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Structural and functional analysis of a chloroplast transit peptide : interactions with the chloroplast translocation apparatus

Chitra Subramanian
University of Tennessee

To the Graduate Council:

I am submitting herewith a dissertation written by Chitra Subramanian entitled "Structural and functional analysis of a chloroplast transit peptide : interactions with the chloroplast translocation apparatus." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Barry D. Bruce, Major Professor

We have read this dissertation and recommend its acceptance:

Otto J Schwarz, Beth C. Mullin, Albrecht von Arnim

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Structural and Functional Analysis of a Chloroplast Transit Peptide: Interactions with the Chloroplast Translocation Apparatus

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Chitra Subramanian
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DEDICATION

This dissertation is dedicated to my parents,

Mr. K. N. Subramanian and Mrs. Shanta Subramanian,

my mother-in-law, Prof. R. Maya Sundari,

and my dear husband, Anand.

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First of all, I wish to thank my research advisor Dr. Barry D. Bruce for his support and for the training I received during my graduate career. I also wish to express my deep gratitude to him and Prof. Otto J. Schwarz for having stood by me in 1996 when I had to join graduate school.

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ABSTRACT

Protein targeting into organelles is a central cellular process that occurs in all-living organisms. Proper cellular targeting is essential for the functioning of most proteins within a cell, yet the mechanism by which this process is mediated is not clearly understood. Plastids from plants provide an excellent model system for studying protein targeting as they are semi-autonomous organelles with a wide variety of structural and functional diversity. Although, plastids have their own genome they strongly rely on imported proteins that are encoded in the nuclear genome and translated in the cytoplasm. The proteins synthesized in the cytoplasm have an N-terminal extension called the transit peptide, which is considered to be necessary and sufficient for the import of proteins into plastids.

Binding of precursors to the plastid surface probably involves both proteins and lipids of the envelope membrane. Several proteins in the outer and inner membrane of chloroplasts have been identified as components of the chloroplast translocation machinery. These proteins form the Toc (Translocon at the outer-envelope-membrane of chloroplasts) components and the Tic (Translocon at the inner-envelope-membrane of chloroplasts) components.

Although, early reports have demonstrated that precursors directly interact with individual components of the two translocators, mechanistic details have been hampered by the inability to trap an early translocation intermediate. We have created a novel reagent that places a dual-epitope tag (His-S) at the N-terminus of the transit peptide

(SStp) derived from the pea Rubisco small subunit precursor (prSSU). These two epitope tags permit facile purification of the fusion protein from *E.coli* via the His-tag, and a highly sensitive detection via the interaction of the S-protein with the S-tag. The S-tag/S-protein interaction may be utilized in a multitude of detection assays including RNase enzymatic assays, fluorescent detection, and chemiluminescent blotting.

Subsequent to the successful binding of His-S-SStp to the chloroplast surface we can detect the transit peptide by both laser-scanning confocal microscopy (LSCM) and flow cytometry using a S-protein/FITC conjugate. These two methodologies in combination provide both high-resolution spatial information about the distribution of the bound transit peptide and a statistically significant measurement of the total number of transit peptides bound. More traditional techniques such as blotting and organelle fractionation were used to verify these results. Moreover, label transfer cross-linking experiments have shown that His-S-SStp is indeed bound to the Toc apparatus.

To further our understanding about transit peptide interaction we made C-terminal deletions of His-S-SStp and found specific motifs (H(P/G)H(R/K) present in the 19 amino acid region from F36 to R54 that are essential for the recognition of the transit peptide by the chloroplast. Disruption of the structure of the transit peptide by insertion of proline residues revealed the presence of five domains with specific functions in the transit peptide. Taken together, these approaches allow for sensitive and quantifiable evaluation of the *in vitro* binding of SStp to the chloroplast translocation apparatus, as well as the first direct visualization of a transit peptide bound to the chloroplast translocation apparatus.

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LIST OF ABBREVIATIONS

APDP	N-[-(p-azidosalicylamido)butyl]-3'(2-pyridyldithio)propionamide
BCIP	*Bis*-chlorophhenol indophenol
CBD	Chitin binding domain
CBPS	Cellulose-based peptide screening
CD	Circular dichroism
CSS1	Chloroplast Stress Seventy 1
DGDG	Digalactosyldiacylglycerol
DHFR	Dihydrofolate reductase
DPC	dodecylphosphocholine
DPG	dodecylphosphoglycol
DTT	Dithiothreitol
EGTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
HPLC	High pressure liquid chromatography
Hsp70	Heat shock protein 70kDa
IB	Import Buffer
IC_{50}	50% Inhibition concentration
IMAC	Immobilized metal affinity chromatography

IPTG	Iso-propyl-β-thio-galactopyranoside
LSCM	Laser scanning confocal microscopy
MGDG	Monogalactosyldiacylglycerol
mSSU	Mature small subunit of Rubisco after removal of the transit peptide from prSSU
NBT	Nitro blue tetrazolium chloride
NMR	Nuclear magnetic resonance
NTP	Nucleotide tri phosphate
OM	Outer membrane of chloroplast
PBS	Phosphate buffered saline
PC	Phosphatidyl choline
PCR	Polymerase chain reaction
pFd	Precursor of ferredoxin
PG	Phosphatidyl gycerol
PI	Phosphatidyl Inositol
pLHCP	Precursor light harvesting chloroplast protein
PMSF	Phenyl methyl sulfonyl fluoride
pOE33	Precursor of oxygen evolving complex 33 kDa
pPC	Precursor of plastocyanin
prSSU	Precursor of small subunit of Rubisco
RNase	Ribonuclease
Rubisco	Ribulose bisphosphase carboxylase/oxygenase

SB	Sample Buffer
SDS-PAGE	Sodium deodecyl sulfate-poly acrylamine gel electrophoresis
SPP	Stromal processing peptidase
SStp	Transit peptide of small subunit of Rubisco
TBS	Tris buffered saline
Tic	Translocon at the inner envelope membrane of chloroplast
Tim	Translocon at the inner membrane of mitochondria
Toc	Translocon at the outer envelope membrane of chloroplast
Tom	Translocon at the outer membrane of mitochondria
trFd	Transit peptide of ferredoxin

Chapter 1

General Introduction

Plastids are semi-autonomous organelles with a wide variety of structural and functional diversity and unique biochemical pathways. In plants, they are formed by differentiation of a progenitor, the pro-plastid. This process involves not only the synthesis of a variety of proteins inside the chloroplast but also the import of several proteins from the cytosol. Proteins that are synthesized in the cytosol are made as precursors with N-terminal targeting sequences that direct the newly synthesized precursor to the chloroplast.

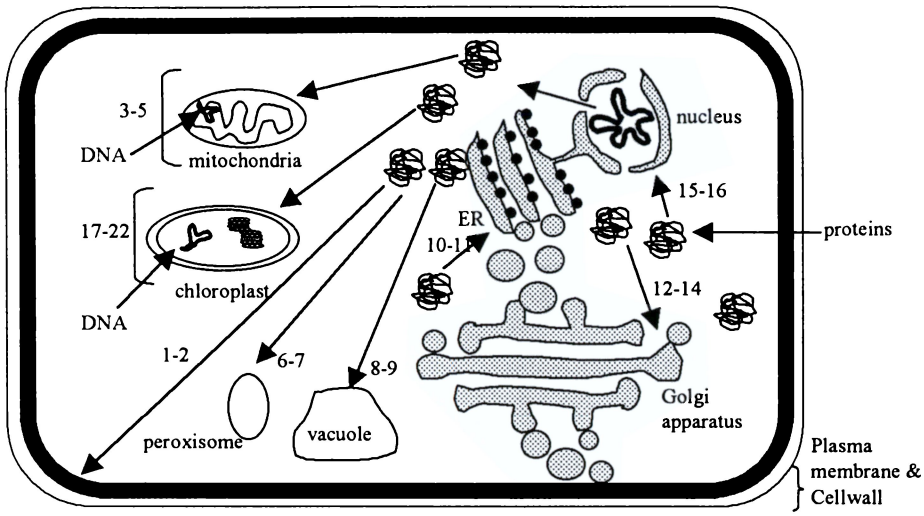
THE PLANT CELL AND PLASTIDS

The plastid genome and membranes

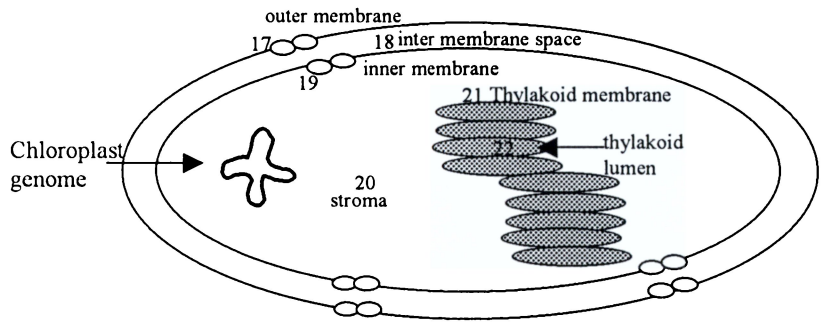
Plant cells contain no fewer than 17 different membrane systems that are formed to provide the cell with functionally specialized aqueous space (Figure 1-1a and b). This is one of the major features that differentiate eukaryotes from prokaryotes. A typical plant chloroplast has two bounding membranes but chloroplasts of many algae have three or four membranes (reviewed in McFadden, 2001). Chloroplasts are thus believed to be derived from prokaryotes like cyanobacteria by secondary endosymbiosis. Among the organelles of a plant cell, plastids and mitochondria are different because they possess their own genome and protein synthesis machinery. Plastids and mitochondria share many traits with prokaryotic organisms, which include the similarity of their genomes to the bacterial genome.

Figure 1-1. Protein trafficking in plant cells and chloroplasts. **(a)** The protein synthesized in the cytosol has at least 22 different destinations inside a cell. The different destinations are numbered 1 through 22. **(b)** The different destinations of a chloroplast precursor protein that is encoded by the nucleus and translated in the cytoplasm. There are at least 6 different destinations numbered 17 through 22 for proteins inside the chloroplast.

(a)



(b)



Chloroplast trafficking

Figure 1-1

A typical chloroplast DNA is circular with genes organized in operons and is multi copy per cell (Zhang et al., 1999). Complete sequencing of the *Synechocystis* genome revealed 3,229 genes while the chloroplasts from higher plants had only about ~100 genes, indicating that the chloroplast from plants had lost most of their genes (Kaneko et al., 1996). Supplemental material of Initiative 2000 indicates that cyanobacteria are the most active source of genes from prokaryotes (Initiative, 2000). Conservation of the large operons present in many prokaryotes indicated that chloroplasts were reduced bacterial genomes (Ohyama et al., 1986). Similar to the bacteria the DNA present in these organelles is circular and does not form supramolecular complexes with proteins. The plastid DNA contains all of the genes for the rRNAs and a full complement of genes for the tRNAs. However, they encode only around a 100 proteins that are almost exclusively involved in photosynthesis and protein synthesis. Due to the loss of a large portion of their genome chloroplasts are semi-autonomous organelles. The complete analysis of the *Arabidopsis* genome revealed that ~3,574 proteins are targeted to the chloroplasts and they range in a variety of functions. These proteins have an N-terminal targeting sequence that targets these proteins to the chloroplast. Chloroplasts thus require an import machinery at the envelope membrane for the entry of proteins that are encoded by the nucleus.

Gene transfer to the nucleus

Several hypotheses have been proposed to explain the migration of genes from the chloroplast to the nucleus. One hypothesis proposes that the spontaneous insertion of genes into the nuclear genome is a common phenomenon (Blanchard &

Lynch, 2000). Due to this the organelles would loose their genes, as it would be advantageous for the organelle during intra-organellar competition to have a smaller genome. Another hypothesis proposes that nucleus is the regulatory organelle of the cell and so naturally all organelles come under the control of the nuclear regulatory genes (Michl et al., 1999). The redox-associated functions in the organelles have been proposed to increase the free radical induced mutagenesis in genes. Thus, this would favor the transfer of genes to the nucleus (Allen & Raven, 1996). The most convincing hypothesis is the Muller's ratchet phenomenon (reviewed in Martin & Herrmann, 1998). It proposes that the chloroplasts reproduce asexually via budding or division and so there is no recombination of genetic material. This results in a greater chance of mutation due to the incapability to remove the mutation by recombination. Hence, if the genetic material had remained in the chloroplast, with time the chloroplasts would have become extinct due to deleterious mutations. Nevertheless, due to the transfer of most of the genes to the nucleus, the chloroplasts are free from the fate of mutational meltdown. The genes that are lost encode proteins involved in the some following functions: transport; regulation; biosynthesis; energy metabolism; DNA replication, transcription, translation, restriction and repair; cellular processes etc.

Need for translocators

Recent analysis of the *Arabidopsis thaliana* genome sequence has predicted that of the 25,489 nuclear genes that code for proteins, 4,467 enter the secretory pathway, 3,574 are targeted to the chloroplast and 2,897 are targeted to the mitochondria (Initiative, 2000). Prediction of signal peptides was done using default

settings on TargetP prediction program (http://www.cbs.dtu.dk/services/TargerP/). For a cell to function properly, it must direct the proteins synthesized in the cytoplasm to their specific organelles. Most proteins that are transported to the different organelles have one or more targeting domains that act as recognition sequences for a specific organelle. Most proteins consist of hydrophilic regions and so cannot pass easily through the hydrophobic core of the lipid bilayer. Translocation involves a proteinaceous channel through which proteins get translocated across the membrane (Chua & Schmidt, 1978; Jensen & Kinnally, 1997; Plath et al., 1998). The process of protein transport across any membrane involves several components. Targeting domains are usually located at the N-terminal end of the protein, e.g. precursor of small subunit of Rubisco (prSSU). An exception to this rule is the precursor of phytohemagglutinin whose targeting sequence is present at the C-terminus, and is targeted to the endoplasmic reticulum (Buchanan et al., 2000). Specific cellular machinery at the organelle membrane interacts with this information and translocates the protein into the proper compartment. Each compartment and membrane system requires a different targeting domain and sorting machinery. Although the targeting domain is essential for protein sorting and transport, it is usually not a part of the active protein. Proteases present in the target compartment remove the targeting domain to create a functional mature protein.

Targeting signals

The signal sequences are called "signal peptides" in proteins that are targeted to the endoplasmic reticulum, "pre-sequences" in proteins that are transported to the mitochondria, "nuclear localization signals (NLS)" in proteins that are transported to

the nucleus and "transit peptides" in proteins that are targeted to the chloroplasts (Buchanan et al., 2000; Schatz & Dobberstein, 1996). Mitochondrial presequences are rich in basic amino acids, generally lack acidic amino acids and can form an amphiphilic α-helix (Roise et al., 1988; von Heijne, 1986). The stromal targeting domain of the chloroplast transit peptides on the other hand are neither strongly basic nor amphiphilic and their "distinguishing features" are still unresolved. There are also composite bipartite signal sequences in the chloroplast transit peptide that act in tandem, for example in the precursor of plastocyanin there are two targeting domains. Initially, the protein is targeted from the cytoplasm to the chloroplast stroma where the transit peptide is cleaved by the stromal processing peptidase (SPP) and then the protein uses the second targeting sequence, which is like the signal peptide, to enter the thylakoids (Hageman et al., 1986). The entry of the protein into the thylakoids utilizes a variety of pathways (reviewed in Keegstra & Cline, 1999). Hence, the chloroplasts have at least 6 destinations for any protein that is targeted to the chloroplast: the outer membrane, the inner membrane space, the inner membrane, the stroma, the thylakoid membrane and the thylakoid lumen as shown in Figure 1-1b. Thus, the transport of proteins into and within the chloroplast is extremely complex and intriguing.

PROTEIN IMPORT INTO THE CHLOROPLAST

Requirements and stages of protein import

The process of protein import into the chloroplast is believed to be initiated soon after the newly synthesized protein emerges from the ribosome. Unlike the transport of precursors to the endoplasmic reticulum that are translocated both co-

translationaly and post-translationaly, the precursors that are targeted to the chloroplast are translocated only post-translationaly (Robinson & Austen, 1987). Several components are required for protein transport into chloroplast. Among them are the *cis*-acting components that include:

1. An N-terminal sequence which acts as a recognition domain for the protein to be recognized by the chloroplast.

2. Soluble factors that act as chaperones and guide the precursor to the chloroplast for example Com70 and 14-3-3 proteins form the 'guidance complex' and guide the protein to the translocon at the chloroplast outer envelope as indicated in Figure 1-2.

3. An alternative pathway, which does not involve the 'guidance complex', involves the chloroplast membrane lipids. Lipids at the membrane of the chloroplast provide a suitable media for the interaction of the precursor with the organelle. Specific lipids like MGDG (mono galactosyl diacyl glycerol), DGDG (digalactosyl diacyl glycerol) and SL (sulpholipids) are believed to play a significant role in transit peptide recognition and precursor import.

4. Usually a receptor protein at the membrane recognizes the pre-protein due to the N-terminal recognition domain. This process is usually energy dependent (in the form of <100μM nucleotide triphosphate (NTP). Several proteins of the translocon at the outer envelope membrane of chloroplasts (Toc). There are three major family of Toc proteins: Toc86/159, Toc120 and Toc132 form the first family, Toc34 and Toc33 form the second family and Toc64 may be forms the third family of Toc receptor proteins.

The *trans*-acting factors include:

1. After engaging with the receptor the precursor then passes through a translocation channel consisting of Toc75 at the outer membrane and the components of the translocon at the inner envelope membrane of chloroplast (Tic), namely, Tic110, Tic22, and Tic20. This process is also energy (ATP) dependent (>1mM).

2. The precursor is then with the help of soluble factors (Cpn60, CSS1 and ClpC) pulled across the membrane in an energy (ATP) dependent fashion.

Evidence for the general import pathway

The process of import of proteins into chloroplasts has been reviewed extensively by various researchers (Archer & Keegstra, 1990; Chen & Schnell, 1999; Gray & Row, 1995; Keegstra et al., 1995; Lubeck et al., 1997; May & Soll, 1999; Schleiff & Soll, 2000; Schnell, 1995). A schematic of the current working model is illustrated in Figure 1-2. The major route for the precursor protein's entry into the plastids is through the general import pathway consisting of a translocon at the outer envelope membrane of chloroplasts called the Toc complex and a translocon at the inner envelope membrane of chloroplasts called the Tic complex.

Several lines of evidence suggest that there is only one general import pathway for the entry of precursors into chloroplasts.

(1) The use of Competitiors like peptides and precursors gave the initial evidence for this. The 20 amino acid synthetic peptides corresponding to the transit peptide of small subunit of Rubisco (SStp) could inhibit the import of several

Figure 1-2. A schematic illustration of precursor import into chloroplast via the general import pathway. The precursor protein either directly interacts with the chloroplast membrane lipids or when its transit peptide is phosphorylated at the serine residue it binds to the 14-3-3 class of proteins and that mediates the precursor interaction with the chloroplast. The interaction of the transit peptide with specific lipids in the chloroplast membrane perturbs the lipid membrane and may lead to a change in the transit peptide structure that influences its recognition by the Toc 86/159 receptor. In the presence of NTP the precursor enters the translocon consisting of Toc86/159, Toc34, Toc75. Com70 is also believed to play role in this initial interaction. Then in the presence of higher levels of ATP the precursor translocates via the outer and the inner membrane translocon. The inner membrane translocon consists of Tic110, Tic55, Tic22 and Tic 20 in the membrane and soluble factors like CSS1, Cpn60 and ClpC where the soluble factors may act as molecular motors.

Placeholder

REPLACE IMAGE

precursors. The precursor of the light-harvesting chlorophyll a/b-binding protein (pLHCP), the precursor of ferredoxin (prFd) and the precursor of plastocyanin (pPC) were inhibited to a similar extent as the precursor of the small subunit of Rubisco (prSSU) in *in vitro* import competitions (Perry et al., 1991). This indicated that all these precursors used a common pathway for their entry into the chloroplast. Recently, the import of precursor of small subunit of Rubisco (prSSU), ferredoxin NADP+ reductase (pFNR), porphobilinogen deaminase, LHCP, Rieske iron-sulfur protein (pRieske protein), ferrochelatse, the γ subunit of ATP synthase, plastocyanin and phosphate translocator were analyzed (Row & Gray, 2001). It was found that all of these precursors used the same import pathway as the other two precursors: the precursor of the 33kDa oxygen-evolving complex (pOE33) and the (pLHCP). The IC_{50} of these proteins required to inhibit the import of other precursors was found to range from 0.2μM to 4.9 μM. It is interesting to note that although all of these proteins are targeted to the chloroplasts they do not have the same destination inside the chloroplast. prSSU, pFNR and porphobilinogen deaminase are destined to the stroma; pLHCP, pRieske, ferrochelatase and γ subunit of ATP synthase protein are targeted to the thylakoid membrane and plastocyanin and phosphate translocator are targeted to the thylakoid lumen.

(2) Another study by the same researchers was done in order to identify functionally important amino acid residues in the Toc apparatus (Row & Gray, 2001). The chloroplasts were incubated with several amino acid modifying reagents and then tested for chloroplast import activity. Most of the amino acid modifying agents inhibited the import of several precursors and so it was concluded that there was only one general import pathway in chloroplasts. In spite of the fact that

proteins are targeted to multiple compartments in the chloroplast, all the evidence indicates that proteins use the general import pathway to translocate into the chloroplast.

(3) The precursor protein has been cross-linked to the same components of the translocon that form the general import pathway (Perry & Keegstra, 1994; Ma et al., 1996; Kouranov & Schnell, 1997; Nielsen et al., 1997; Subramanian et al., 2001).

(4) Mutant *Arabidopsis* plants that are deficient in Toc86/159 or Toc34 have been identified and are found defective in chloroplast biogenesis indicating that protein import into chloroplasts is a limiting process in chloroplast biogenesis (Bauer et al., 2000; Jarvis et al., 2000). However since these plants could import certain proteins it indicates the existence of alternative routes.

While most precursors use the general import pathway for their entry into chloroplasts, most components of the translocon, Toc86/159, Toc34, Toc75 and Tic22, were found to use an energy dependent pathway that was different but yet to be identified (Chen & Schnell, 1997; Hirsch et al., 1994; Kouranov et al., 1999; Li & Chen, 1997; Tranel & Keegstra, 1996). Moreover, recently a second site for chloroplast import specific for the cytosolic precursor of the NADPH:protochlorophyllide (Pchlide) oxidoreductase A (pPORA) has been identified in the chloroplasts isolated from barley, tobacco, *Arabidopsis*, oat, wheat, pea, bean and spinach (Reinbothe et al., 2000). Whether this site can import other proteins or a class of proteins yet remains to be identified.

The N-terminal transit peptide is considered necessary and sufficient for the translocation of nuclear-encoded precursors into chloroplasts (Keegstra, 1989; Keegstra & Bauerle, 1988; Keegstra et al., 1995). Although this is true for the most

part, the mature domain of some precursors has been shown to modulate the translocation efficiency (Dabney-Smith et al., 1999; Kavanagh et al., 1988; Lubben et al., 1989). The C-terminal deletions in the mature sequence of prSSU showed reduced efficiency in inhibiting the import of ^{35}S labeled full-length prSSU indicating the importance of the mature sequence in the precursor-chloroplast interaction (Dabney-Smith et al., 1999).

Structure and function of the chloroplast transit peptides

The recent sequence analysis using specialized searches of the *Arabidopsis thaliana* genome revealed that approximately 3,574 genes code for proteins that have putative transit peptides (Initiative, 2000). Chloroplast transit peptides are highly divergent in length, composition and organization. Although, the transit peptides lack primary sequence homology, it is believed that they use a common import pathway. It is intriguing as to how the chloroplast can recognize such a divergent set of proteins. Several researchers have suggested that multiple structural domains in the transit peptides are the key to the entry into the chloroplast (Pinnaduwage & Bruce, 1996; van't Hof et al., 1991; van't Hof et al., 1993; Wienk et al., 1999; Wienk & de Kruijff, 1999; Wienk et al., 2000). Chloroplast transit peptides have been predicted to form a random coil devoid of any secondary structure in an aqueous environment (von Heijne et al., 1991; von Heijne & Nishikawa, 1991). Although, rapid progress has been made to elucidate the transit peptide structure in membrane mimetic solvents most of the work has been done with algal transit peptides that are shorter and can be easily synthesized than the transit peptides in higher plants. Experimental evidence using circular dichroism and NMR have revealed that the

chloroplast transit peptides are indeed a random coil in an aqueous environment but possess an α-helical conformation in the presence of lipids or a lipid mimicking environment (van't Hof et al., 1991; van't Hof et al., 1993; Wienk et al., 1999; Wienk & de Kruijff, 1999). NMR studies on the ferredoxin transit peptides suggested that the N-terminus formed a α-helix and the C-terminus was unstructured while the NMR data on rubisco activase transit peptides suggested that the N-terminus was unstructured while the C-terminus formed a α-helix (reviewed in Bruce, 2000). Secondary structure formation in the presence of a lipid environment could lead a diverse variety of transit peptides to recognize the common Toc receptor. Several lines of evidence have suggested that the structure of the transit peptide was changed in the presence of a hexagonal II (H_{II}) phase forming lipid (Pinnaduwage & Bruce, 1996; van't Hof et al., 1991). Thus, lipid-induced secondary structures (one or more) could very well be the key to chloroplast recognition and translocation.

Analysis of the primary sequence of transit peptides revealed the presence of three major homology blocks (Karlin-Neumann & Tobin, 1986). Some preliminary experimental evidence suggested that there are certain conserved (H(P/G)H(R/K)) motifs or domains in the chloroplast transit peptides (Pilon et al., 1995). The importance of such motifs in protein recognition and translocation by the chloroplast has not been characterized in detail.

Only a few transit peptides have been analyzed y NMR and CD analysis, but certain general conclusions can be made regarding their structure. They are largely unstructured in aqueous environments but one or more regions of the transit peptide forms α-helices in membrane mimetic environments. Analysis of transit peptides by circular dichroism spectrometry suggests the presence of three distinct domains: an

uncharged N-terminus, a central domain lacking acidic residues but enriched in S/T and a C-terminal domain enriched in K/R which is predicted to form an amphiphilic β-strand. The helix-coil-helix might be a universal organization of transit peptides but the position and the degree of helicity of the transit peptides may vary significantly. The recent discovery of alternate Toc receptors Toc120 and Toc132 other than Toc86/159 leads one to believe that these proteins are present to either recognize the divergent transit peptides in the alternative routes of protein import into chloroplasts (Bauer et al., 2000).

Several cross-linking studies have revealed that the transit peptide region of the precursor protein interacts with several Toc components (Perry & Keegstra, 1994; Ma et al., 1996; Subramanian et al., 2001). The N-terminus of the SStp has been implicated in the interaction with Toc86/159 (Ma et al., 1996; Perry & Keegstra, 1994) and the C-terminus was found to have maximum lipid interaction (Pinnaduwage & Bruce, 1996). *In vivo* analysis of different domains of transit peptides revealed that removal of the N-terminus caused impaired import (Kindle & Lawrence, 1998; Lawrence & Kindle, 1997; Rensink et al., 1998). Transit peptides have been shown to interact with a member of the 14-3-3 class of molecular chaperones (May & Soll, 2000). It has been shown that the transit peptides have to be phosphorylated at S/T present in the central region of the transit peptides in order to be recognized by this class of chaperons (Muslin et al., 1996; Waegemann & Soll, 1996). However, as purified transit peptides and precursors can be translocated across the chloroplast membrane *in vitro* in the absence of this protein, it indicates that this is not an obligate requirement for protein import into chloroplasts (Perry &

Keegstra, 1994; Ma et al., 1996; Kouranov & Schnell, 1997; Nielsen et al., 1997; Subramanian et al., 2001).

COMPONENTS OF THE TRANSLOCON

Cis-acting components

Soluble factors

It has been observed that cytosolic factors bind to the transit peptide of the precursor protein and direct it to the chloroplasts (Kourtz & Ko, 1997; Waegemann et al., 1990). It was also observed that the chloroplast transit peptides are phosphorylated by a cytosolic serine/threonine protein kinase (Waegemann & Soll, 1996). Hence, it was proposed that phosphorylation of the transit peptide leads to the formation of a hetero-oligomeric cytosolic 'guidance complex' consisting of 14-3-3 proteins, cytosolic Hsp70 and the precursor proteins. (reviewed in May & Soll, 1999; Schleiff & Soll, 2000). This phosphorylated precursor complex is guided to the chloroplast Toc apparatus where the precursor is believed to be dephosphorylated by a chloroplast outer membrane phosphorylase to make the precursor competent for import. Chloroplasts are known to contain at least four Hsp70s: one associated with the outer envelope (Ko et al., 1992), one in the inner membrane space (Marshall et al., 1990; Waegemann & Soll, 1991) and two in the stroma (Marshall et al., 1990). Com70 is a cytosolic Hsp70 homologue but it is found associated with the outer membrane of the chloroplast during precursor binding and translocation (Kourtz & Ko, 1997; Wu et al., 1994). Toc64 was found to be co-purified with the components of the outer envelope translocon in sucrose density gradient centrifugation (Sohrt & Soll, 2000). It has been proposed to be the docking protein for the binding of the

cytosolic soluble factors that are essential for early interaction of the precursor protein with the chloroplast.

Envelope lipids

Lipids form the building blocks of all the membranes that surround the cells and the organelles. There are a variety of lipids found in bio-membranes and this suggests that their function is much more than just providing a hydrophobic barrier (Dowhan, 1997). Protein translocation is a universal phenomenon that takes place within a cell where the proteins cross the lipid barrier with the help of protein translocases. Biochemical characterization of the protein translocases not only revealed that they were composed of several individual protein components but also that lipids play an important role in the functioning of these translocases (de Kruijff, 1997). Examples of this are the bacterial Sec system, the Sec system present in the endoplasmic reticulum of eukaryotic cells, translocases at the thylakoid membrane etc.

Direct association of the transit peptide with the chloroplast membrane lipids is believed to mediate the early stages of chloroplast import. Several *in vitro* experiments have shown the transit peptide–lipid interaction using lipid monolayers or lipid vesicles (Chupin et al., 1994; Horniak et al., 1993; Pinnaduwage & Bruce, 1996; van't Hof & de Kruijff, 1995; van't Hof et al., 1991; van't Hof et al., 1993; Wienk et al., 2000). This step is believed to be NTP independent in which the precursor protein is bound to the Toc apparatus with low affinity and so this interaction is fairly reversible. This is probably the reason why it is almost impossible to trap this binding intermediate at this stage. Moreover, there is

evidence that lipid biosynthetic mutants that have defective MGDG or DGDG synthesis, or are deficient in fatty acid desaturase enzymes are defective in chloroplast biogenesis (Chen & Li, 1998; Dormann et al., 1995; Falcone et al., 1994; Jarvis et al., 2000). In one case, there is *in vivo* evidence that an *Arabidopsis* plant that was deficient in digalactosyldiacylglycerol was defective in protein import (Chen & Li, 1998). Lipids are believed to act as molecular chaperones in protein folding (Landau & Rosenbusch, 1996; Surrey & Jahnig, 1992). The α-helical protein bacteriorhodopsin was the first membrane protein to be crystallized in a detergent/lipid micelle in its native folded state (Landau & Rosenbusch, 1996; Pebay-Peyroula et al., 1997). The interaction of the hydrophobic proteins with lipids increases the solubility of folding intermediates and thus prevents protein aggregation promoting proper folding (von Heijne, 1997). Although, much of the evidence on lipid mediated protein folding is from *E.coli* a similar phenomenon could exist in the chloroplast as it is believed to have originated from the prokaryotes.

The chloroplast membrane is composed of some unique lipids like monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfolipid (SL) (reviewed in Bruce, 1998). The evidence for the role of lipids in protein translocation in chloroplasts comes from the fact that lipid-altering agents like Phopholipase C impair protein import (Kerber & Soll, 1992). Moreover, a mutant *Arabidopsis* plant deficient in DGDG showed impaired protein import (Chen & Li, 1998). MGDG is a lipid that does not form a bilayer but forms a reversed hexagonal phase (H_{II}) and such lipids have been found to facilitate precursor–artificial membrane interaction (Chupin et al., 1994; Pinnaduwage & Bruce, 1996;

van't Hof et al., 1991; van't Hof et al., 1993). The transit peptides of the precursor proteins are unstructured in an aqueous environment, but are seen to adopt an α-helical conformation in one or more regions in the presence of a lipid environment (Chupin et al., 1994; Horniak et al., 1993; Krimm et al., 1999; Wienk et al., 1999).

Anionic lipids like phosphatidic acid and phosphatidyl glycerol have been implicated in the electrostatic interaction of pre-sequences and transit peptides and the membrane. Monolayer experiments have shown that anionic lipids like (phosphatidylglycerol) PG and SL interact significantly with the basic residues present in the transit peptide (van't Hof & de Kruijff, 1995; van't Hof & de Kruijff, 1995). These results suggest that the transit peptide sticks to the anionic lipids on the chloroplast membrane initially due to the presence of basic amino acids and then the galactose head group of the galactolipids interact with the hydroxylated amino acids in the transit peptide. The hydroxylated amino acids could also interact with the water molecules present in the membrane interface and cause a reduction in the hydration shell of the galactolipids. This could result in non-bilayer formation of MGDG and allow the transit peptide to import into the envelope (Bruce, 1998). The lipid interaction analysis of the N- and C-terminal regions of the small subunit transit peptide (SStp) revealed that the 20 amino acid of the C-terminus had maximum interaction with outer membrane-mimicking liposomes while the N-terminus was only half as active as the C-terminus (Pinnaduwage & Bruce, 1996). However, the N-terminus of the ferredoxin transit peptide showed maximum interaction with MGDG lipids while the C-terminus showed maximum interaction with anionic lipids (Pilon et al., 1995). This indicates that maybe both the N- and the C-terminus of the transit peptides contribute to the lipid interaction.

Contact sites

Contact sites are believed to be a major site for protein import between the two-mitochondrial membranes (Hartl et al., 1987) Electron microscopic studies have identified 100 to 500 translocation sites in the mitochondria (Rassow et al., 1990; Vestweber & Schatz, 1988). Immunocytological studies have localized import receptors like Tom19 (mitochondrial outer membrane protein, 19kDa) and Tom72 (mitochondrial outer membrane protein, 72kDa) in the contact sites (Pon et al., 1989). It was found that the mitochondrial contact sites are formed only when precursors simultaneously associate with both the complexes (Horst et al., 1995). Recently, analysis of fluorescently labeled DHFR pre-protein binding to the Tom (translocation apparatus at the outer membrane of the mitochondria) complex was done using fluorescence correlation spectroscopy. It was observed that the equilibrium for binding of DHFR to the Tom holo complex (with receptors Tom20 and Tom70) was 1.4 nM, while that for the core complex (without receptors Tom20 and Tom70) was 3.4 nM, indicating that the precursor was stabilized in the presence of the receptors. Further analysis of the translocation complex by size exclusion chromatography and SDS-PAGE revealed that 8 or 9 precursors were bound per Tom core complex (Stan et al., 2000).

Immunoelectron microscopic studies have shown the presence of contact sites between the inner and outer membrane in chloroplast (Dobberstein et al., 1977). Immunolocalizations done using anti-import complex antibodies showed that the import complexes were concentrated in the region around the contact sites. Chloroplasts were found to have ~3000 translocation sites (Friedman & Keegstra, 1989). The *Chlymadomonas* chloroplasts were found to have 8.1 +/- 4.1 $\times$ 10^4

binding sites per chloroplasts (Su et al., 1992). The precursors for the chloroplast that are synthesized in the cytoplasm also have to interact with both the inner and outer envelope membranes of the chloroplast. Protein translocation has been implicated to be localized to contact sites in which both the outer and inner envelope membranes are in close proximity (Pain et al., 1990; Schnell & Blobel, 1993; Schnell et al., 1990). Label transfer cross-linking experiments revealed that the precursors and transit peptides resolved with mixed envelope fraction on a sucrose gradient (Perry & Keegstra, 1994; Subramanian et al., 2001). It is unclear whether these contact sites are permanent structures or whether these are transiently formed during protein translocation. When chloroplast containing precursor proteins like prOE23 (precursor to the oxygen evolving complex) were trapped at an early stage of transport by ATP limitation and treated with chemical cross-linkers, large complexes containing the radiolabeled precursor prOE23 were observed (Akita et al., 1997). Immunoprecipitations of the cross-linked complex with αTic110 antisera showed that a part of Tic110 was present on the outer surface of the inner envelope. Immunoprecipitations using αClpC (a member of hsp100 family) gave results similar to Tic110, indicating that ClpC is a part of the translocation complex involved in protein import into chloroplast, possibly as a molecular chaperone. This indicated that at least some portion of the translocation complex involved both the outer and inner membrane and so may be it was present in the region of contact sites (Akita et al., 1997; Scott & Theg, 1996). The accumulated evidence supports a model where translocation thus occurs concurrently across the two membranes.

Proteins of the translocon - General Import Receptor, Toc86 or Toc159

The use of a non-chloroplast penetrating enzyme like thermolysin revealed that there are several proteins associated with the outer membrane of chloroplasts (Cline et al., 1984; Joyard et al., 1983). Chloroplasts treated with thermolysin could not support protein import *in vitro,* indicating that the proteinaceous components of the outer membrane of chloroplasts play a role in protein translocation (Perry & Keegstra, 1994). To identify some of these proteins, label transfer cross-linking studies were performed using a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase (prSSU) that was cross-linked at an early stage of the transport process. Two envelope proteins were cross-linked, an 86 kDa protein and a 75 kDa protein and both resolved with the outer membrane fraction in a sucrose gradient but only the 75 kDa protein resolved with the contact sites. Hence, it was concluded that the 86 kDa protein was the receptor while the 75 kDa protein was the translocation channel. Another study using label transfer cross-linkers showed that although the precursors could cross-link in the absence of ATP, addition of ATP or GTP enhanced the binding (Ma et al., 1996). Another outer membrane protein, Toc34 has been shown to be cross-linked to the precursor protein in the early stages of import (Kouranov & Schnell, 1997; Seedorf et al., 1995). Both Toc34 and Toc86/159 are GTPases and Toc34 was believed to function as a regulatory protein during chloroplast import. However, recently Toc34 has been proposed to be a receptor, and was shown to be regulated by both GTP and phosphorylation (Sveshnikova et al., 2000).

Recently, two separate laboratories have demonstrated that Toc86 is extremely susceptible to proteolysis and is in fact a 159 kDa protein (Bolter et al.,

1998; Chen et al., 2000) and hence now designated as Toc159. The 598 N-terminal amino acids are exposed to the cytosol and hence that domain is easily cleaved off as a result of proteolysis. This cytosolic domain was found to be not essential for the binding of the precursor to the chloroplast (Chen et al., 2000). Since this domain is extremely sensitive to proteolysis almost all the *in vitro* cross-linking studies have cross-linked only a 86kDa protein of the outer envelope. Several studies have shown that Toc86/159 is the receptor protein at the translocon (Bauer et al., 2000; Bolter et al., 1998; Caliebe et al., 1997; Chen et al., 2000; Kouranov & Schnell, 1997; Perry & Keegstra, 1994). The direct evidence for this comes from the study where antibodies against Toc86/159 blocked the binding of the pre-proteins to the chloroplast (Hirsch et al., 1994).

Recently, two related receptors AtToc120 and AtToc132 have been identified from *Arabidopsis thaliana* (Bauer et al., 2000). The two proteins have ~65% identity with each other in the GTP-binding domain and the C-terminal domain while the N-terminal acidic domains vary considerably in length and have only ~20% identity to AtToc86/159. The AtToc86/159 and psToc86/159 (*Pisum sativum*) have 48% overall homology and are 74% identical in the GTP binding domain and the C-terminus. The divergence in the N-terminal acidic domains among AtToc86/159, AtToc132 and AtToc120 makes it likely that these proteins have distinct roles. Acidic residues in the mitochondrial receptor have been implicated to play an important role in the mitochondrial pre-sequence recognition (Iwata & Nakai, 1998; Komiya et al., 1998; Lithgow et al., 1995; Pfanner et al., 1997). The "acid-chain" hypothesis proposes that the acidic patches of import components guide the import of positively charged mitochondrial pre-sequences (Komiya et al., 1998). A similar

mechanism could exist in the chloroplast since the chloroplast transit peptides have basic residues. It has been suggested that AtToc132 and AtToc120 might help maintain basal protein import and so constitute components of alternate Toc86/159-independent pathways. Further, T-DNA mutagenesis of AtToc86/159 revealed that it was essential for plastid biogenesis as the plastids development in these mutants was very rudimentary, indicating that Toc86/159 was a important component of the general import pathway (Bauer et al., 2000).

Regulator/Receptor, Toc34

Toc34 was first isolated and characterized from a cDNA clone (Kessler et al., 1994) and was found to be closely associated with Toc75 (Seedorf et al., 1995). Toc34 has a single transmembrane domain at its C-terminus and the N-terminus is exposed to the cytosol. It also like Toc86/159 has a GTP binding site and is found cross-linked to pre-proteins during the binding stage. Toc34 was found to interact not only with the transit sequence but also with the mature sequence of the pre-protein and so it was proposed to be more of a regulatory protein rather than a receptor (Kouranov & Schnell, 1997). Toc34 has now been proposed to act as a receptor that is regulated by both GTP and phosphorylation (Sveshnikova et al., 2000). Toc34 is found to interact with precursors in its GTP-bound state but not in its GDP bound state. GTP binding itself was regulated by phosphorylation of Toc34. A mutant deficient in Toc34 (*ppi1*) was not lethal but had a pale green phenotype (Jarvis et al., 1998). This suggested an alternate pathway for import that does not require Toc34 or there was another homologue of Toc34 that could perform the function albeit not so efficiently. Recently, two Toc34 homologues were identified

from *Arabidopsis* namely AtToc33 and AtToc34 (Gutensohn et al., 2000). Both these proteins although possessed structural similarities showed differential pattern of expression and function. Two homologues of Toc34 have also been isolated from *Zea mays* ZmToc34-1 and ZmToc34-2 that show 95% identity to each other and about 60% identity to other Toc34 proteins (Hirohashi & Nakai, 2000). However, no homologues of Toc34 have been isolated from *Pisum sativum.*

The chaperone docking protein of the outer membrane, Toc64

A 64 kDa protein was found to co-purify with the components of the translocon on a sucrose density gradient (Sohrt & Soll, 2000). Using a 0 Å cross-linker this protein was cross-linked to a high molecular weight species containing both the Toc and the Tic complex in the presence of ^{35}S prSSU. Toc64 was found to contain three tetratricopeptide (TPR) repeat motifs that were found to be exposed to the cytosol. These motifs have been found to play a very important role in protein-protein interaction. Many components of the translocation machinery have also been found to have these TPR motifs for example the translocation components of the outer membrane of mitochondria like Tom70 has seven such repeats (Komiya et al., 1997). Hence, Toc64 was proposed to be the cytosolic soluble factor docking protein. Analysis of Arabidopsis genome sequence revealed the presence of three homologues of Toc64 in chromosomes I, III and V; AtToc64-I, AtToc64-III, AtToc64-V (Jackson-Constan & Keegstra, 2001).

The translocation channel, Toc75

Almost all the early import intermediates were cross-linked to Toc75 (Akita et al., 1997; Kouranov & Schnell, 1997; Ma et al., 1996; Nielsen et al., 1997; Perry & Keegstra, 1994; Subramanian et al., 2001). Toc 75 was found to be a voltage sensitive ion conductor that was regulated by the presence of an authentic chloroplast precursor protein (Hinnah et al., 1997). Whether Toc 75 is the only outer membrane constituent of this protein-conducting channel is yet to be determined. Electrophysiological analysis indicated that for the precursor to be translocated across chloroplasts it had to be in an unfolded and extended conformation in order to be able to go through the narrow translocation pore of diameter 8-9 Å (Hinnah et al., 1997). This size was less than the mitochondrial protein-conducting channel or the endoplasmic reticulum protein-conducting channel (Hanein et al., 1996; Kunkele et al., 1998; Romisch, 1999). Moreover, cross-linking experiments indicated that more than one protein (Toc86/159, Toc34, Toc75) of the translocon was cross-linked to the precursor protein, which implied that these proteins directly participated in the process of protein translocation across the chloroplast membrane. Analysis of Arabidopsis genome sequence revealed the presence of three homologues of Toc75 in chromosomes I, III and IV; AtToc75-I, AtToc75-III, AtToc75-IV (Jackson-Constan & Keegstra, 2001).

Several other Toc proteins have been identified over the years, Toc 6, Toc 14, Toc40, Toc70 (Fischer et al., 1994; Ko et al., 1992; Ko et al., 1995; Ko & Ko, 1999; Li & Chen, 1996; Li et al., 1991; Salamon & Tollin, 1992; Seedorf et al., 1995) but none have been characterized in great detail like Toc86/159, Toc75 and Toc34.

Trans-acting components

Components in the inner membrane - The Tic apparatus

The final step involves higher levels of ATP (1-3 mM) in which the precursor protein translocates across both the membranes simultaneously. Translocation across the chloroplast membrane is believed to occur in these contact sites. Label transfer cross-linking experiments have revealed that during the later stages of import the precursor protein is in contact with components of the Tic machinery (Kouranov & Schnell, 1997; Nielsen et al., 1997; Subramanian et al., 2001).

Three inner membrane proteins Tic110, Tic20 and Tic22 have been found to be members of the Toc-Tic translocation complex (Kouranov & Schnell, 1997; Nielsen et al., 1997). Tic110 has been observed to be associated with the pre-protein in later stages of import. Tic110 was the first inner membrane protein to be implicated in protein translocation but the translocation channel in the inner membrane is believed to be formed by Tic20 and Tic22. The large hydrophilic domain (> 90 kDa) of Tic110 was found to be exposed in the stromal compartment (Jackson et al., 1998). Tic110 was found to co-immunoprecipitate with two stromal proteins ClpC (Hsp100 homologue) (Nielsen et al., 1997) and Cpn60 (GroEL homologue) (Cloney et al., 1992; Kessler & Blobel, 1996; Madueno et al., 1989; Tsugeki & Nishimura, 1993) under low ATP conditions. Hence, it has been suggested that Tic110 acts as a stromal chaperone-docking site for the folding of the pre-proteins once they emerge from the translocon (Chen & Schnell, 1999).

Tic20 and Tic22 have been identified as essential components of the core of the Tic apparatus (Kouranov et al., 1998; Kouranov & Schnell, 1997; Kouranov et al., 1999). Tic22 is a 22kDa protein located in the inner membrane space between

the outer and inner membranes and is peripherally associated with the outer face of the inner membrane (Kouranov et al., 1998). Thus, it has been proposed that Tic22 would be the first Tic protein to contact the pre-protein as it emerges from the Toc. Tic22 is synthesized in the cytoplasm with a 50-amino acid N-terminal pre-sequence but was observed not to follow the general import pathway, while Tic20 is a 20 kDa integral membrane protein in the inner envelope (Kouranov et al., 1999). Tic20 thus becomes an excellent candidate to form the protein-conducting channel in the inner membrane (Kouranov et al., 1998). Another protein of 52 kDa was found to co-fractionate with the trapped precursor protein in association with the proteins of the translocon (Toc86/159, Toc75, Toc34 and Tic110), on a two-dimensional native Blue Native PAGE gel (Caliebe et al., 1997). This 52 kDa protein was called Tic55 and was shown to have a Rieske-type iron-sulfur cluster and a mononuclear iron-sulfur binding site but there is no evidence that it participates in the import process (Caliebe et al., 1997). Tic40 is covalently linked to Tic110 by a disulfide bond under oxidizing conditions, indicating its close physical proximity to an established translocon component (Stahl et al., 1999). The Tic40 is synthesized with an N-terminal cleavable chloroplast targeting signal in the cytosol as a precursor and imported into the organelle via the general import pathway. Tic40 has a domain for the binding of CSS1 (chloroplast stress seventy 1).

Soluble factors in the stroma—The molecular motor

Molecular chaperones of the Hsp70 and chaperonin families are basic constituents of the cellular machinery that mediates protein folding. Hsp70 chaperones cooperate with DnaJ and GrpE homologues to ensure a productive

folding cycle. Precursor proteins destined for the chloroplasts traverse inner and outer organelle membranes in an extended conformation. Translocation events are therefore integrally coupled to the processes of protein unfolding in the cytosol and protein refolding in the stroma. To successfully import proteins from the cytoplasm into chloroplasts, cells have recruited a variety of molecular chaperone systems and folding catalysts.

Soluble factors in the stroma have been identified to be associated with the translocating precursor. Cpn60 a GroEL homologue co-immunoprecipitated with the precursor using Tic110 antibody (Kessler & Blobel, 1996). ClpC a Hsp100 homologue co-immunoprecipitated with translocating precursors (Caliebe et al., 1997; Nielsen et al., 1997). There are two models that have been proposed for the role of soluble factors in the translocation of pre-proteins: the Brownian ratchet model (Simon & Blobel, 1992) and the molecular motor model (Glick, 1995). According to the Brownian ratchet model, the precursor would be anchored to the stromal Hsp70 homologue and random thermal fluctuations would allow the protein to slowly move across the membrane, which allows a longer stretch of pre-protein to anchor to the Hsp70 homologue, and this process continues until all of the pre-protein is translocated. The molecular motor model also proposes that the pre-protein is anchored to a Hsp70 homologue but here the Hsp70 pulls the precursor proteins with repeated ATP hydrolysis, and with each molecule of ATP hydrolyzed more and more of the pre-protein is pulled into the stroma. Which of these is the functional model in the chloroplast membrane remains to be determined.

One of the two stromal Hsp70s has been cloned and is called CSS1 (Chloroplast Stress Seventy 1 (Marshall et al., 1990; Marshall & Keegstra, 1992).

Ferredoxin NADP+ reductase (FNR) imported into chloroplasts *in vitro* could be immunoprecipitated with antisera raised against GroEL from *E.coli*, which is a bacterial homologue of chaperonin 60 (Cpn60), in an ATP-dependent manner indicating that newly imported FNR interacts physically with homologues GroEL in chloroplasts (Tsugeki & Nishimura, 1993). These results suggest that Cpn60 in the chloroplasts might assist in the maturation of newly imported FNR in an ATP-dependent manner. ClpC was co-immunoprecipitated with both the outer and inner envelope membranes even in the absence of a precursor (Nielsen et al., 1997). A 350 kDa ClpP protease complex was found associated with the thylakoids in *Arabidopsis thaliana* (Peltier et al., 2001). The role of ClpC is still not clear although it has been implicated to play a role in ATP dependent protein turnover (Shanklin et al., 1995), while it has been suggested that Cpn60 promotes protein translocation across the inner envelope and also plays a role in post import protein folding (Kouranov et al., 1998).

Signal Processing Peptidase

The final step of import involves the cleavage of the transit peptide from the precursor protein. The signal processing peptidase (SPP) is capable of recognizing a number of precursors and removes the transit peptide from them creating the mature protein. Analysis of the amino acid specificity for the SPP in the vicinity of the processing site of 32 precursors that are targeted to the stroma indicated a loosely conserved consensus motif consisting of (V/I)-X-(A/C)$^{\downarrow}$A (Gavel & von Heijne, 1990). After cleavage of the transit peptide the mature protein is then folded into its functional form, which then completes the journey of the protein from its site of

synthesis in the cytoplasm to its destination in the chloroplast stroma. Proteins that are destined to the thylakoids are exported to thylakoids with the help of their thylakoid targeting domain.

EVIDENCE FOR AN ENDOSYMBIOTIC ORIGIN OF THE CHLOROPLASTS

Initially, it was proposed that there were five independent origins of the chloroplasts from five different photosynthetic bacteria, but now it is accepted that chloroplast originated only once (Cavalier-Smith, 2000; Kohler et al., 1997; Race et al., 1999). Comparison of the ribosomal RNA sequences from the organelles and prokaryotes suggested that they shared a common ancestor (Reumann & Keegstra, 1999). This supports the endosymbiont hypothesis that chloroplast probably evolved from prokaryotes that were engulfed by the proto-eukaryotic cells during evolution as shown in Figure 1-3. This evolutionary scheme also explains the present day architecture of cells and the presence of double membranes in chloroplast, mitochondria and nucleus (Cavalier-Smith, 2000). It is possible that the double-layered nucleus originated by deep invagination of the plasma membrane that finally engulfed the genetic material and so the eukaryotes have their genetic material enclosed in an organelle.

Analysis of the import machinery of the plastids reveals that several proteins of the chloroplast import machinery have been derived from organisms similar to the cyanobacterium *Synechocystis* (Heins et al., 1998; Heins & Soll, 1998; Reumann & Keegstra, 1999). Genes similar to the genes encoding the proteins, Toc75, Tic20 and Tic22, have been found in the cyanobactrerial genome of *Synechocystis sp.* (Chen &

Figure 1-3. Endosymbiotic origin of the plant cell. The top right of the diagram depicts the evolution of the nucleus in a eukaryotic cell from a prokarytotic cell. The lower part depicts the evolution of chloroplasts in a plant cell. A photosynthetic prokaryotic cell was engulfed by a eukaryotic cell during evolution and a photosynthetic plant cell was evolved.

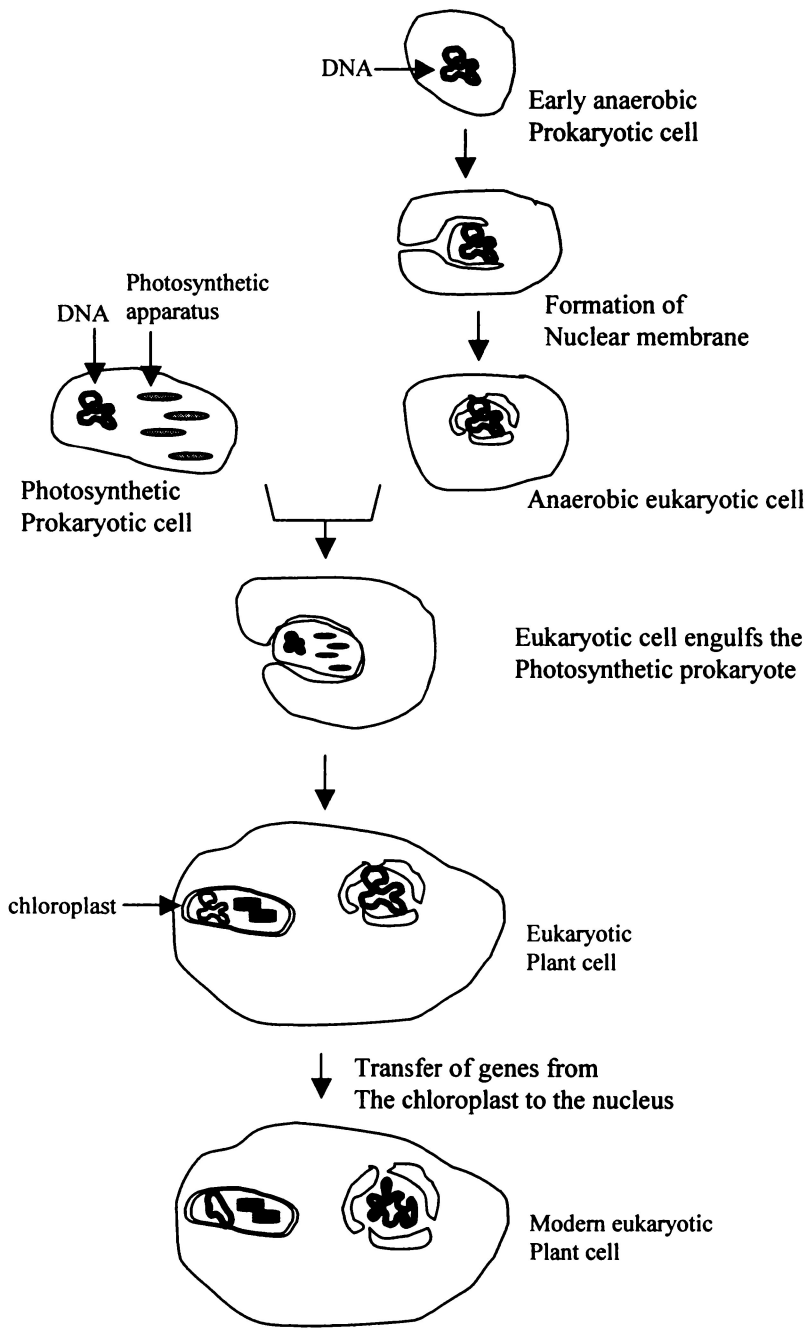

DNA
Early anaerobic
Prokaryotic cell
Formation of
Nuclear membrane
Photosynthetic
DNA apparatus
Photosynthetic
Prokaryotic cell
Anaerobic eukaryotic cell
Eukaryotic cell engulfs the
Photosynthetic prokaryote
chloroplast
Eukaryotic
Plant cell
Transfer of genes from
The chloroplast to the nucleus
Modern eukaryotic
Plant cell

Figure 1-3.

Schnell, 1999; Reumann et al., 1999). This photosynthetic cyanobacterium species is probably the origin of the modern endosymbiont. In support of this hypothesis it was found that SynToc75 had structural and functional homology to a family of prokaryotic ion channels (Bolter et al., 1998). The SynToc75 has also been shown to be related to a specific group of prokaryotic secretion channel proteins that transfer virulence factors such as hemolysins and adhesins across the outer membrane in gram negative bacteria. Some of the chloroplast import components (Toc86/159, Tic110 and Toc34) do not have any homologues in *Synechosystis* indicating that the chloroplast protein import apparatus is of a dual evolutionary origin (Reumann & Keegstra, 1999). Neither the chloroplast nor the mitochondria can exist outside of an eukaryotic cell. During evolution most of the DNA present in these organelles was transferred to the nuclear genome (reviewed in Blanchard & Lynch, 2000).

Recent analysis of the *Arabidopsis* genome has not only revealed high similarity with the pea chloroplast import apparatus but also multiple homologues of each translocation component have also been identified (Jackson-Constan & Keegstra, 2001). This suggests that the protein translocation machinery is conserved between *Arabidopsis* and *Pisum sativum,* thus making *Arabidopsis* a model system to study protein targeting in chloroplasts. Previous work has revealed that more than one of these homologues is expressed within the *Arabidopsis* cells (Bauer et al., 2000; Gutensohn et al., 2000; Jarvis et al., 1998). This implies that there might be multiple import pathways into the chloroplast, but as the stoichiometry of the translocation apparatus is unknown it is possible that all of them exist in the same complex.

INSERTION OF THE COMPONENTS OF THE TRANSLOCON—THE PARADOX

During evolution chloroplasts have relinquished most of their genes to the nucleus including the genes that encode the translocon. This is paradoxical as one would wonder how the translocon would be inserted into the chloroplast membrane in the absence of a translocation machinery. Analysis of the translocation of Toc86/159 revealed that it does not follow the general import pathway for translocation (Hirsch et al., 1994). Import of Toc86/159 was seen to require ATP and other protease-sensitive chloroplast surface components. prSSU did not compete with the import of Toc86/159 indicating that Toc86/159 used a different translocation pathway than prSSU. The processing peptidase for Toc86/159 is yet to be identified, but it is believed to be associated with the outer membrane as the N-terminus of Toc86/159 seems to be exposed to the cytosol.

Toc34 is synthesized in its mature form in the cytosol and it does not have a cleavable transit sequence. It has a hydrophobic transmembrane domain at the C-terminus, which targets the protein to the chloroplast outer membrane. This C-terminal region was found to be essential for its insertion into the outer envelope (Chen & Schnell, 1997; Li & Chen, 1997). Moreover, it was observed that it does not use the general import pathway for its insertion into the chloroplast outer envelope.

Like Toc86/159, Toc75 has N terminal pre-sequences for integration into the chloroplast envelope. Toc75 has a bipartite transit peptide consisting of two functional targeting domains (Tranel & Keegstra, 1996). The N-terminus of the transit peptide targets the protein to the stroma, whereas the C-terminus acts as a stop

transfer sequence and prevents its translocation. The mature Toc75 when sequenced by Edman degradation showed that the cleavage site was between the 35th and 36th amino acid from the N-terminus of the pre-sequence. This was consistent with the predicted stromal processing peptidase cleavage site (V/I)-X-(A/C)$^{\downarrow}$A, indicating that the precursor of Toc75 got translocated to the stroma, where the transit sequence was cleaved and then was integrated into the chloroplast outer membrane. It was also found that like the other outer envelope proteins, Toc75 also did not use the general import pathway (Tranel et al., 1995; Tranel & Keegstra, 1996).

Thus, it can be concluded that the components of the Toc machinery do not utilize the general import pathway to form the general import pathway.

ROLE OF NUCLEOTIDES IN CHLOROPLAST IMPORT

In the first stage of import, the precursor is believed to interact with the chloroplast membrane in an energy-independent and reversible fashion. In this stage the precursor proteins interact with the outer membrane lipids (reviewed in Bruce, 1998) and subsequently bind to the import receptor (Perry & Keegstra, 1994). As described earlier the binding of the precursor proteins to the Toc apparatus requires <100 μM ATP (Olsen & Keegstra, 1992; Olsen et al., 1989). The hydrolysis of ATP at this stage promotes the insertion of the precursor proteins into the protein-conducting channel, thus forming an early import intermediate. At this stage, although the precursor proteins are partially translocated they are susceptible to protease treatment (Ma et al., 1996; Nielsen et al., 1997). GTP is believed to support some binding but cannot substitute for ATP (Olsen & Keegstra, 1992; Olsen et al., 1989). The role of GTP in this process is still not understood. As described earlier

both Toc86/159 and Toc34 have GTP binding motifs. It was proposed that Toc34 might use GTP in the later stages of import and not in the early import-intermediate formation (Kouranov & Schnell, 1996). However, recently Young and co-workers proposed that the role of GTP was distinct from ATP and that both Toc86/159 and Toc34 used GTP in the early intermediate formation. They also observed that GTP did not play a role in later stages of chloroplast import (Young et al., 1999). Toc34 has been proposed to be regulated by both phosphorylation and GTP (Sveshnikova et al., 2000). According to this model dephosphorylation of inactive Toc34 makes it active and it binds GTP, which allows the phosphorylated precursor to bind to Toc34. Then, GTP/GDP exchange causes the precursor to release from Toc34 that moves the protein towards the translocation channel. The third and final stage of import across the chloroplast membrane requires higher levels of stroma ATP (1-3 mM) for the complete translocation of precursors across the chloroplast membrane (Fluegge & Hinz, 1986; Pain & Blobel, 1987; Theg et al., 1989). The Hsp70 homologues and other stromal factors present in the stroma also require ATP to pull the precursors that emerge from the translocon. (Glick, 1995). Thus, NTP is utilized at various stages of protein translocation across the chloroplast membrane.

RESEARCH OBJECTIVES

As stated earlier chloroplast transit peptides are necessary and sufficient for the targeting of proteins to the chloroplast membrane. Nevertheless, most of the studies using *in vitro* chloroplast import have been done with precursor proteins that consist of both the transit peptide, and the mature protein. Moreover, the "distinguishing features" of a chloroplast transit peptide are still not known. Hence,

my goal was to find out if there are special motifs or domains in the transit peptide that mediate its recognition by the chloroplast. In addition, by using just the transit peptide instead of a precursor I eliminated any contributions from the mature domain. I used the transit peptide of small subunit of Rubisco (SStp) for my studies. I have made C-terminal deletions and single amino acid changes in order to identify the specialized features of a chloroplast transit peptide. The transit peptide I used for my studies had dual N-terminal epitope tags. The His tag which allowed facile purification of the recombinant transit peptide and the S-tag allowed its easy non-radioactive detection. The several advantages of using such an epitope-tagged transit peptide are described in the following section.

Advantages of using an epitope-tagged transit peptide

Several researches have attempted to trap the early import intermediate but due to the robust activity of the translocation apparatus they have not been successful (Keegstra et al., 1989; Ma et al., 1996; Pain et al., 1990; Perry et al., 1991; Schnell et al., 1994; Smeekens et al., 1986). A variety of different approaches have been taken to trap the early translocation intermediate. One of which was to use a hybrid (pS/proteinA) construct that consisted of prSSU fused at its C-terminus to the IgG binding domain of staphylococcal protein A (Schnell & Blobel, 1993). Using this method Schnell and Blobel identified two early import intermediates that were susceptible to protease action indicating that they were bound to the outer membrane. One of these intermediates although partially translocated remains associated with the envelope membrane. They proposed that chloroplast translocation had two

distinct protein conducting channels in the outer and inner envelope membranes, each gated and opened by the distinct subdomain of the pS signal sequence.

Another study used avidin and biotinylated precursors to generate translocation intermediates that occupy functional transport sites (Froehlich & Keegstra, 1997). Chemically biotinylated prSSU modified at the cysteine residue was readily imported into chloroplasts, but addition of avidin resulted in the formation of an avidin-biotinylated precursor complex that could not be imported into the chloroplasts even when the precursors had already engaged the transport apparatus before avidin was added. On fractionation, the avidin-biotinylated precursor complex was found to be associated with the envelope membranes. Titration of transport sites with avidin-biotin precursor complex revealed that saturation was reached at 2,000 molecules/chloroplast. Import of radiolabeld prSSU after formation of the biotin-avidin complex reduced the import of prSSU by 35%.

In another study a chimeric precursor PCDHFR was used wherein the transit peptide of plastocyanin was fused to the mouse cytosolic dihydrofolate reductase (DHFR) (America et al., 1994). Addition of methotrexate, an artificial substrate for DHFR, slowed down the import of the precursor but did not inhibit import as seen in mitochondrial import. The addition of methotrexate impaired thylakoid import as the precursor accumulated in the stroma.

All these approaches used chimeric precursors wherein the transit peptide was always at the N-terminus, which is the normal placement of a transit peptide in any known natural precursor. Moreover, placing the transit peptide at the N-terminus makes the chimeric precursor behave more like a natural precursor and allows its rapid translocation. Blocking the N-terminus of the transit peptide by

adding an epitope tag at its N-terminus totally blocks its import into the chloroplasts, but does not prevent it from binding to the chloroplasts (Subramanian et al., 2001). This novel reagent has a dual epitope tag (His-S) at the N-terminus of SStp. The His-tag allows the rapid one step purification of the recombinant transit peptide made in *E.coli.* In addition, the S-tag allows highly sensitive detection of the transit peptide bound to the chloroplast translocation apparatus. There are several ways by which the S-tagged transit peptide can be detected as shown in table 1-1.

This approach allows sensitive and quantifiable evaluation of the *in vitro* binding of the transit peptide to the chloroplast. The S-tag/S-protein interaction has several other advantages. Some of these are as follows:

- ❖ Rapid, non-radioactive detection and measurement (hrs Vs days).
- ❖ Can be used for high throughput analysis (30 samples/hr).
- ❖ LSCM can provide a high resolution image of the distribution transit peptide bound to the Toc components.
- ❖ Analysis of a large number of chloroplasts by FACS (>10,000) gives a statistically significant measurement of the bound transit peptides.
- ❖ Results are obtained only from intact plastids as FACS can separate intact chloroplasts from broken ones.
- ❖ FACS has the ability to analyze chloroplasts at different age or development.
- ❖ Can be used to evaluate transport properties of non-green plastids.

Table 1-1. Different methods of detection for the S-tagged transit peptide.

Application	Reagent	Method of detection	Sensitivity
Qualitative detection	S-protein alkaline Alkaline phosphatase conjugate	Far-western blot	1 nmol
Qualitative Detection	S-protein-FITC	Fluorescence activated cell sorting (LSCM)	—
Quantitative detection	S-protein-FITC	Laser scanning confocal Microscopy (FACS)	50 FITC molecules/S-protein
Purification	S-protein-Agarose	—	—
Quantitative detection	S-protein + substrate poly C	Spectrometry (zymogram)	20 fmol
Quantitative detectin	S-protein + substrate RNA	Fluoremetry (FRET)	1 fmol

- ❖ Can be used to recover individual plastids via cell sorting (FACS) for molecular or biochemical analysis.

Using all my preliminary results obtained thus far, a model for the binding of the transit peptide to the chloroplast translocation apparatus has been proposed in Figure 1-4. In this model the C-terminal transit peptide initially interacts with the chloroplast membrane lipids, and the N-terminus of the transit peptide is exposed outside the chloroplast, as S-protein-FITC could be used to detect the chloroplast bound transit peptide. Alternatively, the transit peptide interacts with the Toc components with its N-terminus exposed outside the chloroplast. The latter was found to be case, as in binding reactions performed using chloroplasts that were pre-treated with thermolysin no binding of the transit peptide was observed, indicating the involvement of the proteinaceous components of the chloroplast outer envelope in the import mechanism. Moreover, label transfer cross-linking studies showed that the transit peptide was cross-linked to the components of the Toc apparatus (Toc86/159 and Toc75) (Subramanian et al., 2001).

SIGNIFICANCE OF PROTEIN IMPORT STUDIES IN CHLOROPLASTS

Protein transport is a central cellular process that occurs in all living organisms starting from a unicellular prokaryote to multicellular eukaryotes. It is important to identify the 'distinguishing features' of a transit peptide to completely understand the process of protein import into plastids. Over the years several successful attempts have been made to target unique gene products to plastids. Transgenic *Arabidopsis* plants were generated by introducing three bacterial gene

Figure 1-4. A schematic representation of His-S-SStp binding to the chloroplast. His-S-SStp, due to the presence of a dual His-S epitope tag has a charged N-terminus and so cannot translocate across the chloroplast membrane. Thus, His-S-SStp can be detected by using S-protein-FITC. His-S-SStp detected in such a manner is either bound to the chloroplast lipids or is bound to the Toc apparatus.

Placeholder

REPLACE IMAGE

products for PHB polymer synthesis (*phbA, B and C*) into the plastids, using the small subunit of Rubisco transit peptide (Nawrath et al., 1994). Although their result was ground breaking it was considered to be merely a stepping-stone due to several problems in the quality of the PHB polymer produced. Another important biotechnological advance was the production of genetically engineered rice rich in provitamin A (β-carotene) (Ye et al., 2000). These investigators successfully introduced the genes for encoding phytoene synthase, phytoene desaturase and lycopene β-cyclase into the rice genome. Improving the nutrient quality of rice and other crops in this manner would thus require further understanding of protein translocation into the plastids.

Understanding the mechanism of protein transport in plastids may also help us in the combat with certain human and animal associated diseases. Organisms in the phylum *Apicomplexa* have two highly conserved organellar genomes: one is of plastid origin, and the other is mitochondrial origin (Wilson et al., 1996; Wilson & Williamson, 1997). Parasites of the phylum *Apicomplexa* include many important human and veterinary pathogens such as *Plasmodium falciparum* (a pathogen that causes malaria), *Toxoplasma* (a pathogen responsible for a leading opportunistic infection associated with AIDS and congenital neurological birth defects), and *Eimeria* (an economically significant disease associated with poultry and cattle). Recent studies have identified an unusual organelle in these parasites: a plastid that appears to have been acquired by secondary endosymbiosis of a green alga. Although approximately half of the plastid-like DNA of *P. falciparum* has been sequenced to date, no function has yet been ascribed to this DNA or its putative

organelle. Understanding protein transport in plastids will thus be extremely useful in designing drugs that can be targeted to the plastid-like organelle in parasites.

Understanding the mechanism of the transit peptide mediated recognition of precursor proteins by the chloroplasts will facilitate the biotechnological advances in agro-biotechnology. The chloroplast genome has many features that make it an ideal site for the insertion of genes. Since the chloroplast DNA is multi-copy per cell it ensures high gene dosage and generally high expression levels for transgenes. Genes can be targeted to specific sites in the chloroplast unlike in plant nuclear transformations where due to "position effects" the transgene shows unpredictable expression.

SUMMARY

Identification and characterization of the chloroplast translocation apparatus has revealed the disparity between the mitochondrial and chloroplast translocation apparatus. Both systems use cleavable recognition sequences at the N-terminus of the precursor proteins, but the Toc-Tic complexes are very different from Tom-Tim complexes. Thus, this indicates that the endosymbiotic acquisition of chloroplast and mitochondria by a modern eukaryotic cell was from two distinct origins. The use of *in vivo* and *in vitro* analysis to study protein import into the chloroplast provides a powerful tool for understanding the process of protein targeting into the chloroplast. This information would be very useful in generating genetically improved crops in the future.

Chapter 2

A novel technique to identify the chloroplast bound transit peptides

ABSTRACT

Chloroplast transit peptides are necessary and sufficient for the targeting and translocation of precursor proteins across the chloroplast envelope. However, the mechanism by which transit peptides engage the translocation apparatus has not been investigated. To analyze this interaction, I have developed a novel epitope-tagged transit peptide derived from the precursor of the small subunit of pea Rubisco. The recombinant transit peptide, His-S-SStp, contains a removable dual-epitope tag, His-S, at its N-terminus that permits both rapid purification via immobilized metal affinity chromatography and detection by blotting, flow cytometry, and laser-scanning confocal microscopy. Unlike other chimeric precursors, which place the passenger protein C-terminal to the transit peptide, His-S-SStp bound to the translocation apparatus yet did not translocate across the chloroplast envelope. This early translocation intermediate allowed non-radioactive detection using fluorescent and chemiluminescent reporters. The physiological relevance of this interaction was confirmed by protein import competitions, sensitivity to pre- and post-import thermolysin treatment, photochemical cross-linking, and organelle fractionation. The interaction was specific for the transit peptide since His-S alone did not engage the chloroplast translocation apparatus. Quantitation of the bound transit peptide was determined by flow cytometry, showing saturation of binding yet only slight ATP-dependence. Addition of GTP showed inhibition of the binding of His-S-SS-tp to the chloroplasts indicating an involvement of GTP in the formation of this early

translocation intermediate. In addition, direct visualization of His-S-SStp and Toc75 by confocal microscopy revealed a patch-like labeling, suggesting a coordinate localization to discrete regions on the chloroplast envelope. These findings represent the first direct visualization of a transit peptide interacting with the chloroplast translocation apparatus. Furthermore, identification of a chloroplast-binding intermediate may provide a novel tool to dissect interactions between a transit peptide and the chloroplast translocation apparatus.

INTRODUCTION

The post-translational targeting and translocation of proteins to eukaryotic organelles involves both specific targeting information in the transported protein and specific translocation machinery in the target membrane (Schatz & Dobberstein, 1996). The majority of chloroplast-destined proteins are nuclear encoded and synthesized in the cytosol as higher molecular weight precursors containing an N-terminal extension known as a transit peptide, which is both necessary and sufficient to direct the successful translocation of the passenger protein (reviewed in Bruce & Keegstra, 1994). The mechanism by which proteins are targeted to the chloroplast has been largely determined by careful analysis of *in vitro* import assays using purified chloroplasts. The chloroplast translocation machinery consists of discrete components in both envelope membranes, the Toc components (Kessler et al., 1994; Ma et al., 1996; Schnell et al., 1997; Schnell et al., 1994; Soll & Alefsen, 1993) and the Tic components (Kouranov et al., 1998; Schnell, 1995). Translocation is believed to occur at specialized contact sites where these two complexes function

coordinately to move proteins across both membranes in a single step (Schnell & Blobel, 1993; Schnell et al., 1990).

Although early reports have demonstrated that precursors directly interact with individual components of the two translocators, mechanistic details have been hampered by the inability to trap an early translocation intermediate (Keegstra, 1989; Ma et al., 1996; Pain et al., 1990; Perry et al., 1991; Schnell et al., 1994; Smeekens et al., 1986). Despite several attempts to develop a chimeric chloroplast precursor that arrests at a specific step during translocation (America et al., 1994; Froehlich & Keegstra, 1997; Schnell & Blobel, 1993), a true translocation intermediate has not been identified. In each of these previous studies, a fusion partner was placed C-terminal to the transit peptide, which mimics the normal placement of the transit peptide. Unfortunately, however, these efforts were foiled by the unusually robust activity of the chloroplast translocator, resulting in complete translocation of the chimeric precursor into the stroma (America et al., 1994).

In contrast, we have created a novel reagent that places a dual-epitope tag (His-S) at the N-terminus of the transit peptide (SStp) derived from the pea Rubisco small subunit precursor (prSSU). These two epitope tags permit facile purification of the fusion protein from *E.coli* via the His-tag, and a highly sensitive, specific means of detection via interaction of the S-protein with the S-tag (Kim & Raines, 1993). The S-tag/S-protein interaction may be utilized in a multitude of detection assays including RNase enzymatic assays, fluorescent detection, and chemiluminescent blotting. Placement of the dual epitope introduces 21 charged residues at the N-terminus of SStp, however, which alters the proposed domain structure of transit peptides (von Heijne et al., 1991; von Heijne et al., 1989). Subsequent to successful

binding at the chloroplast surface, these charged residues potentially prevent the transit peptide from productively interacting with one or more components of the translocation apparatus, thereby inhibiting translocation across one or both of the envelope membranes.

We have begun to characterize the His-S-SStp fusion protein as a novel reagent for visualizing and quantifying the transit peptide bound to the translocation apparatus of intact organelles. Using an S-protein/FITC conjugate, we can detect the transit peptide by both laser-scanning confocal microscopy (LSCM) and flow cytometry. These two methodologies in combination provide both high-resolution spatial information about the distribution of the bound transit peptide and a statistically significant measurement of the total number of transit peptides bound, due to the large numbers of chloroplasts analyzed. More traditional techniques such as far-Western blotting, organelle fractionation and photochemical cross-linking were used to verify these results. In addition, detailed studies of the interaction between the transit peptide and the translocation apparatus like the binding kinetics, the role of ATP, the involvement of protease-sensitive components in the outer membrane were determined. Taken together, these approaches allow for sensitive and quantifiable evaluation of the *in vitro* binding of SStp to the chloroplast translocation apparatus, as well as the first direct visualization of a transit peptide bound to the chloroplast translocation apparatus.

MATERIALS AND METHODS

Plant growth and chloroplast isolation

Chloroplasts were isolated from dwarf pea (*Pisum sativum*) seedlings that were 11-13 days old. The pea seeds (Progresso #9) were imbibed overnight and planted on horticulture Vermiculite, approximately 300 mL of dry seeds per flat. Plants were grown in EGC growth chambers under 160 $\mu E/m^2/sec$ of cool white fluorescent light. To reduce starch accumulation the plants were grown on a 12 hr light/12 hr dark cycle. Leaves were harvested from the seedlings and intact chloroplasts were isolated as described by Bruce *et. al.* (Bruce et al., 1994). Intact chloroplasts were resuspended at a concentration of 1 mg/ml total chlorophyll and used for all labeling/import reactions.

Cloning of full-length small subunit transit peptide into pET30a

The pea prSSU gene in pSP65 (Promega, Madison, WI) was cloned into pGEX-2T (Pharmacia, Piscataway, NJ) to form pGEX-2T-SStp (Ivey et al., 2000). Primers corresponding to regions flanking the SStp-encoding region were used to amplify the insert via standard PCR protocol, which engineered BamH I, and EcoR I restriction sites at the 5′ and 3′ ends of the amplified product, respectively. Both the amplified product and the vector pET30a (Novagen, Madison, WI) were doubly digested and ligated to yield pET30a-SStp. The construct was confirmed by automated DNA sequencing.

E.coli protein expression

The construct (pET30a-SStp), which placed the sequence encoding SStp in frame at the C-terminus of the dual epitope tag His-S, was transformed into *E.coli* [BL (21) (DE3)]. The cells were grown to a cell density corresponding to an OD_{600} of 0.6 and induced with 1mM [IPTG] Iso-Propyl-β-Thio galactopyranoside for three hours. The cells were pelleted, resuspended, and lysed by French press in ice cold binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0) containing, 2 μM leupeptin, 2 μM pepstatin, 1 mM PMSF and 6 M urea. The lysate was centrifuged at 50,000 *g* and the supernatant was loaded on a Ni^{2+}-Sepharose IMAC column and washed with 10 column volumes of wash buffer (60 mM Imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0) containing 6 M Urea. The protein was eluted with elution buffer (1 M Imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0) containing 6 M Urea. The samples containing the protein were pooled and dialyzed against Import Buffer (1X IB) (50 mM HEPES-KOH, pH 8.0, 330 mM Sorbitol) containing 6 M Urea. Most of our work was done using the denatured His-S-SStp as the native His-S-SStp would precipitate on freezing and thawing. In addition, during the purification a large fraction of the His-S-SStp was lost in the 50k pellet unlike the denatured His-S-SStp.

In vitro binding assays and import competitions

Protein import reactions were performed as described previously (Bruce et al., 1994) except for some modifications, which are mentioned here. Intact chloroplasts equivalent to 25 μg of chlorophyll were incubated in the dark for 30 minutes at room temperature to deplete the endogenous ATP levels. Then, Mg-ATP

was added to a final concentration of 30 μM for binding and 3 mM for import reactions except in the case of ATP titration where Mg-ATP was added to a final concentration as indicated in the figure. In some cases where GTP was used, Na-GTP was used and an excess of Mg-acetate was added. Young *et al.* also observed that it was essential to HPLC purify their nucleotides in order to remove any contaminant nucleotides. We did not HPLC purify our nucleotides but we found the same trend with different batches of high quality GTP, purchased from different companies (USB, Cleveland, OH; Fluka, Milwaukee, WI; Sigma, St. Louis, MO), although the extent of inhibition of His-S-SStp binding varied with different batches of GTP. The protein to be imported was added to a final concentration of 5 μM unless otherwise indicated. 1X IB was added to make the final volume of the reaction. After 8 minutes of incubations intact chloroplasts were purified on a 40% Percoll cushion at 3,500 *x g* for 6 minutes at 4°C. For time course studies the samples were diluted with cold 1X IB at indicated time intervals to dilute the precursor and ATP levels and then the intact chloroplasts were purified on a 40% Percoll cushion as mentioned earlier. The pellet obtained was resuspended to 1 ml with 1x IB and 50 μL was kept aside for protein estimation. The samples were centrifuged again similarly as mentioned earlier and the pellets were resuspended in 25 μL of 1x IB and 25 μL of 4XSample Buffer (SB) (4% SDS, 400mM DTT, 40 mM Tris-HCl pH 6.8, 10% Glycerol). Equal protein concentration was subjected to SDS-PAGE and Western Blotting. Import competitions were done similarly as mentioned above except ^{35}S radiolabeled prSSU was added to the import reactions at a final concentration of 50 nM along with the competitor protein. The gel was imaged by

filmless autoradiography using an Instant*Imager* (Packard, Canberra, Canada) and each band was quantified.

Thermolysin treatment of chloroplasts

Pre-thermolysin treatment was performed to dark adapted chloroplasts, where thermolysin (Boehringer Mannheim, Germany) was added prior to import reactions to a final concentration of 10 μg/ml and the chloroplasts were at 250 μg of chlorophyll, which was comparable to earlier work (Cline et al., 1984). Similarly, the post-thermolysin treatment was performed on intact chloroplasts after an import reaction, where the samples were resuspended in 1 ml of 1X IB + 1 mM $CaCl_2$. Half of it was used for post import thermolysin treatment while the other half was used as no thermolysin treated import reaction. Both pre- and post- thermolysin treatments were done for 30 minutes on ice. Samples were then centrifuged and resuspended in 25 μL of 1X IB + 25 μL of 4X SB.

S-protein FITC labeling of the chloroplast-bound His-S-SStp

The samples processed as indicated above were centrifuged at 1,500 *g* for 6 minutes to wash the unbound His-S-SStp and resuspended in 300 μL of Blocking Solution (1X IB containing 1% BSA (Bovine Serum Albumin) + 1% non-fat milk). S-protein FITC (Novagen, Madison, WI) was added at 1:500 dilution and incubated for 30 minutes on ice. Intact chloroplasts were purified over a 40% Percoll cushion as mentioned earlier and resuspended in 1X IB used for FACS analysis and/or Laser Scanning Confocal Microscopy.

Chloroplast subfractionation

Chloroplast envelope membranes were prepared by lysing intact chloroplasts under hypertonic conditions and separating them into soluble and membrane fractions by differential centrifugation as described earlier (Keegstra & Yousif, 1986; Bruce, 1994). Samples after a binding reaction were repurified on 40% percoll in 1X IB. The intact chloroplasts were resuspended in a hypertonic buffer, 0.6 M sucrose in 25 mM HEPES, pH 8.0 such that the final concentration was 2 mg/mL. Lysis was allowed to proceed for 10 minutes on ice and then the samples were frozen at -20°C overnight. Next day the chloroplasts were thawed and gently homogenized using a Dounce homogenizer after addition of an equal volume of 25mM HEPES, pH 8.0. The sample was centrifuged at 4000 *g* for 5 minutes. The supernatant was saved and the pellet was resuspended in 25 mM HEPES, pH 8.0 and homogenized to break up any clumps and centrifuged at 4,000 *x g* for 5 minutes. The supernatants were pooled and centrifuged at 50,000 *x g* to pellet the membranes. The membranes were resuspended in 0.3 M Sucrose in 25mM HEPES, pH 8.0 and loaded on to of a discontinuous sucrose gradient consisting of 1 mL 1.2 M, 1.0 mL 1.0 M, 1 mL 0.8 M and 1.0 mL 0.6 M sucrose in 25mM HEPES, pH 8.0. The gradient was centrifuged at 28,500 rpm in a Beckman SW50.1 rotor for ~ 17 hours. Sample was separated into stroma, outer envelope, inner envelope, combined envelope, and thylakoid membranes by discontinuous sucrose density gradient centrifugation. 0.5 mL fractions were collected and diluted with 1 mL 25 mM HEPES to remove the sucrose and the membranes were centrifuged at 48,000 *g* for 1 hour. The membrane pellets were resuspended in a small volume of 1 M sucrose and boiled with 4X reducing

sample buffer for SDS-PAGE. Except in case of cross-linked sample 4X non-reducing sample buffer was used in order to see the whole cross-linked product.

Flow cytometry

A dual-laser Becton Dickinson FACStar Plus was used for the flow cytometric analyses. Data were acquired and analyzed using Becton Dickinson CellQuest V. 3.1 software. The argon ion laser in the first position was tuned for 488 nm output running on a constant output mode at 250 milliwatts. Forward angle light scatter was collected with the forward photodiode without a neutral density filter in place. The right angle light scatter used a 488/10 nm band pass filter. Dot plot analysis of forward and right angle light scatter was used to determine the percent intact plastids (~70-80% of total plastids) (Kausch & Bruce, 1994). Total chloroplasts were gated to include only the intact plastids for both FL1 (chlorophyll autofluorescence) and FL2 (S-protein FITC). Detection by FL1 was selected via a dichroic mirror and a DF 530/30 nm filter. FL2 was selected via the same dichroic mirror and a DF 660/20 nm filter. Fluorescence analysis of 25,000 intact chloroplasts was analyzed per sample. The side scatter, chlorophyll fluorescence, and FITC fluorescence were collected in log mode with four-decade log amplifiers. The extent of positive labeling was set manually with unlabeled chloroplasts for each experiment to yield value of 2-3% FACS positive. Flow rate was usually maintained at ~1200 chloroplasts/min. Sheath buffer was composed of 1X PBS (Phosphate Buffered Saline).

Laser scanning confocal microscopy

The S-protein FITC stained samples after a binding reaction were analyzed using a Lieca TCS-NT laser confocal microscope. FITC and chlorophyll autofluorescence were simultaneously visualized (two channel collection) using 488 excitation (Ar ion laser) into a 530/30 BP filter (FITC) and a 660 LP filter (chlorophyll auto-fluorescence). Images were collected with a 100X, 1.4 NA oil immersion lens at an Airy disc setting of 0.91. The data are presented as extended focus projections.

Cross-linking of His-S-SStp to the import apparatus

The heterobifunctional cross-linking reagent, N-[-(p-azidosalicylamido) butyl]-3'(2-pyridyldithio)propionamide (APDP) was purchased from Pierce, Inc. (Rockford, IL). 100 nmol of APDP was iodinated with 1 mCi of Na[^{125}I] in Iodogen pre-coated iodination tubes (Pierce, Inc. Rockford, IL) for 10 minutes at room temperature. All iodination and precursor modification reactions were performed in the dark. 85 nmol of His-S-SStp in 1X IB + 6M Urea was reduced with 2% vol/vol β-mercaptoethanol at 37°C for 15 minutes. Excess β-mercaptoethanol was removed by filtering the mixture through a PD-10 (G-25) gel filtration column, (Pharmacia, Piscataway, NJ). 500 μL fractions were collected and a small fraction (of which) was subjected to SDS-PAGE. The fractions containing reduced His-S-SStp were pooled. The pooled fractions were immediately mixed with 100 nmol of [^{125}I]APDP and incubated on ice overnight. The sample was then filtered through another PD-10 (G-25) gel filtration column to remove the unbound [^{125}I]APDP and Na[^{125}I]. 500 μL fractions were collected, a small fraction of which was subjected to SDS-PAGE

and visualized via filmless autoradiography using an Instant*Imager* (Packard, Canberra, Canada). The fractions containing [^{125}I]-APDP-His-S-SStp were pooled again and protein concentration determined by BCA measurement (Pierce, Inc. Rockford, IL). This modified form of His-S-SStp was stored at 4°C in the dark prior to use in cross-linking reactions.

Cross-linking reaction was performed similar to other binding reactions except [^{125}I]APDP-His-S-SStp was used. After re-isolation of intact chloroplasts over a 40% Percoll cushion the samples were transferred to a plastic petri dish and irradiated from above with UV light of wavelength 325 nm using a Stratalinker (Stratagene, La Jolla, CA) for 10 minutes twice with 5 minute cooling in between . Samples were then sub-fractionated into outer, inner mixed inner and outer and thylakoid membrane fractions as described earlier. Each fraction was subjected to SDS-PAGE using sample buffers with and without DTT and gels were visualized by phosphor-imaging (Molecular Dynamics, Foster City, CA).

SDS-PAGE and far-western blotting

Samples were boiled in 4X SB and were resolved on an 18% SDS-PAGE gel. The gel was electro-blotted on to a pre-wetted Immobilon-P membrane (Millipore, Bedford, MA) using a transfer buffer for small molecular weight proteins (48 mM Tris Base, 39 mM Glycine, 0.04% SDS, 20% Methanol). The blot was blocked for 15 minutes with TBS (Tris Buffered Saline) containing 1% gelatin + 0.5 % Tween-20 for 15 minutes. The S-peptide containing proteins were detected by incubation for 15 minutes with an S-protein alkaline phosphatase conjugate (Novagen, Madison, WI) diluted 1:100,000 in TBS followed by extensive washing with TBS + 0.2%

Tween-20. Visualization was conducted using a 1:1 dilution of the chemiluminescent substrate LumiPhos Plus (Lumigen Inc., Southfield, MI) and subjected to film autoradiography. Exposure time was optimized for maximum signal to noise. In some cases chromogenic (NBT and BCIP) substrate was used.

Protein and chlorophyll measurements

Protein was measured using BCA reagents (Pierce, Rockford, IL). Chlorophyll concentration was measured after chlorophyll extraction using 80% acetone. To calculate the total mg of chlorophyll in the chloroplast preparation 50 μL of the chloroplast suspension was added to 5 mL of 80% Acetone and the following equation was used (Bruce 1994).

$$Chlorophyll(mg/mL) = \frac{\{(8.02 \times A_{663}) + (20.2 \times A_{645})\}}{0.05mL \times 1000\mu g/mg} \times 5mL$$

RESULTS AND DISCUSSION

Cloning, expression and purification of His-S-SStp

The SStp was cloned from pGEX2T-SStp into the BamH I-EcoR I site of pET30a (Ivey et al., 2000). This construct, pET30a-SStp, places a dual-epitope tag, consisting of a His-tag and S-tag, at the N-terminus of SStp. The His-tag allows rapid one-step purification of His-S-SStp using a Ni^{2+} IMAC column. The S-tag is useful in permitting sensitive detection of the fusion protein by its ability to interact with the S-protein. Utilization of commercially available conjugates such as S-protein FITC or S-protein alkaline phosphatase permits both fluorescent and enzymatic detection of the fusion protein. The extreme sensitivity of this detection

system enables detection of the transit peptide on a femtomole scale. In addition, this construct contains cleavage sites for the proteases, enterokinase and thrombin, which can be used to yield either SStp or S-SStp, respectively. The organization of this construct is shown in Fig. 2-1a.

After transformation of this construct into competent *E.coli* cells (BL21[DE3]), induction of expression with IPTG yielded a single prominent band with an apparent molecular weight of 12.1 kDa in the total cell lysate, as is shown in Figure 2-1b, lane 2. This mobility agrees well with the predicted molecular weight of 11.6 kDa. The 12.1 kDa band was evident in the supernatant after cell disruption by a French pressure cell press and centrifugation at 50,000 *x g* (Figure 2-1b, lane 3). Purification of His-S-SStp was performed using a single Ni^{2+}-Sepharose column under denaturing conditions (6M urea). This affinity matrix was effective in capturing most of the fusion protein since very little of the 12.1 kDa protein was observed in the flow-through (Figure 2-1b, lane 5). However, some of the fusion protein was lost when the column was washed in the presence of both 6 M urea and 60 mM imidazole (Figure 2-1b, lane 6). The fusion protein was eluted with elution buffer containing 1 M imidazole and most of the protein was eluted in one to two column volumes of elution buffer (Figure 2-1b, lane 7-18). This purification routinely yielded between 25-60 mg of the fusion protein per liter of *E.coli* culture. Enterokinase or thrombin was not used to cleave either epitope tag since the complete fusion protein, His-S-SStp, was required to do the experiments. The higher molecular weight bands probably represent additional fusion protein that resulted from translational read through the opal stop codon of the vector (MacBeath & Kast, 1998). Placement of the dual epitope introduced charged residues at the N-terminus

Figure 2-1. The amino acid sequence and purification profile of His-S-SStp. **(a)** The SStp was subcloned into the BamH I- EcoR I site of vector pET30a, the recombinant SStp has a dual epitope tag at its N-terminus the His tag and the S-peptide which are cleavable using thrombin and enterokinase respectively. The N-terminal His tag is useful in the purification of the proteins by IMAC Ni^{2+} Sepharose column. The N-terminal S-peptide permits detection the transit peptide via either S-Protein AP conjugates or S-protein FITC conjugates. **(b)** A Coomassie stained SDS-PAGE gel showing the purification of recombinant His-S-SStp. MW stands for the broad range marker (Bio-Rad) and the molecular weights are as specified. SM is the total starting material after the cells were induced with 1mM IPTG for 3 hours. Induced cells resuspended in denaturing buffer (6M Urea) were lysed using a French Press. The 50 K Sup. represents the supernatant after centrifugation of the cell lysate at 50,000 x g. FT is the flow-through from loading the supernatant on the Ni^{2+}Sepharose column. The wash is obtained by washing the column using wash buffer. Fractions in lanes 6-18 represent the eluted fractions.

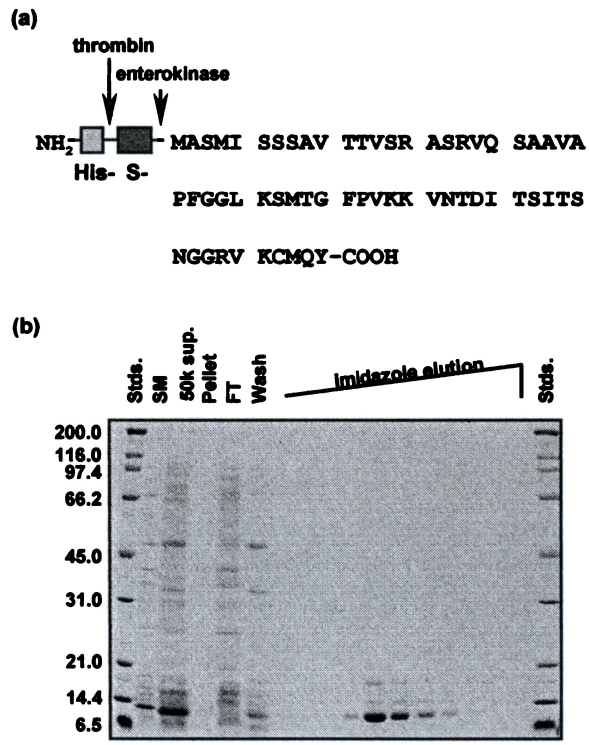

(a)
thrombin
enterokinase
NH2
His- S-
MASMI SSSAV TTVSR ASRVQ SAAVA
PFGGL KSMTG FPVKK VNTDI TSITS
NGGRV KCMQY-COOH
(b)
Stds.
SM
50k sup.
Pellet
FT
Wash
Imidazole elution
Stds.
200.0
116.0
97.4
66.2
45.0
31.0
21.0
14.4
6.5

Figure 2-1.

of SStp, increasing the total number of charged residues in His-S-SStp to 31, compared to 10 in SStp. These additional 21 charged residues at the N-terminus are inconsistent with the accepted domain structure of chloroplast transit peptides, which normally contain uncharged 9-13 N-terminal residues (von Heijne et al., 1991; von Heijne et al., 1989).

Competitive inhibition of chloroplast import by His-S-SStp

The most direct way to determine if the epitope-tagged form of SStp productively engages the chloroplast translocation apparatus is to test its ability to function as a competitive inhibitor of chloroplast protein import. Using ^{35}S-labeled prSSU as the substrate, *in vitro* import competitions were performed. As shown in Figure 2-2a, the import of the prSSU decreased as the amount of His-S-SStp in the import reaction was increased. Addition of ~7 μM His-S-SStp reduced the level of prSSU that was imported and processed to the mature form to approximately 50% of the no-competitor control (Figure 2-2b). Import inhibition is believed to result from specific interaction of the tagged transit peptide with one or more components of the translocation apparatus since the purified epitope tag alone (His-S) was unable to compete for the import of prSSU (data not shown).

However the level of His-S-SStp competitor (7 μM) required for 50% import inhibition was two orders of magnitude greater than the concentration of the prSSU substrate (50 nM). A similar phenomenon was observed previously with non-epitope labeled SStp (Dabney-Smith et al., 1999). Equal molecules of prSSU are required to inhibit the import of ^{35}SprSSU. In addition, the IC_{50} value measured for His-S-SStp was much lower than the IC_{50} values reported previously for shorter synthetic

Figure 2-2. Competition of ^{35}S prSSU import with His-S-SStp. **(a)** Filmless autoradiogram of an import competition of prSSU with His-S-SStp done using Instant Imager. Competition import assays were performed by incubating freshly prepared chloroplasts with 50nM ^{35}S-prSSU and increasing concentrations of cold competitor His-S-SStp. The prSSU and the His-S-SStp were added at the same time. The assay was terminated by rapid dilution in ice-cold 1x IB (3-fold) and placed on ice in the dark and intact plastids were reisolated over 40% Percoll. The plastid proteins were resolved on an 18% SDS-PAGE gel. Equal concentration of protein was loaded in each lane. The first lane shows control prSSU that was not subjected to an import reaction. The rest of the lanes show the import of prSSU with increasing concentrations of the competitor His-S-SStp. **(b)** Graphical representation of the quantitation of individual mSSU bands shown in panel (a) quantified using Instant Imager. The values were normalized to % of zero competitors added. Each data point is an average of 3-5 experiments and the error bars represent the standard deviation.

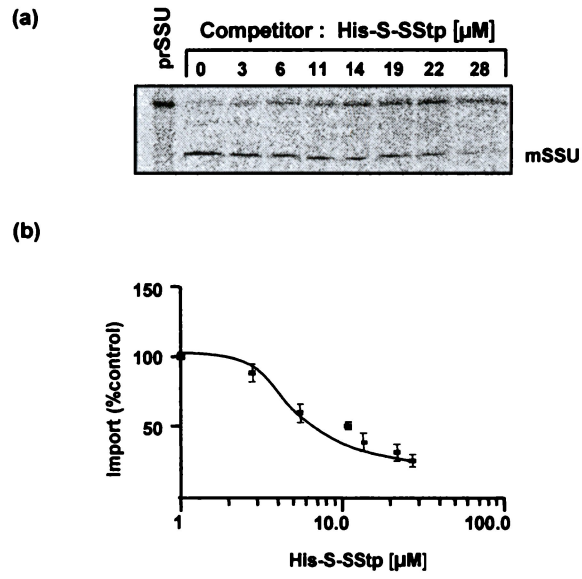

(a)
prSSU
Competitor : His-S-SStp [μM]
0 3 6 11 14 19 22 28
mSSU
(b)
Import (%control)
150
100
50
1
10.0
100.0
His-S-SStp [μM]

Figure 2-2.

peptides derived from SStp (Perry et al., 1991; Schnell et al., 1991). Therefore, the disparity between the amounts of His-S-SStp competitor required to block import of a given amount of prSSU appears to be complex in nature. Factors include sequence-specific or structural interactions between the transit peptide and the mature domain (Dabney-Smith et al., 1999), as well as potential interference from the charged His-S tag itself. The observation that at high levels of His-S-SStp, we detect an accumulation of bound prSSU, suggests that His-S-SStp is somehow blocking steps subsequent to binding, possibly by partially occupying the Toc75 translocon.

Detection of His-S-SStp binding to intact and fractionated pea chloroplasts

To determine the utility of the epitope-tagged transit peptide in terms of specific and sensitive detection, a far-Western blotting assay with commercially available S-protein alkaline phosphatase conjugate was performed (Figure 2-3). The time dependence of binding demonstrated that the epitope-tagged transit peptide bound to intact chloroplasts rapidly (Figure 2-3a). This association was stable, as it withstood multiple washes that are associated with organelle recovery. As shown in the control lane, little to no cross-reactivity was observed with total chloroplast protein in the absence of His-S-SStp. After incubation in the presence of His-S-SStp (5 μM), however, a prominent cross-reactive band was observed at the expected molecular weight. At no time point did we observe a smaller molecular weight species that would reflect transit peptide processing or degradation (van't Hof & de Kruijff, 1995). With extended incubation times (30-60 min), however, a slight

Figure 2-3. Time course and membrane fractionation of His-S-SStp binding. **(a)** Chemiluminescent detection of a time course of His-S-SStp binding. Binding reactions were performed as described in experimental procedures. Samples were then analyzed by SDS-PAGE and blotting. S-protein alkaline phosphatase was used for detection. **(b)** Chemiluminescent detection of a blot of an envelope preparation done using His-S-SStp bound to chloroplasts. Binding reactions were performed as described in experimental procedures. The chloroplasts were then hypertonically lysed and separated on a discontinuous sucrose gradient [0.6 M, 0.8 M, 1.0 M and 1.2 M] centrifuged at 30,000 x g for 20 hours. The fractions representing the interfaces and the thylakoids were then analyzed by SDS-PAGE and blotting. S-protein alkaline phosphatase was used for detection.

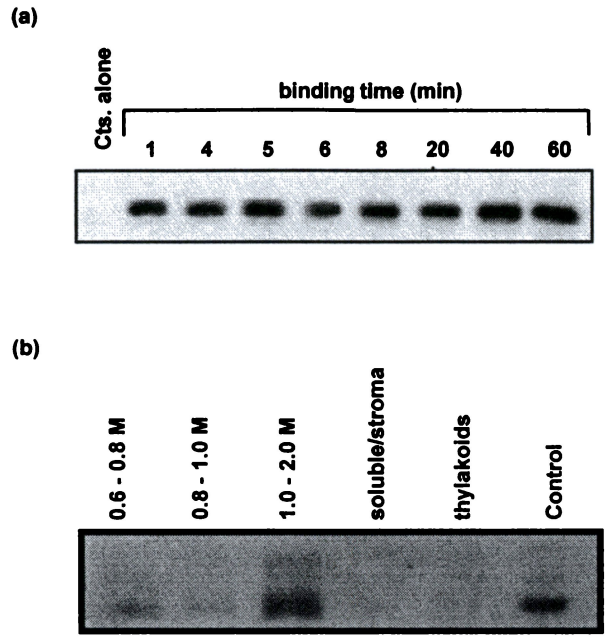

(a)
Cts. alone
binding time (min)
1
4
5
6
8
20
40
60
(b)
0.6 - 0.8 M
0.8 - 1.0 M
1.0 - 2.0 M
soluble/stroma
thylakoids
Control

Figure 2-3.

increase in the amount of bound His-S-SStp was detected. These results clearly illustrate both the exquisite specificity and sensitivity of this epitope tag-based detection system. Using controlled amounts of protein, we were able to detect as little as ~0.4 pM quantities of His-S-SStp (data not shown). In addition, the use of other S-peptide-based detection systems, such as the recently reported fluorometric RNase assays could be used to further increase the sensitivity of this method (Kelemen et al., 1999; Kim & Raines, 1993).

In order to determine the sub-organellar location of the bound His-S-SStp, chloroplast fractionation was performed. Chloroplasts were subjected to a binding reaction (30 μM ATP) and subsequently lysed hypertonically. Membrane fractionation was performed on a discontinuous sucrose gradient, allowing for separation of the inner and outer envelope membranes, thylakoids, and a soluble fraction. The soluble fraction consists of the stroma and the inter-membrane space between the inner and outer membranes of the envelope (Bruce et al., 1994). The fractions Toc86/159were confirmed to have outer membrane/thylakoid/stroma by western blots using α-, α-cyt f, α-SSU antisera respectively (data not shown). A blot revealed that most of the His-S-SStp was bound to a combined inner/outer envelope fraction (Figure 2-3b, lane 3) and to the outer envelope fraction (Figure 2-3b, lane 1). The ability of His-S-SStp to remain associated with these membrane fractions throughout extended centrifugation indicated a stable interaction. The His-S-SStp was not detected either in the soluble fraction or in the thylakoids (Figure 2-3b, lanes 4 and 5, respectively), confirming that the His-S-SStp was neither translocated into the stroma nor trapped within the inter-membrane space. Fractionation of control

chloroplasts, to which no His-S-SStp was added, resulted in no detectable S-protein alkaline phosphatase cross-reactivity (data not shown).

Analysis of chloroplast staining by flow cytometry

The far-Western blotting analysis above (Figure 2-3) indicated that (1) His-S-SStp binds to chloroplasts and (2) the detection system via the S-protein alkaline phosphatase conjugate is both highly sensitive and specific. However, attempts to quantify the extent of chloroplast labeling based on chemiluminescent detection of bound His-S-SStp were not consistently reproducible (data not shown). To facilitate a more sensitive and accurate means of quantifying the amount of bound His-S-SStp, we developed a flow cytometric assay utilizing the S-protein FITC conjugate. This assay allows direct fluorometric measurement of the amount of His-S-SStp bound to individual chloroplasts. Unlike conventional methods, one advantage of using flow cytometry is that it measures each organelle individually instead of measuring an average signal that results from a population of organelles. By testing a large number of individual particles (>10,000), a labeling distribution is determined and statistically significant measurements are reportable, such as mean peak channel of labeling. With the proper negative controls, minimal signal intensity can be determined which establishes a threshold level for identifying positively stained organelles.

Intact versus broken chloroplasts were easily distinguishable based on their different light-scattering properties (Kausch & Bruce, 1994), as shown in the bivariate analysis of total chloroplasts using forward and right angle light scatter (Figure 2-4a). The intact chloroplast region is shown in red and represents the gate

Figure 2-4. A typical graphical representation of FACS analysis. **(a)** Difference in the profile of intact and broken chloroplasts as shown by forward light scatter. The values are plotted against number of chloroplasts. **(b)** Dot plot representation of a typical FACS analysis. The distinct separation of intact chloroplasts (described in the enclosed region) from the broken ones due to the difference in light scattering properties. The intact chloroplasts were gated, and only the chloroplasts in this region that were stained positive with FITC were used in the later experiments. **(c)** Controls used in the FACS analysis: The intact unlabelled chloroplasts where no protein was bound to the chloroplasts are shown in red; the intact chloroplasts that had His-S bound to them and were stained with SP-FITC are shown in blue; the intact chloroplasts that had no protein bound to them but were stained negative with SP-FITC are shown in green.

Placeholder

REPLACE IMAGE

used for both FITC and chlorophyll autofluorescence analysis (Figure 2-4b). Although the ratio of intact to broken chloroplasts varied between preparations, on average the organelles were >70% intact. To determine the specificity of the FITC-S-protein detection system, three control samples were analyzed (Figure 2-4c) and were found to be negative. Chloroplasts that were not treated with either His-S-SStp or S-protein FITC displayed no FL-1 fluorescence, indicating that chlorophyll autofluorescence did not spillover into this channel. Chloroplasts treated with only the S-protein FITC conjugate were also negative in the FL-1 channel, verifying that the S-protein does not bind non-specifically to the chloroplast surface. Finally, incubation of chloroplasts with purified His-S, followed by incubation with S-protein FITC conjugate, were also negative in the FL-1 channel, demonstrating that the His-S epitope tag itself does not show affinity for either the chloroplast translocation apparatus nor some other exposed chloroplast protein. These negative controls confirm that the positive staining of His-S-SStp described below is truly mediated by sequences within the transit peptide.

Concentration dependent and saturable binding of His-S-SStp to chloroplasts

Previous analysis of the binding of radiolabeled precursors to chloroplasts have demonstrated that binding is concentration dependent and saturable (Friedman & Keegstra, 1989), suggesting that chloroplast binding is mediated by a membrane receptor. To determine if the binding of His-S-SStp was also receptor mediated, we investigated the concentration dependence of binding by both the far-Western blotting and flow cytometry (Figure 2-5). To support binding these experiments

Figure 2-5. Concentration titration of His-S-SStp binding. **(a)** Chemiluminescent detection of a blot after a binding reaction with increasing [His-S-SStp]. Binding reactions were performed as described earlier at 30 μM ATP. The intact chloroplasts were subjected to SDS-PAGE and blotting. S-protein alkaline phosphatase was used for detection. **(b)** FACS analysis shows the interaction of increasing [His-S-SStp] with chloroplasts. Binding reactions were performed as described earlier at 30 μM ATP. S-protein FITC was used for detection. The concentrations of His-S-SStp that showed positive staining are indicated on the graph. S-protein FITC was used for detection. **(c)** Quantitative analysis of the positive FITC-stained chloroplasts as a function of increasing [His-S-SStp]. The positive staining was seen to saturate at 8 μM His-S-SStp.

Placeholder

REPLACE IMAGE

were conducted in the presence of 30 μM ATP. Incubation of chloroplasts with increasing concentrations of His-S-SStp, followed by re-isolation and far-Western blotting (Figure 2-5a), indicated that the *in vitro* interaction of His-S-SStp with chloroplasts was concentration dependent and saturable, as expected. The band of higher molecular weight than His-S-SStp was probably due to a co-purified translational read-through product from *E.coli*, as discussed above.

By utilizing the S-protein FITC conjugate, the extent of His-S-SStp binding to chloroplasts was also measured by flow cytometry. Histograms of labeled chloroplasts are shown as a function of His-S-SStp concentration in Figure 2-5b. As the concentration of His-S-SStp is increased, a population of positively staining chloroplasts with brighter FL-1 fluorescence was observed that was not present in the unstained control. Figure 2-5c shows that the percentage of chloroplasts in this population was proportional the amount of His-S-SStp, which reflects an increase in the binding of the labeled S-protein to the chloroplast-bound transit peptide. As expected, the level of staining became saturated at high levels of His-S-SStp (~8-10 μM). The maximum percentage of positive staining chloroplasts varied, however, depending upon both the chloroplast preparation and the His-S-SStp protein preparation.

These binding assays were done in a manner such that the level of urea in the assay mixture never exceeded the 400 mM level, which we have observed to be deleterious to the chloroplasts. We have also investigated whether the initial denaturation of the transit peptide with urea, could in some way, influence its binding properties. To test for some spurious effects of chemical denaturation, we directly tested the binding properties of His-S-SStp isolated under both native and the

denaturing conditions. Native His-S-SStp protein and chemically denatured (6M Urea) His-S-SStp showed a similar trend with respect to concentration dependence and saturable binding (Figure 2-6a vs. 2-6b) indicating that the observed results were not an artifact resulting from chemical denaturation. His-S-SStp has a single cysteine residue at its C-terminus it runs as dimer of ~23 kDa on a non-reducing SDS-PAGE gel (data not shown). Since we use non-reduced His-S-SStp for our experiments we wanted to see if the reduced form of His-S-SStp had different binding properties. Hence, we compared the concentration titration of His-S-SStp with (10mM) and without DTT. The non-reduced form of His-S-SStp showed higher binding than the reduced form of DTT as shown in Figure 2-7. This may be because the non-reduced His-S-SStp is dimerized and so more His-S-SStp would bind to the translocon. Recently, it has been shown that 8-9 precursors bind per translocon in mitochondria (Stan et al., 2000). If a similar mechanism is true in chloroplast then 8-9 dimers would show higher binding than 8-9 monomers.

Thermolysin-sensitivity of His-S-SStp association with chloroplasts

To further characterize the biochemical nature of the His-S-SStp/chloroplast interaction, we investigated whether His-S-SStp association with chloroplasts is dependent on protease-sensitive components, as reported previously for full-length precursors (Cline et al., 1985; Perry & Keegstra, 1994). Treatment of the chloroplasts with thermolysin removes components only on the outer cytosolic surface of the outer envelope, leaving the inner envelope and the inter-membrane space intact (Cline et al., 1984). When thermolysin treated chloroplasts were assayed by far-western blotting and flow cytometry for their ability to bind His-S-SStp, the

Figure 2-6. Comparison of native Vs urea-denatured His-S-SStp. **(a)** Chemiluminescent detection of a blot of a binding reaction with increasing denatured [His-S-SStp]. **(b)** Chemiluminescent detection of a blot of a binding reaction with increasing native [His-S-SStp]. Binding reactions were performed as described earlier at 30 μM ATP. The intact chloroplasts were subjected to SDS-PAGE and blotting. S-protein alkaline phosphatase was used for detection. The band of higher molecular weight than His-S-SStp was probably due to a co-purified translational read-through product from *E. coli.*

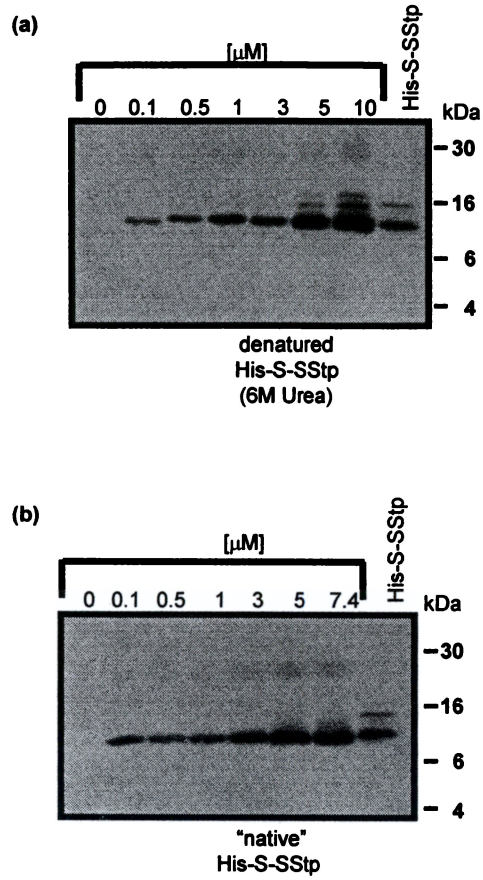

(a)
[μM]
0 0.1 0.5 1 3 5 10
His-S-SStp
kDa
30
16
6
4
denatured
His-S-SStp
(6M Urea)
(b)
[μM]
0 0.1 0.5 1 3 5 7.4
His-S-SStp
kDa
30
16
6
4
"native"
His-S-SStp

Figure 2-6.

Figure 2-7. Effect of DTT on the binding of His-S-SStp to chloroplasts. Binding of increasing concentration of His-S-SStp was performed in the presence and absence of DTT. *Top* panel shows the binding of His-S-SStp in the absence of DTT and *bottom* panel shows the binding of His-S-SStp in the presence of DTT. The band of higher molecular weight than His-S-SStp was probably due to a co-purified translational read-through product from *E. coli.*

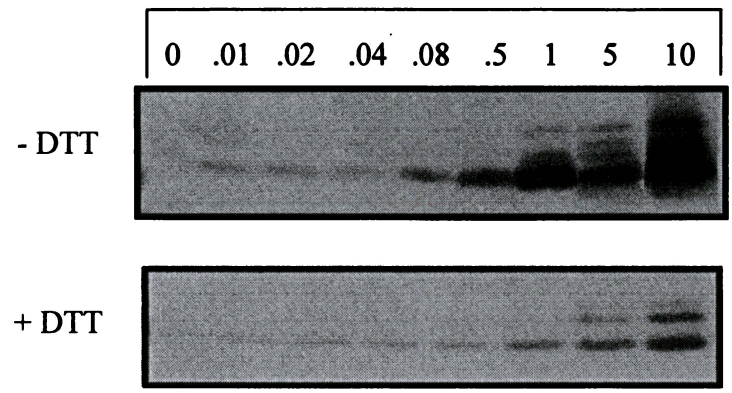


Figure 2-7.

histogram of S-protein FITC labeling showed that binding was reduced to the level of the unlabeled control (Figure 2-8 and 2-9). Whereas non-thermolysin treated chloroplasts were over 60% FACS positive, thermolysin treatment of chloroplasts prior to His-S-SStp addition reduced the FACS positive population to <5% (Figure 2-9). Similar results were obtained by far-western blotting (Figures 2-8). To control for thermolysin activity, identical treatment of intact chloroplasts with thermolysin abolished both the binding and import of ^{35}S-prSSU (Figure 2-10). This analysis indicates that recognition and binding of His-S-SStp to chloroplasts requires a thermolysin-sensitive, proteinaceous component(s) of the outer envelope, as observed for authentic precursors.

To explore the proteolytic accessibility of the chloroplast-bound His-S-SStp intermediate, we treated intact chloroplasts with thermolysin after the His-S-SStp binding step (post-treatment). Flow cytometric and far-western blotting analysis of post-thermolysin treated chloroplasts indicated that most of the bound transit peptide was associated with the outer envelope such that it remained accessible to thermolysin (Figure 2-8 and 2-9). Post-treatment with thermolysin reduced the extent of positive labeling to <5% compared to the initial control labeling of >60% in the absence of thermolysin (Figures 2-9). To ensure that thermolysin treatment was not disrupting the chloroplasts, an identical treatment was performed on chloroplasts that were pre-incubated with ^{35}S-prSSU. The bound precursor was removed by thermolysin treatment but did not reduce the level of the radiolabeled mature form (Figure 2-10). Data from these post-treatment thermolysin experiments are consistent with our finding that His-S-SStp binds the chloroplast surface but that the His-S epitope is not translocated across the outer membrane. The additional charged

Figure 2-8. Thermolysin treatment on His-S-SStp binding to chloroplasts at varying concentrations of ATP. The l*eft* panel shows thermolysin treatment prior to a His S-SStp binding reaction. Lane 9 shows the chloroplast binding reaction of His-S in the presence of 3mM ATP. The m*iddle* panel shows the bound His-S-SStp after thermolysin treatment after a binding reaction. Lane 1 shows the chloroplast binding reaction of His-S in the presence of 3mM ATP using thermolysin treated chloroplasts. The r*ight* panel shows the binding of His-S-SStp without any thermolysin treatment. Lane 9 shows the chloroplast binding of His-S in the presence of 3mM ATP. The samples were subjected to SDS-PAGE and far-western blotting. S-protein-alkaline phosphatase was detected chromogenically.

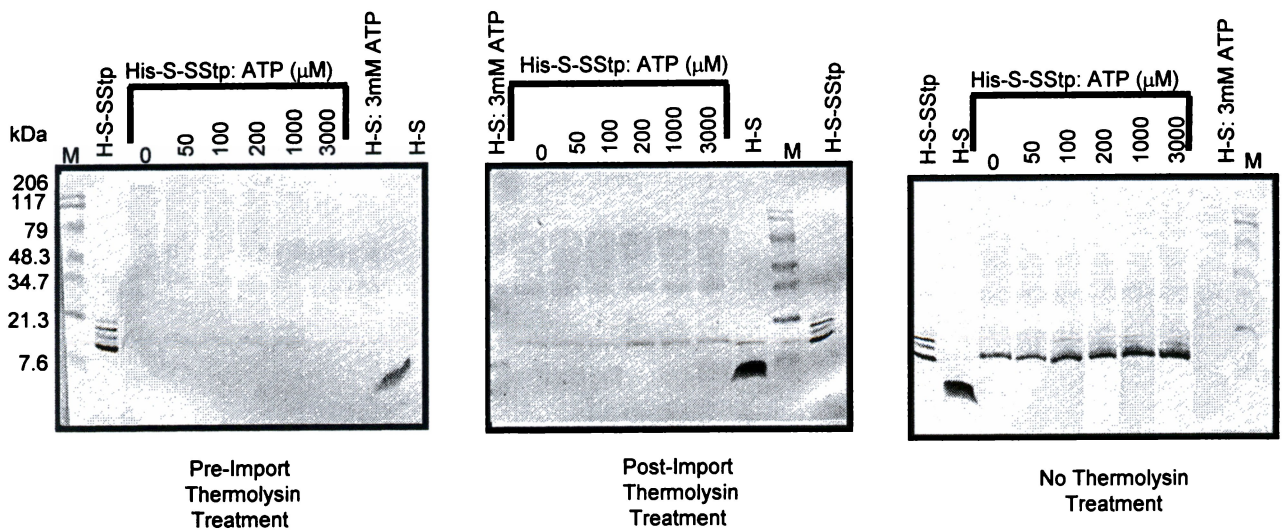

kDa
M
H-S-SStp
His-S-SStp: ATP (μM)
0
50
100
200
1000
3000
H-S: 3mM ATP
H-S
206
117
79
48.3
34.7
21.3
7.6
Pre-Import Thermolysin Treatment
H-S: 3mM ATP
His-S-SStp: ATP (μM)
0
50
100
200
1000
3000
H-S
M
H-S-SStp
Post-Import Thermolysin Treatment
H-S-SStp
H-S
His-S-SStp: ATP (μM)
0
50
100
200
1000
3000
H-S: 3mM ATP
M
No Thermolysin Treatment

Figure 2-8.

Figure 2-9. FACS analysis of thermolysin treatment on His-S-SStp binding. **(a)** FACS analysis shows the FITC staining of a binding reaction of His-S-SStp at different conditions. Pre-import thermolysin treatment of the binding of His-S-SStp was done using dark adapted pea chloroplasts that were pre-treated with thermolysin. Both no thermolysin and post-import thermolysin treatment of the binding reaction were done using dark adapted pea chloroplasts that were not pre-treated with thermolysin but thermolysin treatment was done after the import reaction. S-protein FITC was used to detect the chloroplast bound His-S-SStp. Chloroplasts alone, pre-import TL treatment and post-import TL treatment are all negative whereas no TL treatment shows positive staining with SP-FITC. **(b)** Quantitative histogram analysis of the data shown in (a).

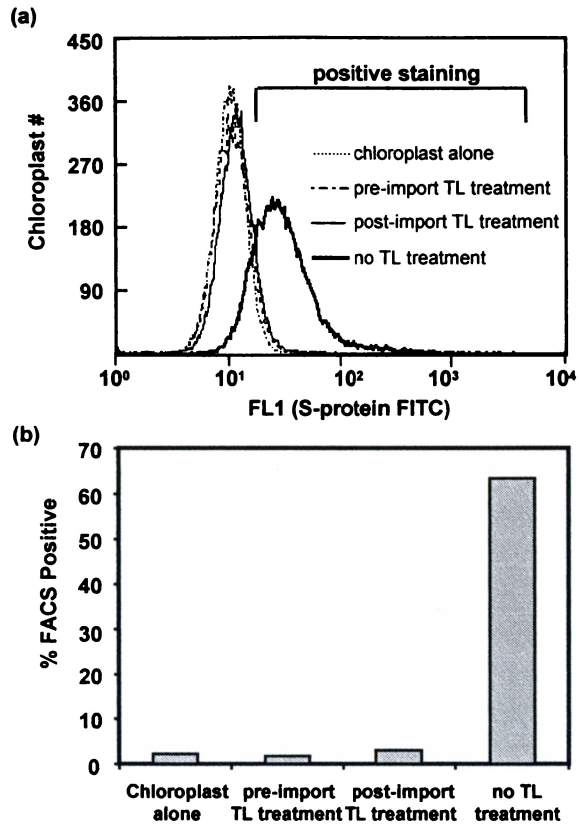

(a)
450
360
270
180
90
Chloroplast #
positive staining
chloroplast alone
pre-import TL treatment
post-import TL treatment
no TL treatment
10⁰
10¹
10²
10³
10⁴
FL1 (S-protein FITC)
(b)
70
60
50
40
30
20
10
0
% FACS Positive
Chloroplast alone
pre-import TL treatment
post-import TL treatment
no TL treatment

Figure 2-9.

Figure 2-10. Thermolysin treatment on ^{35}S prSSU binding and import into chloroplasts. The *left* panel shows thermolysin treatment prior to an import reaction. The *middle* panel shows the thermolysin treatment after a binding reaction. The *right* panel shows the binding reaction without any thermolysin treatment. The control and the experimental lanes are as indicated in the figure. The samples after an import reaction were subjected to SDS-PAGE and the blotted on PVDF membrane, the radiolabeled bands were detected using Instant Imager.

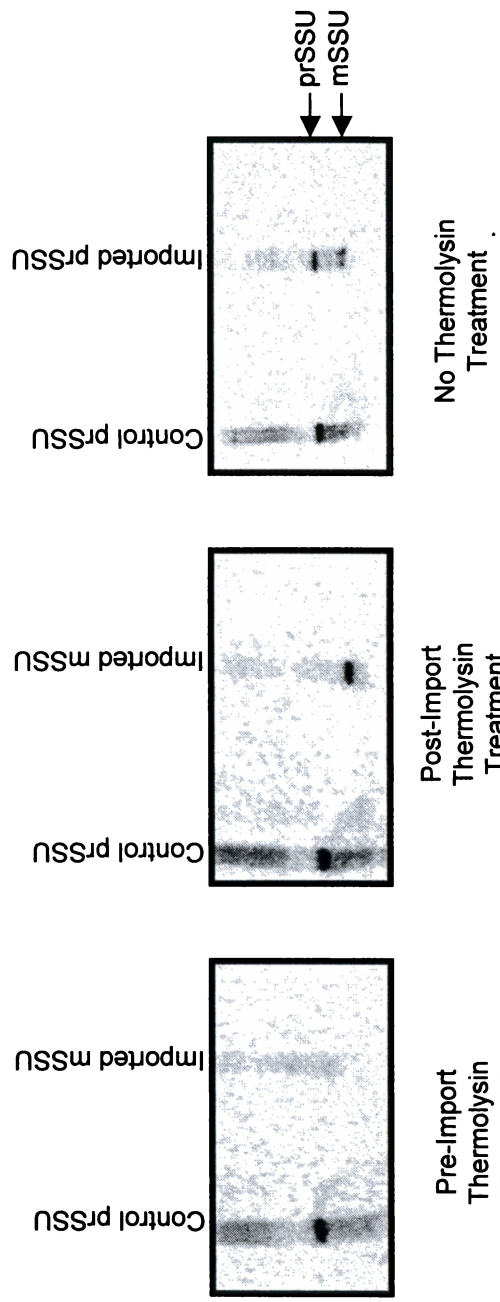

prSSU
mSSU
Imported prSSU
Control prSSU
No Thermolysin Treatment
Imported mSSU
Control prSSU
Post-Import Thermolysin Treatment
Imported mSSU
Control prSSU
Pre-Import Thermolysin Treatment

Figure 2-10.

residues at the N-terminus of His-S-SStp, compared to SStp or prSSU, may either prevent complete insertion of the transit peptide into the translocon or interfere with translocation, but apparently does not interfere with the initial binding process.

Cross-linking of His-S-SStp to the chloroplast translocation apparatus

Although we have shown that His-S-SStp is associated with protease sensitive components of the outer envelope and that it fractionates with the inner/outer envelope fraction, we have not directly shown that this association is mediated via interactions with components of either the Toc or Tic apparatus. In order to confirm that the transit peptide is interacting with the chloroplast translocation apparatus, we used an iodinated heterobifunctional photoactivatable cross-linker, APDP, to cross-link His-S-SStp to one or more components of the translocation apparatus. The [^{125}I]APDP is covalently attached to the transit peptide via the single cysteine (C57) at C-terminus of the His-S-SStp. After the [^{125}I]APDP-His-S-SStp is bound to the chloroplast, exposure to UV light induces cross-linking to the nearest protein. Upon exposure to a reducing agent, the [^{125}I]-labeled cross-linker group is transferred to the adduct resulting in a radiolabeled form of the translocation apparatus component as shown in Figure 2-11.

When the cross-linked samples where subjected to SDS-PAGE in the absence of DTT, the majority of the [^{125}I]APDP-His-S-SStp was cross-linked to a very high (>250 kDa) molecular weight complex of proteins (Figure 2-12b). Upon reduction of the [^{125}I]APDP, the high molecular weight complex disappeared. This higher molecular weight complex could be due to the formation of network of cross-linked transit peptides at the translocon as multiple transit peptides are believed to bind per

Figure 2-11. A schematic representation of the cross-linking protocol. The steps in the cross linking protocol are as follows: 1. The His-S-SStp is first reduced by DTT and excess DTT is removed using a desalting column. 2. The APDP is iodinated with Na[^{125}I]. 3. The samples from step 1. and step 2. are mixed and kept at 4 °C O/N. Unbound [^{125}I]APDP is removed using a desalting column. 4. The His-S-SStp covalently bound to [^{125}I]APDP is used for a binding reaction with intact chloroplasts. 5. Cross-linking is achieved using UV light. 6. The label is transferred to the cross-linked protein using DTT.

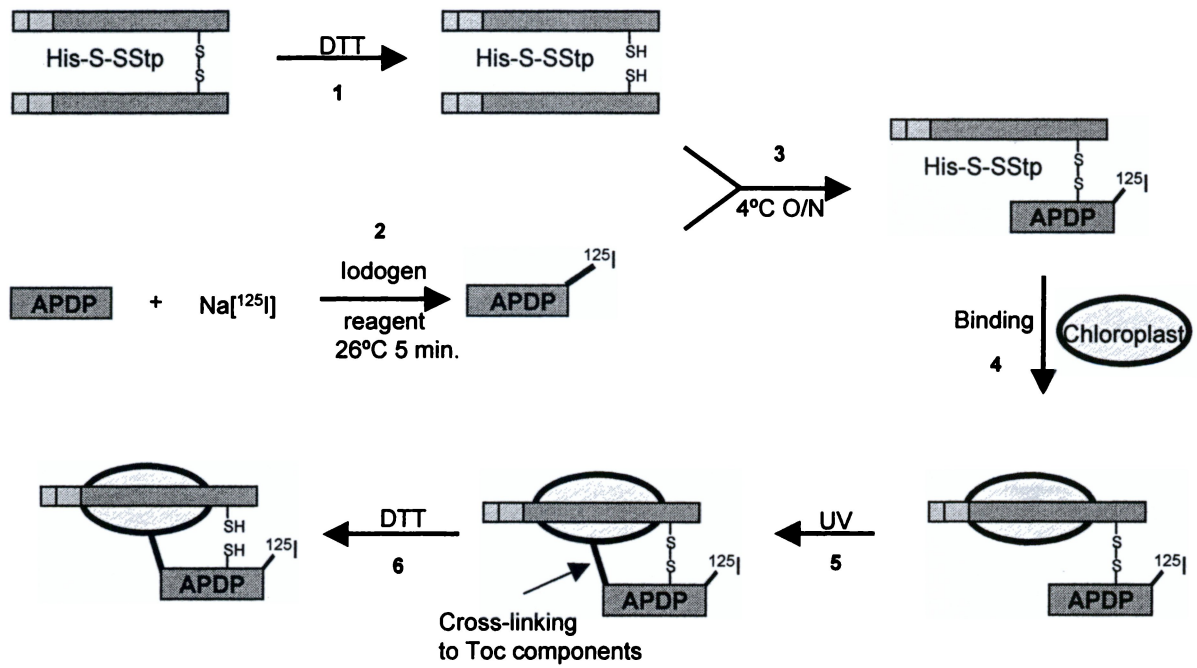

His-S-SStp
DTT
1
His-S-SStp
SH
SH
3
4°C O/N
His-S-SStp
125I
APDP
2
Iodogen
reagent
26°C 5 min.
APDP
+
Na[125I]
APDP
125I
Binding
4
Chloroplast
SH
SH
125I
APDP
DTT
6
APDP
125I
Cross-linking
to Toc components
UV
5
APDP
125I

Figure 2-11.

Figure 2-12. Label transfer reaction of [^{125}I]-APDP-modified His-S-SStp bound to the chloroplast translocation apparatus. **(a)** [^{125}I]-APDP-His-S-SStp were bound to chloroplasts in the presence of 30 μM ATP, chloroplasts were exposed to UV light for cross-linking. Envelopes were then purified by sucrose density centrifugation. The different membrane fractions were then treated with DTT-containing sample buffer to transfer the isotope to the receptor and proteins were resolved by SDS-PAGE. Equal counts were loaded per lane. **(b)** In the absence of DTT, only a single, high molecular weight complex was resolved on SDS-PAGE. Envelopes were not purified from the samples that were not UV treated.

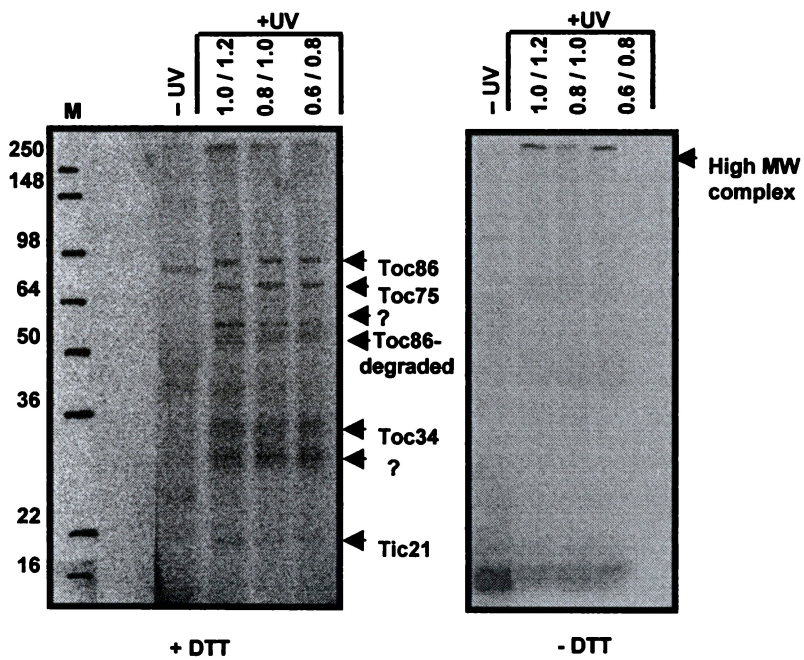

+UV
- UV
1.0 / 1.2
0.8 / 1.0
0.6 / 0.8
M
250
148
98
64
50
36
22
16
Toc86
Toc75
?
Toc86-
degraded
Toc34
?
Tic21
High MW
complex
+ DTT
- DTT

Figure 2-12.

translocon. Analysis of the reduced envelope fractions in Figure 2-12a revealed labeling of several lower molecular weight proteins including bands at 86 kDa, 75 kDa, 34 kDa and 21kDa. Similar higher molecular weight complexes have been observed by (Ma et al., 1996; Perry & Keegstra, 1994). The 86 kDa protein was identified to be as Toc86/159 and the 75kDa protein was identified to be as Toc75 by calculating the molecular weights according to their R_f values in western blots with α-Toc86/159 and α-Toc75 antiseras respectively. The R_f values of the 86 kDa and 75 kDa proteins in the cross-linking gel were only ~1 kDa different from the R_f values calculated in the western blots (data not shown). The samples from the three fractions of the sucrose gradient were loaded at equal radioactive counts. Therefore, the outer membrane fraction, the inner membrane fraction and the contact sites fraction all show similar levels of Toc86/159, Toc75 and other Toc/Tic proteins. Association of His-S-SStp to both the Toc and Tic proteins reveals that the transit peptide has engaged the chloroplast translocation machinery of both the inner and outer envelope yet is unable to be completely translocated into the stroma. The fact that S-protein FITC can be used to detect the chloroplast bound transit peptide it is obvious that the N-terminal His-S part of His-S-SStp is exposed to the cytosolic side of the chloroplast. The ability to cross-link His-S-SStp to Toc75 and Tic21 indicates that the transit peptide part of His-S-SStp is inserted into the outer envelope translocon and at least partially exposed to the inner membrane space. These results in light of the placement of the cross-linking group at the extreme C-terminus (C57), suggests that the bound transit peptide may adopt a N_{out}-C_{in} orientation in the outer envelope translocon.

APDP has a long spacer arm of 21.02 Å, which enhances the efficiency of photolabelling by increasing the distance between the photoaffinity azido and pyridyldithio groups (Pierce technical literature). When His-S-SStp is covalently bound to the cross-linker, His-S-SStp replaces the pyridyldithio group and so the distance between the photoaffinity azido group and His-S-SStp is 21.02 Å. Cross-linking is established using UV light where the proteins that are in vicinity of the azido group are cross-linked. Hence, the distance between the C-terminus of His-S-SStp and the cross-linked protein is ~21 Å. Therefore, any component of the translocon that is ~21 Å away can be cross-linked to His-S-SStp. Since we cross-linked multiple Toc proteins (Toc86/159, Toc75, Toc34 and may be Tic21), it means that different molecules of His-S-SStp are in different stages of binding. Some His-S-SStp is interacting with Toc86/159, while others are with Toc75 or Toc34.

These results are distinguished from another recently described chimeric precursor, $pS34C_{inv}$, that was shown to simultaneously use the N-terminal information of the prSSU domain to engage the Tic components in an ATP-dependent manner while the $Toc34C_{inv}$ sequence is functioning as stop-transfer domain mediating release of the C-terminus into the lipid bilayer of the outer envelope (May & Soll, 1998). The additional His-S sequence associated with Hi-S-SStp only appears to prevent the completion of translocation of SStp yet by itself does not engage components of either Tic or Toc.

Effect of ATP on His-S-SStp binding to chloroplasts

Productive interaction of a chloroplast precursor with the translocation apparatus requires energy in the form of ATP. The energetics of chloroplast protein

import can be broken down into a binding step that requires micromolar levels of either ATP or GTP (Olsen et al., 1989), and a translocation step that requires millimolar levels of ATP (Theg et al., 1989). To compare the energetic requirements of our fusion peptide to the accepted energetics of precursor binding and import, we investigated the role of ATP on the association of His-S-SStp with the chloroplast translocation apparatus by both far-western and flow cytometric analyses. Figure 2-13a shows chemiluminescent detection of His-S-SStp binding in the presence of ATP concentrations ranging from 0 to 2.5 mM. Although ATP was added to dark-adapted chloroplasts, significant levels of binding were observed in the absence of exogenous ATP, suggesting that either depletion of endogenous ATP was incomplete or that binding was only weakly ATP dependent. An attempt to lower endogenous ATP levels was done by using nigericin (40 nM). Nigericin treatment of chloroplasts, decreased the % FACS positive at 0 μM ATP from 58% to 43% but the % FACS positive at 25μM or 75μM ATP did not change. The trends of % FACS positive were similar in nigericin treated and untreated chloroplasts (data not shown).

Both the far-western blotting (Figure 2-13a) and the flow cytometric assays (Figure 2-13b), provided similar results revealing that the extent of His-S-SStp binding increased only slightly as exogenous ATP levels were increased, and then slowly dropped off. Analyzing the percent FACS-positive staining as a function of ATP concentration indicated that maximum binding of His-S-SStp occurred at 25 μM ATP and then slowly decreased at higher ATP concentrations (Figure 2-13c). This decline could represent import and degradation of His-S-SStp or, alternatively, at high ATP concentrations, a nucleotide-dependent decrease in the affinity of one or

Figure 2-13. Effect of ATP and GTP on binding of His-S-SStp to chloroplasts. **(a)** Chemiluminescent detection of a blotting analysis showing the interaction of His-S-SStp with intact chloroplasts as a function of [ATP] except in lane 6, where 500μM of both ATP and GTP were added. S-Protein Alkaline Phosphatase was used to detect the chloroplast bound transit peptide in panels (a) and (d). A control where no His-S-SStp was added in a binding reaction is shown as Cts. alone in (a) and (d). **(b)** FACS analysis showing the interaction of His-S-SStp with intact chloroplasts as a function of [ATP]. The intact chloroplasts stained positive with SP-FITC are shifted to the right compared to the control with no His-S-SStp (black). **(c)** Quantitative graph representing the data in (b and e). **(d)** Chemiluminescent detection of a blotting analysis showing the interaction of His-S-SStp with intact chloroplasts as a function of [GTP] except in lane 6, where 500μM of both ATP and GTP were added. **(e)** FACS analysis showing the interaction of His-S-SStp with intact chloroplasts as a function of [GTP]. The intact chloroplasts stained positive with SP-FITC are shifted to the right compared to the control with no His-S-SStp (black). **(f)** LSCM of His-S-SStp bound to chloroplast in the presence of ATP, GTP or both. *Top three* panels show positive staining in green in the presence of 30 μM ATP. In the *bottom row* the *left* panel is a negative control, the *middle* panel shows binding of His-S-SStp to chloroplasts is inhibited in the presence in the presence of 500 μM GTP. The *right* panel in the *bottom row* shows the binding of His-S-SStp to chloroplasts is inhibited when both ATP (500 μM) and GTP (500 μM) are present.

Placeholder

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more components of the translocation apparatus for His-S-SStp, could result in lower binding levels.

Effect of GTP in the binding of His S-SStp

Previous results on the requirement of GTP for precursor binding have been contradictory. Kouranov and Schnell have proposed that GTP is essential for transition of the precursor protein to later stages of import via a GTP binding and hydrolysis by Toc34 (Kouranov & Schnell, 1997). Young *et al.* has postulated that GTP promotes the early import intermediate formation, which is facilitated by both Toc34 and Toc86/159. They also observed that GTP has no role in the translocation stage of import rather it influences the early-import formation (Young et al., 1999).

All three of our assays (far-Western blotting, flow cytometry, and LSCM) demonstrate that GTP inhibits the early intermediate formation with His-S-SStp as seen in Figure 2-13 c-f. Addition of ATP along with GTP does not relieve the inhibition of the early translocation intermediate formation of His-S-SStp as seen by both the chemiluminescent detection (Figure 2-13a and 2-13d) and by confocal microscopy (Figure 2-13f). Hence, we propose that probably Toc 159 and/or Toc34 is influenced by the GTP-binding status potentially functioning as a molecular switch (Bourne et al., 1991). According to our results, one or both of the GTPase subunits of the Toc complex (Toc 159 and/or Toc34) switches to a low affinity form upon binding GTP. Upon either hydrolysis of GTP to GDP or by direct GDP binding the GTPase is converted to new form, which displays high transit peptide affinity. Such a change in affinity is common for many GTP-dependent receptors (Bourne et al., 1991). According to our model Toc86/159 or Toc34 are in an "inactive" state in the

presence of GTP and in an "active" state when GTP is hydrolyzed to GDP. This model is supported by experiments, which indicate an increase in His-S-SStp binding in the presence of added GDP (data not shown). Further experimentation will be required to evaluate the precise role for each nucleotide triphosphate in the chloroplast binding activity of this novel reagent. The speed and sensitivity of this detection system may allow important insights into the role of ATP/GTP binding and hydrolysis in the early stages of precursor binding and translocation. Moreover, the inability of His-S-SStp to undergo complete translocation may permit a unique energetic state that may allow the different roles of the Toc 159 and Toc 34 to be differentiated. Hence, we propose a model for the role of GTP in the binding of His-S-SStp to the chloroplast translocation apparatus as shown in Figure 2-14. According to our model addition of GTP would cause a normal chloroplast precursor to be further inserted into the translocon due to a change in the conformation of Toc34 in the presence of GTP. However, due to the presence of a charged N-terminus, the His-S-SStp is unable to enter the translocon and so upon GTP addition it is released from the Toc complex.

Laser scanning confocal microscopy detection of bound His-S-SStp

Although the flow cytometric analysis of His-S-SStp binding to the chloroplasts has provided an accurate measurement of the total number of transit peptides bound to individual chloroplasts, it did not provide any information regarding the distribution of bound molecules to the chloroplast surface. Utilizing LSCM of intact chloroplasts after a binding reaction with His-S-SStp and labeling with S-protein FITC conjugate, we could directly visualize the distribution of transit

Figure 2-14. A model for GTP modulation of His-S-SStp binding to chloroplast. The *top* panel shows the binding of any natural chloroplast precursor, where addition of GTP supports the binding and import of the precursor. The *bottom* panel shows the binding of His-S-SStp, where the addition of GTP releases the His-S-SStp from the translocon.

Placeholder

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peptides bound to the outer chloroplast envelope (Figure 2-15). The top left panel shows a superimposition micrograph of the two panels on the right the FITC channel and the chlorophyll autofluorescence. These chloroplasts have been labeled with His-S-SStp and S-protein FITC. The second row shows an immunofluorescent staining of Toc75. The third row is an immunofluorescent staining of a precursor protein prSSU. The bottom most row shows the staining of control chloroplasts where S-protein FITC was added but not His-S-SStp. The intense green staining indicates the regions of His-S-SStp binding to the chloroplast surface. Comparison of the first two rows with the third row indicates that both the His-S-SStp and Toc75 show a similar punctate staining pattern compared to the prSSU. This observation and the APDP cross-linking results suggest that the His-S-SStp is probably associated with the Toc75 complex.

These images represent an extended focus image where each optical series was compressed to a single section. The extent of labeling was not evenly distributed over the surface of the chloroplast, suggesting that the 2000-3000 different translocation sites are not uniformly distributed over the chloroplast surface (Friedman & Keegstra, 1989; Froehlich & Keegstra, 1997). Regions of intense fluorescence appeared isolated from one another and in some cases, seemed to form protuberances extending out from the chloroplast surface. Whether these fluorescent, transit peptide-binding protuberances represent the newly described tubular projections that have been observed to emanate from individual chloroplasts *in vivo* (Kohler et al., 1997) cannot be determined from this analysis. Previous studies of a translocation intermediate of prSSU also noted a patch-like immunofluorescent staining pattern, which was similar to our observation (Schnell &

Figure 2-15. LSCM of His-S-SStp bound to chloroplast. *Top row* shows the LSCM of control intact chloroplasts from a His-S-SStp binding reaction. The *second row* shows the immunofluorescence staining of Toc75. The *third row* shows LSCM of individual intact chloroplasts showing the interaction with prSSU. The *fourth row* is a negative control where no His-S-SStp was added but S-Protein FITC was added. S-protein FITC was used to detect the chloroplast bound His-S-SStp. The chlorophyll auto fluorescence is shown in red and the FITC stained bound His-S-SStp is shown in green. FITC/Chlorophyll is an overlay of the chloroplasts seen in the FITC channel and in the chlorophyll channel.

Placeholder

REPLACE IMAGE

Blobel, 1993). The biochemical basis of this patch-like labeling is not entirely clear. One interpretation is that extended regions of contact form between the inner and outer envelope membranes, which enables precursors to translocate across both membranes in a concerted manner. The very bright staining that we observed suggests that possibly hundreds of transit peptides bind to a supra-aggregation of contact sites in the envelope. Current models of protein translocation fail to account for this additional level of organization of the translocation apparatuses.

In addition to the staining pattern described above, not all chloroplasts labeled to the same extent, as a population we also observed heterogeneity in staining. Staining heterogeneity was also observed in the flow cytometric analyses, which often identified two populations of labeled chloroplasts, the hump in the positive stained region represents more brightly stained chloroplast compared to the peak to its left (Figure 2-5b). The biological basis of this heterogeneity is not known, however the chloroplast preparations used in these studies are made from 13-day old pea seedlings, which contain not only leaves at different developmental stages, but also the leaf petioles and stem tissue. Plastids derived from one or more of these tissues may reflect a developmental regime that does not require highly active chloroplast protein transport (Dahlin & Cline, 1991), and therefore, the envelopes of these plastids may not contain as many active translocation complexes. Clearly, the ability to visualize and locate the translocation apparatus on intact plastids as well as the ability to physically sort out different plastid sub-classes will provide a powerful new insights into the development, assembly, and function of the protein translocation apparatus.

CONCLUSIONS

In this chapter, it has been demonstrated and characterized an early interaction between the chloroplast translocation apparatus and a novel fusion protein containing a full-length transit peptide. The epitope tags associated with this fusion protein enabled both facile purification as well as a sensitive and specific detection of the transit peptide. Although the fusion protein was able to successfully engage the translocation apparatus, it differs from authentic precursors and transit peptides by its inability to translocate into the stroma. Unlike other chimeric precursors, the translocation defect of His-S-SStp results in a long-lived early translocation intermediate that accumulates at or within the outer envelope translocation apparatus (America et al., 1994; Froehlich & Keegstra, 1997). Detection of the bound transit peptide by a fluorescent reporter facilitated novel cytometric analysis by both flow cytometry and LSCM.

The use of site-directed and deletional mutagenesis of the transit peptide used in the following chapters, in combination with the assays described in this chapter, provides a powerful approach for evaluating the role(s) of different regions of the transit peptide. In addition, the combination of chemical cross-linking of this intermediate followed with membrane solubilization and recovery via IMAC may provide a methodology to isolate new components of the translocation machinery. Finally, these tools may be highly relevant to developmental studies of the regulation and assembly of the translocation apparatus in chloroplasts as well as other plastid subtypes.

Chapter 3

Identification of the region essential for binding of the transit peptide to the chloroplast

ABSTRACT

The apparent lack of primary sequence homology among chloroplast transit peptides suggests that the chloroplast specificity may depend upon a common secondary or tertiary structure of the transit peptides. However, not much is known about the secondary or tertiary structure of the transit peptides. Analysis of the transit peptide sequence suggests the presence of one or more conserved H(P/G)H(R/K) (Hydrphobic amino acid (Proline/Glycine)Hydrophobic amino acid (Arginine/Lysine)) motif in transit peptides, which contributes to the targeting of the precursor to chloroplasts (Karlin-Neumann & Tobin, 1986; Pilon et al., 1995). In addition, the work on transit peptides from ferredoxin (Fd) suggests that there are at least four domains and each domain has a role in chloroplast recognition and translocation (Pilon et al., 1995). Further, it has also been hypothesized that chloroplast transit peptides form specific secondary structures when they interact with specific lipids in the outer membrane of chloroplasts. Using synthetic peptides that mimic the different regions of the transit peptide of the small subunit of Rubisco (SStp) it has been demonstrated that the C-terminal region has the maximum lipid interacting capability (Pinnaduwage & Bruce, 1996). Hence, we made C-terminal deletions of SStp and tested them for chloroplast binding and lipid interacting capability. We used an epitope tagged transit peptide (His-S-SStp) for our studies as I then could directly monitor the effect of the transit peptide without the

contributions of the mature domian. The removal of amino acids from the C-terminus of His-S-SStp causes certain structural and functional changes in the transit peptide of SStp like lowering of α-helical content. Here I have shown that removal of one or both H(P/G)H(R/K) motifs prevents the interaction of the transit peptide with the chloroplasts. I have used calcein dye release assay to study the role of the C-terminus in lipid interaction and have shown that the C-terminal deletion mutants have increased lipid interaction than the full length transit peptide. In addition, I have analyzed the structure of the transit peptide by circular dichroism spectroscopy and shown that deleting residues from the C-terminus of His-S-SStp reduced the α-helical content. This study provides direct evidence on the role of the H(P/G)H(R/K) motif in the interaction of transit peptide with the chloroplast translocation apparatus.

INTRODUCTION

Recent genome sequence analysis of *Arabidopsis thaliana* by Target P prediction analysis predicted that 3,574 different protein encoded by the nucleus are targeted to the chloroplasts, of these only 2085 show >0.95 specificity to the chloroplasts (Initiative, 2000). The apparent lack of primary sequence homology among chloroplast transit peptides suggests that the chloroplast specificity may depend upon a common secondary or tertiary structure of the transit peptides. However, due to the limited structural information on the transit peptides it is difficult to emphasize on the significance of the structure of transit peptides in precursor targeting and translocation in chloroplasts. Previous analysis of transit peptides revealed the presence of a conserved H(P/G)H(R/K) (Hydrphobic amino acid (Proline/Glycine)Hydrophobic amino acid (Arginine/Lysine)) motif in transit

peptides, which contributes to the targeting of the precursor to chloroplasts (Karlin-Neumann & Tobin, 1986; Pilon et al., 1995). In addition, the work on transit peptides from ferredoxin (Fd) suggests that there are at least four domains and each domain has a particular function (Pilon et al., 1995). It is possible that both of these play a role in the recognition and translocation of precursor proteins in the chloroplasts.

Phosphorylation of the precursor has been identified as one of the essential factors that influence its recognition by the chloroplast translocation apparatus (Waegemann & Soll, 1996). However, for the pre-protein to be translocated it had to be dephosphorylated by a yet unidentified protein import phosphatase. The phosphorylation motif was identified as (P/G)X_n(R/K)X_n(S/T) Xn(S*/T*), where n = 0-3 amino acid spacer and S*/T* represents the phosphate acceptor. Recently, chloroplast precursors synthesized in wheat germ lysate were found to interact with 14-3-3 plant proteins (May & Soll, 2000). This complex consisted of the precursor protein, 14-3-3 protein (probably as a dimmer) and a heat shock protein Hsp70 isoform. This complex had an apparent molecular weight of 200 kDa. It was observed that precursors in such a complex form are in a highly import competent state and showed 3 to 4 fold higher import efficiency compared to free precursor. The binding motif of the 14-3-3 complex was identified to be related to the phosphorylation motif. Moreover, it has been identified that the GTP mediated phosphorylated form of Toc34 recognizes only the phosphorylated form of the precursors (Sveshnikova et al., 2000). It is interesting to note that this phosphorylation site in SStp is present between the two H(P/G)H(R/K) motifs.

However, since all our experiments are done *in vitro* the 14-3-3 complex is not present in the assay conditions.

The work done by Pilon and co-workers suggests that the whole sequence of Fd transit peptide is important as most deletions in the transit peptide region interfered with the overall efficiency of the precursor Fd protein import (Pilon et al., 1995). They concluded that there are four major functional domain in the transit peptide of Fd. The *first* domain was identified to comprise of the first 14 N-terminal amino acids, which are mainly uncharged. They concluded that this region had the information for targeting to the correct membrane, probably to interact with the lipids like monogalactosyldiacylglycerol (MGDG) in the chloroplast outer membrane. The *second* domain was observed to be in the 15 to 25 amino acid region, which they concluded to be essential for *in vitro* chloroplast import and not recognition by the chloroplast. The loss of flexibility due to the removal of this domain prevented the recognition of the pre-sequence with other Toc proteins. The *third* region was identified to be from amino acids 26 to 38 amino acids, which they concluded to be responsible for the overall efficiency of translocation. This region consists of mainly hydrophobic amino acids and the H(P/G)H(R/K) motif. The *forth* domain was observed to be the C-terminal region which controlled the processing of the pre-sequence to mature form.

It has also been hypothesized that chloroplast transit peptides form specific secondary structures when they interact with specific lipids in the outer membrane of chloroplasts. Structural analysis of transit peptides by circular dichroism spectrometry using membrane-mimetic solvents such as TFE (2,2,2-trifluoroethnol), detergent micelles and liposome vesicles have shown that both the N- and the C-

terminus of transit peptides have α-helical structures (Bruce, 2000; Bruce, 1998; Endo et al., 1992; Krimm et al., 1999; Wienk et al., 1999; Planque 1999). Structural analysis of *Silene pratensis* ferredoxin transit peptide (trFd) by NMR in the presence of TFE and micelles containing MGDG and PG revealed that trFd had mainly two α-helices, one at the N-terminus from V9 to S12 and the other at the C-terminus from M29 to G50. The trFd was mainly unstructured in a random coil conformation in aqueous solution but attained a helical conformation in the presence of lipids and lipid mimicking environment (Wienk et al., 1999). Spin-label NMR experiments have revealed that the transit peptide domain of prFd is able to completely reside in the hydrophobic interior of a micelle (Wienk et al., 2000). The N-terminus was found to form a dynamic α-helix, the central region was devoid of any structure while the C-terminus was found to adopt the most stable α-helical structure. Studies using synthetic peptides, mimicking the different regions of the transit peptide from small subunit of Rubisco (SStp) has revealed that the C-terminal region has the maximum lipid interacting capability (Pinnaduwage & Bruce, 1996). This interaction was observed to be highly lipid class (MGDG) dependent. Binding competitions using similar synthetic transit peptides revealed that the region from S21 to K40 inhibited the binding of prSSU by 80% compared to the extreme C-terminal peptide that inhibited binding by only 20% (Perry et al., 1991). Taking the data from Pinnaduwage & Bruce and Perry et. al. one can conclude that the C-terminal region at least had two domains, one or both of which were responsible for lipid interaction.

Hence, we made C-terminal deletions of SStp and tested them for chloroplast binding and lipid interacting capability. We have used an epitope tagged transit

peptide (His-S-SStp) for our studies as we then could directly monitor the binding of the transit peptide without the contributions of the mature domian. We have previously proved by label transfer cross-linking studies that His-S-SStp is bound at the translocon in Chapter 2. We have also proved the validity of binding and detection of the bound His-S-SStp using various assays (Subramanian et al., 2001). We have also shown previously that His-S-SStp, due to the charged N-terminus, is unable to cross the outer membrane and hence can be detected by S-protein FITC staining in FACS and LSCM. The deletion of some amino acid residues at the C-terminus of His-S-SStp causes certain changes in the structure and function of the transit peptide that we have identified in this chapter. The three C-terminal deletions His-S-SStp$_{\Delta 5}$, His-S-SStp$_{\Delta 25}$ and His-S-SStp$_{\Delta 36}$, have 2, 1 and 0 of the H(P/G)H(R/K) motifs remaining respectively. We have analyzed the role of H(P/G)H(R/K) motifs in the interaction of the transit peptide with the chloroplasts. We have also used calcein dye release assay and circular dichroism to study the role of the C-terminus in chloroplast–lipid interaction. This study provides direct evidence on the role of the H(P/G)H(R/K) motif in the interaction of transit peptides with a proteinaceous the chloroplast translocation apparatus.

MATERIALS AND METHODS

Plant growth and chloroplast isolation

Chloroplasts were isolated from dwarf pea (*Pisum sativum*) seedlings that were 11-13 days old. The pea seeds (Progresso #9) were imbibed overnight and planted on horticulture Vermiculite, approximately 300 mL of dry seeds per flat. Plants were grown in EGC growth chambers under 160 $\mu E/m^2/sec$ of cool white

fluorescent light. To reduce starch accumulation the plants were grown on a 12 hr light/12 hr dark cycle. Leaves were harvested from the seedlings and intact chloroplasts were isolated as described in literature (Bruce et al., 1994). Intact chloroplasts were resuspended at a concentration of 1 mg/ml total chlorophyll and used for all labeling/import reactions.

Cloning of SStp into the IMPACT CN vector

Primers corresponding to regions flanking the SStp-encoding region were used to amplify the insert via standard PCR protocol that engineered Nde I and Sma I restriction sites at the 3′ and 5′ ends of the amplified product. The sense primer used was 5′-TCT CTC TTC ATA TGA (Nde I) TGG CTT CTA TGA TAT CCT CTT CCGC-3′ and the anti-sense primer used was 5′-TCC CCC GGG (Sma I) CTG CAT GCA CTT TAC TCT TCC-3′. The SStp gene from the pET 30a tp construct was PCR amplified and restricted with Nde I/Sma I restriction enzymes. This restricted product was ligated into the Nde I/Sma I site of the pTYB2 vector (NewEngland Biolabs, Beverly, MA). The presence of the gene in the vector was confirmed by sequencing.

Purification of SStp from *E.coli* cells

The construct pTYB2tp placed a splicing element that has an inducible self-cleavage activity (termed intein) and a chitin binding domain at the C-terminus of SStp. This novel purification system allows the purification of SStp without the involvement of an external protease. The DNA was transformed into *E.coli* [BL(21)(DE3)] cell line. The cells were grown at 30°C to an OD_{600} of 0.6, induced with 0.5

mM isopropylthio-β-D-galactoside [IPTG] for 6 hours. Cells were harvested and frozen overnight at –20°C. Cells were lysed in a French press with ice cold 1X column buffer (500 mM NaCl, 20 mM HEPES pH 8.0, 1 mM EDTA) containing 200 μM PMSF, 2 μM Leupetin and 2 μM Pepstatin as protease inhibitors. The lysate was centrifuged at 50,000 *g* and the supernatant was loaded on to a chitin column at a rate of 1 mL/min. The column was washed with 3 column volumes of 1X column buffer containing 0.2 % Triton X-100 at a flow rate of 2 mL/min to remove all the unbound proteins. The column was then washed with 8 column volumes of 1X column buffer at a flow rate of 2 mL/min to remove all the Triton X-100. The column was then incubated with 2 column volumes of 1X column buffer containing 50 mM DTT for the cleavage of the fusion protein. The column was then plugged and kept aside at 4°C overnight. The protein was eluted using 5 column volumes of 1X column buffer at a rate of 1mL/min and 500μL fractions were collected. The protein concentration was determined using Bradford protein assay.

Competition of His-S-SStp binding with SStp

His-S-SStp binding assays were performed as mentioned earlier. The His-S-SStp concentration was kept constant at 5μM and the SStp concentration was varied from 0 to 15μM. The ATP concentration was maintained at 30 μM and the binding reaction was performed for 8 minutes in the dark. Intact chloroplasts were isolated on 40% percoll and subjected to SDS-PAGE and far-western blotting. The bands on the film were quantitated using a scanning densitometer (UVP Inc. Upland CA).

C-terminal deletions of His-S-SStp

C-terminal deletions of His-S-SStp were made using the Erase-a base system purchased from Promega (Madison, WI). The clone pET-30atp was first restricted with Hind III and the overhang ends were Klenow filled with α-phosphorothioate dNTP's to protect the 3′ end from Exonuclease III digestion. The sample was then restricted with EcoR I to create a 5′ overhang end for Exonuclease III. Exonuclease III reaction was performed at 4°C. Aliquotes were removed every 30 seconds into tubes containing S1 nuclease. All samples were incubated at room temperature for 30 minutes. Then the reaction was stopped using S1 stop buffer and the enzyme was heat inactivated. The ends were Klenow filled to ensure proper blunt ends and then liagated at room temperature using T4 DNA Ligase. The ligated products were transformed into XL-1 Blue high efficiency competent cells purchased from Promega (Madison, WI). Universal T7 promoter and universal T7 terminator primers were used to screen colonies by direct colony PCR . The colonies that showed shorter PCR fragments compared to the wild type were sequenced to confirm the extent of C-terminal deletion.

***E.coli* protein expression**

The deletion proteins were purified similar to His-S-SStp IMAC purification as described in Chapter 2.

***In vitro* binding of His-S-SStp C-terminal deletions to intact chloroplasts**

Binding reactions of the C-terminal deletions of His-S-SStp were performed similar to the binding reactions of His-S-SStp. The C-terminal deletion mutants were

added to a final concentration of 5 μM. The ATP concentration was maintained at 30μM and the binding reaction was performed for 8 minutes in the dark. Intact chloroplasts were isolated on 40% percoll and subjected to SDS-PAGE and far-western blotting. The samples were stained with S-protein FITC and analyzed by FACS and LSCM. The FACS and LSCM analyses were performed as mentioned earlier.

S-protein FITC labeling of the chloroplast-bound His-S-SStp

The samples post-binding reaction as described above were centrifuged at 1,500 *g* for 6 minutes to wash the unbound His-S-SStp/ His-S-SStp deletions and resuspended in 300 μL of Blocking Solution (1X IB containing 1% BSA (Bovine Serum Albumin) + 1% non-fat milk). S-protein FITC (Novagen, Madison, WI) was added at 1:500 dilution and incubated for 30 minutes on ice. Intact chloroplasts were purified over a 40% Percoll cushion as mentioned earlier, resuspended in 1X IB and used for FACS analysis and/or Laser Scanning Confocal Microscopy.

Flow Cytometry

A dual-laser Becton Dickinson FACStar Plus was used for the flow cytometric analyses. Data were acquired and analyzed using Becton Dickinson CellQuest V. 3.1 software. The argon ion laser in the first position was tuned for 488 nm output running on a constant output mode at 250 milliwatts. Forward angle light scatter was collected with the forward photodiode without a neutral density filter in place. The right angle light scatter used a 488/10 nm band pass filter. Dot plot analysis of forward and right angle light scatter was used to determine the percent

intact plastids (~70-80% of total plastids) (Kausch & Bruce, 1994). Total chloroplasts were gated to include only the intact plastids for both FL1 (chlorophyll autofluorescence) and FL2 (S-protein FITC). Detection by FL1 was selected via a dichroic mirror and a DF 530/30 nm filter. FL2 was selected via the same dichroic mirror and a DF 660/20 nm filter. Fluorescence of 25,000 intact chloroplasts was analyzed per sample. The side scatter, chlorophyll fluorescence, and FITC fluorescence were collected in log mode with four-decade log amplifiers. The extent of positive labeling was set manually with unlabeled chloroplasts for each experiment to yield value of 2-3% FACS positive. Flow rate was usually maintained at ~1200 chloroplasts/min. Sheath buffer was composed of 1X PBS (Phosphate Buffered Saline).

Laser scanning confocal microscopy

The S-protein FITC stained samples after a binding reaction were analyzed using a Lieca TCS-NT laser confocal microscope. FITC and chlorophyll autofluorescence were simultaneously visualized (two channel collection) using 488 excitation (Ar ion laser) into a 530/30 BP filter (FITC) and a 660 LP filter (chlorophyll auto-fluorescence). Images were collected with a 100X, 1.4 NA oil immersion lens at an Airy disc setting of 0.91. The data are presented as extended focus projections.

SDS-PAGE and far-western blotting

Samples were boiled in 4X SB and were resolved on an 18% SDS-PAGE gel. The gel was electro-blotted on to a pre-wetted Immobilon-P membrane (Millipore,

Bedford, MA) and blocked with TBS (Tris Buffered Saline) containing 1% gelatin + 0.5 % Tween-20 for 15 minutes. The S-peptide containing proteins were detected by probing for 15 minutes with an S-protein alkaline phosphatase conjugate (Novagen, Madison, WI) diluted 1:100,000 in TBS and then washed extensively with TBS + 0.2% Tween-20. Visualization was conducted using a 1:1 dilution of the chemiluminescent substrate LumiPhos Plus (Lumigen Inc., Southfield, MI) and subjecting the blot to film autoradiography. Exposure time was optimized for maximum signal to noise.

Protein and chlorophyll measurements

Protein was determined using BCA reagent (Pierce, Rockford, IL). Chlorophyll concentration was determined after chlorophyll extraction using 80% acetone (Bruce 1994) as described earlier.

Liposome preparation

Liposomes were prepared as described earlier (Pinnaduwage & Bruce, 1996) by mixing appropriate concentration of individual lipids to a final concentration of 5mM. The liposome composition of the different liposomes is given in the result section. The solvent was then evaporated under a stream of N_2 gas and was vacuum desiccated overnight to remove any trace solvents. The dried lipid film was then hydrated with 0.1X phosphate buffered saline (pH 7.8) containing 1 mM EGTA, 0.02% Na-azide and 200 mM calcein. The mixture was vortexed vigorously and then sonicated in a bath sonicator (Laboratory Supplies Inc.) for 10 minutes. The sonication was repeated several times at 6-12 hour intervals until the lipid mixture

was clear and no longer turbid indicating liposome formation. Free unincorporated calcein and lipids were removed using a Bio-Gel A-0.5m column equilibrated with 0.1X phosphate buffered saline containing 1 mM EGTA, 0.02% Na-azide. Liposomes that eluted in the void volume fractions had a calcein fluorescence quenching of >70-80%. All liposome assays were performed in an 8 X 12 format 96 well glass plate (Kontes glass, Vineland, NJ). Fluorescence of the liposomes was analyzed using a Wallac Victor^2 1420 Multilabel counter (Turku, Finland) fluorescence plate reader. Excitation wavelength was set at 485 nm using a fluorescein excitation filter and emission wavelength was set at 514.5 nm using a narrow band pass (10 nm) filter at 514.5 nm. % Quenching was calculated from the formula below.

$$\%Quenching = \left(1 - \frac{F_0}{F_t}\right) \times 100\%$$

F_0 and F_t are the fluorescence of the liposome samples before and after addition of 0.1% Triton X-100, respectively.

Protein/Peptide-induced calcein release liposome assay

Calcein entrapped liposomes showing >70-80% fluorescence quenching were incubated at room temperature with various proteins at varying concentrations as indicated in the figure legends for 1 hour. All liposome assays were performed in an 8 X 12 format 96 well glass plate (Kontes glass Vineland, NJ). Fluorescence of the liposomes was analyzed using a Wallac Victor^2 1420 Multilabel Counter (Turku, Finland) fluorescence plate reader. Excitation wavelength was set at 485 nm using a

fluorescein excitation filter and emission wavelength was set at 514.5 nm using a narrow band pass (10 nm) filter at 514.5 nm. The percentage of calcein release was calculated using the formula below.

$$\%\mathrm{Re}\,lease = \left(\frac{F - F_0}{F_t - F_0}\right) \times 100\%$$

F_0 and F are the calcein fluorescence before and after addition of the protein/peptide, respectively, and F_t is the total fluorescence after addition of 0.1% Triton X-100. All measurements were done in triplicate and the graph indicates the standard deviation as error bars.

Competition of His-S-SStp binding with OM liposomes

His-S-SStp binding assays were performed as mentioned earlier. The His-S-SStp concentration was kept constant at 5 μM and the amount of lipids added to the reaction was varied from 0 to 1000 μM. The ATP concentration was maintained at 30 μM and the binding reaction was performed for 8 minutes in the dark. Intact chloroplasts were isolated on 40% percoll, stained with S-protein FITC and analyzed by FACS. The FACS analysis was performed as mentioned in Chapter 2.

Circular dichroism spectrometry

Circular dichroism spectrometry was performed using an Aviv-202 Circular dichroism Spectrometer (Aviv Instruments, Lakewood, NJ) at 25°C using a quartz cell with 0.1cm path length. Protein/Peptide was added to a final concentration of 1

μM with varying amounts of 2,2,2-trifluoroethnol (TFE). The CD spectra are reported in Molar Ellipticity after baseline correction for the buffer (0.1 X phosphate buffered saline, pH 7.8). The CD data was deconvoluted using *CDNN* CD spectra deconvolution program (version 2.1) obtained from http://bioinformatik. biochemtech.uni-halle.de/cdnn/ web site (ref). The data was also analyzed using the K2d program for predicting the protein secondary structure obtained from http://www.embl-heidelberg.de/~andrade/k2d.html (ref). The final graph of % alpha helix Vs % TFE was plotted after comparison of the data from both the analyses.

RESULTS AND DISCUSSION

Purification of SStp

We have shown that His-S-SStp is a valid chloroplast substrate (Subramanian et al., 2001). The His-S-domain at the N-terminus of SStp does not prevent it from interacting with the chloroplast but it seems to prevent its translocation. In order to obtain SStp without any epitope tag SStp from pET30atp was subcloned into pTYB2 vector. The sequence of His-S-SStp and SStp are given in Figure 3-1a. This vector allows one-step purification of SStp using a self-cleavable affinity tag (Chong et al., 1998). This novel protein purification system utilizes the inducible self-cleavage activity of a protein splicing element (intein) to separate native SStp from the chitin binding domain (CBD, affinity tag) without the use of a separate protease. Upon induction with IPTG, the fusion protein $^{+}NH_3$-SStp-Intein tag-CBD-COO^{-} is made in *E.coli* cells. The CBD allows the affinity purification of the fusion protein on a chitin column. In the presence of thiols like DTT or β-mercaptoethanol or cysteine,

Figure 3-1. Sequence and purification profile of SStp from *E.coli* **(a)** The SStp sequence is given, the SStp was subcloned into the Nde I-Sma I site of vector pTYB2 from pET30a-tp. The intein tag at the C-terminus has self-cleavable activity inducible by DTT. **(b)** A Coomassie stained SDS-PAGE gel showing the purification of recombinant SStp. MW stands for the broad range marker (Bio-Rad) and the molecular weights are as specified. SM is the total starting material after the cells were induced with 1mM IPTG for 3 hours. The cell lysate was obtained by French Pressing the induced cells under non-denaturing conditions. The Sup. represents the supernatant after centrifugation of the cell lysate at 50,000 x g. FT is the flow-through from loading the supernatant on the chitin column. The wash is obtained by washing the column using wash buffer. Fractions in lanes 7-19 represent the eluted fractions after DTT cleavage. The SStp (5.8 kDa), intein-CBD (56 kDa) and DnaK (70k Da) are indicated in the figure.

(a)

$_{2}^{HN}$-MASMI SSSAV TTVSR ASRVQ SAAVA PFGGL

KSMTG FPVKK VNTDI TSITS NGGRV KC$_{-COOH}$-Intein-CBD

(b)

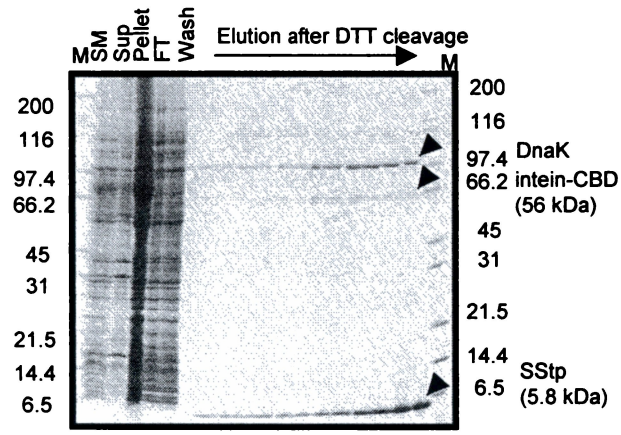


Figure 3-1.

the intein undergoes specific self-cleavage that releases native SStp from the chitin bound intein tag.

After transformation of pTYB2-SStp into competent *E.coli* cells (BL21[DE3]), induction of expression with IPTG yielded a prominent band with an apparent molecular weight of ~63 kDa in the total cell lysate when run on a non-reducing SDS-PAGE gel (lane 2 Figure 3-1b). This mobility agrees well with the predicted molecular weight of the fusion SStp-intein-CBD protein of ~62.9 kDa. The purification profile of SStp is shown in Figure 3-1b. A 63 kDa band was evident in the supernatant and pellet after cell disruption using a French pressure cell press followed by centrifugation at 50,000 *g*, (Figure 3-1b, lane 3 and 4). Since, the SStp purification is done under native conditions there is some loss of protein in the pellet. Purification of SStp was performed using a single Chitin column. This affinity matrix was effective in capturing most of the fusion protein since very little of the 63 kDa fusion protein was observed in the flow through (Figure 3-1b, lane 5). The binding of the fusion protein to the column was very tight as there was very little or no loss of protein in the 0.2% Triton X-100 wash (Figure 3-1b, lane 6). The fusion protein was then incubated with 50mM DTT to activate the intein-mediated cleavage. The elution profile of protein is shown in Figure 3-1b, lanes 7-22. It can be seen that all the lanes that contain a ~6 kDa protein also contain a ~70 kDa band and a band at ~45 kDa. The band at ~70 kDa is *E.coli* dnaK which has been shown to have affinity for SStp in *E.coli* (Ivey et al., 2000) and the ~45 kDa band is most likely the intein-CBD fusion protein. In order to remove all the contaminating proteins, the fractions containing SStp were pooled and 1 mM Mg^{2+}ATP was added to dissociate the hsp70 from SStp. The sample was then purified further using a

diaflow apparatus with a YM30 MWCO membrane (Millipore, Bedford MA) (Figure 3-2a). The purity of the protein is shown in Figure 3-2b. The flow-through contained pure SStp, which was frozen at -80°C in the presence of 2% glycerol. The purified SStp was subjected to electro-spray mass spectrometry and the mass was determined to be 5915.12 g. The mass of SStp calculated using molecular weight prediction programs was 5921.92 g. Therefore, the mass of SStp calculated by mass spectrometry is 6.8 g less than the mass predicted by DNAstar. DTT is believed to be added to the free carboxyl group during elution (Chong et al., 1997) and the first methionine is believed to be removed. If this was the case then the predicted mass of SStp would be 5919.92 g that is still less than the calculated mass by 4.72 g. The reason for the discrepancy between the mass of SStp calculated by mass spectrometry and the mass calculated by prediction programs is not clear.

Competitive inhibition of His-S-SStp binding to intact chloroplasts

The most direct way to determine if the epitope tag in His-S-SStp contributes/reduces its interaction with the chloroplasts is to test the ability of SStp to function as a competitive inhibitor for His-S-SStp binding. Using His-S-SStp as the substrate, *in vitro* binding competitions were performed. As shown in Figure 3-3a, the binding of His-S-SStp that was provided at 5μM decreased as the amount of SStp in the binding reaction was increased. Addition of ~5 μM of SStp reduced the level of bound His-S-SStp to approximately 50% of the no-competitor control (IC_{50}). (Figure 3-3a, compare lanes 2 and 6). This indicates that the binding of His-S-SStp results from specific interactions of the transit peptide with the components of the chloroplast translocation apparatus and the epitope tag (His-S) itself does not

Figure 3-2. Further purification of SStp (removal of DnaK). **(a)** The pooled fractions after an chitin were subjected to diaflow using a YM30 cut-off membrane. The flow through was pooled and concentrated using a YM5 cut-off membrane. The retentate after YM30, the retentate after YM5 and the flow-through (FT) after YM5 were run on a SDS-PAGE gel and stained with Commassie stain. Pre-stained Bio-Rad marker is shown in the last lane. **(b)** A Commassie stained 4-12% NuPAGE gel (Novex) run with purified SStp and different molecular weight markers. See Blue, See Blue Plus, Multi mark and Mark12 (from Novex), Peptide standards and Broad range markers (from Bio-Rad) were used to calculate the molecular weight of SStp.

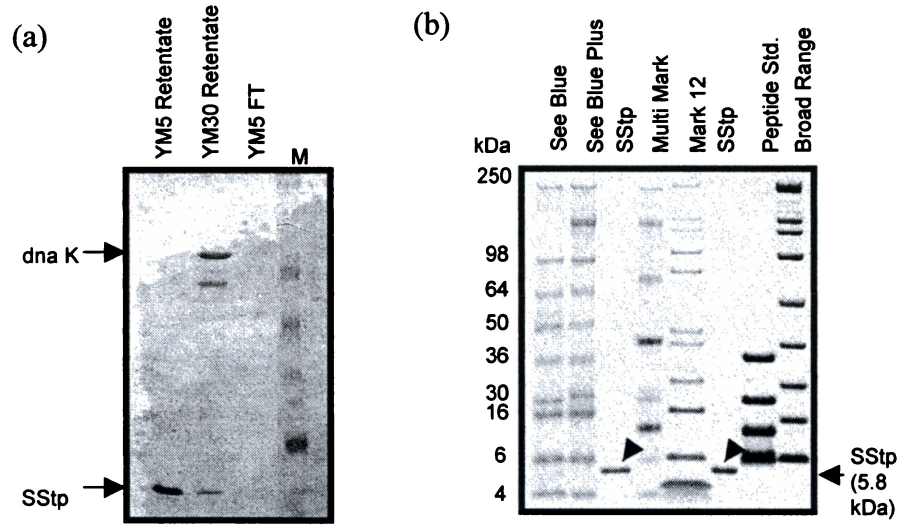
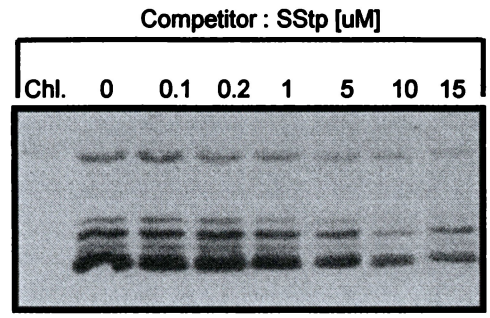

(a)
YM5 Retentate
YM30 Retentate
YM5 FT
M
dna K
SStp
(b)
kDa
See Blue
See Blue Plus
SStp
Multi Mark
Mark 12
SStp
Peptide Std.
Broad Range
250
98
64
50
36
30
16
6
4
SStp
(5.8
kDa)

Figure 3-2.

Figure 3-3. Competition of His-S-SStp binding to chloroplasts with SStp. **(a)** Chemiluminescent detection of a far-western blot showing the competition of His-S-SStp binding to the chloroplasts with increasing concentrations of SStp. Chl. is the control where no His-S-SStp was added **(b)** Quantitiation of the gel in (a) using a scanning desitometer. The 50% inhibition level is indicated.

(a)


Competitor : SStp [uM]
Chl. 0 0.1 0.2 1 5 10 15

(b)

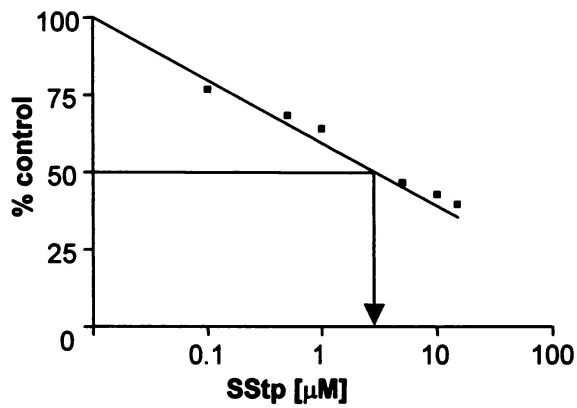

100
75
50
25
0
% control
0.1
1
10
100
SStp [μM]

Figure 3-3.

contribute in any way to the specific interaction as equal molecules of SStp competed with the binding of His-S-SStp. The quantitation of the bands in Figure 3-3a using a scanning densitometer is shown in Figure 3-3b. The binding of His-S-SStp at 5 μM is reduced to 50% of no competitor control at 5 μM of SStp, indicating that both His-S-SStp and SStp are interacting with the chloroplast in a 1:1 ratio. This therefore further re-enforces the fact that His-S-SStp is a valid transit peptide analogue and that even if the epitope tag is blocking the N-terminus of the transit peptide it can still specifically interact with the chloroplasts.

Role of the C-terminus of His-S-SStp in chloroplast interaction

Comparison of the amino acid sequences of various transit peptides in *Pisum sativum* is shown in Figure 3-4. Although these sequences have only 20% protein sequence homology, specific motifs consisting of H(P/G)H(R/K) can be identified as shown in the figure. It is observed that all transit peptides have at least one such motif. Specific domains in the transit peptide have been identified using the Ferredoxin transit peptide (trFd) from *Silene* and the small subunit of Rubisco transit peptide (SStp) from *Pisum sativum* (Archer & Keegstra, 1993; Ostrem et al., 1989; Pilon et al., 1995; Reiss et al., 1987; Reiss et al., 1989; Rensink et al., 2000; Schnell et al., 1991; Wasmann et al., 1989).

Analysis of deletion mutants of SStp indicated that the C-terminus was essential for transport and processing (Reiss et al., 1987). The same group later showed that parts of the N- and C-terminus of SStp are essential for binding of the transit peptide to the chloroplast as mutants PSd1/24, PSd6/25, PSd6/29, PSd42/58 and PSd45/57 transported inefficiently into the chloroplast (Reiss et al., 1989).

Figure 3-4. Comparison of transit peptide sequences of precursors from *Pisum sativum*. Transit peptide sequences from the precursors of carbonate dehydratase (carb), early light-induced protein (elip), ferredoxin (fd), ferritin, ferredoxin-NADP reductase (fnr), heat shock protein 21 kDa (hsp21), oxygen evolving complex 16 kDa (oe16), phospho glucomutase (pgm), plastocyanin (pc), photosystem II oxygen evolving complex (psII oe), small subunit of Rubisco (ss), ribosomal protein 9kDa (rp9), ribosomal protein 25 kDa (rp25) are compared. The H(P/G)H(R/K) identified in the transit peptides is underlined. The (-) region indicates the region that is not homologous between the various transit peptide sequences.

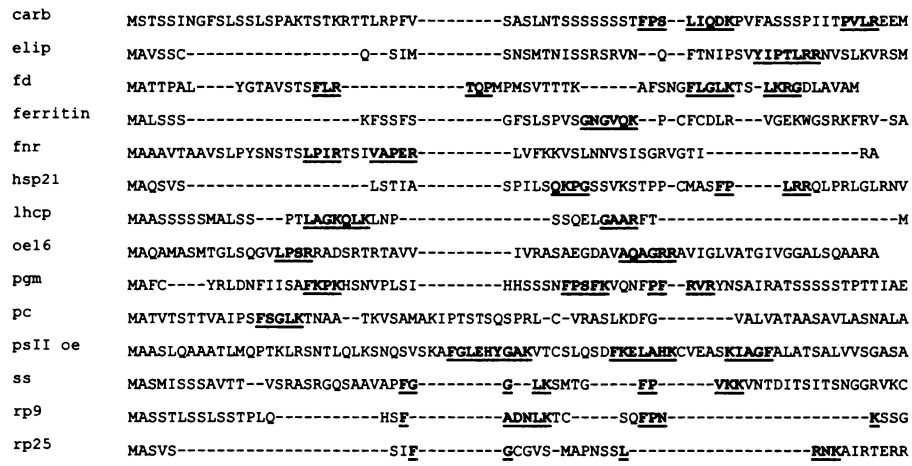

carb MSTSSINGFSLSSLSPAKTSTKRTTLRPFV---------SASLNTSSSSSSSTFPS--LIQDKPVFASSSPIITPVLREEM
elip MAVSSC------------------Q--SIM---------SNSMTNISSRSRVN--Q--FTNIPSVYIPTLRRNVSLKVRSM
fd MATTPAL----YGTAVSTSFLR-------------TQPMPMSVTTTK------AFSNGFLGLKTS-LKRGDLAVAM
ferritin MALSSS------------------KFSSFS---------GFSLSPVSGNGVQK--P-CFCDLR---VGEKWGSRKFRV-SA
fnr MAAAVTAAVSLPYSNSTSLPIRTSIVAPER----------LVFKKVSLNNVSISGRVGTI----------------RA
hsp21 MAQSVS------------------LSTIA---------SPILSQKPGSSVKSTPP-CMASFP-----LRRQLPRLGLRNV
lhcp MAASSSSSMALSS---PTLAGKQLKLNP----------------SSQELGAARFT------------------------M
oe16 MAQAMASMTGLSQGVLPSRRADSRTRTAVV----------IVRASAEGDAVAQAGRRAVIGLVATGIVGGALSQAARA
pgm MAFC----YRLDNFIISAFKPKHSNVPLSI---------HHSSSNFPSFKVQNFPF--RVRYNSAIRATSSSSSTPTTIAE
pc MATVTSTTVAIPSFSGLKTNAA--TKVSAMAKIPTSTSQSPRL-C-VRASLKDFG--------VALVATAASAVLASNALA
psII oe MAASLQAAATLMQPTKLRSNTLQLKSNQSVSKAFGLEHYGAKVTCSLQSDFKELAHKCVEASKLAGFALATSALVVSGASA
ss MASMISSSAVTT--VSRASRGQSAAVAPFG---------G--LKSMTG-----FP------VKKVNTDITSITSNGGRVKC
rp9 MASSTLSSLSSTPLQ----------HSF----------ADNLKTC-----SQFPN----------K SSG
rp25 MASVS----------SIF---------GCGVS-MAPNSSL----------RNKAIRTERR

Figure 3-4.

Previous work done in our laboratory (Pinnaduwage & Bruce, 1996) using synthetic peptides showed that the C-terminal region (20 amino acid) of SStp was responsible for lipid interaction. Since this experiment was done with just the C-terminal 20 amino acid region we wanted to test the chloroplast binding and lipid interaction of the SStp that had only its C-terminus removed but had the rest of the peptide.

We made C-terminal deletions of the His-S-SStp using the Erase-a base system from Promega. Due to the inefficient activity of the Exonuclease III only three useful C-terminal deletions could be generated. Figure 3-5 shows a 4% acrylamide gel after direct colony PCR of the transformants using universal T7 promoter and universal T7 terminator primers. It can be seen that in most cases the Exonuclease III was either too efficient or too inefficient. The deletions that were used are shown in Figure 3-5. The pET 30a vector containing the insert ligated in the "b frame" causing all the three deletions to have five extra amino acids (LAALE) in addition to a hexa Histidine tag at the C-terminus (Figure 3-6a). Most of the earlier work involving deletion mutants of SStp was done by introducing restriction sites in the coding region of the gene, which introduced extra amino acids in middle of the protein (Reiss et al., 1987; Wasmann et al., 1989). Our approach to create deletions does not introduce extra amino acids in the middle of the protein but introduces them at the C-terminus. Thus, although His-S-SStp$_{\Delta 5}$ (12.29 kDa) has 5 amino acids deleted from the C-terminus, it is larger is size than wt His-S-SStp (11.64 kDa). Similarly, although His-S-SStp$_{\Delta 25}$ has 25 amino acids deleted and His-S-SStp$_{\Delta 36}$ has 36 amino acids deleted from the C-terminus their molecular weights are 10.27 kDa and 9.22 kDa respectively (Figure 3-6b). The Commassie stained SDS-PAGE gel shows that the proteins were purified to homogeneity.

Figure 3-5. Direct colony PCR samples run on 4% acrylamide gel. The PCR products that showed C-terminal deletions in His-S-SStp are indicated, the PCR product of His-S-SStp is also indicated. GIBCO 100 bp ladder is shown in the two corner lanes.

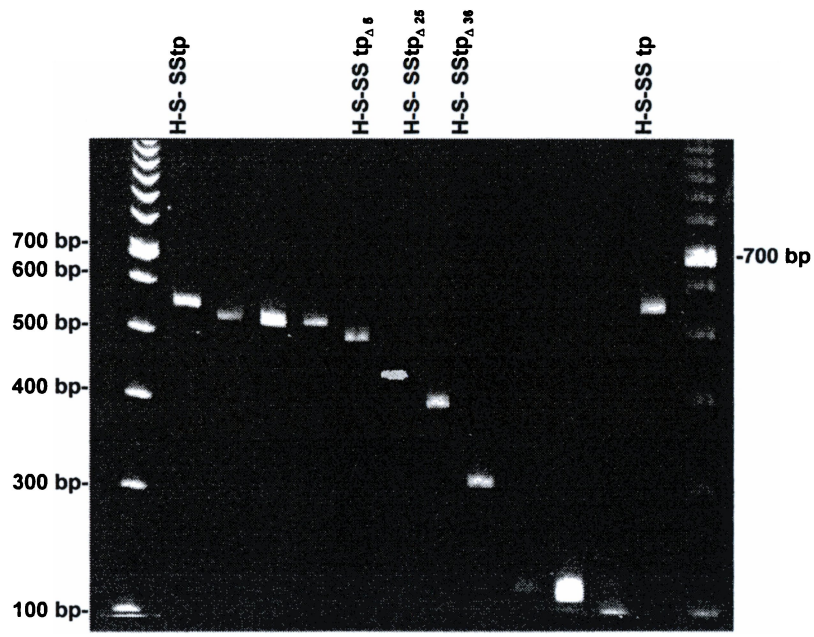

H-S- SStp
H-S-SS tp$_{\Delta 8}$
H-S- SStp$_{\Delta 25}$
H-S- SStp$_{\Delta 36}$
H-S-SS tp
700 bp-
600 bp-
500 bp-
400 bp-
300 bp-
100 bp-
-700 bp

Figure 3-5.

Figure 3-6. Sequence, Commassie stained gel and binding of C-terminal deletion mutants of His-S-SStp to chloroplasts. **(a)** Sequence information of His-S-SStp and its C-terminal deletions. **(b)** Commassie stained SDS-PAGE showing purified His-S-SStp, His-S-SStp$_{\Delta 5}$, His-S-SStp$_{\Delta 25}$ and His-S-SStp$_{\Delta 36}$. **(c)** Chemiluminescent detection of a far-western blot showing the binding of His-S-SStp and the C-terminal deletions.

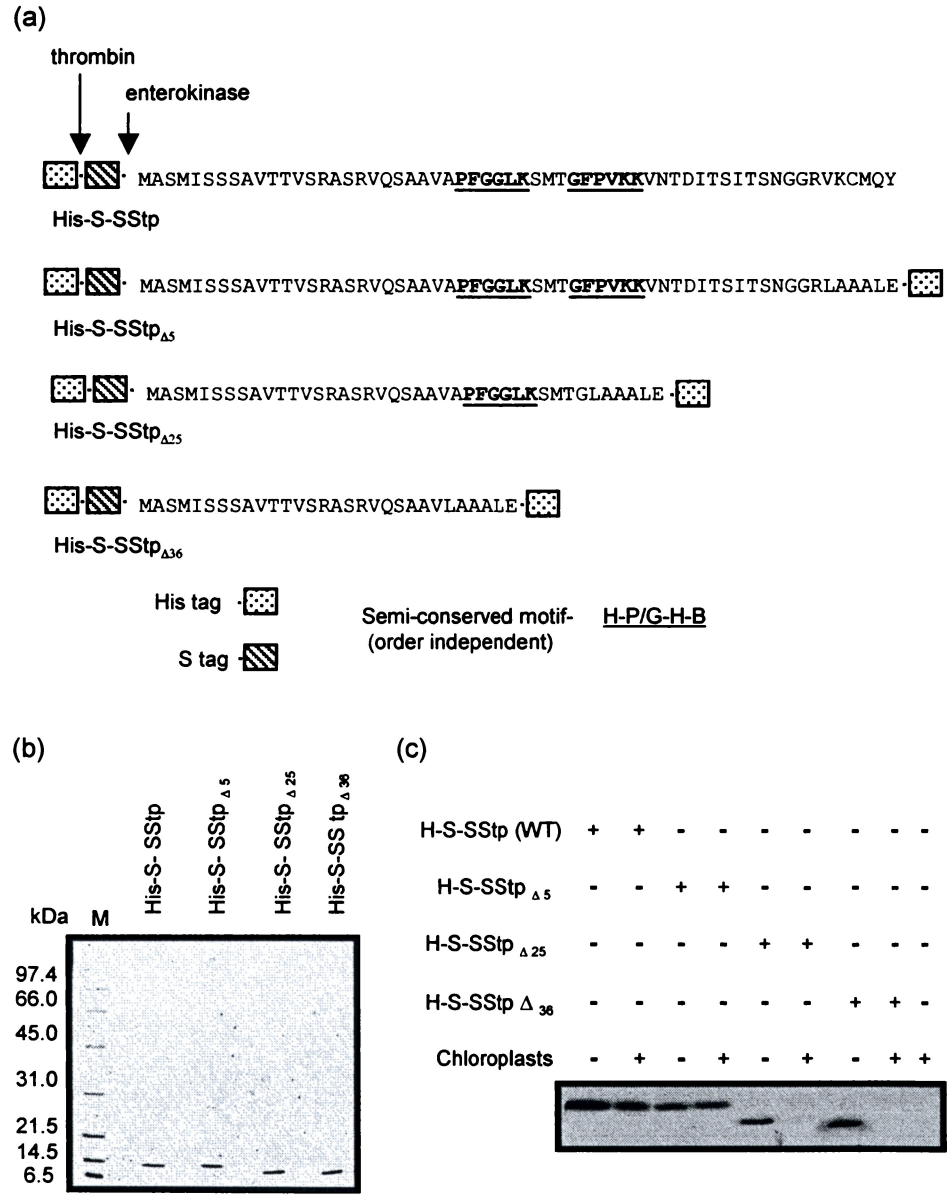

(a)
thrombin
enterokinase
MASMISSSAVTTVSRASRVQSAAVAPFGGLKSMTGFPVKKVNTDITSITSNGGRVKCMQY
His-S-SStp
MASMISSSAVTTVSRASRVQSAAVAPFGGLKSMTGFPVKKVNTDITSITSNGGRLAAALE
His-S-SStpΔ5
MASMISSSAVTTVSRASRVQSAAVAPFGGLKSMTGLAAALE
His-S-SStpΔ25
MASMISSSAVTTVSRASRVQSAAVLAAALE
His-S-SStpΔ36
His tag
S tag
Semi-conserved motif- H-P/G-H-B
(order independent)
(b)
kDa M
His-S- SStp
His-S- SStp Δ 5
His-S- SStp Δ 25
His-S-SS tp Δ 36
97.4
66.0
45.0
31.0
21.5
14.5
6.5
(c)
H-S-SStp (WT)
H-S-SStp Δ 5
H-S-SStp Δ 25
H-S-SStp Δ 36
Chloroplasts

Figure 3-6.

Binding of the deletion mutants to chloroplasts

The label transfer cross-linking experiments revealed that deleting amino acids from the C-terminus of trFd lead to reduced affinity for Toc86/159 (Rensink et al., 2000). Several studies have shown that removing or mutating the H(P/G)H(K/R) domain prevents the transit peptides from recognizing the translocation apparatus (Perry et al., 1991; Pilon et al., 1995; Schnell et al., 1991).

We have used far-western blotting, FACS and LSCM assays to analyze the binding of C-terminal deletion mutants of His-S-SStp. *In vitro* binding studies using the far-western blotting revealed that the binding of the His-S-SStp$_{\Delta 5}$ mutant was similar to that of the wt. However, the His-S-SStp$_{\Delta 25}$ and His-S-SStp$_{\Delta 36}$ mutants did not bind to the chloroplasts (Figure 3-6c). Similar results were observed by FACS and LSCM, which revealed that the peptides His-S-SStp$_{\Delta 25}$ and His-S-SStp$_{\Delta 36}$ were not bound to the chloroplast (Figure 3-7a and 3-7b). Although all the three mutants His-S-SStp$_{\Delta 5}$, His-S-SStp$_{\Delta 25}$ and His-S-SStp$_{\Delta 36}$ have the extra residues and a His tag at the C-terminus, only His-S-SStp Δ5 binds almost as efficiently as the wild type His-S-SStp to the chloroplast but the other two deletion mutants His-S-SStp$_{\Delta 25}$ and His-S-SStp$_{\Delta 36}$ do not bind. This indicates that the deletion of specific residues at the C-terminus of His-S-SStp prevented it from interacting with the chloroplast and not the addition of the C-terminal residues. The far-western blot data indicates that these two mutants were not bound to the chloroplasts. One could argue that the two deletion mutants were translocated and degraded immediately and so could not be detected by the far-western analysis. However, that is highly unlikely because earlier work with C-terminal deletions of transit peptides have revealed that this region is

Figure 3-7. LSCM and FACS of C-terminal deletion mutants of His-S-SStp binding to chloroplasts. **(a)** LSCM showing the binding of His-S-SStp and the C-terminal deletions. FL is the full length His-S-SStp. **(b)** FACS analysis of the full length and the C-terminal deletions.

Placeholder

REPLACE IMAGE

essential for transport (Kuntz et al., 1986; Perry et al., 1991; Pilon et al., 1995; Reiss et al., 1989; Schnell et al., 1991; Wasmann et al., 1989). Analysis of the supernatant after the binding reaction revealed that most of the His-S-SStp$_{\Delta 25}$ and His-S-SStp$_{\Delta 36}$ was present in this fraction and none in the pellet bound to the chloroplast (data not shown). It is interesting to note that His-S-SStp$_{\Delta 25}$ does not have one of the two H(P/G)H(K/R) motifs and the His-S-SStp$_{\Delta 36}$ does not have both the H(P/G)H(K/R) motifs. Thus, removal of H(P/G)H(K/R) motif prevented His-S-SStp from interacting with the chloroplasts.

Schnell and co-workers have shown that some of the N-terminal deletions of trFd do not compete with the binding/import of prFd, while the C-terminal 30 amino acids of both trFd and SStp could successfully inhibit the import of their precursor proteins (Schnell et al., 1991). When these peptides were analyzed by me looking at their published results, the 30 C-terminal amino acids of both trFd and SStp had the H(P/G)H(R/K) motif and so could function a competitors (Schnell et al., 1991). On the other hand the C-terminal 19 amino acid peptide of SStp and the N-terminal 30 amino acid peptide of trFd did not have the H(P/G)H(R/K) motif and these could not inhibit the import of their precursors (Schnell et al., 1991).

In vitro competitions of prSSU, prFd, prLHCP and prPC import with 20-residue synthetic peptides representing the N-terminal, middle and C-terminal regions of SStp revealed that the central region of SStp competed with the binding while the ends competed with translocation of the precursors (Perry et al., 1991). It is interesting to note that the 20 amino acid synthetic peptide pS(21-40) which has both the H(P/G)H(R/K) motifs, was the most efficient competitor for the binding of prSSU to the chloroplasts. The pS(31-50) which had only one of the two

H(P/G)H(R/K) motifs also competed with the binding of prSSU but was a little inferior to pS(21-40). The other two pS(1-20) and pS(41-60) did not compete as well.

In vitro competitions using deletions of trFd in prFd from *Silene* revealed that the N-terminal domain was necessary for outer membrane translocation, the middle region was required for inner membrane translocation and the C-terminal was mainly involved in processing (Rensink et al., 2000). The same domains have been shown to be required for efficient import *in vivo* (Rensink et al., 1998). However, none of the deletions Rensink and co-workers used affect the H(P/G) H(R/K) motifs in trFd. Some of the deletions made in prFd from *Silene* by Pilon and co-workers do not have the H(P/G)H(R/K) motifs. All these deletion mutants do not compete well with prFd binding/import (Pilon et al., 1995). All these data indicate that the H(P/G)H(R/K) motif is at least one of the essential features required for binding of the transit peptide to the chloroplast, which is in agreement with our hypothesis.

Analysis of the data obtained by Schnell et. al., Perry et. al. and Pilon et. al. agrees with our data. It shows that the H(P/G)H(R/K) motif is clearly responsible for the binding of the transit peptide with the chloroplast.

Competitive inhibition of His-S-SStp binding with OM liposomes

The outer membrane of chloroplast is composed of some common and some unusual lipids. The composition of lipids in a chloroplast outer membrane is as follows: 30% phosphatidyl choline, 20% monogalactosyldiacylglycerol (MGDG), 30% digalactosyldiacylglycerol (DGDG), 6% sulfolipid (SL), 10% phosphatidyl inositol (PI) and 10% phosphotidylglycerol (PG). PC, MGDG, DGDG lipids are the

major components while SL, PG and PI are minor constituents (Joyard et al., 1991; Joyard et al., 1982). Hence, to mimic the outer chloroplast membrane, we prepared artificial bilayer (liposomes) at the following molar ratio: MGDG:DGDG:PC:PG = 2:3:4:1. Several studies suggest that the early stages of protein import into chloroplasts are lipid-mediated (Endo et al., 1992; Horniak et al., 1993; Pilon et al., 1995; Pinnaduwage & Bruce, 1996; van't Hof & de Kruijff, 1995; van't Hof et al., 1991; van't Hof et al., 1993), reviewed in (Bruce, 1998). This intimate relationship between lipids and protein translocation has also been discovered in other organelles like mitochondria and endoplasmic reticulum (Endo et al., 1989; Rapoport, 1992; Roise et al., 1986; Roise et al., 1988; Van Voorst & De Kruijff, 2000). The most definite way to prove if lipids play an important role in the interaction of the transit peptide with chloroplasts is to compete the interaction of the transit peptide and chloroplasts with liposomes of similar composition as the chloroplast outer envelope. We increased the amount of OM liposomes [MGDG/DGDG/PC/PG (20/30/30/10)] in a binding reaction keeping the chloroplast and His-S-SStp concentration constant. Interestingly, we observed that as we increased the amount of the OM liposomes, lesser His-S-SStp bound to chloroplasts as observed using S-protein-FITC staining in FACS analysis (Figure 3-8). The IC_{50} (50% inhibition) was found to be approximately 120 μM of total lipids. The PC liposomes that have only PC as lipids, did not compete as well, the 50% inhibition was found to be approximately >100mM total lipids indicating that specialized lipids in the outer membrane are essential for the interaction of the transit peptide with the chloroplast. Hence, PC lipids are required at ~1000 fold higher concentration than the OM lipids

Figure 3-8. OM liposomes (MGDG:DGDG:PC:PG/15:30:45:10) compete with the interaction of His-S-SStp with the chloroplast membrane. ■ indicates OM liposomes and ▲ indicates PC liposomes. The graphs were plotted after three repeats of each experiment and the significance of the results is indicated using error bars.

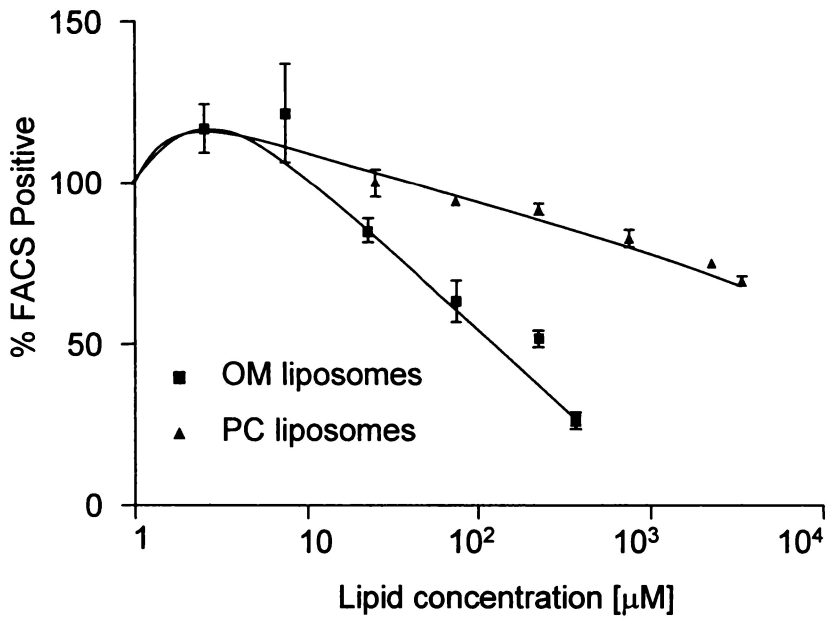

150
100
50
0
% FACS Positive
OM liposomes
PC liposomes
1
10
10²
10³
10⁴
Lipid concentration [μM]

Figure 3-8.

(MGDG/DGDG/PC/PG) indicating that the lipids like MGDG, DGDG and PG play a very important role in transit peptide interaction.

It has been proven that non-bilayer forming lipids play a crucial role in protein translocation (reviewed in Bruce, 1998; Van Voorst & De Kruijff, 2000). Phosphatidyl ethanolamine (PE) in *E.coli*, Cardiolipin (CL) in endoplasmic reticulum and mitochondria (Cullis & de Kruijff, 1979) and MGDG in the chloroplast are the unique non-bilayer forming lipids. CL was shown to be essential for the interaction of mitochondrial pre-sequence with artificial liposomes (Leenhouts et al., 1994; Torok et al., 1994). *E.coli* mutants lacking in PE had an increased content of the other non-bilayer forming lipid CL in their membrane and required Mg^{2+} for their growth, Mg^{2+} is known to promote bilayer formation (Vasilenko et al., 1982). The ratio of time averaged volume occupied by the hydrophobic acyl chain domain versus the volume of the hydrophilic head group domain predicts the molecular shape of that individual lipid molecule. The lipids whose volume ratio is 1.0 prefer bilayers like phosphatidyl serine (PS) and saturated phosphotidylethanolamine (PE), detergents have this ratio less than one and form micelles while those lipids that have this ratio >1.0 like MGDG form the reversed hexagonal array known as H_{II} phase (reviewed in Bruce, 1998). Monolayer experiments have shown that the N-terminus of trFd interacts with the MGDG via hydrogen bonding while the C-terminus interacts with anionic lipids via electrostatic interactions (Pilon et al., 1995). Addition of SStp to liposomes with the composition of outer membrane of chloroplast transformed from a homogenous spherical population to aggregated structures comprised of many elongated and appressed sacs indicating that the transit peptides definitely interact with the outer membrane lipids and perturb the lipid

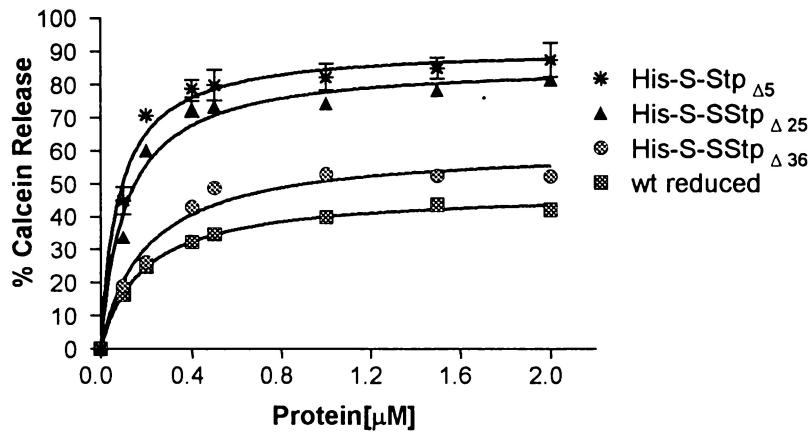
membranes (Pinnaduwage & Bruce, 1996). MGDG in the chloroplast membrane forms a H_{II} phase that has been hypothesized to play a role in converting relatively unstructured transit peptide into an α-helical molecules.

Testing the lipid interaction of the deletion mutants

Previous work on the *in vitro* interaction of chloroplast transit peptide with the outer envelope lipids using vesicle disruption assay indicated that the interaction was lipid class (MGDG) dependent. Moreover, using chemically synthesized peptides it was shown that although the 20 amino acid C-terminal region (41-60) was responsible for lipid interaction, it required at a ~6-fold higher concentration compared to full length SStp (Pinnaduwage & Bruce, 1996). This suggests that either different regions of the transit peptide act cooperatively in lipid interaction or that the lipid interacting region spans through a longer region from the C-terminus towards the N-terminus of SStp. Interactions with prSSU and mSSU clearly indicate that the SStp alone cannot account for all the lipid interaction since the calcein dye release by prSSU was significantly more compared to SStp (Pinnaduwage & Bruce, 1996). Probably the mature domain interacts in *cis* with SStp and enhances the vesicle disruption activity of the transit peptide. Hence, we performed calcein entrapped dye release assay with the three C-terminal deletions of SStp we had generated. The deletions were found to perform better (in releasing the dye) than the full length His-S-SStp (Figure 3-9a). The oxidized full length His-S-SStp is a dimer as it has one cysteine residue at its C-terminus as seen on a non-reducing SDS-PAGE (data not shown). However, addition of DTT did not affect % calcein release by full length His-S-SStp indicating that the decrease in % calcein release by non-reduced

Figure 3-9. Calcein entrapped dye release assay of OM liposomes by His-S-SStp and C-terminal deletion mutants. **(a)** ✳ indicates Calcein dye release assay by His-S-SStp$_{\Delta 5}$, ▲ indicates His-S-SStp$_{\Delta 25}$, ● indicates His-S-SStp$_{\Delta 36}$ and ■ indicates full length His-S-SStp. **(b)** Clacein entrapped dye release of OM liposomes by Hid-S-SStp is compared in the presence and absence of DTT. The dye release by His-S is also shown in the figure. ■ indicates the dye release in the presence of DTT ▲ indicates the dye release in the absence of DTT and ✳ indicates the dye release by His-S. The graphs were plotted after three repeats of each experiment and the significance of the results is indicated using error bars.

(a)


100
90
80
70
60
50
40
30
20
10
0
% Calcein Release
0.0
0.4
0.8
1.2
1.6
2.0
Protein[μM]
His-S-Stp Δ5
His-S-SStp Δ 25
His-S-SStp Δ 36
wt reduced

(b)

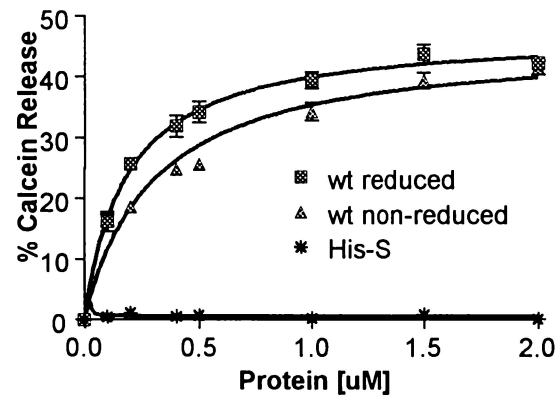

50
40
30
20
10
0
% Calcein Release
0.0
0.5
1.0
1.5
2.0
Protein [uM]
wt reduced
wt non-reduced
His-S

Figure 3-9.

His-S-SStp compared to the C-terminal deletion mutants was not as a result of dimer formation by full length His-S-SStp. His-S and DTT did not affect the dye release assay significantly (Figure 3-9b). Urea also did not affect the dye release assay (data not shown).

On analyzing the structure of His-S-SStp using peptide structure analyzing software, Pep tools (version 2.01) and DNAstar, it was observed that deleting the C-terminal region of His-S-SStp and adding an extra His tag and five amino acids at the C-terminus (due to the 'b frame') made the deletions more α-helical. The α-helical content increased from 42.5% in His-S-SStp to 46.6% in His-S-SStp$_{\Delta 5}$, to 58.8% in His-S-SStp$_{\Delta 25}$, to 60.5% in His-S-SStp$_{\Delta 36}$. Both the % β-sheet and % random coil formation decreased from His-S-SStp to His-S-SStp$_{\Delta 36}$ in this prediction analysis. It is essential to point out that the binding of the C-terminal deletions is not compromised due to the extra residues created at the C-terminus of His-SStp. We have found that His-S-SStp$_{\Delta 5}$ binds to the chloroplasts even though it possesses the extra residues at the C-terminus. Moreover, even though the α-helical content is predicted to increases the binding of His-S-SStp$_{\Delta 25}$ and His-S-SStp$_{\Delta 36}$ is inhibited (Figure 3-6c, Figure 3-7a and 3-7b), indicating that specific regions from F36 to R54, mainly the H(P/G)H(R/K) motifs are essential for the binding of the transit peptide to the Toc components. The interaction of the transit peptides with the chloroplast membrane lipids is a reversible process. Hence, we propose that although the deletion mutants, His-S-SStp$_{\Delta 25}$ and His-S-SStp$_{\Delta 36}$, interact with the chloroplast lipids they are released as they cannot interact with the Toc components due to the absence of the H(P/G)H(R/K) motif.

Structural flexibility of the transit peptide, His-S-SStp and His-S-SStp deletions

Chloroplast transit peptides have a random coil with minimum α-helical or β-sheet content (von Heijne & Nishikawa, 1991). It was hypothesized that the unstructured transit peptide assumes an α-helical conformation in the presence of lipids or a lipid-mimicking environment (Bruce, 1998; Pinnaduwage & Bruce, 1996; Wienk et al., 1999; Wienk et al., 2000). The formation of a particular secondary structure may enable them to interact with the Toc components. This could be an essential feature in the transit peptide recognition by the Toc receptor as there is no sequence homology among the chloroplast transit peptides.

The α-helical content of SStp increased from 2 to 60% in the presence of 60% TFE as seen in Figure 3-10a and 3-10b. The % α-helical content was obtained from the CD data using the *CDNN* deconvolution program. The α-helical content of His-S-SStp when going from an aqueous phase to 60% TFE increased from 6% to 40% as seen in Figure 3-11a and 3-11b. The slight decrease in α-helical content of His-S-SStp compared to SStp in the presence of 60%TFE may be the result of changes in the protein structure due to the presence of the His-S tag at the N-terminus.

Use of TFE in the study of conformation and dynamics of proteins and polypeptides is sometimes controversial (Buck, 1998). TFE is known to preferentially stabilize proteins and peptides in a α-helical conformation. Studies with various polypeptides suggest that this stabilization is not indiscriminate but relies on the underlying structural preferences of the amino acid residues (Dyson et

Figure 3-10. α-helical content of SStp in the presence of TFE. **(a)** Far UV-CD spectra of SStp at 25 °C in the presence of increasing concentrations of TFE in water. TFE concentrations were increased from 0-60% with 10% increments as indicated in the figure. **(b)** α-helical content of SStp in the presence of increasing concentrations of TFE shown in (a) was calculated using CDNN CD deconvoluting program.

Placeholder

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Figure 3-11. α-helical content of His-S-SStp in the presence of TFE. **(a)** farUV-CD spectra of SStp at 25 °C in the presence of increasing concentrations of TFE in water. TFE concentrations were increased from 0-60% with 10% increments as indicated in the figure. **(b)** α-helical content of His-S-SStp in the presence of increasing concentrations of TFE shown in (a) was calculated using CDNN CD deconvoluting program.

Placeholder

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al., 1992; Dyson et al., 1992; Kemmink & Creighton, 1995; Lehrman et al., 1990; Yang et al., 1995). TFE promotes the polypeptide chain to express its local and intrinsic conformational preference. Although, several studies have shown highly significant correlations between predicted helix content and the helix signal measured by CD at 222nm in the presence of TFE (Luidens et al., 1996; Shiraki et al., 1995), several other studies have shown that β-sheet formatting proteins adopt an α-helical conformation in the presence of TFE (Buck et al., 1993; Shiraki et al., 1995).

The α-helical content of the C-terminal deletions was seen to decrease with the increase in amino acid deletions as shown in Figure 3-12. This indicates that the C-terminal region in the transit peptide is the major α-helix forming region. This is in total agreement with the previous observation that transit peptides are in a random coil conformation in aqueous environment but gain helical structures at the N- and C-terminus in the presence of a hydrophobic solvent like TFE (Wienk et al., 1999). The calcein dye release assay indicated that the C-terminal deletions of His-S-SStp had more lipid interaction that the wt His-S-SStp (Figure 3-9a). The circular dichroism analysis indicated that the α-helical content decreased with the increase in the C-terminal deletions (Figure 3-12). Therefore, it can be concluded that the decrease in the flexibility at the C-terminus increases the lipid interaction of the transit peptide with the chloroplast lipids. The CD results do not agree with the prediction results from pep-tools described in the previous section. Although the prediction analyses use many algorithms it is possible that they cannot accurately predict the conformation of the peptide or it is possible that the presence of TFE forced the peptide into an α-helical conformation. However, it has been observed

Figure 3-12. α-helical content of C-terminal deletions of His-S-SStp in the presence of increasing concentrations of TFE. ◆ indicates full length His-S-SStp, ✱ indicates His-S-SStp$_{\Delta 5}$, ■ indicates His-S-SStp$_{\Delta 25}$, indicates His-S-SStp$_{\Delta 36}$.

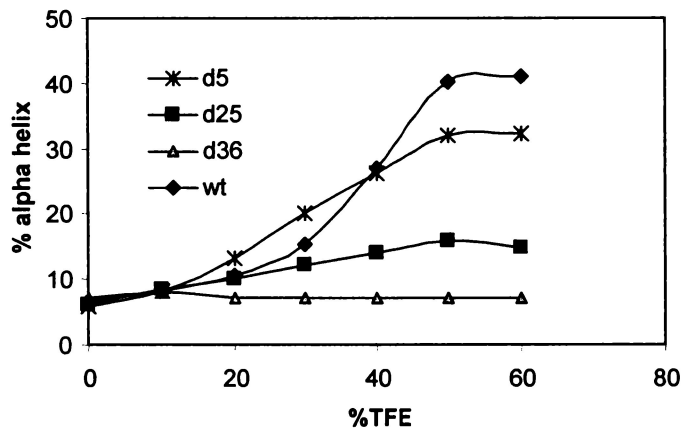


Figure 3-12.

that the intrinsic helical propensity for peptides is sometimes over predicted compared to extent of helical structures stabilized by TFE (reviewed in Buck, 1998).

Our results are in agreement with the circular dichroism results of the Ferredoxin transit peptides from *Silene pratensis.* This revealed that the α-helical content increased from 6% in the presence of no TFE to 24%in the presence of 50% (v/v) TFE and random coil content decreased form 74% in the presence of no TFE to 50% in the presence of 50% TFE (Wienk et al., 1999). The ^{15}N-labeled NOESY NMR experiment showed that the transit peptide is in a random coil form in aqueous solution but in the presence of 50% TFE the transit peptide forms two α-helices, one from residues 2 to 14 and the other from residues 27 to 50 (Wienk et al., 1999). Further circular dichroism analysis by the same group using mixed micelles (molar ratio of MGDG: DPG: DPC = 1:2:97) revealed that the unstructured trFd gained a helical structure in the presence of micelles. The NMR data showed that the trFd interacted with the micelles and gained helical features at the N- and C-terminus. The central proline rich region was largely unstructured. The use of hydrophobic spin label indicated that this region of the transit peptide is able to completely reside in the hydrophobic interior of the micelle. It is possible that it is forced to insert into the hydrophobic phase when bound to the micelle (Wienk et al., 2000). Our results also indicate that C-terminus is the major helix forming domain of the transit peptide. Its removal leads to a decrease in the α-helical content that in turn increases the lipid interaction. Hence, we conclude that the decrease in α-helical content at the C-terminus of the transit peptide increases its ability to interact with the membrane lipids.

CONCLUSIONS

In this chapter we have clearly demonstrated that the two H(P/G)H(R/K) motifs present in the C-terminus of His-S-SStp are essential for the binding of the transit peptide to the Toc apparatus. The interaction of His-S-SStp like SStp is lipid class (MGDG) dependent as both His-S-SStp and SStp interact best with outer membrane mimicking liposomes. An inverse relationship between flexibility at the C-terminus of the transit peptide and lipid interaction was observed. To further support this hypothesis ^{15}N NOESY NMR experiments have to be performed as the circular dichroism analysis does not give much information about the spatial presence of the α-helical structures.

Chapter 4

Structural flexibility of the transit peptide plays an important role in its interaction with the chloroplasts

ABSTRACT

The nuclear encoded, chloroplast destined precursors have their targeting information in the N-terminal transit peptide region. These transit peptides show no sequence homology among them but share a common import pathway into the chloroplasts. It has been implicated that the transit sequences interact with the chloroplast membrane lipids and change their conformation such that they can be recognized by the chloroplast import receptor. Several studies have analyzed the structure of the transit peptides and indicated that both the N- and the C-terminus of the transit peptide have a tendency to form α-helices in a membrane mimicking environment. In this study we have investigated the role of the structure of transit peptides by systematically changing the small aliphatic residues into helix breaking proline residues all along His-S-SStp at positions A9, A16, A22, M33, V41, I48, V55. *In vitro* chloroplast binding studies indicated that introduction of flexibility in the N-terminal region prevented the transit peptide from interacting with the chloroplast while this did not impair the lipid interaction. Introduction of flexibility at the C-terminus does not effect the chloroplast interaction as seen by *in vitro* binding studies while the lipid interaction is enhanced in these mutants. Removing the only two prolines present in SStp at position P26 and P37 gave interesting results. Removing the proline at the P26 position inhibited both chloroplast binding and lipid interaction while removing the proline at the P37 position did not change the binding

or lipid interaction significantly compared to the wild type. These results indicate that the structure of the transit peptide plays a very important role in its interaction with the chloroplast. The change in the structure probably affects the interaction of the transit peptide with both the proteinaceous component of the translocon and the membrane lipids.

INTRODUCTION

Majority of the chloroplast proteins are synthesized in the plant cell cytosol as precursor proteins (Reiss et al., 1987; Smeekens et al., 1989; Van den Broeck et al., 1985). Although the transit peptides greatly differ in length and share no sequence homology among them, they are imported via one general import pathway. The import of a precursor into chloroplast involves several steps and transit peptides are believed to play a crucial role in each of these stages. The first step probably involves the interaction of the transit peptide with the membrane lipids. It has been predicted that although the transit peptides have a random coil conformation (von Heijne et al., 1989) in aqueous solution, they assume an α-helical conformation when they interact with membrane lipids (Bruce, 1998; Chupin et al., 1994; van't Hof et al., 1991; van't Hof et al., 1993; Wienk et al., 1999; Wienk et al., 2000). Lipids are believed to act as molecular chaperons that direct folding, prevent mis-folding and even prevent unfolding of proteins (reviewed in Bogdanov & Dowhan, 1999). Lipids are believed to assist proteins in folding (reviewed in Bogdanov & Dowhan, 1999). There are several examples in *E.coli* where the absence of a certain lipid type lead to improper folding, for example absence of PE in *E.coli* cells lead to improper folding of LacY (Bogdanov & Dowhan, 1998).

Due to the limited information available on the structure of transit peptides during membrane interaction it has been difficult to rule out the role of transit peptide structure in the chloroplast-recognition and import process. Several researchers have attempted to study the structure of transit peptide in membrane mimetic environments such as TFE (2,2,2-trifluoroethanol) and micelles made from lipids/detergents (Krimm et al., 1999; Lancelin et al., 1994; Wienk et al., 1999; Wienk et al., 2000). Analysis of the transit peptide structure by circular dichroism and NMR indicated that the transit peptide had a random coil structure in aqueous environment but attained a significant α-helical content in a lipid or lipid mimicking environment. This work was done on the transit peptides of ferredoxin (trFd) from *Silene pratansis* (Wienk et al., 1999; Wienk et al., 2000) and Rubisco activase (Krimm et al., 1999) and ferredoxin (Lancelin et al., 1994) from *Chlamydomonas*. Monolayer studies showed that the trFd could interact with the lipids extracted from the outer membrane of chloroplast (van't Hof et al., 1993). Monolayer experiments using 20 amino acid fragments of SStp indicated that the central region interacted the most with lipids as the change in the surface tension (pressure) was in the following order: 21-40>41-60>1-20 (van't Hof et al., 1991). Calcein dye release assays have shown that the C-terminus and N-terminus of SStp have the maximum lipid interacting ability while the central region does not interact well with the lipids (Pinnaduwage & Bruce, 1996). This lipid interaction of the transit peptide depended upon MGDG and anionic lipids (Horniak et al., 1993; Pinnaduwage & Bruce, 1996). Hence, we analyzed the lipid interacting capacity of our proline mutants.

Various amino acids are thought to have different tendencies to form α-helices. When the relative helix forming tendency was measured by substituting

each amino acid residue into a solvent-accessible position in a synthetic peptide that forms a α-helix, it was found that proline required ~3 kcal/mol for relative stabilization of the α-helical conformation compared to alanine which required –0.77 kcal/mol (O'Neil & DeGrado, 1990). The five-membered ring of the proline residue imposes constraints on the rotation of the N—C^{α} bond of the peptide backbone. Comparison of α-helical preferences of the various amino acids shows that Proline has very poor preference to form α-helices when present in the central region of the peptide (Williams et al., 1987).

In order to study the role of the structure of the transit peptides in chloroplast interaction we have systematically made single amino acid mutations in the transit peptide of small subunit of Rubisco (SStp). We have introduced proline residues all along the length of the transit peptide from the N to C terminus changing aliphatic residues like Ala, Val, Leu and Ile to Pro residues. In addition, we have analyzed the structure and function of the transit peptide after removal of the only two proline residues present in the central region of SStp. We have used an epitope tagged transit peptide (His-S-SStp) for our studies since we can directly monitor the binding of the transit peptide to the chloroplast translocation apparatus. Our results indicate that disruption of the α-helix at the N-terminus of SStp inhibits the binding of the transit peptide to the chloroplasts while disruption of the α-helix at the C-terminus effects the lipid interaction. We have also analyzed the structure of these mutants using circular dichroism spectroscopy.

MATERIALS AND METHODS

Site-directed mutagenesis of His-S-SStp

Site-directed mutagenesis of His-S-SStp was performed using the Quickchange site-directed mutagenesis kit from Stratagene Cloning Systems (La Jolla, CA). We designed the primers corresponding to the mutation and synthesized them at IDT (Coralville, IA). The primer sequences are given in Table 4-1. The annealing temperature for each primer was calculated from the formula below.

$$Tm = 81.5 + 0.41(\%GC) - {}^{675}\!/\!_{N} - \%Mismatch$$

Where, N is the primer length and Tm is the annealing temperature.

The PCR reaction was performed in a Perkin Elmer thermal cycler (Perkin Elmer, Norwalk, CN). The PCR conditions used were as follows:

Step 1. Denaturation : 94°C for 1 minute.

Step 2 Denaturation : 94°C for 30 seconds.

Step 3 Annealing : 65°C or as calculated for 30 seconds.

Step 4 Extension : 68°C for 14 minutes.

20 cycles of steps 2 to 4 were performed.

The PCR product was restricted with Dpn I enzyme to digest the methylated parental DNA template. The PCR products were transformed into Nova-Blue competent cells (Novagen, Madison, WI). Four colonies were picked and the DNA was purified using a SV mini-prep kit from Promega (Madison, WI). The mutation was confirmed by automated sequencing. The average mutation efficiency was 80-90%.

Table 4-1. Primer sequences of the proline mutations generated using the Quick change kit.

Primer Name	Strand	Length (bases)	Sequence 5′ ⟶ 3′
His-S-SStp A9P	+	26	CCTCTTCC**C**CTGTGACAACAGTCAGC
His-S-SStp A9P	–	26	GCTGACTGTTGTCACAG**G**GGAAGAGG
His-S-SStp A16P	+	34	GCTGTGACAACAGTCAGCCGT**C**CTTCTAGGGTGC
His-S-SStp A16P	–	34	GCACCCTAGAAG**G**ACGGCTGACTGTTCTCACAGC
His-S-SStp A22P	+	22	GGGTGCAATCC**C**CGGCAGTGGC
His-S-SStp A22P	–	22	GCCACTGCCGG**G**GATTGCACCC
His-S-SStp M33P	+	26	GGCCTGAAATCC**CC**GACTGGATTCCC
His-S-SStp M33P	–	26	GGGCCTCCAGTC**GG**GGATTTCAGGCC
His-S-SStp V41P	+	34	CCCAGTGAAGAAG**CC**CAACACTGACATTACTTCC
His-S-SStp V41P	–	34	GGAAGTAATGTCAGTGTTG**GG**CTTCTTCACTGGG
His-S-SStp I48P	+	39	GGTCAACACTGACATTACTTCC**CC**TACAAGCAATG GTGG
His-S-SStp I48P	–	39	CCACCATTGCTTGTA**GG**GGAAGTAATGTCAGTGTT GACC
His-S-SStp V55P	+	37	GCAATGGTGGAAGA**CC**AAAGTGCATGCAGTATTG ACC
His-S-SStp V55P	–	37	GGTCAATACTGCATGCACTTT**GG**TCTTCCACCATT GC
His-S-SStp P26A	+	21	GCAGTGGCT**G**CATTCGGCGGC
His-S-SStp P26A	–	21	GCCGCCGAATG**C**AGCCACTGC
His-S-SStp P37A	+	27	CCATGACTGGATTC**G**CAGTGAAGAAGG
His-S-SStp P37A	–	27	CCTTCTTCACTG**C**GAATCCAGTCATGG

Plant growth and chloroplast isolation

Chloroplasts were isolated from dwarf pea (*Pisum sativum*) seedlings that were 11-13 days old. The pea seeds (Progresso #9) were imbibed overnight and planted on horticulture Vermiculite, approximately 300mL of dry seeds per flat. Plants were grown in EGC growth chambers under 160 $\mu E/m^2/sec$ of cool white fluorescent light. To reduce starch accumulation the plants were grown on a 12 hr light/12 hr dark cycle. Leaves from the plants were harvested and intact chloroplasts were isolated as described in literature (Bruce et al., 1994). Intact chloroplasts were resuspended at a concentration of 1 mg/ml total chlorophyll and used for all labeling/import reactions.

***E.coli* protein expression**

The His-S-SStp proline insertion and removal mutants were purified similar to wt His-S-SStp as described in Chapter2 except that pre-packed His-bind columns (Novagen, Madison, WI) and a vacuum manifold (Novagen, Madison, WI) were used as at least 6 different mutant proteins could be purified at the same time. The proteins were expressed in [BL21(DE3) PR] *E.coli* cells after induction with IPTG as described earlier. The cells were resuspended in 1X binding buffer (5 mM Imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0) containing, 2 mM leupeptin, 2 mM pepstatin, 1 mM PMSF and 6 M Urea and lysed using a French press. The lysate was centrifuged at 50,000 *g* and the supernatant was loaded on to a His-bind column. The column was first washed with 5 column volumes of 1X wash buffer (5 mM Imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0) containing 6 M Urea and then with 3 column volumes of 1X wash buffer (20 mM Imidazole, 0.5 M NaCl, 20 mM

Tris-HCl, pH 8.0) containing 6 M Urea. The protein was eluted with elution buffer (1 M Imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0) containing 6 M Urea and five 2 mL fractions were collected. The samples containing the protein were pooled and stored at -80°C until further use.

***In vitro* binding of His-S-SStp mutants (Proline insertions/removals) to intact chloroplasts**

Binding reactions of the His-S-SStp mutants were performed similar to the binding reactions of His-S-SStp. The mutant proteins were added to a final concentration of 5 μM. The ATP concentration was maintained at 30 μM and the binding reaction was performed for 8 minutes in the dark. Intact chloroplasts were isolated on 40% percoll and subjected to SDS-PAGE and far-western blotting.

SDS-PAGE and far-western blotting

Samples were boiled in 4X SB and were resolved on an 18% SDS-PAGE gel. The gel was electro-blotted on to a pre-wetted Immobilon-P membrane (Millipore, Bedford, MA) and blocked with TBS (Tris Buffered Saline) containing 1% gelatin + 0.5 % Tween-20 for 15 minutes. The S-peptide containing proteins were detected by probing for 15 minutes with an S-protein alkaline phosphatase conjugate (Novagen, Madison, WI) diluted 1:100,000 in TBS and then washed extensively with TBS + 0.2% Tween-20. Visualization was conducted using a 1:1 dilution of the chemiluminescent substrate LumiPhos Plus (Lumigen Inc., Southfield, MI) and subjecting the blot to film autoradiography. Exposure time was optimized for maximum signal to noise.

Protein and chlorophyll measurements

Protein was determined using BCA reagent (Pierce, Rockford, IL). Chlorophyll concentration was determined after chlorophyll extraction using 80% acetone as described in chapter 2.

FRETworks RNAase A S-protein assay

The Fluorescence resonance energy transfer (FRET) assay reagents were purchased from Novagen (Madison, WI). Samples used for SDS-PAGE were diluted 100-fold in 1X assay buffer (200 mM Tris-HCl, pH 7.5, 1M NaCl, 1% Triton X-100). 2 μL of the 1:100 diluted sample was used in 200 μL reactions giving a final dilution of 1:10,000. Reactions were performed in a 12 X 8, 96-well Microflour, Dynex (Millopore, Bedford, MA) plate and incubated at room temperature for 30 minutes in the dark. The reaction was stopped after 30 minutes using 1X stop buffer. The plate was read using a Wallac Victor^2 1420 multi label counter (Turku, Finland) plate reader. A standard curve was plotted from 50 fmol to 0.78 fmol using the S-tag standard provided with the kit. Excitation wavelength was set at 485 nm and emission wavelength was set at 535 nm.

Liposome preparation

Liposomes were prepared as described by Pinnaduwage *et. al.* by mixing appropriate concentration of individual lipids to a final concentration of 5 mM (Pinnaduwage et. al. 1996). The solvent was then evaporated under a stream of N_2 gas and the sample was vacuum desiccated overnight to remove any trace solvents. The dried lipid film was then hydrated with 0.1X phosphate buffered saline (pH 7.8)

containing 1 mM EGTA, 0.02% Na-azide and 200 mM calcein. The mixture was vortexed vigorously and then sonicated in a bath sonicator (Laboratory Supplies Inc.) for 10 minutes. The sonication was repeated several times at 6-12 hour intervals until the lipid mixture was clear and no longer turbid indicating liposome formation. Free unincorporated calcein and lipids were removed using a Bio-Gel A-0.5m column equilibrated with 0.1X phosphate buffered saline containing 1 mM EGTA, 0.02% Na-azide. Liposomes eluted in the void volume fractions and had a calcein fluorescence quenching of >70-80%. All liposome assays were performed in a 8 X 12-format 96 well glass plate (Kontes glass Vineland, NJ). Fluorescence of the liposomes was analyzed using a Wallac Victor^2 1420 multi label counter (Turku, Finland) fluorescence plate reader. Excitation wavelength was set at 485 nm using a fluorescein excitation filter and emission wavelength was set at 514.5 nm using a narrow band pass (10 nm) filter at 514.5 nm.

% Quenching was calculated from the formula below.

$$\%Quenching = \left(1 - \frac{F_0}{F_t}\right) \times 100\%$$

F_0 and F_t are the fluorescence of the liposome samples before and after addition of 0.1% Triton X-100, respectively.

Protein/Peptide-induced calcein release liposome assay

Calcein entrapped liposomes showing >70-80% fluorescence quenching were incubated at room temperature with mutant proteins at varying concentrations as indicated in the figure legends for 1 hour. All liposome assays were performed in a 8

X 12 format 96 well glass plate (Kontes glass, Vineland, NJ). Fluorescence of the liposomes was analyzed using a Wallac Victor^2 1420 multi label counter (Turku, Finland) fluorescence plate reader. Excitation wavelength was set at 485 nm using a fluorescein excitation filter and emission wavelength was set at 514.5 nm using a narrow band pass (10 nm) filter at 514.5 nm. The percentage of calcein release was calculated using the formula below.

$$\%\mathrm{Re}\,lease = \left(\frac{F - F_0}{F_t - F_0}\right) \times 100\%$$

F_0 and F are the calcein fluorescence before and after addition of the protein/peptide, respectively, and F_t is the total fluorescence after addition of 0.1% Triton X-100. All measurements were done in triplicate and the graph indicates the standard deviation as error bars.

Circular dichroism spectrometry

Circular dichroism spectrometry was performed using Aviv 202 circular dichroism spectrometer (Lakewood, NJ) at 25°C using a quartz cell with 0.1cm path length. Protein/Peptide was added to a final concentration of 1 μM with varying amounts of Tri fluoro ethanol (TFE). The CD spectra are reported in Molar Ellipticity after baseline correction for the buffer (0.1 X phosphate buffered saline, pH 7.8). The CD data was deconvoluted using *CDNN* CD spectra deconvolution program (version 2.1) obtained from http://bioinformatik.biochemtech.uni-halle.de/cdnn/ web site. The data was also analyzed using the K2d program for predicting the protein secondary structure (obtained from http://www.embl-

heidelberg.de/~andrade/k2d.html). The final graph of % alpha helix Vs % TFE was plotted after comparison of the data from both the analyses.

RESULTS AND DISCUSSION

Mutagenesis, expression and purification of the His-S-SStp mutants

The SStp in pET30a-SStp was subjected to site directed mutagenesis as described in material and methods. The 0.8% agarose gel in Figure 4-1 represents a typical DNA gel after PCR mutagenesis of the His-S-SStp DNA. The methylated parental pET30a-tp DNA was removed by Dpn I restriction enzyme (lane 3), compare lanes 2, 3 and 4 in Figure 4-1. The supercoiled parental DNA runs faster than the circular DNA after mutagenesis by PCR. Dpn I enzyme cleaves only methylated DNA and so only the parental DNA is digested. This increases the probability of isolating colonies with mutated DNA after transformation. The mutants were confirmed by automated sequencing and the mutagenesis efficiency was found to be 80-90%. The protein sequence of all the mutants generated from His-S-SStp are shown in Figure 4-2a. All the proteins were purified to near homogeneity as shown in a commassie stained SDS-PAGE gel (Figure 4-2b).

In vitro binding of His-S-SStp proline insertion mutants to chloroplast

Most of the studies on the structure of transit peptides have involved analysis of the structure of the transit peptide by circular dichroism or NMR in a membrane mimetic environment (Krimm et al., 1999; Lancelin et al., 1994; Pinnaduwage & Bruce, 1996; Wienk et al., 1999; Wienk et al., 2000). Their analyses essentially indicated that the transit peptide probably assumes an α-helical structure when it

Figure 4-1. 0.8% Agarose DNA gel after quick change PCR. Lane1. GIBCO 1kb ladder, lane 2. DNA after quick change PCR, lane 3. DNA after Dpn I digestion for the removal of template DNA strand, lane 4. template DNA before PCR.

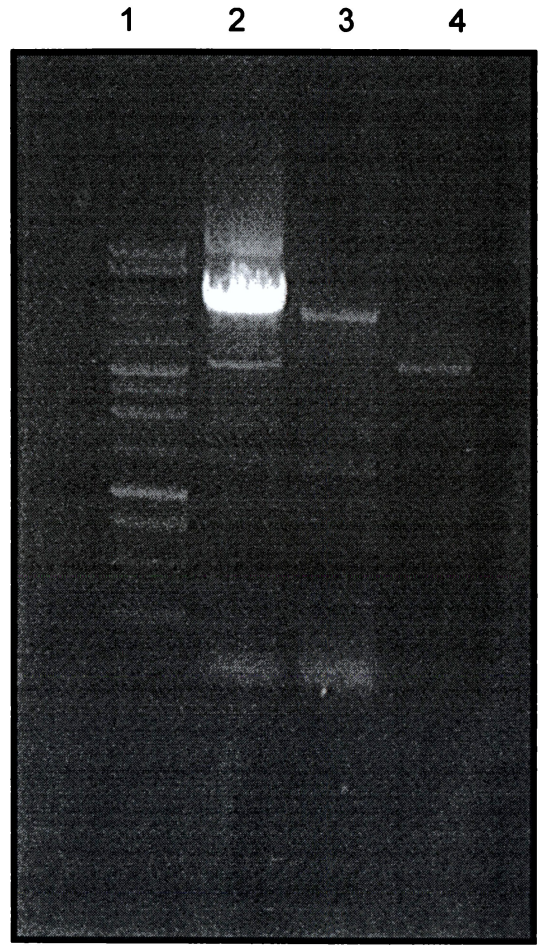


Figure 4-1.

Figure 4-2. Sequence information and Commassie stained SDS-PAGE gel of His-S-S-SStp mutants **(a)** Sequence information of His-S-SStp proline mutants. The base changed is indicated. **(b)** A Commassie stained SDS-PAGE gel of all the His-S-SStp proline mutants after IMAC purification. 2μg of protein was loaded in each lane. A part of the Bio-Rad broad range marker is shown in lane 1.

(a)

```
wt      MASMISSSAVTTVSRASTVQSAAVAPFGGLKSMTGFPVKKVNTDITSITSNGGRVKC
A9P     --------P---------------------------------------------------
A16P    ---------------P--------------------------------------------
A22P    ----------------------P--------------------------------------
M33P    ---------------------------------P-----------------------
V41P    ------------------------------------------P----------------
I48P    ---------------------------------------------P---------
V55P    ------------------------------------------------------P--
P26A    -------------------------A-----------------------------
P37A    ------------------------------------A--------------------
```

(b)

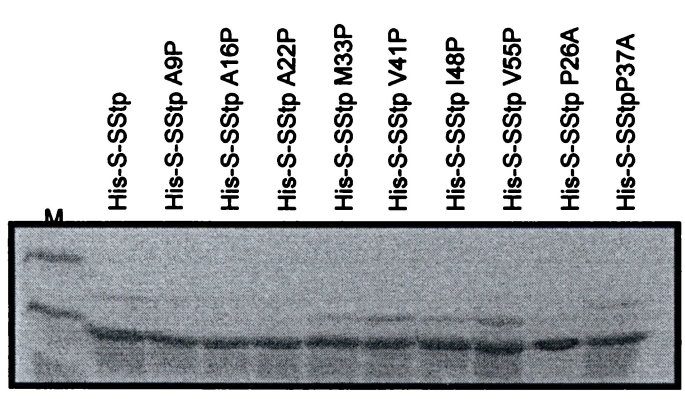


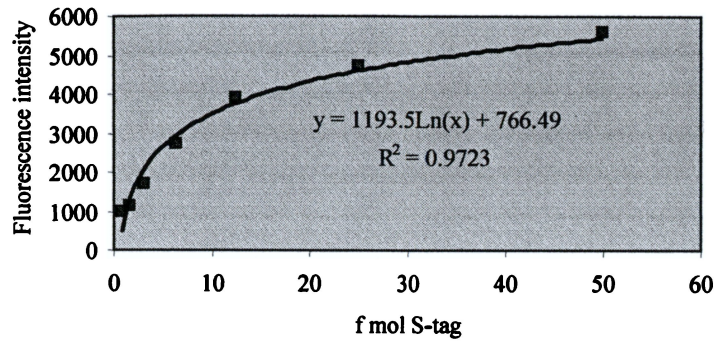
Figure 4-2.

interacts with the chloroplast lipids. However, none of these experiments show that the α-helices of the transit peptide contribute to its interaction with the Toc complex. Proline, due to its bulky side chain causes breaks in α-helices while aliphatic residues like A, V, L and I are excellent helix formers. Therefore, we have mutated aliphatic residues like A, V, L and I to P such that the helices that maybe formed during the interaction of the transit peptide with chloroplast lipids are disrupted. We have systematically made these single amino acid mutations from the N terminus to the C-terminus of His-S-SStp.

A typical standard curve obtained using the S-tag standard is given in Figure 4-3a. The functional RNase acts on a novel fluorescent substrate 5′-(6-FAM)-ArUAA-(6-TAMRA)-3′ as shown in Figure 4-3b. The fluorescence of 6-carboxy fluorescein label (6-FAM) is quenched by the 6-carboxytetramethylrhodamine (6-TAMRA) label due to their proximity. However, when functional RNase is formed it cleaves the substrate separating the two labels and the fluorescence of 6-FAM is manifested as shown in Figure 4-3b. This increase in fluorescence was determined to be 180 fold more than that for the un-cleaved substrate (Kelemen et al., 1999). *In vitro* binding reactions analyzed by far-western and FRET analysis indicated that proline mutations at the N-terminus of the transit peptide inhibited the binding of the transit peptide to the chloroplasts (A9P, A16P, A22P) as shown in Figure 4-4a and b). However, proline mutations at the C-terminus do not affect the binding of the mutants to the chloroplasts (V41P, I48P, and V55P) as seen by far-western blotting after the binding reaction (Figure 4-4a). The amount of transit peptide bound to the chloroplasts was quantified by calculating the fmols of S-tag present using FRET analysis (Figure 4-4b). The FRET assay is based on the measurement of RNase

Figure 4-3. FRET works RNase A assay. **(a)** A typical standard curve using S-tag standard done using FRET works RNase A assay. **(b)** A schematic representation of detecting chloroplast bound epitope tagged transit peptide using FRETworks RNase A assay. The S-tag in the epitope tagged transit peptide interacts with the S-protein and forms a functional RNase A protein, which splits Fl-dArUdAdA-Rh RNA substrate. The fluorescence of Fluorescein is quenched by Rhodamine due to their close proximity. The cleaving of the substrate allows the fluorescence of Fluorescein to be manifested which can be measured by exciting at 485 nm and then emission is measured at 535 nm.

(a)


Fluorescence intensity
6000
5000
4000
3000
2000
1000
0
y = 1193.5Ln(x) + 766.49
R2 = 0.9723
0
10
20
30
40
50
60
f mol S-tag

(b)

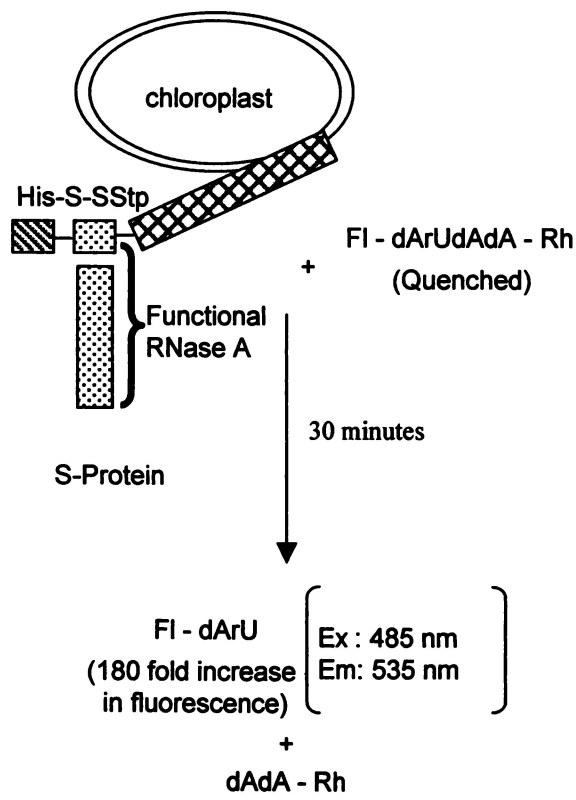

chloroplast
His-S-SStp
Fl - dArUdAdA - Rh
+
(Quenched)
Functional
RNase A
30 minutes
S-Protein
Fl - dArU
(180 fold increase
in fluorescence)
Ex : 485 nm
Em: 535 nm
+
dAdA - Rh

Figure 4-3.

Figure 4-4. Binding of proline addition mutants to chloroplasts. **(a)** Chemilumeniscent detection of a far-western blot after a binding reaction of the proline insertion mutants to intact chloroplast. The proteins were added at increasing concentrations from 0 to12 μM with 3 μM increments. **(b)** Quantitation of the binding reaction in (a) using FRET works RNase A assay. **(c)** % binding with change in proline position was calculated from (b). Each marker is indicated in the figure legends. The graphs were plotted after three repeats of each experiment and the significance of the results is indicated using error bars.

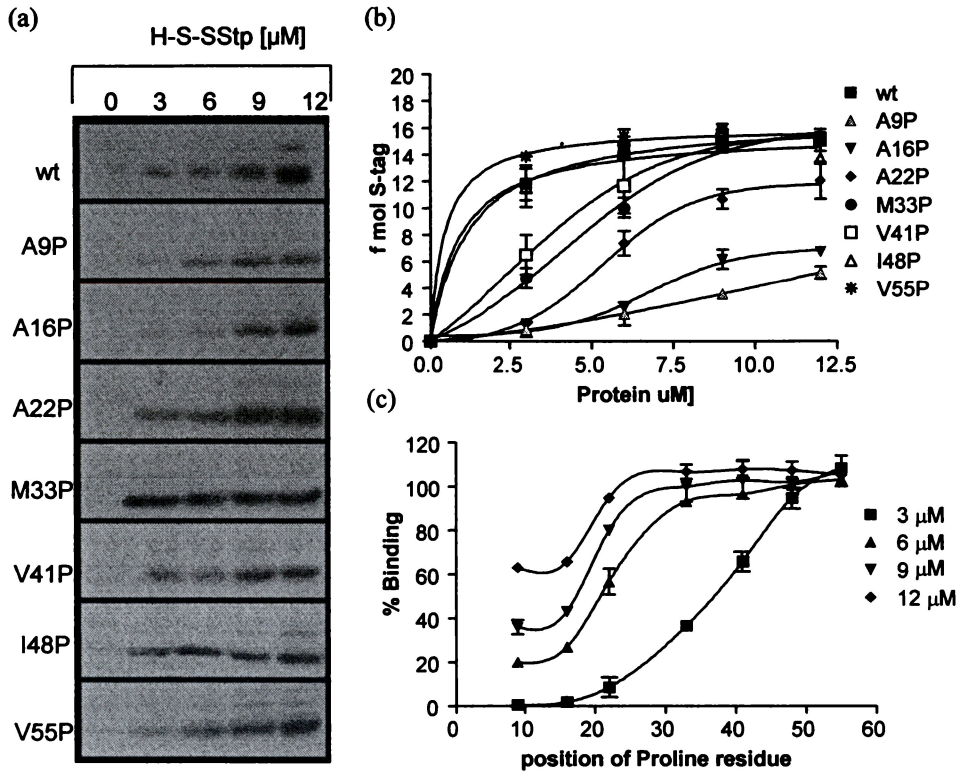

(a)
H-S-SStp [μM]
0 3 6 9 12
wt
A9P
A16P
A22P
M33P
V41P
I48P
V55P
(b)
f mol S-tag
Protein uM]
wt
A9P
A16P
A22P
M33P
V41P
I48P
V55P
(c)
% Binding
position of Proline residue
3 μM
6 μM
9 μM
12 μM

Figure 4-4.

activity reconstituted by the binding of S-tag in the transit peptide with purified S-protein supplied in the assay. It is clear from Figure 4-4c that as the proline insertion mutations progress from the N-terminus towards the C-terminus the binding of transit peptide increases and the mutants behave more like wild type His-S-SStp. The concentration titration of His-S-SStp indicated that its binding saturated at ~8-10 μM. (Subramanian et al., 2001). Therefore, we performed the titration from 3μM to 12μM. It can be seen from the concentration titration that the effect of proline insertion at lower concentration (3μM) is more severe than at higher concentrations (6μM, 9μM, 12μM). The two N terminal proline insertions A9P and A16P show the least binding to the chloroplast by both far-western and FRET analysis (Figure 4-4a and 4-4b) while the two C-terminal proline insertions I48P and V55P show at least the same if not more binding to the chloroplast than the wild type His-S-SStp.

Previous studies that have used deletions of SStp have shown that residues 6 to 24 are essential for binding of prSSU to the chloroplasts (Reiss et al., 1989). Moreover, the work with the Fd shows that deleting residues 6-14 at the N-terminus of trFd prevents the interaction of prFd with the chloroplasts (Pilon et al., 1995). Their results indicated that the N-terminal region from 1-25 was essential for the binding of trFd to the chloroplast. Recently, work in our laboratory has shown that the N-terminal region (residues ~ 1-16) binds to Hsp70 (Ivey et al., 2000). The algorithm described by Rudiger, is based on the cellulose-bound peptide screening assay (CBPS). According to this the free energy change for high Hsp70 sites is <– 4.0. It was observed that the wt SStp had a free energy change of ~ –4.9, while A9P mutant that showed the least binding to the chloroplast had a free energy change of – 0.18. All the three His-S-SStp C-terminal deletions (described in Chapter 3) showed

interaction with *E.coli* DnaK proving the predicted Hsp70 binding site to be experimentally correct (Ivey et al., 2000). This interaction of the transit peptide with the Hsp70 (Com 70) may be essential for the formation of a successful binding intermediate. The insertion of proline residues in this region probably changes the conformation of the protein such that it reduces binding of the transit peptide to some proteinaceous component, which may be an Hsp70 of the chloroplast membrane, as the lipid interaction is not affected in these mutants. An Hsp70 homologue (Com70) has been identified in the chloroplast envelope as a component of the chloroplast translocation machinery (Kourtz & Ko, 1997; Wu et al., 1994). Com70 was accessible to thermolysin, but its association with the envelope could not be disrupted by stringent washes. Com70 was found to be involved in the early stages of import as seen by chemical cross-linking, and co-immunoprecipitation analysis. It was found cross-linked to the plastid transit peptide under binding conditions and under not complete translocation conditions further indicating that it was a component of the translocation machinery of the chloroplast outer membrane and associated with the pre-protein while it was still partly exposed to the cytosol.

Several researchers have mutated the C-terminus of SStp to see if the processing of the precursor to mature form is in any way affected (Archer & Keegstra, 1993; Ostrem et al., 1989; Reiss et al., 1987; Reiss et al., 1989). It was observed that the cysteine residue at the C-terminus was not important for processing, but mutating the R residue resulted in aberrant processing. His-S-SStp, due to its charged N-terminus is not imported into the chloroplasts, and therefore does not get processed (Subramanian et al., 2001). Due to the presence of two glycine residues, the C-terminus of SStp is already flexible and hence adding proline

residues would only increase the flexibility of this region. Therefore, it was not surprising that the proline insertions at the C-terminus had binding activity similar to wild type His-S-SStp. Moreover, the C-terminus has been implicated in lipid interaction rather than the interaction with the chloroplast translocation apparatus (Pinnaduwage & Bruce, 1996).

In vitro binding of His-S-SStp proline removal mutants to chloroplast

Each of the semi-conserved H(P/G)H(R/K) motif of SStp has a proline residue. Removal of a single proline residue, P26A from one of the motifs resulted in decreased binding the to chloroplasts while the other mutant P37A behaved like wild type His-S-SStp as shown by the far-western blot analysis in Figure 4-5a and the FRET assay in Figure 4-5b. This indicated that the first H(P/G)H(R/K) motif from the N-terminus is probably more sensitive then the second one. Moreover, it is possible that removal of a proline residue makes the central domain less flexible and this flexibility may be important for the proper interaction of the transit peptide with the Toc components. Hence, the flexibility at the central region may be one of the important factors influencing transit peptide interaction with the chloroplasts. Decreasing flexibility around the central region (P26A) seems to decrease the binding of the transit peptide (Figure 4-5a and 4-5b), while increasing more flexibility around the central region does not increase the binding, as M33P behaves more like wild type His-S-SStp (Figure 4-4a and 4-4b). This shows that the rigid N-terminus and flexible middle region are probably important determinants of a chloroplast transit peptide. By analyzing the sequence of the various chloroplast

Figure 4-5. Binding of proline removal mutants to chloroplasts. **(a)** Chemilumeniscent detection of a far-western blot after a binding reaction of the proline removal mutants to intact chloroplast. The proteins were added at increasing concentrations from 0-12 μM with 3 μM increments. **(b)** Quantitation of the binding reaction in (a) using FRET works RNase A assay. Each marker is indicated in the figure legends. The graphs were plotted after three repeats of each experiment and the significance of the results is indicated using error bars.

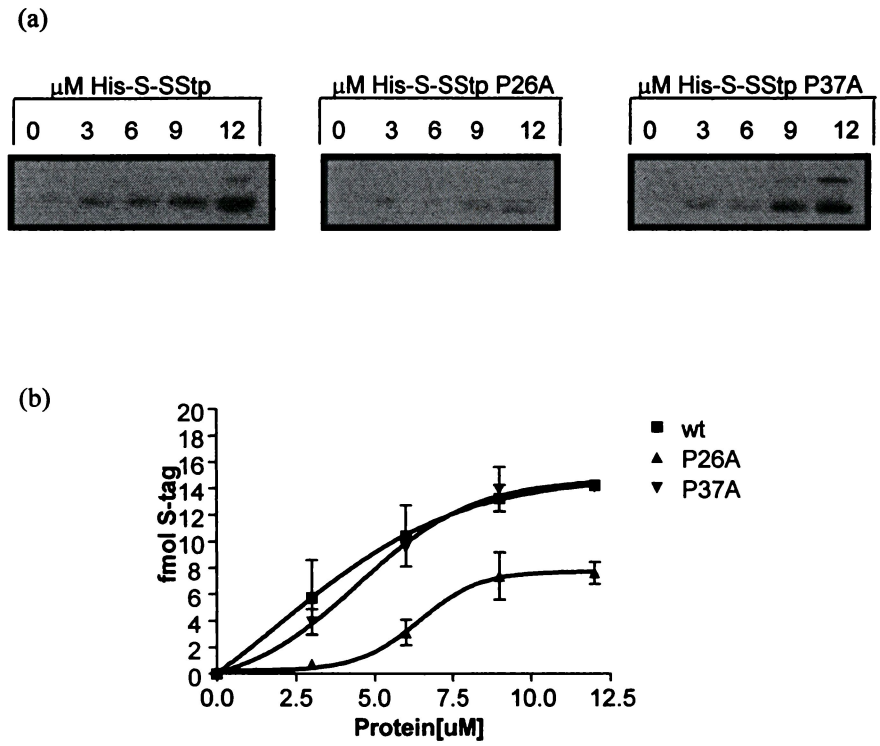

(a)
μM His-S-SStp
0 3 6 9 12
μM His-S-SStp P26A
0 3 6 9 12
μM His-S-SStp P37A
0 3 6 9 12
(b)
fmol S-tag
20
18
16
14
12
10
8
6
4
2
0
0.0 2.5 5.0 7.5 10.0 12.5
Protein[uM]
wt
P26A
P37A

Figure 4-5.

transit peptides it can be seen that the N-terminus is usually rigid, the middle region is flexible and the C-terminus is semi-flexible.

Interaction of His-S-SStp with lipids using calcein dye release assay

Most of the current models for protein translocation across the chloroplast propose that the first stage is an energy independent interaction of the transit peptide with the membrane lipids, which is reversible (reviewed in Keegstra et al., 1989). Several studies have shown that transit peptides can interact with micelles composed of only lipids (Chupin et al., 1994; Horniak et al., 1993; Pinnaduwage & Bruce, 1996; van't Hof & de Kruijff, 1995; van't Hof et al., 1991; van't Hof et al., 1993; Wienk et al., 2000). Lipid alteration studies using Phospholipase C (Kerber & Soll, 1992) and lipid depletion studies using cyclodextrin (Bruce unpublished result) have shown that transit peptide–lipid interaction decreases with changes in the lipid composition. An *Arabidopsis* mutant deficient in digalactosyldiacylglycerol showed defective chloroplast import (Chen & Li, 1998). Another *Arabidopsis* mutant deficient in the prokaryotic MGDG synthesis pathway was found to have defective chloroplast ultrasturcture strongly suggesting a unique role for MGD in the structural organization of plastidic membranes (Jarvis et al., 2000).

We studied the interaction of His-S-SStp with lipid micelles mimicking the chloroplast outer membrane (MGDG/DGDG/PC/PG: 20/30/40/10) and other micelles that were composed of either only PC or PC/PG (90/10) or PC/DGDG (70/30) or PC/MGDG (80/20) lipids. We observed that His-S-SStp interacted the best with liposomes mimicking the chloroplast outer membrane (OM liposomes) as this interaction led to a 42% dye release as shown in Figure 4-6a. The next best

Figure 4-6. Calcein entrapped liposome assay of SStp and His-S-SStp. **(a)** Calcein entrapped liposome assay of SStp. ▲ indicates OM, ● indicates PC/MG, ■ indicates PC/PG, ✳ indicates PC/DG and ◆ indicates PC liposomes. **(b)** Calcein entrapped liposome assay of His-S-SStp. ■ indicates OM, ◆ indicates PC/PG, ▲ indicates PC/MG, ✳ indicates PC/DG, ● indicates PC liposomes. **(c)** Comparison of calcein entrapped dye release by SStp and His-S-SStp. ▲ indicates interaction of SStp with OM liposomes, ■ indicates interaction of His-S-SStp with OM liposomes. The graphs were plotted after three repeats of each experiment and the significance of the results is indicated using error bars.

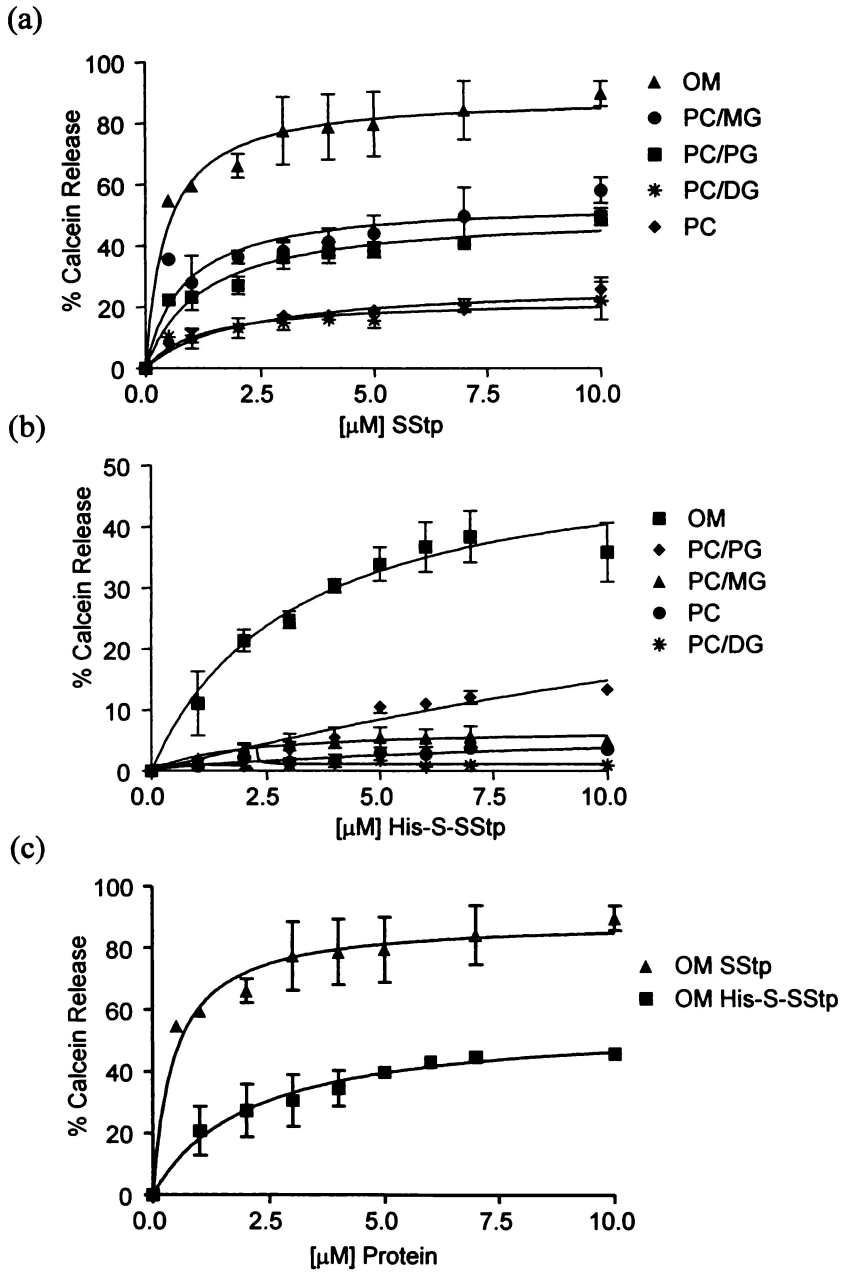

(a)
% Calcein Release
100
80
60
40
20
0
0.0
2.5
5.0
7.5
10.0
[μM] SStp
OM
PC/MG
PC/PG
PC/DG
PC
(b)
% Calcein Release
50
40
30
20
10
0
0.0
2.5
5.0
7.5
10.0
[μM] His-S-SStp
OM
PC/PG
PC/MG
PC
PC/DG
(c)
% Calcein Release
100
80
60
40
20
0
0.0
2.5
5.0
7.5
10.0
[μM] Protein
OM SStp
OM His-S-SStp

Figure 4-6.

interaction was observed with PC/PG liposomes (12% dye release), followed by PC/MGDG liposomes that had 6% dye release while PC/DGDG and PC liposomes had negligible dye release (~2%) as seen in Figure 4-6a. This shows that MGDG, PG, DGDG and PC together form the ideal environment for the interaction of the transit peptides. It has been proposed that the interaction of unstructured transit peptides with lipids induces certain regions to assume an α-helical conformation (reviewed in Bruce, 2000; Bruce, 1998). Previous work from our laboratory (Pinnaduwage & Bruce, 1996) and others (van't Hof et al., 1993) have shown that the interaction of chloroplast transit peptides is lipid class (MGDG) dependent. MGDG along with PE and CL are non-lamellar phase forming lipids. These lipids tend to form a hexagonal array known as H_{II} that has been hypothesized to aid in α-helix formation. Lipid interaction studies with Fd have shown that anionic lipids (like PG) and sulfolipids play an important role in the interaction of transit peptides with lipids. The positively charged basic residues in the transit peptides can interact with the anionic phospholipids (van't Hof et al., 1993). In order to compare His-S-SStp with SStp under the same conditions, liposome assays were performed with SStp obtained using the Chitin column as described in chapter 3. We observed that SStp interacted the best with OM liposomes showing >75% dye release, PC/PG and PC/MGDG showed ~45% dye release while PC/PG and PC liposomes showed <10% dye release (Figure 4-6b). This data indicated that the MGDG and PG act synergistically in creating a suitable environment so that the transit peptide can interact with the chloroplast membrane. It is possible that the basic amino acids in the transit peptide first interact with the anionic PG lipids and then the H_{II} phase formed by MGDG aids in the α-helix formation. It is also clear that His-S-SStp is less efficient in

interacting with OM liposomes compared to SStp as shown in Figure 4-6c. The N-terminal 20 amino acids of SStp showed ~30% dye release with OM liposomes (Pinnaduwage & Bruce, 1996). Thus, blocking the N-terminus of SStp with the His-S epitope tag, may have decreased the accessibility of this domain and hence decreased its lipid interaction. His-S itself does not have any lipid interacting activity with OM liposomes as shown in Figure 3-9b in Chapter 3.

Interaction of His-S-SStp proline mutants with OM liposomes

Structural analysis of the chloroplast transit peptides predict that they are to be devoid of any secondary structure in an aqueous environment (von Heijne & Nishikawa, 1991). Prediction analyses divide the transit peptide into three distinct domains: an uncharged amino-terminal domain, a central domain lacking acidic residues and a carboxy-terminal domain with the potential to form an amphiphilic β-strand (von Heijne et al., 1989). Several researchers have shown by Circular dichroism and NMR analysis that the chloroplast transit peptides are unstructured in aqueous solution but tend to gain an α-helical conformation when they interact with lipid micelles, SDS micelles or TFE (Horniak et al., 1993; Krimm et al., 1999; Lancelin et al., 1994; Wienk et al., 1999; Wienk et al., 2000).

Hence, we analyzed the lipid interacting properties of His-S-SStp with proline insertion mutants that would prevent α-helix formation during lipid interaction. After analyzing the lipid interacting activity of all our mutants we divided them into three categories: lower lipid interaction than wild type (A22P), similar to wild type interaction (A9P, A16P and M33P) and higher lipid interaction than wild type (V41P, I48P and V55P) as shown in Figure 4-7a-c. The N-terminus

Figure 4-7. Lipid interaction of proline insertion and removal mutants. **(a)** Calcein dye release by A22P and His-S-SStp. **(b)** Calcein dye release by A9P, A16P, M33P and His-S-SStp. **(c)** Calcein dye release by V41P, I48P, V55P and His-S-SStp. **(d)** Calcein dye release by P26A, P37A and His-S-SStp. Each marker is indicated in the figure legends. The graphs were plotted after three repeats of each experiment and the significance of the results is indicated using error bars.

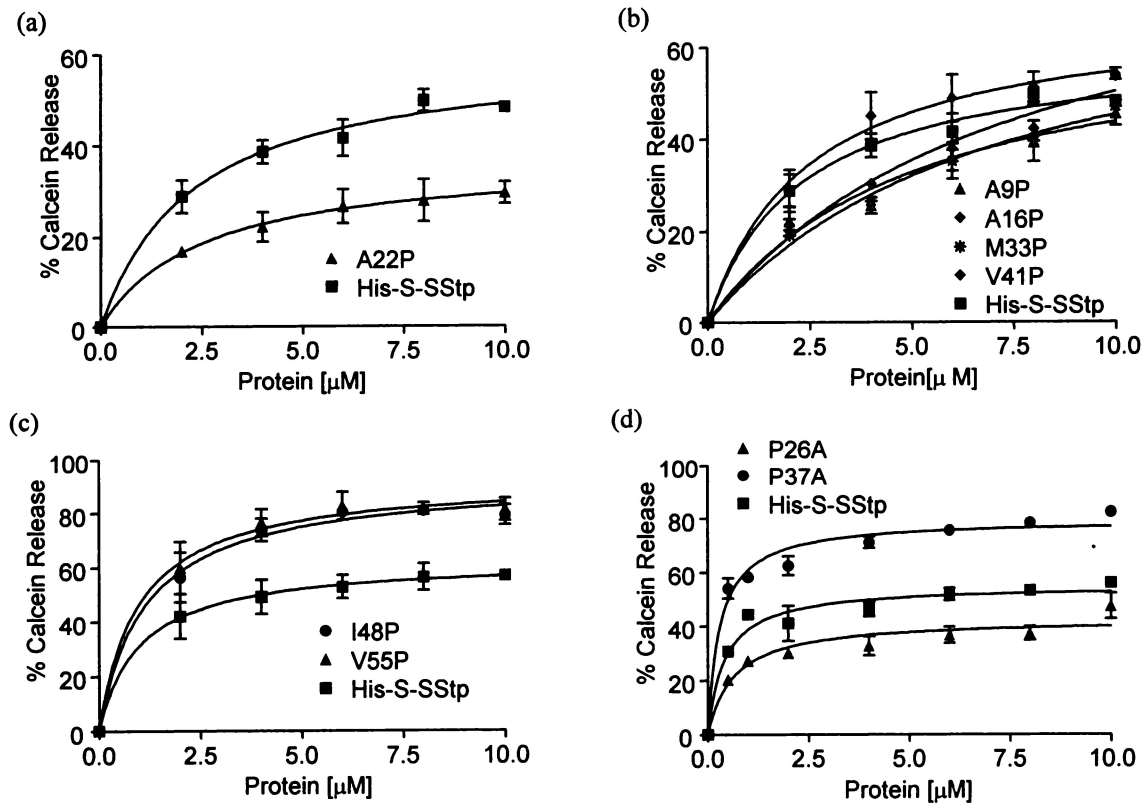

(a)
% Calcein Release
Protein [μM]
A22P
His-S-SStp
(b)
% Calcein Release
Protein[μ M]
A9P
A16P
M33P
V41P
His-S-SStp
(c)
% Calcein Release
Protein [μM]
I48P
V55P
His-S-SStp
(d)
% Calcein Release
Protein [μM]
P26A
P37A
His-S-SStp

Figure 4-7.

of SStp (1-20 residues) has previously been shown to interact with lipids (Pinnaduwage & Bruce, 1996). We have seen that increasing flexibility in this 20-amino acid region of the N-terminus decreases the lipid interaction only slightly but substitution with a proline residue at 22A position greatly decreases the interaction of the transit peptide with the OM liposomes (Figure 4-7a). Our *in vitro* chloroplast binding experiments showed that increasing the flexibility of the N-terminus (A9P, A16P, A22P) led to impaired binding to the chloroplasts. This would indicate that the 15 amino acids of the N-terminus are more essential for the interaction of the transit peptide with some protein component in the chloroplast, like Com70 the hsp70 homologue in the Toc apparatus as proposed earlier (Ivey et al., 2000; Kourtz & Ko, 1997; Wu et al., 1994) rather than the chloroplast membrane lipids. However, when a proline residue is introduced at the A22 position it is possible that it extends the flexible central domain towards the N-terminus and this in turn decreases the interaction of the transit peptide with the chloroplast membrane lipids as shown in Figure 4-7a. Earlier work from our laboratory has shown that the central region of SStp is less membrane interactive (Pinnaduwage & Bruce, 1996), and so extending the central region would cause decreased interaction with the chloroplast lipids. Alternatively, it is possible that there are two inter-domain regions: inter-domain-1, between the N-terminus and the central domain and inter-domain-2, between the central domain and C-terminus as proposed earlier by Karlin-Neuman and Tobin (Karlin-Neumann & Tobin, 1986) and these domains may also be involved in lipid interaction. Considering the later hypothesis, it would mean that the inter-domains are extremely sensitive to changes in the position of the proline residues. The A22P mutation showed decreased transit peptide–lipid interaction, and decreasing the

flexibility as in P26A also decreased the lipid interaction (Figure 4-7a and 4-7d). Similarly, V41P showed a slight increase in the lipid interaction and so did P37A; again indicating that the position of the proline residue in the interdomain-2 plays an important role in transit peptide–lipid interaction (Figure 4-7c and 4-7d). Increasing flexibility in the already flexible central region (M33P) does not in any way effect the binding or lipid interaction of the transit peptide as shown in Figure 4-7b. Previously work from our laboratory showed that the C-terminal 20 amino acids (41-60) had the maximum lipid interaction. The C-terminus of SStp is semi-flexible and increasing flexibility in this region (I48P and V55P) increased the lipid interaction of the transit peptide with OM liposomes as compared to the wild type transit peptide (Figure 4-7c). Based on all these results and previous work SStp can be divided into five main domains, as shown in Table 4-2, that play different roles during the initial interaction of the transit with the chloroplasts.

Analysis of the structure of the transit peptide by circular dichroism spectrometry

Due to the lack of homology among the various transit peptides, the presence of specific structural motifs has been suggested to be the recognition criterion (von Heijne & Nishikawa, 1991; von Heijne et al., 1989; Wienk et al., 1999). In order to investigate this hypothesis we studied the structure of the transit peptide mutants by circular dichroism. Proline residues are known to be excellent helix breakers (Chou-fassman) and so by analyzing the structure of the transit peptides with proline residues inserted at various positions of the transit peptide would give us a relationship between the transit peptide structure and function. Alcohol based

Table 4-2. Domain organization of the transit peptide from prSSU.

Domain	**Amino acids**	**Structure**	**Function**
N-terminal	1-20	Rigid	hsp70 interaction
Inter-domain-1	21-26	Semi-flexible	Lipid interaction
Central	27-41	Flexible	Toc interaction (Toc34)
Inter-domain-2	42-51	Semi-flexible	Lipid interaction
C-terminal	52-60	Semi-flexible	Lipid interaction & SPP

solvents such as TFE have been used for decades to stabile the structures in peptides (Buck, 1998). Although the mechanism of this is not clearly understood but various hypothesis have been proposed. It has been implicated that TFE either acts as a hydrogen bond donor (Rajan & Balaram, 1996) or effects the non-polar side chains of the peptides by weakening the hydrophobic-hydrophobic interactions (Albert & Hamilton, 1995).

We analyzed the structure of the proline insertion and deletion mutants in the presence of increasing concentration of TFE. The %α-helical content of the N-terminal proline insertions (A9P, A16P, A22P) at lower concentration of TFE was slightly higher than wt His-S-SStp but at higher concentrations of TFE the %α-helix was similar to wt His-S-SStp (Figure 4-8a). But the C-terminal proline insertions (V41P, I48P, V55P) showed decreased α-helical content at 50% and 60% TFE (Figure 4-8b). These C-terminal proline insertion mutants showed increased lipid interaction (Figure 4-7c). Hence, this indicates that there is an inverse relationship between the α-helical content at the C-terminus of the transit peptides and lipid interaction. This corroborates the results seen in Chapter 3 where decrease in α-helical content at the C-terminus by deleting portions of the C-terminus caused an increase in lipid interaction. Increasing the flexibility in the central region (M33P) caused an increase in α-helical content (Figure 4-8c) but this did not effect the lipid interaction (Figure 4-7b). Decreasing flexibility by removing either of the two proline residues was seen to decrease the α-helical content that was surprising as by removing helix breaking proline residues one would expect to see an increase in the α-helical content. This effect may be due to the presence of glycine residues in the

Figure 4-8. Circular Dichroism of His-S-SStp proline mutants. **(a)** α-helical content of A9P, A16P, A22P and His-S-SStp. **(b)** α-helical content of M33P, V41P, I48P, V55P and His-S-SStp. **(c)** α-helical content of P26A, P37A and His-S-SStp. Each marker is indicated in the figure legends.

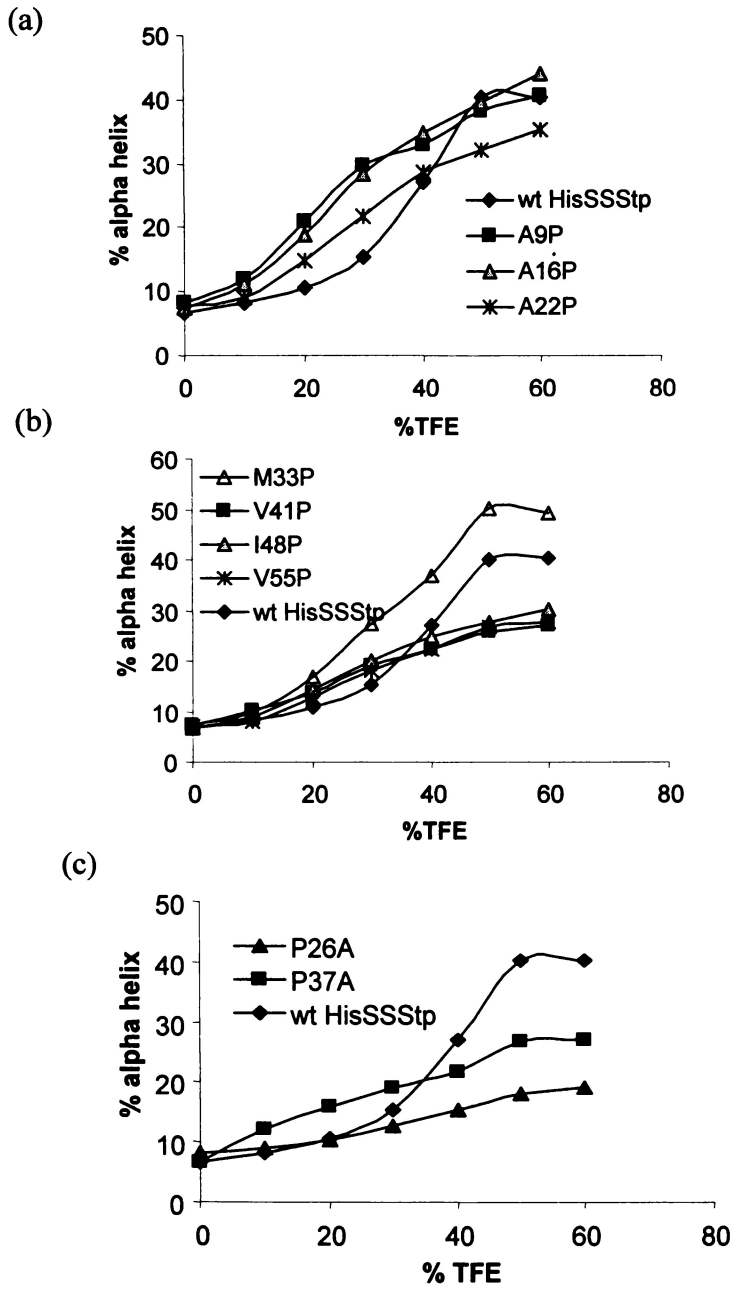

(a)
% alpha helix
%TFE
wt HisSSStp
A9P
A16P
A22P
(b)
% alpha helix
%TFE
M33P
V41P
I48P
V55P
wt HisSSStp
(c)
% alpha helix
% TFE
P26A
P37A
wt HisSSStp

Figure 4-8.

central region that are also helix breakers. The structure of the peptides may have to be analyzed again in order to see if changes observed are significant. ^{15}N labeled NOESY-NMR experiments may have to be performed to resolve this issue, as circular dichroism does not give any spatial information regarding the helices.

CONCLUSIONS

In this chapter by inserting proline residues from the N- to the C-terminus of His-S-SStp we have shown that there are five possible domains with distinct functions. The N-terminus is mainly involved in the interaction with some protein component in the chloroplast membrane that is essential for the formation of a stable binding intermediate. The central region interacts with the Toc apparatus and the C-terminus interacts with the chloroplast membrane lipids. In addition there may be two inter-domains that contribute to the interaction of the transit peptide with the chloroplast membrane lipids. We have also demonstrated that there is a inverse relationship between the flexibility at the C-terminus and the lipid interaction. NMR experiments have to be performed to further understand the role of structure of the transit peptide with its function.

Chapter 5

Conclusions and Future Directions

By using a non-natural chloroplast precursor, an N-terminally tagged transit peptide we succeeded in trapping an early binding intermediate. Due to the presence of a charged N-terminus we were able to trap a transit peptide bound to the translocation apparatus at a very early stage of import. This epitope tagged transit peptide could not cross the outer membrane even when provided with import conditions. Several methods were used to prove the validity of this binding intermediate. His-S-SStp like the precursor prSSU, binds to the proteinaceous components in the chloroplast membrane that are thermolysin sensitive. Moreover, we have cross-linked this epitope tagged transit peptide to the components of the general import pathway. Confocal Microscopy has been used to show for the first time a transit peptide bound to the chloroplast translocation apparatus.

C-terminal deletions of the transit peptide indicate that specific motifs in the C-terminus are essential for the interaction of the transit peptide with the chloroplast. The calcein entrapped dye release assay indicated that the deletions interacted better with the liposomes compared to the full-length control. Comparison of the structure of the full length His-S-SStp with its C-terminal deletions indicated that in the C-terminal deletions there is a decrease in α-helicity. This decrease in α-helicity probably corresponds to an increase in lipid interaction. Since, all the three C-terminal deletions were in the 'b-frame' unlike the full length His-S-SStp, it would have been more appropriate to have used a control His-S-SStp that was also in the 'b-frame' for direct comparison. Nevertheless, the C-terminal residues in the deletions

due to the 'b-frame' do not seem to contribute to or inhibit the binding of the epitope tagged transit peptide as His-S-SStp$_{\Delta 5}$ that has the extra residues at the C-terminus binds to the chloroplast like His-S-SStp.

Proline insertion studies have helped us to map the domains in the transit peptide. The N-terminus seems to be essential for the formation of an early import intermediate by interacting with probably Com70, the central region seems to play an important role in the interaction with the Toc components, while the C-terminus seems to be involved in lipid interaction. Increasing the flexibility at the C-terminus increases the lipid interaction. Decreasing the flexibility at the central region affects both the Toc interaction as well as the lipid interaction. In addition to the above-mentioned domains there are two inter-domains, one between the N-terminus and central region and the other between the central region and the C-terminus and both contribute to lipid interaction.

IN VIVO Vs *IN VITRO*

Since all these studies have been done *in vitro* it would be interesting to study mutations in the transit peptide *in vivo*. Some researches have used the *in vivo* approach to study the interaction of the transit peptide with the chloroplast translocation apparatus (Bauer et al., 2000; de Boer et al., 1991; Gutensohn et al., 2000; Jarvis et al., 1998; Kindle & Lawrence, 1998; Lawrence & Kindle, 1997; Rensink et al., 1998; Silva-Filho et al., 1997). There are several differences between *in vitro* and *in vivo* protein import studies: Firstly, *in vitro* targeting is probably not as efficient as *in vivo* due to the absence of several cytosolic factors present in the cytosol. Secondly, an *in vitro* assay occurs over a period of minutes in isolated

chloroplasts whereas *in vivo* targeting is studied over a period of hours to days. Thirdly, synthesis of the precursor proteins or transit peptide is usually done in *E.coli* using a prokaryotic protein synthesis machinery while the plant cytosol uses a eukaryotic protein synthesis machinery. Sometimes precursors are synthesized *in vitro* using wheat germ or rabbit reticulocyte translation systems, but even in this case the co-factors bound to the precursors may be different compared to the ones normally found in a plant cytosol. Fourthly, although transit peptides are considered necessary and sufficient for the targeting of proteins to the chloroplast, we do not know if there are other factors present *in vivo* that help to avoid mis-targeting to other organelles. Finally, *in vitro* targeting studies do not account for the competition between several other precursors present *in vivo*, all of which use the same general import pathway to enter the chloroplast. Moreover, the *in vitro* studies use precursors in non-physiological quantities.

Due to the differences in *in vivo* and *in vitro* targeting most researchers find differences while studying targeting properties of the same precursor protein *in vivo* and *in vitro* (Kindle, 1998; Kindle & Lawrence, 1998; Lawrence & Kindle, 1997; Rensink et al., 1998). Protein accumulation in the chloroplasts in transgenic plants is seen to occur over a period of hours to days and is found to be dependent upon other cellular processes like transcription, translation, assembly of co-factors as well as protein turn over. Small changes in *in vivo* chloroplast import might not lead to detectable changes in the accumulation of the mature protein. Moreover, mis-targeted and unusual intermediates might be unstable and may be degraded before they can be detected. Mutant *Arabidopsis* plants defective in Toc86/159 and Toc34 showed defective chloroplast biogenesis indicating that protein import is a essential

for chloroplast biogenesis (Bauer et al., 2000; Jarvis et al., 2000). However, due to the ability of these chloroplasts to import some of the nuclear encoded precursors, it is evident that alternative routes for import into the chloroplast are present.

FUTURE DIRECTIONS

As mentioned in the previous section there are several differences in *in vivo* and *in vitro* protein targeting into chloroplasts. Hence, it would be interesting to analyze the binding of His-S-SStp *in vivo*. Since, His-S-SStp is bound at an early intermediate stage to the chloroplast and as it cannot import further into the translocon it is possible that it blocks the translocation channel. It would be interesting to see the growth and development of transgenic *Arabidopsis* plants transformed with His-S-SStp. We predict that these plants will have poor chloroplast biogenesis since their chloroplast translocation channel would be blocked by His-S-SStp.

Analysis of *in vitro* binding of His-S-SStp by FACS and LSCM has revealed three distinct populations of chloroplasts differing in their precursor binding properties (Figure 5-1). As seen by LSCM some chloroplasts bind precursor proteins with high efficiency while others bind with moderate efficiency and some do not bind precursors at all. This indicates that either there is a difference in the number of translocons present per chloroplast or some chloroplasts have modified their translocon to prevent further import as they have all the proteins they need for their viability. In order to see if this decrease in chloroplast binding was related to the age of the plastid, we performed *in vitro* binding studies with chloroplast of different

Figure 5-1. LSCM of His-S-SStp bound to chloroplast. The chloroplasts are shown in red (auto-fluorescence). The FITC staining is shown in green. The stained chloroplasts have the green FITC staining on red chloroplasts while the unstained chloroplasts do not have the green FITC staining.

Placeholder

REPLACE IMAGE

ages. Interestingly, the younger chloroplasts had lower chlorophyll content but higher import efficiency compared to the older chloroplasts, which had higher chlorophyll content but lower import efficiency (Figure 5-2 a, b and c). Analysis of the translocation complex in the plastids of different ages would shed light on their varying import capabilities. In addition, such an analysis can be performed with other classes of plastids such as leucoplasts, amyloplast, chromoplasts etc.

Further characterization of the transit peptide can be done by mutating and deleting certain domains and residues. The total understanding of the "distinguishing features" of a transit peptide would be useful not only in the *de novo* synthesis of a transit peptide, but it would also help us understand the fundamentals of protein translocation into organelles. This would enable us to generate transgenic crops with increased targeting efficiency to various types of plastids.

Figure 5-2. Effect of plastid age on plastid import. (a) FACS analysis showing the amount of chlorophyll in chloroplasts of different ages. (b) FACS analysis showing the amount of His-S-SStp bound by measuring FITC fluorescence. (c) Quantitative analysis of (a) and (b) showing the decrease in His-S-SStp binding and increase in chlorophyll fluorescence with age of the plastid.

Placeholder

REPLACE IMAGE

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