Cysteine Scanning Mutagenesis and Disulfide Mapping Studies of the TatA Component of the Bacterial Twin Arginine Translocase*

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The Tat (twin arginine translocation) system transports folded proteins across the bacterial cytoplasmic membrane and the thylakoid membrane of plant chloroplasts. The integral membrane proteins TatA, TatB, and TatC are essential components of the Tat pathway. TatA forms high order oligomers and is thought to constitute the protein-translocating unit of the Tat system. Cysteine scanning mutagenesis was used to systematically investigate the functional importance of residues in the essential N-terminal transmembrane and amphipathic helices of Escherichia coli TatA. Cysteine substitutions of most residues in the amphipathic helix, including all the residues on the hydrophobic face of the helix, severely compromise Tat function. Glutamine 8 was identified as the only residue in the transmembrane helix that is critical for TatA function. The cysteine variants in the transmembrane helix were used in disulfide mapping experiments to probe the oligomeric arrangement of TatA protomers within the larger TatA complex. Residues in the center of the transmembrane helix (including residues 10–16) show a distinct pattern of cross-linking indicating that this region of the protein forms well defined interactions with other protomers. At least two interacting faces were detected. The results of our TatA studies are compared with analogous data for the homologous, but functionally distinct, TatB protein. This comparison reveals that it is only in TatA that the amphipathic helix is sensitive to amino acid substitutions. The TatA amphipathic helix may play a role in forming and controlling the path of substrate movement across the membrane.

Protein export across the cytoplasmic membrane of prokaryotes used two parallel, but mechanistically distinct, pathways. The Sec system (1) transports proteins in an extended conformation, whereas the Tat apparatus (2–6) functions to translocate folded proteins. Both Sec and Tat pathways are conserved in the thylakoid membrane of plant chloroplasts (5, 6).

The structure of the Sec translocase has been determined (7), and rapid progress is being made in understanding the molecular basis of Sec transport. By contrast, the mechanism by which the Tat pathway is able to undertake the challenging task of transporting folded proteins across a membrane while maintaining the ionic permeability barrier of that membrane is still almost completely obscure.

In Escherichia coli the Tat system is minimally composed of the three integral membrane proteins TatA, TatB, and TatC (8–11). TatA and TatB are sequence-related proteins. They are predicted to share a common structure comprising an N-terminal transmembrane α-helix, followed by a basic amphipathic α-helix, and then a water-soluble C-terminal region of variable length (Fig. 1A). Genetic analysis has shown that TatA and TatB have discrete roles in the E. coli Tat pathway despite their sequence similarity (8, 10). TatB forms a complex with TatC (12, 13). This TatBC complex acts as the substrate receptor for the Tat pathway (13, 14). TatA, by contrast, forms homo-oligomeric complexes that contain varying numbers of TatA protomers (11, 13, 15, 16). Electron microscopy of these TatA complexes shows ring-like structures of variable diameter that are likely to constitute the protein translocating channels of the Tat system (16). Because TatA is proposed to be the protein-conducting element of the Tat system, understanding structure-function relationships in TatA will be key to elucidating how the Tat apparatus is able to transport folded proteins across ionically tight membranes.

Currently only limited information is available about the functionally important regions of the TatA molecule. Truncation experiments (17) as well as sequence conservation (2) indicate that the essential portions of TatA reside within the N-terminal transmembrane and amphipathic helices. Site-directed (18–20) and random mutagenesis (21) studies have enabled the identification of some functionally important residues. Mutagenesis studies have also been reported for the chloroplast TatA ortholog Tha4 (22, 23).

Little information is available on the arrangement of the TatA protomers within TatA complexes, although protein engineering experiments show that the transmembrane helix is essential for the formation of stable oligomers (24). Gene fusion experiments have been used to suggest that the amphipathic
helix of TatA may change topology in the course of protein translocation (25), whereas disulfide cross-linking experiments carried out with the chloroplast Tha4 protein also suggest a rearrangement of the amphipathic helix during transport (23). It is likely that the amphipathic helix either forms or controls access to the pathway of transmembrane substrate movement (6, 16, 23).

An alternative approach to understanding TatA structure-function relationships is to identify which features of the TatA molecule distinguish it from the homologous, but functionally distinct, TatB protein. Domain swapping experiments between the two proteins have been used to infer that the transmembrane helix confers the distinctive properties of TatA, whereas the amphipathic helix is necessary specifically for TatB function (17). More recently, a genetic suppressor selection was used to isolate TatA variants that could simultaneously substitute for both wild-type TatA and TatB functions (26). These variants have amino acid substitutions in the early part of the TatA transmembrane helix.

To obtain a more complete picture of structure-function relationships within the TatA protein we have carried out systematic cysteine scanning mutagenesis of the entire functionally essential region of the TatA molecule. These studies have identified so far unrecognized residues that are important for TatA function. Scanning disulfide mapping experiments have been used to identify regions of the TatA molecule that are involved in forming protomer-protomer interactions. We have also compared the cysteine scanning mutagenesis and disulfide mapping data obtained with TatA to equivalent data that we previously reported for the homologous, but functionally distinct, TatB protein (27). This comparison reveals differences between the two proteins in both functionally important residues and protomer-protomer interactions, which help in understanding the distinct roles of TatA and TatB in the Tat protein transport system.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—**TMAO$^2$ reductase activity and cysteine cross-linking assays were performed in strain DADE-P (as MC4100 (28) and ΔtataBCΔtatE, pcnB1 zad-981::Tn10d (Km$^r$) (27)). Strain GB100 (as MC4100, pcnB1 zad-981::Tn10d (Km$^r$), dsbA::Cm, ΔtatABC::aac3(III)IV (27)) was used to test the possible involvement of the Dsb system in the in vivo formation of disulfide cross-links between certain single cysteine TatA variants.

Plasmid pUNITATCC4 (27) is pQE60 (Qiagen) carrying a tatABC gene cluster modified to replace all Cys codons (which are all located in tatC) with Ala codons and to contain silent mutations that introduce convenient restriction sites. Plasmid pUNITATA contains the tatA gene in pBluescript II SK$^-$ (Stratagene). pUNITATA was constructed by amplifying the tatA gene with primers TATA5 (8) and UNIA1 (5), introducing site-specific mutations are available on request. Plasmid pUNITATA contains the tatA gene in pBluescript II SK$^-$ (Stratagene). pUNITATA was constructed by amplifying the tatA gene with primers TATA5 (8) and UNIA1 (5), digesting the product with EcoRI and BamHI, and then cloning it into pBluescript II SK$^-$ that had been previously digested with the same enzymes. Site-specific cysteine mutations were initially introduced into the tatA gene in pUNITATA using the QuikChange$^TM$ method (Stratagene). Each mutation was subsequently subcloned into pUNITATCC4 for cysteine cross-linking and TMAO reductase assays. All clones obtained from PCR-amplified DNA were sequenced to ensure that no undesired mutations had been introduced. The primer sequences for introduction of site-specific mutations are available on request.

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*2 The abbreviations used are: TMAO, trimethylamine-N-oxide; CuP, copper(II) phenanthroline; NEM, N-ethylmaleimide; MOPS, 4-morpholinepropane-sulfonic acid.*
Cell Growth, Fractionation, and Protein Methods—During all genetic manipulations, and for cysteine cross-linking experiments, *E. coli* strains were grown aerobically in Luria-Bertani (LB) medium at 37 °C. Concentrations of antibiotics were as described previously (10, 30).

Membranes for cysteine cross-linking experiments were prepared from cells harvested by centrifugation and then resuspended in 20 mM MOPS·NaOH, pH 7.2, 200 mM NaCl supplemented with DNase I, 0.6 mg ml⁻¹ lysozyme, and protease inhibitors (Roche Applied Science, Complete EDTA-free protease inhibitor mixture). Cells were disrupted by three passages through a French press unit at 8,000 p.s.i., and cell debris was removed by centrifugation at 10,000 × g and 4 °C for 15 min. The total membrane fraction was then collected by centrifugation at 150,000 × g and 4 °C for 90 min. The membrane pellet was resuspended in 50 mM Tris·HCl, pH 7.5, 5 mM MgCl₂, 10% (v/v) glycerol, and aliquots were stored at −80 °C until use.

For TMAO reductase assays cells were cultured anoxically at 37 °C in 30 ml of modified Cohen and Rickenberg medium (31) supplemented with 0.2% glucose and 0.4% TMAO. Subcellular fractions were prepared using a cold osmotic shock protocol (32), and TMAO:benzylviologen oxidoreductase activity was measured as described (33).

SDS-PAGE and immunoblotting analyses were as described before (34, 35), and immunoreactive bands were visualized with the ECL detection system (Amersham Biosciences). Polyclonal TatA antibodies were as described (36). Protein concentrations for the TMAO reductase assays were estimated by the method of Lowry et al. (37) and for the cysteine cross-linking experiments using the Bio-Rad DC protein assay kit.

RESULTS

Cysteine Scanning Mutagenesis—We employed cysteine scanning mutagenesis to systematically probe the importance of residues in the *E. coli* TatA protein. Previous studies have shown that the C-terminal tail of TatA is not essential for Tat transport (17, 21). We therefore restricted our analysis to residues 2–43 within the critical N-terminal region. This range of substitutions contains the predicted transmembrane and amphipathic helices (Fig. 1A). Each mutant *tatA* allele was introduced into an expression plasmid carrying a *tatABC* operon that had been modified to remove all endogenous cysteine codons (i.e. the four in TatC). It has previously been demonstrated that a cysteineless TatC protein does not display significant defects in Tat-dependent transport (38).

The effect of individual cysteine substitutions on the transport activity of the Tat system was examined by assessing the extent to which the variant-coding plasmid could restore the Tat transport activity of a strain lacking Tat function. Tat transport activity was quantified in these experiments by measuring the amount of the Tat substrate trimethylamine-N-oxide reductase that reaches the periplasm.

All variants with cysteine substitutions in the predicted transmembrane helix retained significant levels of Tat transport activity with the notable exception of the Gln-8 to Cys substitution (Fig. 2A). The distinctive phenotype of the Gln-8 substitution prompted us to test the side-chain requirement at this position in more detail (Fig. 3). Replacement of the glutamine by asparagine, which retains the functionality of the side chain but decreases its length by one carbon atom, did not significantly affect the activity of the TatA protein. Substantive transport activity was also retained upon substitution of the glutamine by glutamate, a change that modifies the hydrogen-bonding potential of the side chain and introduces a potentially ionizable group. However, a variant in which the side-chain functionality was removed by an alanine substitution was, like the cysteine substitution, not able to support Tat transport.

In marked contrast to the tolerance of the transmembrane helix to cysteine substitutions the majority of the cysteine substitutions in the amphipathic helix abolished Tat activity (Fig. 2B). Of the seven mutants that retained significant activity, three were conservative serine to cysteine substitutions. Disulfide Cross-linking—The availability of a set of single cysteine variants of TatA presented an opportunity to use disulfide cross-linking to probe the interactions between the protomers in TatA complexes in their native membrane environment. This approach is expected to be particularly useful in analyzing the interactions of the predicted transmembrane helix, because...
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FIGURE 3. Relative Tat transport activities of variants of TatA with substitutions at position 8. Tat transport activity was assessed by measuring the activity of the Tat substrate TMAO reductase in the periplasmic fraction of cells grown in TMAO-containing medium. Data are shown for strain DADE-P (ΔtatABCDΔtatE pcnB) containing no plasmid ("None"), transformed with plasmid pUNITATCC4, which expresses cysteineless TatABC ("WT"), or containing derivatives of pUNITATCC4 in which the tatA gene encodes specific single amino acid variants at position 8 (the nature of the substitution in the TatA protein is shown under each column). All activities are quoted relative to that of the strain carrying the parental plasmid pUNITATCC4. Three independent cultures of each strain were each assayed in triplicate. Error bars indicate the standard error of the mean.

This helix is essential for TatA oligomerization (24) and is, thus, presumably in direct contact with the transmembrane helices of other TatA protomers. A cysteine residue in the transmembrane helix of one protomer should be at the same vertical height in the membrane as the same cysteine residue in another protomer. Whether the two cysteine residues can approach closely enough to form a disulfide bond will, therefore, depend on the relative arrangement of the helices within the plane of the membrane (Fig. 1B).

To determine the disulfide-crosslinking patterns of single cysteine TatA variants we co-expressed each variant with TatBC using the same plasmids that were employed for the activity studies. Subcellular fractionation of the strains followed by immunoblotting with anti-TatA serum established that all our cysteine variants were expressed and localized to the membrane fraction of the cells although some variation in TatA levels was noted (Figs. 4–6).

It was immediately apparent for variants with cysteine substitutions at the N terminus of the transmembrane helix (residues 2–8) that TatA was present in the purified membrane fractions predominantly in the form of a cross-linked dimer (Fig. 4A). To test whether these cross-links had been formed in vivo we treated cells expressing the TatA variants with the membrane-permeant thiol-specific reagent N-ethylmaleimide (NEM) prior to harvesting. NEM will react with any free cysteine thiols present in the TatA variants in vivo and thus prevent these residues forming disulfide bridges during cell fractionation. NEM treatment decreased the proportion of TatA present in disulfide-linked dimers (Fig. 4B). We, therefore, conclude that the close to quantitative cross-linking exhibited in purified membranes by the cysteine variants at positions 2–8 takes place partially in vivo and partially during preparation of the membranes.

The observation that cysteine variants at positions 2–8 exhibit substantive in vivo disulfide bond formation suggests that this region of TatA may be exposed to the oxidizing environment at the periplasmic side of the membrane. The major route of disulfide bond formation in E. coli is the periplasmic DsbAB system in which DsbA is the primary oxidant (39). However, the cross-linking pattern of the position 2–8 variants was not altered when the variants were expressed in a dsbA null background (data not shown). The observation that all cysteine variants at positions 2–8 show strong disulfide cross-linking suggests that this region of the protein has high conformational flexibility. Because the position 2–8 variants are almost fully cross-linked following isolation of the membranes, no further analysis of the cross-linking propensity of this region of the TatA molecule was possible.

Cysteine variants in the amphipathic helix also generally exhibited significant dimer formation following membrane purification (Fig. 5A). However, in complete contrast to the behavior of the position 2–8 variants no disulfide cross-links were seen for the amphipathic helix variants following in vivo NEM treatment (Fig. 5B). We conclude that the disulfide bonds seen in the membrane samples of the amphipathic helix variants form during cell fractionation. Importantly, this allows us
to exclude the possibility that the lack of Tat activity exhibited by the amphipathic helix cysteine variants (Fig. 2B) is due to formation of cross-links between protomers. The poor functionality of the cysteine substitutions at most positions in the amphipathic helix means that it is not justified to use the disulfide cross-linking pattern in this region to infer details of the structure of the parental TatA protein. For this reason no further disulfide cross-linking studies of the amphipathic helix were carried out.

Cysteine variants in both the amphipathic helix and the N terminus of the transmembrane helix show small populations of a TatA-containing species with a mobility slightly greater than that of monomeric TatA (Figs. 4 and 5). This species is unchanged by treatment with the oxidation catalyst copper(II) phenanthroline (CuP) or with chemical reductants such as dithiothreitol. From these observations we infer that the cysteine residue in these molecules forms a thioether linkage to a lipid component.

Variants with cysteine substitutions at residues 9–20 were not significantly cross-linked in isolated membranes (Fig. 6). We were, therefore, able to assess the ability of these variants to form self-self disulfide cross-links under controlled conditions using CuP as a membrane-permeant catalyst of the oxidative cross-linking reaction. The extent of cross-linking in these experiments was assessed by non-reducing SDS-PAGE analysis of the treated samples followed by immunoblotting with TatA-specific antiserum. It was important to avoid the possibility that the TatA variants could become artificiually cross-linked when removed from their native membrane environment following solubilization under denaturing conditions for the SDS-PAGE analysis. To this end samples were treated with NEM before solubilization to derivatize any remaining free thiol groups and thereby prevent them forming disulfide bonds. EDTA was also added to chelate the Cu(II) cofactor from the CuP catalyst. Control experiments of the type shown for the Ile12Cys variant added to chelate the Cu(II) cofactor from the CuP catalyst. Control experiments of the type shown for the Ile12Cys variant (Fig. 2B) were performed with the quenching agents for 10 min prior to CuP addition, and the sample was then incubated for a further 60 min. The extent of cross-linking was assessed by SDS-PAGE analysis under non-reducing conditions followed by immunoblotting with anti-TatA serum. The monomer and dimer of TatA are indicated with one or two stars, respectively. The uncharacterized TatA adduct is indicated by a horizontal bar.
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FIGURE 7. Time courses of CuP-dependent cross-linking of cysteine residues in the core of the TatA transmembrane helix. Membrane samples were prepared from E. coli strain DADE-P (ΔtatABCDΔtatE puc8) containing plasmid pUNITATCC4 encoding cysteineless TatBC together with the indicated single cysteine TatA variant. Samples (30 μg of total protein) were incubated at 20 °C for the indicated time points in the presence of 1 mM of the oxidation catalyst CuP. Reactions were quenched by the addition of 25 mM NEM and 50 mM EDTA. The extent of cross-linking was assessed by SDS-PAGE under non-reducing conditions followed by immunoblotting with anti-TatA serum. The TatA monomer and dimer are indicated with one or two stars, respectively.

To differentiate more fully between the cross-linking efficiencies of these cysteine substitutions, the relative kinetics of disulfide bond formation were determined by running time-course experiments (Fig. 7). TatA variants containing cysteine substitutions at residues 9, 12, and 17–20 exhibited virtually complete cross-linking after only 30 s (only data for positions 9 and 12 are shown in Fig. 7). Variants with substitutions at residues 10 and 11 were also quantitatively cross-linked but on a slightly longer time scale. Cysteine variants at position 13–16 exhibited slower rates of cross-linking than residues 9–12 with cross-linking at position 13 having the slowest kinetics of any of the residues in the transmembrane helix. Some variants showed small variations in cross-linking rates between repeat experiments. Nevertheless, an order of reaction rates in which 9/12 > 10/11 > 14–16 > 13 was consistently obtained.

DISCUSSION

Cysteine Scanning Mutagenesis—We have used cysteine scanning mutagenesis to systematically examine the functional importance of each residue within the functionally essential N-terminal half of the E. coli TatA protein. We find that cysteine substitutions can be tolerated at all positions in the predicted transmembrane helix except Gln-8 (Figs. 2A and 8A). By contrast, cysteine substitutions at the majority of positions in the proposed amphipathic helix are incompatible with retention of Tat function (Figs. 2B and 8A). This difference in behavior between the two helices is unexpected given the similar levels of sequence conservation exhibited by each region (2) but is consistent with the results of an earlier, less comprehensive, random mutagenesis study of tatA (18). Control experiments exclude the possibility that the negative effects of any of the cysteine substitutions are due to formation of disulfide links in vivo (Fig. 5B).

The cysteine scanning data are consistent with the view that the primary function of the transmembrane helix is oligomerization of TatA (24). Such packing interactions are likely to involve multiple contacts and therefore may be relatively resistant to disruption by single amino acid substitutions. Gln-8 was the only residue within the transmembrane helix that could not be functionally substituted with cysteine (Fig. 2A). This residue is highly conserved in Proteobacterial TatA molecules as either glutamine or histidine and is the only polar residue in the hydrophobic core of the transmembrane helix (2). We were able to functionally substitute this residue with asparagine or glutamate but not alanine (Fig. 3), observations that confirm the importance of the side-chain functionality at this position. Polar residues with side chains that can act as both a good hydrogen bond donor and a good acceptor (i.e. Asn, Asp, Gln, Glu, and His) are implicated in driving association of transmembrane helices (40–42). Thus Gln-8 might play a crucial role in helix oligomerization.

The amphipathic helix appears to undergo conformational change during transport (23, 25) and is thought to form or control access to the pathway of transmembrane substrate movement (6, 16). The exquisite sensitivity of the amphipathic helix to cysteine substitutions is remarkable and confirms a key role for this region of the protein in the Tat transport mechanism. We note, in particular, that every cysteine substitution on the hydrophobic face of the amphipathic helix is inactivating. An earlier study using a genetic selection found that point mutations that inactivate Tat transport also cluster on the non-polar face of the helix (21). The cysteine side chain is non-polar. Thus the inactivating cysteine substitutions on the hydrophobic face of the amphipathic helix are unlikely to act by perturbing the amphipathic character of the helix but must, instead, disrupt some more exacting structural constraint. Almost half of the functional cysteine substitutions that are found on the polar face of the amphipathic helix are of positions where serine residues are found in the wild-type protein, which argues for a role in side-chain size in the functionality of the amphipathic region. It is possible that the structural constraints arise from the need to form specific protein-protein packing interactions either with other amphipathic helices or with TatBC. In this context it is worth noting that the glycine residues at positions 29 and 33 form a Gly-Xaa-Xaa-Gly motif and that this, or the related motifs Gly-Xaa-Xaa-Xaa-Ala and Ala-Xaa-Xaa-Gly, are strongly conserved in bacterial TatA sequences. Such Gly-Xaa-Xaa-Xaa-Gly-based motifs have been demonstrated to promote helix-helix interactions in both membrane and soluble proteins (43, 44). Certainly substitution of either Gly-29 or Gly-33 with cysteine blocked Tat function (Figs. 2B and 8A), and it may be significant that substitution of Gly-33 with either serine or aspartate interferes with TatA oligomerization in membranes as judged by chemical cross-linking studies (21). The structural constraints on the TatA amphipathic helix may, alternatively, reflect a need for precise control of the physicochemical properties of the helix to enable appropriate interac-
tions with membrane phospholipids and/or the substrate protein. The amphipathic helix is known to interact with phospholipid bilayers (24). It has been suggested that the amphipathic helix could allow substrate transport by inducing lipid disorder in the membrane (23, 45). Alternatively, it has been proposed that the amphipathic helix of multiple TatA protomers could fold into the bilayer in response to force on the substrate with the polar faces of the helices contacting the substrate (6). It could also be envisaged that membrane bending resulting from insertion of a circle of amphipathic helices into the cytoplasmic leaflet of the bilayer opens an aqueous transmembrane channel. In any of these schemes the ability of the amphipathic helix to carry out its function would depend both on the amphipathic properties of the helix and on phospholipid packing interactions congruent with the observed sensitivity of the helix to structural alterations.

Cysteine mutagenesis data have been recently reported for Tha4, the TatA ortholog of the plant chloroplast Tat system (23). The effects of cysteine substitutions in the transmembrane helix of Tha4 closely parallel those reported here for E. coli TatA with the only inactivating substitution being that of a glutamate at the position corresponding to the essential Gln-8 in TatA (Fig. 8A). This glutamate is sensitive even to substitution to glutamine or aspartate (22). The set of cysteine substitutions reported for the amphipathic helix of Tha4 is less comprehensive than the scanning mutagenesis reported here for the amphipathic helix of E. coli TatA (Fig. 8A) making a comparison of the phenotypes of mutations in this region difficult. Nevertheless, for the cysteine replacements that are common to the two proteins, the Tha4 substitutions always retain Tat transport activity even when the equivalent TatA variant is inactive. This was unexpected but may reflect the fact that TatA and Tha4 are quite divergent at the amino acid sequence level. Indeed, Tha4 exhibits far greater sequence similarity to the thylakoid TatB ortholog Hcf106 than it does to E. coli TatA. Our observations emphasize that care should be exercised in interpolating data obtained for Tha4 onto TatA and vice versa.

**Disulfide Cross-linking**—Site-specific disulfide cross-linking was used to probe the interactions between the transmembrane helices of protomers within TatA complexes. The observed cross-links confirm the earlier conclusion from protein engineering studies that TatA protomers interact through their transmembrane helices (24). Contiguous residues at both helix ends showed similar strong disulfide cross-linking suggesting

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**FIGURE 8. Summary of the cysteine scanning mutagenesis and disulfide cross-linking data obtained for TatA and for TatA homologues.** A, comparison of the effects on Tat transport of single amino acid substitutions between the homologous proteins TatA and TatB proteins of E. coli and the TatA ortholog Tha4 from pea chloroplasts. The sequences of the three proteins are aligned to each other. Each sequence is annotated to show the amino acid positions where cysteine substitutions have been experimentally introduced (this study for TatA; Ref. 27 for TatB; Ref. 23 for Tha4). Residues in blue can be substituted with cysteine without seriously affecting the activity of the Tat pathway. However, cysteine substitutions of red residues severely affect, or abolish, Tat transport. Positions in TatA where inactivating substitutions were identified in a genetic screen for loss of Tat function (21) are shown by a dot over the appropriate position. The secondary structure elements of TatA are indicated schematically above the TatA sequence. The sequence numbering refers to the TatA protein. B, comparative rates of self-self disulfide bond formation between single cysteine variants in the transmembrane helix of E. coli TatA. The core of the predicted transmembrane helix is shown in helical wheel projection. Different colors are used to indicate for each sequence position the relative rates of disulfide cross-linking expressed as the time taken to obtain 50% cross-linking (t1/2). C, comparative propensity for self-self disulfide bond formation between single cysteine variants in the transmembrane helix of E. coli TatB. The core of the predicted transmembrane helix is shown in helical wheel projection. The extent of disulfide cross-linking after incubation for 60 min with 2 mM CuP is given for each position (data from Ref. 27).
that the helix ends have high conformational mobility (Figs. 4A and 6). By contrast, time-course experiments revealed clear differences in strength of the cross-links around the central portion of the transmembrane helix (Fig. 7). This suggests that this portion of the transmembrane has a well-defined structure. If the rates of disulfide cross-linking at different positions in this central core of the transmembrane helix are plotted on a helical wheel, it is clear that TatA is forming self-self interactions on more than one face (Fig. 8B). The fastest cross-linking was found at positions 9 and 12, which are on the same face as the functionally critical residue Glu-8. The helix face containing these residues is likely to be particularly important for TatA self-self interaction.

The possibility has been raised that TatA/Tha4 could function by undergoing transport-related cycles of polymerization and depolymerization (16, 23). The defined disulfide cross-linking patterns we have observed for the core of the TatA transmembrane helix under non-transporting conditions could be taken as evidence that TatA is found as oligomers in the resting state. However, it is also possible that the cross-links stabilize a putative assembled state of TatA. In either case the disulfide links we observe would be expected to faithfully report the modes of interactions between TatA protomers.

Identification of Structural and Function Differences between TatA and TatB Proteins from Comparative Scanning Mutagenesis and Disulfide Cross-linking Data—The Tat system of E. coli requires both TatA and its homologue TatB. Whereas TatA forms a homo-oligomeric complex that is thought to form the protein-conducting channel of the Tat system, TatB forms part of the substrate receptor complex with TatC. The structural features responsible for the distinct functional roles of these two homologous proteins are still unclear. We recently reported cysteine scanning mutagenesis and scanning disulfide cross-linking studies of TatB performed under essentially the same experimental conditions employed in the current studies of TatA (27). Comparison of the data sets obtained for the two proteins gives insight into the structural differences responsible for the differing functions of TatA and TatB.

The cysteine scanning mutagenesis experiments show remarkable differences in the sensitivity of TatA and TatB toward amino acid substitutions. For TatA the majority of substitutions in the amphipathic helix, as well as at Glu-8 in the transmembrane helix, abolish Tat transport activity (Figs. 2 and 8A). By contrast, all substitutions tested in TatB retained Tat transport function (Fig. 8A). Given that the amphipathic helix of TatB is essential for Tat function (17), the observation that this helix is tolerant to single amino acid substitutions suggests that the helix has a structural role that is relatively resistant to disruption by single amino acid replacements. The structure of the TatA amphipathic helix, by contrast, is clearly much more highly constrained by its function. Indeed, the TatA amphipathic helix shows a greater degree of sequence conservation than the TatB amphipathic helix (2). The greater structural constraints on the TatA amphipathic helix would be consistent with the proposed involvement of this helix in the protein transport pathway in TatA (discussed above) but not in TatB. The transmembrane helix of TatB contains a highly conserved glutamate at the same position as critical Glu-8 in TatA (Fig. 1A). Nevertheless, substitution of TatB Glu-8 with either cysteine (17) or alanine (18, 19) is well tolerated showing that, in contrast to TatA Glu-8, this residue does not have a crucial mechanistic or structural role.

As in TatA, the pattern of disulfide cross-linking in the transmembrane helix of TatB suggests that the transmembrane helix has a defined helical core structure with greater conformational flexibility at the helix ends, an interpretation that is consistent with molecular dynamics simulations of the TatB transmembrane helix (27). Strikingly, however, although the core of the transmembrane helix in TatA demonstrates self-self disulfide cross-linking interactions on opposite faces of the helix (Fig. 8B) the self-self interactions in TatB are found on only one side of the helix (Fig. 8C). These differences in the pattern of protomer-protomer contacts between TatA and TatB are consistent with the higher oligomeric state of the TatA protein and the need for TatB to form protein-protein contacts with TatC.

Concluding Remarks—The mutagenesis studies described here reveal that there are exacting constraints on the structure of the amphipathic helix of TatA if it is to fulfill its role in Tat transport. In addition they have identified Glu-8 in the transmembrane N-terminal helix of TatA as critically important for Tat function. Scanning disulfide mapping experiments show that TatA protomers interact through their transmembrane helices and that these interactions occur on more than one face of the helix. These cross-linking studies further indicate that the transmembrane helix contains a stable helical core.

A comparison of the results of this study of TatA with analogous experiments carried out on the homologous TatB protein have enabled us to identify structural differences between the two proteins that may underlie their distinct roles in Tat transport in E. coli. The most notable difference is that, whereas the amphipathic helix in TatA is exquisitely sensitive to amino acid substitutions, the amphipathic helix in TatB is robust to such substitutions. This suggests that the precise structure of the amphipathic helix is critical for TatA function. We speculate that this is because this helix is likely to be involved in forming and controlling the path of transmembrane protein movement in the Tat system.

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