Clustering of C-Terminal Stromal Domains of Tha4 Homo-oligomers during Translocation by the Tat Protein Transport System

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The chloroplast *Twin arginine translocation* (Tat) pathway uses three membrane proteins and the proton gradient to transport folded proteins across sealed membranes. Precursor proteins bind to the cpTatC-Hcf106 receptor complex, triggering Tha4 assembly and protein translocation. Tha4 is required only for the translocation step and is thought to be the protein-conducting component. The organization of Tha4 oligomers was examined by substituting pairs of cysteine residues into Tha4 and inducing disulfide cross-links under varying stages of protein translocation. Tha4 formed tetramers via its transmembrane domain in unstimulated membranes and octamers in membranes stimulated by precursor and the proton gradient. Tha4 formed larger oligomers of at least 16 protomers via its carboxy tail, but such C-tail clustering only occurred in stimulated membranes. Mutational studies showed that transmembrane domain directed octamers as well as C-tail clusters require Tha4's transmembrane glutamate residue and its amphipathic helix, both of which are necessary for Tha4 function. A novel double cross-linking strategy demonstrated that both transmembrane domain directed- and C-tail directed oligomerization occur in the translocase. These results support a model in which Tha4 oligomers dock with a precursor-receptor complex and undergo a conformational switch that results in activation for protein transport. This possibly involves accretion of additional Tha4 into a larger transport-active homo-oligomer.

INTRODUCTION

Plant thylakoids have two major pathways that transport soluble proteins into the lumen (for review, see Cline and Theg, 2007). The first pathway is analogous to the bacterial Sec system and transports unfolded proteins in an ATP-dependent manner. The second pathway, called the chloroplast Tat (cpTat) pathway, transports folded proteins and requires the protonmotive force, which in isolated thylakoids consists primarily of the ΔpH (Braun *et al.*, 2007; Cline and Theg, 2007). *Twin arginine translocation* (Tat) pathways are named for the obligate twin arginines found in the signal peptide of precursors. Genetic and biochemical experiments have identified three components essential for cpTat transport. Hcf106 (Settles *et al.*, 1997) and Tha4 (Walker *et al.*, 1999) each have an amino terminal transmembrane domain (TMD) followed by a stromally located, predicted amphi-

pathic α helix (APH) and a loosely structured carboxy tail (C-tail) (Mori and Cline, 2001). Tha4 (81 residues) and Hcf106 (176 residues) differ primarily in APH and C-tail domains. The third component, cpTatC, is a multispanning membrane protein (Mori *et al.*, 2001) that serves as the initial signal peptide receptor (Alami *et al.*, 2003; Gerard and Cline, 2006).

The cpTat pathway operates by a cyclical mechanism. Precursor proteins bind to a receptor complex consisting of cpTatC and Hcf106 (Cline and Mori, 2001). Binding, in the presence of the ΔpH , triggers the assembly of Tha4 with the precursor-receptor complex, thereby forming the translocase and allowing precursor translocation. After transport, Tha4 dissociates (Mori and Cline, 2002), resetting the system. Several characteristics have suggested that Tha4 serves as the protein-conducting component of the system. These include the cyclical assembly mechanism, the requirement for Tha4 only for translocation (Cline and Mori, 2001), the molar excess of Tha4 over cpTatC (Mori *et al.*, 2001), and observations that TatA, the *Escherichia coli* orthologue of Tha4, occurs as a collection of channel-like structures in detergent extracts (Gohlke *et al.*, 2005; Sargent *et al.*, 2006). This idea has recently gained more support from the findings of Panahandeh *et al.* (2008), who showed that TatA was associated with an apparent translocation intermediate, i.e., a precursor protein that had engaged the Tat apparatus but had not completed translocation.

Knowledge of the structure and organization of Tha4 in the translocase would provide a better idea of Tha4's exact role in translocation. We have taken a biochemical approach to this question with a staged transport assay and a biochemical Tha4 substitution assay. Previous work from our laboratory demonstrated that Tha4-Tha4 interactions occur

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Abbreviations used: APH, amphipathic helix; BMOE, bis-maleimidoethane; BM(PEO)₃, 1,11-bis-maleimido-triethyleneglycol; C-tail, carboxy-terminal loosely structured domain of Tha4; CuP, Cu⁺⁺ 1,10-*o*-phenanthroline; DSP, dithiobis(succinimidyl propionate); DTT, dithiothreitol; IB, import buffer; NEM, *N*-ethyl maleimide; Tat, twin arginine translocation; TMD, transmembrane domain, tpOE17, 31-amino acid synthetic peptide of the lumen-targeting sequence of the 17-kDa subunit of the oxygen evolving complex of photosystem II.

throughout the molecule as evidenced by disulfide cross-linking of single Cys-substituted Tha4 (Dabney-Smith *et al.*, 2006). Cysteines placed in the TMD of Tha4 interacted to form dimers in unstimulated membranes, becoming more pronounced in stimulated membranes, i.e., in the presence of precursor and the Δ pH. Cysteines placed in the APH or C-tail formed dimers only under protein transport conditions. Cross-linking with homobifunctional amine reactive cross-linkers demonstrated that Tha4 oligomers were present during protein transport. However, such oligomers were difficult to characterize because of competing cross-linker reactions with other translocase components and the lack of reactivity with the TMD. Thus, information on the oligomeric state of Tha4 in unstimulated membranes as well as a more representative size of Tha4 oligomers in the translocase could not be obtained with this approach.

Here, we engineered double cysteine substitutions in several domains of Tha4 to examine homo-oligomers under different conditions related to protein transport. Oligomers directed by TMD cysteines and oligomers directed by C-tail cysteines were readily obtained. TMD-mediated tetramers were present in unstimulated membranes and grew to octamers under protein transport conditions. C-tail-mediated oligomers were larger and occurred only under protein transport conditions. The Tha4 oligomer in the translocase could be cross-linked either through the TMD or the C-tail. These results suggest at least two possible Tha4 arrangements in the translocase that are consistent with models for Tat protein translocation. How these arrangements agree with existing data is discussed.

MATERIALS AND METHODS

Source Plants, Chloroplast, and Thylakoid Isolation

Intact chloroplasts were isolated from 9- to 10-d-old pea plants, Laxton's Progress #9 Improved, according to published procedures (Cline *et al.*, 1993). Chloroplasts were resuspended to 1 mg/ml chlorophyll in import buffer (IB: 330 mM sorbitol and 50 mM HEPES-KOH, pH 8.0) and kept on ice until use. Isolated thylakoids were obtained from intact chloroplasts as described using osmotic lysis (Cline *et al.*, 1993). Thylakoids were resuspended to 1 mg/ml chlorophyll in IB, 10 mM MgCl₂ before use. Chlorophyll was measured according to Arnon (1949).

Preparation and in Vitro Expression of Recombinant Tha4

Double Cys substitutions in Tha4 were generated as described previously (Dabney-Smith *et al.*, 2006) by using QuikChange mutagenesis (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Tha4E10A or Tha4 with the C-tail removed or the APH and the C-tail removed were described previously (Dabney-Smith *et al.*). Deletion of the predicted APH was also done using the QuikChange method and a pair of complementary primers that bound to regions on either side of the region to be deleted (Makarova *et al.*, 2000). The primers, 5'-gtcttgttttcggtccaaggcaaggattgagacc-3' and its complement, where the carat indicates the deleted region on the template, were used to produce the Tha4 deletion variant with the amino acid sequence of ... V₂₁F₂₂G₂₃P₂₄K₂₅A₂₆₍₄₅₎K₂₇₍₄₆₎E₂₈₍₄₇₎F₂₉₍₄₈₎E₃₀₍₄₉₎T₃₁₍₅₀₎, where the numbers in parentheses indicate the original residue number. The resultant Tha4 was 63-amino acid residues total. DNA sequencing on both strands at the University of Florida Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Facility verified cloned constructs. In vitro expression of recombinant Tha4 or precursors was as described by translation in a wheat germ extract from capped mRNA in the presence of [³H]leucine (Cline, 1986). Unless otherwise indicated, the translation reactions were diluted with an equal volume of 60 mM unlabeled leucine in 2 × IB before use or further diluted with IB, 30 mM unlabeled leucine.

Overexpression of Precursor Proteins and Solubilization from Inclusion Bodies

Unlabeled precursor proteins DT23 (the 23-kDa subunit of the oxygen evolving complex of photosystem II with a modified lumen-targeting sequence) and KK-DT23 (a nonfunctional DT23 where lysines replace the obligate twin arginines) were overexpressed in *E. coli* and purified as inclusion bodies as described previously (Henry *et al.*, 1997; Mori and Cline, 2002). tpOE17, a synthetic peptide representing the targeting peptide of iOE17, the stromal

intermediate precursor of OE17 (Teter and Theg, 1998), was the generous gift of Dr. Steve Theg (University of California-Davis, Davis, CA). For each experiment, purified DT23 and KK-DT23 inclusion bodies and tpOE17 were dissolved in freshly prepared 8 M urea, 1 mM dithiothreitol (DTT) to 120 μ M at 37°C for 1 h before dilution into assay mixtures.

Functional Replacement of Endogenous Tha4

Cys-substituted Tha4 proteins were assayed for functionality with an in vitro complementation assay to restore transport of the precursor DT23 as described previously (Dabney-Smith *et al.*, 2003). Briefly, isolated thylakoids (1 mg/ml chlorophyll) were pretreated with anti-Tha4 immunoglobulin Gs (IgGs) (or preimmune IgGs as a control) followed by protein A to inactivate endogenous Tha4. In vitro-translated recombinant Tha4 was then integrated into the IgG-treated thylakoids for 20 min at 15°C. After recovery and washing, the Tha4-integrated thylakoids were assayed for protein transport with in vitro translated precursor protein (DT23) in the light (80 μ mol/m²/s) at 15°C for 15 min. Thylakoids recovered from transport assays were treated with or without thermolysin. Samples were analyzed by SDS-polyacrylamide gel

Figure 1. Double cysteine substitutions in Tha4. (A) Predicted structure of Tha4 with primary cysteines shaded black and secondary cysteines shaded gray. (B) Complementation efficiency of recombinant Tha4. Complementation efficiency (the relative amount of precursor transported) was calculated as the amount of DT23 transported in anti-Tha4-treated thylakoids normalized by the relative amount of recombinant Tha4 integrated into thylakoids. Complementation by wild-type (WT) in each experiment was arbitrarily set at 100%, and the amount of DT23 transported in the no-integration control, generally <2%, was subtracted from the test samples and set at 0%. Complementation efficiencies of Cys-substituted Tha4 were calculated relative to that of wild-type Tha4 in the same experiment.

electrophoresis (PAGE) and fluorography, and radiolabeled proteins were quantified by scintillation counting of extracted gel bands (Cline, 1986).

Oxidative Cross-Linking by Disulfide Bond Formation

Thylakoids with Tha4 integrated were used for cross-linking reactions in the presence or absence of light or precursor. Light reactions contained 50 μ M ATP, 0.1 mM DTT, and 10 μ M methylviologen in IB, 3.3 mM $MgCl_2$, whereas dark reactions did not contain ATP or methylviologen. Typical assays were 88 μ l and contained 25 μ g of chlorophyll. Reactions were initiated with precursor (1.5 μ M of either DT23, KK-DT23, or tp-OE17) or 8 M urea and were incubated at 15°C in the light ($\sim 80 \mu$ mol/m²/s) or darkness for 4 min before adding 1.0 mM copper phenanthroline (CuP) or as indicated in figure legends. After an additional 5 min, oxidative cross-linking was stopped with 50 mM *N*-ethylmaleimide (NEM) and 12 mM EDTA. CuP was prepared with $CuSO_4$ and 1,10-phenanthroline as described previously (Dabney-Smith *et al.*, 2003). Thylakoids were recovered by centrifugation, resuspended in nonre-

ducing sample solubilizing buffer (2 $\times$: 100 mM Tris-HCl, pH 6.8, 5 mM EDTA, 5% SDS, 30% glycerol, and 8 M urea) and analyzed by SDS-PAGE.

Where indicated, thylakoids were pretreated with NEM before Tha4 integration as follows. For each experiment, NEM was freshly prepared in 95% ethanol as a 1 M stock. Thylakoids were pretreated with increasing concentrations of NEM as indicated or mock treated with ethanol for 5 min. The reaction was quenched with 2.5 mM DTT. The thylakoids were pelleted and washed with IB containing 0.25 mM DTT and recovered by centrifugation before suspension to 1 mg/ml chlorophyll in IB, 10 mM $MgCl_2$. Tha4 was integrated into the recovered thylakoids as described above, which were then used for cross-linking or transport assays. In addition an untreated, positive control for transport was prepared, receiving IB (instead of NEM).

Dual Cross-Linking of Tha4 and Coimmunoprecipitation

Thylakoids with Tha4 integrated were used for cross-linking reactions. Reactions were initiated with precursor (1.5 μ M DT23) or 8 M urea and incubation in the light for 5 min before addition of the cross-linkers bis-maleimidoethane

Figure 2. Tha4 C-tail oligomerizes in a transport-dependent manner. (A) Thylakoids were treated with 2.5 or 5.0 mM NEM or mock treated (0 NEM) before integration of the indicated in vitro-translated, radiolabeled Tha4. The oligomerization assays were done under transport conditions (precursor and the Δ pH) as described in *Materials and Methods*. Cys-Cys disulfide bond formation was initiated with 0.25 mM CuP and terminated after 5 min with 50 mM NEM and 12.5 mM EDTA as described in *Materials and Methods*. Recovered thylakoids were analyzed by 4–15% acrylamide Tris-tricine SDS-PAGE and fluorography under nonreducing conditions. Molecular weight markers are to the left and Tha4 oligomers are to the right. (B) Image taken from lane 9 in A and enlarged to show detail. Average M_r (relative molecular mass) of oligomers (in kilodaltons) was determined from relative mobilities of bands fit to a curve from the molecular mass standards. (C) Transport efficiency of thylakoids from A with or without recombinant Tha4 integrated as indicated below the chart. Efficiencies were calculated as the amount of DT23 transported in NEM or mock (no NEM)-pretreated thylakoids relative to that of an untreated control, which was arbitrarily set at 100%. (D) Cross-linking of Tha4 induced with different amounts of CuP. Assay conditions of dark (no Δ pH), light (Δ pH), and light + RR (Δ pH and precursor) are shown above the panel and described in *Materials and Methods*. Thylakoids were pretreated with 2.5 mM NEM before Tha4 integration. Disulfide cross-linking was as described in A.

(BMOE) or 1,11-bis-maleimido-triethyleneglycol [BM(PEO)₃] and dithio-bis(succinimidyl propionate) (DSP) to a final concentration of 1 mM. These cross-linkers were obtained from the Pierce Chemical (Rockford, IL). BMOE and DSP were prepared in dry dimethyl sulfoxide as 20 mM stocks, whereas BM(PEO)₃ was prepared in dimethyl formamide as a 20 mM stock. Cross-linking with DSP was for 5 min and stopped by addition of 100 mM glycine (final concentration). BMOE or BM(PEO)₃ cross-linking continued for a total of 10 min. Reactions were then removed from the light and diluted fivefold with ice-cold IB. Thylakoids were recovered by centrifugation, washed, and resuspended to 1 mg/ml chlorophyll. Aliquots were removed directly to sample solubilizing buffer with or without DTT, and the remainder was denatured with SDS and used for denaturing coimmunoprecipitation with either anti-Hcf106 antibodies or anti-psAlb3 antibodies linked to protein A-Sepharose beads as described previously (Mori and Cline, 2002). The beads recovered from the coimmunoprecipitation reaction were transferred to minicolumns (Promega, Madison, WI) and washed with Tris-buffered saline. Bound complexes were eluted from the beads by adding sample solubilizing buffer containing 100 mM DTT to the minicolumns, incubation for 2 h at 25°C, and centrifugation.

RESULTS

Double Cysteine Substitutions of Tha4

Double cysteine substitutions were made in each of the three Tha4 domains: TMD, APH, and C-tail (Figure 1A). The functionality of the Tha4 variants in protein transport was tested with an in vitro Tha4 substitution assay, wherein endogenous Tha4 is sequestered with antibodies and recombinant in vitro translated Tha4 introduced by in vitro integration (Dabney-Smith *et al.*, 2003, 2006). Most of the Tha4 double Cys variants were at least 60% as active as wild-type Tha4 (Figure 1B). The exceptions were G5CP9C, L6CP9C, and G7CP9C, which showed very low ability to support protein transport, suggesting that this region of the TMD is critical for transport function. Of interest is that the L20C-substituted Tha4 variants were even more active than wild-type Tha4 (Figure 1B). The reason for this is unclear, but the same phenomenon was observed previously for single Cys-

substituted Tha4, especially for those residues in the stromal-proximal region of the TMD (Dabney-Smith *et al.*, 2006). Oligomers were obtained with TMD variants and the C-tail variant. Although cysteines placed in the APH did not significantly affect the ability of the Tha4 variant to restore transport, oligomerization was not detected (data not shown), so this study focuses on cysteines in the TMD and the C-tail.

Tha4 C-Tail Oligomerizes in a Transport-dependent Manner

Tha4 with double cysteine substitutions in the C-tail formed ladders with an average step size of 11.0-kDa indicative of Tha4 multimers (Figure 2, A and B), i.e., fully reduced Tha4A65CT78C exhibited a relative molecular mass (M_r) of 11.5 kDa. Because Cys-substituted Tha4 could conceivably also form disulfide bonds with endogenous thylakoid proteins, an experiment was conducted in which free sulfhydryls on endogenous proteins were blocked by treatment of thylakoids with the alkylating agent NEM before integration of in vitro-expressed Tha4 (Figure 2, A and C). Our preliminary experiments showed that NEM pretreatment does not substantially affect the ability of thylakoids to generate a ΔpH , confirming the previous findings of Evron and McCarty (2000). We did observe that the extensive incubation and washing protocol incurred during the NEM pretreatment procedure significantly reduced the transport activity of the thylakoids as can be seen by the thylakoids receiving mock NEM pretreatment (Figure 2C, black bars). However, transport activity was fully restored by integrating recombinant Tha4 (Figure 2C). We have observed that extensive washing lowers the Tha4 content of thylakoids (unpublished data). cpTatC is the only thylakoid Tat component that contains Cys residues, and this result indicates that the

Figure 3. Tha4 TMDs oligomerize in stimulated thylakoids. Disulfide cross-linking of Tha4 directed by double Cys in the TMD was conducted in the presence of precursor and the ΔpH by the procedure described in Figure 2A. (A) Oligomerization via the TMD on the luminal proximal side. An arrowhead indicates the anomalous G7CP9C cross-linking product. The gel in the left panel was a 12.5% Tris-glycine SDS-polyacrylamide gel and those in the right hand panel were 4–15% gradient Tris-tricine SDS-polyacrylamide gels. (B) Oligomerization via the TMD on the stromal proximal side. Samples were analyzed by 4–15% acrylamide Tris-tricine SDS-PAGE. Molecular weight standards are indicated to the left and the Tha4 oligomers to the right. Average M_r of oligomers is indicated in parentheses (in kilodaltons).

cpTatC cysteines are either nonessential for transport or unavailable for NEM modification.

NEM pretreatment eliminated most of the bands larger than Tha4 dimer produced by the Tha4 C-tail single Cys substitutions A65C and T78C (Figure 2A, lanes 1–6) and decreased the intensity of the ladder produced by Tha4A65CT78C (lanes 7–9). Nevertheless, the overall oligomer pattern formed by Tha4A65CT78C was not affected by pretreatment with NEM and yielded unambiguous Tha4 oligomers as large as hexadecamers as determined by relative mobility (Figure 2B). This experiment indicates that NEM pretreatment can eliminate disulfide formation with endogenous proteins, allowing a more accurate assessment of Tha4 oligomer size.

Because Tha4A65CT78C produced oligomers only in the presence of precursor and the Δ pH (Figure 2D; see below), it was important to know whether the oxidation resulting in disulfides was complete, especially because oxidation was only allowed to continue for 5 min to minimize nonspecific cross-linking. Increasing the CuP concentration did not result in oligomers in nonstimulated membranes, nor did it alter the size of the oligomers produced by C-tail (Tha4A65CT78C) cross-linking (Figure 2D). Similarly, longer oxidation times did not significantly increase the yield or size of C-tail oligomers (data not shown).

Oligomerization Mediated by the TMD

For investigation of TMD oligomerization, two sets of Cys pairs were produced. One set used P9C as the common Cys, paired with G5C, L6C, G7C or V8C, i.e., the residues around a turn of the α helix. A second set used L20C as the common Cys, paired with V17C, A18C, A19C, V21C, or F22C. The

cross-linking experiment in Figure 3, which was done in the presence of precursor and the Δ pH, shows that both sets of double Cys substitutions generated oligomers with an average step size of 13 kDa (Figure 3), which is the apparent M_r of fully reduced Tha4 with double Cys residues in the TMD. NEM pretreatment was not effective for the transmembrane substitutions. This may be due to a lower reactivity of NEM with Cys residues buried in the low dielectric environment of the hydrophobic core of the bilayer (Bogdanov *et al.*, 2005). Accordingly, interpretations relied more heavily on comparison with the patterns obtained from the single Cys substitutions and from the M_r of the bands. The single Cys variant P9C yielded a strong dimer and a very small amount of band at the trimer position that was not eliminated by NEM (Figure 3A, lanes 1–3). Nevertheless, Tha4V8CP9C (lanes 4–6), Tha4G5CP9C (lanes 7–9), and Tha4L6CP9C (lanes 13–15) yielded unambiguous oligomers as large as octamers that were clearly distinguishable based on their estimated molecular weights. In subsequent experiments, Tha4V8CP9C consistently gave oligomers as large as octamers, whereas Tha4G5CP9C and Tha4L6CP9C were more variable in oligomer production above tetramer. Tha4G7CP9C seemed to yield cross-linking products as large as tetramers. Tha4G7CP9C also gave a major band at ~55 kDa. However this was unlikely to be a Tha4 oligomer because it clearly migrated between the tetramer and pentamer locations (Figure 3A, arrowhead). Because of the inconsistency of oligomerization of the latter three double Cys variants and because they showed very low functional activity, they could not reliably be used to evaluate potential TMD helix packing.

At the stromal proximal transmembrane region Tha4A18CL20C, Tha4A19CL20C, and Tha4L20CV21C also

Figure 4. Transport-related conditions required for Tha4 oligomerization via the TMD and via the C-tail. (A) Tha4V8CP9C was integrated into isolated, NEM-pretreated thylakoids and oxidative cross-linking was performed in the absence (lane 1) or presence of light (lane 2), light plus methylviologen (lanes 3–7) and either urea (lanes 1–3), 1.5 μ M nonfunctional precursor (KK; lane 4), 1.5 μ M functional precursor (RR; lane 5), or 1.5 μ M synthetic signal peptide (tpOE17; lanes 6 and 7). Nigericin (0.5 μ M) and valinomycin (1.0 μ M) were added to completely dissipate the protonmotive force before cross-linking (lane 7). (B) A densitometry scan of lanes 3 and 5 of the film shown in A. This film was overexposed to detect the pentamer through octamer bands in the unstimulated membrane lanes. The film image was acquired with a scanner by using transmitted light and the peak profile produced by ImageJ (National Institutes of Health, Bethesda, MD). (C) As in A except Tha4A65CT78C was integrated into isolated, NEM-pretreated thylakoids. (D) A densitometry scan of lanes 5 and 6 of the film shown in C to demonstrate the similar oligomer patterns triggered by the signal peptide (SP, tpOE17) versus the full RR precursor DT23. Cross-linking was as described in *Materials and Methods* and recovered thylakoids were analyzed by 4–15% acrylamide Tris-tricine SDS-PAGE under nonreducing conditions.

Figure 5. Tha4 structural requirements for oligomerization. Integration of different variants of Tha4 and cross-linking are as described in *Materials and Methods*. (A) Cross-linking in the TMD of Tha4. Tha4V8CP9C (WT), Tha4V8CP9CE10A (E10A), Tha4V8CP9CΔAPH (ΔAPH), Tha4V8CP9CΔ29 (ΔC-tail), and Tha4V8CP9CΔ54 (ΔC-tail/ΔAPH) were integrated into isolated thylakoids and assayed in the presence of ΔpH alone (–) or ΔpH and either nonfunctional (KK) or functional (RR) precursor as described in Figure 2 except that 1 mM CuP was used for the cross-linking. (B) Cross-linking in the C-tail. Thylakoids were pretreated with NEM. Tha4A65CT78C (WT), Tha4E10AA65CT78C (E10A), or Tha4A65CT78CΔAPH (ΔAPH) were integrated and assayed as described in A. (C) Cartoon diagram depicting the domain organization of Tha4. Deletion of these domains in the Tha4 used in A and B is denoted as Δ.

yielded oligomers as large as octamers under the stimulated conditions of precursor plus the ΔpH (Figure 3B). The Tha4L20C single Cys variant yielded dimers, a band at ~41 kDa migrating slightly above the trimer band and a fainter band at the approximate location of the tetramer (Figure 3B, lanes 1–3). Although, these bands were not eliminated with NEM, they could be discerned from oligomeric bands by a slightly different migration. Tha4V17CL20C and L20CF22C did not show oligomers higher than trimer or tetramer (data not shown). Residues producing oligomers, i.e., A18, A19, L20, and V21, are equally spaced around a turn of the predicted helix and do not suggest a particular helix packing face preference. However, because these residues are in the region where the TMD emerges from the bilayer, the TMD helix may show more torsional flexibility as has been found for other transmembrane proteins (Jones *et al.*, 1998; Greene *et al.*, 2007).

Transport-related Conditions Required for Oligomer Formation Mediated by the TMD and the C-tail

We selected Tha4V8CP9C for further examination as a functional representative of the TMD series because the cysteine substitutions occur in the heart of the TMD near the essential transmembrane glutamate (E10) (Dabney-Smith *et al.*, 2006). As shown in Figure 4A, Tha4V8CP9C formed tetramers under all conditions tested, but these were enhanced by the ΔpH (lane 3) and increased to octamers by the presence of a twin arginine containing precursor or signal peptide (Figure 4A, lanes 5 and 6) but not a mutant twin lysine precursor (Figure 4A, lane 4). Densitometer scans of lanes 3 and 5 in Figure 4A show the substantial increase of the higher oligomers stimulated by the presence of precursor (Figure 4B).

Oligomerization of Tha4 directed by the C-tail, Tha4A65CT78C, required both the ΔpH and a twin arginine-containing precursor or twin arginine signal peptide (Figure 4C). Without precursor (Figure 4C, lanes 1–3) or without the ΔpH (Figure 4C, lane 7), only dimer was produced. Of particular note is that the same pattern and maximum size of oligomers was triggered by the synthetic signal peptide tpOE17 as by the full 23-kDa precursor (Figure 4C, lanes 5 and

6; and D). This was also true for the Tha4V8CP9C-directed oligomeric pattern above tetramer (Figure 4A, lanes 5 and 6).

Structural Elements of Tha4 Required for Oligomerization

Previous work determined that Tha4's transmembrane glutamate (E10) is essential for Tha4 function and its ability to assemble with the receptor complex (Dabney-Smith *et al.*, 2003). In addition, deletion of the C-tail did not eliminate Tha4 function, but deletion of both the C-tail and the APH rendered Tha4 inactive. Similar mutations were placed in Tha4V8CP9C and, where appropriate, Tha4A65CT78C to assess their effects on oligomerization (Figure 5). Substituting alanine for E10 eliminated Tha4V8CP9C oligomerization above tetramer (Figure 5A, compare lane 6 with lane 3) and eliminated Tha4A65CT78C oligomerization (Figure 5B, compare lane 6 with lane 3). Similarly, an internal deletion of the APH, which produced a Tha4 consisting of the TMD and C-tail, eliminated Tha4V8CP9C oligomerization above tetramer (Figure 5A) and all oligomerization of Tha4A65CT78C (Figure 5B). Deletion of the C-tail from Tha4V8CP9C did not prevent oligomerization to tetramers in nonstimulated membranes or the oligomerization to octamers in stimulated membranes (Figure 5A, lanes 13–15). However, deletion of both the C-tail and APH from Tha4V8CP9C completely inhibited oligomerization (Figure 5A, lanes 16–18). Alkaline extraction and protease treatment verified that the Tha4ΔC-tailΔAPH protein was integrated into the bilayer (Supplemental Figure 1). However, it cannot be ruled out that such a drastic truncation alters the precise manner in which the Tha4 TMD associates with the bilayer and with other Tha4 TMDs. These results show that the TMD glutamate and the APH are the same structural elements required for Tha4 function in protein translocation and oligomerization, either above the tetramer in the TMD or at all in the C-tail.

Tha4 Protomers Interact in the Translocase through Both the TMD and the C-Tail

The requirement of precursor and the ΔpH for the TMD oligomerization above tetramer and for C-tail oligomerization suggested that both forms are present in the translocase.

analyzed by SDS-PAGE under reducing conditions. Each immunoprecipitation lane contains the equivalent of 3.6 μ g of chlorophyll of starting material, and the gels were exposed to film for 16 d.

To test this possibility, we used a double cross-linking strategy. The irreversible bis-maleimide cross-linkers BMOE, for Tha4V8CP9C, and BM(PEO)₃, for Tha4A65CT78C, were used to cross-link Tha4 oligomers through the double Cys residues. DSP, a homobifunctional amine-reactive cross-linker that can be cleaved with sulfhydryl reagents, was used to cross-link Tha4 to the precursor bound receptor complex. After cross-linking with both agents, the membranes were solubilized with SDS and subjected to denaturing coimmunoprecipitation with antibodies to the receptor complex component Hcf106. Bound proteins were then released from the antibody beads with SDS buffer containing the sulfhydryl reagent DTT to cleave the DSP cross-link and release Tha4.

As seen in Figure 6 for membrane integrated Tha4V8CP9C, the membranes were incubated in the light to generate the Δ pH, either with or without precursor, and were then

treated with BMOE, DSP, or both. BMOE treatment resulted in oligomers in the absence of precursor (Figure 6A, lane 2, top), and this was increased to octamers by precursor (compare lane 2 with lane 6, top). DSP treatment either with or without BMOE resulted in smears to the top of the gel (Figure 6A, lanes 3, 4, 7, and 8, top). When the samples were treated with reducing agent, the smears largely disappeared and the oligomers cross-linked by BMOE remained (Figure 6A, bottom). Importantly, in the immunoprecipitates with antiHcf106, oligomers were present only in the sample that received precursor, BMOE, and DSP (Figure 6B, lane 9, top), indicating that Tha4 oligomers directed by the TMD are present in the translocase.

DSP cross-linking is prone to some nonspecific cross-linking and coimmunoprecipitation. Here, we controlled for nonspecific cross-linking by immunoprecipitation with antibodies to psAlb3, a membrane component of the chloro-

Figure 7. Tha4 oligomers via the C-tail are in the translocase. Tha4A65CT78C was integrated into isolated thylakoids and subjected to cross-linking in the presence of light and the absence or presence of functional precursor (RR). The sulfhydryl reactive cross-linker BM(PEO)₃ and the amine reactive cross-linker DSP were added simultaneously to 1 mM. Cross-linking and analysis was as described in Figure 6. (A) SDS-PAGE under nonreducing (top) or reducing conditions (bottom) before immunoprecipitation (pre-IP). Each lane contains the equivalent of 0.9 μ g of chlorophyll. (B) The remaining samples were subjected to coimmunoprecipitation with antiHcf106 (α Hcf106 IP, top) and with antiAlb3 (α Alb3 IP, bottom). Samples were eluted from the beads and analyzed by SDS-PAGE under reducing conditions. Each lane contains the equivalent of 3.6 μ g of chlorophyll. Gels in A and B were exposed to film for 16 d.

plast SRP pathway (Figure 6B, bottom). As seen, a small amount of monomeric Tha4 was immunoprecipitated from DSP cross-linked samples, equivalent to the amount of monomeric Tha4 immunoprecipitated by antiHcf106 from reactions lacking precursor (compare bottom lanes 4 and 5 with top lanes 4 and 5). However, oligomeric Tha4 was not immunoprecipitated in the control anti-Alb3 immunoprecipitates.

A similar experiment conducted with Tha4A65CT78C gave similar results (Figure 7). Oligomers were absent from BM(PEO)₃ treatments unless precursor was present. A significant amount of cross-linking occurred without BM(PEO)₃ in the absence of precursor (Figure 7 A, lane 2, top). This was nonspecific auto oxidative disulfide cross-linking to endogenous proteins previously seen (Figure 2). In the presence of precursor but without BM(PEO)₃, a ladder of oligomers occurred (Figure 7A, lane 6, top). This also likely resulted from auto-oxidation. When the samples were reduced, these patterns disappeared (Figure 7A, lanes 2 and 6, bottom). The C-tail oligomers were only present in anti-Hcf106 immunoprecipitates from reactions that received precursor, BM(PEO)₃, and DSP (Figure 7B, lane 9, top). This experiment demonstrates that Tha4 oligomers cross-linked through double Cys residues in the C-tail are also present in the translocase. In fact, it is likely that such C-tail oligomers are exclusively present in the translocase because pretreatment of thylakoids with IgGs directed against cpTatC or Hcf106, which inactivate the receptor complex (Cline and Mori, 2001), prevented C-tail oligomerization (Supplemental Figure S2).

DISCUSSION

Tha4 and its bacterial ortholog TatA have long been thought to provide a protein-conducting passageway during protein transport (Berks *et al.*, 2000; Cline and Mori, 2001; Mori and Cline, 2002). The first evidence for such a role came from Alami *et al.* (2003), who showed that *E. coli* TatA makes contact with the signal peptide under conditions leading to translocation. More recently, Panahandeh *et al.* (2008) found an apparent translocation intermediate associated with TatA. The current challenge is to determine exactly what Tha4/TatA is doing during translocation. Our approach is to characterize the Tha4 conformation and organization under different conditions related to protein transport. Because Tha4 is only transiently present in the translocase, methods that can probe its characteristics in the membrane during its assembly with the receptor complex and during translocation are essential.

Here, we have used cysteine scanning and oxidative disulfide cross-linking as a site directed cross-linking method that provides specific information on the in situ organization and conformation of membrane proteins (Jones *et al.*, 1998; Kaback *et al.*, 2007). By reducing the cross-linking time to 5 min or less, our intention was to capture those events that specifically relate to translocation (Mori and Cline, 2002). We show, with double Cys-substituted Tha4 cross-linking, that Tha4 oligomers are associated through their TMD in unstimulated membranes but undergo an organizational change upon docking with precursor-bound receptor complex that additionally clusters the C-tails. Presumably, this conformational or organizational switch either activates Tha4 for translocation or reflects its conformation during translocation.

Our results indicate that Tha4 protomers are held together by the TMD both in unstimulated membranes and in the translocase. Seven different Cys pairs in the TMD produced

oligomeric bands up to tetramer in unstimulated membranes (Figure 4; unpublished data) and up to octamer in stimulated membranes (Figures 3 and 4). In addition, deletion of either the C-tail or the APH of Tha4V8CP9C still resulted in tetramers in unstimulated membranes. Association of protomers through the TMD was not dependent upon the highly conserved E10 as was predicted for the corresponding residue in TatA, Q8 (Greene *et al.*, 2007). However, the E10A mutation did prevent the oligomerization above tetramer seen in stimulated membranes as well as oligomerization through the C-tail (Figure 5), both of which are characteristic of Tha4 in the translocase (Figures 6 and 7). These observations confirm previous studies (Dabney-Smith *et al.*, 2003) that E10 is essential for Tha4 interaction with the receptor complex rather than for Tha4 oligomerization per se.

The TMD probably also plays an essential role in protomer interaction in the translocase because deletion of the C-tail did not eliminate transport function (Dabney-Smith *et al.*, 2003) nor prevent TMD oligomerization above tetramer (Figure 5). This makes it unlikely that the striking oligomerization exhibited by Tha4A65CT78C is due to specific C-tail interactions. Rather, it is more likely that C-tail oligomerization is simply reporting on the spatial proximity of C-tails in the translocase. Double Cys variants in the Tha4 APH did not yield oligomers in our study, but the APH likely plays a role in protomer association in the translocase because deletion of the APH eliminated C-tail oligomerization and TMD oligomerization above tetramer (Figure 5) and single Cys variants of the APH produced dimers in stimulated membranes (Dabney-Smith *et al.*, 2006).

The size of Tha4/TatA homo-oligomers has been of considerable interest in the context of conceptual models for translocation. Detergent-solubilized *E. coli* TatA homo-oligomers range in size from <100 kDa to >600 kDa (Gohlke *et al.*, 2005; Oates *et al.*, 2005; McDevitt *et al.*, 2006). On blue native gels, *E. coli* TatA migrates as a series of oligomeric bands with step size corresponding to a TatA tetramer. Several mechanistic interpretations have been made based on the size and appearance of detergent-solubilized TatA (Gohlke *et al.*, 2005; Oates *et al.*, 2005; Sargent *et al.*, 2006). However, in a recent study of TatA in situ, a partially functional TatA-yellow fluorescent protein (YFP) expressed at wild-type levels occurred in bacteria as particles with a median size of ~25 TatA-YFP per complex and a step change of ~4 TatA-YFP molecules (Leake *et al.*, 2008). Importantly, such particles were only observed in bacteria that were simultaneously expressing TatB and TatC, implying that they are representative of translocase associated TatA. Without TatBC, a diffuse image was obtained suggesting low molecular weight complexes. This is of interest because very large TatA oligomers were obtained by detergent solubilization of *E. coli* overexpressing TatA but not expressing TatBC (Gohlke *et al.*, 2005). This raises questions regarding the physiological relevance of detergent solubilized TatA (or Tha4) complexes. Nevertheless, the combined studies support a "polymerization" model wherein tetrameric TatA units associate to form a larger complex in the translocase.

The double Cys-disulfide cross-linking approach used in the present study also interrogates Tha4 in situ, and, when interpreted in the context of the TatA-YFP in situ results, leads us to conclude that the basic functional unit of Tha4 oligomer in unstimulated membranes is a tetramer (Figure 4). Double Cys-cross-linking has been used previously for determining oligomer size (Jones *et al.*, 1998). Under ideal conditions, appropriately placed pairs of Cys residues can cross-link all protomers of an oligomer. In practice, several

chain-terminating reactions result in ladders of bands smaller than the in situ oligomer. Even the very stable homooligomer of the c subunit of the ATP synthase F_0 gave a spectrum of oligomeric bands (Fillingame and Dmitriev, 2002). In unstimulated thylakoids, oligomers up to tetramer were produced from all seven Tha4 variants with Cys pairs in the TMD (Figure 4; unpublished data). This result is consistent with studies of bacterial TatA, which also suggest a basic tetrameric unit (Gohlke *et al.*, 2005; Oates *et al.*, 2005; Leake *et al.*, 2008).

Because Cys-mediated disulfide cross-linking requires very close spacing of Cys residues, higher order association of Tha4 tetramers in unstimulated membranes might not be efficiently cross-linked by this method. Indeed, pentamers through octamers could be detected from TMD-double Cys variants in unstimulated membranes at very low levels (Figure 4B). Thus, we cannot rule out the possibility that Tha4 is octameric in unstimulated membranes. However, we note that the Tha4 variant (Figure 5, Tha4E10A) that cannot assemble into the translocase (Dabney-Smith *et al.*, 2003) produced only tetramers.

A higher order organization was clearly apparent in the translocase by TMD-mediated cross-linking, in which the production of pentamer to octamer was greatly stimulated (Figure 4) and by C-tail mediated cross-linking, in which oligomers as large as hexadecamers (187 kDa, Figure 2) could clearly be discerned. The possibility that even larger oligomers are present in the translocase is supported by the observations that smears of radiolabel were frequently present above the hexadecamer band and that on blue native acrylamide gels, by using detergent conditions that disrupt noncovalent Tha4 complexes, an intensified smear of Tha4-containing complexes was present even above 200 kDa (Dabney-Smith and Cline, unpublished data).

The differential cross-linking mediated by the TMD and the C-tails is quite interesting and suggests a model in which Tha4 becomes active only when associated with a precursor-occupied cpTatC-Hcf106 complex. Two feasible scenarios could explain both the enhanced TMD cross-linking and the C-tail clustering in the translocase. The first is that a Tha4 oligomer docks with the receptor complex and undergoes a conformational change to a more ordered structure that packs the TMD domains more tightly and brings the C-tails into contact. Several models propose ordering of these domains in the translocase, e.g., cylindrical pores that arrange around precursors (Gohlke *et al.*, 2005; Sargent *et al.*, 2006) and passive gates composed of Tha4/TatA mats (Bruser and Sanders, 2003; Dabney-Smith *et al.*, 2006).

Another possibility is that a Tha4 tetramer (possibly octamer) initially docks with the receptor complex and undergoes a conformational change that results in accretion of additional Tha4 tetramers (or octamers). C-tails might be in close proximity in the translocase simply because the packing of tetramers increases the local C-tail concentration. This scenario is consistent with the polymerization model evoked by the results with TatA-YFP by Leake *et al.* (2008).

In summary, our results are consistent with the idea that Tha4 exists in unstimulated membranes as an oligomer held together through TMD contacts. On precursor protein binding, Tha4 oligomers dock with the receptor complex and undergo a conformational shift that either alters their packing and organization or results in accretion of additional Tha4 into a larger transport-active oligomer. Previous studies demonstrated that Tha4 dissociates from the receptor complex upon translocation of the precursor and that addition of more precursor triggers another round of Tha4 assembly and translocation (Mori and Cline, 2002). However,

it is not currently clear what triggers translocation and detachment of Tha4. An attractive possibility would be that translocation of the precursor occurs when the associated oligomer becomes sufficiently large. However, as shown in Figure 4, a signal peptide alone induced the same pattern and maximum detectable size of C-tail oligomers as that induced by a full 25-kDa precursor (Figure 4), implying that the mature domain of the precursor does not play a direct role in the size of the associated Tha4 oligomer. Similarly, Leake *et al.* (2008) report that the size distribution of TatA-YFP in *E. coli* was not significantly altered by saturating the system with substrates of different size. Although these observations seemingly argue against the proposed form-fitting channels for substrates, they also emphasize the need to determine the size of the oligomer at the time of translocation. The site-specific Cys-mediated cross-linking reported here is a start toward determining the conformation and organization of Tha4 in the translocase. In addition, our results underscore the need for structural and topological analysis of Tha4 when it is assembled in the translocase.

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