ABSTRACT

INSIGHTS INTO THE CHLOROPLAST TAT MECHANISM OF TRANSPORT

by Aman Gebreyohannes Habtemichael

The chloroplast twin arginine transport (cpTat) system transports folded proteins across the thylakoids in chloroplast using proton motive force as the only source of energy. The cpTat is composed of three components; Tha4, Hcf106, and cpTatC. Hcf106 and cpTatC form a receptor complex where the precursor binds. Tha4 is found as separate homo-oligomers that assemble the receptor-bound substrate to complete the translocation. Though the overall model for the transport cycle is established, detailed sequential events remain to be elucidated. This thesis mainly probes conformational change of Hcf106 upon substrate binding using cysteine accessibility technique and interaction points between cpTatC first stromal domain (S1) and precursor mature domain using disulfide crosslinking to understand their direct contribution to the actual translocation event. We detected a more buried Hcf106 amphipathic helix (APH) after precursor binds. Besides, interactions were detected between cpTatC S1 and precursor mature domain. Altogether, our data supports a model that depicts Hcf106 APH membrane weakening and cpTatC insertase activity to promote the translocation of the precursor.
INSIGHTS INTO THE CHLOROPLAST TAT MECHANISM OF TRANSPORT

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Dedication

This work is dedicated to my wife, son, and parents
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CHAPTER 1

Introduction
1.1 Protein transport and trafficking in cells

All organisms are built on a common structural and functional unit, the cell. Primitive prokaryotic cells, most likely, assumed a non-compartmented, cell wall-less system that later evolved to engulf a self-replicating system hypothesized to be initiated due to metabolic driven syntrophy followed by endosymbiosis (Cavalier-Smith, 2010; Sommer and Schleiff, 2014). The engulfing cell gradually developed an invaginating membrane to surround the highly concentrated and complex components secreted by the engulfed bodies (Cavalier-Smith, 2010). The enclosed contents might then have progressively differentiated into distinct membrane-bound subcellular compartments, organelles, such as chloroplasts and mitochondria, which gave rise to eukaryotic cells. Each organelle has a different protein composition and are highly specialized to perform different metabolic functions (Cavalier-Smith, 2010). Most cellular proteins are encoded in the nucleus and synthesized in the cytoplasm including those for organelles, although they also encode and synthesize a small fraction their own proteins. Such complexity created a new challenge where nascent proteins needed to be shuttled from the cytoplasm to different cell compartments where there they are required to perform their metabolic functions (Sommer and Schleiff, 2014).

More than 50% of all cytosolically-synthesized proteins in eukaryotes make their way to different organelles, sub-organelles, and the plasma membrane, each usually requiring the crossing of at least one membrane barrier (Schatz and Dobberstein, 1996). For example, more than 95% of the proteins that are responsible for the metabolic function of the mitochondria or chloroplasts, are encoded by nuclear genes, synthesized in the cytoplasm from nuclear transcribed mRNAs, and subsequently localized to their respective organelles; the presence of organellar genomes notwithstanding (Cline and Dabney-Smith, 2008; Schatz and Dobberstein, 1996; Wickner and Schekman, 2005). Protein trafficking can be of two types. In co-translational transport, translocation and/or integration occur simultaneously with protein synthesis (Walter, 1995); most proteins destined for the endoplasmic reticulum, plasma membrane, lysosomes, and Golgi apparatus are transported co-translationally into the endoplasmic reticulum (Agarraberes and Dice, 2001). Alternatively, in post-translational transport, proteins are transported after they are synthesized in the cytosol (Sommer and Schleiff, 2014).
1.1.1 **Protein transport systems: fundamental principles and common features**

Eukaryotic cells contain different organelles designed to carry out specific metabolic functions in the cell, and as such each require a unique protein complement to perform its specialized tasks (Sommer and Schleiff, 2014). Because the proteins needed for those metabolic functions are mostly synthesized in the cytosol, cells have developed an elaborate system of targeting proteins to the appropriate transport system in the membrane of the target organelle. For example, proteins destined for an organelle are typically synthesized as higher molecular weight precursor proteins containing an N-terminal, often cleavable, amino acid sequences signaling or targeting sequences (Cline and Dabney-Smith, 2008; Schatz and Dobberstein, 1996; Wickner and Schekman, 2005).

Signal sequences target precursors to their target membrane by interaction with a translocase receptor localized on the surface of the membrane (Agarraberes and Dice, 2001; Wickner and Schekman, 2005). These peptides are often transient and cleaved off by the action of proteolytic enzymes following transport, generating the mature version of the protein. Signal sequences for precursor destined for each organelle do not have a conserved sequence, however, they share common motifs, structure, and polarity that can be characterized into three distinct domains: (1) an N-terminal acidic region, (2) highly hydrophobic region, and (3) a C-terminal proximal polar region containing the cleavage recognition sequence for the signal peptidase (Agarraberes and Dice, 2001; Wickner and Schekman, 2005). Most transport complexes translocate their substrate in an unfolded state, proteins largely composed of hydrophobic residues may also require cytosolic chaperones that prevent newly synthesized proteins from folding prior to transport (Hendrick and Hartl, 1993). Members of the heat shock protein (Hsps) 70 family are the major players in preventing aggregation and stimulating subsequent translocation of proteins destined to the mitochondria, endoplasmic reticulum, and chloroplast (Hendrick and Hartl, 1993; Schatz and Dobberstein, 1996).

Another feature of biological membranes is protein complexes known as ‘translocases’ or ‘translocons’, that mediate the transport of cellular proteins in a highly-organized fashion. Most translocases are protein complexes comprising three functional features: (1) a receptor that recognizes the signal peptide, (2) a hetero-oligomeric transmembrane channel that serves as passage way across the membrane, and (3) a translocation motor powered by an energy source, typically ATP or GTP (Schatz and Dobberstein, 1996). Translocase receptors hand over the
precursor to the channel complex, the central entity, that interacts with all other components of the translocase complex, and the actual route of translocation, where the substrate is transported into or across the membrane (Cline and Dabney-Smith, 2008; Walter, 1995).

Nevertheless, there are a few exceptions to the typical features of translocases. The chloroplast Twin Arginine Transport (cpTAT) pathway which is responsible for importing stromal proteins into the thylakoid uses the protonmotive force (pmf) (Braun et al., 2007; Cline et al., 1992) as the only source of energy. Protein precursors that are targeted for the cpTAT pathway and peroxisomal translocases are transported while they are in their folded state (Clark and Theg, 1997; Schatz and Dobberstein, 1996).

1.1.2 Protein import into chloroplasts
Plastids, characteristic organelles of plant cells, are the foundation of all food chains and key for sustaining life on earth (Leister, 2003). One type of plastid, the chloroplast, plays a crucial part in photosynthesis, cell defense, and intracellular signaling (Bhattacharyya and Chakraborty, 2017). Plastids originated around 1.5 billion years ago, from an endosymbiotic integration of cyanobacteria, a free-living, oxygenic photosynthetic prokaryote, into an eukaryotic host cell (Gould et al., 2008; Gray, 1989; Gray, 1999; Pedroza-Garcia et al., 2016).

The chloroplast contains three membranes—the outer envelope that encloses the intermembrane space; the inner envelope that encloses the stroma; and the thylakoid that harbors the photosynthetic machinery and encloses the lumen. About 95% of the required 3500 – 4000 plastid specific proteins are encoded by nuclear genes and are synthesized in the cytosol (Lamberti et al., 2011). As a result, these proteins need to be targeted to the organelle and routed to their respective sub-organelle compartments in the plastid (Celedon and Cline, 2013a; Kouranov et al., 1999; Race et al., 1999; Soll and Schleiff, 2004). Stromal and thylakoid proteins are translocated through the double-membrane envelope by oligomeric complexes, the translocons of the outer (TOC) and inner (TIC) chloroplast membranes (Celedon and Cline, 2013a; Kouranov et al., 1999). Both translocons are composed of several transmembrane and peripheral proteins. The core TOC subunits include the GTP-binding proteins, Toc159 and Toc34 (Toc33), and the β-barrel translocon pore, Toc75 (Chen et al., 2016; Soll and Schleiff, 2004). These components exist as 550 kDa complex in a 1:4:4 stoichiometric ratio. In Arabidopsis thaliana, there are three small
families of Toc159-like proteins: Toc 90, Toc120, and Toc132. A less well-defined subunit is Toc64 which contains a cytosolically exposed tetratricopeptide motif and is believed to play a role in docking and catalyzes the transfer of Hsp90-chaperoned precursors to the channel (Schlegel et al., 2007).

Most TOC proteins are synthesized as mature products where the signaling sequence is found in transmembrane domains and adjacent regions. These proteins, except Toc75, integrate into the membrane unassisted (Kouranov et al., 1999). Toc75, unlike most other TOC components, is synthesized as a large precursor with an N-terminal signal sequence, called transit peptide for plastid-destined precursors, that consists of two targeting domains: the typical, N-proximal stromal targeting sequence and a C-proximal outer envelope targeting sequences (Baldwin and Inoue, 2006; Tranel et al., 1995; Tranel and Keegstra, 1996). Toc75 contains novel sequences, conserved among diverse plant species, that are necessary for integration. One is a polyglycine stretch in the C-terminal region of its transit peptide that mediates envelope targeting. In addition, an AXA motif and a glutamate, both located near the second cleavage site for a type I signal peptidase, are important for the maturation of Toc75 (Inoue et al., 2003).

TIC is made up of at least seven subunits: Tic110, Tic55, Tic62, Tic20, Tic22, Tic32, and Tic40 (Agarraberes and Dice, 2001; Soll and Schleiff, 2004) of which first three subunits are the core complex (Küchler et al., 2002). Though its exact role is yet to be elucidated, Tic110 is near or perhaps in contact with TOC subunits. At present, there are conflicting results in regard to the exact topology of Tic110 within the envelopes (Inaba et al., 2003; Soll and Schleiff, 2004). It appears though that Tic110 contains a bulky C-terminal stromal protrusion that is proposed to serve as a site where stromal chaperones function to fold translocated mature proteins (Inaba et al., 2003; Kouranov et al., 1998; Soll and Schleiff, 2004). Tic 55 and Tic 62 are believed to be involved in the regulatory redox reactions of the transport process because Tic55 contains a Rieske iron-sulfur center (Caliebe et al., 1997) and Tic 62 binds to ferredoxin-NAD(P)+ oxidoreductase (Küchler et al., 2002). Tic20 is associated with the TIC channel (Becker et al., 2004; Kouranov et al., 1998) and Tic22 is a receptor, peripherally assembled to the TIC complex (Kouranov et al., 1999), to which translocating substrates bind (Becker et al., 2004) and have been recently discovered to co-precipitate with Toc75 (Paila et al., 2016).
A general translocation mechanism of TOC/TIC has been described (Flores-Pérez and Jarvis, 2013). Briefly, proteins destined to the aqueous and membrane compartments in the plastids are directed by their N-terminal, cleavable transit peptides to the TOC complex where they bind in an energy independent manner. Import of precursor through TOC and TIC complexes is energy-dependent, requiring both ATP and GTP. Once precursor is imported, stromal proteases cleave the N-terminal transit peptide to reveal the mature protein, if the protein is destined for the stroma or inner envelope membrane (Flores-Pérez and Jarvis, 2013; Jarvis, 2008). Stromal chaperones, Hsp70 and Hsp60, bind to incoming proteins to prevent misfolding or aggregation and facilitate transit peptide cleavage and proper folding to the native conformation (Flores-Pérez and Jarvis, 2013). A subset of stromal proteins is then either integrated into or translocated across the thylakoid membrane (Flores-Pérez and Jarvis, 2013; Kouranov et al., 1999). **Figure 1.1** shows protein import into the chloroplast by the TOC/TIC apparatus.

There are, however, a few proteins that are imported into the chloroplast through other routes that don’t involve the TOC/TIC apparatus. For instance, Tic32 and the chloroplast envelope
quinone oxidoreductase (ceQORH) don’t possess a directing transient peptide and are imported across the outer envelope through a route other than TOC (Cline and Dabney-Smith, 2008).

1.2 **Protein Targeting to the Thylakoid**

Chloroplasts in plants, algae, and cyanobacteria contain thylakoids that enclose an aqueous lumen (Barber, 2002; Dekker and Boekema, 2005). The thylakoid membrane consists of two structural domains: the grana and stromal lamellae, which are interconnected but differ in structural arrangement. The grana contain stacked thylakoidal membranes housing the photosynthetic complexes, photosystem (PS) II and the Light Harvesting Complex (LHC) II. The stroma lamellae are entirely made up of unstacked thylakoids that interconnect several stacks of grana. The photosynthetic complexes, PSI and ATP synthase, reside on the membrane of stromal lamellae. Another important component of the photosynthetic apparatus, cytochrome b6/f, is, evenly distributed between the grana and stromal lamellae (Dekker and Boekema, 2005).

![Figure 1.2 The Photosynthetic protein complexes in the thylakoid](image-url)

**Figure 1.2 The Photosynthetic protein complexes in the thylakoid:** The photosynthetic electron transport apparatus includes PSII, Cytb/f, PSI, and ATPase. Solid arrows show the electron flow. The electron flow results in a net decrease of luminal pH that fuels ATPase in the generation of ATP. The generation of NADPH supplies the Calvin-Benson cycle. The image is adapted from (Michelet et al., 2013).

The photosynthetic machinery is synchronized by multi-subunit complexes, containing light absorbing pigments and reaction centers, in the thylakoid membrane that oxidizes water to molecular oxygen through the use of light energy (Barber, 2002). This biochemical redox reaction is the synthesis of ATP and NADPH which are catalyzed by ATP-synthase and ferredoxin-
NADP⁺-oxidoreductase respectively, that fuels carbon fixation, the reduction of CO₂ to carbohydrates, in the stroma (Fromme et al., 2001; Krauss, 2003). **Figure 1.2** depicts the photosynthetic protein complexes and electron flow across the thylakoid. The biogenesis of the thylakoid and its components requires hundreds of proteins that require transport from the stroma into/across the thylakoid membrane (Aliev et al., 2001). Translocation and import of stromal proteins across the thylakoid membrane is organized by at least four membrane transport protein pathways (**Figure 1.3**): the spontaneous pathway, the signal recognition particle (SRP) pathway, the general secretory (Sec) pathway and the chloroplast Twin Arginine Translocation (cpTat) pathway (Agarraberes and Dice, 2001).

**Figure 1.3. Protein import and routing in chloroplast:** The Toc/Tic apparatus works in importing cytosolic proteins into the chloroplast. After removal of the targeting sequences by the stromal targeting peptidase, processed proteins are integrated and/or transported by one of the four thylakoid transport pathways. From left to right in the figure: spontaneous, chloroplast twin arginine (cpTat), chloroplast secretory (cpSec), and signal recognition particle (SRP) pathway. Spontaneous and SRP are involved in the insertion while cpTat and cpSec in the translocation of the precursor. Translocated proteins undergo maturation through the cleavage of the signal peptide by the thylakoid processing peptidase in the lumen. The homology of Sec pathway, Sec2, is also believed to be responsible in the integration and translocation of proteins across the inner envelope of the chloroplast. The image is adapted from (Celedon and Cline, 2013).
1.2.1 Spontaneous Insertion Pathway

The spontaneous insertion pathway is responsible for the unassisted insertion of single span membrane proteins into the thylakoid membrane (Aldridge et al., 2009; Schünemann, 2007). Several protein subunits, such as the ATP synthase subunit, CFoII (Michl et al., 1994); the PSII complex subunits, PsbW and PsbX (Aldridge et al., 2009); the Sec translocase subunit, SecE; and the cpTat translocase subunits, Hcf106 and Tha4 (Fincher et al., 2003; Schünemann, 2007) find their way into the membrane in this fashion. It has been proposed that the thylakoidal signal peptide not only serves for directing the proteins to the thylakoid membrane, but also plays an active part in the formation of a loop intermediate that is central to the unassisted insertion process. (Schünemann, 2007; Thompson et al., 1998). Interestingly, an in vitro study showed that positively charged residues play instead the role of completing the insertion process in PsbW and PsbX (Zygadlo et al., 2006). However, little is known about this pathway and much is yet to be discovered (Schünemann, 2007).

1.2.2 The Signal Recognition Particle (SRP) Pathway

The SRP transport systems in bacteria and eukaryotes function by inserting their substrates into the respective membranes co-translationally (Agarraberes and Dice, 2001). SRP, in both prokaryotes and eukaryotes, interacts with their protein substrate to begin the integration process before translation is fully completed (Schatz and Dobberstein, 1996). In eukaryotes, the SRP system is highly complex and absolutely relies on the presence of a 7S RNA (Beckert et al., 2015) to successfully integrate the nascent polypeptide into the endoplasmic reticulum (Agarraberes and Dice, 2001). In gram-negative bacteria, the SRP is associated with a smaller RNA, 4.5S RNA, while gram-positive bacteria or archaea still require the larger RNA, the 7S or 6S RNA, respectively. The SRP pathway in gram-negative bacteria also lacks the Alu domain, thus, losing its competence in arresting protein elongation during translation (Beckert et al., 2015).

Unlike the classical SRP pathway found in the cytoplasm of prokaryotic and eukaryotic cells, cpSRP translocates stromal proteins, post-translationally, into the thylakoid lumen without the involvement of RNA (Li et al., 1995). The bacterial SRP is known to translocate a wide variety of cytoplasmic proteins (Schünemann, 2007); the cpSRP system, on the other hand, is mainly restricted to the large array of substrates from the LHC protein family (Klimmek et al., 2006). There are two cpSRP subunits, cpSRP54 and cpSRP43 (Li et al., 1995; Schuenemann et al., 1998).
cpSRP54 is homologous to its corresponding subunit in both prokaryotes and eukaryotes; although, it performs a different function customized to the needs of the thylakoid system (Li et al., 1995). A study indicated that cpSRP54 contains two prominent domains, the G- and M-domain. The other cpSRP subunit, cpSRP43, is novel to the plastid and doesn’t have a bacterial homologue but is thought to have replaced role of the RNA molecules. It contains 3 chromosome domains (or chromodomain), namely CD1 in the N-proximal (Eichacker and Henry, 2001) and CD2 and CD3 in the C-proximal region (Klimyuk et al., 1999). A chromodomain contains three β-barrels and an α-helix strand situated in the C-terminal (A39). Among those domains, only CD2 is suggested to be involved in the integration pathway by serving as a cpSRP54 binding site and cpSRP complex formation (Hermkes et al., 2006; Sivaraja et al., 2005).

**Figure 1.4** illustrates the working model for cpSRP pathway. During the initial insertion mechanism, LHC substrate binds to these two subunits to form a transient complex (Tu et al., 2000). The three components of the complex have distinct interaction points with one another; the LHC substrate binds to cpSRP43 via the domain that contains a highly conserved 18 amino acid, the TM3 domain of LHC interacts with cpSRP54, and the CD2 of cpSRP43 fits with the M-domain of cpSRP54 (Franklin and Hoffman, 1993; Tu et al., 2000). A third subunit, cpFtsY thylakoid receptor, later engages to the complex by interacting with cpSRP54 (Kogata et al., 1999). The entire complex is then targeted to Alb3, a chloroplast insertase homologous to Oxa1p in mitochondria and YidC in bacteria, for subsequent integration into the thylakoid membrane (Falk et al., 2010).

Other studies have also shown that cpSRP54 integrated plastid components in a co-translational manner (Schünemann, 2007). There is evidence that cpSRP54 associates with the elongating RC protein, D1, while it is still attached to 70S ribosome, in the absence of cpSRP43 (Nilsson and van Wijk, 2002).
1.2.3. The General Secretory (Sec) Pathway

The Sec translocation system is located in the plasma membranes of bacteria and archaea, the eukaryotic endoplasmic reticulum, and the thylakoid membrane in chloroplast (Natale et al., 2008). Bacterial Sec pathway functions post-translationally to transport secretory proteins into the extracellular environment, most of which are integrated into the plasma envelope (Natale et al., 2008). The Sec transporter in the chloroplast, cpSec pathway, functions in translocating preproteins from the stroma into the thylakoid lumen posttranslationally (Aldridge et al., 2009).

The Sec translocase in *E. coli* is composed of at least seven components; SecA, SecB, SecE, SecY, SecG, SecD, SecF, and YajC (Natale et al., 2008; Schünemann, 2007). After complete synthesis and release from the ribosome, the preproteins are directed to SecA by its signal peptide while it is maintained in its unfolded state by the specific chaperone, SecB (Driessen, 2001; Luirink et al., 2005). A few Sec substrates, however, like the signal proteins, HflK and HflC, located in the plasma membrane are not dependent on SecB for translocation (Kihara and Ito, 1998). SecE, SecY, and SecG make up the Sec core conducting channel to which the SecA-precursor complex binds for exporting or integrating the precursors through the cytoplasmic membrane (Mori and Ito, 2001). SecD, SecF, and YajC are proposed to increase the SecA-dependent substrate translocation efficiency (Duong and Wickner, 1997). The Sec export pathway in bacteria operates in a series of SecA insertion and de-insertion cycles to push the precursor through the channel (Economou and
Wickner, 1994; Price et al., 1996). SecA-dependent translocation is energized by the SecA ATPase system, but, under limited SecA, it is more dependent on the proton motor force (PMF) though it varies among different precursors (Driessen, 1992). Proteins secreted extracellularly by the Sec system play a vital role in cell signaling, in excreting unwanted debris, in delivering virulence factors during the pathogenesis of infections, and as structural components of the cell envelope (Lee and Schneewind, 2001; Stathopoulos et al., 2000).

The cpSec pathway contains the homologous components, cpSecA, cpSecE, and cpSecY, housed in the thylakoid membrane (Aldridge et al., 2009; Nakai et al., 1994). CpSec functions in a similar manner compared to its bacterial companion, except that it serves as an import machinery (Aldridge et al., 2009). Structurally and functionally, SecAYE resembles the bacterial Sec pore complex and doesn’t contain the bacterial Sec homologue, SecB, SecG, and SecD/F (Schünemann, 2007). As in E. coli, the cpSec requires ATP to translocate the substrate in an unfolded state (Hulford et al., 1994) and is enhanced by the PMF (Driessen, 1992), thus, transport via the Sec translocase can be inhibited by ATP inhibitors, such as azide, or protonophore reagent, such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Agarraberes and Dice, 2001; Nishiyama et al., 1996). As chloroplasts lack SecB, there are a several other chaperones that maintain the precursor in a transport competent form (Celedon and Cline, 2013a). Prominent luminal proteins translocated by this pathway include plastocyanin (PC) and the 22-, 33-, and 16-kDa proteins of the manganese containing oxygen-evolving complexes (OEC) (Hulford et al., 1994; Nakai et al., 1994). The working model for cpSec pathway is shown in Figure 1.5.

The signal peptide is on average 20 amino acids long and is divided into three charged regions; positively charged N-terminal n-region, a hydrophobic core h-region, and a polar C-terminal c-region (Natale et al., 2008). During or shortly after transport, a signal peptidase recognizes the precursor through a conserved sequence in the c-region that it subsequently cleaves from the transient signal peptide to generate the mature version (Paetzel et al., 2002). In chloroplasts, Sec substrates, like most substrates targeted through the other thylakoid translocases, contain two transient signal peptides: a hydrophilic domain that targets cytoplasmic precursors to the stroma after which it is removed by stromal peptidase and a hydrophobic segment, exposed in
the stroma after removal of the first transient peptide, that is targeted to the thylakoid lumen where it is also subsequently cleaved off by luminal peptidase (Nakai et al., 1994).

**Figure 1.5. Chloroplast Secretory pathway (cpSec):** The cpSec pathways translocates unfolded preproteins across the thylakoid in a ATP- and PMF-dependent process. Unfolded substrate interacts with stromal cpSecA and is directed to the cpSecYE channel for translocation. The signal peptide is then removed by stromal processing peptidase in the lumen.

### 1.2.4. Chloroplast Twin Arginine Translocation (cpTat) Pathway

#### 1.2.4.1 Overview

The Twin Arginine Transport (Tat) system derives its name from the conserved twin arginine motif in the signal peptide of its precursors (Berks, 1996; Mendel et al., 2008). This pathway was first identified in the 1990s in plant chloroplast after numerous observations that some substrates were translocated in a Sec-independent manner and were dependent upon the pH gradient across the thylakoid (Cline et al., 1992; Mould et al., 1991; Santini et al., 1998). Later, Tat pathway was also identified in prokaryotes and archaea (Berks, 1996). To date, it is known that nearly 80% of the bacteria harbor this unique translocation system, but is generally absent in animals and fungi except for the choanoflagellate *Monosiga brevicollis* and the homoscleromorph sponge, Oscarellidae (Cline, 2015; Wang and Lavrov, 2007). In bacteria, the Tat system is in the cytoplasmic membrane and functions in the secretion of proteins (only ~ 10% of the total proteins secreted in *E. coli*) across the bacteria plasma membrane that are involved in energy metabolism, virulence, and cell survival. Considering the high structural similarities between their targeting
signal peptide, it is suggested that the thylakoid import machinery and the bacterial export system are structurally, mechanistically, and evolutionarily related (Berks 1996).

The chloroplast Tat pathway is responsible for the translocation of about 50% of proteins destined to the thylakoid lumen (Albiniak et al., 2012). Tat systems translocate precursor in a fully folded state (Clark and Theg, 1997) without compromising the ionic integrity of the membrane (Teter and Theg, 1998). cpTat transport in the in thylakoids contains three major membrane components: Tha4 (Walker et al., 1999a), Hcf106 (Settles et al., 1997b), and cpTatC (Mori et al., 2001b). Their bacterial homologs are TatA, TatB, and TatC respectively (Albiniak et al., 2012), though, most gram-negative bacteria have a bifunctional TatA and TatC homolog with no TatB (Blaudeck et al., 2005; Sargent et al., 2006). The Tat translocation system is energized by the transmembrane electric potential ($\Delta \Psi$) and protonmotive force (PMF) and does not require NTP hydrolysis (Bageshwar and Musser, 2007; Braun et al., 2007; Mould et al., 1991).

1.2.4.2. CpTat Protein Components

In plant thylakoids, thylakoid assembly 4 protein (Tha4) is the smallest membrane protein among the Tat components and is present at a much higher molar ratio: 1:4:21 cpTatC: Hcf106: Tha4 (Celedon and Cline, 2012b). Spectroscopic (White et al., 2010) and image analysis (Gohlke et al., 2005) of Tha4 homolog, TatA, strongly proposed that it forms the primary translocation channel across the membrane. Hcf106 (high chlorophyll fluorescence 106) was the first cpTat component discovered in maize (Voelker and Barkan, 1995). Tha4 and Hcf106 are similar in structure which can be divided into three distinct regions: a single transmembrane N-terminal domain, a hydrophobic amphipathic helix, and a stromally exposed, loosely structured, acidic C-terminal domain (Figure 1.6). A short, conserved hinge motif, FGPK, links the stromal amphipathic domain to the transmembrane domain. Nevertheless, Hcf106 has a significant longer amphipathic and C-domain which are more acidic than that of Tha4 (Walker et al., 1999a). The N-proximal region of Hcf106 is closely associated to that of Tha4 bearing ~ 65% sequence identity (Cline and Mori, 2001).

The chloroplast TatC (cpTatC) has six transmembrane segments where both the N- and C-terminal is exposed to the stroma (Figure 1.6). cpTatC can be divided into twelve segments: six transmembrane (TM) domains, a stromal N- and C-segment (S1 and S4 respectively), two
stromally exposed hinges (S2 and S3), and two luminal hinges (L1 and L2) (Ma and Cline, 2013; Mori et al., 2001b). cpTatC rests in the thylakoid membrane as a receptor complex along with Hcf106 in a 1:1 molar ratio (Cline and Mori, 2001). TatC crystal structures developed for a hyperthermophilic, rod shaped bacteria, *Aquifex aeolicus*, indicate a glove-like structure with an exposed, substrate binding lipid pocket (Rollauer et al., 2012b).

![Diagram of cpTat components](image)

**Figure 1.6. Structure of the cpTat components**: The cpTat pathway translocates substrates in their fully folded state across the thylakoid membrane. It has three components, namely, Tha4, Hcf106, and cpTatC (from left to right in the image). Tha4 and Hcf106 are homologous having a TMD, APH, and stromally exposed C-tail. The larger component, cpTatC, contains 6 TM domains with both N- and C-terminals exposed to the stroma. In plants, all these three components are crucial for effective translocation.

In the resting state (i.e., no PMF or no precursor bound), cpTatC and Hcf106 coprecipitate as a ~700 kDa complex indicating that these components assemble prior to the recruitment of precursor by the translocase. These membrane proteins make up the receptor complex to which the precursor binds during the initial stage of translocation (Cline and Mori, 2001). A study recently identified key residues in the hinge region of Hcf106 that serve as a contact point between Hcf106 and cpTatC in the resting complex (Ma and Dabney-Smith, unpublished). On the cpTatC side, cpTatC/TatC interacts with Hcf106/TatB through its TM5 (Kneuper et al., 2012). Crosslinking assays also recently discovered other contact site on cpTatC: TM1 and TM2 (Ma and Dabney-Smith unpublished). On the contrary, few studies indicated the less important role of Hcf106/TatB stromal C-tail as complex formation and transport was not reduced in the absence of the domain (Lee et al., 2002) (Ma and Dabney-Smith, unpublished). Tha4, is found mostly separately in the membrane as a homo-oligomer prior to the transport event (Dabney-Smith et al., 2006); although, some Tha4 can be detected in association with the receptor complex even at rest (Aldridge et al., 2014). Despite the significant role of Hcf106 in cpTat system, the functional role...
of its bacterial homolog, TatB, in most gram-positive bacteria and archaeb, is accomplished by TatA (Blaudeck et al., 2005; Sargent et al., 2006).

The bacterial homologs, TatA, TatB, and TatC, respectively, is structurally and mechanistically like the cpTat translocation pathway. The majority of gram negative bacteria contain all three components of which, E. coli is the model system for studying the translocase. (Mendel et al., 2008). Streptomyces species, a gram-negative Actinobacteria, and Bacillus subtilis, a non-pathogenic gram-positive bacterium, however, lack TatB, where TatAC complex execute the translocation task (Jongbloed et al., 2006; Schaerlaekens et al., 2001). In such circumstance, considering the structural similarities between TatA and TatB, it was recently proposed that TatA may take over the function of TatB and complement the function of TatAB. Furthermore, B. subtilis, consists two tatC (tatCd and tatCy) and three tatA (tatAd, tatAy, and tatAc) gene variants. (Jongbloed et al., 2006). Bacterial Tat may also comprise a fourth component, TatE, which is structurally homologues to TatA (Berks et al., 2000). Consistently, mutation analysis have proved that TatB, TatC, and either TatA or TatE make up the functional unit of the Tat transport system (Sargent et al., 1998).

1.2.4.3. CpTat Signal Peptides

The transit peptide of luminal-destined proteins consists of two functional domains: the stromal and luminal targeting domains. Once the cytoplasmic precursor is translocated into the stroma, the stromal targeting domain is cleaved by the stromal peptidase generating the intermediate precursor that harbors an exposed luminal targeting domain (Keegstra and Cline, 1999).

The luminal targeting domain targets the mature domain to the cpTat pathway and is characterized by a twin arginine motif (RR) located in its N-domain. This RR motif is absolutely essential for most precursors to be recognized by the cpTat translocase and for subsequent efficient transport (Summer et al., 2000). The strict requirement of the RR motif was explicitly evident when transport was severely retarded with single lysine mutants (KR/RK) and completely blocked with double lysine mutants (KK) despite high similarly in their structure and $pK_a$ values (Chaddock et al., 1995).

As with Sec signal peptide, the cpTat signal peptide is divided into three functional regions: the charged N-domain, the hydrophobic core H-domain, and a more polar C-domain (Bogshe et
The determinant feature of these N-terminal peptides is the short consensus sequence, \textit{RRXXY}, where X is not conserved and Y is either leucine, valine, methionine, and phenylalanine (Stanley et al., 2000). CpTat signal peptide also bears basic residues in their C terminus, which distinguishes them from Sec signal peptides (Robinson and Bolhuis, 2004).

Conversely, bacterial Tat signal peptides are markedly different from those of their cpTat counterparts. In \textit{E. coli}, Tat substrates contain other characteristic features in addition to the conserved RR motif. For instance, residue +1 and +2 of RR have been identified as critical point for effective translocation of the mature domain. Typically, +1 is either serine, asparagine, threonine, or aspartate, while, +2 is mostly phenylalanine or leucine. Bacterial Tat precursors contains the consensus sequence, S/TRXFLK, where X can be any amino acid (Mendel et al., 2008). The RR-motif and C-domain lysine contribute weakly to the high selectivity of bacterial Tat targeting peptide. A study suggested the presence of a “Sec avoidance signal”, superimposed with in the transfer peptide, might play a substantial role for diverting the precursor away from the Sec pathway, indirectly enhancing substrate recognition by Tat pathway (Bogsch et al., 1997).

\textbf{1.2.4.4. Mechanism of Transport}

The mechanism of protein transport across the thylakoid membranes by the Tat system has been studied extensively. Tat transport cycle involves four steps: substrate recognition and binding, translocase assembly, translocation of the substrate, and disassembly to reset the transport system (Figure 1.7). According to these studies, it is proposed that the precursor first comes in contact with the Tat complexes, Hcf106-cpTatC (Cline and Mori, 2001) where the twin arginine proximal regions comes in close contact with the TM5 of cpTatC and the amphipathic helix of Hcf106 (Albiniak et al., 2012; Gérard and Cline, 2006; Kneuper et al., 2012). Chemical crosslinking assay depicted that Tha4 is not in contact with the precursors during the initial assembly stage with the ~700 kDa receptor complex (Cline and Mori, 2001). Though precursors can associate with the receptor complex in the absence of energy supply, binding is largely enhanced in the presence of PMF (Aldridge et al., 2014; Gérard and Cline, 2007).
The subsequent Tha4 assembly to the tri-complex requires three criteria: substrate binding, PMF, and E10 of Tha4 (Mori and Cline, 2002). Crosslinking assay depicted that Tha4 comes in contact with the receptor complex through TM4 (Aldridge et al., 2014). Tha4 resides in the membrane as a homo-oligomer and only associates prior to the onset of the actual translocation (Mori and Cline, 2002). There is compelling evidence that at least eight Tha4 (TatA) monomers form a ring-shaped oligomeric complex of varying diameter that serves as the channel for catalyzing the translocation of the bounded precursor across the lipid layer (Dabney-Smith et al., 2006; Gohlke et al., 2005; White et al., 2010). The oligomerization of Tha4 monomers may occur before or during its assembly to the precursor-receptor complex, which is driven by pH gradient. New findings, however, are emerging that doubts the previous notion of Tha4 serving as the core translocation element and rather suggests a cooperative work of all Tat components. Though much remains to be shown, it is likely that the receptor complex either interacts with the signal peptide where the precursor subsequently is inserted, unassisted, into the lipid bilayer or TatC may insert the precursor deep into the membrane to the point where Tha4 and Hcf106 further continue the translocation process (Brüser and Sanders, 2003; Fröbel et al., 2012; Patel et al., 2014). The latter scenario appears to be strongly supported by two results: one, Tha4 and Hcf106 have similar cpTatC contact point and (2) both the precursor signal and mature domain binds to Hcf106 and cpTatC (Ma and Dabney-Smith, unpublished).

**Figure 1.7. The Chloroplast Twin Arginine Pathway (cpTat):** The cpTat pathway translocates substrates in their fully folded state across the thylakoid membrane. It utilizes the PMF as the only source of energy. The receptor complex, jointly formed by Hcf106 (green) and cpTatC (blue), serve as a docking site for the precursor signal peptide (black). After recognition, Tha4 (pink) oligomers assemble the precursor-receptor complex to promote passage of the precursor via the predicted channel formation or membrane weakening strategy. The dissociation of Tha4 from the receptor complex resets the cycle.
One of the remarkable aspect of the pathway is that Tha4 pore complex is capable of translocating the substrate without being permeable to ions (Teter and Theg, 1998). To date, detailed mechanism on how Tha4 translocate the precursor or manages to prevent ion leakage is still not well understood. However, a recent study showed that Tat contact with cpTatC TM5 increased as the translocation progressed which might give us an insight on its mechanistic implication (Aldridge et al., 2014). Tha4 then disassembles after the substrate is fully translocated (Mori and Cline, 2002).

cpTat translocates its substrate while in their folded form, which are usually subunits of complexes and occasionally even oligomeric complexes (Clark and Theg, 1997). Some predict that certain substrates must be, at least in a partial folded state, to be effectively transported across the bacterial cytoplasmic or thylakoid membrane. Under such circumstance, Tat system may take over the translocation function of Sec system as the latter pathway only serve to translocate unfolded precursors (Robinson and Bolhuis, 2004; Sargent et al., 1998). Bacterial Tat system, however, appear to have a screening mechanism that recognizes and discriminate unfolded or misfolded precursors from those that meet the folding requirement for effective transport (Rocco et al., 2012).

Tat system translocates precursor that vary in size from 4 to 60 KDa. The 23 and 16 KDa Oxygen evolving complex (OEC) are the predominant substrate for the cpTat mediated translocation (Ifuku et al., 2011; Klosgen et al., 1992; Mould et al., 1991). In E. coli, redox proteins, such as the trimethylamine N-oxide (TMAO) (Santini et al., 1998) and FeS or NiFe containing complexes (Sargent et al., 1998) are inserted into the cytoplasmic membrane via this pathway.

1.2.4.5. Energy system

Another remarkable aspect of the Tat translocase is its sole dependence on the PMF as an energy requirement (Alder and Theg, 2003; Braun et al., 2007; Cline et al., 1992). It is suggested that the energy requirement for Tat-based translocation and insertion depends on the substrate which can be either of three states: total ATP dependence, ATP-PMF combined dependence, and total PMF dependence (Braun et al., 2007). This infers that a certain ΔpH threshold, that varies among different substrates, has to be surpassed for successful translocation. A quantitative analysis also
showed that cpTat transport directly utilizes the H+ leak which was estimated to be as high as 90% (Alder and Theg, 2003). A biochemical assay predicted that $\Delta\psi$ may trigger oligomerization of Tat proteins to form the translocon (Bageshwar and Musser, 2007).

### 1.3 Goals and Objectives

Many of cpTat substrates are crucial for the assembly of thylakoids and proper functioning and maintenance of the photosystem (Järvi et al., 2013). Therefore, understanding the mechanism of cpTat system and its role in photosystem and plastid development is of great significance.

Recent studies have been engaged into the investigation of the structural features of all three Tat components in favor to understand Tat mechanism of transport. Translocation begins with precursor binding to the cpTatC-Hcf106 receptor complex in the absence of energy (Cline and Mori, 2001). In the presence of PMF, primarily $\Delta p\text{H}$, Tha4 assembles the tri-complex to actively transport the precursor. Tha4 then disassembles to regenerate the receptor complex for subsequent cycle of translocation (Mori and Cline, 2002). However, to date, detailed mechanism for cpTat translocation process remains to be discovered.

The major aim of this thesis work was to gather more information toward understanding cpTat mechanism of transport. Briefly, Chapter 2 addressed the topology and conformational change of Hcf106 is resting and precursor binding state using cysteine accessibility labeling technique. In this chapter, a water-soluble and membrane-impermeable methoxypolyethylene glycol (PEG) maleimide reagent was used to label single-cysteine Hcf106 variants previously integrated in the thylakoid membrane. This technique enables us to map the membrane topology of several residues in the transmembrane and amphipathic domain of Hcf106. By contrasting the position of an individual residue with respect to the membrane before and after precursor binding, we were also able to probe the conformational change of Hcf106 upon precursor recognition. Chapter 3 aimed to probe the interaction between cpTatC and precursor mature domain during cpTat translocation process. In this study, mutagenesis and cysteine crosslinking were conducted to map the interaction between the N-proximal region of cpTatC and the mature domain of a Tat substrate. By completing this study, we aimed to answer a recent finding on whether cpTatC plays an active role in the formation of the point of passage during the translocation event.
1.4 References


CHAPTER 2

Topology Study of One CpTat Component, Hcf106, in Resting and Precursor Binding State using Cysteine Accessibility Assay

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2.1 Abstract

The chloroplast Twin arginine transport (cpTat) system transports fully folded proteins across the thylakoid membrane which is energized by the trans-thylakoidal proton motive force. The cpTat pathway consists of three membrane proteins: Tha4, Hcf106, and cpTatC. At resting state, cpTatC and Hcf106 form a receptor complex to which the precursor initially binds. Tha4 is found as separate homo-oligomers that assembles with the receptor-bound substrate to complete the translocation of the cargo protein into the thylakoid lumen. Topologically, Hcf106 is predicted to contain 3 domains: an N-terminal transmembrane domain (TMD), a basic amphipathic helix (APH) separated from the TMD by a short hinge region, and an unstructured C terminus region. To date, detailed topology information of Hcf106 is lacking. In this study, cysteine accessibility labeling techniques were used to characterize the topology of Hcf106 in the absence and presence of precursor. In the resting state, Hcf106 displayed an overall membrane integrated N-proximal domain with a shortened TMD and stromal APH with both ends accessible from the stroma. During precursor binding, though the overall topology of Hcf106 remained unchanged, the N-proximal region of APH became more buried assuming an overall tilted conformation with respect to the membrane surface. We attribute this accessibility change to a precursor-driven conformational change of Hcf106 which likely contributes to a weakening of the thylakoid membrane as the potential mechanism for promoting the passage of the precursor.

2.2 Introduction

In plant chloroplasts, there are two independent pathways, the chloroplast Secretery pathway (cpSec) and the chloroplast Twin arginine transport (cpTat) system, that operate in parallel to transport nuclear encoded, thylakoid localized proteins from their point of synthesis in the cytosol to the thylakoid lumen (Cline and Theg, 2007; Eytan and Ohad, 1970; Green, 1980). These proteins are synthesized as higher molecular weight precursors containing an N-terminal targeting sequence that direct to be transported proteins to their respective translocation machinery (Green, 1980).

Unlike cpSec, the cpTat pathway utilizes the proton motive force (PMF) as the sole source of energy to translocate fully folded proteins without ion leakage (Clark and Theg, 1997; Cline et al., 1992; Teter and Theg, 1998). The precursor bears an obligate twin arginine residues in the H-region of the signal peptide, hence derives its name, that directs the precursor to the cpTat system (Chaddock et al., 1995). Signal peptides are transient that exist until the substrate is translocated.
across the thylakoid (Mori and Cline, 2002). The cpTat pathway operates through three membrane-bound subunits: Tha4 (Walker et al., 1999), Hcf106 (Settles et al., 1997), and cpTatC (Mori et al., 2001).

Hcf106 and Tha4 are topologically homologous with each predicted to contain three domains: a N-terminal transmembrane domain (TMD), followed by a conserved short hinge region, an amphipathic helix (APH), and an unstructured, acidic, C tail (Berks et al., 2003; Mori et al., 2001; Mori et al., 1999; Settles et al., 1997). cpTatC contains six transmembrane domains where both the N- and C-termini are exposed to the stroma (Allen, 1995). CpTat precursors recognize and bind Tat receptor complexes formed by cpTatC and Hcf106 prior the translocation event (Cline and Mori, 2001). The receptor complex is a 700 kDa hetero-oligomer formed by equal molar ratios of Hcf106 and cpTatC (Cline and Mori, 2001). During the initial translocation event, the precursor binds to both subunits of the receptor complex: the signal peptide interacts with cpTatC and the hydrophobic region close to the signal peptide cleavage motif interacts with Hcf106 (Cline and Mori, 2001; Gérard and Cline, 2007). Tha4 oligomers then assemble at precursor-bound receptor complexes once the PMF has been established. Tha4 then disassembles to regenerate the receptor complex for subsequent cycle of translocation (Mori and Cline, 2002). Recent studies proposed that Tha4 forms the translocation pore through the oligomerization of multiple Tha4 monomers (Cline and Theg, 2007; Gohlke et al., 2005). Others, refuted this model by proposing a membrane weakening strategy that causes destabilization of the membrane around the precursor thus facilitating its unidirectional passage across the membrane (Brüser and Sanders, 2003; Dabney-Smith et al., 2006).

Recent studies have been engaged into the investigation of the structural features of all three Tat components in order to understand the mechanism of cpTat transport. Among those, Tha4/TatA has been structurally characterized using NMR as the potential channel forming Tat component (Aldridge et al., 2012; Kuhlbrandt, 1988; Morre et al., 1991). NMR studies conducted by Hu et al. and Walther et al. showed that TatA has two α-helices that form an L-shaped topology (Hu et al., 2010; Walther et al., 2010). A recent cysteine accessibility labeling study also provided detailed topology of Tha4 showing that it contains a short TMD with a slanted APH such that its N-proximal end is partially buried in the membrane (Aldridge et al., 2012; Walther et al., 2010). Two studies were successful in solving the crystal structure of TatC demonstrating a glove-like structure.
with a membrane-exposed pocket (Bruce, 1998; Rollauer et al., 2012a). NMR studies on Hcf106 have been primarily aimed at investigating the peptide-lipid interaction after inserting the proteins in a synthetic phospholipid layer (Zhang et al., 2013; Zhang et al., 2014a). A similar NMR structural study of the homolog TatB in detergent micelles reported four helices: a transmembrane segment, amphipathic domain, and two highly mobile helices (Zhang et al., 2014b). To date, however, Hcf106 lacks detailed structural and topological studies.

In this study, cysteine accessibility labeling techniques were used to identify the location of selected residues in the TMD and APH of Hcf106 with respect to the thylakoid membrane. To understand the conformational change in different regions of Hcf106 in relation to the membrane, the change of cysteine accessibility between resting and precursor binding state was also assessed. A water-soluble and membrane-impermeable methoxypolyethylene glycol (PEG) maleimide reagent, that is capable of covalently binding to sulfhydryl moiety of cysteines exposed to the stroma in intact thylakoids, was used to label in vitro translated Hcf106. Our results indicated a more parallel APH during the resting state where both the N- and C-proximal region of APH are stromally exposed. After precursor binding, the N-proximal end of the APH is more buried into the membrane such that the APH adopts a tilted topology as compared to its parallel conformation during resting state. This conformational shift is in direct opposite to that of Tha4 upon precursor binding (Aldridge et al., 2012). We believe that a localized weakening and disruption of the thylakoid membrane around the precursor that is caused by the mismatch between the Hcf106 TMD and the lipid bilayer, as well as the predicted antimicrobial property of the APH from Hcf106 and/or Tha4, are the factors that drive forward the translocation through cpTat system.

2.3. Experimental Methods

2.3.1 Preparation of chloroplasts and thylakoids

Intact chloroplast were prepared from 10-12-day-old pea seedlings (Pisum sativum L. cv. Laxton’s Progress 9 or Little Marvel) (Schulz et al., 1993). Chloroplasts were suspended to 1 mg chlorophyll mL⁻¹ with import buffer (IB; 50 mM HEPES-KOH, pH 8.0, and 330 mM sorbitol) and kept on ice until use. Isolated thylakoids were prepared from osmotic shock of intact chloroplasts. Briefly, chloroplasts suspensions were centrifuged at 1000 × g for 5 min. The supernatant was discarded and pellets was suspended to 1 mg chlorophyll mL⁻¹ with lysis buffer HKM (50 mM HEPES-KOH, pH 8.0, 10 mM MgCl₂) (Murray and Kohorn, 1991). After incubation on ice for 5 min, the
buffer was adjusted osmotically by addition of $2 \times \text{IB}$. The lysates were centrifuged at $3200 \times g$ for 8 min and then washed once in $1 \times \text{IB}$, 10 mM MgCl$_2$. Isolated thylakoids were adjusted to 1 mg chlorophyll mL$^{-1}$ by IB and 10 mM MgCl$_2$. Chlorophyll concentration was determined according to Arnon (Arnon, 1949).

2.3.2 Generation of Hcf106 cysteine variants and substrate protein

Hcf106 with cysteine substitutions (Hcf106XnC, where the amino acid X at position n was substituted by cysteine) were generated by QuikChange mutagenesis (Agilent Technologies) according to the manufacturer’s instructions. The template used for the mutagenesis was the coding sequence for mature Hcf106 (lacking the targeting peptide) in a pGEM 4Z plasmid. The coding sequence begins with MASLFGVGAEPA…. All amino acid substitutions were confirmed by DNA sequencing on both strands at the Center for Bioinformatics and Functional Genomics (CBFG) at Miami University. The pOE17V-20F substrate protein, which is a modified form of the OE17 precursor protein from maize, was a generous gift from Dr. Kenneth Cline (University of Florida).

2.3.3. Preparation of radiolabeled Hcf106 and precursor protein

Radiolabeled Hcf106 variants and cpTat precursor protein pOE17V-20F were prepared by in vitro translation from capped mRNA in the presence of L-[3,4,5-$^3$H(N)]-Leucine (Perkin-Elmer) according to the manufacturer protocol from Promega Wheat Germ Extract Kit (Madison, WI USA) (Cline, 1986). Translation products were diluted with an equal volume of 60 mM leucine in $2\times$ IB before use.

2.3.4 Precursor binding assay

In vitro translated pOE17V-20F was incubated with equivalent volume of washed thylakoids, integrated with Hcf106, and equivalent volume of IB, 10 mM MgCl$_2$ in ice for 20 min under dark condition. Thylakoids were recovered by centrifugation and washed by resuspending in 5 volumes of IB + 10 mM MgCl$_2$ and centrifuging at $3200\times g$ for 8 min. Pellets were resuspended to a final concentration of 1 mg chlorophyll mL$^{-1}$ with IB, 10 mM MgCl$_2$.
2.3.5 Detecting cysteine location in Hcf106 in resting and binding state

Cys-substituted in vitro translated Hcf106 was integrated into thylakoids under light (100-150 µE/m²/s) for 20 min at 25°C. Samples were washed and divided into two equal aliquots. The first half (not treated with the precursor) was kept in ice until further use. Precursor binding assays were conducted for the other half as mentioned previously. Stromally exposed cysteines in Hcf106 were detected by treatment with 0.5 mM PEG (methoxypolyethylene glycol maleimide 5000, Sigma-Aldrich) at 25 °C for 20 min. Labeling was quenched by addition of 10 mM DTT for 10 min at room temperature. After labeling, the thylakoids were centrifuged, washed, and adjusted for equal chlorophyll concentration. The samples were dissolved in equivalent volume of 2× SSB and analyzed by SDS-PAGE. Labeled samples were cut from the gel, purified and quantified by scintillation counting in a Beckman LS 6500.

2.4 Results

2.4.1 Labeling sulfhydryls of recombinant Hcf106 with PEG maleimide

Translocation of precursors by the cpTat pathway is a transient process and hard to track, however, this “tricky” system is composed of several separate steps that can be detected by manipulating experimental conditions. In this study, an efficient cysteine labeling technique with fast quenching was utilized to investigate the topology of Hcf106 in resting state (no precursor). This modified substituted cysteine accessibility method (Johal and Holaday, 1989) has been used in our lab (Aldridge et al., 2012) to identify the location of cysteine residues of proteins in the thylakoid membrane and was used here to probe the topology of the Hcf106 in the receptor complex. At first, a single cysteine residue was introduced in Hcf106, and Hcf106 variants were integrated into the thylakoid membrane. PEG maleimide, which is a membrane impermeable thiol-modifying reagent, was used to label single cysteines in Hcf106. PEG is a large water-soluble, membrane-impermeable reagent that covalently binds to stromally exposed sulfhydryl groups of cysteines (Figure 2. 1) (Doherty et al., 2005; Dozier and Distefano, 2015). Attachment of PEG (Mw ~5000) to Hcf106 (28 kDa) increases the protein size to ~ 33 kDa (Hcf106-PEG adduct) appearing as a higher molecular weight band of the target protein on SDS-PAGE.
2.4.2 Residues in the TMD of Hcf106 are found in the hydrocarbon core of the bilayer

Wild type Hcf106 protein does not contain native cysteines; therefore, the single cysteine Hcf106 variants were tested in our lab for their ability to insert into membranes and localize to the receptor complex (Ma et al., dissertation). After integration of Hcf106 variants into the thylakoid membrane, recovered thylakoids were treated with PEG maleimide. Figure 2.2 shows the PEG labeling of the predicted TMD of Hcf106. PEG did not react with any of the residues in the N proximal region (Figure 2.2A) as indicated by the very slight adduct band, but very poorly with the C proximal region (Figure 2.2B) of TMD suggesting that the TMD is buried or facing the luminal side of the membrane. The appearance of a small amount of Hcf106-PEG adduct formed in the presence of intact membrane for some residues (Figure 2.2B) could be due to the de-insertion of Hcf106 from the membrane during the washing steps similar to results seen for Tha4 (Aldridge et al., 2012; Evans et al., 1992) or due to contamination of nonintact thylakoid membranes. Our results identified that at least 20 residues in the N-terminal proximal region of Hcf106 are not accessible from the stromal side indicating that the residues are located in luminal and hydrocarbon core region of the membrane bilayer.

2.4.3 Residues in the APH of Hcf106 are found in the stromal side of the thylakoid

Figure 2.3 shows the PEG labeling of the predicted APH of Hcf106. Unlike in the TMD regions, residues in the APH regions reacted strongly with PEG as indicated by the formation of the ~33 kDa Hcf106-PEG adduct. This confirms that the residues located in the APH region are stromally exposed in the thylakoids. Residues in the early N-proximal region of APH (Fig 2.3A; A29C, A32C, and R33C) showed poor labelling by PEG maleimide. The poor labelling could be
attributed not necessarily to the poor cysteine accessibility but rather to the poor reactivity of cysteine located in the interfacial region of the membrane. The poor reactivity is perhaps due to the pKa shift of the cysteines located in the membrane interface as these residues are influenced by the electronegative polar lipid head groups (Bogdanov et al., 2005).

2.4.4 Hcf106 APH displays decreased stromal accessibility from N-proximal to C-proximal portion after precursor binding

Little is known about what role Hcf106 plays in cpTat-dependent precursor trafficking. Previous studies have shown that Hcf106 directly interacts with the precursor in facilitating substrate transport, along with cpTatC, across the thylakoids. Understanding the topology of Hcf106 with respect to the thylakoid membrane and possible conformational changes it may undertake during the precursor binding state may thus provide insight on its role during substrate transport. One way to do this is by probing for changes in stroma-exposed cysteine accessibility it undergoes from its resting to the transport state. To engage more recombinant Hcf106 Cys-mutants in their binding state, we involved several experimental conditions. First, all experimental conditions conducted in this study were in favor of precursor binding, but not transport. As such, assays were conducted in the absence of ATP, in ice, under low-light conditions for 20 minutes. Second, a modified form of the OE17 precursor protein from maize, pOE17V-20F, was used, which contains a valine to phenylalanine mutation that promotes tighter binding to the receptor complex (Gérard and Cline, 2006). Third, during the binding process, an excess amount of precursor was added to saturate every available binding site on the Tat receptor complex and engage more recombinant Hcf106 variants in their binding state (Celedon and Cline, 2012a; Gerard and Cline, 2007).
**Figure 2.2.** PEG-maleimide labeling of representative residues in Hcf106 TMD before/after precursor binding: PEG labeling efficiency was compared in the absence (gray bars) or presence (black bars) of precursor pOE17V-20F binding by observing Hcf106-PEG adduct band intensity. A decrease or unchanged PEG labeling in the presence of precursor is marked by a downward and sideways arrow, respectively. A decrease by at least 1.5-fold is considered to be a decrease. No significant increase (by at least 1.5-fold) was observed. (A) Native Hcf106 (WT) as a negative accessibility control and PEG labeling of cysteines in Hcf106 predicted N-proximal TMD (G8C to E11C). (B) PEG labeling of cysteines in Hcf106 C-proximal TMD (L13C to G24C). (C) Percent PEG labeling of cysteines in Hcf106 predicted TMD expressed as a percentage of total integrated Hcf106 for each variant. All samples were treated with PEG maleimide before or after precursor binding (+ pOE17V-20F) or non-binding in intact thylakoids. Samples were analyzed by SDS-PAGE and fluorography. Hcf106-PEG adduct is marked with an arrow. Gels are representative of three experiments.
All experiments were carried out in intact thylakoid membranes; therefore, the PEG labeling results are a direct reflection of stroma-accessibility of the residues in Hcf106 APH. The PEG labeling efficiency of residues in Hcf106 TMD, hinge, and APH were quantified by gel band extraction of Hcf106 and Hcf106-PEG adducts followed by scintillation measurements. The amount of Hcf106-PEG adducts generated by PEG labeling was normalized to the total amount of each integrated Hcf106 variants. In the presence of precursor, residues in the N proximal region of the APH showed a decrease in the intensity of Hcf106-PEG adduct (Figure 2.3A; compare adjacent lanes for each variant) indicating a decrease in the stromal localization of the residues following precursor binding. Residues in the C-proximal region of the APH showed full PEG accessibility (Figure 2.3B). Exceptions were residues P44C, R47C, and S53C, which showed a decrease in the stromal accessibility after precursor binding state.

By mapping selected cysteine residues in the proposed functional domain of Hcf106, i.e. TMD and APH, we showed that residues located in N proximal APH region (A32C to E41C) demonstrated decrease in stroma accessibility during precursor binding. This suggests that precursor binding induces a conformational shift in the Hcf106 such that the N-proximal portion became more deeply buried in the membrane. Residues in the APH located near the C tail (Q43 to L60C) remained unchanged. These results suggested that Hcf106 undergoes a shift in placement so that residues in the N-proximal APH moved into the membrane to a less stroma-accessible location during precursor binding.
Figure 2.3. PEG-maleimide labeling of representative residues in Hcf106 APH before/after precursor binding: PEG labeling efficiency was compared in the absence (gray bars) or presence (dark bars) of precursor pOE17V-20F binding by observing Hcf106-PEG adduct band intensity. A decrease or unchanged PEG labeling in the presence of precursor is marked by a downward and sideways arrow, respectively. A decrease by at least 1.5-fold is considered to be a decrease. No significant increase (by at least 1.5-fold) was observed. (A) PEG labeling of cysteines in Hcf106 predicted N-proximal APH (A29C to E41C). (B) PEG labeling of cysteines in Hcf106 C-proximal region of the APH (Q43C to L60C). (C) Percent PEG labeling of cysteines in Hcf106 predicted APH expressed as a percentage of total integrated Hcf106 for each variant. All samples were treated with PEG maleimide before or after precursor binding (+ pOE17V-20F) or non-binding in intact thylakoids. Samples were analyzed by SDS-PAGE and fluorography. Hcf106-PEG adduct is marked with an arrow. Gels are representative of three experiments.
2.5. Discussion

Structurally, among the three cpTat components, Hcf106 is the least studied. Solid-state NMR of Hcf106 (Zhang et al., 2013; Zhang et al., 2014a) and solution NMR of the bacterial homolog TatB in micelles (Zhang et al., 2014b) provided clues on the overall structure Hcf106/TatB. TatB is known to contain four helices, two of which are the TMD and APH that adopts an L-shaped conformation (Zhang et al., 2014b). Yet, detailed conformational information of Hcf106 remains unknown. In this study, the topology and conformational change of Hcf106 from resting to precursor bound state was addressed using cysteine accessibility labeling techniques. This study will provide valuable insight into the function of the protein and cpTat mechanism of transport.

A previous study showed that the bacterial homology TatA and TatB comprise a TMD integrated in the inner cytoplasmic membrane with a periplasmic exposed N-terminal and cytoplasmic exposed APH domain (Koch et al., 2012). In the present study, single cysteine introduced into specific locations within the TMD and APH domain of Hcf106 was subjected to PEG labelling to identify the location of the target residue with respect to the thylakoid membrane. Our results indicate that Hcf106 assumes an integrated TMD and stromally exposed APH under non-transporting condition (Figure 2.4). In resting state, the TMD is buried in the lipid bilayer or exposed to the luminal side of the thylakoid (Figure 2.2 and 2.4). The APH is stromally exposed with respect to the thylakoid membrane (Figure 2.3 and 2.4). This agrees with the results from the biocytin-streptavidin accessibility testing previously conducted in our lab (Zhang et al., dissertation). Although functionally distinct, Hcf106 and Tha4 share similar topologies with regard to APH and TMD (Kuhlbrandt, 1988). In plants, one cannot substitute Tha4/Hcf106 for the other, though, the TMD can be exchanged without significantly affecting the translocation process (Dabney-Smith et al., 2003). As evidenced through biochemical accessibility techniques, the TMD of Tha4 is buried into the thylakoid membrane while the APH faces the stroma.

Nevertheless, the gradual increase in stromal accessibility from N- to the C-proximal region of the APH emphasized a tilted alignment of the domain such that the APH shoots out of the membrane at an angle (Aldridge et al., 2012). Though our data showed strong PEG labeling efficiency in the APH region, we were not able to demonstrate meaningful differences in the labeling intensity across the length of the APH. We, however, noticed poor labeling in the hinge and with not more than the first seven residues in the APH region. Cysteines, located in the membrane interface, can
be strongly influenced by the polar lipids leading to a potential shift in its pKa (Bogdanov et al., 2005). Consequently, despite stromally accessible, integrated cysteines would poorly react to PEG maleimide explaining the poor labelling efficiency as evident in the Hinge and N-proximal APH domain. Taken together, our findings potentially support an APH domain that is parallelly aligned on the hydrophobic membrane.

In an effort to analyze the conformational change of Hcf106 after precursor binding, cysteine accessibility assay was carried out to measure PEG labelling efficiency between a single cysteine Hcf106 variant and its precursor pre-treated companion. Even though more than half of the tested residues showed significant decrease (at least by 1.5-fold) in transport-induced labelling, almost all residue in the N-proximal region of APH showed relatively higher differences (decrease) in labelling (Figure 2.5). Tested residues in other segments of the protein, particularly in the C-proximal APH region, showed minimal or no change in labelling upon precursor binding. We

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**Figure 2.4. Cartoon diagram of Hcf106 topology in resting state based on PEG labeling:** The conformation of Hcf106 is at resting state based on PEG labeling. Hcf106 residues tested are bolded and colored. The residues are colored by three colors based up on the PEGylation intensity and therefore the topology with respect to the membrane: dark red indicates high intensity (stromally exposed), orange for moderate intensity (partially buried), and gray for weak or no intensity (completely buried or luminally exposed).
believe that the early region of APH moves into the membrane phase to the point where local residues would react weakly with the maleimide moiety of PEG. A similar finding was also evident with Tha4 where the middle region of APH shifted into the membrane under transport condition (Aldridge et al., 2012). The study also detailed the fact that the APH segment of Tha4 is minimally involved with the membrane prior to the interactions with the receptor complex (Aldridge et al., 2012; Patel et al., 2014), which is an important finding that we also observed with the APH segment of Hcf106 under the resting condition.

The transport-induced accessibility difference is due to the movement of the N-proximal APH region into the lipid interface which would reduce the reactivity of cysteine to PEG maleimide. After precursor binding, APH is potentially undergoing conformational change that would tilt the Hcf106 APH with respect to the plane of the membrane to potentially favor the passage of the precursor (Figure 2.6). Of particular to our interest, Tha4/TatA exhibit a fairly opposite incidence

![Cartoon diagram of Hcf106 differential PEGylation between resting and precursor binding state:](image)

**Figure 2.5.** Cartoon diagram of Hcf106 differential PEGylation between resting and precursor binding state: The conformation of Hcf106 is at resting state based on PEG labeling. Hcf106 residues tested are bolded and colored. The residues are colored by three colors based up on the change of cysteine accessibility to PEG maleimide after precursor binding: dark brown indicates decrease by more than 2-fold, light orange for ~2-fold decrease, very light orange for ~1.5-fold decrease, and gray for no significant change (increase or decrease within 1.2-fold) in stromal accessibility.
where its APH aligns parallel to the bacterial inner cytoplasmic membrane following the binding of the precursor (Aldridge et al., 2012; Patel et al., 2014).

To date, many studies have developed a number of models that propose specific modes of the Tat transport process. A general agreement appears to be with the theory that Tat precursor bind to the cpTatC-Hcf106 receptor complex in the initial step of translocation (Cline and Mori, 2001) after which Tha4 is recruited to complete the transport (Dabney-Smith et al., 2006). Detailed knowledge on how the core translocation event operates at molecular level remains unknown. Most of these models favor two theories: the translocation pore model and the membrane weakening model (Brüser and Sanders, 2003; Gohlke et al., 2005; Hauer et al., 2013; Patel et al., 2014). The translocation core model proposes that Tha4 oligomerizes at the receptor complex to form a hydrophilic protein-conducting channel of varying diameter that transports precursors while still in their folded state (Gohlke et al., 2005). Tha4/TatA associates via the TMD, though it is highly likely that APH could mode the pore formation and directly interact with the substrate to execute its transport in a trapdoor-like fashion (Dabney-Smith et al., 2006). The cpTat pathway can translocate precursors ranging from 2 to 7 nm in diameter (Celedon and Cline, 2013b). In a previous research study, it was shown that the amount of Tha4 recruited to the receptor complex does not vary sufficiently with the size of the precursor (Chang and Walling, 1992). This brought up the challenge to understand how Tha4 oligomers manage to accommodate the translocation of substrate of different sizes without ion leakage. The alternative would be the membrane weakening model which suggests that Tha4/TatA works in transporting the precursor by weakening and destabilizing the membrane (Brüser and Sanders, 2003).

This study agrees with the latter model in which the APH domain of Hcf106 causes membrane compression and destabilization. Amphipathic helices are often known to cause membrane destabilization, bilayer bending, and disruption (Grage et al., 2010). Based on our data, we envision a model that highlights the functional conformational change of Hcf106 during the active translocation event (Figure 2.6). We believe that the APH domain of Hcf106 assumes a parallel conformation prior to precursor recognition. Upon the binding of the precursor, the N-proximal segment moves into the membrane gradually driving membrane compression due to the hydrophobic mismatch. At this point, the thylakoid membrane losses its fluidity and stability such that the TMD sinks down the membrane further dragging the N-proximal APH with it. While this
in action, Tha4 oligomerizes at the precursor-bound receptor complex (Dabney-Smith et al., 2006). Upon recruitment, the APH domain of Tha4 topologically shifts from its tilted to a parallel conformation with respect to the plane of the membrane that facilitates complexation of multiple Tha4 monomers (Aldridge et al., 2012) large enough to augment the weakening and destabilization of the membrane (Brüser and Sanders, 2003). A simulation study demonstrated that 9 Tha4/TatA copies could cause membrane compression and further assembly of up to 25 copies of TatA could potentially disrupt the membrane (Rodriguez et al., 2013). Tha4 assembly and oligomerization is powered by the proton motive force generated from the photosynthetic electron transport system (Dabney-Smith and Cline, 2009; Mori and Cline, 2002). The membrane insertion and resulting tilted orientation of APH Hcf106 upon precursor binding and the subsequent Tha4 complexation, thus, results to the weakening and destabilization of the membrane easing the passage of the Tat precursor by lowering the energy barrier required for its transport.

Our results support the membrane weakening strategy rather than the pore/channel model. Future investigation, if possible, should address the thickness and conformational change of the thylakoid membrane during precursor transport and the amount of energy consumption accompanying the translocation process.
2.6 References


Dabney-Smith, C., and K. Cline. 2009. Clustering of C-Terminal Stromal Domains of Tha4 Homologomers during Translocation by the Tat Protein Transport System.


CHAPTER 3

Interaction Points Between Stromally Exposed, N-proximal Region of cpTatC And Precursor Mature Peptide

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3.1 Abstract

The chloroplast Twin Arginine Translocation (cpTat) system facilitates the transport of about half of the lumen proteins across the thylakoid membranes. Biochemical research has revealed information about the transport of proteins into the thylakoid lumen by the cpTat pathway. The cpTat system comprises three protein components: Tha4, Hcf106, and cpTatC. The cpTatC component is the largest protein of the translocase containing six transmembrane domains with stromally exposed N- and C-terminals. In the resting state, cpTatC usually exists in a heterodimeric receptor complex along with Hcf106. Prior research has identified a cyclical transport process of binding of the signal peptide of the precursor to the receptor, assembly with additional Tha4, transport in the presence of a protonmotive force, and disassembly to reset the system. As part of determining the organization of the receptor complex upon precursor binding and translocation, we are interested in studying contact points between the S1 segment of cpTatC and precursor mature domain. We generated single cysteine-substituted cpTatC variants in the N-proximal region of the S1 domain and a precursor carrying a single cysteine in the mature domain to investigate point interactions through disulfide crosslinking. Our data indicates positive interaction between cpTatC S1 segment and precursor mature domain. We believe that this interaction is an advanced binding, occurring after precursor recognition, to initiate the insertion of the precursor by cpTatC. Our results, thus, supports the potential role of cpTatC in forming the point of passage for the precursor during translocation by the cpTat pathway.

3.2 Introduction

In plant chloroplasts, proteins destined for the thylakoid lumen must be transported across the thylakoid membrane. Two systems, namely the chloroplast secretory and the chloroplast Twin Arginine Transport (cpTat) systems operate independently in translocating stromal substrates (Cline and Dabney-Smith, 2008; Cline and Theg, 2007; Müller and Klösgen, 2005). The cpTat system differs from the cpSec by its ability to transport fully folded stromal proteins across the thylakoid membrane (Clark and Theg, 1997; Hynds et al., 1998). In addition, the cpTat system is unique in that it requires only the transmembrane proton motive force (pmf) to drive the translocation of its substrates while maintaining ion permeability of the membrane (Cline et al., 1992; Teter and Theg, 1998).
Based on biochemical and genetic analysis, the transporter has three components, Tha4 (Walker et al., 1999b), Hcf106 (Settles et al., 1997a), and cpTatC (Mori et al., 2001a). Tha4 and Hcf106 are structurally homologous transmembrane proteins, but with distinct functions (Dabney-Smith et al., 2003). Topologically, they are divided into three distinct regions: an N-terminal transmembrane domain (TMD); a hydrophobic amphipathic helix (APH) connected to the transmembrane domain by a short hinge; and a stromally exposed, acidic C-terminal domain (Mori et al., 1999; Settles et al., 1997a). The largest cpTat component, cpTatC has six transmembrane segments, TM1 to TM6, four stromal segments, S1 (N-proximal domain), S2, S3, and S4 (C-proximal domain) and three luminal hinges, L1, L2, and L3. (Mori et al., 2001a; Zoufaly et al., 2012). At resting state, cpTatC and Hcf106 coprecipitate as a heterodimeric receptor complex which serves as the precursor docking site during the initial stage of the transport (Cline and Mori, 2001; Fröbel et al., 2012).

The cpTat system translocates a subset of luminal proteins in a luminal targeting signal peptides of cpTat (Cline et al., 1992; Ifuku et al., 2011; Klosgen et al., 1992; Mould et al., 1991). Genes for cpTat substrates are nuclear encoded and as such the proteins are cytosolically synthesized as high molecular weight precursors containing cleavable N-terminal amino acid sequences, the transit peptide, that serves to target proteins to the chloroplast and ultimately to the thylakoid lumen. Substrates contain three recognizable regions: the positively charged N-terminal (n-region), a hydrophobic core (h-region), and a polar C-terminal (c-region) regions (Natale et al., 2008). CpTat precursor also contain a conserved twin arginine motif located in the cleavable signal peptide immediately before the H-region (Chaddock et al., 1995; Henry et al., 1997), which gives the pathway its name. In the thylakoidal lumen, the signal peptide is removed by Thylakoid Processing Peptidase (TPP) that exposes the mature domain of the substrate (Halpin et al., 1989; Kirwin et al., 1987).

The translocation process begins with precursor binding to the cpTatC-Hcf106 receptor complex in an energy independent manner (Cline and Mori, 2001). In the presence of the PMF, primarily the ΔpH, Tha4 assembles with the precursor-bound complex to initiate active transport of the precursor. Tha4 then disassembles to regenerate the receptor complex for subsequent cycles of translocation (Mori and Cline, 2002). The regulated assembly and oligomerization of Tha4 at the translocation point during the actual transport stage suggests a critical role of Tha4 in cpTat-
mediated translocation process (Celedon and Cline, 2012b; Cline and Mori, 2001; Dabney-Smith and Cline, 2009). However, to date, a detailed mechanism for the cpTat translocation process is lacking. Understanding the organization of the receptor complex, how the precursor interacts with the receptor complex, how folded proteins make it across the intact membrane, and how it manages to transport the substrate without ion leakage are crucial for understanding the mechanism of transport by the cpTat pathway.

Previous studies using site-specific and random mutagenesis have identified required cpTatC (TatC) residues for interaction with the signal peptide and other Tat components, although, few TatC residues among several bacterial and chloroplast proteins are conserved (Allen et al., 2002). Mutagenic studies, however, have revealed certain conserved residues in the extreme N terminus and second cytoplasmic domain are required for Tat-mediated translocation perhaps involved in TatB/C complex assembly (Allen et al., 2002; Barrett and Robinson, 2005; Bolhuis et al., 2001; Ramasamy et al., 2013). In chloroplasts, crosslinking studies identified that the Hcf106 TMD forms contact points with cpTatC TM5 (Kneuper et al., 2012) (Ma et al., dissertation). In recent studies, however, additional contact points in the Hcf106-cpTatC receptor complex were identified and suggested to play an integral part in its proper assembly. These include interactions between the first two stromal domains of cpTatC and C-proximal APH of Hcf106 as well as cpTatC L3 and N-terminus of Hcf106 (Ma et. al., dissertation). Based on algorithmic analysis of alanine substituted cpTatC, all luminal domains, TM5, and TM6 were also shown to have a significant role in the proper assembly of the receptor complex (Ma and Cline, 2013).

The receptor complex forms the docking site for the precursor binding prior to transport by recognizing the RR proximal region of the signal peptide (Cline and Mori, 2001; Fröbel et al., 2012; Ma and Cline, 2013). The S1 and S2 domains of cpTatC interacts with the RR motif of the signal peptide (Gérard and Cline, 2007; Ma and Cline, 2013; Zoufaly et al., 2012). The highly-conserved glutamate residues in these segments are thought to form the binding site for the signal peptide (Mori and Cline, 2001). Very few studies have investigated interaction between the precursor mature domain and cpTatC. If the precursor signal peptide interacts with the cpTatC S1 domain, the mature domain of the precursor should extend toward the N-proximal region of S1 to achieve within an optimum distance for potential interaction. This could potentially provide us insight for the nature of interaction between the precursor and cpTat receptor complex during
transport as well as the involvement of cpTatC in initiating the insertion and passage of precursor across the thylakoid membrane.

In this study, cysteine substitution and cysteine crosslinking experiments were used to map interactions between the N-proximal region of cpTatC S1 domain and the mature domain of a cpTat substrate. Prior studies detected significant crosslinking between residues of the precursor signal peptide and S1 domain of cpTatC (Ma and Cline, 2013; Zoufaly et al., 2012). We predict, therefore, that the precursor mature domain extends out such that it is in close enough proximity to crosslink with the nonconserved, N-proximal region of cpTatC S1 domain. Here we show a positive interaction between the cpTatC S1 domain and substrate mature domain providing evident that it is involved in substrate binding and contributes to forming the point of passage during the translocation event. Though the cpTat receptor complex recognizes and interacts with the signal peptide of the precursor (Chaddock et al., 1995; DeLille et al., 2000; Ma and Cline, 2013), we also believe that the precursor mature domain contributes to its transport by tightly binding to cpTatC following recognition of the precursor signal peptide.

3.3 Experimental Methods

3.3.1 Generation of single cysteine-substituted cpTatC variants and substrate protein

Two cpTatC single cysteine constructs (D35C and D38C) were prepared from a template containing the complete precursor sequence for cpTatC fused to its N-terminus by the transit peptide and the first 13 residues of the mature ribulose-1,5-bis-phosphate carboxylase/oxygenase (Genebank accession no. AAA33685) and cloned into the pGem4Z vector. These constructs were made by QuickChange mutagenesis (Agilent Technologies) using the following primers: for D35C, 5’ – CGAGAATAAATGTATGATTGATGGTATAAGTG – 3’; and for D38C, 5’ – GATATGTTTGTGGTATAAGTGAAGAAGCATTGG – 3’ and their complimentaries. CpTatC contains 3 native cysteines which were previously changed to alanine, thus, designated as cpTatCaaa (Ma and Cline, 2013).

The precursor used in the study was a truncated version of iOE17 from maize where the strand targeting region and 11 amino-terminal residues are removed (Henry et al., 1997; Ma and Cline, 2000). A C-terminal His6 tag is joined to tOE17 by a polylinker consisting of GGGGS repeated three times, designated by tOE17G3H. A single cysteine version of the modified precursor tOE17-
G3H at A137 in the mature domain was also previously constructed in the lab (Pal et al., 2012). All cysteine mutations were confirmed by Sanger sequencing at the Center for Bioinformatics and Functional Genomics (CBFG) at Miami University.

3.3.2 Preparation of chloroplasts and thylakoids

Intact chloroplasts were isolated from 10-12-day-old pea seedlings (Pisum sativum L. cv. Laxton’s Progress 9 or Little Marvel) as described (Cline et al., 1993). Isolated chloroplasts were resuspended in import buffer (IB, 50 mM HEPES-KOH, pH 8.0, 330 mM sorbitol) to 1 mg/mL and kept on ice until use. Chlorophyll concentration was determined according to Arnon (Arnon, 1949). Following import of in vitro translated radiolabeled cpTatC, intact thylakoids were isolated by osmotic lysis of intact chloroplast. Briefly, suspension of intact chloroplasts was pelleted for 5 min at 1000 × g. The supernatant was removed and pellets were resuspended at 1 mg/mL with 50 mM lysis buffer (HKM, HEPES-KOH, pH 8.0, 10mM MgCl₂) (Mori and Cline, 1998). Following a 5 min ice incubation, lysates were osmotically adjusted with IB, 10 mM MgCl₂ and subsequently pelleted at 3200 × g for 8 min to recover cpTatC integrated thylakoids.

3.3.3 Import of in vitro translated radiolabeled cpTatC

Single cysteine-substituted cpTatC plasmid DNAs were transcribed to mRNA by in vitro transcription in the presence of SP6 polymerase, ribonucleoside triphosphates (rNTPs), and CAP. Radiolabeled cpTatC variants were then prepared from the capped mRNA by in vitro translation in the presence of L-[3,4,5-3H(N)]=Leucine (Perkin-Elmer) according to the manufacturer protocol from Promega Wheat Germ Extract Kit (Madison, WI USA) (Cline, 1986). The translated product was adjusted with 60 mM leucine in IB buffer use in import assays. Briefly, the translated cpTatC was incubated with chloroplast equivalent to 0.40 mg/mL chlorophyll chloroplast, 5 mM DTT, and 5mM Mg-ATP in IB with ~100 µE/m²/s of white light in a 25°C water bath for 40 min. Intact chloroplasts, with the imported protein, were then re-purified by centrifugation through 35% percoll at 3200 × g for 8 min. The thylakoids were harvested as described above and used for transport of cpTat precursor.

3.3.4 Substrate binding assay

The precursor was derivatized by incubating it with 80 µm 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) at room temperature for 30 minutes before use (Pal et al., 2012). Binding reactions were
carried out by incubating imported thylakoids with the precursor in IB and 10 mM MgCl$_2$ at 0°C for 30 min in darkness (Ma and Cline, 2013). Thylakoids were recovered by centrifugation; washed, by resuspending in IB and 10 mM MgCl$_2$ and pelleting at 3200 × g for 8 min; and finally analyzed as binding sampling by crosslinking.

### 3.3.5 Disulfide cross-linking between imported cpTatC and mature tOE17

Formation of the disulfide bond between sulfhydryl moieties from proximal cysteine residues was catalyzed by adding 4 mM copper phenanthroline (CuP) and incubation at room temperature for 10-15 minutes. The reaction was stopped upon adding of 50 mM N-ethylmaleimide (NEM), from a 1 M stock in ethanol in IB + 10 MgCl$_2$. Intact thylakoids were recovered by centrifugation at 3200 × g for 8 min and washed with IB, 5 mM EDTA, and 10 mM NEM. The pellets were resuspended to 50 uL and divided into two equal volumes: the reducing half was mixed with equal volumes of sample solubilizing buffer (2x), 100 mM Tris-HCl (pH 6.8), 0.2M DTT, 5% SDS, and 30% glycerol and the non-reducing half mixed with equal volume of sample solubilizing buffer (2x), 100 mM Tris-HCl (pH 6.8), 8 M urea, 5% SDS, and 30% glycerol lacking reducing agent. Samples were then analyzed by SDS-PAGE and fluorography.

### 3.4 Results

#### 3.4.1. cpTatC S1 domain interacts with RR proximal region of the precursor signal peptide

In the initial stage of cpTat transport, the precursor is targeted to bind to cpTatC-Hcf106 receptor complex by the twin arginine residues in the signal peptide (Cline and Mori, 2001; Fröbel et al., 2012; Ma and Cline, 2013). Biochemical studies show that cpTatC S1 and S2 domains interact with twin arginine proximal region of the precursor signal peptide following recognition (Gérard and Cline, 2007; Ma and Cline, 2013; Zoufaly et al., 2012). Ma et al. showed in a recent study that significant crosslinkings were detected between the stromal S1 domain of cpTatC and RR proximal region of precursor signal peptide (Ma and Cline, 2013). Here, we used disulfide crosslinking assay to test one cpTatCaaa variant with a cysteine-substituted residue in the S1 domain, i.e. E73C (Figure 3.1), to see if we were able to replicate the experiment to find crosslinking between the variant and a cpTat precursor signal peptide as shown in Ma et al. The precursor, we used in this study, was a tOE17-20F variant that carried a cysteine near the RR motif, i.e. at −18 (Ma and Cline, 2013). The precursor also carried a phenylalanine at −20 to provide higher affinity of the precursor signal peptide for the cpTat receptor complex (Gérard and Cline, 2007). Another cpTatCaaa
variant, L247C, was also tested for crosslinking with tOE17-20C-18C (Figure 3.2). The cysteine substitution is located in the S3 domain of cpTatCaaa (Figure 3.1) which was previously concluded for not playing a role in receptor complex assembly or substrate binding (Ma and Cline, 2013).

The disulfide crosslinking assay involves treatment of the binding samples with 4 mM CuP (refer to experimental methods for details) to catalyze the formation of disulfide bond between sulfhydryl moiety in cysteine residues provided they are within an optimum distance. Such covalent interaction shows for potential molecular interaction between proximal residues from tOE17 signal peptide and cpTatC S1 domain. To visualize the crosslinking status, samples were run on a non-reducing polyacrylamide gel electrophoresis (PAGE). The presence of a ~48 kDa band in a non-reducing PAGE corresponds to the cpTatCaaa + tOE17 adduct which is dependent upon disulfide crosslinking if it disappears in a reducing PAGE. Each variant was treated with precursor of two versions: one that was [³H]-Leu labelled and another that was not. This would track any protein bands that could possibly appear from the addition of the precursor.

Our results were able to replicate the ~48 kDa cpTatCaaa + substrate adduct in the non-reducing PAGE for E73C as expected (Figure 3.2, lanes 9 and 10 in a red box). The crosslinking adduct is as a result of disulfide bond formation as it disappeared in the reducing PAGE (Figure 3.2, lanes 11 and 12). We also confirmed that cpTatCaaa L247C did not crosslink with tOE17-20C-18C.
The cysteine-free cpTatC, cpTatCaaa, was used as a negative control for its inability to form a crosslinking product (Figure 3.2, Lanes 3 to 6).
region (the first 67 residues) of cpTatC stromal N-proximal region in the S1 domain as shown in Figure 3.1.

Each variant was treated with [$^{3}$H]-Leu labelled or unlabeled precursor. This would track any protein bands that could possibly appear from the addition of the precursor. By comparing the ~48 kDa band in both lanes for each variant, it is also evident that the band intensity increases, by at least by 2-fold, from the unlabeled to the labeled version strengthening our analysis (Figure 3.4; compare lanes 3 with 4 and 6 with 7 for each gel). The reducing counterpart was able to reduce, thus, eliminate all of the cys-cys crosslinks from appearing in the gel further confirming that the ~48 kDa band was indeed a result of disulfide crosslinking (Figure 3.4; lanes 5 to 7).

The tested cpTatC variants crosslinked with tOE17G3HA137C as evident by the formation of ~48 kDa band formation as visualized by non-reducing PAGE (Figure 3.4; lanes 2 to 4). The strongest interaction was detected between cpTatCD38C and the tOE17G3HA137C precursor (Figure 3.4; lanes 3 and 4). Interaction between cpTatCD35C and the precursor was detected but not to the same intensity as with D38C (Figure 3.4; lane 3 and 4). As expected, the cysteine-free version of cpTatC, cpTatCaaa, was unable to form the adduct (Figure 3.4; lanes 3 and 4).

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**Figure 3.3.** tOE17G3H protein sequence: protein sequence of tOE17G3H. The amino acids are numbered relative to the thylakoid processing signal peptidase (Tpp) cleavage site. The location where cysteine was introduced in highlighted in red.
Figure 3.4. Crosslinking of single cysteine substituted in the N-proximal domain of cpTatC with tOE17 mature domain:

Intact chloroplasts imported with radiolabeled cpTatC cysteine variants were purified and treated with HKM buffer for 5 min on ice. Isolated intact thylakoids containing radiolabeled cpTatC cysteine variants were treated with mock buffer (lanes 2 and 5), or unlabeled precursor, i.e. tOE17G3HA137C (lanes 3 and 6) or labelled precursor, i.e. tOE17G3HA137C [3H] (lanes 4 and 7) for 20 min in dark at 0°C. Samples were then subjected to CuP crosslinking (see experimental method for details). Samples were analyzed by SDS-PAGE and fluorography under non-reducing (lanes 2 to 4) and reducing conditions (lanes 5 to 7). Crosslinked products are indicated at ~48 kDa adduct in the non-reducing lanes.

(A) Labelled cpTatCaaa D38C treated with the precursor (labelled or unlabeled) or mock. The variant interacts with the precursor as evident by the ~48 kDa adduct.

(B) Labelled cpTatCaaa D35C treated with the precursor (labelled or unlabeled) or mock. Weak interaction is evident.

(C) Labelled cpTatCaaa treated with the precursor (labelled or unlabeled) or mock. As expected, no crosslinked product was formed.
3.5 Discussion

Multiple piece of evidence support that Tha4 serves as the primary passage for substrate during transport by the cpTat pathway (Aldridge et al., 2012; Cline and Mori, 2001; Dabney-Smith and Cline, 2009; Dabney-Smith et al., 2006). However, recent work also indicates that cpTatC plays a role in the interaction with the signal sequence, insertion, and subsequent transport of the precursor along with Tha4 (TatA, the bacterial homolog) during the translocation event (Fröbel et al., 2012) (Ma et. al., dissertation). This study shows the direct interaction between the N-proximal region of cpTatC S1 domain and precursor mature domain. Our data provide insight into the undefined mechanism underlying the actual formation of the passage point for Tat precursors by the chloroplast Tat pathway in thylakoids.

In this preliminary study, cpTatC variants containing a single cysteine substitution in the N-proximal region were constructed to characterize contact points with the precursor mature domain by disulfide crosslinking. Physiological precursors, while excellent transport substrates, exhibit poor binding to thylakoids and as such precursors are often engineered to form derivatives with a tighter, more stable binding affinity [for representative examples, see Refs. (DeLille et al., 2000; Gould et al., 2007; Gérard and Cline, 2007; Mori and Cline, 2002; Wexler et al., 1998)]. In this study, preOE17 with two major modifications was used: a truncated version where the entire strand targeting region and 11 amino-terminal residues of iOE17 were removed (designated as tOE17) (Henry et al., 1997; Ma and Cline, 2000) and a C-terminal His-tag joined to tOE17 by a polylinker consisting of GGGGS repeated three times to give the precursor name tOE17G3H. Only one variant of tOE17G3H was tested which contained a cysteine substituted for alanine at the 137th residue. Our data indicate an interaction between cpTatC S1 region and the precursor mature domain (Figure 3.4). It is surprising to see interaction with tOE17G3HA137C and cpTatC considering the recent finding that single cysteine-substituted Tha4 were unable to crosslink with cysteine substituents at pOE17A71 and pOE17A137 because they were thought to be buried in the folded precursor (Pal et al., 2012).

Multiple interaction points between cpTatC and precursor signal peptide have been identified (Ma and Cline, 2013) (Ma et al., dissertation). These points of interactions were most prominent in the S1 and S2 domains of cpTatC. For initial precursor binding to the receptor complex, these stromal domains interact with the twin arginine proximal region in the signal peptide of the precursor.
Our results identified novel contact points between these proteins; the N-proximal segment of cpTatC interacted with residues in the mature domain of the precursor. These findings are of keen of interest because the S1 segment of cpTatC interacts with the residues in the precursor signal peptide and the mature domain that are separated by at least 150 amino acids (Balsera et al., 2005; Ma and Cline, 2010). Our lab previously demonstrated interaction points between the precursor signal peptide with both Hcf106 TMD and APH which, at first, was also seemed unlikely to occur simultaneously, considering that the tested residues from TMD and APH are separated by approximately 30 Å (Ma et al. dissertation).

In the present study, the S1 domain of cpTatC interacted with A137C in the mature domain of tOE17. S1 also interacts with the residues in the twin arginine proximal region of tOE17 (Ma and Cline, 2013). A possible interpretation for this interaction is the structural flexibility of the precursor which allows to form turns such that its signal peptide and mature domain are in proximity to interact with the N- and C-proximal region of cpTatC S1 domain. The most likely explanation is, however, the step wise positioning of the precursor by cpTatC during the Tat-mediated translocation. In the initial stage of transport, the precursor binds to the receptor complex, in this manner to cpTatC S1, via its signal peptide. Beyond the recognition stage, cpTat positions the precursor mediating the translocation of its signal peptide across the membrane. While this is progress, the precursor mature domain slides down toward the binding pocket such that it comes into close proximity with the stromal segment of cpTatC. During experimental conditions that favors recognition and subsequent positioning of the precursor, we were able to freeze the complete translocation of the precursor and trap the interaction between the cpTatC S1 domain and precursor mature domain beyond the recognition stage of transport. Frobel et. al demonstrated by a biochemical study that cpTatC/TatC catalyzes the transmembrane insertion of Tat precursors following recognition (Fröbel et al., 2012). Our finding is in line with the idea that cpTatC continues to interact with the substrate to promote insertion of the precursor and thus likely plays a role in forming the point of passage during translocation.

Several studies in plants and bacteria focus on elucidating actual mechanism of cpTat transport. The translocation process begins with the precursor binding to the receptor complex, jointly formed by Hcf106 and cpTatC, in an energy independent manner (Cline and Mori, 2001).
presence of PMF, Tha4 assembles with the complex to initiate active transport of the precursor (Dabney-Smith et al., 2006). Tha4 then disassembles to regenerate the receptor complex for subsequent cycles of translocation (Mori and Cline, 2002). Currently, a number of Tat transport models generally agree with the hypothesis that Tat precursors bind to the receptor complex in the initial step of translocation (Cline and Mori, 2001). The mechanism outlining the actual passage of the precursor, following Tha4 recruitment, across the thylakoid is, however, explained by two models. According to the translocation core model, Tha4/TatA forms a conducting channel that allows passage of the precursor (Gohlke et al., 2005). The membrane weakening model, however, suggests that Tha4/TatA transports the precursor by weakening and destabilizing the membrane (Brüser and Sanders, 2003; Patel et al., 2014).

Though Tha4 dominates the core of the translocation site, we believe that Tha4 is working along with Hcf106 and cpTatC to translocate the precursor (Fröbel et al., 2012) (Ma et al., dissertation). Based on our present study, we thereby envision a model (Figure 3.5) that depicts the insertase role of cpTatC and the membrane weakening role of Tha4 and Hcf106 to translocate the precursor across the thylakoid membrane. Briefly, the precursor first binds to the binding pocket that is jointly formed by Hcf106 and cpTatC (Figure 3.5, left model) (Cline and Mori, 2001). The conserved region (beyond L67) in cpTatC S1 domain, close to the N-proximal region of TM1, interacts with the signal peptide near the twin arginine motif while Hcf106 APH interacts with the hydrophobic region close to the signal peptide cleavage motif (Cline and Mori, 2001; Ma and Cline, 2013). The APH domain of Hcf106, which originally assumes a parallel conformation, gradually perturb itself into the lipid layer via its N-proximal region to drive membrane compression (for more details, refer chapter 2 discussion). While this in action, Tha4 oligomerizes at the precursor-bound receptor complex (Figure 3.5, right model) (Dabney-Smith et al., 2006) which also undergo topological change to facilitate complexation of multiple Tha4 monomer (Aldridge et al., 2012). The tilted Hcf106 APH and the recruitment of multiple Tha4 monomers at the translocation site collectively causes weakening and destabilization of the membrane (Brüser and Sanders, 2003) that potentially favors cpTatC-mediated insertion of the precursor signal peptide (Fröbel et al., 2012). During the insertion of the precursor signal peptide, the mature domain of the precursor slides down toward the membrane and gains access for interaction with the N-proximal region of cpTatC S1 domain (Figure 3.5, right model). The signal peptide also interacts with cpTatC TM5 and Hcf106 TMD (Aldridge et al., 2014) (Ma et al., dissertation). By
this stage, the increasing assembly of Tha4 at the site of translocation potentially disrupts the membrane reducing the threshold for energy barrier to an extent that eases the complete passage of the precursor (Brüser and Sanders, 2003; Patel et al., 2014).

For future studies, many other regions in the cpTatC domains need to be tested for possible crosslinking with the precursor mature domain. For example, interaction points can be mapped between precursor mature domain and C-proximal region of cpTatC S1 domain and even cpTatC TM domains. As Hcf106 TMD is closely associated with cpTatC TM5 (Kneuper et al., 2012) and TM1 and TM2 (Ma et al., dissertation), we also anticipate potential crosslinking between Hcf106 TMD and precursor mature domain. In general, these future studies would provide valuable information in sketching the transport path of the precursor and confirm the insertase activity of cpTatC as proposed by Fröbel et al. 2012 and this study.
3.6 References


Dabney-Smith, C., and K. Cline. 2009. Clustering of C-Terminal Stromal Domains of Tha4 Homologomers during Translocation by the Tat Protein Transport System.


CHAPTER 4

Conclusions
4.1 Conclusions

The chloroplast is the primary organelle in plants and algae that houses the photosynthetic machinery (Leister, 2003). With evolution, chloroplasts lost more 90% of their genetic information to the nucleus. Majority of the proteins present in the chloroplast are thus synthesized in the cytosol as higher molecular weight proteins and translocated into the chloroplast to perform their function. With the elaborate system for maintaining diverse proteins in membrane compartments, cells developed complex membrane protein translocases that traffic proteins in, out, and within the organelle. Among the protein translocases that transport thylakoid proteins, the chloroplast Twin Arginine Translocation, cpTat, attracts scientists for its unique features of translocating proteins while in their fully folded state in the presence of the proton motive force (PMF) (Bageshwar and Musser, 2007; Braun et al., 2007; Clark and Theg, 1997).

The cpTat precursor contain a conserved twin arginine motif located in the cleavable signal peptide (Chaddock et al., 1995; Henry et al., 2007), which gives the pathway its name. Based on biochemical and genetic analysis, the transporter has three components; Tha4, Hcf106, and cpTatC. Tha4 and Hcf106 are structurally homologous proteins, but with distinct functions, that contain a single transmembrane domain (TMD), amphipathic helix (APH), and stromal C-tail (Dabney-Smith et al., 2003; Mori et al., 1999; Settles et al., 1997). The largest component, cpTatC has six transmembrane segments with stromally exposed N- and C-terminals (Mori et al., 2001). At resting state, Hcf106 and cpTatC join to form the receptor complex that forms the docking site for the precursor (Frobel et al., 2012; Ma and Cline, 2013).

During the past few decades, much effort has been made to fully understand the mechanism of transport by the cpTat pathway. Though the overall model for the transport cycle is established, detail sequential events remain to be elucidated. The translocation process begins with precursor binding to the cpTatC-Hcf106 receptor complex in an energy independent manner (Cline and Mori, 2001). In the presence of PMF, primarily ΔpH, Tha4 assembles with the complex to initiate active transport of the precursor. Tha4 then disassembles to regenerate the receptor complex for subsequent cycles of translocation (Mori and Cline, 2002). Detailed mapping for functional interactions among cpTat components and substrates and how the cpTat components topologically rests with respect to the membrane would greatly contribute to the organization and mechanism of transport.
My thesis focused on supplementing more information toward understanding cpTat mechanism of transport; Chapter 2 aimed to understand the topology and conformational change of Hcf106 upon precursor binding and Chapter 3 aimed in studying contact points between the N-proximal region of cpTatC S1 domain and precursor mature domain.

Topologically, Hcf106 is predicted to contain 3 domains: an N-terminal transmembrane domain (TMD), a basic amphipathic helix (APH) connected to TMD by a short hinge region, and a free spanning C-tail. To date, detailed topology information of Hcf106 is lacking. In Chapter 2, cysteine accessibility labeling techniques were used to characterize the topology of Hcf106 in the absence and presence of precursor. In resting state, Hcf106 displayed an overall membrane integrated N-proximal domain with a shortened TMD and stromal APH with both ends accessible from the stroma. During precursor binding, though the overall topology of Hcf106 remained unchanged, the N-proximal region of APH became more buried assuming an overall tilted conformation with respect to the membrane surface. Our research is consistent with the previously proposed membrane weakening model, where the cpTat translocase partially perturb into the thylakoid membrane to weaken and destabilize the membrane to favor passage of the precursor (Brüser and Sanders, 2003; Patel et al., 2014). We, therefore, attribute this accessibility change to a precursor-driven conformational change of Hcf106 that likely contributes to membrane weakening as potential mechanism for promoting the passage of the precursor.

In Chapter 03, single cysteine was introduced in the N-proximal region of cpTatC S1 domain. The precursor, also carrying a single cysteine in the mature domain, was used to investigate point of interactions with the cpTatC variants through disulfide crosslinking. Our data indicates positive interaction between the S1 domain of cpTatC and precursor mature domain. We believe that this interaction is an advanced binding, occurring after precursor recognition, to initiate the insertion of the precursor by cpTatC. This study supports Fröbel et al. finding which suggests cpTatC-mediated insertion of the precursor as part of the cooperative work by all cpTat components in the formation of the active point of passage for the precursor (Fröbel et al., 2012).

The membrane insertion and resulting tilted orientation of APH Hcf106 upon precursor binding and the subsequent Tha4 complexation at the receptor complex, thus, results to membrane weakening and destabilization and lowering of the energy barrier, easing cpTatC-mediated
insertion of the precursor across the thylakoid membrane. For future studies, crosslinking and time-course analysis would provide valuable information in sketching the transport path of the precursor and confirm the insertase activity of cpTatC as proposed by Fröbel et al. 2012 and this study.

4.2 References


