The Chloroplast Twin Arginine Transport (Tat) Component, Tha4, Undergoes Conformational Changes Leading to Tat Protein Transport*

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Background: Tha4 is the assumed cpTat protein conduit for transporting folded precursors across thylakoid membranes.

Results: During assembly into active Tat translocases, the Tha4 amphipathic helix (APH) and adjacent C-tail undergo accessibility changes.

Conclusion: Translocase assembly involves more extensive membrane partitioning of Tha4 APH and packing of C-tails into a mesh-like network.

Significance: Conformational change of Tha4 within active translocases leads to Tat protein transport.

Twin arginine transport (Tat) systems transport folded proteins using proton-motive force as sole energy source. The thylakoid Tat system comprises three membrane components. A complex composed of cpTatC and Hcf106 is the twin arginine signal peptide receptor. Signal peptide binding triggers assembly of Tha4 for the translocation step. Tha4 is thought to serve as the protein-conducting element, and the topology it adopts during transport produces the transmembrane passageway. We analyzed Tha4 topology and conformation in actively transporting translocases and compared that with Tha4 in nontransporting membranes. Using cysteine accessibility labeling techniques and diagnostic protease protection assays, we confirm an overall N\textsubscript{OUT}-C\textsubscript{IN} topology for Tha4 that is maintained under transport conditions. Significantly, the amphipathic helix (APH) and C-tail exhibited substantial changes in accessibility when actively engaged in protein transport. Compared with resting state, cysteines within the APH became less accessible to stromally applied modifying reagent. The APH proximal C-tail, although still accessible to Cys-directed reagents, was much less accessible to protease. We attribute these changes in accessibility to indicate the Tha4 conformation that is adopted in the translocase primed for translocation. We propose that in the primed translocase, the APH partitions more extensively and uniformly into the membrane interface and the C-tails pack closer together in a mesh-like network. Implications for the mode by which the substrate protein crosses the bilayer are discussed.

Plant thylakoid membranes, like many bacterial cytoplasmic membranes, contain two pathways to export soluble proteins (1). The well described Sec system transports unfolded substrate proteins coupled to ATP-binding and hydrolysis. The twin arginine transport (Tat) system operates in parallel but transports folded precursor proteins, coupled to the proton-motive force. Tat systems are so-named by the presence of obligate twin arginine residues in the signal sequences of precursors. Three membrane-bound components in plant thylakoids (and most bacteria) are required for Tat transport: Tha4 (TatA), Hcf106 (TatB), and cpTatC (TatC). Tha4 and Hcf106 are similar proteins, both possessing a predicted N-terminal transmembrane domain (TMD) followed by a hinge region, a predicted amphipathic \alpha helix (APH), and a loosely structured carboxyl domain (C-tail). cpTatC contains six predicted transmembrane domains (2). Tha4 and Hcf106, although structurally very similar, are functionally distinct (3). The thylakoid Tat system (cpTat) operates in a cyclical fashion whereby the signal peptide-containing precursor first binds to a large cpTatC-Hcf106 receptor complex in which cpTatC forms the primary twin arginine receptor (3). Precursor binding and the proton-motive force trigger assembly of Tha4 for translocation, during which Tha4 organizes into higher order homo-oligomers (4, 5). After transport Tha4 dissociates, thus resetting the system for subsequent rounds of transport (6). A large oligomer of Tha4 is thought to form the point of passage for the precursor and the manner by which this is achieved has been the subject of considerable speculation. Early studies suggested a N\textsuperscript{OUT}-C\textsubscript{IN} topology for Tha4 (TatA) (7–9). This topology is inconsistent with channel models of the Tat mechanism that propose TatA oligomers form channels lined by the hydrophilic faces of the APH. However, two studies indicate that TatA undergoes...

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3 The abbreviations used are: Tat, twin arginine transport; TMD, transmembrane domain; APH, amphipathic helix; C-tail, carboxyl tail; Br-PEG, branched-PEG (methyl-polyethylene glycol\textsubscript{12}–polyethylene glycol\textsubscript{4}–maleimide cis, stromal (in) side of thylakoid membrane or cytoplasmic side of bacterial plasma membrane; \textit{trans}, lumen (out) side of thylakoid membrane or periplasmic side of bacterial plasma membrane; SA, streptavidin; IB, import buffer; Tricine, \(N\)-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
dynamic topology changes during transport of precursors (10, 11). In both studies, the C-tail of TatA was reported to be accessible from both the periplasm and cytoplasm of Escherichia coli suggesting a dual topology of the C terminus that is postulated to be dependent on the stage of transport. Both groups proposed that TatA exists in two states: a single membrane-spanning state in which only the N-terminal TMD spans the plasma membrane, and a double membrane-spanning state in which the amphipathic helix also spans the membrane. This double membrane-spanning state could potentially form the channels postulated in the channel model. A more recent study confirms an N_OUT topology for TatA but did not detect any topological reorganization upon overexpression of a Tat precursor (12). In all of these previous studies, the topologies reported could not be directly linked to protein transport and the topology of TatA in an actively transporting translocase remains under speculation.

Here we investigated the topology of Tha4 in thylakoid membranes undergoing active transport in an effort to demonstrate the conformation of Tha4 in the active translocase. One advantage of the cpTat system is the ability to assay topological or conformational changes and directly link those changes to stages of the transport reaction. In addition, recombinant Tha4 can functionally replace endogenous Tha4 for in vitro assays, thereby speeding the tagging and mutagenesis process (4, 13, 14). Thus we have substituted single Cys residues at diagnostic locations throughout the Tha4 protein and determined their accessibility to membrane permeant and impermeant Cys reactive reporters. Similarly, specific protease cleavage sites were placed in Tha4 domains and tested for protease accessibility. Our results confirm that in energized but nontransporting membranes, Tha4 is organized with the C-tail exposed to the stromal (cis or IN) side of the membrane; the amphipathic helix is also located on the stromal side but is only partially partitioned into the membrane-bulk phase interface. The putative transmembrane domain is buried in the bilayer with its N terminus exposed to the thylakoid lumen (trans or OUT). In actively transporting translocases, the amphipathic helix and carboxyl proximal flanking region undergo accessibility shifts, with a “masking” of previously accessible Cys residues in the central portion of the APH and a reduction in protease accessibility of the adjacent C-tail. We interpret these results to mean that in the active translocase the APH partitions more uniformly and deeply into the membrane interface and that the C-tails pack closer together, possibly forming a net around the precursor protein. We suggest that these conformational changes lead to localized disruptions of the membrane bilayer and provide directionality for precursor membrane crossing.

EXPERIMENTAL PROCEDURES

Preparation of Chloroplasts and Thylakoids

Intact chloroplasts were prepared from 10–12-day-old pea seedlings (Pisum sativum L. cv. Laxton’s Progress 9 or Little Marvel). Chloroplasts were suspended to 1 mg/ml of chlorophyll in lysis buffer (50 mM HEPES-KOH, pH 8.0, and 300 mM sorbitol) and kept on ice until use. Isolated thylakoids were obtained by osmotic lysis of intact chloroplasts. Briefly, intact chloroplast suspensions were pelleted at 1000 × g, supernatant was removed and suspended at 1 mg/ml of chlorophyll in lysis buffer (50 mM HEPES-KOH, pH 8.0, 10 mM MgCl₂), and incubated on ice. After 5 min, lysates were adjusted to IB and 10 mM MgCl₂, the thylakoids were pelleted at 3300 × g for 8 min and washed. For biotinylation or protease treatments, thylakoids were suspended in IB, 10 mM MgCl₂ and stored on ice until use. For Br-PEG labeling (see below), thylakoids were suspended in IB, pH 7.5. Chlorophyll was measured according to Arnon (15).

Generation of Cysteine-substituted cpTatC, Mature Tha4, and Tha4 Containing Protease Cleavage Sites

Tha4 proteins with cysteine substitutions (Tha4X_C) were generated by QuikChange mutagenesis (Agilent Technologies) according to the manufacturer’s instructions. The template used for the mutagenesis was the coding sequence for Tha4 (lacking the target peptide) from pea as described (7). The coding sequence begins MAFFGLGVPE…, QuikChange was also used to insert a thrombin cleavage site (LVPRGS) at the very end of the C-tail of Tha4 followed by a His₁₆ affinity tag (Tha4CT-Throm) and to insert a thrombin cleavage site at the amino proximal end of the C-tail after residue Ile⁶² giving the sequence: ⁶²ILVPRGS⁶³ (Tha4I62-Throm). All amino acid substitutions were confirmed by sequencing both strands of the DNA at the Center for Bioinformatics and Functional Genomics, Miami University, or at the University of Florida Interdisciplinary Centre for Biotechnology Research DNA Sequencing Core Facility. All modified Tha4 proteins were assayed for functionality with an in vitro complementation assay to restore transport of the precursor DT23 or tOE17V-20F as described previously (4, 13).

Generation of Other Constructs Used in this Study

The pre-cpTatC used here as a control protein is a modified version where the pSSU transit peptide is fused to the amino terminus of the native pre-cpTatC transit peptide to improve import efficiency, additionally, the three native Cys residues of the mature cpTatC have been replaced with alanines. tOE17V-20F is as described previously (16). The precursor protein SufI is from E. coli, except that the native signal peptide was replaced with the tOE17V-20F signal peptide using standard molecular biology techniques. SpF16, SpF12, and SpF8 are truncated versions of TOE17V-20F containing only the first 16, 12, or 8 residues of the mature protein, respectively. Truncations were generated using QuikChange mutagenesis (Agilent Technologies) by inserting stop codons into tOE17V-20F immediately after the indicated positions.

Preparation of Radiolabeled Mature Tha4 and Precursor Proteins

Radiolabeled mature Tha4, Tha4 variants, pre-cpTatC, and Tat precursor proteins, TOE17V-20F, SufI, SpF16, SpF12, and SpF8, were prepared by in vitro translation from capped mRNA using a wheat germ extract in the presence of [³H]leucine (17). Translation products were diluted 2-fold with 60 mM leucine in...
Tha4 Topology Shift Leading to Tat Precursor Transport

2× IB before use. Tat precursors were incubated with apyrase (0.15 units/μl) for 10 min at 0°C before use to hydrolyze ATP contained in the wheat germ extract. ATP hydrolysis by the ATP synthase can energize the thylakoid membranes and lead to premature transport of precursors.

Preparation of Overexpressed and Purified Monovalent Streptavidin

Monomeric dead (D) or alive (A) streptavidin was prepared according to the protocol by Howarth et al. (18, 19) from pET21a plasmid containing a dead (D; Addgene plasmid 20859) or alive (A; Addgene plasmid 20860) version of the monomer streptavidin. The A version also contained a His₆ affinity tag. Briefly, each form of streptavidin was overexpressed in BL21(DE3) cells and purified as inclusion bodies. The proteins were solubilized in 6 mM guanidinium chloride and mixed together at a 3:1 molar ratio, D:A. The tetramer was folded by rapid dilution out of guanidinium chloride with cold PBS (phosphate-buffered saline, pH 7.5). After dialysis and concentration, the tetramer was analyzed by SDS-PAGE at 0–4°C to avoid dissociation. Monovalent tetramer was purified by Ni²⁺-affinity chromatography, using standard procedures, eluting with 50 mM Tris-HCl, pH 7.8, 300 mM NaCl, 70 mM imidazole, followed by dialysis to equilibrate the monovalent tetramer in PBS (50 mM Na₂PO₄, pH 7.5, 500 mM NaCl). Protein concentration of the monovalent tetramer was determined using the BCA method (Pierce) according to the manufacturer’s instructions. The monovalent tetramer was subsequently used to detect biocytin labeling of Tha4 in thylakoids either before or after solubilization with detergent as described below.

Chloroplast Import

Import of radiolabeled pre-cpTatC was conducted with intact chloroplasts at 300 μg/ml in IB supplemented with 5 mM Mg₂⁺ATP and 5 mM dithiothreitol in the light (100–150 μE/m²/s) at 25°C for 15 min as described (20). After import, intact chloroplasts were isolated by centrifugation through 35% Percoll in IB at 3300 × g and the supernatant was removed. The pellet was suspended in excess IB and centrifuged at 1000 × g and the supernatant was removed. Chloroplasts were processed to isolate thylakoids as described above. Isolated thylakoids were used for detection of cysteines according to the method below.

Detecting Cysteine Location by Chemical Modification

Method I—Recombinant Cys-substituted Tha4 was integrated into thylakoids in the light (100–150 μE/m²/s) as described (4) and subject to centrifugation (3200 × g, 8 min), suspended in 5 volumes of IB, 10 mM MgCl₂, centrifugated, and a final suspension in IB, 10 mM MgCl₂ to ~1 mg/ml of chlorophyll (washing step). Washed thylakoids with integrated Tha4 were subjected to treatment with 500 μM biocytin maleimide (N⁴-(3-maleimidylpropionyl)biocytin, Invitrogen) at room temperature. Labeling was for 20 min followed by a 10 mM dithiothreitol (DTT) quench for 10 min at room temperature. Thylakoids were recovered from labeling reactions, washed with IB, 10 mM MgCl₂, suspended in IB, 10 mM MgCl₂ to ~1 mg/ml of chlorophyll, and then treated with monovalent streptavidin (0.42 mg/ml) at room temperature for 15 min before or after solubilization in 1% digitonin for 1 h. Samples were adjusted for equal chlorophyll concentration and one-tenth total volume of a 10× sample buffer (33.3 mM HEPES, pH 8.0, 75% sucrose, 50 mg of Serva G (Coomassie G)) was added. Samples were subjected to analysis by SDS-PAGE on ice followed by fluorography.

Method II—The water-soluble and -membrane impermeant (methyl-PEG)₃-PEG₄-maleimide (Pierce, Br-PEG) was used to detect stroma-exposed cysteines in Tha4. Cys-substituted in vitro translated Tha4 was integrated into thylakoids in the presence of tris(2-carboxyethyl)phosphine (1 mM final concentration) in 150 μE/m²/s white light for 20 min at 25°C. Thylakoids were then recovered by centrifugation (3300 × g, 8 min), washed with 1× IB, and suspended in 1× IB + 1 mM tris(2-carboxyethyl)phosphine at ~1 mg/ml of chlorophyll. Labeling with 0.5 mM Br-PEG was performed in the light at 20°C for 20 min in Figs. 1–3 and 5. For comparisons of Br-PEG labeling before and during protein transport (Figs. 6 and 7), after Tha4 integration, 62.5 μl of diluted in vitro translated precursor (or mock precursor) was added to 25 μl of thylakoids containing radiolabeled Tha4, and samples were incubated in the dark on ice for 15 min. Samples were transferred to 15°C for 3 min to equilibrate and then transferred to light at 15°C for 2.5 min to initiate transport. Br-PEG was added to a final concentration of 0.5 mM and labeling was performed for 2.5 min at 15°C. Labeling was stopped by the addition of 10 mM DTT. After labeling, thylakoids were washed, dissolved in sample buffer (125 mM Tris-HCl, pH 6.8), 4% SDS, 66% glycerol, 2 mM EDTA, 20 mM DTT), and separated on 12% Tris-Tricine gels followed by fluorography.

Protease Cleavage of Tha4

After integration of Tha4 containing protease cleavage sites, thylakoids were collected by centrifugation, washed with IB, and resuspended in IB, 10 mM MgCl₂ to ~1 mg/ml of chlorophyll. Precursor protein (100 μl of diluted in vitro translated precursor or 1 × IB) was added to 40 μl of thylakoids and samples were incubated on ice in the dark for 15 min. Samples were transferred to the light at 15°C for 2.5 min to initiate transport. Five units (Tha4I62-Throm) or 1.5 units (Tha4CT-Throm) of thrombin or 60 units of factor Xa were added and samples were taken at the time points indicated. Thrombin was inactivated by the addition of 10 mM PMSF (phenylmethylsulfonyl fluoride) + 10 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Sigma). To inactivate factor Xa, samples were mixed 1:1 with 2× SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 66% glycerol, 2 mM EDTA, 20 mM DTT) and heated at 95°C. Cleaved and uncleaved Tha4 were separated on 12% Tris-Tricine gels and detected by fluorography. Thrombin cleavage of Tha4I62-throm was quantified by scintillation counting in a Beckman LS 6500. Thrombin cleavage of Tha4CT-Throm was quantified by quantitative immuno blotting. Gels were electroblotted to 0.2-μm pore size polyvinylidene fluoride membranes and the His₆ tag on uncleaved Tha4CT-throm was detected with His₆ polyclonal antibody (Thermo Scientific) and enhanced chemiluminescence (ECL, GE Healthcare) according to the manufacturer’s instructions.
Films were scanned at 600 dpi in translucent mode using an Epson Perfection 3170 scanner followed by densitometry analysis with Quantity One software (Bio-Rad).

RESULTS

Labeling Solvent-exposed Sulfhydryls—The Tat translocase is a very transient structure, existing only during the translocation process (6). Analysis of Tha4 topology within the translocon requires methods that speedily label the protein and are rapidly quenched. We validated our approach by first investigating Tha4 topology under nontransporting conditions with a battery of methods as described below. The idea was to establish the topology of Tha4 before it assembles into the translocon as a solid foundation upon which to interpret the labeling results obtained under transport conditions. We used a modification of the substituted cysteine accessibility method (21) for identifying Cys residues exposed to the cis side of the membrane. The thiol-modifying reagents, biocytin maleimide or Br-PEG maleimide, were used to label single cysteines on recombinant Tha4 that were integrated into isolated thylakoid membranes. Biocytin maleimide is membrane permeable (22) and labels cysteines on either side of the membrane, but reacts poorly with Cys residues in the nonpolar hydrocarbon core of the membrane bilayer (23). To differentiate residues facing the stroma from those exposed to the thylakoid lumen, biocytin-treated thylakoids were subsequently tested for accessibility to streptavidin (SA) either with intact thylakoids to indicate stroma-accessible biocytin, or with digitonin-solubilized thylakoids to measure total biocytin labeling. SA binding is easily scored by a large band shift of the target protein on SDS-PAGE. Br-PEG was used to label stroma-accessible sulfhydryl groups directly because it is unable to cross the membrane (24) and produces a detectable band shift of the target protein on SDS-PAGE.

To test the efficacy of these approaches, they were applied to single Cys-substituted cpTatC, which has known topology (25). The three native cysteines of cpTatC were replaced with alanines and single cysteines were introduced into the stromal N-tail, a lumen-facing loop region, or the transmembrane domain (Fig. 1A). The precursor to cpTatC was imported into intact chloroplasts resulting in a mature sized protein that was localized to the thylakoid membrane. Thylakoids were then isolated and treated with biocytin (Fig. 1B) or Br-PEG (Fig. 1C). The location of biocytin was probed directly with SA in intact or digitonin-solubilized membranes. Biocytin covalently attached to the cysteine in the stromal facing N-terminal tail (cpTatCK72C) and was bound by SA in both intact and digitonin-solubilized thylakoids (Fig. 1B). Biocytin also reacted with the luminal loop cysteine (cpTatCL126C), but this was only bound by SA after digitonin solubilization, confirming that biocytin can access the aqueous luminal compartment, but streptavidin cannot. Biocytin did not react with the thylakoid membrane cysteine (cpTatCL92C), consistent with its location in the nonpolar region of the bilayer. As expected, Br-PEG labeled only the stroma-exposed cysteine (cpTatCK72C, Fig. 1C). Taken together, these data verify that both methods are a suitable way to map the topology of different regions of Tha4.

Tha4 Adopts an NOUT Topology—We employed the same substituted cysteine accessibility methods described above to analyze Tha4 in nontransport conditions. Tha4 contains no native cysteines; singly substituted Cys variants of Tha4 were tested in a functional assay and shown to be mostly active (Ref. 4 and supplemental Fig. S1A). Cys-substituted Tha4 was integrated into thylakoids under energized conditions (i.e. in the light) and the recovered thylakoids were treated with either biocytin or Br-PEG. Fig. 2A shows a survey with biocytin treatment of Cys residues in Tha4 domains. Biocytin reacted with residues at the N terminus (lanes 2 and 4), in the APH (lane 10), and C-tail (lane 12), as evidenced by the appearance of a SA adduct in the + digitonin lane, which reports on total biocytin-Tha4. SA added to intact thylakoids bound biocytin-reacted cysteine in the APH (lane 9) and at the C terminus of Tha4 (lane 11). SA labeling of the N terminus was primarily observed after digitonin disruption of the membrane, indicating that the N terminus is on the luminal side of the membrane. The small

FIGURE 1. Testing Cys labeling approaches using cpTatC. Two topology-labeling strategies were employed: biocytin/streptavidin (SA) labeling in intact or detergent-solubilized thylakoid membranes (B) and direct Br-PEG labeling (C). A, topology of the control protein cpTatC (25). Amino acid residues substituted with cysteine are indicated in black with the residue number. B, cpTatC biocytin/SA labeling: demonstration of SA accessibility patterns for cysteines in the stromal region (K72C), the transmembrane domain (L92C), and the lumen region (L126C) of cpTatC in thylakoid in the presence (+) or absence of digitonin, where accessibility is indicated by the appearance of a higher molecular weight cpTatC:SA adduct (arrow). C, cpTatC branched-PEG labeling: demonstrated accessibility of Br-PEG (+) where accessibility is indicated by the appearance of the cpTatC:Br-PEG adduct (arrowhead). Samples were analyzed by SDS-PAGE and fluorography. Gels are representative of at least two experiments.
amount of SA adduct formed with intact membranes (lanes 1 and 3) is likely due to either the presence of contaminating nonintact membranes or de-insertion of Tha4 during the SA reaction, i.e. Tha4 is somewhat susceptible to release from thylakoids during washing steps (26). Similar to the results with the cpTatC control, biocytin followed by SA either in the absence or presence (+) of digitonin solubilization of the membrane. B, Tha4 labeling: survey of Br-PEG accessibility to cysteine for the same residues tested in B. The Tha4:Br-PEG adduct is marked with an arrowhead. Samples were analyzed by SDS-PAGE and fluorography. Gels are representative of at least two experiments. C, amino acid sequence of Tha4 as modeled from B. subtilis TatAα (29). Amino acid residues substituted with cysteine are indicated in black with the residue number.

**FIGURE 3.** Tha4 emerges from the hydrophobic membrane core at the N-proximal APH. A, biocytin/SA labeling: accessibility of cysteines placed in the hinge region of Tha4 to added SA with intact thylakoids or after (+) digitonin solubilization. B, branched-PEG labeling: accessibility of cysteines placed in the hinge region of Tha4 to Br-PEG. Assays were as described in the legend to Fig. 1 and under "Experimental Procedures." Samples were analyzed by SDS-PAGE and fluorography. Gels are representative of at least two experiments.
A similar pattern of Br-PEG labeling confirmed the gradient of accessibility from the N-proximal to C-proximal APH residues (Fig. 5C). Taken together these data suggest that the APH is angling out of the membrane from the hinge region toward the C terminus or is highly flexible at the C terminus allowing access to the hydrophobic face of the helix.

Important for subsequent analysis of Tha4 topology in the active translocase is that all of the methods used were internally consistent; i.e. both substituted cysteine accessibility methods and factor Xa cleavage reported the same topology and were also in agreement with previous reports of Tha4 (7, 8) and TatA topology (9, 12). This validates the use of the Br-PEG and specific proteases as reliable reporters of Tha4 topology and allows us to apply them with confidence to analyze the conformation of Tha4 within the active translocases.

Differential Accessibility of APH Residues Induced by Shift to Protein Transport Conditions—Due to the simplicity and speed of the assay, Br-PEG labeling was used to assess topology changes in the APH during transport (Fig. 6). Tha4 containing cysteine substitutions throughout the APH were integrated into thylakoid membranes. The Tat precursor tOE17V–20F was used and was present in amounts known to saturate all of the cpTatC-Hcf106 receptor complex binding sites (14) in an effort to engage all of the Cys-substituted Tha4 in the transport reaction (14, 16). With precursor-saturated receptor complexes and a moderate amount of Tha4, there is a lag period of ~2.5 min, followed by a rapid rate of precursor transport that lasts for at least 5 min at the temperature of these assays (15 °C) (14). Reactions were transferred to the light to initiate transport. Br-PEG was added after a 2.5-min interval and allowed to react 2.5 min with Tha4 before the reaction was quenched. Transport is evidenced by the production of the mature form of OE17 (Fig. 6).

Cysteines at the N terminus of the APH (i.e. K25C–E29C) remained inaccessible to Br-PEG with or without transport (Fig. 6). Cysteines V30C, S33C, and I34C demonstrated increased labeling with transport (Fig. 6), indicating a move toward a more accessible location, whereas G35C and Q36C remained unchanged. Residues in the middle portion of the APH (T37C to F48C) exhibited reduced labeling to varying degrees with transport, with the notable exceptions of F41C and E47C, which appear to be labeled to the same extent with or without transport (Fig. 6). This indicates that, in general, residues in the middle portion of the APH shift toward a less accessible conformation under transport conditions. This behavior would be consistent with partitioning of the middle APH region into the membrane interface. Labeling of residues nearer to the C-terminal end of the APH (T50C–L52C) did not differ with transport. No periodicity for transport-induced labeling differences was detected, indicating that the changes were not restricted to a particular “face” of the APH but were correlated to the relative positions of the residue in the APH. Residues throughout the C-tail (T59C, A65C, and T78C) were strongly labeled with or without transport conditions (Fig. 6). N-terminal Cys substitutions F3C and F4C were not labeled during transport (data not shown), indicating the N terminus remains at the trans side of the membrane.

To assess whether the reduced labeling with transport is due to precursor “footprinting,” we tested the effect of different
sized precursors on the labeling of T37C and F41C (Fig. 7). Pre-SufI is a ~50 kDa substrate of the *E. coli* Tat system. We removed the native signal peptide and replaced it with the tOE17V-20F signal peptide to make it compatible with the thylakoid Tat apparatus. SpF16, SpF12, and SpF8 are C terminally truncated versions of tOE17V-20F containing only the first 16, 12, or 8 residues of the mature protein, respectively. As can be seen in Fig. 7, all five precursors led to reduced labeling of T37C during transport ([left panel](#)) but no change in labeling was observed for F41C ([right panel](#)). A reduction in T37C labeling was also produced with precursors tOE23 and a tOE17 that lacked the F-20 substitution (data not shown). tOE23 and tOE17 have a reduced affinity for the receptor binding site (16, 27), indicating that the specific signaling peptide is not responsible for reduction in Br-PEG labeling.

**Protease Accessibility of the C-tail Under Transport Conditions**—The virtually identical amount of Br-PEG labeling of C-tail cysteine substitutions with or without transport suggested that the C terminus of the C-tails remains exposed to the stroma throughout transport. To confirm this, a thrombin cleavage site was placed at the extreme C terminus between Tha4 and a His<sub>6</sub> tag (Fig. 8A, *Tha4CT-Throm*). The majority of

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**FIGURE 5.** A gradient of accessibility of residues in the APH. A, total biocytin labeling of Tha4 single cysteine residues placed along the entire APH as determined by SA binding with digitonin-solubilized thylakoids calculated from scintillation counting of radiolabeled proteins extracted from gel bands as described (17). Biocytin labeling is expressed as a percentage of total integrated Tha4 for each Tha4 Cys variant. B, percent stromal accessibility of biocytin-labeled APH residues as determined by SA binding to intact thylakoid membranes. Percent stromal accessibility is expressed as a percentage of the total amount of biocytin-labeled Tha4. Percent of biocytin labeling was assigned relative to the totally accessible A65C control. Includes data from at least two separate trials for each residue. C, Br-PEG labeling: accessibility of Br-PEG to select hydrophobic and hydrophilic substitutions within the APH of Tha4 measured with intact thylakoids. Samples were analyzed by SDS-PAGE and fluorography. Gels are representative of at least two experiments.
Tha4 was cleaved within 1 min of thrombin addition both with and without transport (Fig. 8A), suggesting that the C-tail remains exposed to the stroma during protein transport.

Because changes in accessibility were observed for Cys residues in the APH during transport, a thrombin site was engineered in the C-tail closer to the APH, i.e., after residue Ile62 (Fig. 8B, Tha4I62-Throm). Thrombin quickly cleaved Tha4I62-Throm when assayed in the light without transport, e.g., more than 60% cleavage within 1 min, reaching a maximum of 80% cleavage by 4 min, similar to that seen with Tha4CT-Throm. Under transport conditions, however, thrombin cleaved Tha4I62-Throm at a substantially slower rate, with only 50% of Tha4 cleaved after 10 min (the duration of the assay). This indicates that the Ile62 region is much less accessible to protease during protein transport, even though this region is very accessible to Br-PEG.

Either the full tOE17 V20F precursor or truncated precursors SpF16 and SpF8 were able to induce a reduced accessibility of Tha4I62-Throm to the protease (Fig. 8B).

DISCUSSION

This study shows for the first time the conformation of Tha4 within the active translocase with simultaneous protein transport. Several lines of evidence suggest Tha4 (TatA) is the protein-conducting element of the Tat translocase. First, it is found in the membrane in a much higher molar ratio than cpTatC or Hcf106 (14). Second, it is only required for the transport step.
Tha4 Topology Shift Leading to Tat Precursor Transport

A. Tha4CT-Throm

![Tha4CT-Throm Diagram]

B. Tha4I62-Throm

![Tha4I62-Throm Diagram]

FIGURE 8. Reduced accessibility of the APH proximal C-tail to protease during protein transport. Effect of transport on Tha4 C-tail accessibility was studied by insertion of two different thrombin cleavage sites; one placed at the extreme C terminus followed by a His tag; the other near the APH after Tha4 residue 62. Thrombin was added during the period of maximum precursor transport and Tha4 cleavage was monitored over time as described under "Experimental Procedures." A, Tha4CT-Throm: thrombin cleavage of a site inserted at the C terminus of Tha4 in the light or in the light with precursors tOE17V-20F, SpF16, or SpF8. Samples were analyzed by SDS-PAGE and fluorography. Cleavage was calculated from scintillation counting of radio-labeled proteins extracted from gel bands as described (17). Error bars represent mean ± S.E. of 3 experiments (light, light + tOE17V-20F) or mean ± range of two experiments (light + SpF16). SpF8 represents a single experiment. Nonlinear regression analysis was performed using GraphPad Prism, version 5.0d for Mac (GraphPad Software, San Diego, CA).

(2). Third, upon assembling into the translocase, it forms higher order homo-oligomers (5). Finally, recent studies show that the mature domain of the precursor protein makes contact with TatA (28) or Tha4 during transport. The conformation Tha4 adopts in the translocase has significant implications for the mode of Tat transport and may help distinguish between different models for the Tat mechanism. All mechanistic models include a cpTatC-Hcf106 receptor complex that initially binds the incoming precursor in an energy-independent manner. The current models also agree that, for transport to occur, Tha4 (TatA) must be recruited to the precursor-bound receptor complex in a proton-motive force-dependent manner, however, the mechanism by which recruitment of Tha4 facilitates transport is unknown.

In the present study, we have carried out a systematic examination of domains of Tha4 in both resting and actively transporting membranes. Our approach used two different cysteine-labeling methods and was verified by specific protease accessibility to engineered or natural cleavage sites. These three methods were internally consistent and unambiguously demonstrate that Tha4 adopts a N<sub>OUT</sub>–C<sub>IN</sub> topology in nontransporting thylakoids and does not change that overall topology under protein transport conditions. We do not attribute the slight SA labeling of cysteines at positions Phe<sup>3</sup> or Phe<sup>4</sup> to a dual topology, but rather to possibly contaminating nonintact thylakoids. If a dual topology exists, we would have expected to see similar labeling using the Br-PEG, which did not label membrane-bound F3C or F4C. This is in agreement with the N<sub>OUT</sub>–C<sub>IN</sub> topology recently reported for TatA in native E. coli membranes (12). In the TatA study, Koch et al. (12) analyzed TatA from the trans (OUT) side of the membrane, and therefore could only label the N terminus of TatA and did not observe any topological or conformational reorganization of TatA over a period in which transport occurred. In contrast, we analyzed Tha4 from the cis side of the membrane and observed accessibility changes with transport of the APH and APH-proximal C-tail.

Recent NMR spectroscopy studies of the Bacillus subtilis TatA<sub>4</sub> in bicelles (29) and micelles (30) demonstrate an unusually short N-terminal TMD, a hinge that is buried in the membrane, and an APH that is tilted with respect to the plane of the membrane (29). Our biochemical approaches with Tha4 in native membranes are consistent with this structure. We mapped the TMD hydrophobic core to contain residues 11–24, consistent with residues 8–22 of TatA<sub>4</sub> (29). Our data imply that the glutamate at position 10 of Tha4 is in a more energetically favorable location, e.g. closer to the hydrophilic head groups, than previously predicted. This would make it more easily protonated when the membrane is energized with a proton gradient, a requirement for Tha4 assembly with the precursor-bound receptor complex (6). Both biochemical approaches used here indicate that the hinge residues are not accessible to the stromal compartment and appear to be in the nonpolar environment of the membrane bilayer.

Of particular interest is that the C-terminal portion of the APH is more accessible to Cys-directed labeling than the N-ter-

5 D. Pal, K. Fite, and C. Dabney-Smith, unpublished results.
minal portion of the APH. That both the hydrophobic and hydrophilic residues at the C-terminal APH are accessible may mean that this region is not fully partitioned into the membrane interface and possibly unfolded. It may also mean that this region is more flexible and on average allows access of Cys-targeted reagents, which is not inconsistent with NMR data showing divergent traces for the APH carboxyl-proximal region (30). Regardless of the underlying reason, these results strongly suggest that when not in the translocase, the Tha4 APH is less membrane active. Because APH domains are well known to cause bilayer bending, destabilization, and pores (31), this may be a key factor regulating Tha4 activity until assembled into the translocase. Thus although our results with Tha4 in nontransporting native membranes are generally consistent with early Tha4 topology studies (7, 8), recent studies of E. coli TatA topology (12), and the detailed structural results of B. subtilis TatA in artificial membranes (29, 30), they take on a special functional meaning when viewed in the context of Tha4 conformation in the translocase.

**Differential Accessibility of APH and APH-Proximal C-tail Residues during Transport**—Although, for the most part, consensus has been reached over the topology of Tha4 (TatA) in resting conditions, the conformation of Tha4 in the translocase remains unresolved. At least two models for the mechanism by which Tha4/TatA accomplishes precursor transport imply topologies different from the N\textsubscript{OUT}-C\textsubscript{INT} topology shown here. One model proposes that TatA oligomers form aqueous channels that are presumably lined by TatA APHs (32), requiring TatA to adopt an N\textsubscript{OUT}-C\textsubscript{OUT} topology as suggested by previous work (10). The second model suggests the APH of Tha4 acts like helical cationic antimicrobial peptides (33), to induce aqueous pores requiring the APH to reorient from the membrane interface to across the bilayer. Such APH reorientation would likely cause a transient movement of stroma accessible domains to the trans side of the membrane. A third model, however, proposes that Tha4 simply “weakens” or destabilizes the membrane around the precursor protein, reducing the energy barrier to the precursor protein crossing the membrane (34). This model does not necessarily result in topology change of Tha4 (TatA).

In an effort to analyze the conformation of Tha4 within an active translocase, we designed Br-PEG labeling and protease treatment assays to measure accessibility to the APH and C-tail during a time window in which protein transport was maximal (14). Both methods showed that the extreme C terminus of Tha4 remained accessible to the stromal face of the membrane throughout the transport reaction (Figs. 6 and 8). However, in the presence of a proton-motive force and precursor proteins, we saw substantially delayed cleavage of the Ile62 thrombin cleavage site and masking of previously accessible regions of the APH to Br-PEG.

It is tempting to view our results as defining the status of Tha4 during precursor protein transit across the membrane bilayer. Certainly, accessibility measurements were made during that time window. However, two processes are induced when precursor proteins bind to the Tat receptor. First, Tha4 assembles with the precursor-bound receptor complex to form the translocase; then the precursor protein is translocated. The precursor proteins in the present study have been shown to trigger Tha4 assembly (5, 6). However, the truncated precursor proteins SpF16 and especially SpF8 are not efficiently transported. Thus the changes we have captured here most likely represent the conformation of assembled Tha4 within an active translocase rather than translocation per se. We consider four possible explanations for the observed accessibility changes based on previous studies and models for Tha4 assembly.

**Precursor Footprinting**—Contacts between the APH of E. coli TatA and the precursor protein have recently been documented (35) and have also been observed between Tha4 APH and precursor. These contacts could occlude Br-PEG or thrombin access to cysteine in the APH of Tha4, based on the footprint of the precursor. We reasoned that we would see differences in the residues affected based on the size of the precursor. Interestingly, all precursors tested, from the 55-kDa precursor SufI to the smallest truncated peptide of OE17 (SpF8), produced an identical reduction in access of Tha4T37C to Br-PEG, whereas OE17, SpF16, and SpF8 caused delayed cleavage of thrombin at position Ile62. The amino terminus of the OE17 mature protein is relatively unstructured (36–38) and in an extended conformation could conceivably obstruct access to APH or the APH-proximal region of the C-tail. However, the mature domains of SpF16, or especially, SpF8-truncated precursors are unlikely to be long enough to restrict access to both the Ile62 thrombin cleavage site and T37C simultaneously. Additionally, ~25 Tha4 protomers are estimated to be required for transport of a single precursor (14, 39). For many APH residues, restricted Br-PEG access affects a significant portion of the Tha4 population (Fig. 6). It seems unlikely that one SpF precursor could smother sites on all 25 protomers. Thus, we believe precursor footprinting is only a minor contributor to the observed accessibility changes.

**Topology Changes**—Earlier models and studies indicated Tha4 (TatA) may adopt a N\textsubscript{OUT}-C\textsubscript{OUT} topology concurrent with protein transport. Although precursor proteins were transported during the duration of our assays, we see no evidence for Tha4 C-tail movement to the lumenal (trans) side of the thylakoid membrane. Thrombin proteolysis of Tha4CT-Throm (Fig. 8) and persistent accessibility of Br-PEG to C-tail cysteines (Fig. 6) supports a continued stromal (cis) localization of the C-tail throughout translocation. Our assays were unable to exclude the possibility that the C-tail makes very quick flips to the trans side of the membrane during transport, but they do indicate that the resident time for such a putative excursion is insignificant compared with time of access on the cis side of the membrane. A recent study in E. coli also failed to detect the C-tail or APH of TatA exposed to the periplasm (trans side of the membrane). Thus our study and the E. coli study (12) agree on this point and neither supports a model in which Tha4 (TatA) forms structural channels lined with APHs.

**Conformation of Tha4 and Its Interaction with the Membrane Interface**—The middle region of the APH exhibits significantly reduced labeling with Br-PEG under transport conditions (Fig. 6). However, the labeling patterns of flanking regions argue

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6 F. Gérard and K. Cline, unpublished results.
Tha4 Topology Shift Leading to Tat Precursor Transport

against large-scale flipping of the entire APH as being the cause of the reduced labeling. Rather, we think that reduced accessibility of the central APH is due to an altered association of the APH with the membrane in which the APH uniformly partitions into the membrane interface rather than just the N-proximal end. Movement of Cys residues into the membrane interface would decrease their reaction with the maleimide moiety of Br-PEG (21). Such a partitioning would tilt the Tha4 APH back toward the membrane normal; more importantly, it would increase the surface activity of the APH, which may be essential for translocation activity of Tha4. This shift could lead to a thinning of the membrane as has been reported for some cationic antimicrobial peptides (31). A “thinning” or “weakening” of the bilayer near the cpTatC-tethered precursor protein may facilitate transmembrane movement of the Tat precursor.

Tha4 Packing—The delayed thrombin cleavage at Tha4I62-Throm under transport conditions can be interpreted as resulting from either the Ile62 C-tail region leaving thestromal compartment (a topology change) or being sterically hindered by proximity of other Tha4 protomers (Tha4 packing). T95C and A65C cysteine substitutions flanking the Ile62 thrombin cleavage site remained very accessible to Br-PEG with or without transport conditions (Fig. 6), counter indicative of topology change and indicating that other factors are restricting thrombin access, such as steric hindrance. Br-PEG may gain access in this region because it is small (~2.4 kDa) and contains a spacer region of 26.4 Å that separates the Cys-reactive maleimide bin access, such as steric hindrance. Br-PEG may gain access in change and indicating that other factors are restricting thrombin.

The thrombin active site. This argues that there is a structural constraint of the C-tail that restricts thrombin access.

A Cys-Cys disulfide cross-linking study showed that the Tha4 C-tails form clusters of at least 18 Tha4 protomers (4, 5). The arrangement would poise the precursor protein to move in only one direction, into the APH destabilized bilayer. This model for the arrangement of the translocase is highly speculative, but is also testable, and it is our intention to subject its major tenets to rigorous examination.

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