Oligomers of Tha4 Organize at the Thylakoid Tat Translocase during Protein Transport*

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The Tat (twin arginine translocation) systems of thylakoids and bacteria transport fully folded protein substrates without breaching the permeability barrier of the membrane. Two components of the thylakoid system, cpTatC and Hcf106, compose a precursor-bound receptor complex. The third component, Tha4, assembles with the precursor-bound receptor complex for the translocation step and is thought to compose at least part of the protein-conducting channel. Here, we used two different cross-linking approaches to explore the organization of Tha4 in the translocase. These cross-linking techniques showed that transition to an active protein transport state resulted in an alignment of the Tha4 amphipathic helix and C-terminal tail domains to form Tha4 oligomers. Oligomerization required functional Tha4, a twin arginine signal peptide, and an active cpTatC-Hcf106 receptor complex. The spectrum of oligomers obtained was independent of the mature folded domain of the precursor. We propose a trapdoor mechanism for translocation whereby aligned oligomers of Tha4 amphipathic helices fold into the membrane to allow formfitting passage of precursor proteins.

Plant thylakoid membranes possess two systems to transport proteins to the lumen (1). The thylakoid Sec pathway is homologous to the bacterial Sec pathway and uses ATP and chloroplast SecA to thread unfolded precursor proteins through a SecY/E channel (2–4). Conversely, the thylakoid Tat² (twin arginine translocation) pathway uses only the transmembrane proton motive force to transport fully folded precursor proteins through an uncharacterized channel (see Refs. 5–8 for review). Targeting to either pathway is mediated by cleavable hydrophobic signal peptides. However, Tat pathway signal peptides also contain an essential twin arginine motif immediately before the hydrophobic helix (9). Three membrane proteins appear to be the only components required for thylakoid Tat transport (5, 8). Hcf106 and Tha4 are homologous proteins with similar structures. Each is anchored by an N-terminal transmembrane domain, which is followed by a flexible hinge, an amphipathic α-helix, and a divergent C-terminal tail (5, 10). cpTatC is a multimembrane-spanning protein (11). The Tat system, originally described in chloroplasts (12, 13), is also widely represented in eubacteria and Archaea (8, 14–16). This suggests that the Tat system is of ancient design and likely employs basic, possibly simple mechanisms. The bacterial and archaeal orthologs of Hcf106, Tha4, and cpTatC are called TatB, TatA, and TatC, respectively (5, 6).

Transport by the thylakoid Tat pathway operates by a cyclical assembly and transport mechanism (17, 18). Precursor proteins bind to a large cpTatC-Hcf106 receptor complex via their signal peptides (18). In the presence of the proton motive force, which consists primarily of the Δψ of isolated thylakoids (19), Tha4 then assembles with the precursor-bound receptor complex to form the presumptive translocase (17). After protein translocation, Tha4 disassembles from the receptor complex, thereby resetting the path for additional transport events. The tight correlation between Tha4 assembly and protein translocation implies that Tha4 plays a specific critical role in the translocation step.

Several models for the Tat translocase propose that Tha4 (TatA) serves as the protein-conducting channel. Specifically, multiples of Tha4 (TatA) transmembrane domains and/or amphipathic helices are envisioned to form a ring around the mature folded domain of the precursor protein (5, 7, 14, 20, 21). Such a formfitting channel could explain the ability of Tat systems to transport folded proteins from ~2 kDa to well over 100 kDa without permitting uncontrolled ion flow (22, 23). The large molar excess of Tha4 (TatA) proteins over cpTatC (TatC) proteins (11, 24) and the appearance of a purified TatA complex as a large ring-forming structure (21) are consistent with such a model. However, it is not currently known what form Tha4 (TatA) adopts in an active translocase. If Tha4 functions as the envisioned channel, then, in the translocase, it should be present in multiples in which the transmembrane domains and/or amphipathic helices align with each other to encompass the substrate. Cross-linking studies verified the association of Tha4 with the cpTatC-Hcf106 precursor complex during transport, but provided no information on the number of Tha4 proteins or the organization of Tha4 in this complex (17).

Here, two cross-linking approaches were used to examine Tha4 interactions and organization during the protein translocation step. Cysteine scanning/oxidative disulfide cross-linking showed that interactions between Tha4 proteins occur across all domains of the Tha4 protein. Notably, the stroma-facing amphipathic helix and C-terminal tail appear to interact only during protein transport. The use of homobifunctional lysine-reactive cross-linkers showed that Tha4-Tha4 interactions during protein transport result in the formation of multimers or oligomers. Two observations indicate that the Tha4 oligomers are present in the translocase. First, Tha4 mutants inactive for translocase assembly did not form oligomers. Second, Tha4 oligomerization was prevented by pretreating thylakoids with transport-inhibiting anti-cpTatC IgG antibodies, arguing that Tha4 must dock with the precursor-bound receptor complex to oligomerize. Surprisingly, oligomer size appeared to be independent of the size of the precursor. These results suggest that Tha4 docking to the precursor-bound receptor complex

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2. The abbreviations used are: Tat, twin arginine translocation; DTT, dithiothreitol; CuP, copper(II)-1,10-phenanthroline; NEM, N-ethylmaleimide; DSS, disuccinimidyl suberate; DSP, diithiobis(succinimidyl propionate); Tricine, N-[2-hydroxy-1,1-bis(hydroxyethyl)-ethyl]glycine.
either concentrates or reorganizes Tha4 oligomers at the site of translocation. Implications of this finding for the mechanism of translocation are discussed.

**EXPERIMENTAL PROCEDURES**

Preparation of Chloroplasts and Thylakoids—Intact chloroplasts were prepared from 9–10-day-old pea seedlings (Pisum sativum L. cv. Laxton’s Progress 9) (25). Chloroplasts were resuspended to 1 mg/ml chlorophyll in import buffer (50 mM HEPES-KOH (pH 8.0) and 330 mM sorbitol) and kept on ice until used. Chloroplast lysates and isolated thylakoids were obtained as described (25) by osmotic lysis of intact chloroplasts. Thylakoids were resuspended in import buffer and 10 mM MgCl₂ before use. Treatment of thylakoids with antibodies to Tha4, cPTatC, Hcf106, and Alb3 was conducted as described (18, 26).

**Generation of Cysteine-substituted Mature Tha4—Tha4 proteins with cysteine substitutions (Tha4(XnC)) were generated by QuikChange mutagenesis (Stratagene) according to the manufacturer’s instructions. The template used for mutagenesis was the coding sequence for mature Tha4 (lacking the targeting peptide) from pea as described (10); the coding sequence begins with MAFFGLGVPE...Cloned constructs were verified by DNA sequencing to change the coding sequence such that the resulting protein started with cysteine substitutions (Tha4(XnC)).

Preparation of Radiolabeled Mature Tha4 and Precursors—Radiolabeled mature Tha4, Tha4 mutants, and precursor proteins were prepared by *in vitro* translation in a wheat germ extract from capped mRNA in the presence of [³H]leucine (9). Tha4 translation products were diluted with an equal volume of 60 mM leucine in 2X import buffer before use. Unlabeled DT23 and KK-DT23 (the nonfunctional variant of DT23) were overexpressed in *Escherichia coli* as described previously (9, 17). tPOE17, a synthetic peptide representing the signal peptide of the OE17 precursor, was the generous gift of Dr. Steven Theg (University of California-Davis). Purified DT23 and KK-DT23 inclusion bodies and tPOE17 were freshly dissolved in 8 M urea and 1 mM dithiothreitol (DTT) to 120 µM at 37 °C for 1 h before use.

In *Vitro* Assay for Functional Tha4 Complementation by Recombinant Tha4 Proteins—Cys-substituted Tha4 proteins were assayed for functionality with an *in vitro* complementation assay to restore transport of the precursor DT23 as described previously (26). Briefly, isolated thylakoids were treated with anti-Tha4 IgG antibodies to inactivate endogenous Tha4, and the bound antibodies were capped with protein A. *In vitro* translated recombinant Tha4 was then integrated into the resulting thylakoids. Tha4-integrated thylakoids were assayed for transport of *in vitro* translated DT23. Complementation transport assays were conducted for 15 min at 15 °C in white light (~100 µmol/m²/s) and stopped by transfer to ice and centrifugation to recover thylakoids. Thylakoids were resuspended in 300 µl of 1X import buffer and divided into two aliquots. Thylakoids in one aliquot were analyzed directly, whereas thylakoids in the second aliquot were treated with thermolysin before analysis by SDS-PAGE/fluorography. Quantification of radiolabeled proteins was done by scintillation counting of radio-labeled proteins extracted from gel bands (27). Untreated samples were used to calculate the amount of recombinant Tha4 in the membrane, whereas thermolysin-treated samples were used to calculate the amount of DT23 transported. The amount of DT23 transported was corrected for background transport that occurred in the negative control for each experiment. The negative transport control was a transport reaction conducted with anti-Tha4 antibody-treated thylakoids in the presence of 8 µM Tha4 stromal domain antigen to control for possible release of antibodies. Transport in the negative control was generally <4% of the transport that occurred in the wild-type Tha4 complementation assay. Complementation efficiency was calculated as described in the legend to Fig. 1.

**Cross-linking—** *In vitro* translated [³H]Tha4(XnC) was integrated into isolated thylakoids or anti-Tha4 antibody-treated thylakoids for 20 min at 15 °C. Recovered thylakoids (50 µg of chlorophyll in 175 µl of import buffer and 10 mM MgCl₂) were incubated under transport conditions (5 mM MgATP, 0.5 mM DTT, and 50 µM methyl viologen; white light at ~100 µE/m²/s) or non-transport conditions (absence of energy, darkness) at 15 °C for 2 min before saturating levels of DT23 precursor (1.5 µM; dispensed from a 120 µM stock in 8 M urea and 1 mM DTT) or an equivalent volume of 8 M urea (no-precursor control) was added to initiate transport. After an additional 2 min, 0.25 mM copper(II)-1,10-phenanthroline (CuP; from a 15 mM stock) or 1 mM CuP (from a 150 mM stock) was added to catalyze disulfide formation between proximal cysteine residues. The CuP stock solution (150 µm) contained 150 µm CuSO₄ and 500 mM 1,10-phenanthroline (28). After cross-linking for 2 min (or for the times indicated in the figures), oxidation was quenched with 50 mM N-ethylmaleimide (NEM; from a 1 M stock in Me₂SO) and 0.5 ml of 14 mM EDTA in import buffer. Thylakoids recovered by centrifugation were washed with 1X import buffer, 5 mM EDTA, and 10 mM NEM. Thylakoid pellets were resuspended in 125 mM Tris-HCl (pH 6.8), 8 M urea, 5 mM EDTA, 5% SDS, and 15% glycerol and analyzed by SDS-PAGE and fluorography under nonreducing conditions.

For homobifunctional cross-linking with the cross-linker disuccinimidyl suberate (DSS) or dithiobis(succinimidyl propionate) (DSP), [³H]Tha4 was integrated into washed thylakoids or antibody-treated thylakoids as specified in the figure legends and described previously (10). Thylakoids were recovered by centrifugation, washed and then resuspended in 100-µl aliquots containing 33 µg of chlorophyll in 1X import buffer and 3.3 mM MgCl₂. For cross-linking under transport conditions, aliquots received 5 mM MgATP, 50 µM methyl viologen, and 1.5 µM unlabeled DT23 precursor. For cross-linking in the absence of energy or precursor, aliquots received an equivalent amount of 8 M urea and 1 mM DTT without ATP and methyl viologen. Aliquots were incubated at 25 °C in white light at ~70 µE/m²/s or darkness for 5 min and then received Me₂SO or varying amounts of the cross-linker DSS or DSP (dispensed from a 5 or 25 mM stock in Me₂SO). After 5 min, cross-linking reactions were quenched with 90 µM Tris-HCl (pH 7.6) for 5 min at 25 °C, followed by 15 min at 0 °C. Each reaction was then diluted with 0.8 ml of 1X import buffer and 3.3 mM MgCl₂, and thylakoids were recovered by centrifugation. Thylakoids were dissolved in SDS sample buffer with or without β mercaptoethanol (DSS or DSP cross-linker, respectively) and analyzed by SDS-PAGE/fluorography.

**Immunoblotting and Immunoprecipitation—** Antibodies against Tha4, cPTatC, Hcf106, or OE23 have been described (11, 29). Immunodetection was performed following standard methods using the ECL reagents and protocol (Amersham Biosciences) for detection. For immunoprecipitation, samples were resuspended in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% SDS; heated to 37 °C for 5 min; and centrifuged to remove insoluble material. Immunoprecipitation under denaturing conditions was performed as described previously with anti-IgG antibodies covalently cross-linked to protein A-Sepharose (17).
Impair Tha4 Function in Vitro

Tha4 is inactivated with anti-Tha4 IgG antibodies, and it in an substituted Tha4 (Tha4) mediated by different domains of the protein. A library of single Cys-substitution models were undertaken to characterize Tha4-Tha4 interactions thereby. Cys-substituted Tha4 does not affect function. A schematic representation of Tha4 based on experimental (10, 29) and predictive structural features. Residues in black circles are those at which cysteines were substituted. The N-terminal cysteine shown was present only in the Tha4MAC construct. All other Tha4 constructs begin with MAF (see “Experimental Procedures”). B, functional replacement of endogenous Tha4 with Cys-substituted Tha4. Biochemical complementation assays were performed as described under “Experimental Procedures.” The amino acid residues in Tha4 substituted with cysteine are shown across the x axis. Complementation efficiency (amount of transported mature protein) was calculated as the amount of DT23 transported divided by the amount of recombinant Tha4 integrated into thylakoids. Complementation by wild-type Tha4 (WT) in each experiment was arbitrarily set at 100%, and the amount of DT23 transported in the non-integration control, which was generally ~4% of the wild type, was set at 0%. Transport of Cys-substituted Tha4 proteins was normalized to that of wild-type Tha4. Data presented are the means ± S.E. of at least three separate experiments. PI, preimmune IgG.

RESULTS

Introduction of Single Cysteine Residues throughout Tha4 Does Not Impair Tha4 Function in Vitro

Cysteine scanning and oxidative disulfide formation were undertaken to characterize Tha4-Tha4 interactions mediated by different domains of the protein. A library of single Cys-substituted Tha4 (Tha4(XxC)) was prepared and tested for functionality in an in vitro Tha4 replacement assay (26). In this assay, endogenous Tha4 is inactivated with anti-Tha4 IgG antibodies, and in vitro translated recombinant Tha4 is introduced into the membrane by in vitro integration. Transmembrane and hinge domain residues are systematically substituted with cysteine, whereas representative residues in the amphipathic helix and C-terminal tail were selected for substitution (Fig. 1A). Nearly all Cys-substituted Tha4 proteins retained some transport function, although the range of functionality varied from 25 to 200% of wild-type Tha4 (Fig. 1B and supplemental Fig. S1). Only Tha4(E10C) was completely nonfunctional (Fig. 1B). This confirms the previously reported Glu requirement for Tha4 function (26).

Conditions for Tha4-Tha4 Disulfide Cross-linking Analysis—Tha4-Tha4 interactions were mapped by integrating radiolabeled Cys-substituted Tha4 into thylakoids and catalyzing disulfide formation between proximal cysteine residues with CuP. Because single Cys substitutions were made, Tha4-Tha4 disulfide formation was scored by the presence of a Tha4 dimer band on SDS-PAGE fluorograms. Preliminary experiments calibrated cross-linking conditions to reliably report on interactions that occur during protein transport. The experiment in Fig. 2 shows the results from analysis of Tha4 proteins with Cys substitutions at transmembrane domain residues Pro and Val and stromal domain residue Thr, as these are representative of the behavior of Cys substitutions in those regions (Table 1).

Inclusion of 0.5 mM DTT in assays was necessary to minimize cross-linking without CuP (Fig. 2, lanes 2 and 3). Two different concentrations of CuP were examined. Tha4(V12C) was not cross-linked by either CuP concentration (middle panel). Tha4(P9C)-Tha4(P9C) was cross-linked under both resting state and active transport conditions with the relatively high 1 mM CuP concentration (upper panel). Tha4(P9C) cross-linking occurred almost as quickly as the quenching agent NEM could be added (lanes 4–8). Control experiments showed that NEM quenched the reaction within 10 s of its addition (data not shown). When 0.25 mM CuP was used, the Tha4(P9C)-Tha4(P9C) dimer was produced at a slower rate and only under transport conditions (upper panel, lanes 9–13). Tha4(T59C) was specifically cross-linked only under active transport conditions with either the low or high CuP concentration and exhibited similar kinetics compared with Tha4(P9C) (lower panel, lanes 4–13).

Tha4 Interacts through Its Stromal Domain under Conditions of Active Protein Transport—Table 1 summarizes the results of oxidative disulfide cross-linking at single sites across the Tha4 molecule with high

![FIGURE 1](image.png)

**FIGURE 1.** Cys-substituted Tha4 does not affect function. A, schematic representation of Tha4 based on experimental (10, 29) and predictive structural features. Residues in black circles are those at which cysteines were substituted. The N-terminal cysteine shown was present only in the Tha4MAC construct. All other Tha4 constructs begin with MAF (see “Experimental Procedures”). B, functional replacement of endogenous Tha4 with Cys-substituted Tha4. Biochemical complementation assays were performed as described under “Experimental Procedures.” The amino acid residues in Tha4 substituted with cysteine are shown across the x axis. Complementation efficiency (amount of transported mature protein) was calculated as the amount of DT23 transported divided by the amount of recombinant Tha4 integrated into thylakoids. Complementation by wild-type Tha4 (WT) in each experiment was arbitrarily set at 100%, and the amount of DT23 transported in the non-integration control, which was generally ~4% of the wild type, was set at 0%. Transport of Cys-substituted Tha4 proteins was normalized to that of wild-type Tha4. Data presented are the means ± S.E. of at least three separate experiments. PI, preimmune IgG.

![FIGURE 2](image.png)

**FIGURE 2.** Cys-Cys oxidative cross-linking of Tha4. A time course analysis of cross-linking at high and low CuP concentrations was conducted in the absence or presence of protein transport. Tha4 containing the indicated cysteine substitution (P9C, V12C, or T59C) was integrated into isolated thylakoids as described under “Experimental Procedures.” The resulting thylakoids were incubated under transport conditions (white light, ATP, methyl viologen, and 1.5 μM DT23) or in darkness without energy or precursor as described under “Experimental Procedures.” Transport was initiated by addition of precursor and cross-linking by addition of CuP. Aliquots (removed at the indicated times) received NEM (50 mM final concentration) to quench the reaction. Thylakoids were recovered by centrifugation, and samples were analyzed by SDS-PAGE under nonreducing conditions. Cross-linking is evident by the appearance of the Tha4 dimer (arrowheads). Panels represent fluorograms of gels processed in parallel.}


and low CuP concentrations and in the presence or absence of active protein transport. The greatest variation in Tha4-Tha4 interaction was observed for transmembrane domain residues: some residues did not cross-link under any conditions; some cross-linked under both resting and transport conditions; and some cross-linked only under transport conditions. In addition, cross-linking was dependent on the concentration of CuP used to catalyze disulfide formation. A number of Tha4 proteins with Cys substitutions at transmembrane domain residues cross-linked under both conditions with the high CuP concentration and transport conditions; and some cross-linked only under transport conditions. These results indicate that Tha4-Tha4 interactions occur throughout the entire protein molecule. The transmembrane domain showed some interaction under most conditions, but interacted more strongly in the presence of the pH gradient and a functional precursor. The Tha4 stromal domain, i.e. the amphipathic helix and C-tail, interacted almost entirely in response to conditions leading to protein transport (Deff and precursor).

**Tha4 Forms Higher Oligomers during the Transport Reaction**—The site-specific cross-linking through single residues used above can assess interaction, but cannot reveal the presence of oligomers. However, because 9 lysine residues are present in the Tha4 stroma-facing domains, the lysine-reactive homobifunctional cross-linkers DSS and DSP were used to stabilize any oligomers present during protein transport. A preliminary experiment in which endogenous Tha4 was cross-linked with DSS showed Deff and precursor-dependent formation of Tha4 dimer- and higher order Tha4-containing products, but was limited by a high background staining.

**Summary of cross-linking of Cys-substituted Tha4**

| Residue       | Dimerization* | ≥1 mM CuP | ≤0.25 mM CuP |
|---------------|---------------|-----------|--------------|
| N terminus   |               |           |              |
| MAC          | C*            | V         |              |
| F3C          | C             | V         |              |
| F4C          | T*            | T         |              |
| Transmembrane|               |           |              |
| G5C          | C             | C         |              |
| L6C          | T             | T         |              |
| G7C          | —             | —         |              |
| V8C          | T             | T         |              |
| P9C          | C             | T         |              |
| E10C         | C             | C         |              |
| L11C         | T             | T         |              |
| V12C         | —             | —         |              |
| V13C         | T             | T         |              |
| I14C         | —             | —         |              |
| A15C         | —             | —         |              |
| G16C         | —             | —         |              |
| V17C         | —             | —         |              |
| A18C         | —             | —         |              |
| A19C         | C             | T         |              |
| L20C         | C             | T         |              |
| V21C         | C             | T         |              |
| Hinge        |               |           |              |
| F22C         | C             | T         |              |
| G23C         | C             | T         |              |
| P24C         | C             | T         |              |
| K25C         | C             | T         |              |
| Amphilathic helix and C-terminal tail |               |           |              |
| K26C         | T             | T         |              |
| P28C         | T             | T         |              |
| E29C         | T             | T         |              |
| K39C         | T             | T         |              |
| S40C         | T             | T         |              |
| Q43C         | T             | T         |              |
| K46C         | T             | T         |              |
| Q47C         | T             | T         |              |
| T59C         | T             | T         |              |
| A65C         | T             | T         |              |
| Q68C         | C             | T         |              |
| T78C         | T             | T         |              |

*Cross-linking experiments were conducted at low and high CuP concentrations as described under “Experimental Procedures.” Results are the average of at least three separate experiments.

*Constitutive dimerization occurred under all conditions.

*Variable; dimerization varied from experiment to experiment as either constitutive or transport-induced.

*Transport-induced dimerization occurred only in the presence of precursor and the ΔpH.

*− No dimerization under any condition.
Tha4 Oligomerization during Tat Protein Transport

FIGURE 4. Transport-related Tha4 oligomers. In vitro translated [3H]Tha4 was integrated into washed thylakoids in the presence of 5 mM ATP for 15 min at 25 °C, and the resulting thylakoids were subjected to cross-linking with DSS under transport or non-transport conditions as described under “Experimental Procedures.” A, thylakoids were incubated at 25 °C either with 1.5 μM unlabeled precursor DT23 in white light and ATP to generate a ΔpH (lanes 3–8) or without precursor in darkness (lanes 1 and 2) for 5 min and then received MeSO or varying amounts of the cross-linker DSS (dispensed from stocks in MeSO). After the reactions were quenched, the recovered thylakoids were analyzed by SDS-PAGE. Panels represent fluorograms of two 11.5% gels processed in parallel. B, membranes recovered from cross-linking of thylakoids incubated under transport conditions with 0.5 mM DSS were dissolved in 1% SDS and then subjected to immunoprecipitation under denaturing conditions as described under “Experimental Procedures.” The antibody-linked beads used are depicted above each lane. The sample in the αTatC/106 lane was immunoprecipitated with a mixture of anti-cpTatC and anti-Hcf106 antibody-linked beads. The upper panels show proteins that were bound by the beads, whereas the lower panel shows proteins that were not bound by the beads. The JX and JX panels of antibody-bound samples were obtained from fluorograms of the same gel exposed to film for 30 and 90 h, respectively. The panel of unbound samples represents a fluorogram exposed for 20 h.

Because Tha4 can be cross-linked to Hcf106 and cpTatC under transport conditions (17, 26), co-immunoprecipitations were conducted to determine whether any of the higher molecular mass bands resulted from Tha4 cross-linking to other components or precursor (Fig. 4B). The radiolabeled proteins that bound to the antibody-linked beads are shown in upper panel of Fig. 4B, and those that did not are shown in the lower panel. All of the putative Tha4 oligomers were efficiently immunoprecipitated by anti-Tha4 antibody (lane 2), but not by anti-cpTatC (lane 3) or anti-Hcf106 (lane 4) antibody. Upon extended exposure, bands representing Tha4-cpTatC and Tha4-Hcf106 cross-linking products could be detected migrating just above the Tha4 trimer band and near the Tha4 tetramer bands (lanes 3–5), but these represented very minor amounts of cross-linked Tha4 species. None of the bands were immunoprecipitated by antibodies to the mature form of DT23 (OE23) (lane 6) or to Alb3 (lane 7), a translocase component of the chloroplast signal recognition particle pathway (30). These results strongly suggest that the major Tha4 cross-linking products are Tha4 homo-oligomers.

FIGURE 5. Nonfunctional Tha4 mutants do not form transport-related oligomers. In vitro translated [3H]labeled wild-type Tha4 (WT), Tha4E10Q, Tha4E10D, and Tha4L20I) were integrated into thylakoids pretreated with anti-Tha4 IgG antibodies to inactivate endogenous Tha4 (see “Experimental Procedures”). The resulting thylakoids were used for cross-linking as described in the legend to Fig. 4, except that integration was conducted with chloroplast lysate rather than with washed thylakoids in darkness without ATP; integration and cross-linking reactions were conducted at 15 °C in white light; and the cross-linking reactions were conducted with DSP, the cleavable analog of DSS. Cross-linking reactions contained 5 mM ATP, 50 μM methyl viologen, and 1.5 μM DT23 or urea as described in the legend to Fig. 4. Precursors were prepared as described under “Experimental Procedures.” Samples recovered from cross-linking reactions were analyzed by SDS-PAGE and fluorography in the absence of reducing agent. Panels represent fluorograms of two 11.5% gels processed in parallel.

Radiolabeled Tha4 integrated into thylakoids and analysis by SDS-PAGE and fluorography (Fig. 4) produced much cleaner banding profiles compared with immunoblotting (compare Fig. 4A with supplemental Fig. S3).

DSS cross-linking of thylakoids incubated in the presence of the ΔpH (light) and the precursor DT23 stabilized a ladder of larger species with average molecular mass on several gel systems of 28, 41, 52, and 64 kDa (Fig. 4A, lanes 4–8). The ladder step size of 12 kDa corresponds roughly to the 14 kDa of the Tha4 monomer, suggesting that these products correspond to the Tha4 dimer, trimer, tetramer, and pentamer, respectively. The oligomer bands were largely absent in DSS-cross-linked thylakoids actively transporting protein and is likely a nonspecific cross-linking product. Essentially the same results were also obtained with the water-soluble DSS analog bis(sulfosuccinimidyl) suberate (data not shown) and the cleavable DSS analog DSP (below).
with the precursor-bound receptor complex. To directly address the involvement of the cpTatC-Hcf106 complex in Tha4 oligomerization, thylakoid membranes were pretreated with anti-cpTatC or anti-Hcf106 IgG antibodies, and the resulting membranes were then analyzed for precursor transport and ability to form cross-linked oligomers (Fig. 6). Increasing amounts of anti-cpTatC (Fig. 6A, lanes 4 and 5) or anti-Hcf106 (lanes 7 and 8) IgG antibodies inhibited precursor transport, although anti-Hcf106 IgG antibodies were less effective. Treatment with preimmune IgG (lanes 3 and 6) or anti-Alb3 IgG antibodies (lane 9), which served as negative controls for this experiment, did not inhibit transport. Anti-Hcf106 IgG antibodies had only a minor effect on Tha4 oligomerization in the experiment shown in Fig. 6B (lanes 7 and 8). In other similar experiments, an effect of anti-Hcf106 IgG antibodies on oligomerization was not apparent (data not shown). In contrast, anti-cpTatC IgG antibodies strongly inhibited the amount of Tha4 oligomers produced in this experiment (compared lanes 4 and 5 with lanes 1 and 2) and other similar experiments (data not shown). Neither preimmune IgG nor anti-Alb3 IgG antibodies inhibited the cross-linking of Tha4 oligomers (lanes 3, 6, and 9). This result indicates that Tha4 oligomerization is not the result of direct binding of precursor to Tha4; rather, Tha4 oligomerization likely results upon Tha4 docking to the precursor-bound receptor complex.

Tha4 Oligomerization Depends upon a Twin Arginine-containing Precursor, but Oligomer Size is Independent of the Size of the Precursor—

One model for Tha4 (TatA) channel function suggests that a Tha4 (TatA) oligomer forms around the mature domain of the precursor (5, 20). For example, Berks et al. (20) estimated that 21–23 TatA (Tha4 ortholog) molecules would be required to form a channel large enough to accommodate a folded protein with a diameter of 7 nm. This same logic predicts that ~14 Tha4 protomers would be required to accommodate the ~4-nm OE23 protein (31). To address this aspect of the model, thylakoids were cross-linked in the presence of different precursors. In preliminary experiments, we were unable to detect differences in Tha4 banding patterns resulting from reactions containing the OE23 precursor protein versus the 2–4.5-nm OE17 precursor protein (32) (data not shown). In the experiment shown in Fig. 7, the Tha4 oligomer pattern induced by the precursor DT23 was compared with that induced by tpOE17, a 33-residue synthetic signal peptide of the OE17 precursor, i.e. lacking any mature domain (22). As a negative control, we also used the nonfunctional precursor KK-DT23. As expected, oligomers were not induced by KK-DT23 (Fig. 7A, lanes 5–7). Surprisingly, the oligomer pattern induced by tpOE17 (lanes 11–13) was nearly identical to that induced by DT23 (lanes 8–10). A Tricine gel of these samples resolved oligomers as large as octamers, with no differences between the pattern induced by DT23 and that induced by tpOE17 (data not shown).

The actual size of an oligomeric protein is unlikely to be determined with chemical cross-linkers because the yield of the oligomeric cross-linking was not apparent (data not shown). In contrast, anti-cpTatC IgG antibodies strongly inhibited the transport. Anti-Hcf106 IgG antibodies on oligomerization was not apparent (data not shown). In the experiment shown in Fig. 7, the Tha4 oligomer pattern induced by the precursor DT23 was compared with that induced by tpOE17, a 33-residue synthetic signal peptide of the OE17 precursor, i.e. lacking any mature domain (22). As a negative control, we also used the nonfunctional precursor KK-DT23. As expected, oligomers were not induced by KK-DT23 (Fig. 7A, lanes 5–7). Surprisingly, the oligomer pattern induced by tpOE17 (lanes 11–13) was nearly identical to that induced by DT23 (lanes 8–10). A Tricine gel of these samples resolved oligomers as large as octamers, with no differences between the pattern induced by DT23 and that induced by tpOE17 (data not shown).
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linking product diminishes with size. And, in the case of Tha4, at higher cross-linker concentrations, Tha4 oligomers also cross-link to the cpTatC-Hcf106 complex. However, to examine the possibility that some putative channel oligomer might be present in the DT23 sample, the cross-linking products were analyzed on a 5–15% linear gradient SDS gel that can resolve species at least as large as ~300 kDa (Fig. 7B).

No differences could be seen between the pattern induced by the signal peptide alone and the DT23 precursor (with little material in sample wells).

DISCUSSION

Tha4 appears to be intimately involved in the Tat translocation step for several reasons. First, if Tha4 is inactivated with antibodies, precursors are not translocated, but remain bound to the receptor complex (18). Second, Tha4 can be cross-linked into a complex that contains cpTatC, Hcf106, and precursor only under transport conditions (17). Finally, a time course analysis showed that Tha4 assembles with the receptor complex just before the onset of translocation and disassembles when the precursor has been translocated (17). This last observation suggests that Tha4 assembly prepares the translocase for protein transport. The specific function that Tha4 plays in the translocation step is not known, although several models propose that Tha4 or the bacterial ortholog (TatA) form at least part of the protein-conducting channel (8). Therefore, knowledge of the structure and organization of Tha4 in the translocase is important for evaluating this proposed function. Because the thylakoid Tat translocase is a transient and unstable complex (17), we used cross-linking approaches to probe Tha4 organization during translocation. Both cross-linking approaches demonstrated that Tha4-Tha4 associations occur during transport.

Tha4-Tha4 cross-linking mediated by cysteine substitution in the N terminus, transmembrane domain, and hinge occurred to some extent under resting state conditions (Fig. 3 and Table 1). This result might reflect the presence of a Tha4 complex that can be observed when thylakoid membranes are solubilized with mild detergent and analyzed by blue native PAGE (18). The instability of this complex during attempts at purification suggests that such Tha4 complexes are loose assemblages rather than tight associations. This contrasts with E. coli TatA complexes, which appear to be very stable to purification procedures (21, 33). The nature of interactions mediated by the Tha4 transmembrane domain is not presently clear. The variability of cross-linking may reflect geometric constraints imposed by preferred helix-helix interactions. This is currently being pursued with double residue-substituted Tha4 proteins. Regardless of the exact organization of Tha4 under resting conditions, Tha4 clearly undergoes a conformational rearrangement as thylakoids transition to active protein transport. Cross-linking of Tha4 with cysteine substitutions in the amphipathic helix or the C-terminal tail was strongly dependent on the presence of precursor and the ΔpH (Fig. 3 and Table 1). This suggests that, during transport, Tha4 stroma-exposed domains become aligned with each other such that comparable domains are in contact.

The analysis of Tha4-Tha4 interactions detected by single cysteine substitutions was extended by chemical cross-linking with DSS or DSP. This showed that the alignment of Tha4 stromal domains during transport resulted in Tha4 oligomers (Fig. 4). Tha4-Tha4 cross-links were by far the most prevalent observed. Tha4-Hcf106 and Tha4-cpTatC cross-linking products were present at a very low level. This suggests that Tha4-Tha4 contacts are more abundant in the translocase compared with direct Tha4-cpTatC-Hcf106 contacts and might be expected if the Tha4 oligomer attaches to the precursor-bound receptor complex at only one or a limited number of locations. Similarly, cross-linking products between Tha4 and precursor were observed only upon very long exposure of immunoprecipitation gels. Although these and previous studies focused on the interactions of Tat components during transport, critical future experiments should address direct interactions between the precursor mature domain and Tat component(s), as this would directly identify all components that contribute to the structure of the protein-conducting channel.

Tha4 oligomerization could conceivably occur either before or during translocation. However, it is notable that oligomerization shares several features in common with the Tha4 assembly step. These include the requirement for the transmembrane ΔpH and a functional (but not twin lysine-containing) precursor (Fig. 7) (17). This is relevant as twin lysine-containing precursors have been reported to interact with the Tat machinery (23, 34, 35). Oligomerization also required assembly-active Tha4 (Fig. 5) (26). Finally, a twin arginine signal peptide without the mature folded domain of the precursor was sufficient to induce oligomerization (Fig. 7) and to trigger assembly of Tha4 (17). The observation that antibodies to cpTatC inhibited Tha4 oligomerization (Fig. 6) indicates that Tha4 must interact with the precursor-bound receptor to oligomerize. This is important because TatA of Bacillus subtilis has been shown to directly bind precursor protein (36). Thus, our data suggest that signal peptide binding to the receptor complex in the presence of the ΔpH opens up a docking site for Tha4 that promotes its concentration and conformational reorganization. We suggest that this event sets the stage for translocation.

Our results combined with previous studies (10) point to an ordered Tha4 arrangement of multiple stromal domains anchored by N-proximal transmembrane domains. Several considerations lead us to suggest an arrangement with the amphipathic helices at least partially embedded in the membrane. First, the Tha4 amphipathic helix residues mapped in an Eisenberg plot (37) to the same regions as surface-active peptides. Second, circular dichroism experiments with the bacterially expressed Tha4 stromal domain showed a transition from an unstructured conformation to a largely helical secondary structure as the trifluoroethanol content increased from 10 to 30%. Similarly, E. coli TatA has been shown to adopt α-helical structure in the presence of membrane-mimetic environments (38). Such transitions are characteristic of amphipathic helices that embed in the lipid bilayer (39, 40). Thus, docking to the precursor-bound receptor complex may concentrate an ordered collection of Tha4 amphipathic helices at the site of translocation. This is reminiscent of the concentration of antimicrobial amphipathic peptides, which precedes their forming a transmembrane permeation pathway (41). Our cross-linking experiments indicated that oligomers with at least eight Tha4 protomers assembled in the chloroplast Tat translocase (Fig. 7), but this is almost assuredly an underestimate. E. coli TatA, even under non-transport conditions, is cross-linked with DSS into dimers, trimers, and tetramers (42), but the isolated TatA homo-oligomer is very large, at least 460 kDa (21, 38).

How might oligomers of Tha4 (TatA) function in translocation? A recent single particle electron microscopic study of E. coli TatA complexes provides the most compelling evidence that TatA forms structures that might function as channels (21). Purified TatA homo-oligomers appeared as a collection of ring-shaped structures with sufficient variation in diameter to accommodate the known range of E. coli Tat substrates. Although the idea of a pre-existing pool of chan-

5 C. Dabney-Smith, H. Mori, and K. Cline, unpublished data.

6 C. Dabney-Smith, B. Bruce, and K. Cline, unpublished data.
nels with varying diameters is intriguing, it poses the serious mechanistic challenge of recruiting specific channels to match the translocating substrate. This would be required because the TatA structures of varying size were isolated from bacteria lacking a functional Tat system (21). Our results suggesting that oligomer size is not dictated by the mature domain of the precursor appear to rule out the possibility that the folded domain recruits an appropriately sized channel. Another possible explanation for the range of TatA particle sizes is that a larger complex undergoes partial breakdown during isolation. Such breakdown appears to readily occur during blue native PAGE analysis (21, 43). The implication is that the precursor-bound receptor complex recruits a large Tha4 (TatA) complex regardless of precursor size.

In this context, we suggest that oligomeric Tha4 stromal domains provide a passive conduit for the precursor only in response to a mechanical force that pushes or pulls the substrate across the membrane. This would open a transient channel, similar to a trapdoor, lined with the hydrophilic faces of the amphipathic helices to interact with similar surfaces on the substrate. A similar mechanism has been proposed by Bruser and Sanders (44), termed a concentrated TatA-induced “weakening” of the membrane. This speculative mechanism could address at least two requirements of the translocation process. First, it would circumvent the need for substrate-specific channel selection because the substrate would push through the minimum number of Tha4 protomers required to make an opening. Thus, a wide range of channels or conduits could be formed from a sufficiently large Tha4 oligomer. Second, because the opening would be transient and tight to the substrate, any ion or proton flow across the membrane would be minimized. This model is also consistent with evidence that TatA undergoes topology inversion as a result of Tat protein transport (45). Still left unanswered is the mechanical force for moving the precursor across the membrane, which is necessary in any model for Tat transport. Although this and other mechanistic aspects of the Tat protein transport process are unclear, our suggestive model offers a framework for future investigation.

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