 100:6115–20
98. Cline K, McCaffery M. 2007. Evidence for a dynamic and transient pathway through the TAT protein transport machinery. *EMBO J.* 26:3039–49
99. Richter S, Lindenstrauss U, Lucke C, Bayliss R, Brüser T. 2007. Functional Tat transport of unstructured, small, hydrophilic proteins. *J. Biol. Chem.* 282:33257–64
100. Rocco MA, Waraho-Zhmayev D, DeLisa MP. 2012. Twin-arginine translocase mutations that suppress folding quality control and permit export of misfolded substrate proteins. *PNAS* 109:13392–97
101. Panahandeh S, Maurer C, Moser M, DeLisa MP, Müller M. 2008. Following the path of a twin-arginine precursor along the TatABC translocase of *Escherichia coli*. *J. Biol. Chem.* 283:33267–75
102. Blobel GA. 2000. Protein targeting [Nobel lecture]. *ChemBioChem* 1:86–102
103. Mikhaleva NI, Santini CL, Giordano G, Nesmeyanova MA, Wu LF. 1999. Requirement for phospholipids of the translocation of the trimethylamine *N*-oxide reductase through the Tat pathway in *Escherichia coli*. *FEBS Lett.* 463:331–35
104. Sikdar R, Doerrler WT. 2010. Inefficient Tat-dependent export of periplasmic amidases in an *Escherichia coli* strain with mutations in two DedA family genes. *J. Bacteriol.* 192:807–18
105. Ma X, Browse J. 2006. Altered rates of protein transport in *Arabidopsis* mutants deficient in chloroplast membrane unsaturation. *Phytochemistry* 67:1629–36
106. Brüser T, Sanders C. 2003. An alternative model of the twin arginine translocation system. *Microbiol. Res.* 158:7–17